ECOLOGIA BALKANICA

2026, Vol. 18, Issue 1 June 2026 pp. 021-033

Influence of the urban environment on the metabolic activity and functional diversity of phyllospheric microbial communities in linden trees

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Abstract. It has been shown that microorganisms associated with the crown of trees (phyllosphere) can improve their ability to purify the air from pollutants. On the one hand, this is due to the metabolites released by the microorganisms that stimulate the development of trees and their resistance to stress, and on the other hand, the microorganisms themselves also are able to degrade some of the atmospheric pollutants. The aim of the present study was to assess the influence of the urban environment on the metabolic activity and functional diversity of microbial communities in the phyllosphere of linden trees, planted in four experimental plots within the city of Plovdiv (Bulgaria). Each plot is characterized by different anthropogenic load, thus allowing for detection of potential specificity of microbial metabolism. A total of 12 saplings of *Tilia tomentosa* Moench were planted (3 individuals per plot) and leaf samples were collected after 3-month period in the urban environment. Biolog EcoPlate™ of the BIOLOG system (Biolog, Hayward, CA, USA) was used for estimation of metabolic activity of microbial communities, associated with linden trees. The epiphytic communities isolated from leaf samples of Plot 4 (lowest degree of urbanization) showed the highest average-well color development (AWCD) and substrates` metabolic activity. It was found that microorganisms in the two more strongly affected by the traffic locations (Plot 1 and Plot 3) have a higher rate of carbohydrate assimilation and a lower rate of phenolic compounds assimilation compared to the other two locations. Most of the analyzed functional indices showed higher biodiversity and better distribution of substrate utilization in the epiphytic microflora of the leaves of trees planted on Plot 4 (lowest urbanization intensity).

Key words: air pollution, urban environment, phyllosphere microbiome, Biolog Ecoplate, phytoremediation.

Introduction

Air pollution is one of the most serious environmental problems in urban centers, affecting the population and economy, causing loss of agricultural production, and significant loss of flora and fauna biodiversity (Alberti et al., 2005; Yurukova et al., 2013). Many chemical substances, including greenhouse gases, organic compounds, and fine particles, are emitted from natural and anthropogenic sources. Moreover, after their release, these pollutants undergo physical, chemical, and photochemical transformations that ultimately determine their behavior and concentrations in the atmosphere (Atanassov et al., 2006; Petrova et al., 2015). Ambient air contains a variety of both primary and secondary pollutants of a chemical, physical or biological nature, mainly carbon monoxide (CO), lead (Pb), nitrogen oxides (NO_x), ground-level ozone (O_3), sulfur oxides (SO_x), particulate matters (PMs), volatile organic

compounds (VOCs), polyaromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (Supreeth, 2022).

Plants are autotrophic organisms that perform intensive gas exchange to carry out cellular processes, in which air pollutants can be absorbed or accumulated internally. Due to the large total surface area of their leaves, trees are generally considered effective in removing particulate matter (PM) and gases from the surrounding air (Alahabadi et al., 2007; Kumar et al., 2021; Petrova, 2024). Phytoremediation of air pollution is considered a widely applicable and sustainable technique, as plants eliminate environmental pollutants in an ecological, cost-effective and non-invasive way (Yang et al., 2005; Yin et al., 2011; Petrova et al., 2024). Plants reduce the mobility, toxicity and volume of pollutants through various mechanisms, such as accumulation, immobilization, volatilization and degradation (Matic et al., 2023). Stomata on plant surfaces and leaves are major structures that absorb pollutants, the surface of plant leaves can accumulate PM and effectively filter the air (De Nicola et al., 2008).

It is well known that plants effectively remove CO_2 through photosynthesis, as well as degrade organic compounds with the support of rhizosphere and/or phyllosphere microorganisms (Kumar et al., 2023; Shilev et al., 2019). Some studies have shown that phyllosphere associated with the crown of trees can improve their ability to clean the air of pollutants (Babu et al., 2013; Shin et al., 2012). This effect results from metabolites released by microorganisms that promote tree development and increase stress resistance, and from the microorganisms' direct involvement in the immobilization and degradation of atmospheric pollutants (Kumar et al., 2023). In addition to indirectly reducing of the particulate matters concentrations by promoting plant growth, urban air pollution can be reduced directly through interactions between plants and microorganisms, resulting in the breakdown of ultrafine particles and soot (Ho et al., 2013; Horemans et al., 2013), thus promoting the plant and microbes-assisted remediation of air pollutants in the environment. Phyllosphere microbial communities could participate in atmospheric pollutants degradation through the metabolic capacities of colonizing microbial cells (Imperato et al., 2019), as specific taxa are influenced by environmental factors including NO_2 concentration. For example, research has demonstrated a significant association between fungal community composition and NO_2 concentration in the atmosphere (Faticov et al., 2024). Bacterial phyllosphere populations degrade organic air pollutants such as polycyclic aromatic hydrocarbons through metabolic pathways that utilize these compounds as carbon sources (Espenshade et al., 2019). Recent research on urban phyllosphere bacterial communities reveals that their composition and diversity are shaped by urbanization gradients and environmental variables such as particulate matter pollution and landscape characteristics (Muyshondt et al., 2022; Perreault & Laforest-Lapointe, 2021). CLPP in the phyllosphere of linden trees has been shown to vary significantly along urbanization gradients, suggesting that microbial metabolic potential is directly influenced by anthropogenic stressors such as traffic emissions (Imperato et al., 2019; Perreault & Laforest-Lapointe, 2021).

