

METABOLIC FINGER PRINTS OF SOIL BACTERIA: INFLUENCE OF ORGANIC AND INORGANIC AMENDMENTS

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ABSTRACT

The effect of organic and inorganic manure amendments on the metabolic finger prints of bacteria from soil samples taken from 0-20 cm depth of a humid tropical agricultural soil (University of Nigeria, Nsukka research farm) was assessed. The manures (rice straw, (RW), poultry manure (PM) and fertilizer N. P.K. (12: 12: 17) were incorporated into the soil in the field and samples were collected after three, six, nine and twelve months for Biolog ecoplate analysis in the laboratory. Data of average well color density (AWCD) from the ecoplates at twelve hourly readings were subjected to multivariate analysis of variance (MANOVA) after normalization with the Garland-mills scaling. Results indicated that the combined application of RW+ NPK increased the type and number of microorganisms in the soil by increasing the community of generalist for the carbon source utilization. The principal component analysis (PCA) of F1 and F2 using the standardized AWCD values indicated a shift from generalist to specialist C-source utilization with increased incubation period. There was a temporal and spatial difference in C availability and utilization pattern of C in the unamended or amended ecoplate samples.

Keywords: Organic and inorganic manures, Metabolic finger prints, AWCD, C- source utilization, PCA.

INTRODUCTION

Microbial communities have great potential for temporal or spatial changes. These changes in microbial community structure affect ecosystem processes such as nutrient recycling, decomposition, and growth of pathogens and release of genetically engineered microorganisms. Several methods employed in describing microbial communities are unpopular because they are either time-consuming or biased due to their reliance on cultural procedures. Methods like the phospholipid fatty acids (PFLA), DNA and RNA eliminate the bias associated with culturing microorganisms but are also time consuming. A comparatively fast method of microbial community characterization that relies on metabolic traits, is the community level physiological profiling (CLPP), (Garland *et al.* 1997).

The CLPP is a functional index for microorganisms based on the fact that it measures metabolic traits, but it is disputed as an indicator of an *in situ* carbon source utilization because of the fact that colour development in CLPP wells may not be a function of the proportion of organisms in the community able to utilize the specific carbon source (because of the differential growth rate among organisms). The lack of a direct link-

age between growth and tetrazolium dye reduction reported by Winding and Hendrickson (1997), as well as cross feeding among organisms within a single well substantiated this view.

However, Garland *et al.* (1997), strongly contested this view and explained that changes in phenotypic potential may be the result of selective forces such as difference in carbon sources, pH sensitivity, resistance to predation, which are not directly measured in CLPP, thus a shift in profile may be structurally relevant but functionally misleading. Besides, Lehman *et al.* (1995), observed that the limited numbers of C-sources (that is amino acids and carboxylic acids) utilized by subsurface communities were the same types used by surface microbial isolates, suggesting a link between CLPP and functional potential of the community. Also the use of a more diverse or unusual set of carbon sources allows for a more functionally relevant characterization of microbial community (Garland, 1997).

Therefore, in this study, the effect of different sources of nutrient carbon on the functional ability of soil bacteria was investigated. The aim was to ascertain the extent to which sole carbon sources in Biolog ecoplates can indicate structurally and functionally relevant nutrients in the soil and on the long run determine the

