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Effects of powdery mildew (*Sawadaea bicornis* (Wallr.) Miyabe) on photosynthetic process in Tatarian maple (*Acer tataricum* L.)

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Abstract. Damage to woody plants caused by phytopathogenic fungi in urban environments leads to disturbances in photosynthetic processes and reduced plant vitality, which necessitates the search for effective methods for the early diagnosis of physiological changes. The aim of this study was to determine the effectiveness of the chlorophyll fluorescence induction (CFI) method for assessing the functional state

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of the photosynthetic apparatus in leaves of *Acer tataricum* L. infected with *Sawadaea bicornis* (Wallr.) Miyabe. The study was conducted in September 2025 in the city of Dnipro using a portable fluorometer "FLS 10s" to diagnose disturbances in native chlorophyll photosynthesis in living leaves of *A. tataricum*. Measurements were performed on completely undamaged leaves and leaves affected by powdery mildew by more than 50% within the same tree, followed by analysis of the Kautsky curve parameters. The degree of leaf blade damage by phytopathogen was determined visually. Fo, Fm, Fv, the ratio of Fv/Fo and Fv/Fm, as well as the indices (Fm – Fst)/Fst and (Fp –Fo)/Fv were revealed as the most significant parameters. Analysis of these indicators allowed to identify specific changes associated with pathogenic exposure and assess the degree of photosynthetic activity violation. The results obtained indicate that CFI method using the portable device "FLS 10s" is an effective technique for rapid diagnostics of the plant general condition, and allows to perform timely detection of the negative effect of biotic factors. Thus, the analysis of chlorophyll fluorescence parameters can be considered as a powerful and reliable tool for determining the degree of parasitic fungi influence on the host plant and assessing its functional state in urbanised environment

Keywords: plant fungal diseases; fluorescence induction; urban ecosystems; photosynthetic apparatus; biosensors

INTRODUCTION

Urban trees are increasingly exposed to combined climatic and anthropogenic pressures that alter their physiological condition, reduce their vitality, and complicate the management of green spaces. Climate change-induced aridisation affect negatively the growing conditions of trees, restricting their ability to perform environmental and social functions (Blanco *et al*., 2021). A qualitative assessment of the response of trees to extreme climatic conditions and related changes in their growing sites is an important aspect of urban green space management, as noted by O.M. Kunakh *et al*. (2022). In addition, the urban environment creates a wide range of stressors that have a serious negative impact on vegetation. B. Cui *et al*. (2021) argued that trees growing within urbanised space are affected by multiple unfavourable factors: air pollution from exhaust gases, heavy metal accumulation in the soil, and specific aerodynamics of the city. According to C.E. Aucique-Perez & A.E. Román Ramos (2024), this causes depletion of plant organisms, making them more vulnerable to disease (viral, bacterial, fungal) and pest damage. Despite this, urban ecosystems need to enrich plantings and expand their species composition. In such conditions, the care of green spaces should include modern methods of rapid plant health diagnostics, specifically chlorophyll fluorescence induction (CFI). The CFI method constitutes a critical analytical tool for assessing the physiological status and photosynthetic performance of plants subjected to environmental stressors, as emphasised by Y. Liu *et al*. (2024) and B. Park *et al*. (2024). Maples are some of the most valuable in green construction, forestry, and forest stands among the deciduous tree species growing in the temperate zone. D. Korányi *et al*. (2022) pointed out, that in urban development, plants of the genus *Acer* L. are widely used for construction of park and forest-park phytocenoses, in cluster and single plantings, in landscaping city squares, avenues, and boulevards. Maples are widely considered premier landscaping trees used in industrial, residential and recreational areas due to its high decorative qualities with a seasonal aspect. Their massive crown can perform a sanitary and hygienic function, adsorbing dust and aerogenic pollutants through the leaf surface structures. Today, the species composition of maples is very limited in plantings of various functional purposes, and especially in industrial areas. In Ukraine, Tatarian maple is one of the most common species of the genus *Acer* used in urban landscaping. This is a frost-resistant, shade-tolerant woody species highly tolerated to drought and soil salinity, and is also a nectar-bearing plant. However, like as other species of the genus *Acer*, Tatarian maple is more vulnerable to pathogens in urban conditions. A study by A. Alexeyeva *et al*. (2024) demonstrated that damage by phytopathogenic fungi reduces the decorative effect of maples, and also results in violation of photosynthetic and transpiration

processes, causing drying and death of both individual branches and entire trees.

Sawadaea bicornis (Wallroth) Miyabe 1937 is a parasitic fungus causing powdery mildew on young seedlings and adult plants of the genus *Acer*; it is one of the most common plant diseases. At the leaf level, effect of powdery mildew infection strongly depends on the infection time and severity, since the leaf susceptibility to phytopathogen depends on their age. Young foliage and tops of shoots are more vulnerable to damage by *S. bicornis*; so, powdery mildew initially occurs on the tops of plants. In case of severe infection, the foliage and stems are covered with coating followed by their yellowing and drying up. According to R.A. Ramut & W. Pusz (2023), powdery mildew is one of the most destructive plant diseases, especially in the early ontogenesis stages, leading to stunted growth of seedlings, significantly reduced winter hardiness, causes complete premature leaf drop and significantly weakens the plant. Photosynthesis is a universal process of plant survival, and immune defence is a key process of plant adaptation to growing conditions. Various studies have shown that these two processes are interconnected in a complex network. Photosynthesis can influence signalling pathways and provide materials and energy for immune defence, while the immune defence mechanisms can also have feedback effects on photosynthesis (Cheaib & Killiny, 2025).

Modern techniques allow obtaining information on the state of the plant photosynthetic health and assessing the level of environmental factors impact on the plants (Chang *et al.*, 2025). The important advantage of these techniques over others is that they can determine even minor alterations in photosynthetic apparatus activity, and can be applied in the field, quite rapidly and simply. The technique of chlorophyll fluorescence induction is based on detection of changes in chlorophyll fluorescence under intense light following short period of dark adaptation. This technique is enough sensitive and allows to perform a rapid access of alterations in photosynthetic apparatus under the stress and the regeneration potential of intact plants. Thus, it can be used in large-scale ecological research and benefits forestry, agriculture, horticulture and arboristics (Swoczyna *et al.*, 2022). The purpose of the paper was to establish the effectiveness of applying a technique based on estimating the intensity of chlorophyll fluorescence induction to determine the functional state of the photosynthetic apparatus in *S. bicornis*-affected foliage of *A. tataricum*.

