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EVALUATION OF SOIL MICROBIAL ACTIVITY AFTER APPLICATION OF *PAENIBACILLUS POLYMYXA*-BASED BIOFERTILIZER IN WHEAT FIELD EXPERIMENT

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Abstract

The establishment of eco-friendly principles in agriculture requires, from one side, a reduced dependence on chemical fertilizers, pesticides and herbicides, and on the other, a maintenance of consistent yield and high plant productivity. Such substantial shift in the contemporary agricultural practices can be achieved only if a wide variety of alternative options is available. Furthermore, in order to become part of agricultural practices these options have to be affordable in a broad scale and effective against the loss of biodiversity and reduced soil fertility. The current study presents data about the metabolic activity and microbial community` structure in two soil samples – one treated with chemical fertilizer and another treated with chemical fertilizer reduced by 25% dose and supplemented with commercially available biofertilizer. Metabolic activity and properties of microbial soil communities were assessed by the BIOLOG® EcoPlate technique. The estimated parameters included average well-color development (AWCD) and utilisation of six guilds of substrates. Several functional indexes that are used for evaluation of community diversity and evenness were also estimated. The AWCD, expressed either as total activity or as guilds utilisation during the EcoPlate incubation period of seven days did not reveal significant difference between variants. However, most of the estimated functional indexes consistently indicated higher microbial diversity and more balanced structure of microbial community in the biofertilizer-supplemented variant. The results clearly implied that the biofertilizer based on nitrogen-fixing bacteria can positively influence the structure of soil communities and its application could be beneficial for soil fertility and plant productivity.

Keywords: diazotroph, biofertilizer, BIOLOG® EcoPlate, functional indexes, metabolic activity, microbial communities

INTRODUCTION

Biofertilizers contain selected strains of bacteria and fungi which can affect plant growth and productivity by influencing processes in the soil and in the plant (Kumar et al., 2021). The use of biofertilizers is considered an eco-friendly approach, which complies with the aims set in the "farm to fork" strategy of the EU for 20% reduction of mineral fertilisers and 50% of pesticide until 2050 (Kurniawati et al., 2023). The biofertilizers usually contain either a single strain or a combination of strains and species which belong primarily to the genera *Pseudomonas*, *Azospirillum* and *Bacillus* (Zhao et al., 2024) and are applied in the soil, on the leaves or by seeds imbibition (Nosheen et al., 2021). The positive effects of biofertilizers application is towards nutrients supply, microbial activity, changes in the ratio between different microorganisms, organic matter turnover, plant stress alleviation, control of pathogens (Vessey, 2003, Bargaz et al., 2018, Kumar et al., 2022). The biological activity of selected bacteria which are used as biofertilizers constituents usually includes one or more traits

such as: nitrogen fixation, phosphorus solubilisation and mobilisation, potassium solubilisation and mobilisation, micronutrients supply and plant-growth promoting properties (Saha et al., 2023). Due to complexity of interactions between plants and their intrinsic microorganisms, the underlying mechanisms are not revealed yet and are one of the most active area of contemporary research (Ramakrishna et al., 2019). The properties of selected species and strains assessed in laboratory conditions will govern the effectiveness of biofertilizers a priory, but the combined use of biofertilizers and mineral fertilisers in field conditions will require additional information. It will be used for establishing the optimal ratio between participating components, for practical recommendation about the time and method for their combined application and for assessment of effects on soil microbiota and plants productivity (Bargaz et al., 2018).

The species *Penibacillus polymyxa* (formerly *Bacillus polymyxa*) possess a variety of properties that justified its inclusion in the group of plant-growth promoting rhizobacteria (Padda et al., 2017a, Daud et al., 2019). The bacterium is considered a promising alternative to chemical fertilizers and pesticides and is part of the contemporary eco-friendly approach for sustainable agriculture (Govindasamy et al., 2010, Padda et al., 2017b). The strains which are considered as beneficial for agriculture usually exhibit antimicrobial activity, nitrogen fixation and phosphate solubilisation abilities, soil porosity enhancing properties, plant hormone production potential, or capacity for lignocellulose degradation (Lebuhn et al., 1997, Timmusk et al., 1999). However, the isolated and characterized strains usually showed one or several of abovementioned activities but not all of them (Weselowski et al., 2016).

Timmusk et al. (2005) summarized that *Paenibacillus polymyxa* has been isolated from rhizosphere of a variety of crops such as wheat and barley, white clover, perennial ryegrass, crested wheatgrass, lodgepole pine, Douglas fir, green bean and garlic. Currently, *Paenibacillus* is considered as an important constituent of the rhizosphere of staple crops such as wheat (Bezzate et al., 2000) and maize (Von der Weid et al., 2000), but it has been isolated also from peanut (Haggag, 2007) and pepper (Phi et al., 2010). Some authors considered *P. polymyxa* as part of root rhizosphere rather than the endophyte (Bent & Chanway, 2002, da Mota et l., 2002) while the others stressed on its close contact with roots or its presence in the endorhizosphere (Timmusk et al., 2009). Several years ago, *P. polymyxa* colonization in the roots of wheat, maize and cucumber has been visualised by a confocal laser scanning microscope (Hao & Chen, 2017). Bezzate et al. (2000) succeeded to prove that levan (a polysaccharide) synthesised by *P. polymyxa* was responsible for its better adherence to soil and plant roots. Due to *P. polymyxa* characteristic, the application of biofertilizer which contains selected stain *P. polymyxa* can be expected to affect soil fertility, microbial activity and plant productivity.

The objective of this study was to compare metabolic activity and structure of microbial community in soil samples either treated with chemical fertilizer or with reduced by 25% dose accompanied with diazotroph-based biofertilizer.