The aim of the present study was to assess the influence of the urban environment on the metabolic activity and functional diversity of the microbial communities in the phyllosphere of linden trees, planted in four experimental plots within the city of Plovdiv (Bulgaria).

Materials and methods

Study area and experimental design

This research was carried out in the city of Plovdiv, Bulgaria, characterized with a plenty of ecological pressures such as air contamination, dense vehicular traffic, and local industrial emissions (Petrova et al., 2022). Four experimental plots with different anthropogenic load have been selected within the city's boundaries as follows: Plot 1 – heavy traffic and high level of air pollution; Plot 2 – moderate traffic and moderate level of air pollution; Plot 3 – very heavy traffic (motor and railroad) and very high level of air pollution; Plot 4 – low traffic and no air pollution (Fig. 1).

A total of 12 standardized saplings (8 years old) of silver linden (*T. tomentosa* Moench) were purchased from certified nursery and planted by our team at the four experimental plots - 3 individuals per plot as a group planting. Three months after planting, representative leaf samples have been collected, each one consisting of at least 30 fully developed leaves per each individual tree.

Fig. 1. Map of the city of Plovdiv (Bulgaria) and locations of the four experimental plots.

Analysis of the metabolic activity of microbial communities

Biolog EcoPlate™ is broadly used in the analysis of samples of various sources - soil samples, presence of xenobiotics in the environment, samples of fresh and salt water, etc. and provide the information necessary for the assessment of microbial communities. Although the assessment is based on certain substrates, which may be absent in the natural habitats from which the samples for analysis were taken, the method is sensitive and in combination with functional indices provides an idea of the metabolic activity in the studied samples and permits meaningful comparisons (Dimitrova, 2024).

20 g of fresh leaves with petioles were used, sampled no later than 24 hours before the analysis. The leaves were rinsed thoroughly with water, washed with distilled water and briefly dried on filter paper. They were placed in flasks with 100 ml of sterile saline solution (0.85% NaCl) and shaken at 180-190 rpm for 1 hour. The solution was transferred to centrifuge tubes and centrifuged briefly (2 min) to separate leaf fragments or other contaminants. The supernatant was centrifuged for 20 min at 9000 rpm. Due to the lack of visible sediment, 25 ml was removed from the tube, the remaining 5 ml at the bottom was transferred to another tube. 15 ml of sterile saline solution was added, mixed well and used to inoculate the Ecoplates (150 µl per well). The plates are incubated at 25°C±2°C for seven days.

The metabolic activity of microbial communities was determined by the utilization of different substrates. The Biolog EcoPlate™ of the BIOLOG system (Biolog, Hayward, CA, USA) contains 96 wells, which are organized into three blocks (three replicates) of 31 substrates and three control wells (without added substrate). The reduction of an indicator dye (tetrazolium blue) was used as an indicator for substrates utilization. Optical density (OD) measurements were performed spectrophotometrically immediately after plates inoculation and after that on every 24 hours until the end of the incubation period using the BIOLOG - MicroStation™ System automatic reader, which provides values at two wavelengths - 590 and 750 nm. The obtained optical density values were used to: (1) calculate the average color change in the wells of the Ecoplate (Average well-color development, AWCD) and (2) calculate functional indices as indicators of the structure of the formed microbial communities.

Calculation of the average change in optical density in the wells of the plate (AWCD) was according to the methodology of Garland & Mills (1991), Urakawa et al. (2013) and Sofo & Ricciuti (2019). In cases of negative values after the correc-

tions made, they were recorded as zero (Garland, 1996) and the calculation of AWCD for each hour of reading was according to the formula of Chen et al. (2020):

$$AWCD = \sum \frac{C_i}{31}.$$

Analysis of the functional diversity of microbial communities

For the calculation of the metabolic diversity, the optical density data measured at 96 hours was used and all values above 0.250 were included in the calculations according to Sofo & Ricciuti (2019). Estimation of microbial metabolic diversity was done with utilization of several ecological indices, which was explained below.

The Shannon-Weaver index, also known as the Shannon diversity index, combines two quantifiable measures, which in the case of Ecoplate estimates richness (the number of substrates utilized by the bacterial community) and equitability (how the number of positive wells in the plate corresponds to the total number of substrates). It is a fundamental ecological measure of biodiversity that combines richness and evenness into a single value, with higher numbers indicating greater diversity. It is calculated using the formula (Jurkšienė et al., 2020):

$$H' = -\sum p_i \times (ln\ p_i)$$

where p_i is C_i, divided by the sum of C_i, wells with value ≥ 0.250.

