

Use of Biolog® for the Community Level Physiological Profiling (CLPP) of environmental samples

HERIBERT INSAM¹ and MARTA GOBERNA²

¹*Institute of Microbiology, Technikerstr. 25, A-6020 Innsbruck, Austria;* ²*CIDE, Camí de la Marjal s/n, 46470, Albal (Valencia), Spain*

Introduction

Carbon is a key factor governing microbial growth in soil, and functional aspects related to substrate utilisation can provide important information beyond that afforded by taxonomic level investigations or structural investigations based on rRNA or rRNA analysis [11]. The functional diversity of microorganisms, particularly as defined by the substrates used for energy metabolism, is integral to our understanding of biogeochemistry [16]. Indeed, it has been argued that it is diversity at the functional level rather than at the taxonomic level that is crucial for the long-term stability of an ecosystem [24].

The method involves direct inoculation of environmental samples into Biolog® microtiter plates (containing different C sources, nutrients, and a redox dye), incubation, and spectrometric detection of heterotrophic microbial activity. The method was originally developed [3] for medical strain identification, and has only later been adapted for use with inocula from extracted microorganisms from environmental samples [7]. Its simplicity and speed of analysis are attractive to the microbial ecologist, but the technique requires careful data acquisition, analysis, and interpretation.

Several approaches have been used to account for biases related to inoculum density, incubation time, and microenvironment [6]. For instance, standardisation of initial inoculum density is commonly used [7], although it is laborious and choice of cell enumeration method remains a subject of debate. Normalisation of optical density (OD) readings by dividing by average well colour development (AWCD) is restricted to a number of conditions [13, 14, 21]. Single time point readings [7] and integration of the OD over time are still the most widely used measuring strategies, but can be compromised by the effect of inoculum density. Use of continuous plate reading for analysing the kinetics, rather than the degree of colour development at a given time [13, 6], has led to the development of a sigmoidal type of growth model [22]. Nevertheless, the kinetic parameters are not independent of inoculum density [22, 9] and need to be normalised prior to statistical analysis [6]. Recently,

a normalisation procedure that employs integrated OD values derived from four dilutions of the same sample rather than a single dilution level has been developed as a cell density- and time-independent method of analysis [4]. Most of these approaches, however, have yet to be applied often enough to allow robust critical comparison.

Sampling, extraction of microbes and microplate incubation

As for most biological investigations, fresh samples are superior to any stored samples. If storage is necessary, samples may be stored up to 10 days at 4 °C, for longer periods freezing is recommended, which, however may cause a certain bias towards frost tolerant organisms. Before extraction, we usually let the samples equilibrate at room temperature for 2 days, and sieve samples with a 2 mm mesh sieve.

The literature contains many methods for bacterial extraction from soil, sediment and compost samples [see chapter 1.10]. The extraction procedures vary widely among each other but they all include two main steps: 1) aggregate dispersion and 2) separation between cells and organic and inorganic particles. Dispersion techniques can be physical, chemical or both. Separation is carried out by centrifugation, according to sedimentation velocities (low-speed centrifugation), buoyant density (high-speed centrifugation), or both [1].

Two microplate options are possible, either use of Biolog EcoPlates [18] or GN plates [3], containing 31 or 95 different C sources, respectively, plus a water control. The EcoPlates contain 3 replications of the C and control wells. The EcoPlates contain substrates that are known to be plant root exudates [5] or that have previously been found to have a high discriminatory power among soil communities [15]. The plates are inoculated with 130 µl suspension, diluted (in 1/4 strength Ringer solution) to obtain a cell density of approximately 1×10^8 cells ml⁻¹ (acridine orange direct count, AODC) [2]. The plates are then incubated at 20 °C in the dark (other incubation temperatures may also be selected), and subsequent colour development is measured every 12 hours for 5 d (592nm) using an automated plate reader. Readings may be terminated if the average well colour density reaches an optical density of 2. To avoid problems with different inoculum densities among soil samples, the determination of microbial biomass (e.g. with substrate induced respiration or a fumigation-extraction procedure) may be used for an initial comparison, and subsequent dilution of the extracts so as to obtain similar microbial biomass for each sample inoculum.