MATERIALS AND METHODS

The field experiment was conducted in the Experimental Field of the Agricultural University in Plovdiv (AUP), Bulgaria with the wheat variety KWS LAZULI using a randomized complete block design with four replicates/plots with size of 18 m^2 (12.5×1.44) per variant (Table 1). The control treatment was fertilized with nitrogen at dose of 160 kg N ha^{-1} and had the abbreviation 100 % N or control. The other treatment (75% N+BF) was fertilized with reduced by 25% dose of chemical fertilizer with addition of commercially available

biofertilizer – Azofix*Plus* (Bioenergy LT, Italy). The biofertilizer contains strain *Paenibacillus polymyxa* MVY-024. The complete presentation of experiment and the data related to soil parameters, plant physiology and productivity will be published elsewhere and the discussion herein is focused on the soil communities` comparison.

Table 1. Fertilization plan during wheat phenological stages

Treatments abbreviations	N fertilization, kg N ha^{-1}			
	Before sowing	**Germination**	**Early vegetation (27th of February)**	**Middle vegetation (7th of April)**
100 % N (control)	30	-	50	80
75% N+BF	30	*biofertilizer*	50	40

Metabolic activity of microbial soil communities

Metabolic activity of soil communities was assessed using EcoPlates™ of BIOLOG (Biolog Inc., USA). Each EcoPlate is comprised of 31 different substrates organized in the following guilds - carbohydrates (ten components), carboxylic acids (seven components), polymers (four components), amino acids (six components), amines (two components) and phenolic compounds (two components). Each EcoPlate contains three sets of guilds.

One gram of carefully brushed from the plant roots rhizosphere soil was suspended in 9 ml sterile distilled water thoroughly mixed and left to settle for 5 min. The inoculation of Biolog® EcoPlates was done with 150 µl of 10^{-3} dilution and plates were incubated at 25±1°C. The plates were read spectrophotometrically immediately after inoculation and consequently at 24 hour intervals for 7 days (168h) with the MicroStation™ Reader provided by the BIOLOG® System. The calculations for average well-color development (AWCD) and separately for each guilds were based on the optical density (OD) measured at 590 nm and 750 nm according to the procedure described by Sofo & Ricciuti (2019) except the formula for AWCD which was according to Huang et al. (2012) as follows:

$$\mathrm{AWCD} = \sum(C_i - R_i)/31,$$

where R is the control well (water) and C_i is the value of each substrate well. The data normalisation was done by subtracting each measurement from the corresponding OD at 24th hour in order to remove the background noise according to Urakawa et al. (2013). The negative values obtained at any stage of data normalisation was set to zero (Garland, 1996).

Functional indexes

Only the wells with an OD ≥0.250 were taken as a positive response for substrates utilisation according to Sofo & Ricciuti (2019) and were used in indexes calculation. The corresponding formulas and used source materials are listed in Table 2.

Data analysis

Average values of AWCD expressed as total activity and as per substrates guilds, and graph visualization was calculated with Microsoft Excel considering the three sets of substrates in each EcoPlate as replicates (n=3). Functional indexes were compared applying independent t-test with equal variances assumed by SPSS program (IBM, ver. 26). The heatmap of optical density values measured on the 120th hour of EcoPlate incubation of two variants was created with Heatmapper according to Babicki et al. (2016), http://www.heatmapper.ca/.

Table 2. Formulas for functional indexes calculation

Functional index	***Formula***	***References***
Shannon-Weiner diversity index, H'	$H' = -\sum p_i \times (ln\, p_i)$ *where p_i is C_i, divided by the sum of C_i, values ≥ 0.250*	*Jurkšienė et al. (2020)*
Pielou index, E	$E = \frac{H'}{ln\, S}$ *where H' is Shannon-Weiner diversity index and **S** – number of values ≥ 0.250*	*Pielou (1966), Jurkšienė et al. (2020)*
Simpson diversity index, D	$D = 1 - \sum P_i^2$ *where p_i is C_i, divided by the sum of C_i, values ≥ 0.250*	*Chen et al. (2020)*
Mardalef diversity index, d	$d = \frac{(S-1)}{lnN}$ *where S – number of wells ≥ 0.250 N – number of substates i.e. 31*	*Türkmen & Kazanci (2010)*
McIntosh diversity index, U	$U = \sqrt{\sum P_i^2}$ *where pi is Ci, divided by the sum of Ci values ≥ 0.250*	*McIntosh (1967) Huang et al. (2012)*
McIntosh evenness, McI	$McI = N - U/N - (N/\sqrt{S})$ *where U – McIntosh diversity index, N – sum of values ≥ 0.250, S – number of substrates i.e. 31*	*Xu et al. (2015)*
Gini coefficient, G	$G = \frac{\sum_{i=1}^{N}\sum_{j=1}^{N}\lvert x_i - x_j\rvert}{2N^2\bar{x}}$ *where x_i and x_j represent each pair of OD readings, $\bar{x}$ is AWCD, N- number of substrates. The final value was further multiplied n/(n-1) to give unbiased estimates of the true population G.*	*Weiner and Solbrig (1984), Harch et al., (1997)*

RESULTS AND DISCUSSION

The current study aimed to explore the differences in soil microbial activity and changes in the structure of microbial community triggered by biofertilizer application. The comparison between the two variants – one treated with chemical fertilizer and another treated with reduced by 25% dose accompanied with diazotroph-based biofertilizer did not reveal any significant difference in the overall metabolic activity of soil microbial communities (Figure 1).