The Shannon's evenness index measures how equally abundant different substrates are in a community, calculated by dividing the Shannon diversity index (which considers both richness and evenness) by the maximum possible diversity for that richness, yielding a value between 0 and 1, where 1 means perfect evenness and lower values indicate more dominance by a few substrates. The formula used for calculation is derived by Jurkšienė et al. (2020):

$$E = \frac{H'}{ln\ S}$$

where H' is Shannon-Wiener index, S – number of wells with value ≥ 0.250

Simpson's diversity index (D) measures biodiversity, considering both the number of substrates (richness) and their relative abundance (evenness). The value of D ranges between 0 and 1, where a higher value (closer to 1) indicates greater diversity, while a lower value (closer to 0) suggests less diversity. It is calculated by the following formula (Chen et al., 2020):

$$D = 1 - \sum P_i^2$$

where P_i is C_i, divided by the sum of C_i, wells with value ≥ 0.250.

The Margalef diversity index (d) is a measure of substrates richness that accounts for sample size. It helps compare biodiversity across different samples with higher values generally indicating greater richness. The index is calculated according to formula by Türkmen & Kazanci (2010):

$$d = \frac{(S-1)}{lnN}$$

where S – number of wells with value ≥ 0.250, N – number of substrates, i.e. 31.

McIntosh evenness index (*McI*) is an ecological metric, with higher values indicating more uniform abundance and lower values pointing to dominance by a few substrates or habitat degradation. It is derived from concepts by Shannon and Simpson, and is calculated by the formula (Xu et al., 2015):

$$McI = N - U/N - (N/\sqrt{S})$$

where U – McIntosh diversity index, N – sum of wells with value ≥ 0.250, S – number of substrates, i.e. 31.

The Gini coefficient (G) is a key measure of income or wealth inequality, ranging from 0 (perfect equality) to 1 (perfect inequality), indicating how much a distribution deviates from uniform sharing. In ecology it is widely used to measure inequality in the distribution of ecological resources, species abundance, or ecosystem services, adapting its original economic use (income inequality) to quantify disparities in areas like urban green spaces, waste discharge permits, or species sizes within populations. It assesses how far a system deviates from perfect equality (Gini=0) or total inequality (Gini=1), revealing uneven access to parks, disproportionate pollution, or skewed biomass, helping understand ecological fairness and sustainability. The formula used is derived by Weiner & Solbrig (1984) and Damgaard & Weiner (2000):

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

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).

Statistical evaluation

The mathematical processing of the data and charts were performed using Excel. The average value, standard deviation and statistical analysis were calculated using SPSS (IBM, ver.26). To establish statistically significant differences, a one-way analysis of variance (ANOVA) was applied with the outcome variable being the studied parameter and the factor - location, with the application of Tukey's criterion and a significance level of $p < 0.05$.

Results and Discussion

The dynamics of the metabolic activity of epiphytic microbial communities by average well-color development (AWCD) is presented on Fig. 2. The graph shows a short lag phase and a typical sigmoid curve of increase in optical density during the utilization of substrates in the Ecoplate. After the 48th hour until the end of the incubation period, a clear difference between microbial activity of samples collected from selected locations was observed. The highest metabolic activity showed microbial community on the leaves collected from Plot 4. At the end of the incubation period, the optical density (AWCD) at Plot 4 was significantly higher than that of microflora from the leaves collected from the other locations. In descending order, they can be presented as follows: Plot 4 (0.890 ±0.051) > Plot 1 (0.511 ±0.053) > Plot 3 (0.370 ±0.043) > Plot 2 (0.276 ±0.113).

With the exception of amine-compound utilization, for which the highest activity was observed in the epiphytic microflora on leaves from Plot 1, the utilization of all other substrate groups was higher in the sample from Plot 4 (Fig. 3–8).

Amino acids were more actively utilized by microbial communities inhabiting leaves collected from Plot 4 (Fig. 3). On the 168 hours of incubation optical density reached 1.123±0.225. The metabolic activity in other samples was almost twice lower with the lowest value for Plot 2 (0.321 ±0.229), and relatively small difference was found between Plot 3 and Plot 1, with values of 0.467 ± 0.139 and 0.498 ±0.212, respectively.

Analysis revealed that microbial community of leaves, collected from trees at the moderately polluted location (Plot 2) was not able to utilized amines. At the end of the incubation period the estimated metabolic activity was higher in samples collected from Plot 1 – 0.125 ±0.071. The metabolic activity of microbiota from the other two plots – Plot 4 with value 0.067 ±0.018 and Plot 3 with value 0.039 ±0.060 was lower, which affected also the standard deviation of the mean which can be seen on the graph (Fig. 4).

The metabolic utilization of carboxylic acids by the leaf-associated microbial community in Plot 4 exhibited a distinct exponential phase, followed by a transition into a stationary phase. At the end of the incubation period optical density reached value of 0.943 ±0.058. A slightly lower was the activity in samples from Plot 1 – 0.738 ±0.125. In contrast, the metabolic activity of microbial communities from Plot 3 and Plot 2 was comparatively low, with values of 0.389 ± 0.067 and 0.326 ± 0.136, respectively (Fig. 5).