MATERIALS AND METHODS

The study was a field comparative study and was aimed at assessing the impact of the biotrophic pathogen *Sawadaea bicornis* (Wallr.) Miyabe on the functional state of the photosynthetic apparatus of *Acer tataricum* L. leaves. The object of the study was the leaves of the Tatar maple, and the subject was changes in the parameters of chlorophyll fluorescence induction upon powdery mildew infection. The study design involved comparing chlorophyll fluorescence indices in unaffected and infected leaves within the same trees. The research presented was conducted during the 2025 growing season in Dnipro city. The region's climate has distinct continental features, and is characterised by seasonal high-temperature droughts and dry, hot winds. A small average precipitation amount (472 mm) decreases in dry years to 250 mm, and the total annual evaporation exceeds annual precipitation by 2-3 times. Here, woody plants grow in conditions of ecological inconsistency and show high sensitivity even to minor changes in climatic factors, as well as to anthropogenic pollution. The Botanical Garden of Oles Honchar Dnipro National University was founded on Dnipro city territory (48°26' N, 35°02' E; 127 m above sea level) in 1931. In the Garden, *A. tataricum* is cultivated, that naturally grow in the Steppe zone of Ukraine. The soil cover is represented by urban soil developed on zonal ordinary low-humus middle loamy chernozems on loess-like loams (Lovynska *et al.*, 2022).

To study the impact of powdery mildew on photosynthesis processes, 10 *A. tataricum* trees, about 10 years old, growing under the same conditions on the territory of the Botanical Garden of Oles Honchar Dnipro National University, were selected. The trees were characterised by a satisfactory general condition, similar morphometric indicators and the same level of crown illumination. The selection of trees was purposeful and was carried out within the same area of plantations. The measurements were carried out in September 2025, which corresponded to the period of maximum activity of *S. bicornis* development on the leaves of Tatar maple. From each tree, 10

completely unaffected and 10 affected leaves were selected, thus the total number of leaves examined was 200. Comparisons of affected and unaffected leaves were performed within the same trees (paired study design). The analysis included fully formed leaves of the same phenological stage without mechanical damage or signs of additional stress. Leaves with visually pronounced development of powdery mildew mycelium on more than 50% of the leaf blade area were considered affected. The degree of damage was determined visually. Leaves were collected from the middle part of the crown on the illuminated side of the tree with the same orientation relative to the light source. Measurements were performed in situ on live leaves between 12:00 and 13:00 under conditions of maximum natural light. Light intensity was measured using a PCE-174 lux meter (PCE Instruments, Germany). Temperature and humidity were recorded using an HE-173 thermohygrometer (Huato Electronic Co. Ltd, China).

To diagnose photosynthetic process disorders, a portable fluorimeter "FLS 10s" (manufacturer: V.M. Glushkov Institute of Cybernetics of the National Academy of Sciences of Ukraine) was used. Before measurement, the leaves were subjected to dark adaptation for 300 s. Chlorophyll fluorescence induction was recorded over a 10-second interval, during which the device automatically generated a Kautsky curve from 470 consecutive signal measurements, in accordance with the method of O. Voronenko (2024). To interpret the Kautsky curve, its established critical parameters were utilised, including Fo – the initial value of fluorescence induction measured immediately after switching on the illumination; Fp – the "plateau" of chlorophyll fluorescence induction; Fm – the maximum value of chlorophyll fluorescence induction; Fst – the steady-state value of chlorophyll fluorescence induction reached after light adaptation of the plant leaf. In addition to the critical parameters of the Kautsky curve, the following calculated variables were employed: Fv = Fm – Fo – variable chlorophyll fluorescence; Fv/Fm – maximum quantum efficiency of photosystem II (PSII); Fv/Fo – maximum yield of PSII primary photochemistry; (Fm – Fst)/Fst – coefficient of photochemical processes efficiency; (Fp – Fo)/Fv – the share of QA acceptors in non-renewable reaction centres PSII (Kautsky & Hirsch, 1931; Kalaji *et al.*, 2016). Statistical comparisons between damaged and undamaged leaves were performed separately for each chlorophyll fluorescence and environmental parameter using linear mixed-effects models. Leaf condition (damaged vs undamaged) was treated as a fixed effect, whereas tree identity was included as a random intercept to account for non-independence among leaves sampled from the same tree. Models were fitted using restricted maximum likelihood, and the significance of leaf condition was assessed by analysis of variance with Satterthwaite's approximation. Model assumptions were evaluated using residual diagnostic plots (Q-Q plots and residual vs fitted plots), and no major violations of normality or homogeneity of variance were detected. For each parameter, the estimated mean difference (undamaged – damaged) ±SE, t-values, F-values, p-values, and marginal R^2 were extracted. To account for multiple comparisons among fluorescence parameters, p-values were additionally adjusted using the Benjamini-Hochberg false discovery rate procedure. All statistical analyses were conducted in R Core Team (n.d.) using the packages lme4, lmerTest, emmeans, and performance.

RESULTS AND DISCUSSION

Undamaged tree leaves showed higher values of the main chlorophyll fluorescence parameters, including Fo, Fm, Fp, Fst, and Fv, as well as the ratios Fv/Fo and Fv/Fm compared to damaged leaves (Fig. 1). The most pronounced differences were observed for Fm, Fp, Fst, and Fv, indicating that leaf damage was associated with a marked decline in the functional activity of the photosynthetic apparatus. In particular, lower Fm and Fv values in damaged leaves suggest a reduced capacity of photosystem II for excitation energy capture and photochemical conversion. The decrease in Fv/Fm further indicates a reduction in the potential quantum efficiency of photosystem II, which is commonly interpreted as a sign of physiological stress or photoinhibition. The variability within groups differed among parameters. For the main fluorescence parameters, damaged and undamaged leaves showed some overlap, but their medians and interquartile ranges were clearly shifted, indicating a consistent effect of leaf condition rather than only the influence of individual outliers. Greater within-group variability may reflect differences in the degree of leaf

injury, local microenvironmental conditions, leaf age, or tree-level heterogeneity. This supports the use of tree identity as a random effect in the mixed models.