The average well-color development (AWCD) (Figure 1) showed a short lag phase during the first 24 hours of EcoPlate incubation. After that, until 120th hour, there was a relatively proportional increase of approximately 0.2 units of OD per each reading interval. After 120th hour until the end of the incubation period the curve was almost flat. These results are in agreement with Ge at al. (2018) who observed a lag phase at the beginning of EcoPlate incubation and a gradual increase in the metabolic activity. In general, the changes in the OD have to be assigned predominantly to bacterial utilisation of substrates since the length of incubation period is not sufficient for fungi development. The reason for this is related to prevention of mycelium and fungal fruiting bodies formation which ultimately will block the wells surface and thus the OD readings will be incorrect. As a result, the contribution of fungi to OD change in the EcoPlate is actually ignored. Usually, if there are no inhibitory factors that could suppress the growth and

metabolic activity of bacteria the curve of AWCD keeps a typical sigmoid shape (Stefanowicz, 2006, Lima et al., 2015). However, either depending on the analysed samples and the participating species a longer lag phase (Koner et al., 2021) or specific differences in the trend can also be observed (Li et al., 2012).

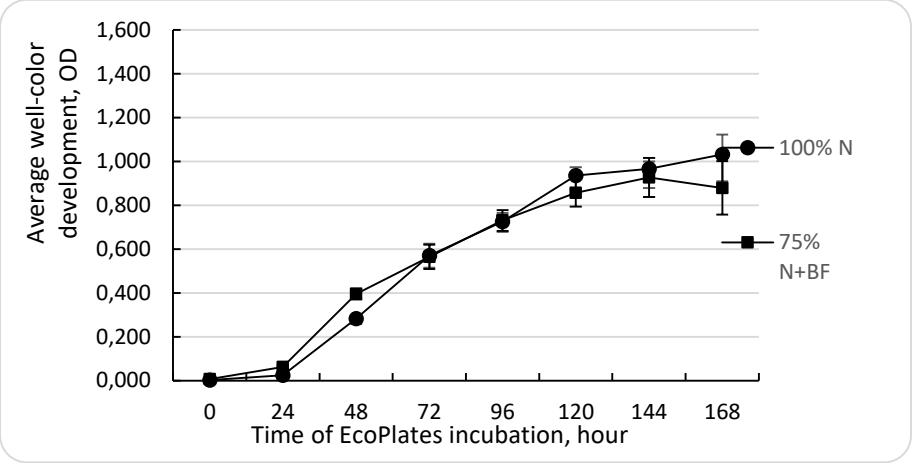

Figure 1. Microbial metabolic activity in the Biolog® EcoPlate

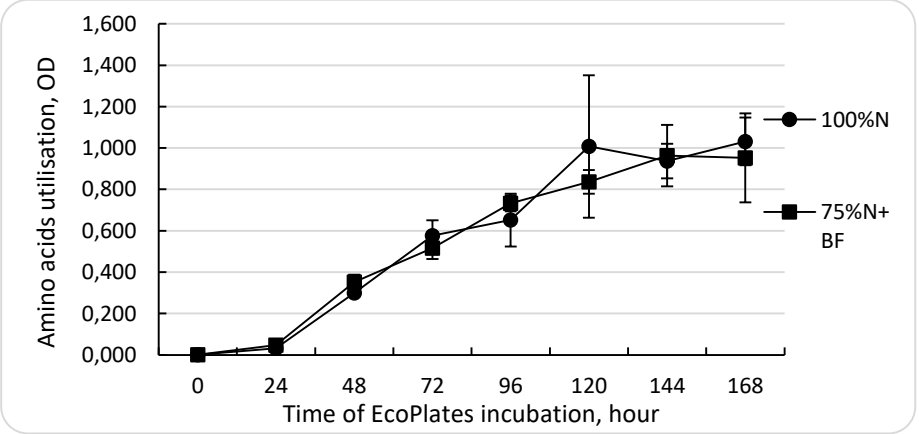

Figure 2. Dynamics of amino acids utilisation in the Biolog EcoPlate

The Biolog EcoPlate substrates are organized in three sets that can be summarized in six guilds (amino acids, carbohydrates, carboxylic acids, amines, polymers, and phenolic compounds) and calculation of AWCD separately per each guild provided a more detailed information about utilisation of specific compounds.

The utilisation of amino acids (Figure 2) did not show difference between two variants. Gomez at al. (2000) who study used the Biolog GN microplates reported that 35 % (seven of twenty amino acids) present in the microplate were not utilized. Furthermore, the authors did not observe any difference of microbial communities in the samples which was taken from four locations with different native vegetation, cultivated crop and management system. In the current study the analyses soil samples shared a relatively high similarity in many aspects and this can explain the lack of significant difference between amino acids utilisation of microbial communities in the variants. However, it has been noticed that during the first hours of incubation (from 24 to 96 hour) the utilisation of L-phenylalanine, L-threonine and glycyl-L-glutamic acid was relatively low in comparison to other amino acids in the EcoPlates but after that they also were metabolised in both variants.

The utilisation of amines, after 96th hour, was more active for the variant that was supplemented with biofertilizer and the difference remained until 144th hour (Figure 3). However, due to considerable range of standard deviations the difference between variant was not proven statistically. It has been observed that utilisation of amines included in the Biolog EcoPlate is relatively low (Gomez et al., 2000). However, the mean values for amines utilisation in the current study reached – 0.774 ± 0.190 (168th hour) and 0.783 ± 0.447 (144th hour) for variant 100% N and 75% N+BF, respectively. Ge et al. (2018) considered amines and amides as the least preferable substrates for microbial utilisation and reported similar to the current study values for optical density 0.600 – 0.800 with some exceptions in which the OD reached higher value (1.00).

