STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 71, 1, 2026 (p. 161-184)
doi:10.24193/subbbiol.2026.1.08

Evaluating soil microbial metabolic activity in response to two plant biostimulants using the Biolog Ecoplate™ method

Katya Dimitrova✉

Agricultural University – Plovdiv, Plovdiv, Bulgaria
✉***E-mail: katia_dimitrova@au-plovdiv.bg***

Article history: Received 16 February 2026; Revised 17 June 2026;
Accepted 19 June 2026; Available online 25 June 2026

Abstract. Biostimulants are emerging as viable alternatives to chemical fertilizers, offering a pathway toward more sustainable and environmentally friendly agricultural practices. Their effectiveness, however, is influenced by a range of environmental and biological conditions, making outcomes variable. Evidence suggests that biostimulants may exert their influence by modifying the metabolic activity and composition of soil microbial communities – key drivers of nutrient cycling. This study aimed to assess microbial activity in untreated soil and soil treated with two distinct biostimulants: a seaweed-derived product (PStim) and a microbial-based formulation (MbPB), under greenhouse conditions. Microbial dynamics were evaluated using the Biolog EcoPlate™ method, focusing on substrate utilization pattern. Soil respiration rate was also estimated. No significant changes in soil chemical properties were observed following biostimulant application. Nonetheless, all variants demonstrated high metabolic activity, as indicated by average well-color development (AWCD), with notable differences in substrate preferences. Functional indices pointed to increased metabolic diversity and evenness in biostimulant-treated soils, potentially linked to the targeted stimulation of specific microbial taxa.

Keywords: soil microbial activity, Biolog EcoPlate™, plant biostimulants, microbial biostimulants, substrate utilization patterns, functional indices

Introduction

Agriculture is of paramount importance for providing food, materials, and resources for industry and society. However, due to its immense intensification and the adoption of environmentally detrimental practices, it is currently considered responsible for a third of greenhouse gas emissions, 80% of deforestation, and 70% of terrestrial biodiversity loss (FAO, 2021). It is expected that the problems along the food chain and its social impact will increase in the future and warrant efforts and action in order to address sustainability challenges (Pe'er *et al.*, 2020; Verfuerth *et al.*, 2023). Among the European Union tasks to alleviate the negative effects of agricultural practices is the reduction of mineral fertilizers with 20% by 2050. Such policies aim to stimulate the transition from contemporary, highly intensified agriculture towards adoption of more eco-friendly approaches and provision of sustainability. The concept of sustainable agriculture is based on four main principles: land management, resource management, human interaction, and the ecosystem interface (Shelef *et al.*, 2018). Sustainable agriculture is a holistic approach which purpose is to meet the current food production needs while preserving environment, support economic viability, and ensure the social equity for future generations. In general, it involves practices that promote soil health, biodiversity, water conservation, and energy efficiency, while minimizing the use of synthetic inputs. The assessment of various approaches toward sustainable agriculture and their potential short- or long-term impact on the environment, economy, society, and politics is underway (Zhang and Drury, 2024). One of the possible approaches of reducing reliance on chemical fertilizers is the application of plant biostimulants.

Plant biostimulants encompass substances and microorganisms aimed at enhancing natural processes within plants, improving nutrient uptake and efficiency, stress tolerance, and overall crop quality (EU Regulation 2019/100). The main substances in the biostimulants are humic and fulvic acids, seaweed extracts, protein hydrolysates, chitosan and other biopolymers, inorganic compounds and beneficial microorganism (Tejada *et al.*, 2011; Pichyangkura and Chadchawan, 2015; Tiwari *et al.*, 2019; Rathor *et al.*, 2023; Mughunth *et al.*, 2024). The application of biostimulants in horticulture is increasing due to their potential to strengthen plants, improve commercial standards, enhance product quality, increase plant vitality, and facilitate harvesting (Kisvarga *et al.*, 2022). Biostimulants, including protein hydrolysates and seaweed extracts, have been successfully utilized in horticultural crops promoting plant growth and improving crop quality (Paglialunga *et al.*, 2022). These substances have been shown to promote the accumulation of carbohydrates and biosynthesis of compounds like anthocyanins in plants, contributing to improved fruit yield and quality (Lu *et al.*, 2023).

Additionally, biostimulants have been found to increase mineral content, yield, and reduce nitrate content in horticultural crops, demonstrating their potential to enhance plant nutrition while reducing reliance on traditional fertilizers (El-Nakhel *et al*., 2023). Furthermore, the application of biostimulants derived from seaweed and yeast extracts has been shown to improve fruit development and quality in tomato plants, highlighting their positive impact on crop production (Mannino *et al*., 2020). Furthermore, microbial biostimulants have been proven to enhance plant growth, increase nutrient availability, improve soil quality, and boost plants' tolerance to abiotic stress in various horticultural crops (Shahrajabian *et al*., 2023). Plant biostimulants have been shown to positively influence soil health by modulating soil biological and physicochemical properties (Koleška *et al*., 2017, Shayen, 2023, Wadduwage, 2023). By leveraging the benefits of biostimulants derived from natural sources and microorganisms, horticultural practices can optimize plant performance and contribute to sustainable agricultural production. Despite the active role of soil microorganisms in organic matter decomposition, nutrient cycling, and crop production, the mechanisms behind their activity remain largely unknown. Moreover, the effects of their application are often inconsistent, highlighting the need for further research to develop more precisely targeted products (Fadiji *et al*., 2022). Research on shifts in microbial community structure following the use of various biostimulants can facilitate the development of best management practices for agroecosystems.