Similar to the utilization of carboxylic acids was the trend for carbohydrates utilization in Plot 4 (Fig. 6). However, metabolic activity was comparatively higher and consistent for the whole incubation period in comparison to the other plots. As a results of this higher activity, at the end of the incubation period OD value for Plot 4 (0.882 ± 0.026) was almost twice higher than the OD estimated for Plot 3 – 0.441 ±0.079. The lowest activity was observed for Plot 2 – 0.286±0.149, while the Plot 1 was characterized with intermediate activity and OD value of 0.537 ±0.057.

Polymers were more actively utilized by microorganisms in leaf samples collected from Plot 4 (Fig. 7), where OD reached 1.036 ±0.325. Microbial communities from other plots utilized in a similar manner polymers and the difference of their optical density at the end of the incubation period was insignificant with values of 0.359±0.206 (Plot 3), 0.367±0.071 (Plot 2) and 0.452±0.077 (Plot 1), respectively.

Microbial communities of leaves collected from Plot 2 and Plot 3 were not able to utilized phenolic compounds and there OD did not exceed the base value of control well in the inoculated Ecoplates. Microorganism from Plot 4 showed the highest activity and at the end of the incubation period OD reached 0.584±0.188. For samples, collected from Plot 1, OD reached value of only 0.133±0.159. However, it is worth mentioning that the estimated activity for both plots, i.e. Plot 1 and Plot 4, was due to utilization of primarily 4-hydroxy benzoic acid. The uneven utilization of phenolic compounds of Ecoplate wells affected the standard deviation of the mean which can be observed on Fig. 8.

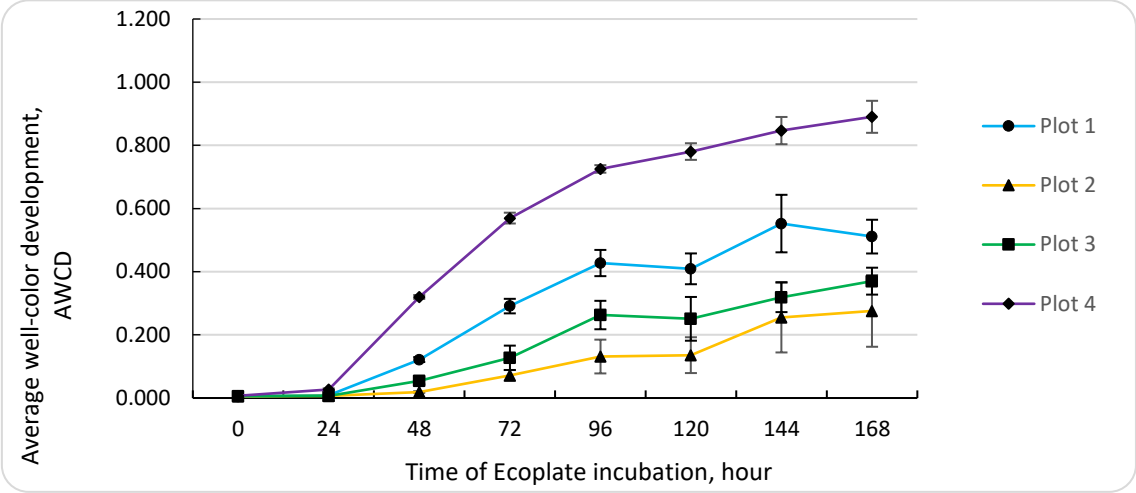

Fig. 2. Average well-color development representing the metabolic activity of epiphytic microbial communities collected from plots with varying level of pollution.

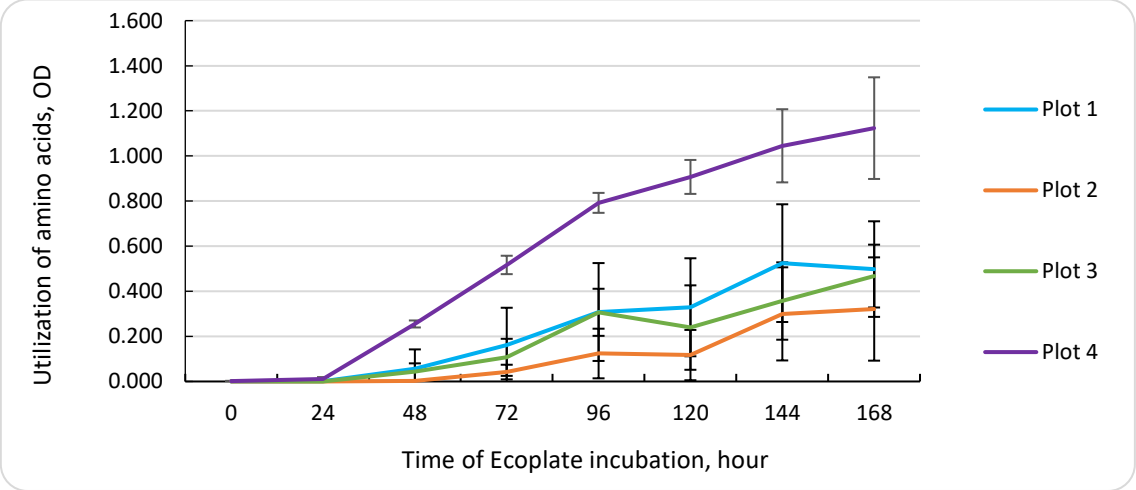

Fig. 3. Assimilation of amino acids by epiphytic microbial communities collected from plots with varying level of pollution.