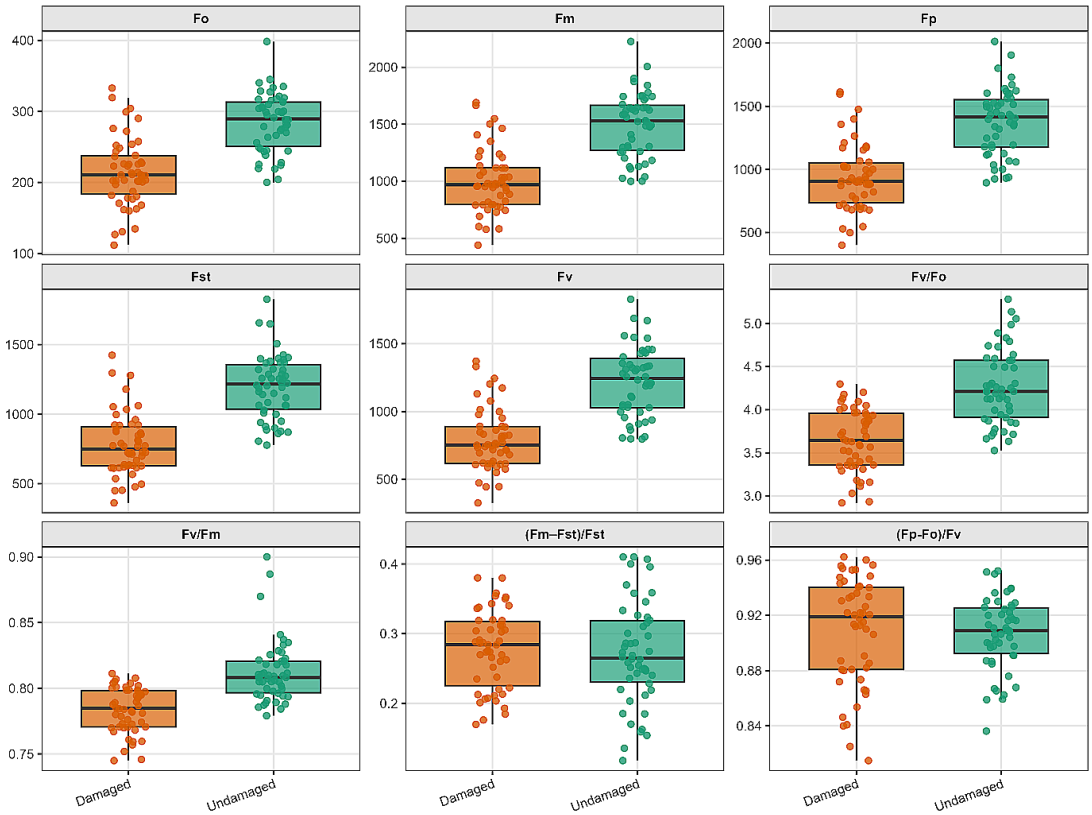

Figure 1. Variation in chlorophyll fluorescence parameters between damaged and undamaged leaves of *A. tataricum*

Source: developed by the authors

The stronger response of Fm, Fst, and Fv may be explained by their direct dependence on the functional state of the chlorophyll fluorescence emission and PSII activity. In contrast, the derived indices (Fm – Fst)/Fst and (Fp – Fo)/Fv showed substantial overlap between damaged and undamaged leaves and were not statistically significant. This may indicate that these ratios are less sensitive to the type of damage observed in this study, or that proportional relationships among fluorescence components were partly preserved despite the overall reduction in absolute fluorescence values. The observed differences are not only statistically significant but also physiologically meaningful. They indicate that visible leaf damage was accompanied by a decline in photosynthetic performance, especially in parameters related to PSII efficiency and variable fluorescence. At the same time, the absence of significant changes in some derived indices suggests that damage affected the overall intensity and efficiency of fluorescence responses more strongly than the relative balance among selected fluorescence components. The statistical analysis showed significant effects of leaf damage on Fo, Fm, Fp, Fst, Fv, Fv/Fo, and Fv/Fm (all $p < 0.001$) (Table 1). Positive mean differences (undamaged – damaged) indicated that these parameters were higher in undamaged leaves vs damaged ones. The strongest effects were observed in Fst, Fv, and Fm, as reflected by the highest F-values and coefficients of determination ($R^2 = 0.45, 0.44,$

and 0.43, respectively). In contrast, no significant effect of leaf damage was found for the derived indices (Fm – Fst)/Fst and (Fp – Fo)/Fv (p = 0.66 and 0.76, respectively).

Table 1. Comparison of chlorophyll fluorescence parameters between undamaged and damaged leaves of *A. tataricum*

Parameter	(Undamaged – damaged) ± SE	t-value	F-value	p-value	R^2
Fo	69.72 ± 7.92	8.81	77.56	<0.001	0.37
Fm	493.96 ± 50.95	9.70	94.01	<0.001	0.43
Fp	436.60 ± 48.38	9.02	81.43	<0.001	0.40
Fst	416.20 ± 41.26	10.09	101.76	<0.001	0.45
Fv	428.84 ± 44.03	9.74	94.84	<0.001	0.44
Fv/Fo	0.62 ± 0.07	8.36	69.87	<0.001	0.37
Fv/Fm	0.03 ± 0.00	7.18	51.48	<0.001	0.32
(Fm – Fst)/Fst	-0.01 ± 0.01	-0.44	0.19	0.66	0.00
(Fp – Fo)/Fv	-0.00 ± 0.01	-0.31	0.10	0.76	0.00

Note: application of the Benjamini-Hochberg correction for multiple comparisons did not alter the significance pattern of the results

Source: developed by the authors

Slightly higher temperature values were associated with undamaged leaves, whereas damaged leaves tended to appear under somewhat higher humidity (Fig. 2). Light intensity varied broadly in both groups and showed substantial overlap, although undamaged leaves were characterised by a slightly higher median and a wider range of values. Overall, the distributions of the environmental parameters overlapped considerably between damaged and undamaged leaves, indicating only moderate separation between the two leaf conditions.

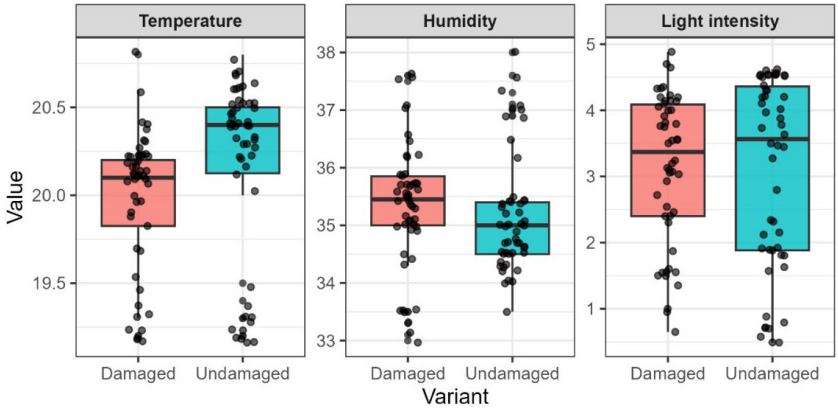

Figure 2. Variation in temperature, humidity, and light intensity parameters between damaged and undamaged leaves of *A. tataricum*

Source: developed by the authors

The assessment of temperature, air humidity, and light intensity was necessary to account for the possible influence of microclimatic conditions on chlorophyll fluorescence parameters, since these factors directly affect the photochemical activity of photosystem II and the course of photosynthetic processes. Temperature may alter the rate of electron transport, the activity of Calvin cycle enzymes, and the level of thermal energy dissipation, whereas air humidity affects stomatal conductance and transpiration. Light intensity determines the degree of excitation of the

photosynthetic apparatus and is one of the key factors influencing the parameters of the Kautsky curve. Statistical analysis identified temperature as the only environmental variable parameter significantly associated with leaf condition (Table 2). The positive mean difference (undamaged – damaged = 0.19 ± 0.04) indicated slightly higher temperatures for undamaged leaves, and this effect was significant ($p < 0.001$), although the explained variance was low ($R^2 = 0.04$). In contrast, humidity and light intensity were not significantly affected by leaf condition ($p = 0.32$ and 0.60, respectively), indicating substantial overlap between damaged and undamaged leaves for these parameters.