The aim of the current study was to evaluate the metabolic activity and functional indices in soil treated with two different types of biofertilizers: seaweed-based and microbial, by taking samples at times when a greater effect on soil microorganisms was expected.

Materials and methods

Greenhouse experiment

The experiment was carried out in the private greenhouse located near Asenovgrad, Plovdiv Municipality. The greenhouse measured 13.50 m in length, 2.60 in width and 3 m in height (latitude: 42.013371, longitude: 24.874939). The two biofertilizers were compared to the untreated control in a completely randomized design with three replicates of five plants per variant. The space between rows were 70 cm and between plants in the row – 40 cm. Soil tillage included deep plowing and harrowing. The previous crop was spinach. The tomato seedlings (*Solanum lycopersicum*) var. *Kalina* F1 was transferred in the greenhouse in March 2024.

Biostimulants

The soil-applied biostimulants used in the current study are available on the Bulgarian market with the commercial name *RaisaMix* (*Daymsa*, Spain) and *AzoFixPlus* (*Bioenergy* LT, Lithuania), respectively. According to the manufacturer, *RaisaMix* is defined as a root's enhancer in the group of physioStimulants with a main content of free amino acids – 10.7% w/w (12.0% w/v) and total nitrogen (N) 4.0% w/w (4.5% w/v). *RaisaMix* contains also oligopeptides, polypeptides, alginates, mannitol, oligosaccharides and polysaccharides, natural growth hormones, betaines, polyamines and vitamins which are derived from special high-quality extract of the sea plant *Ascophyllum nodosum*.

AzoFixPlus contains the free-living diazotroph *Paenibacillus polymyxa* MVY-024 at 1.2×10^{12} CFU/l concentration, B-group vitamins (B1, B3, B6), macroelements such as potassium (7.14 g/l), sodium (1.88 g/l), calcium (1.50 g/l), sulfur (1.17 g/l), phosphorus (0.278 g/l), magnesium (0.275 g/l), and microelements (Cu, Co, Fe, Mn, Mo, Zn) with a maximum quantity of 0.02%.

In this study, both biostimulants were applied at a rate of 3 L/ha, following the manufacturers' recommendations. To achieve the prescribed dosage, 2 mL of the original stock solution was diluted in 10 mL of tap water and administered at 0.5 L per plant directly into the root zone. Applications were carried out twice, at ten-day intervals, during growth stages 19–22 as defined by the BBCH scale (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie). For clarity, the treatment variants are referred to as follows: RaisaMix is abbreviated as PStim, and AzoFixPlus as MbPB.

Soil sampling

Soil samples for both chemical and microbiological analyses were taken after 60 days of the first application of biostimulants. Soil was collected from the rhizosphere of two uprooted plants and pooled to generate a composite sample for each treatment. This approach was intended to reduce the influence of local spatial heterogeneity within the rhizosphere and to obtain a representative sample of each treatment. However, sample pooling may reduce the ability to assess plant-to-plant variability and could lead to an underestimation of within-treatment variability. The soil was cleaned from stones, debris and biological materials, air-dried at room temperature, sieved using a 2-mm sieve and further processed according to requirements of analyses.

Soil chemical analyses

Soil analysis was done at the Accredited Laboratory complex of the Agricultural University, Plovdiv, Bulgaria and here the corresponding analyses are presented briefly. Total mobile nitrogen was determined using the modified

Kjeldahl method. Soil pH was determined potentiometrically in a soil-water solution. Soil phosphorus analysis was conducted according to the Egner *et al.* (1960) method. The applied method, with slight modification, includes $SnCl_2$ as an indicator and measurement of wavelength at 700 nm (BG GOST 26209-91). Available potassium was determined with a flame photometer (GOST 26209:1992).

Soil respiration

Soil respiration was determined according to Isermeyer method (1952) as described in Alef & Nannipieri (1995). The method is based on soil respiration in closed jars and the quantity of CO_2 absorbed by NaOH solution and consequently titrated with HCl. The following formula is used:

$$CO_2(mg)/SW/t = \frac{(V_0 - V)\times 1.1}{dwt},$$

where SW – the amount of soil dry weight in g, t is incubation time in hours, V_0 is quantity (in ml) of HCl for blanks sample titration (average), V is quantity (in ml) of HCl for soil sample titration, dwt is the dry weight of 1 g moist soil, 1.1 is the conversion factor (1 ml 0.05 M NaOH equals 1.1 mg CO_2).

Biolog EcoPlate technique

Biolog EcoPlate™ (Biolog Inc., USA) was used for assessment of metabolic activity of microbial communities. Each EcoPlate is comprised of three replicates of 31 different substrates organized in the following guilds - carbohydrates, carboxylic acids, polymers, amino acids, amines and phenols (Table 1).