Data Management

Raw OD data are corrected by blanking each response well against its own first reading (immediately after inoculation). This blanking not only avoids the intrinsic absorbance of the carbon sources but also the negative values when compared to subtracting the control well from the response well. The AWCD is calculated from each plate at each reading time. For each plate, those time points of reading are

selected that have an AWCD closest to 0.6. Alternatively, also other AWCDs (e.g. 0.30, 1.00) may be chosen. To diminish biases by different inoculum densities, data may be normalised by dividing each well OD by the AWCD [8]. This is particularly important when inoculum densities are not standardised prior to inoculation.

Data analysis may be further elaborated by calculating the area under the curve for each well OD for the entire period of incubation [12], or by estimation of kinetic parameters (K, r, s) by fitting the curve of OD versus time to a density dependent logistic growth equation [22],

$$Y = OD_{592} = \frac{K}{1 + e^{-r(t-s)}}$$

where K is the asymptote (or carrying capacity), r determines the exponential rate of OD change, t is the time following inoculation of the microplates, and s is the time when the mid point of the exponential portion of the curve (i.e., when $y = K/2$) is reached.

Diversity parameters, such as Shannon's diversity index (H) and Evenness (E) [25], can also be calculated as a means of evaluating microbial community functional diversity [27], using the following equations:

$$H = - \sum p_i (\ln p_i) \quad \text{and} \quad E = \frac{H}{H_{\max}} = \frac{H}{\log S},$$

where p_i is the ratio of the corrected absorbance value of each well to the sum of absorbance value of all wells, S (substrate richness) is the number of different substrates used by the community (counting all positive OD readings). H encompasses both the richness and evenness of substrate utilisation, E (substrate evenness) is a measure of the uniformity of activities across all substrates.

Procedures

The following procedure is the basic protocol for CLPP analysis. According to specific needs, extraction procedures may be modified, inoculation densities and temperature may be altered, or single-point data reading may be replaced by continuous readings (see above).

Cell Extraction

We suggest two alternatives for the extraction procedure. The first one combines physical and chemical agents for soil dispersion and a low-speed centrifugation for separating bacterial cells from soil particles according to their size. The second one includes a physical dispersion and a high-speed centrifugation step, using a non-ionic substance (Nycodenz[®]) to generate a density barrier between cells

and soil colloids. The latter extraction method is more expensive and time-consuming than the former, but ensures the maintenance of the CLPP of the extracted and purified bacterial community [23].

Extraction protocol 1 (modified from [17])

1. Blend 5 g fresh soil/sediment/compost with 20 ml of 0.1% (w/v) sodium cholate solution, 8.5 g cation exchange resin (Dowex 50WX8, 20–50 mesh, Sigma) and 30 glass beads.
2. Shake the suspension on a head-over-head shaker (2 hours, 4 °C).
3. Centrifuge at $800 \times g$ for 2 minutes.
4. Decant the supernatant into a sterilised flask.
5. Re-suspend the pellet in 10ml Tris buffer (pH 7.4) and shake for 1 hour.
6. Centrifuge as above and add the supernatant to the first step extract.
7. (optional). If the extract is turbid or dark (due to clay or humus particles) centrifuge the resulting supernatant another time.

Extraction protocol 2 (as described in [1])

1. Add 20 ml of Milli-Q water to 2 g soil wet weight in 40 ml centrifuge tubes.
2. Disperse the suspension by using a Waring blender for 1 minute (3 times, 4 °C).
3. Pipette carefully 6–7 ml of Nycodenz[®] (60% w/v) below the soil suspension.
4. Centrifuge at $10,000 \times g$ for 30 minutes (4 °C) with a swing-out rotor, for establishing the density barrier.
5. Transfer 10 ml of the supernatant to a 250-ml centrifuge bottle containing 100 ml of Milli-Q water.
6. Centrifuge at $16,000 \times g$ for 60 minutes (4 °C).
7. Resuspend the bacterial pellet in $\frac{1}{4}$ strength Ringer solution (Merck, Darmstadt, Germany).

Inoculation and incubation

1. If you want to perform statistical testing of single substrate utilisation with MANOVA, inoculate a sufficient number of replicates (one EcoPlate contains 3 replications) according to $n_i \times q = n \geq 31 + q + 2$, where q is the number of groups to be compared, n_i is

the replicate number required per group (sample sizes are equal in each group). For example, if two groups are compared, an $n_i > 17$ is required (i.e. 6 EcoPlates) [20].