Table 2. Comparison of temperature, humidity, and light intensity parameters between undamaged and damaged leaves of *A. tataricum*

Parameter	(Undamaged – damaged) ± SE	t-value	F-value	p-value	R^2
Temperature	0.19 ± 0.04	4.54	20.59	< 0.001	0.04
Humidity	-0.15 ± 0.15	-1.00	1.00	0.32	0.00
Light intensity	-0.08 ± 0.15	-0.53	0.28	0.60	0.00

Note: application of the Benjamini-Hochberg correction for multiple comparisons did not alter the significance pattern of the results

Source: developed by the authors

Over the past decade, the research has increasingly focused on using changes in CF parameters to interpret plant physiological mechanisms. The CF technique is used not only to determine the plant response to stress factors, but also as an indicator of feedback when developing strategies for their mitigation. The analysis of chlorophyll fluorescence (CF) changes revealed a distinct effect of the biotrophic pathogen *S. bicornis* on individual fluorescence induction (CFI) indicators of *A. tataricum* leaves. Furthermore, this effect extended to the coefficients characterising the phases of light-dependent photosynthesis and the efficiency of photochemical processes underlying the dark-dependent stages of light energy absorption. The results obtained indicate a negative effect of powdery mildew infection on the photosynthetic activity in Tatarian maple. Under conditions of biotic stress, CO_2 uptake by the host plants changes significantly due to pathogenic infection, regardless of the pathogen lifestyle (Silva *et al.*, 2021). Biotrophic pathogens grow and feed off living plant tissues, colonising cells through mechanisms that inhibit plant defence responses. This strategy, known as obligate parasitism, involves establishing molecular and physiological interactions with the host cell, which provides the pathogen with energy for growth (Salcedo-Sarmiento *et al.*, 2021; Mapuranga *et al.*, 2022). During the host plant-pathogen interaction, the affected plants show a decrease in CO_2 assimilation, dynamic stomatal response (in particular, stomatal closure), chronic inhibition of photosystems, increased energy dissipation manifested as chlorophyll *a* fluorescence (non-photochemical quenching), changes in the content of photosynthetic pigments, structural damage to chloroplasts, a decrease in the leaf area index, a violation of the source/absorber ratio, as well as changes in the expression profiles of genes, proteins and metabolites associated with photosynthesis. Pathogenic infection of leaf tissue reduces light conductivity due to cell damage, which negatively affects photosynthetic activity and causes the development of chlorotic and necrotic spots (Cheaib & Killiny, 2025). In particular, coffee and eucalyptus, being affected by biotrophic fungi, such as *Hemileia vastatrix* and *Austropuccinia psidii* respectively, showed a decrease in CO_2 assimilation by 20-50% subsequent to manifestation of symptoms. At the same time, a significant inhibition of stomatal conduction and transpiration rate by 30-50% was found in the leaves of affected plants (Einhardt *et al.*, 2020; Salcedo-Sarmiento *et al.*, 2021). L. Shupranova *et al.* (2022) found that feeding by *Parectopa robiniella* larvae significantly altered antioxidant enzyme activities and soluble protein content in *Robinia pseudoacacia* leaves under urban industrial conditions, with variable responses depending on tree age and pollution exposure, indicating substantial biochemical stress effects of this invasive phytophage. I. Ivanko *et al.* (2025) demonstrated that infection of *Quercus robur* seedlings by the foliar pathogen Erysiphe alphitoides significantly

disrupted photosynthetic processes, as evidenced by pronounced alterations in Kautsky chlorophyll fluorescence parameters, indicating severe impairment of the photosynthetic apparatus in infected leaves compared with healthy controls.

The results of this research also indicated a significant decrease in Fo and Fm parameters of chlorophyll fluorescence induction in Tatarian maple leaves covered with *S. bicornis* mycelium by 50% or 75% of the area. A decrease in the Fv parameter, defined as the difference between Fm and Fo, is associated with a violation of the energy transformation in photosystem II, as well as with partial closure of reaction centres. This leads to a fragmented disruption of their functioning in the process of converting light energy into chemical potential (Stefanov *et al.*, 2022), which indicates a decrease in photosynthetic competence per unit of chlorophyll and, accordingly, a decrease in PSII activity. Studies aimed at analysing energy dissipation using CF have made it possible to better understand the physiological processes that occur in both the asymptomatic and symptomatic stages of the disease development. According to Q. Xia *et al*. (2023), the Fv/Fm index is widely used as one of the most common parameters of chlorophyll a fluorescence for assessing the stress level caused by abiotic and biotic factors. In the present research, powdery mildew-caused biotic stress led to a significant decrease in this indicator. Under normal conditions, the Fv/Fm value for undamaged leaves was about 0.80-0.83, while its decrease indicates damage to component of the PSII reaction centres that cause photoinhibition of the photosynthetic apparatus and is often accompanied by the oxidative stress development, as noted by B.O. Calvo *et al*. (2025). Thus, the Fv/Fm parameter is a reliable indicator of photochemical activity and the general physiological state of plants. A. Cheaib & N. Killiny (2025) emphasised that in phytopathology, its use has proven effective for assessing the degree of damage to the photosynthetic apparatus, depending on the type of pathogen. Another relevant indicator of PSII efficiency is the maximum quantum yield of water photolysis, Fv/Fo. This parameter is more sensitive to stress effects, since it is normalised by the Fo, and not by the Fm, as in the case of Fv/Fm. A decrease in Fm and an increase in Fo cause significant changes in Fv/Fo, even when the changes in Fv/Fm are less pronounced (Jat *et al*., 2024).

The efficiency ratio of dark-dependent photochemical processes (Fm – Fst)/Fst is also an important indicator of the leaf functional state. It reflects the level of fluorescence quenching developed under the influence of both photochemical processes (CO_2 fixation) and non-photochemical mechanisms (thermal energy dissipation in excited chlorophyll molecules). The parameter (Fm – Fst)/Fst is closely correlated with the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase, a key enzyme of the Calvin cycle, and reflects the adaptive capacity of plants to environmental conditions, which is consistent with the findings of X. Song *et al*. (2023). Based on the Fm, Fst indicators and calculated parameter (Fm – Fst)/Fst, which determine the shape of the descending part of the Kautsky curve, the activation and flow of Calvin cycle reactions and the transport of substances through the leaf membranes and vessels might be argued to be more efficient in *A. tataricum* plants not affected by powdery mildew (undamaged). Being parameter that characterises the relative proportion of inactive reaction centres to their total number, photosynthetic rate (Fp – Fo)/Fv reflects the saturation rate of inactive PSII centres responsible for water photolysis and oxygen release, according to O. Vasylenko *et al*. (2021). An increase in the value of this parameter indicates a violation of both energy migration and electron transport, while its decrease may indicate an acceleration of electron transport processes. The results confirmed the high sensitivity and informative value of CF/CFI methods in studies of plant-pathogen interaction, and indicate the feasibility of their use for early diagnosis of biotic stress, plant phenotyping and development of effective approaches to the control of phytopathogens in combination with other physiological and biochemical methods of analysis.