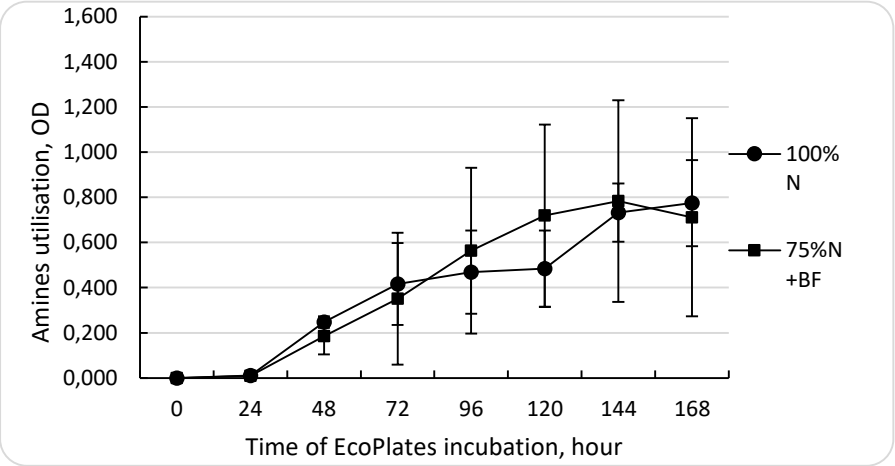

Figure 3. Dynamics of amines utilisation in the Biolog EcoPlate

The utilisation of carboxylic acid was similar for both variants until 48th hour (Figure 4). After that the variant supplied with biofertilizer showed a relatively low utilisation in comparison to the control. According to Cai et al. (2010) the guild of carboxylic acids in the EcoPlate is the most representative one in regards to the microbial beneficial effects on plant` health. The authors linked a higher carboxylic acids utilisation of microbial communities with the lower level of damages on the trees which were objects of the study. Some studies indicated that organic acids secreted by roots could play an important role in bacterial root colonization. Ling et al. (2011) conducted an *in vitro* experiment and found that some intermediate products of the tricarboxylic acid cycle could increase the population of *P. polymyxa* SQR-21 in the rhizosphere. Additionally, the field conditions accommodate a much more complex plant-bacterial interactions and they could have an impact on utilisation of selected carboxylic acids provided in the EcoPlate.

In the current study, the higher utilisation of carboxylic acids was observed in the sample which is supposed to have sufficient supply of nitrogen. Contradictory to the observed trend was the study of Obernosterer et al. (1999) who reported a high utilisation of carboxylic acids in seawater samples of the Mediterranean Sea due to a low availability of nitrogen. Additionally, the authors suggested that in some ecological niche carboxylic acids, as part of organic matter, serve as important carbon source for microorganisms (Obernosterer et al. 1999). It is highly possible that not only availability of nitrogen but also some other organic components and the principal difference of constituents in soil and water samples can significantly affect microbial metabolism and the observed trends of substrates utilisation.

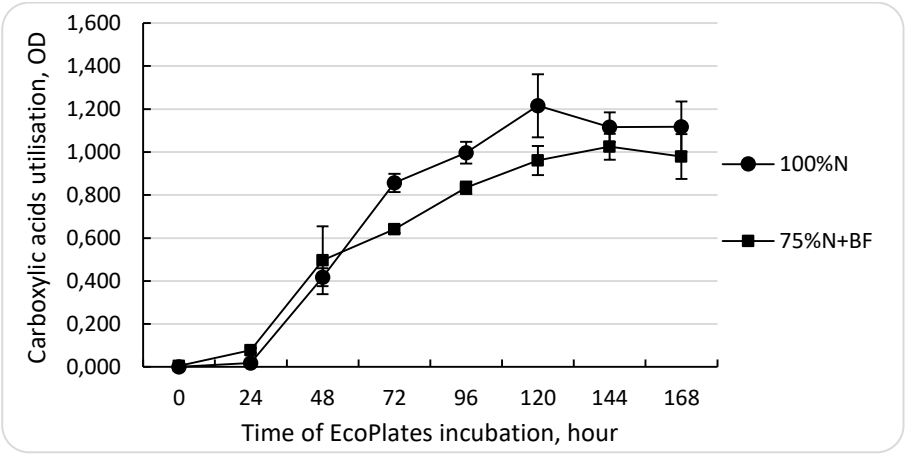

Figure 4. Dynamics of carboxylic acids utilisation in the Biolog EcoPlate

Up to 120th hour of incubation, the carbohydrates were more intensively utilised by the biofertilizer-supplemented variant (Figure 5). Von der Weid et al. (2000) studied phenotypic properties of sixty-seven stains of *P. polymyxa* and the type of substrates that they can utilise. The authors reported that 67% of strains produced acid from glycerol and 11 strains (16%) showed production of acid from rhamnose. Furthermore, 60% of the isolates were able to utilise glycerol, xylose and arabinose but not rhamnose. Utilisation of carbohydrates in *P. polymyxa* was associated with its ability to synthesize exopolysaccharides that participate in bacterial adhesion to root and soil particles (Bezzate et al., 2000). The ability *P. polymyxa* to metabolise sorbitol was estimated as a positive adaptation towards establishment of the rhizobacteria in the vicinity of wheat roots (Mavingui et al., 1992). Yegorenkova et al. (2012) also stressed on *P. polymyxa* exoglycan as important intermediary for bacterial-plant root interaction. It could be hypothesised that the biofertilizer strain could affect utilisation of naturally present carbohydrates in the soil and thus to be responsible for higher utilisation of substrates provided in the EcoPlate. Li et al. (2012) considered that carbohydrates and carboxylic acids were the main C sources utilised by the soil microbial community in the experiment with legumes.