2. Dilute the samples with 1/4 strength Ringer solution 10 (sediment)- to 1000 (composts)- fold and check the cell density by acridine orange direct counting (AODC).
3. Dilute your samples appropriately to obtain a cell density of approximately 1×10^8 cells ml^{-1} .
Note: In case of background colouration of the extract, further dilutions are recommended.
4. For environmental investigations the use of Biolog[®] EcoPlates[™] [18] is suggested. The plates are inoculated with 130 μl of the diluted cell suspension.
5. Cover the plates with a lid and incubate at 20 °C in the dark (other incubation temperatures may also be used; if you chose >30 °C, more frequent readings are suggested).
6. Measure colour development (592nm) every 12 hours for 5 d using an automated plate reader. Readings may be terminated if the average well colour density reaches an optical density of 2.

Note

If curve parameters or the area under the curve are to be determined make sure you always have the same reading intervals and the same number of readings (e.g. [11]). In case of a long lag-time, or low incubation temperature, incubation may be prolonged (reading interval up to 24 hours). Make sure that desiccation is avoided by placing the plates in polyethylene bags if you incubate at temperatures >30 °C.

Data Management

1. Raw OD data are corrected by blanking each response well against its own first reading. This blanking not only avoids the intrinsic absorbance of the carbon sources but also the negative values when compared to subtracting the control well from the response well.
2. Two data management alternatives are suggested:
 - a) Single-point absorbance readings. There are three possibilities:
 - Select a single reading time (e.g. 24 hours, 48 hours, 72 hours). This is only feasible when inoculum densities are previously standardised.
 - Calculate the AWCD from each plate at each reading time. For each plate, the time point where the AWCD is closest to 0.6

- is chosen. Alternatively, other AWCDs (e.g. 0.30, 1.00) may also be chosen.
- Normalise data by dividing each well OD by AWCD [8]. This is particularly important when inoculum densities are not standardised prior to inoculation.
- b) Kinetic approach: This requires continuous readings. It yields more information, but is more dependent on inoculum density than the approach using the AWCD normalised data [9]
3. Use discriminant analysis (DA) or principal component analysis (PCA) for exploratory data analysis, and MANOVA for statistical testing (applied on DA or PCA factors)
 4. From the data, functional diversity parameters may be deduced from single substrates or substrate groups (like carbohydrates, amino acids, carboxylic acids, etc.). Use discriminant analysis (DA) or principal component analysis (PCA) for exploratory data analysis, and MANOVA for statistical testing (applied on DA or PCA factors)
 5. Data analysis may be further elaborated by calculating the area under the curve for each well OD for the entire period of incubation [12], by the estimation of kinetic parameters [22] or by studying different dilution levels as outlined above.

Applications of the method, limitations and final remarks

Community level physiological profiling is a fast screening method to detect differences among treatments. Since first published, *in vitro* community level physiological profiles (CLPPs) have been used widely to characterise microbial communities of different habitats, ranging from sediments to seawater, and from oligotrophic groundwater to soils and composts [7, 10, 11, 19].

However, it is not possible to draw conclusions on cause-effect relationships because the utilisation of a certain substrate is not necessarily coupled to a change in that substrate's availability. Also, links to structural changes of a community may not be made, since changes in CLPPs can either be due to an adaptation of the prevalent microflora, or to a change in community composition. The researcher must always keep in mind that it is not the utilisation of single substrates, but

the change in the substrate utilisation pattern that is important. It also must be emphasized that CLPP is not a culture-independent method, but rather biases towards fast-growing, easily culturable species [26]. Thus, CLPP should not be seen as a stand-alone method, but it can be highly complementary to other approaches (e.g. classical and molecular) in the polyphasic analysis of microbial communities.