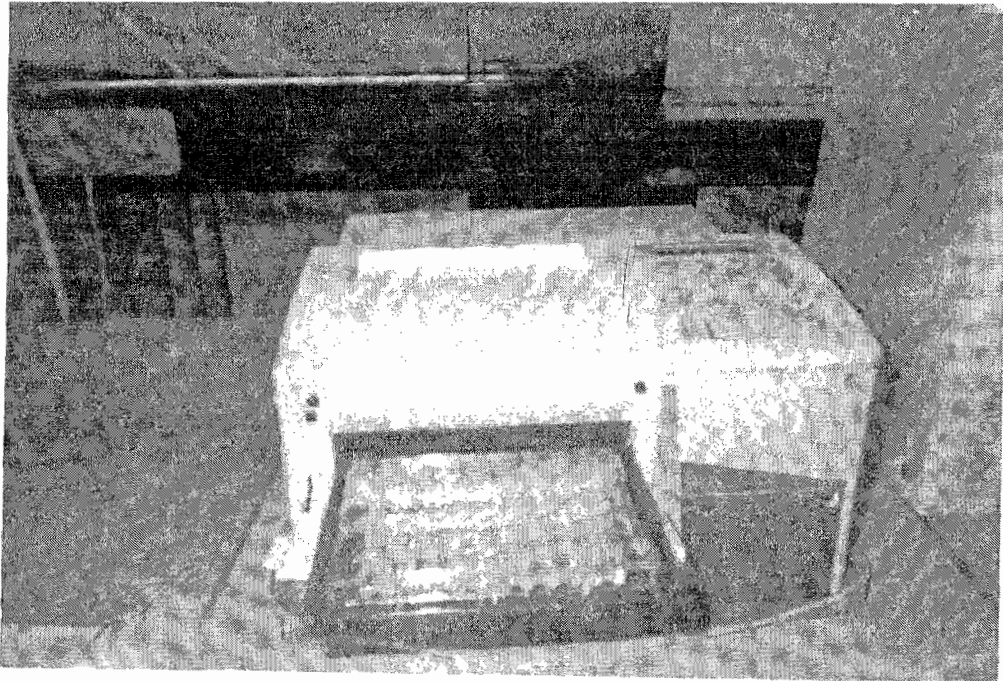


Fig. 1 Biolog ecoplate in microplate reader.

organic or inorganic nutrients or their combination that will be decomposed relatively fast enough to release carbon and other mineral nutrients needed for microbial and plant growth.

MATERIALS AND METHODS

Field sampling

Soil samples for Biolog ecoplate analysis were collected from 0-20 cm depth of a field experiment laid out in a randomized complete Block Design (RCBD). The soil classified as Typic Kandistult was treated with organic and inorganic amendments at the rates of 10 t/ha for the organic and 0.48t/ha for the inorganic amendments. The organic amendments applied were rice-mill wastes (RW), poultry manure (PM), a mixture of both PM and RW (PM + RW), while the inorganic amendment was NPK (12: 12: 17) fertilizer alone, and a mixture of NPK + RW. Each treatment was replicated three times and samples collected for three, six, nine and twelve months respectively.

Laboratory methods

Biolog ecoplate

The biolog ecoplate comprising 96 wells employs only 31 substrates and a control each replicated three times (Fig. 1). The ecoplates can be used to describe and distinguish different microbial communities and ensures sound statistical analysis (Hitzl et al., 1997). The substrate in each of the biolog ecoplate wells contains specific C sources, nutrient salts, a small amount of peptone and a redox dye-tetrazolium violet. When a respiring cell oxidizes the carbon source in the well, NADH (Nicotina-

mid Adenine Dinucleotide Hydrogenase) is formed and it donates electrons to the electron transport chain. The redox dye taps the electrons from the flow, converting the tetrazolium to a highly coloured formazan (Bochner, 1989). Thus the redox dye tetrazolium violet is reduced during the respiratory activity of bacteria producing a violet colour in the ecoplate well.

Preparation of microbial cell suspension for biolog ecoplates

The extraction of bacterial cells from the soil samples was by the method of Hopkins et al. (1991), with some modifications. Five grammes soil fresh weight was added into a centrifuge tube containing 8.5 g Dowex and thirty glass beads and blended mechanically with 20 ml of 0.1% w/v sodium cholate solution. The tube's content was then mechanically shaken for 2 hrs at 4°C and then centrifuged at 500g (2200 rpm) for 2 min at 5°C after which the pellets was resuspended in 10 ml Tris buffer (pH 7.4) and mechanically shaken for 1 hr at 4°C before centrifuging at 500g for 1 min. To obtain a clearer suspension, high-density centrifugation with Nycodenz was carried out. A solution containing 0.75 ml of Nycodenz was transferred with pipettes into the bottom of centrifuge vials about 10 to 15 ml capacity with the supernatant solution of the preceding centrifugation and centrifuged for 1 hr at 9000 rpm. The clear suspension was then diluted with Ringer solution.