Table 1. List of the substrates in the Biolog EcoPlate

Guild	Substrates
Amino acids	L-Arginine L-Asparagine L-Phenylalanine L-Serine L-Threonine Glycyl-L-glutamic acid
Amines	Phenylethylamine Putrescine

Guild	Substrates
Carboxylic acids	Pyruvic acid methyl ester
	D-Glucosaminic acid
	D-Galacturonic acid
	γ-Amino butyric acid
	Itaconic acid
	α-keto Butyric acid
	D-Malic acid
Phenols	2-Hydroxybenzoic acid
	4-Hydroxybenzoic acid
Carbohydrates	D-Cellobiose
	α-D-Lactose
	β methyl D Glucoside
	D -Xylose
	i-Erythritol
	D-Mannitol
	N-acetyl-D-glucosamine
	Glucose-1-phosphate
	D, L-α-Glycerol phosphate
	D-Galactonic acid γ-lactone
Polymers	Tween 40
	Tween 80
	A-Cyclodextrin
	Glycogen

Ten grams of preliminary prepared soil was suspended in 90 ml sterile physiological solution (0.85% NaCl), thoroughly mixed for 30 min at 190 rpm and left to settle for 10 min, after which a 10^{-3} dilution was prepared. One hundred and fifty µl were used for the Biolog EcoPlates inoculation as recommended by the producer`s protocol. The plates were incubated at 24±1°C and read spectrophotometrically at 24-hour intervals for 7 days (168 hours) with the MicroStation™ Reader provided by the Biolog system. The calculations for average well-color development (AWCD) and separately for each substrate guild 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:

$$\text{AWCD} = \sum(C_i - R)/31,$$

where R is the control well (water) and C_i is the value of any substrate-containing well. The measurement at 24-hour was used for data normalization by subtracting each consecutive measurement with the corresponding values at 24-hour in order to avoid so called background noise according to Urakawa *et al*. (2013). The negative numbers obtained during normalization were set to zero (Garland, 1996).

Functional indices

According to recommendation of Sofo & Ricciuti (2019) functional indices should be calculated based on wells with an OD ≥0.250. Due to very high metabolic activity of microorganisms in collected soil samples the current study implemented a different approach and the indices were calculated using the measurements from 48th to 168th hour of EcoPlate incubation taking into account wells with OD ≥0.350. The indices calculation was done with the formulas listed in the Table 2.

Table 2. Formulas for functional indices calculation

Functional indices	Formula	References
Shannon-Wiener index, H'	$\text{H}' = -\sum p_i \times (\ln p_i)$ where pi is Ci, divided by the sum of Ci, wells with value ≥ 0.350	Jurkšienė *et al*. (2020)
Pielou index, E	$\text{E} = \frac{H'}{\ln S}$ where H' is Shannon-Wiener index S – number of wells with value ≥ 0.350	Pielou (1966), Jurkšienė *et al*. (2020)
Simpson index, D	$D = 1 - \sum P_i^2$ where Pi is Ci, divided by the sum of Ci, wells with value ≥ 0.350	Chen *et al*. (2020a)
Margalef index, d	$d = \frac{(S-1)}{\ln N}$ where S – number of wells with value ≥ 0.350, N – number of substrates i.e. 31	Türkmen & Kazanci (2010)
McIntosh index, U	$U = \sqrt{\sum P_i^2}$ where Pi is Ci, divided by the sum of Ci wells with value ≥ 0.350	McIntosh (1967) Huang *et al*. (2012)

Functional indices	Formula	References
McIntosh evenness, McI	$McI = N - U/N - (N/\sqrt{S})$ where U – McIntosh diversity index, N – sum of wells with value ≥ 0.350, 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, $\bar{x}$ - AWCD, N - number of substrates. The final value was further multiplied with n/(n-1)	Weiner & Solbrig (1984) Harch *et al.* (1997)

Data analysis

The average values of AWCD, expressed both as total activity and per guild, were visualized using Microsoft Excel. Each EcoPlate's three sets of substrates were treated as replicates (n=3). Error bars indicate the standard deviation. Functional indices were compared using ANOVA, followed by Tukey's Honest Significant Difference (HSD) post-hoc test in SPSS (IBM, version 26).

Results

Soil analysis

Soil pH, ammonium and nitrate content of the soil were not affected by application of biostimulants (Table 3). There was a slight increase in the phosphorus content of soil samples treated with biostimulants but the potassium content was the same in the control and treated with microbial biostimulant sample and slightly lower in the variant which was treated with a seaweed-based biostimulants.

Table 3. Soil chemical parameters after application of two different biostimulants

Soil parameter	Units	Variant		
		Control	**PStim**	**MbPB**
pH	-	8.15	8.30	8.26
Ammonium (NH_4^+)	mg/kg	7.64	8.34	7.00
Nitrate (NO_3^-)	mg/kg	15.93	18.24	14.59
Phosphorus (P_2O_5)	mg/100g	98.61	100.16	107.91
Potassium (K_2O)	mg/100g	93.48	86.62	93.87

Soil respiration

Soil respiration was higher for variants that were treated with biostimulants, with corresponding values of 0.174 ± 0.010 and 0.162 ±0.018 CO_2(mg)/SW/24h for MbPB and PStim treatment, respectively. The control variant had a soil respiration of 0.124 ±0.014 CO_2 (mg)/SW/24h (Figure 1).

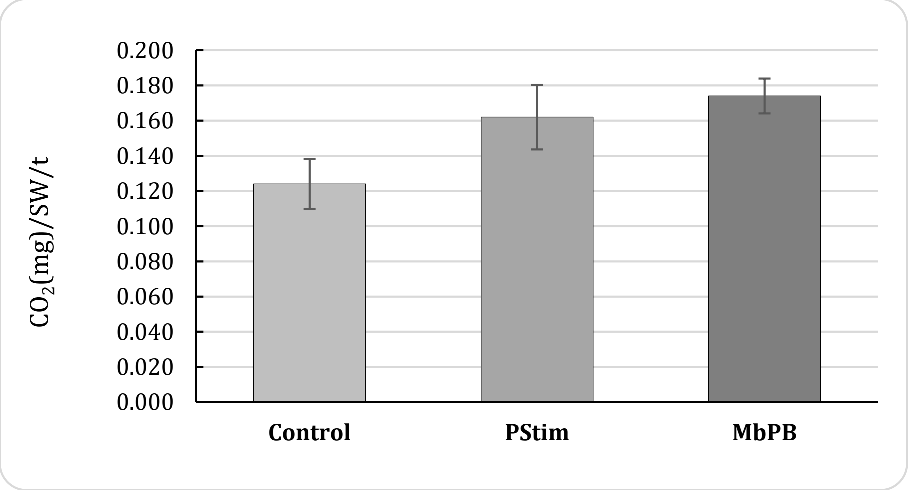

Figure 1. Soil respiration (PStim=RaisaMix; MbPB= AzoFixPlus).