CONCLUSIONS

The damage of the *A. tataricum* foliage by biotrophic pathogen *S. bicornis* was found to be accompanied by a significant decrease in the main CFI parameters (Fo, Fm, Fp, Fst, Fv) and indicators of photochemical efficiency of PSII (Fv/Fo and Fv/Fm); it indicates the functional state inhibition of the photosynthetic apparatus. The parameters Fst,

Fv and Fm were the most sensitive to pathogenic damage, in which the highest values of variance and statistical significance were recorded ($p < 0.001$; $R^2 = 0.43$-0.45); it indicated their high informative value in assessment of plant physiological state. A decrease in variable fluorescence (Fv) and the FV/Fm ratio confirms a violation of the efficiency of converting light energy into PSII and partial inactivation of reaction centres, which is indicative of the development of photoinhibition under the influence of biotic stress. The derived parameters (Fm – Fst)/Fst and (Fp – Fo)/Fv showed no statistically significant changes between damaged and undamaged leaves ($p > 0.05$), which indicates their lower sensitivity to pathogen action under the research conditions. Among environmental factors, only temperature had a significant, though weak, effect on leaf condition ($p < 0.001$; $R^2 = 0.04$), while humidity and light intensity showed no statistically significant differences between the variants. The technique of chlorophyll fluorescence induction was proved to be an effective tool for diagnosing *S. bicornis*-caused disorders of the photosynthetic apparatus in *A. tataricum*, and it might be used for early detection of biotic stress and assessment of the physiological state of plants in urban ecosystems. Further research should focus on evaluating seasonal dynamics of chlorophyll fluorescence parameters and integrating fluorescence analysis with biochemical and molecular markers of plant stress resistance.

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Особливості впливу борошнистої роси (*Sawadaea bicornis* (Wallr.) Miyabe) на процес фотосинтезу клену татарського (*Acer tataricum* L.)

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Анотація. Ураження деревних рослин фітопатогенними грибами в умовах урбанізованого середовища призводить до порушення фотосинтетичних процесів і зниження життєздатності рослин, що зумовлює необхідність пошуку ефективних методів ранньої діагностики фізіологічних змін. Метою роботи було встановлення ефективності застосування методу індукції флуоресценції хлорофілу (ІФХ) для оцінки функціонального стану фотосинтетичного апарату листків *Acer tataricum* L., уражених *Sawadaea bicornis* (Wallr.) Miyabe. Дослідження проводилося у вересні 2025 році на території м. Дніпро із використанням портативного флуориметра «FLS 10s» для діагностики порушення фотосинтезу нативного хлорофілу в живих листках *A. tataricum*. Вимірювання виконували на абсолютно не уражених та уражених понад 50 % борошнистою росою листках клену татарського у межах одного дерева з подальшим аналізом параметрів кривої Каутського. Ступінь ураження листкової пластинки фітопатогеном визначали візуально.

Найбільш значущими параметрами виявилися Fo, Fm, Fv, співвідношення Fv/Fo та Fv/Fm, а також індекси (Fm – Fst)/Fst і (Fp – Fo)/Fv. Аналіз цих показників дозволив виявити специфічні зміни, пов'язані з патогенним впливом, та оцінити ступінь порушення фотосинтетичної активності. Отримані результати свідчать, що метод ІФХ із використанням портативного приладу «FLS 10s» є ефективним підходом для швидкої діагностики загального стану рослин і дає змогу своєчасно виявляти негативний вплив біотичних чинників. Таким чином, аналіз параметрів флуоресценції хлорофілу можна розглядати як потужний і надійний інструмент для визначення ступеня впливу паразитичних грибів на рослину-хазяїна та оцінки її функціонального стану в урбанізованому середовищі

Ключові слова: грибкові захворювання рослин; індукція флуоресценції; міські екосистеми; фотосинтетичний апарат; біосенсори

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Identification of lipopeptide synthesis genes by RT-qPCR in endophytic strains *Bacillus pumilus* and *Bacillus amyloliquefaciens*

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Abstract. Endophytic bacteria of the genus *Bacillus* are promising agents for biological plant protection due to their broad spectrum of antimicrobial metabolites, in particular cyclic lipopeptides (CLPs). The endophytic strains *Bacillus pumilus* E11 and *Bacillus amyloliquefaciens* E12, isolated from *Pinus sylvestris* L. seeds, exhibited antagonistic activity against phytopathogenic microorganisms; however, the molecular-genetic basis of this activity remained unestablished. The aim of the study was to characterise the transcriptional activity of CLPs synthesis genes (fengycin, surfactin, iturin A) in *B. pumilus* E11 and *B. amyloliquefaciens* E12 using RT-qPCR with specificity verification via melting curve analysis. Total RNA was isolated from pure cultures in the stationary growth phase using GuSCN buffer with DTT and proteinase K; DNA was eliminated by treatment with DNase I; reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit, and RT-qPCR analysis was carried out on a CFX96 Opus (Bio-Rad) amplifier with SYBR Green. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with the 16S rRNA reference gene. During the study, the *fenD* gene of the fengycin operon was identified and its specificity was confirmed by a melting peak (74.0-74.5°C) in both strains studied. The mean C_q

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value for *fenD* amplification was 30.3 for *B. pumilus* E11 and 29.8 for *B. amyloliquefaciens* E12, and the mean C_q values for 16S rRNA were 21.8 and 23.8 respectively. The relative transcriptional activity of the fengycin biosynthesis gene in *B. amyloliquefaciens* E12 exceeds that of *B. pumilus* E11 by a factor of 5.7 ($\Delta\Delta C_t$ = -2.5). The surfactin (*srfA*) and iturin A (*ituA*) synthesis genes were not detected in any of the strains. Thus, fengycin proved to be the only transcriptionally confirmed antifungal CLP among the studied endophytic strains, confirming their activity against the microfungi *Fusarium oxysporum* and *Alternaria alternata*, as determined in previous studies. The high level of *fen* operon transcription in *B. amyloliquefaciens* E12 confirms the potential of this strain as a producer of antifungal metabolites for the biocontrol of plant pathogens

Keywords: microorganisms; fengycin; biosynthesis; operon; biocontrol; antifungal activity; *Pinus sylvestris*