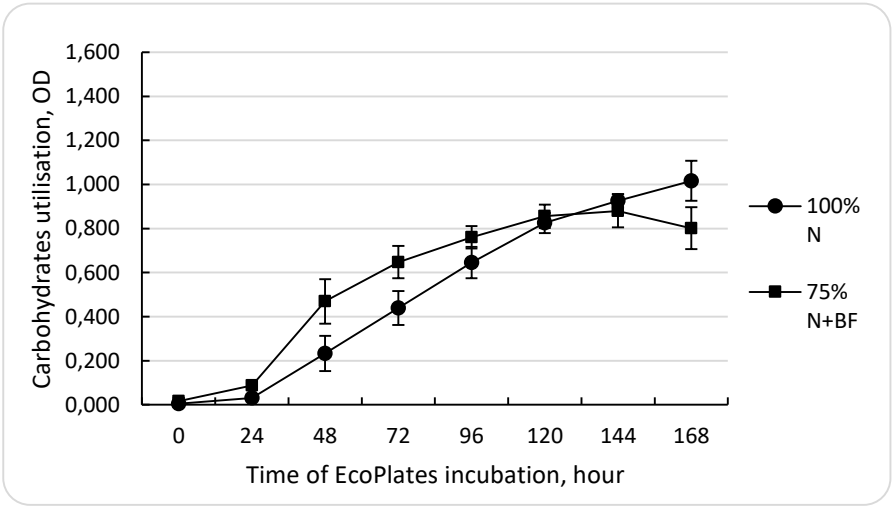

Figure 5. Dynamics of carbohydrates utilisation in the Biolog EcoPlate

The utilisation of polymers in both variants was very similar and uniform during the incubation period (Figure 6). Pessi et al. (2012) who studied soil microbial communities from Wanda Glacier in Antarctic Peninsula, hypothesised that the high utilisation of Tween 40 and Tween 80 provides adaptation at low temperatures, and their accumulation could play role in cell protection against freeze-and-thaw damages.

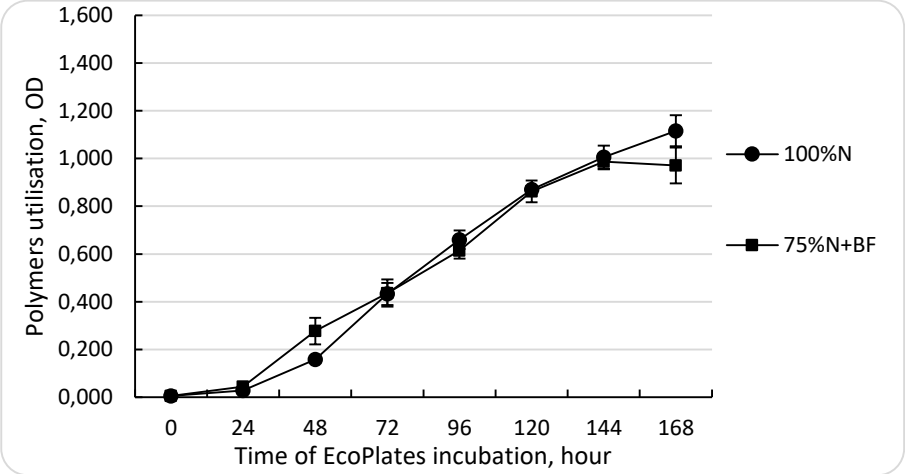

Figure 6. Dynamics of polymers utilisation in the Biolog EcoPlate

In the current experiment, at the end of the incubation period, the mean values for polymer utilisation reached 1.116 and 0.971 for variant 100% N and 75% N+BF, respectively. This OD was comparable to the corresponding mean values for carbohydrates utilisation – 1.016 and 0.802, and for carboxylic acids – 1.118 and 0.979 for variant 100% N and 75% N+BF, respectively. In some cases, the overall utilisation of polymers was low due to insignificant utilisation of (wells E 1-5-9). The metabolism of α-cyclodextrin in bacterial cells was related to the existence of a relatively rare biochemical pathway. It has been described in hyperthermophile archaea such as *Thermococcus sp*., *Pyrococcus furiosus* and *Archaeoglobus fulgidus* and in a few mesophilic bacteria such as *Klebsiella oxytoca* and *Bacillus subtilis* (Centeno-Leija et al., 2022). This imply that the cyclodextrin utilisation could be assigned mainly to bacteria with a relatively low abundance in the soil. However, Oros et al. (1990) who tested twenty-four bacterial strains reported that their relative degradability of selected cyclodextrins and derivatives could reach almost 80% for α-cyclodextrin and at least 60% for β-cyclodextrin. Similarly, Sala et al. (2005) reported that α-cyclodextrin was among the ten substrates with higher utilisation in samples taken from Antarctic water. The results from the current study are in agreement with these data since utilisation of all polymers was quite proportional without any preferences towards specific substrates. The relative contribution of α-cyclodextrin to the average OD of polymer`s guild reached 28% and 25% for variant 75% N+BF and 100% N, respectively.