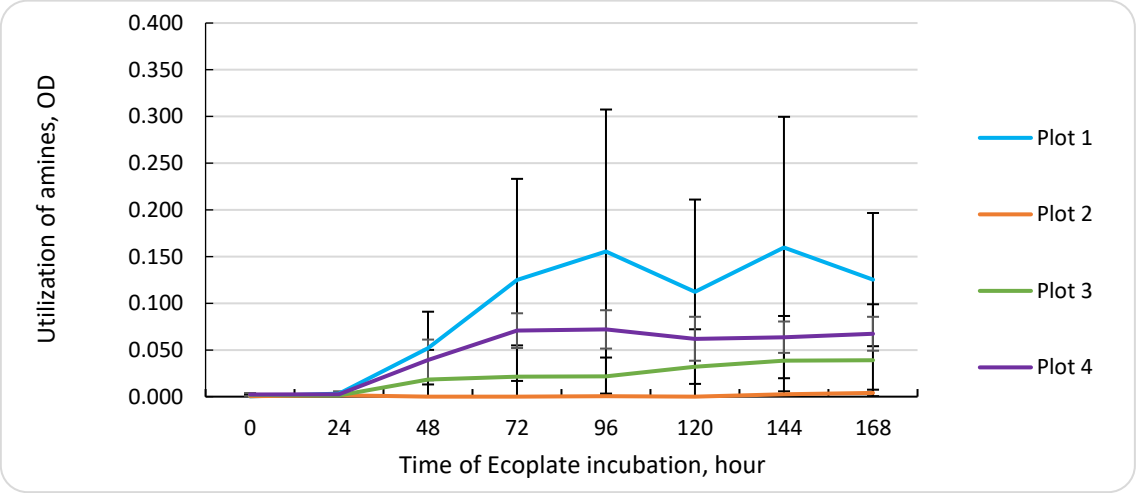

Fig. 4. Assimilation of amines by epiphytic microbial communities collected from plots with varying level of pollution.

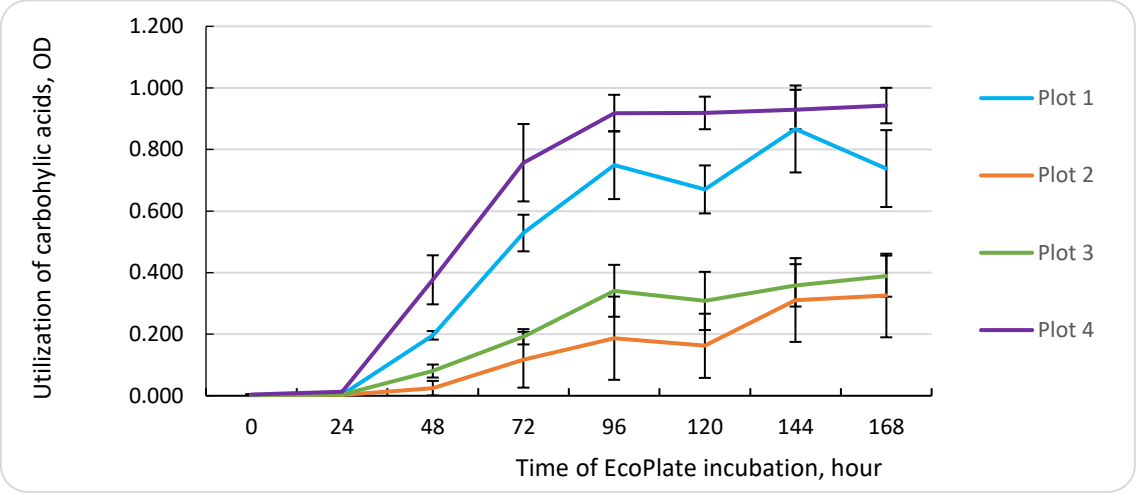

Fig. 5. Assimilation of carboxylic acids by epiphytic microbial communities collected from plots with varying level of pollution.