INTRODUCTION

Microorganisms of the genus *Bacillus* are the most efficient producers of structurally diverse antibiotic compounds among soil prokaryotes: some strains produce between five and eight chemically unrelated classes of secondary metabolites simultaneously. A. Ortiz *et al.* (2025) identified a particularly pronounced antimicrobial metabolome in two species, *Bacillus pumilus* and *Bacillus amyloliquefaciens*. As noted by N. Vasilyeva *et al*. (2025), the *B. pumilus* genome contains up to 15 functional biosynthetic gene clusters (BGCs), three of which are responsible for the synthesis of bacteriocins. This genetic structure accounts for the specific multifunctionality of these microorganism species as effective producers of antimicrobial agents. Among the secondary metabolites of microorganisms of the genus *Bacillus*, cyclic lipopeptides (CLPs) play a key role in the biological defence of plants; these are amphiphilic compounds synthesised by multi-enzyme complexes of non-ribosomal peptide synthetases (NRPS), encoded by a gene cluster. Three main classes of CLPs are distinguished: surfactins (peptides that cyclise with a β-hydroxyfatty acid carbon chain containing 13 to 15 carbon atoms to form a lactone structure), iturins (cyclic heptapeptides that form a ring via an amide bond with a β-amino fatty acid carbon chain containing 14 to 17 carbon atoms) and fengycins, also known as plipastatins (cyclic lipopeptides with a complex structure in which the peptide moiety consists of 10 amino acid residues, 8 of which form an internal lactone ring between the amino acids). The biosynthesis of each class of CLP is controlled by a separate cluster of genes: surfactin – *srfAA*, *srfAB*, *srfAC* and *srfAD*; iturin – *ituA*, *ituB*, *ituC* and *ituD*; fengycin – *fenA*, *fenB*, *fenC*, *fenD* and *fenE*. Importantly, not all *Bacillus* strains carry all three operons in their genomes, and the composition and repertoire of BGCs vary significantly even within a single species (Dobrzynski *et al*., 2023; Markelova *et al*., 2025). G. Balleux *et al*. (2025) determined that *B. pumilus* and *B. licheniformis* typically produce only one type of CLP belonging to the surfactin class, whereas *B. subtilis* and *B. amyloliquefaciens* can synthesise two types of CLPs, and the plant-associated species *B. velezensis* and *B. siamensis* can produce all three classes. Fengycin is of particular practical interest as a lipodecapeptide containing a β-hydroxy fatty acid chain (C14-C18) incorporated into a cyclic structure through a lactone bond between tyrosine and isoleucine. The antifungal mechanism of action of fengycin involves the inhibition of ergosterol synthesis, damage to membrane structure and the induction of apoptosis in fungal cells. According to N. Markelova *et al*. (2025), fengycin exhibits lower haemolytic activity than iturins and surfactins, which is a significant advantage in the development of biological plant protection products. N. Markelova *et al*. (2025) and C.-Y. Cao *et al*. (2025) emphasised that the spectrum of fungicidal activity of fengycin covers a wide range of pathogenic filamentous fungi: *Fusarium* spp., *Sclerotinia* spp., *Alternaria solani*, *Magnaporthe grisea*, *Botrytis cinerea*, *Rhizoctonia solani* and others. According to S. Wang *et al*. (2022), the fengycin operon (*fenA-fenE*) and the iturin operon (*ituA-ituD*) encode fengycin synthase and iturin synthase, respectively. Transcription of these operons is activated by the regulators ComA, SigA, DegU, DegQ, and Spo0A, while deletion of the repressor gene abrB further increases fengycin and iturin yields.

Iturin A is an effective antifungal agent whose mechanism of action involves the formation of ion

channels in the fungal cytoplasmic membrane, inducing apoptosis-like changes. J. Dobrzynski *et al*. (2023) and N. Markelova *et al*. (2025) argued that surfactin (the *srfAA-srfAD* cluster) plays a key role in biofilm formation and the induction of systemic plant resistance; its direct antifungal activity is lower than that of iturins and fengycins, yet it is critically important for effective colonisation of the root zone. Analysis of the transcriptional activity of genes involved in the biosynthesis of antimicrobial lipopeptides in the studied strains is a crucial step, facilitating the transition from the observed antimicrobial effect to the elucidation of its molecular basis and the scientifically grounded optimisation of the fermentation process. The increasing resistance of phytopathogenic fungi to chemical fungicides, linked to changes in ergosterol biosynthesis and the activity of transport systems, necessitates the search for new (alternative) means of biological control. Microorganisms of the genus *Bacillus*, by producing CLPs (surfactin, iturin A, fengycin), exhibit pronounced antifungal activity. As noted by C. Bakker *et al*. (2024), their action is associated with disruption of the integrity of the cell membrane, interaction with its lipid components, including ergosterol, as well as the induction of metabolic disturbances in fungal cells. The multifunctionality of CLPs is one of the factors that potentially reduces the development of resistance in phytopathogens compared to fungicides with a narrow-spectrum mechanism of action.

The endophytic microbiota of conifer seeds is an under-researched source of new strains capable of producing CLPs. In previous studies by V. Shopinskyi *et al*. (2025) and V. Shopinskyi & L. Butsenko (2026) the endophytic strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 were isolated from *Pinus sylvestris* L. seeds and identified using MALDI-TOF and VITEK® 2 Compact methods. For *B. pumilus* E11, antagonistic activity against phytopathogenic bacteria was detected: growth inhibition zones against *Pseudomonas syringae* UCM B-1027 were 35 ± 2 mm, against *Clavibacter michiganensis* subsp. *michiganensis* 10_2 – 33 ± 2 mm, against *Xanthomonas campestris* UCM B-1049 – 32 ± 2 mm, against *Pseudomonas fluorescens* 8573 – 25 ± 1 mm and against *Pectobacterium carotovorum* UCM B-1075 – 20 ± 1 mm (Shopinskyi *et al.*, 2025). Antagonistic activity against phytopathogenic bacteria was also detected for *B. amyloliquefaciens* E12: growth inhibition zones against *P. syringae* UCM B-1027 were 37 ± 2 mm, against *C. michiganensis* subsp. *michiganensis* 10_2 – 22 ± 1 mm, against *X. campestris* UCM B-1049 – 42 ± 2 mm, against *P. fluorescens* 8573 – 26 ± 1 mm and against *P. carotovorum* UCM B-1075 – 35 ± 2 mm. It was also determined that *B. amyloliquefaciens* E12 exhibits antagonistic activity against phytopathogenic microfungi: the strain under investigation inhibited the growth of *Fusarium oxysporum* GTF1 and *Alternaria alternata* GTA2 with a zone of inhibition of 15-18 mm (Shopinskyi & Butsenko, 2026). Given the detected biocontrol potential of the endophytic microorganisms *B. pumilus* E11 and *B. amyloliquefaciens* E12 against plant pathogens, the molecular mechanisms of their antagonistic activity, in particular the expression profile of genes responsible for the biosynthesis of lipopeptides, remain insufficiently studied. To characterise the antifungal phenotype and to develop targeted fermentation strategies, it is necessary to identify actively transcribed BGCs. The aim of this study was to establish the transcriptional activity profile of cyclic lipopeptide genes (*fenD*, *srfA*, *ituA*) in *B. pumilus* E11 and *B. amyloliquefaciens* E12 using RT-qPCR with SYBR Green detection and verification of specificity via melting curve analysis.