Microbial metabolic activity

After the initial lag phase (0–24 hours), which is typical for microorganisms adapting to a new environment and newly available substrates, the microbial communities in all three tested samples exhibited a rapid and pronounced increase in metabolic activity, as measured by the Biolog EcoPlate (Figure 2). Between 48 and 120 hours, the increase in optical density (OD) was proportional, with a margin of approximately 0.2 units. Statistically, the mean values during this period showed no significant differences between the variants. However, after 96 hours, the control treatment displayed slightly higher values compared to the biostimulant-treated samples.

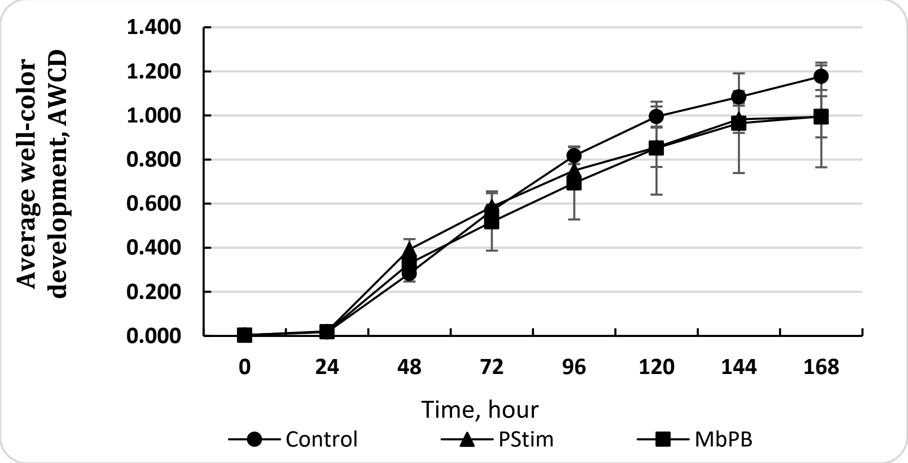

Figure 2. Overall metabolic activity (AWCD) of microbial communities in soil treated with two different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus).

Despite the lack of a significant difference in the metabolic activity expressed as AWCD between treatments, they revealed some differences in the utilization of different substrates. Utilization of amino acids after the 72nd hour until the end of the incubation period was higher in the control, and at the 96th hour, this difference was even statistically significant (Figure 3).

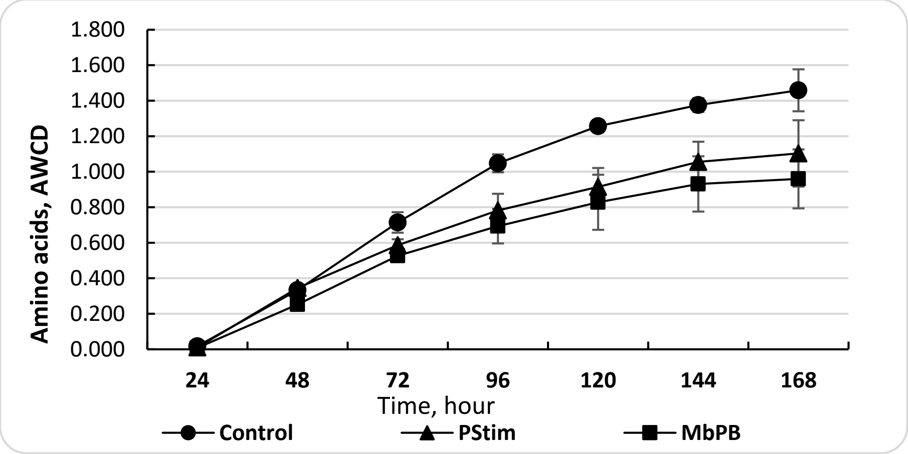

Figure 3. Amino acids utilization by microbial communities in soil treated with different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus).

Utilization of amines was relatively higher in all treatments, and at the end of the incubation period, the highest values were measured for the community in the sample treated with microbial-based biostimulant – 1.280 ±0.379, followed by the control and PStim with mean values of 1.159 and 1.009, respectively (Figure 4). A statistical difference was not obtained, probably because of the similar mean values and relatively wide range of standard deviation.

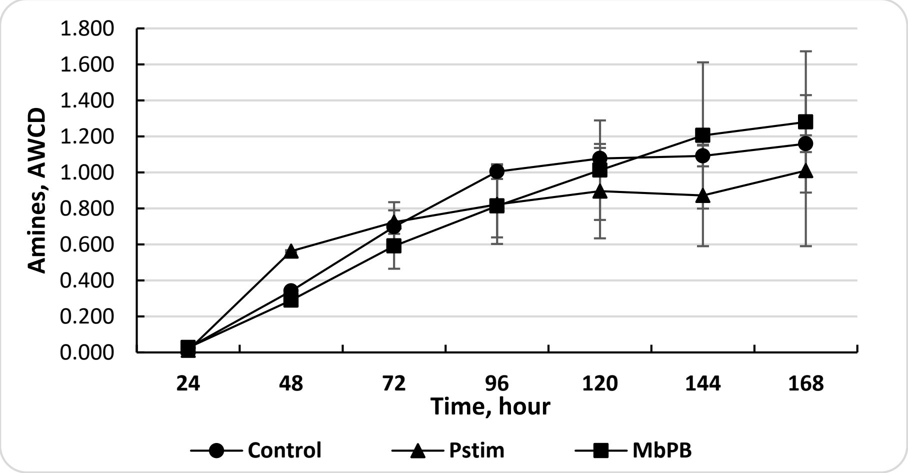

Figure 4. Amines utilization by microbial communities in soil treated with different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus).