References

1. Bakken LR, Lindahl V (1995) Recovery of Bacterial Cells from Soil. In: Trevors JT, Van Elsas JD (eds) *Nuclei Acids in the Environment. Methods and Applications*. pp. 9–27. Springer-Verlag, Berlin.
2. Bloem J, Bolhuis PR, Veninga MR, Wieringa J (1995) Microscopic methods for counting bacteria and fungi in soil. In: Alef, K and Nannipieri P (eds) *Methods in Applied Soil Microbiology and Biochemistry*. pp. 162–191. Toronto: Academic Press.
3. Bochner B (1989) Instructions for the use of Biolog GP and GN microplates. Biolog Inc., Hayward, CA, USA.
4. Breure AM, Wind B, Bogte JJ, Rutgers M (1999) Development of an indicator for functional diversity of microbial communities. *Proceedings of the BAGECO Meeting, Florence, Italy*.
5. Campbell CD, Grayston SJ, Hirst D (1997) Use of rhizosphere C sources in sole C source tests to discriminate soil microbial communities. *J. Microbiol. Methods* 30, 33–41.
6. Garland JL (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. *FEMS Microbiol Ecol* 24: 289–300.
7. Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level-sole-carbon-source-utilization. *Appl Environ Microbiol* 57: 2351–2359.
8. Garland JL, Mills AL (1996) Patterns of potential C source utilization by rhizosphere communities. *Soil Biol Biochem* 28: 223–230.
9. Garland JL, Mills AL, Young JS (2001) Relative effectiveness of kinetic analysis vs. single point readings for classifying environmental samples based on community-level physiological profiles (CLPP). *Soil Biol Biochem* 33: 1059–1066.
10. Grayston SJ, Campbell CD (1995) Functional biodiversity of microbial communities in the rhizospheres of hybrid larch (*Larix eurolepis*) and Sitka spruce (*Picea sitchensis*). *Tree Physiol* 16: 1031–1038.
11. Grayston SJ, Wang S, Campbell CD, Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol Biochem* 30: 369–378.
12. Guckert JB, Carr GJ, Johnson TD, Hamm BG, Davidson DH, Kumagai Y (1997) Community analysis by Biolog: curve integration for statistical analysis of activated sludge microbial habitats. *J Microbiol Meth* 27: 183–197.
13. Haack SK, Garchow H, Klug MJ, Forney LJ (1995) Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilizations. *Appl Environ Microbiol* 61: 1458–1468.
14. Heuer H, Smalla K (1997) Evaluation of community-level catabolic profiling using BIOLOG GN microplates to study microbial community changes in potato phyllosphere. *J Microbiol Meth* 30: 49–61.
15. Hitzl W, Rangger A, Sharma S, Insam H (1997) Separation power of the 95 substrates of the Biolog system determined in various soils. *FEMS Microbiology Ecology* 22, 167–174.
16. Hooper D, Hawksworth D, Dhillon S (1996) Microbial diversity and Ecosystem Processes. In: VH. Heywood, RT Watson (eds) *Global Biodiversity Assessment*, Cambridge Univ. Press.

17. Hopkins DW, MacNaughton SJ, O'Donnell AG (1991) A dispersion and differential centrifugation technique for representatively sampling microorganisms from soil. *Soil Biol Biochem* 23: 217–225.
18. Insam H (1997) A new set of substrates proposed for community characterization in environmental samples In: Insam H, Rangger A (eds) *Microbial Community*. Springer-Verlag, Berlin. pp. 259–260.
19. Insam H, Amor K, Renner M, C Crepaz (1996) Changes in functional abilities of the microbial community during composting of manure. *Microb Ecol* 31: 77–87.
20. Insam H, Hitzl W (1999) Data evaluation of community level physiological profiles: A reply to the letter of PJA Howard. *Soil Biol Biochem* 31: 1198–1200.
21. Konopka A, Oliver L, Turco JR (1998) The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbiol Ecol* 35: 103–115.
22. Lindstrom JE, Barry RP, Braddock JF (1998) Microbial community analysis: a kinetic approach to constructing potential C source utilization patterns. *Soil Biol Biochem* 30: 231–239.
23. Mayr C, Winding A, Hendriksen, NB (1999) Community level physiological profile of soil bacteria unaffected by extraction method. *J Microbiol Meth* 36: 29–33.
24. Pankhurst CE, Ophel-Keller K, Doube BM, Gupta VVSR (1996) Biodiversity of soil microbial communities in agricultural systems. *Biodiv Conserv* 5: 197–209.
25. Shannon, PO, Weaver W (1969) *The mathematical theory of communication—Fourth Edition*. The University of Illinois Press, Urbana, IL.
26. Smalla K, Wachtendorf U, Heuer H, Liu W, Forney L (1998) Analysis of Biolog GN substrate utilization patterns by microbial communities. *Appl Environ Microbiol* 64: 1220–1225.
27. Zak JC, Willig MR, Moorhead DL, Wildman HG (1995) Functional diversity of microbial communities: a quantitative approach. *Soil Biol Biochem* 26: 4404–4408.

<http://www.springer.com/978-1-4020-2176-3>

Molecular Microbial Ecology Manual

Kowalchuk, G.A.; de Bruijn, F.; Head, I.M.; Van der Zijpp, A.J.; van Elsas, J.D. (Eds.)

2004, XVI, 1778 p. In 2 volumes, not available separately., Hardcover

ISBN: 978-1-4020-2176-3