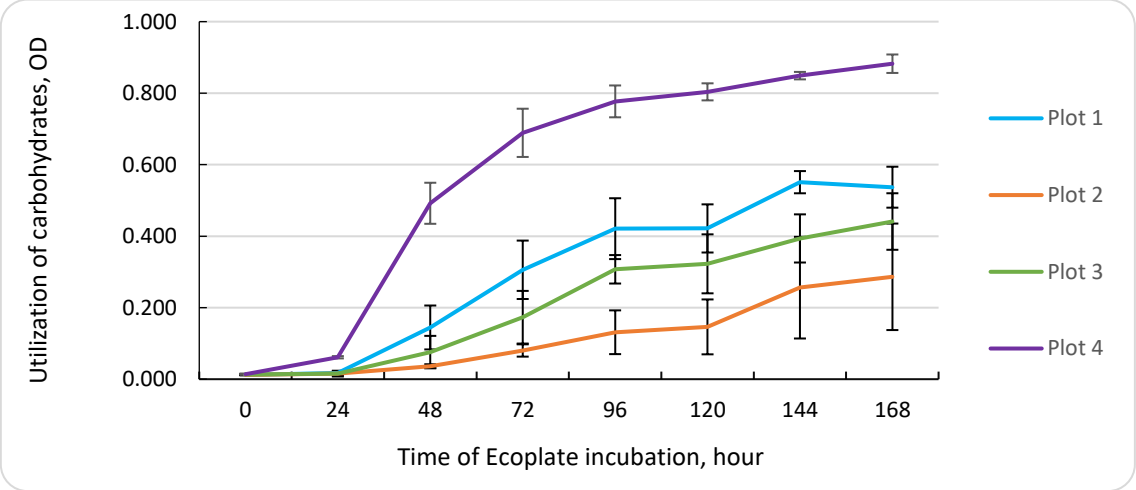

Fig. 6. Assimilation of carbohydrates by epiphytic microbial communities collected from plots with varying level of pollution.

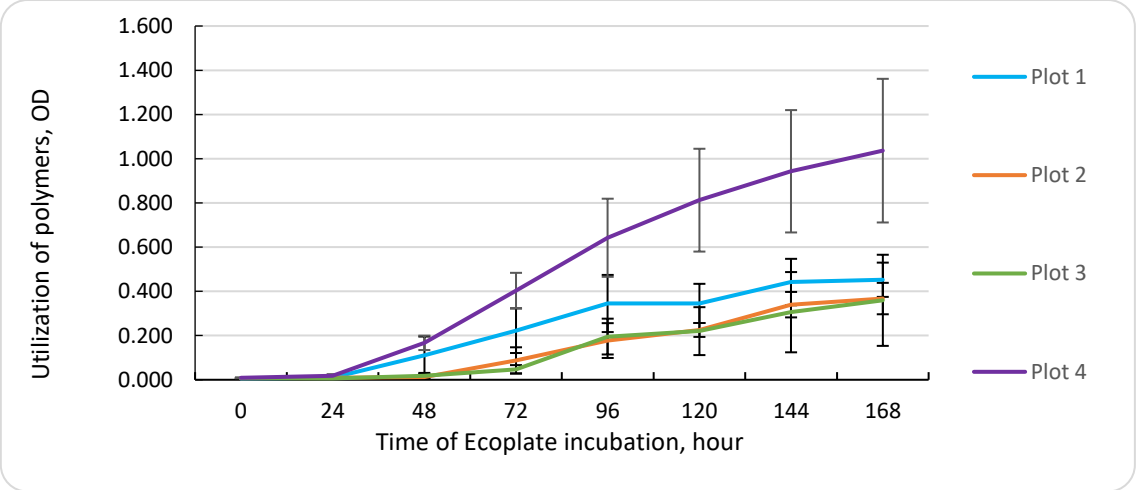

Fig. 7. Assimilation of polymers by epiphytic microbial communities collected from plots with varying level of pollution.

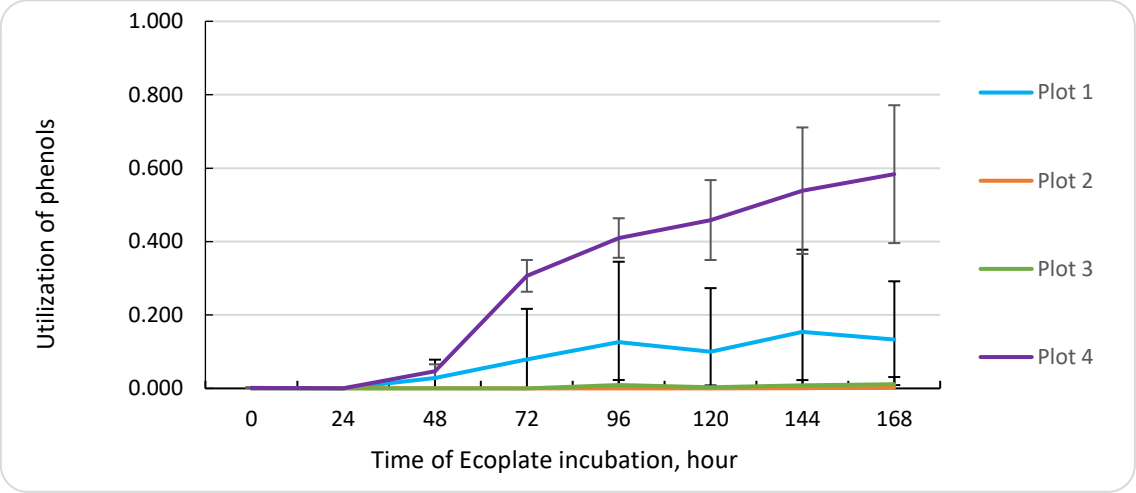

Fig. 8. Assimilation of phenolic compounds by epiphytic microbial communities collected from plots with varying degrees of pollution