Carboxylic acids were utilized quite uniformly across the different treatments up to the 48th hour. After this point, the curve became steep and then plateaued from the 120th hour onward, with the last two optical density readings in the control variant remaining nearly constant (Figure 5). Microbial communities collected from soil that received biostimulants treatment utilized carboxylic acids less intensely in comparison to the control (1.260 ±0.108 at the 168th hour) but very uniformly. A statistical difference between the control and biostimulants-treated variants was proven for measurements taken from the 46^{th} to the 96^{th} hour and at the end of the incubation period (p=0.006).

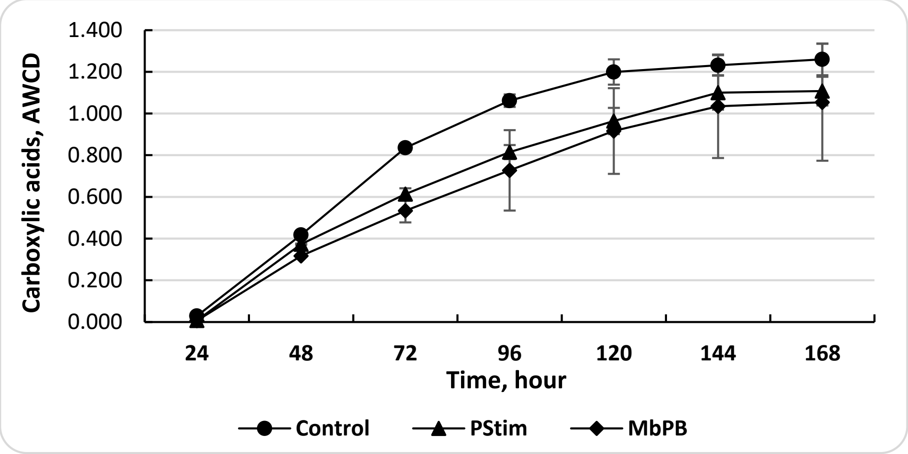

Figure 5. Carboxylic acids utilization by microbial communities in soil treated with different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus).

Contrary to the use of carboxylic acids, the communities treated with biostimulants showed significantly higher carbohydrate utilization (see Figure 6).

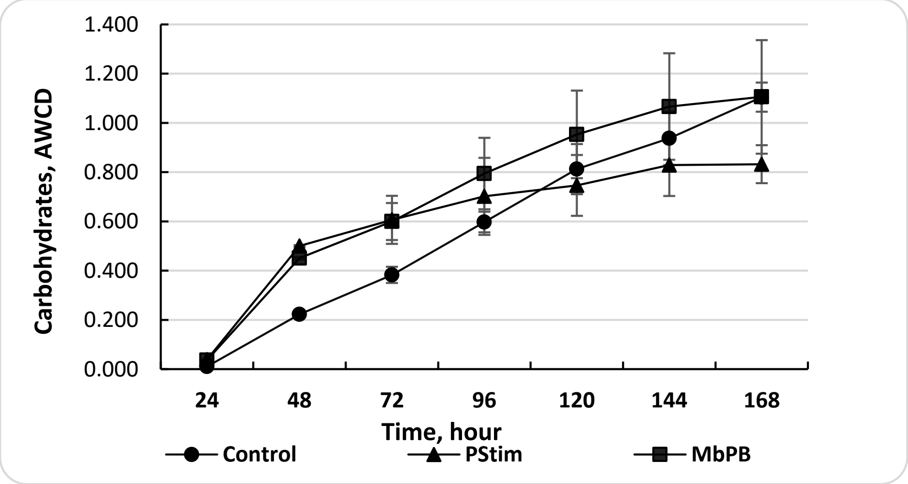

Figure 6. Carbohydrates utilization by microbial communities in soil treated with different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus).

By the 48th hour of incubation, the mean optical density (OD) was twice as high as that of the control treatment (0.222 ±0.033), and the difference was statistically significant. After the 96th hour, the communities treated with seaweed-based biostimulants slowed their carbohydrate utilization, reaching the lowest value of 0.832 ± 0.08 by the end of the incubation period. Carbohydrate utilization in the control community was consistent and exponential throughout the incubation period. From the 96th to the 144th hour, the highest metabolic activity was observed in the community treated with microbial biostimulant. However, the final measurement showed that the mean values for the control and microbial biostimulant-treated samples were almost the same –1.105 ±0.130 and 1.106 ±0.240, respectively.