Campbell et al. (1997) are among the researchers who explored the appropriateness of different substrates and who have a significant contribution to the contemporary design of BIOLOG plates. The researchers noticed that the long chain aliphatic acids and phenolic acids were the most slowly utilised and had much lower AWCD even after 144 hours of incubation. It has been suggested that the discrimination power of analysis could be significantly increased if the root exudates are included as compounds in the substrate` set. However, Sala et al. (2005) reported that phenolic compounds - 2-hydroxy benzoic acid

and 4-hydroxy benzoic acid were among the most intensely used substrates for arctic seawater microbial communities. Pessi et al. (2012) considered D-xylose and 2-hydroxy benzoic acid as constituents of root exudates and assign them in the group of extensively metabolised substrates. On the contrary, in the current study neither of the variants utilised 2-hydroxy benzoic acid and its contribution to the AWCD was less than 1%. Very often after data normalisation the wells with 2-hydroxy benzoic acid provided negative values and has to be set to zero. Thus, the average mean value for phenolic compounds was based mainly on the OD of 4-hydroxy benzoic acid well which utilisation was dominant in the guild. The graph which depicted a phenolic compounds utilisation showed that in the current study the control variant was more active than the biofertilizer-supplemented variant (Figure 7).

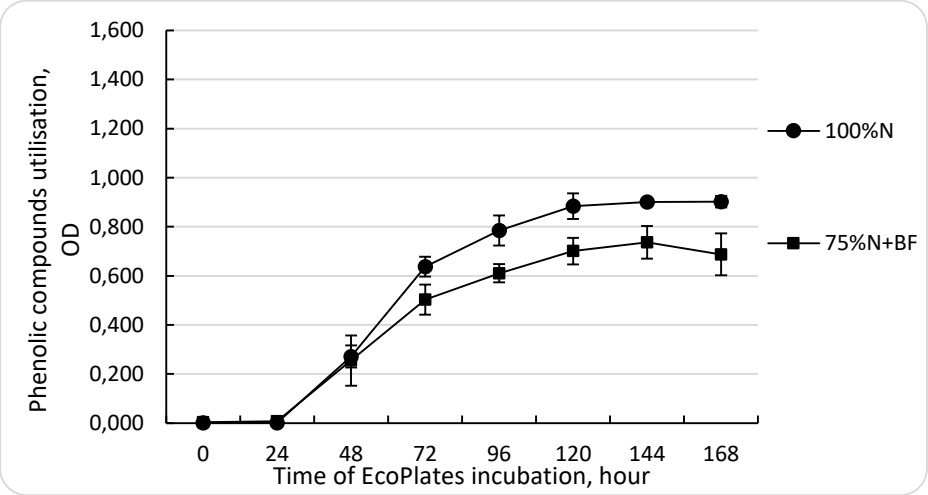

Figure 7. Dynamics of phenolic compounds utilisation in the Biolog EcoPlate

The number of positive wells and specific metabolic pattern of substrates utilisation are the central concept for functional indexes calculation based on the OD values obtained by the Biolog EcoPlates. Each index has a specified range and presents two aspects of community structure – diversity and evenness. Except the McIntosh index all other indexes in the current study clearly indicated that application of biofertilizer positively affect soil microbial biodiversity (Table 3). Janniche et al. (2012) reported Shannon-Wiener and Simpson's indexes in the range of 2.97–3.24 and 0.94–0.96, respectively and concluded that both values indicated high diversity. The values of McIntosh index and Simpson in the current study are similar to those reported by Huang et al. (2012). In comparison to the current study, Zhang et al. (2013) presented slightly lower Simpson index – 0.650-0.873 and much wider range for Shannon-Wiener index - 0.076-2.343. According to Jurkšienė et al. (2020) the Shannon index below 1.00 indicates pollution or imbalance in the community structure and values higher than 3.00 indicate stable and balanced community.

According to Türkmen & Kazanci (2010) the Mardalef index does not have a particular range because it is dependent upon the number of participating in the calculation species (in the current usage it is dependent on the number of positive wells in the EcoPlate) but it is useful and representative for comparison of different samples. The higher values of Mardalef index correspond to the higher biodiversity that in the current study was held by the biofertilizer-treated soil (8.060 ±0.168). However, in this particular case the statistical difference was not proven ($p=0.064$).

The Gini coefficient was originally employed by economists to measure income and wealth inequalities but currently it is broadly

used with variety of biological, anthropological and demographic data (Ceriani & Verme, 2011). In biology, the Gini coefficient is used in a very broad range of studies related to assessment of supplements or management approaches which can resulted either in disturbance or to be beneficial for microbial species (Wittebolle et al., 2009). Beaugrand & Edwards (2001) considered that, among the other indices, the Gini coefficient is the most accurate diversity estimator and the comparisons based on it are reliable and trustworthy. In the interpretation of Biolog EcoPlate data the lower Gini coefficient is an indicator of higher microbial diversity (Harch et al., 1997). In the current study, the Gini coefficient showed statistically significant difference between the samples with a clear indication of higher biodiversity in biofertilizer-supplemented soil.

Table 3. Functional indices of soil microbial community`s diversity and evenness in samples treated either with mineral fertilizer or with *Paenibacillus polymyxa*-based biofertilizer

Functional indexes	**Variant**		***p* value**
	100% N	*75% N+BF*	
Shannon-Weiner diversity	3.150 ±0.087	3.300 ±0.026	0.052
Pielou index	0.970 ±0.007	0.980 ±0.04	0.036
Simpson diversity	0.950 ±0.005	0.960 ±0.002	0.059
McIntosh index	0.220 ±0.013	0.200 ±0.004	0.058
McIntosh evenness	1.210 ± 0.00	1.210 ±0.001	1.000
Margalef index	7.280 ±0.504	8.060 ±0.168	0.064
Gini coefficient	0.270 ±0.038	0.170 ±0.018	0.016

Legend: *Indexes are presented as: mean, ± stand. deviation, n=3*

The relative utilisation (%) provides a general overview of metabolic activity of microbial communities in the experimental samples in a proportional scale based on the six guilds (Figure 8). However, it does not possess significant discrimination power and does not contain the details about the preferences towards specific substrates. However, Teng et al. (2020) used the same approach to compare four types of wetland soils and succeeded to present a very clear visual distinction between samples. The heatmap (Figure 9) is another approach and in the current study it was employed for more precise difference between samples and allowed a more detailed comparison based on the normalised data of OD on the 120th hour of EcoPlate incubation. In this case, the heatmap add value to elucidation and clarification of calculated functional indexes.