The difference in the metabolic activity of the microbial communities from four locations in the city of Plovdiv can be presented also as a proportion based on the utilization of the groups of substrates present in the Ecoplate (Fig. 9). Carbohydrates were the most utilized substrates among the six groups and this was most pronounced in the samples from Plot 3 and Plot 1, both subjected to a higher influence by the traffic in comparison with the other two plots. Simultaneously, the phenolic compounds and the polymers were the less assimilated by the epiphytic microorganisms in these plots, and the level of utilization of amino acids and amines was also lower than in Plot 2 and Plot 4. These data confirm that the local urban environment shapes the microbiome of the phyllosphere of linden trees which reflect on the microbial metabolic activity and functional diversity.

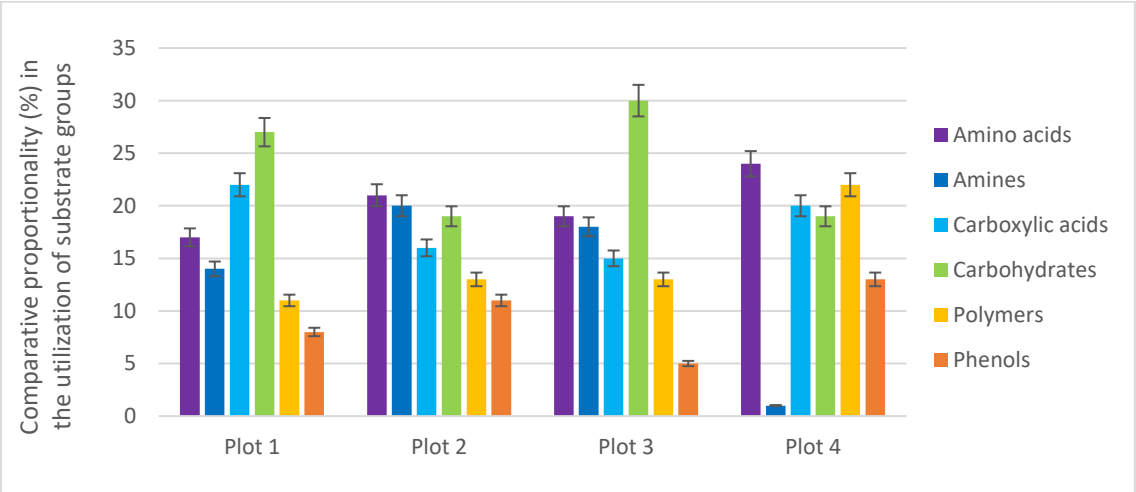

Fig. 9. Comparative proportion (%) in the utilization of substrate groups by epiphytic microbial communities collected from plots with varying degrees of pollution at the 168th hour of incubation of the Ecoplate

Table 1 presents functional indices that are used as indicators for the structure of microbial communities. Of the six functional indices presented, three show a significant difference in diversity and evenness of microbial metabolic profile at individual sampling plots. According to the Shannon index, the lowest metabolic diversity was estimated for the microbiome of Plot 2 (1.484±0.735) and the difference is statistically significant when comparing with samples from Plot 1 and Plot 4.

The Margalef index, which uses the value for the number of wells reported as positive (OD≥0.250) in the Biolog data, also indicates a significant difference between the microbiome of studied locations, with the epiphytic microflora of the leaves at Plot 4 being characterized by the greatest biodiversity, followed by Plot 1, Plot 3 and Plot 2. The Gini coefficient confirms the more even distribution of the utilization of substrates by the microflora at Plot 4, followed by Plot 1, Plot 3 and Plot 2. The functional indices confirmed that the differences in the local urban environment within four plots resulted in the adaptation of epiphytic inhabitants of phyllosphere that are specialized for a particular niche and can either have beneficial (mutualism), neutral (commensalism), or harmful (pathogenic) effects on the fitness of plants (Nair & Padmavathy, 2014).

Table 1. Functional indices for the structure of microbial communities, based on the optical density data of the 96th hour of incubation of the Ecoplates.

Plot	Functional indices					
	Shannon-Wiener index	**Shannon's evenness index**	**Simpson's diversity index**	**Margalef diversity index**	**McIntosh evenness index**	**Gini coefficient (G)**
	H′	**E**	**D**	**d**	**McI**	**G**
1	2.591^{b} ±0.040	0.957 ±0.014	0.918 ±0.004	4.077^{c} ±0.291	0.286 ±0.006	0.475^{b} ±0.008
2	1.484^{a} ±0.735	0.914 ±0.232	0.815 ±0.018	1.165^{a} ±0.583	0.404 ±0.178	0.645^{c} ±0.059
3	2.304^{ab} ±0.085	0.962 ±0.009	0.892 ±0.029	2.912^{b} ±0.291	0.329 ±0.015	0.484^{b} ±0.042
4	3.069^{b} ±0.073	0.979 ±0.002	0.951 ±0.085	6.407^{d} ±0.505	0.221 ±0.007	0.292^{a} ±0.043