In comparison to the utilization of other available substrates in the Biolog EcoPlate, the polymers utilization was probably the most intriguing (Figure 7). Between the 24th and 48th hours, there was no difference between treatments. The community from the sample treated with microbial biostimulant showed almost the same OD mean values from the 48th to the 72nd hour, and after that, until the end of the incubation period, the increase did not exceed 0.1 units. As a result, the value at the final measurement was the lowest: – 0.497 ±0.124. In the current study, this was valid not only in comparison to the other experimental variants but also in comparison to the utilization of all other substrates' guild in the EcoPlate. The communities in the control variant and the treatment which received the seaweed-based biostimulant showed a stable and consistent increase in the OD, which reached almost 0.3 units from the 72nd to 96th hours, and then decreased to only 0.1-0.2 units. At the end of the incubation period, the variant treated with seaweed-based biostimulant had the highest mean value of 1.178 ±0.143, and the control reached 1.056 ±0.159.

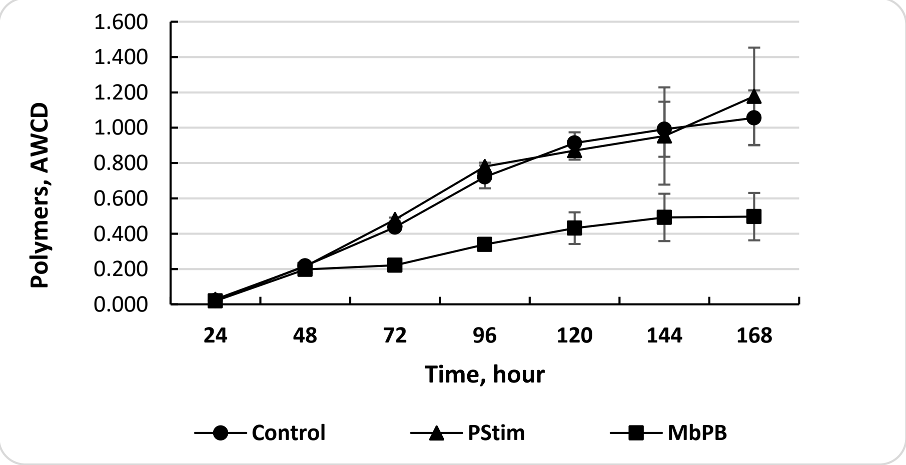

Figure 7. Polymers utilization by microbial communities in soil treated with different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus).

The metabolic activity of soil microbial communities towards phenolic compounds was primarily based on the utilization of 4-hydroxybenzoic acid, which greatly surpassed that of the second compound in the guild, 2-hydroxybenzoic acid, except in the variant treated with microbial biostimulant. Despite the highest utilization of phenols observed in the community collected from the MbPB variant, the uneven distribution of positive values in the wells containing 2-hydroxybenzoic acid caused a high standard deviation, as shown in the graph (Figure 8). As expected, at the end of the incubation period, the MbPB variant showed the highest utilization of phenols, reaching a value of 1.069 ±0.0558. This can be compared to its metabolic activity towards carboxylic acids and amino acids, with end values of 1.054 ±0.298 and 0.960 ±0.172, respectively. Microbial communities in samples that received no treatment and those treated with seaweed extract showed lower metabolic activity, with similar OD values at the end of the incubation period – 0.677 ±0.070 and 0.698 ±0.183, respectively.

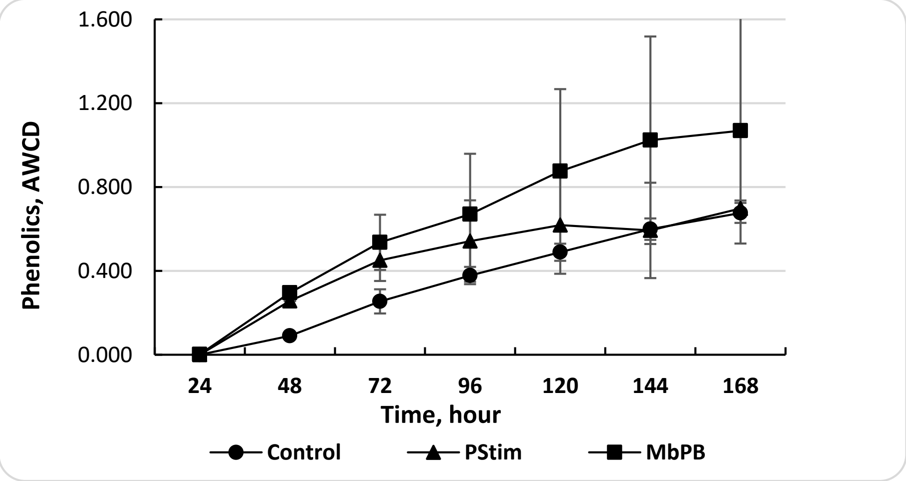

Figure 8. Phenols utilization by microbial communities in soil treated with different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus).

Functional indices

Despite the absence of statistically significant differences, some functional indices showed numerical increases in the biostimulant-treated samples, suggesting a potential trend toward enhanced metabolic diversity and evenness. However, indices such as Shannon-Weiner, Pielou, McIntosh evenness, and the Gini coefficient indicated higher functional diversity and evenness for variants treated with seaweed-based and microbial biostimulants (Table 4). According to the Margalef index, higher biodiversity was observed in substrate utilization from sample treated with seaweed-based biostimulant, with the highest value of 6.49 ± 1.38. Conversely, the McIntosh index indicated higher metabolic diversity from soil treated with microbial biostimulant, with the highest value of 0.229 ±0.037.