Bacterial cell density and inoculation of biolog ecoplates

To obtain meaningful comparisons between communities,

Table 1: Summarised manova of the significant effects of strawy manure (RW) and fertilizer NPK alone and in combination and time of sampling on metabolic finger prints of bacteria in a cultivated soil.

Factor	Ecoplate substrate	Level of significance
RW	L- Serine	*
NPK	L-Asparagine	*
	4- Hydroxy benzoic acid	*
	L-Serine	*
	D-Glucosaminic acid	***
	α -Keto butyric acid	**
	D- Malic acid	**
RW+NPK	Tween 40	*
	l- Erythritol	*
	Glycogene	*
	D- Glucosaminic acid	*
	Glucose- l- phosphate	*
Time	D-L- α - Glycerol phosphate	*
	β -Methyl-D- glucoside	***
	L- Arginine	***
	Pyruvic acid methyl ester	*
	D- xylose	*
	Tween 40	***
	l- Erythritol	*
	L- serine	*
	N- acetyl-D-glucosamine	**
	α -D-lactose	*
	D-Glucosaminic acid	***
	L- phenylalanine	**
	Tween80	*
	G-hydrobuteric acid	**
	Glucose-l-phosphate	***
	phenylethylamine	***
	D-cellobiose	***
	D-L- α -glycerol phosphate	**
	L-phenylalanine	**

similar inoculation density was a prerequisite and was estimated by quantifying the number of bacteria in the sample through acridine orange direct count (AODC) as described by Binnerup et al. (1993). One milliliter of the diluted extract was collected into a 0.2 µm bluish poly-carbonate film (poretics) through a luer tip syringe. The filter was then washed with 2 ml particle-free water and placed with the bacteria containing side upwards on the surface of a 0.01% acridine orange solution for 3 min. The filter was subsequently washed in particle free water for some seconds to remove excess colour. Excess water droplets were carefully removed from the backside of the filter before it was placed on a microscopic slide. Water retained on the surface and within the filter was allowed to evaporate before it was covered with paraffin oil and a cover slip. The total number of bacteria was counted by fluorescence microscopy and the samples, were then diluted accordingly using the equation:

$$B = N * F * D / M * V \quad \dots\dots\dots (1)$$

where,

- B = number of bacteria in 1 ml
- N = Number of bacteria counted
- F = diameter of filter (78.5 mm)
- D = dilution factor (depending on N)
- M = microscopic field area (0.01mm²)
- V = volume of diluted solution passed through filter (100 ml)

An estimated bacterial cell density of 2×10^5 to 2×10^7 cells for each well of the Biolog ecoplates was used for inoculation. Portions of the samples (with estimated bacterial cell density) were pipetted into the Biolog ecoplates with a multipipette. Approximately 12.5 ml of the samples was required to inoculate one plate with a capacity of 0.13 ml per well of the estimated cell density. The inoculated plates were incubated at 21°C (corresponding to average temperature during the growth season) for 150 hrs. An initial scan or absorbance reading was performed immediately after inoculation, after which twelve hourly readings with a micro plate reader (Fig. 1) was carried out for one week.

Data analysis

The Biolog plate analysis of the community level physiological profile (CLPP) essentially measures the overall rate of colour development. This is estimated by the rate of average well colour density (AWCD), which is a function of inoculum density (Garland, 1996). Though Garland (1997), also observed that the differences in the overall rate of colour development among samples (i.e. inoculum density) could produce variations in the diversity or pattern of colour development independent of the change in the type of organisms present. However, in this study, the normalization of inoculum density was achieved through acridine orange staining and cell count (which ensured standard inoculum density) and a 12 hourly incubation reading which ensured equivalent incubation time). Also the variation in the overall extent

of colour development among samples was accounted for by the Garland-Mills scaling which normalized the data of the AWCD prior to multivariate analysis using the equation of Garland and Mills as follows (Howard 1997):

$$AWCD = \sum (R - C) / 31 \quad \dots\dots\dots (2)$$

$$\text{Garland-Mills scaling} = R - C / AWCD \quad \dots\dots (3)$$

where,

- C = Amount of colour developed in control cell
- R = the value for an experimental cell

This relationship between inoculum density (R-C) and AWCD indicates that differences in the multivariate pattern of colour response among samples, determined by principal component analysis (PCA), are strongly influenced by the relative abundance of bacteria in the samples. The scaling of data ensures that samples of equivalent AWCD are used in the MANOVA. This allows for effects caused by differences in inoculum density and effects due to differences in the type of activities of organism to be assessed.