In the current study, the most actively utilised substrates in both samples estimated on the 120th hour of EcoPlate incubation were L-arginine and L-serine, γ-amino butyric acid and itaconic acid, D-cellobiose, D-mannitol and N-acetyl-D-glucosamine, glycogen and 4-hydroxy benzoic acid. None of the samples utilised 2-hydroxybenzoic acid and the usage of substrates such as α-keto-butyric acid, D-xylose and D,L-α-glycerol phosphate was very low.

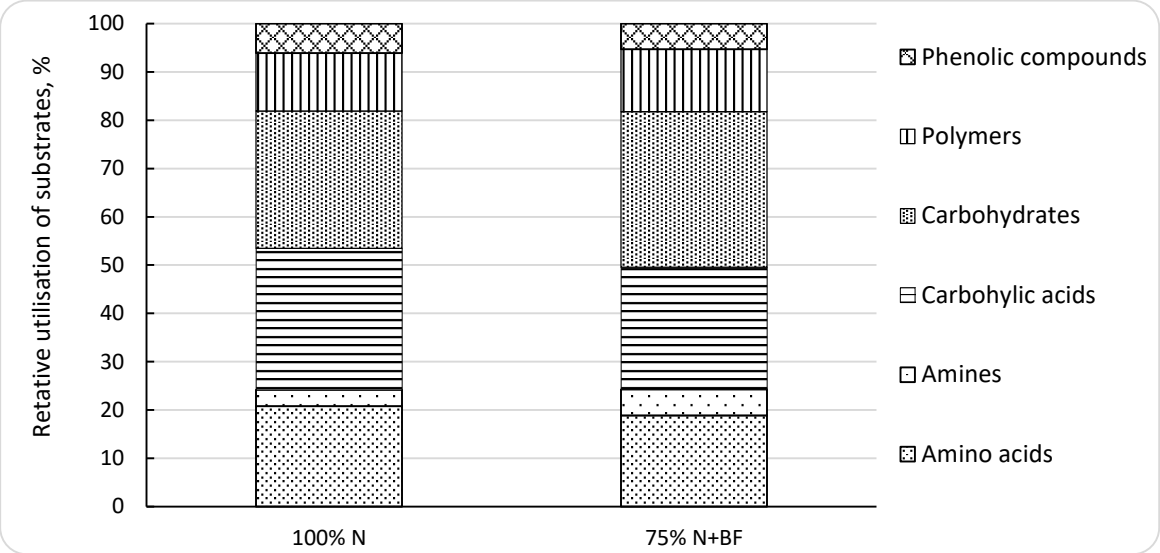

Figure 8. Relative utilisation (%) of guilds on 120th hour of incubation

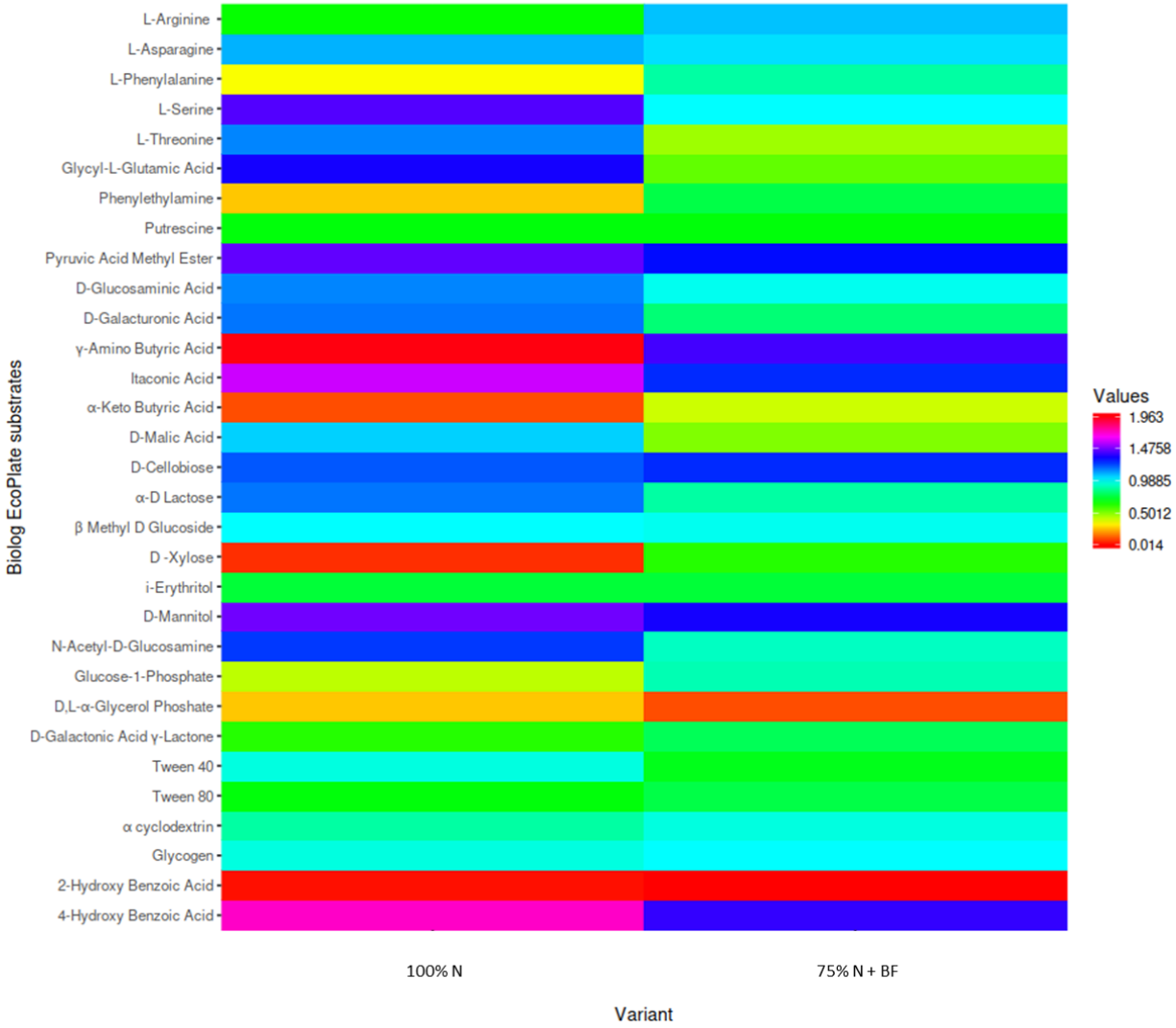

Figure 9. Heatmap of average OD values of substrates at 120 hours of EcoPlate incubation

Janniche et al. (2012) studied communities in water samples and observed a relatively stable preference towards utilisation of some substrates. The authors reported that only nine carbon sources were utilised in all surveyed samples and these substrates were not proportionally distributed among guilds. In general L-asparagine was the only preferred amino acids, D-Mannitol and N-acetyl- D-glucosamine were the most preferred carbohydrates, putrescine was the only one utilized amine compound, D-galacturonic acid and itaconic acid were the most preferred carboxylic acids, 4-hydroxy benzoic acid was the only one utilised phenolic compound, and tween 40, tween 80 the most preferred polymers. In all water samples the microorganisms utilised more actively D-mannitol, D-galacturonic acid, tween 40, and 4-hydroxy benzoic acid, whereas none metabolised 2-hydroxy benzoic acid, α-D-lactose, D,L-α-glycerol phosphate, α-keto-butyric acid, L-threonine and glycyl-L-glutamic acid. Such preference could be assigned to the presence of particular species which association is under both biotic and abiotic factors. However, Sala et al. (2005) reported utilisation of more substrates across all guilds – two phenolic compounds (2-hydroxy benzoic acid and 4-hydroxy benzoic acid), three polymers: tween 40, glycogen and α-cyclodextrin, both of the included in the EcoPlate amines – phenylethylamine and putrescine, 2 carbohydrates: N-acetyl-D-glucosamine and D-cellobiose and one amino acid: L-phenylalanine. Furthermore, the substrates that did not show a positive response in any sample were D-galactonic acid, γ-lactone, I-erythritol, L-arginine, L-asparagine and D-malic acid. Similarly, Pessi et al. (2012) and Sala et al. (2006) observed specific metabolic pattern for substrates utilisation in collected samples. Abundancies of such data clearly indicates the usefulness of EcoPlate technique in discriminating samples from different sources, provides information for community preferences towards specific substrates and allow a substrate profile modelling.