Table 4. Functional indices of the characteristics of microbial communities in soil treated with two different biostimulants

Treatments	**Functional indices**						
	Shannon-Wiener index, H'	Pielou index, E	Simpson index, D	Margalef index, d	McIntosh index, U	McIntosh evenness, McI	Gini coefficient, G
Control	2.994 ±0.367	0.975 ±0.040	0.946 ±0.026	6.37 ±1.95	0.227 ±0.049	1.200 ±0.023	0.246 ±0.089
PStim	3.066 ±0.244	0.981 ±0.007	0.867 ±0.238	6.49 ±1.38	0.222 ±0.030	1.204 ±0.010	0.259 ±0.077
MbPB	3.015 ±0.291	0.984 ±0.005	0.946 ±0.019	6.16 ±1.58	0.229 ±0.037	1.201 ±0.015	0.256 ±0.083

Relative utilization of substrates

The relative utilization of substrates in the EcoPlate, based on measurements taken from the 48th to 168th hour of incubation, indicated higher utilization of amino acids and carboxylic acids at 22% and 21%, respectively, compared to the biostimulants-treated samples (Figure 9). The utilization of polymers in the control sample was comparable to those treated with the seaweed extract – 15% and 14%, respectively. However, the control sample showed significantly lower utilization of phenols – only 9% – compared to the samples treated with microbial biofertilizer (17%) and seaweed extract (13%). Both communities treated with either seaweed extract or microbial biostimulant showed similar metabolic activity towards utilization of amino acids (17% and 16%), amines (20%), carboxylic acids (18%), and carbohydrates (18% and 19%), respectively. The main difference between substrate utilization of communities in biostimulants-treated soil was utilization of polymers and phenolic compounds. In the former case, the variant that received the seaweed extract surpassed the sample with microbial biostimulant by 5%. Conversely, for phenolic compounds utilization, the sample with microbial biostimulant surpassed the sample that received the seaweed extract by 4%.

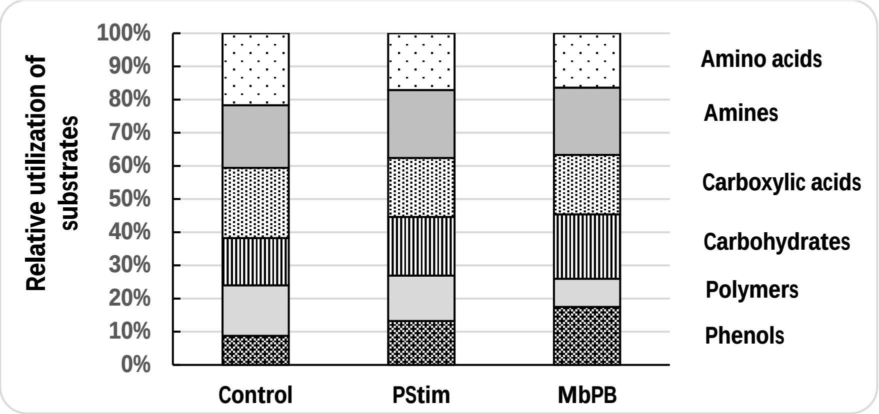

Figure 9. Relative utilization of substrates (%) in the Biolog Ecoplate by microbial communities in soil treated with two different biostimulants (PStim=RaisaMix; MbPB= AzoFixPlus)

Discussion

The application of biostimulants did not significantly affect the measured soil parameters. Similarly, none of the differences in the calculated functional indices characterizing substrate utilization pattern were statistically significant.

Although numerical differences were observed among treatments, these should be interpreted as trends rather than as evidence of treatment effects. The results should also be considered in light of certain limitations, including the controlled greenhouse conditions and the relatively small number of biological replicates, which may have limited the statistical power to detect subtle treatment effects. Despite the absence of significant changes in soil parameters under the conditions of the present study, contrasting results have been reported in the literature. For example, Ioppolo et al. (2020) observed an initial decrease of 2–3 pH units and higher electrical conductivity in samples treated with biofertilizer. However, the authors used in their study a citrus fruit processing wastewaters which supposedly could affect the aforementioned soil parameters. Chen et al. (2020b) also reported that application of seaweed-based extract increased the ammonium and nitrate content in comparison to the control. Conversely, Hellequin et al. (2018) reported a pH neutralizing effect of biostimulants application and increased organic carbon mineralization. The authors considered that these changes were linked to changes in microbial communities.

Currently, agricultural practices are evolving to achieve sustainable soil management, health, and productivity, while also supporting biodiversity through alternatives to common approaches. In this effort, agricultural biostimulants are being developed and used as alternatives to mineral fertilizers. However, the ways in which these biostimulants enhance soil biological functions and indirectly boost crop yields remain unclear (Hellequin *et al*., 2018). The summarized net metabolic activity of microbial communities can be represented by changes in the OD throughout the incubation period as AWCD. In principle, in the absence of inhibitory supplements or other factors that can suppress microbial activity, the AWCD curve, similar to the observed in the current experiment, has a typical sigmoid shape (Stefanowicz, 2006; Lima *et al*., 2015). On the other hand, the length of the lag phase and the pace of the OD increase entirely depend on metabolic activity of community (Li *et al*., 2012; Koner *et al*., 2021). The very short lag phase observed in the current study is similar to the results of Ge *et al*. (2018) and contrasts with the study of Sun *et al*. (2012) who observed a lag phase of 24 and more than 60 hours, respectively. Despite the provision of summarized information, the AWCD cannot represent the specifics of substrate utilization. The calculation of AWCD separately per each of the six substrates guilds in the Biolog EcoPlate addresses this gap. Across the utilization of substrates, some specificities have been observed in the current study. In the case of utilization of amino acids and carboxylic acids the communities which were collected from samples treated with biostimulants firstly were quite similar and despite being considered as the main carbon and nitrogen sources they were metabolized less intensely in the biostimulants supplemented variants. This could be explained with changes in carbon and nitrogen microbial metabolic dynamics. This implication was made by Chen *et al*. (2002) who investigated the effects of two commercially available soil biostimulants through short-term (1 week) and longer-term (8 weeks) soil incubations under laboratory conditions. They found that in the short-term soil incubations, the two biostimulants had different effects on microbial activity and stimulated different properties and in the long-term experiment the biostimulants significantly affected soil nitrogen dynamics. The results of the study indicated that the two biostimulants could stimulate both the breakdown and mineralization of soil organic materials and the observed trend was related to the possibility of selective inhibition or stimulation of particular species in the microbial community (Chen *et al*., 2002). Ioppolo *et al*. (2020) suggested that the application of biostimulants changed the total and labile C pools, thus stimulate soil microbial activity and biomass. However, they assigned this effect predominantly on the transient soil acidification caused by the biofertilizer used. In similar cases of changes in soil pH after biofertilizer application the authors recommended soil electrical conductivity and soil pH monitoring.