MATERIALS AND METHODS

The endophytic strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 under investigation were isolated from surface-sterilised seeds of *Pinus sylvestris* L. by serial dilution and identified based on morphological and cultural characteristics, MALDI-TOF mass spectrometry and the VITEK® 2 Compact analyser (Shopinskyi *et al.*, 2025). For gene expression analysis, pure cultures of both strains were grown in LB liquid nutrient medium (tryptone 10 g/L; yeast extract 5 g/L; NaCl 10 g/L) at 28°C with stirring at 240 rpm under aerobic conditions. Samples were collected during the stationary phase of growth after 48 hours of cultivation, as it is during this period that the maximum transcriptional activity of the genes encoding CLPs synthesis, under the regulation of the *Spo0A* and *DegU*/*ComA* cascades, is observed in most *Bacillus* strains. RNA was isolated from a pure bacterial culture. A bacterial colony was placed in a 1.5 mL sterile tube, 100 µL of RNase-free water was

added, and the sample was resuspended. Cell lysis was carried out sequentially: 10 µL of lysozyme (1 mg/mL) was added, followed by incubation for 10 minutes at 37°C, followed by 400 µL of 4 M GuSCN buffer with DTT and 10 µL of proteinase K; the samples were incubated for 10 minutes at room temperature, then centrifuged for 1 minute at 10,000 rpm. The supernatant was transferred to a new sterile microtube, 0.7× the volume of isopropanol was added, and the mixture was incubated at -20°C for 30 minutes to precipitate the RNA. After centrifugation at 12,000 rpm for 5 minutes, the pellet was washed with 70% ethanol followed by a similar centrifugation, dried for 5 minutes, and resuspended in 40 µL of RNase-free water. Residual genomic DNA was removed by treatment with DNase I (Thermo Scientific). The reaction mixture (20 µL) contained: 10 µL RNA, 1 U DNase I, 2 µL 10×$MgCl_2$ buffer and 7 µL DEPC-treated deionised water. The mixture was incubated for 30 minutes at 37°C; DNase I was inactivated by adding 2 µL of 0.5 M EDTA and heating at 65°C for 15 minutes. The concentration and purity of the RNA were determined on a Denovix 11FX spectrophotometer: based on the A_{260} (concentration) and the A_{260}/A_{280}, A_{260}/A_{230} ratios (purity). The absence of DNA contamination was verified by PCR without reverse transcription. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Samples were incubated at 25°C for 5 minutes and at 42°C for 60 minutes. Enzyme inactivation was carried out at 70°C for 5 minutes. The composition of the reaction mixture is shown in Table 1.

Table 1. Composition of the reverse transcription reaction mixture

Component	Volume, µL
RNA (concentration 0.1 ng – 5 µg)	5
Random primer	1
5× reaction buffer	4
RNAse inhibitor	1
10 mm dTTP	2
Reverse transcriptase	1
Deionised water (DEPC-treated)	6
Total volume	20

Source: developed by the authors according to the manufacturer's instruction

cDNA samples were stored at -20°C for no longer than 1 week. For reverse transcription, 5 µL of the total RNA preparation, corresponding to 0.1 ng – 5 µg of RNA depending on the sample, was used as input in a 20 µL reaction; random hexamer primers were chosen to ensure unbiased, whole-transcriptome conversion independent of the position of the target genes within the lipopeptide operons. The reverse transcription was carried out in duplicate for each sample. The efficiency and integrity of the reverse transcription were assessed indirectly through the reproducible amplification of the 16S rRNA reference gene from the resulting cDNA: consistent low Cq values for 16S rRNA across replicates confirmed that intact, amplifiable cDNA was obtained, while the parallel no-reverse-transcriptase control excluded genomic DNA carry-over. Analysis of the transcriptional activity of CLP genes was performed using the RT-qPCR method on a CFX96 Opus amplifier (Bio-Rad), with data analysis carried out in Bio-Rad CFX Maestro. The iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) was used. The composition of the reaction mixture is shown in Table 2.

Table 2. Composition of the RT-qPCR reaction mixture

Component	Volume, µL
iTaq™ Universal SYBR® Green mix	10
Forward primer (300 nM final concentration)	0.5
Reverse primer (300 nM final concentration)	0.5
cDNA (100 ng)	2

Table 2. Continued

Component	Volume, μL
Deionised water	7
Total volume	20

Source: compiled by the authors according to the manufacturer's instruction

A separate reaction mixture was prepared for each sample and gene studied. The reaction was carried out in triplicate. Amplification temperature profile: initial denaturation for 3 minutes at 95°C; 40 cycles: 20 s at 95°C and 30 s at 58°C (fluorescence signal detection was performed on the FAM/SYBR channel); melting curve analysis: 10 s at 65-95°C with a temperature increase of 0.5°C per cycle. The primers for the fengycin (*fenD*), surfactin (*srfA*) and iturin A (*ituA*) genes were designed specifically for this study in Primer-BLAST (NCBI) on the basis of the corresponding biosynthetic operon sequences of reference strains *Bacillus amyloliquefaciens* FZB42 (GenBank CP000560) and *Bacillus velezensis* (reference lipopeptide synthetase genes), with the target amplicon length constrained to 180-220 bp and the melting temperature of the primers set to 60°C to ensure uniform annealing under a single thermal profile. Primer specificity was verified in silico against the NCBI nucleotide database, and only primer pairs yielding a single predicted product for the intended target were retained. The reference-gene primers (16S rRNA) were taken from H. Younas *et al*. (2023). The nucleotide sequences of the primers are given in Table 3.

Table 3. Primers used for RT-qPCR analysis of cyclic lipopeptide synthesis genes

Lipopeptide	Gene	Primers (5′→3′)	Expected amplification product, bp
Fengycin	*fenD*	F: ATGGGAAATGTTCGGAGCGT R: CTGTAAAGCTTCTCCGCCGA	220
Surfactin	*srfA*	F: CCGCACCAAAAGAAGAACGG R: GCTCGCCCTTCCTAGACTTC	182
Iturin A	*ituA*	F: AAGACGCCATTGCCACTGTA R: CGAGTTCCTCCGCTCTGATC	187

Source: compiled by the authors based on H. Younas *et al*. (2023)

Amplification specificity was confirmed by three independent criteria: a single, narrow peak in the melting-curve analysis; the correspondence of the melting temperature to the value predicted for the target amplicon; and the absence of any signal in the negative control (NTC). Since the study addressed the qualitative detection of transcripts and their relative quantification by the $2^{-\Delta\Delta Ct}$ method, in which the target and reference genes are amplified from the same cDNA under identical conditions, amplification efficiency was taken as equivalent for the compared genes; the amplicons were kept short (180-220 bp) precisely to keep efficiency close to the theoretical maximum, as recommended for SYBR Green assays (Bustin *et al*., 2009). A limitation of the present design is that no external standard dilution series was run to determine the exact PCR efficiency and the coefficient of determination (R^2) of a standard curve, and no positive control template was available for *srfA* and *ituA*; consequently, the negative results for these two genes are interpreted as the absence of a detectable transcript under the conditions studied rather than as definitive proof of the absence of the corresponding gene clusters, which will be verified by whole-genome sequencing. The relative level of transcriptional activity of CLP genes was calculated using the $2^{-\Delta\Delta Ct}$ method described by K.J. Livak & T.D. Schmittgen (2001). 16S rRNA was used as the reference gene, as its stable transcription has been confirmed under various cultivation conditions for strains of the genus *Bacillus* (Younas *et al*., 2023). ΔC_t was defined as: $\Delta C_t = C_q$ (target gene) – C_q (16S rRNA). The $\Delta\Delta C_t$ value was calculated relative to the calibrator strain *B. pumilus* E11: $\Delta\Delta C_t$ (*B. amyloliquefaciens* E12) = ΔC_t (*B. amyloliquefaciens* E12) – ΔC_t (*B. pumilus* E11). The relative expression level was calculated as $2^{-\Delta\Delta Ct}$ (Livak *et al*., 2001). Statistical analysis of the results was performed using CFX Maestro (Bio-Rad) and Microsoft Excel software. Each C_q measurement was performed in three