RESULTS AND DISCUSSION

More substrates of the ecoplates were greatly influenced by fertilizer NPK compared to the strawy RW alone. As shown from MANOVA summarized in Table 1, the addition of fertilizer NPK in combination with RW enhanced the substrate utilization pattern of containing wells by microorganism. This observation indicate that the application of RW + NPK directly increased the type and number of microorganisms in the soil.

The community level physiological profile assessed with the ecoplate samples essentially measures the overall rate of colour development, which is a function of inoculum density. The type of organism present in the sample also would influence the rate of average well colour density (AWCD) on a per cell basis because communities comprising of a greater percentage of generalist for C source utilization would have faster rates of AWCD relative to communities of greater percentage of specialist for C source due to the utilization of ecoplate substrates on selective basis. This view is supported by the observation of Haack et al. (1995), that some bacteria produced no response in Biolog plates because the cells failed to maintain viability or because they do not use the substrate.

The community profile pattern visualized by the PCA F1 and F2 of the standardized AWCD values of the different ecoplate substrates indicate that the effects of community-generalists for carbon source utilization was prominent at early stage of the organic amendment application (Fig. 2) as all the amendments induced a C utilization pattern that clustered within -4 and 4 of F1 and F2 axes. With increased incubation period there was specialization in this pattern as indicated in F2, 3, and 4 where ecoplate samples amended with RW+ NPK, RW and unamended control, respectively encouraged differ-

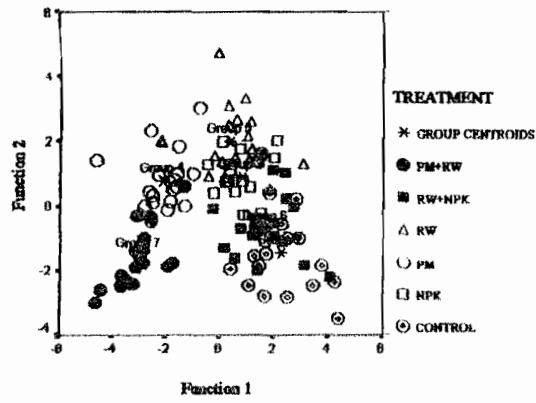


Fig 2: Grouped effect of cultivation on substrate utilization pattern of microbes after three months.

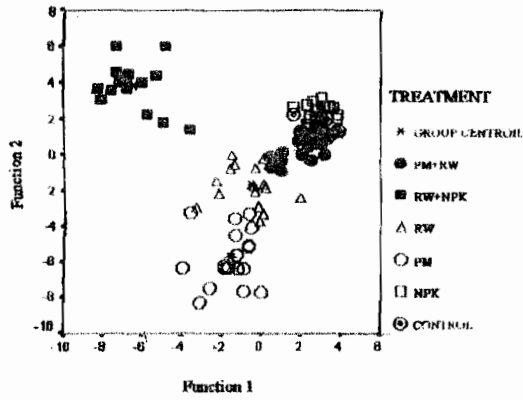


Fig. 3: Grouped effect of cultivation on substrate utilization pattern of microbes after six months.