Chen *et al* (2020b) studied the effect of seaweed fertilizer application and found that the overall functional pattern was similar between the different soil samples. However, the authors stressed on the fact that the relative abundance of functional proteins involved in carbohydrate transport and metabolism in the treatment groups have increased when compared to the control. This result corresponds with the higher utilization of carbohydrates which was observed in the current study in the biostimulants treated samples. Based on the observations and analysis Chen *et al*. (2020b) made the conclusion that the relative abundance of the microbiota varied significantly after biofertilizer application and there were also some changes in the enzyme activity.

Functional diversity constitutes a crucial aspect of overall metabolic diversity in soil, encompassing a wide range of activities. Biodiversity plays a vital role in maintaining ecosystem stability and resilience. However, due to the immense abundance and species diversity of microbial communities in soil, the precise relationship between diversity and metabolic activity remains largely unknown (Torsvik and Øvreås 2002). Li *et al.* (2022) conducted a meta-analysis on the effects of biofertilizers and concluded that the benefits of biostimulant application are greater under unfavorable soil and environmental conditions, particularly in non-neutral, saline, nutrient-poor sandy soils with low organic matter content. This suggests that the effects of biostimulant application may be less pronounced in fertile soils and under favorable growing conditions. Estimating bacterial functional diversity based on substrate utilization has been reported as a sensitive approach for detecting changes in soil conditions (Pessi *et al*., 2012; Zhang *et al*., 2013). Additionally, it allows for the comparison of microbial communities from different sources if certain considerations are taken into account (Preston-Mafham *et al*., 2002). Roesti *et al*. (2006) found that a biofertilizer application caused significant modifications in microbial community structure. In contrast, Baldi *et al*. (2021) and Wang *et al*. (2021) did not detect significant changes in soil microbial communities following biofertilizer application, or the observed effects were inconsistent. The results obtained by Wadduwage *et al*. (2023) also implied that biostimulant treatment did not significantly affect total bacterial and fungal abundance or their alpha diversity. Dal Cortivo *et al*. (2020) did not observe alterations in microbial biodiversity. Nevertheless, they argue that optimizing biofertilizer use in sustainable wheat cultivation necessitates attention to the composition of microbial consortia. Furthermore, Mickan *et al.* (2021) reported that microbial biostimulants influenced soil biodiversity but did not affect the growth of the plant species studied. Their results also revealed an interaction between plant type and soil amendments. The authors emphasized the complexity of interactions between soil amendments and microbial biostimulants, which can affect soil bacterial communities and warrant caution when interpreting the relationship between soil biodiversity and plant growth. Hellequin *et al.* (2018), who conducted soil microcosm experiments with the addition of crop residues and a biostimulant, found that the increase in microbial biomass and crop residue mineralization was linked to changes in the soil microbial communities. However, due to the significant prevalence of decomposer species, the overall soil microbial richness and diversity actually decreased. Some authors have reported that soil application of microbial biostimulants increased bacterial diversity, whereas seed treatment reduced the diversity of indigenous plant-associated bacteria. The observed changes in bacterial diversity were attributed primarily to the response of r-strategist bacteria (Ciccillo *et al*., 2002). Similarly, other studies have shown that different fertilization practices increased the abundance of soil microorganisms while exerting only limited effects on microbial community structure. Nevertheless, these practices may influence the catabolic activity of fast-growing bacterial populations (Guanghua *et al*., 2008). The results of the present study suggest that the applied biostimulants may have selectively affected specific functional groups within the soil microbial community. However, because no taxonomic analyses were conducted, the possible predominance of fast-growing copiotrophic microorganisms remains speculative.

Conclusions

The growing use of biostimulants in agriculture is driven by sustainability goals and scientific research. As partial or full alternatives to chemical fertilizers, they support eco-friendly farming, though their effectiveness varies with environmental and biological conditions. Soil-applied biostimulants can alter metabolic activity and substrate utilization, influencing nutrient turnover and soil health. While no changes in chemical composition were observed in the current experiment, treated and untreated soils showed very high metabolic activity, limiting detection of subtle differences. Biostimulant-treated soils showed a tendency toward greater metabolic diversity and evenness in substrates utilization, although these differences were not statistically significant. These effects may stem from selective stimulation of specific microbial species, warranting further research into their role in soil fertility.

Competing interests. The author declares no competing interests

Acknowledgements. This study was financially supported by the Centre for Science Research, Technology Transfer, and Protection of Intellectual Property at the Agricultural University – Plovdiv, within the scope of Project 03/23.

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