In general, the use of Biolog with plant materials is not very common approach. However, there are several studies that justified the use of the method for estimation of epiphytic microbial communities (Ellis et al., 1995; Heuer & Smalla, 1997; Feckler et al., 2016). The study of Heuer & Smalla (1997) evaluated for the potato phyllosphere with Biolog GN microplates. The important contribution of that work is that by investigating reproducibility, sensitivity, and impact of different potato phyllosphere populations on the catabolic profiles authors concluded that variability of the BIOLOG pattern was mainly caused by the phyllosphere heterogeneity. As a result, the method can be considered reliable for estimation of difference between various plant-associated microbial communities (Heuer & Smalla, 1997). Similarly, Ellis et al. (1995) reported that the observed carbon-utilization patterns allowed the grouping of communities according to the habitats from which they were isolated.

Yang et al. (2001) who examined the phyllosphere of leaves from field grown plant species applied a combined methodological approach. Suspensions from the positive Biolog wells were subjected to DGGE analysis. The results showed that epiphytic microbial communities were strongly shaped by the host plant. Even when identical carbon sources were supplied in the BIOLOG wells, the DGGE banding patterns differed among tree samples, indicating that each plant harbors a distinct microbial community. In contrast, when different carbon sources were tested within the same phyllosphere sample, the DGGE profiles remained highly similar, suggesting that a stable microbial community within a single plant can utilize a range of carbon substrates.

Feckler et al. (2016) evaluated the functional properties of microbial communities associated with leaf material either treated or not with the fungicide epoxiconazole. They observed consistently higher metabolization of all C-substrates in exposed communities compared with the control. Carbohydrates, carboxylic acids, and amino acids were the C-guilds contributing most to these differences. However, after 72 hours, the metaboliza-

tion of complex carbon sources, phosphate-containing substrates, and amines became similar between the two treatments, i.e., the control and the fungicide-treated samples. Overall, the findings of Feckler et al. (2016) indicated higher metabolization of carbohydrates and amino acids in the presence of the pesticide compared with uncontaminated reference locations. Conducting a principle component analysis (PCA), Cai et al. (2010) found a link between the decrease in the utilization of carbohydrates, carboxylic acids, amino acids and amines and crown health. The changes in physiological responses of microbial communities were related to declining tree health.

Our findings correspond well to the results of Gandolfi et al. (2017), who studied microbial diversity on the leaves of *Platanus* trees and revealed that the location (urban vs. rural) caused the greatest variation. By this study, they suggested that the pollutants into the atmosphere control the choice of air pollutant-degrading bacteria in a plant's phyllosphere. In general, the urban areas have larger concentrations of hydrocarbon-degrading bacteria due to a significant exposure of plant leaves to the pollutants, which correlates with our results from Plot 1 and Plot 3. The possible explanation of the observed trend is that the phyllosphere microbiome is affected by the atmospheric air pollution and particular bacterial taxa are more or less stimulated depending upon the type of pollutants. In the phyllosphere, endophytic and epiphytic microorganisms interact with the host in the aerial parts of urban trees, adapting to the local environment and depending directly on physicochemical, biotic, and abiotic factors and constraints (Sivakumar et al., 2020).

Conclusions

The metabolic pattern of the epiphytic microbiota of urban trees is more diverse due to the exposure of the phyllosphere to the external environment with significant dynamic. The estimated total and substrate metabolic activity of epiphytic communities from leaves collected from the four locations shows higher activity of the microflora at Plot 4 (lowest anthropogenic load). An individual-location specific curve of optical density change during substrates assimilation was observed, supposing significant differences in microbiome composition. Most of the analyzed functional indices confirm the higher biodiversity and better distribution of the assimilation of the substrates in the epiphytic microflora of the leaves from Plot 4. It was found that microorganisms in the two plots more strongly influenced by traffic (Plot 1 and Plot 3) had a higher rate of carbohydrate assimilation and a lower rate of phenolic compound utilization compared to the other two plots. In this regard, our results could take place in some nature-based solutions which relies on biological techniques for remediation, with a focus on the converging/degradation/reduction of pollutants by plants and the accompanying microorganisms that inhabited different plant organs. The bioremediation of air pollutants by the phyllosphere, could be regarded as a promising approach to address urban air pollution, so discovering the plant-microbes' interactions and their common response to the local environmental factors is crucial.

Acknowledgements

This research was funded by the Centre for Science Research, Technology Transfer, and Protection of Intellectual Property at the Agricultural University – Plovdiv, within the scope of Project 04-24. The authors gratefully acknowledge the support of the project BG16RFPR002-1.014-0012-C01 "Establishment and sustainable development of a Center of Competence AgriFood Systems and Bioeconomy", financed by the European Regional Development Fund through the "Program for Research, Innovation and Digitalisation for Smart Transformation" (PRIDST).

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Received: 29.11.2025
Accepted: 25.01.2026