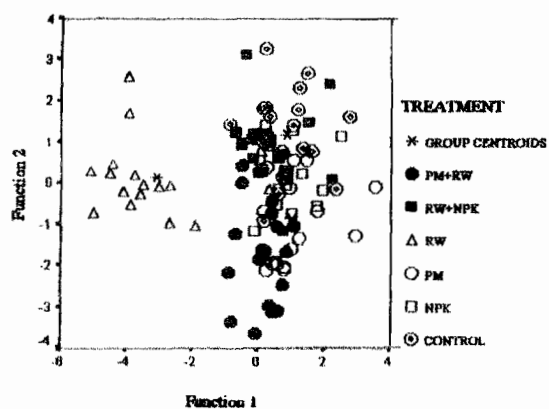


Fig. 4 : Grouped effect of cultivation on substrate utilization pattern of microbes after nine months.

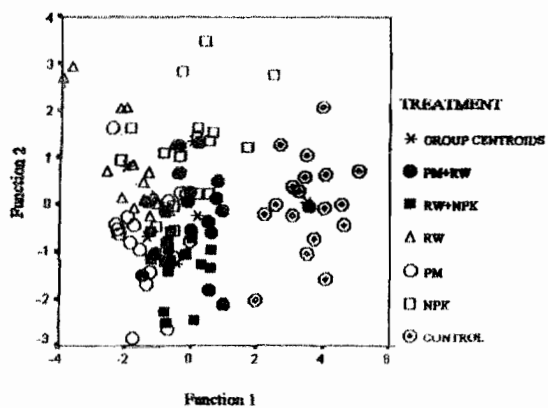


Fig. 5 : Grouped effect of cultivation on substrate utilization pattern of microbes after twelve months.

ent community activity indicating a prominence of specialist C source utilization.

At six months, the amendment of ecoplate samples with RW+NPK produced metabolic pattern clearly different from all other amendments as defined by F1 and F2, falling within the axis - 6 and 8 respectively (Fig. 3). This suggest that the RW+ NPK reached a peak of C release and availability to microorganisms after six months of incorporation into the soil while the other amendments (RW, PM, and PM+ RW) were of similar profile pattern falling mainly along the opposite axes of F1. The strawy RW alone also showed a different pattern of C utilization at nine months (Fig. 4). This difference in time of peak C utilization of RW+ NPK and RW, suggest that the addition of mineral nitrogen to strawy amendment greatly enhanced its decomposition such that availability of carbon which strongly influence the activity of microorganisms invariably result in the enrichment of soil nutrients. This observation is supported by the reports of Bossio and Scow (1995) who reported significant treatment effect of field incorporation of rice straw on metabolic diversity of microbial community measured by Biolog analysis. They observed significant effect irrespective of time of incubation and date of sampling and stressed that the Biolog result was more sensitive to carbon input compared to microbial biomass carbon analysis where probably several years of carbon input is needed to change soil microbial biomass.

Therefore, the benefit from the addition of strawy manure mixed with a nitrogen source should be harnessed after six months of incorporation and incubation, while strawy manure applied alone require a longer time of incubation to avoid loss and waste of agricultural input. Also the admixture of strawy manure with nitrogen source should be carried out with caution, as the admixture of strawy manure with organic nitrogen source (PM+ RW) was at all sampling times in this study insensitive to carbon availability in the soil and not reflected in the C utilization pattern of soil microbial community in the ecoplate, unlike the admixture of RW+ NPK.

Generally, there was a clearer difference in the C utilization pattern of the amended ecoplate samples compared to the unamended control (Fig. 5). Thus indicating firstly, a difference in the microbial community response to the original ecoplate substrates and that due to organic and inorganic amendments. Secondly, there was a temporal and spatial difference in C availability in the RW, PM, NPK, PM+ RW and RW+ NPK amended soil compared to the control even a year later and the metabolic finger print of bacteria on substrates containing mineral and inorganic amendment differed from that of organic amendments contained in ecoplate substrate wells.

CONCLUSION

Organic carbon is linked to the availability of other soil nutrients needed for plant growth, and influences the decomposition rate of most applied manures, hence the activity of microorganism. Therefore, from this study it

can be inferred that the combination of organic and mineral fertilizer, would give better performance in agricultural productivity compared to organic amendment in combination or either used alone. Also a longer time of incubation rather than one week should be allowed before seed planting to allow maximum mineralization of nutrients and its availability at the most crucial point of plant growth.

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