Gilbert et al (1993) tried to determine whether introduction of a single bacterial species could alter microbial communities associated with roots. The authors considered that the communities of rhizosphere bacteria on control plants and treated plants sometimes can be different and this effect could be significant even when the introduced bacterium did not persist as a common member of the community. The author expressed the opinion that bacteria from the bulk soil were more likely to degrade complex carbon sources than were rhizospheric ones. This difference could be explained easily with the adaptation of rhizosphere bacteria to their host plant`s root exudates. Xiao et al. (2022) reported that application of *Rhodopseudomonas palustris* and *Bacillus subtilis* strains showed a synergy and have an influence on the structure of microbial communities. The authors observed that this effect was particularly pronounced on the presence of rare species than on the typical ones. The possible explanation for this trend was related either to the increase of beneficial microorganisms in the soil or to the soil processes associated with the supply of nutrients (Xiao et al., 2022).

However, some studies did not find a significant effect of biofertilizer on soil microbial communities or the effect was very limited (Baldi et al., 2021, Wang et al., 2021). The functional indexes in the current experiment indicated that application of biofertilizer could alter soil microbial communities. Similarly, Roesti et al. (2006) reported a significant modification in the structure of microbial communities after biofertilizer application. Microbial communities are dynamic and sensitive entity that respond to variety of environmental signals. However, the changes in microbial community structure and metabolic activity should convey a positive influence on the plant growth and productivity. The combination of analyses that

assess both microbial communities and crop productivity will provide more comprehensive understanding about the underlying mechanisms and relationships and will further encourage the use of biofertilizers as part of sustainable agriculture.

CONCLUSION

The current study employed an average well-color development (AWCD), substrates utilisation and several functional indexes aiming to reveal the effect of biofertilizer application on soil microbial communities. The AWCD and graphical representation of substrates utilisation based on OD changes did not show any particular difference between the analysed samples but the functional indexes and the scrutinised analysis on usage of substrates revealed the notable shifts in microbial community structure. Despite the fact that not all analysed functional indexes were statistically different, there was a strong implication about the favourable effect of biofertilizer application on diversity of soil microbial communities. These observations give grounds for further research towards revealing the long-term effects of biofertilizers application and their rational use as part of sustainable management practices.

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