technical replicates for each sample studied. Reproducibility between replicates was assessed by the difference in C_q values between duplicates: a difference of $\Delta C_q \leq 0.5$ between technical replicates was considered acceptable. For the 16S rRNA reference gene and the genes of the lipopeptides under investigation, the mean C_q value was calculated as the arithmetic mean of the three technical replicates (Bustin *et al.*, 2009). The $2^{-\Delta\Delta Ct}$ method was used for the relative quantification of the target gene's expression level, using the *B. pumilus* E11 strain as the primary calibrator ($2^{-\Delta\Delta Ct} = 1.0$). Dispersion was assessed via the standard deviation of technical replicates. The absence of non-specific amplification products was confirmed by the correspondence of melting peak temperatures for each target gene and the absence of a signal in the NTC. Genes were considered absent or as having undetectable transcripts in the absence of an amplification signal ($C_q = 0$ or failure to reach the threshold) after 40 cycles and in the absence of a melting peak in all technical replicates.

RESULTS AND DISCUSSION

The total RNA preparations obtained from strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 were of sufficient quality for RT-qPCR analysis, and the synthesised cDNA enabled reproducible amplification of the 16S rRNA reference gene in all samples studied. The amplification curves in Figure 1 provide the primary evidence for the transcriptional status of each target gene.

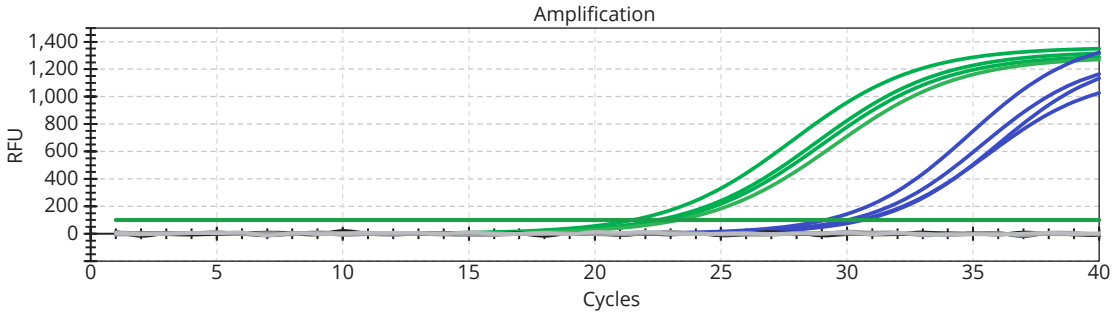

Figure 1. Summary amplification curves (obtained by RT-qPCR) of the target genes for strains *B. pumilus* E11 and *B. amyloliquefaciens* E12
Note: RFU – fluorescence units; green – 16S rRNA (reference gene); blue – fengycin (*fenD*); black – surfactin (*srfA*); grey – iturin A (*ituA*)
Source: compiled by the authors

The sigmoidal profile of the *fenD* and 16S rRNA curves, with a clearly defined exponential phase, indicates efficient template amplification, whereas the flat, sub-threshold traces of *srfA* and *ituA* over the full 40 cycles reflect the absence of the corresponding transcripts rather than reaction inhibition, since the reference gene amplified normally from the same cDNA. The earlier exponential rise of the *fenD* curve in *B. amyloliquefaciens* E12 relative to *B. pumilus* E11 already anticipates the quantitative difference in transcriptional activity established below, and the flat NTC baseline across all channels excludes genomic DNA carry-over and primer-dimer artefacts. The negative control did not produce an amplification signal for any of the analysed genes, confirming the absence of reagent contamination. To verify the specificity of the amplification, melting curve analysis was performed separately for each gene under investigation. For the 16S rRNA reference gene, a single symmetrical melting peak at 83.0-83.5°C, reproduced in both strains with comparable peak heights, confirmed that amplification yielded one homogeneous product without secondary peaks or shoulders (Fig. 2).

The narrow width of the derivative peak and its stable position between strains confirm both the specificity of the reference primers and the uniform quality of the cDNA preparations, which is a prerequisite for valid normalisation in the $2^{-\Delta\Delta Ct}$ calculation. For the surfactin biosynthesis gene *srfA*, the amplification traces of both technical replicates for each strain remained at baseline and did not cross the fluorescence threshold

within 40 cycles, while the corresponding melting-curve derivatives showed no peaks across the 65-95°C range, and no peak was observed in the no-template control (NTC) (Fig. 3).

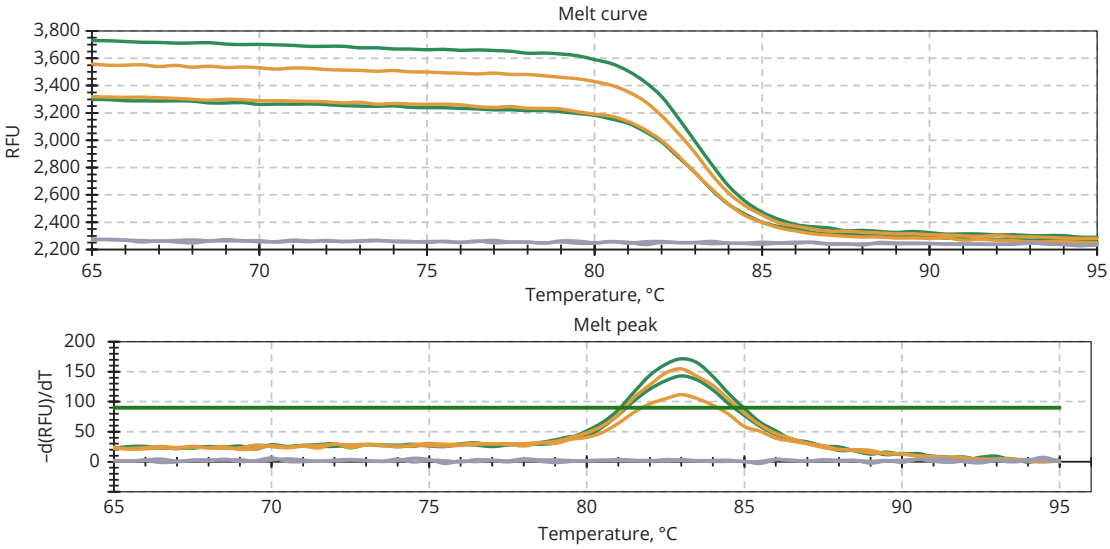

Figure 2. Melting curves and their derivatives for the 16S rRNA reference gene
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

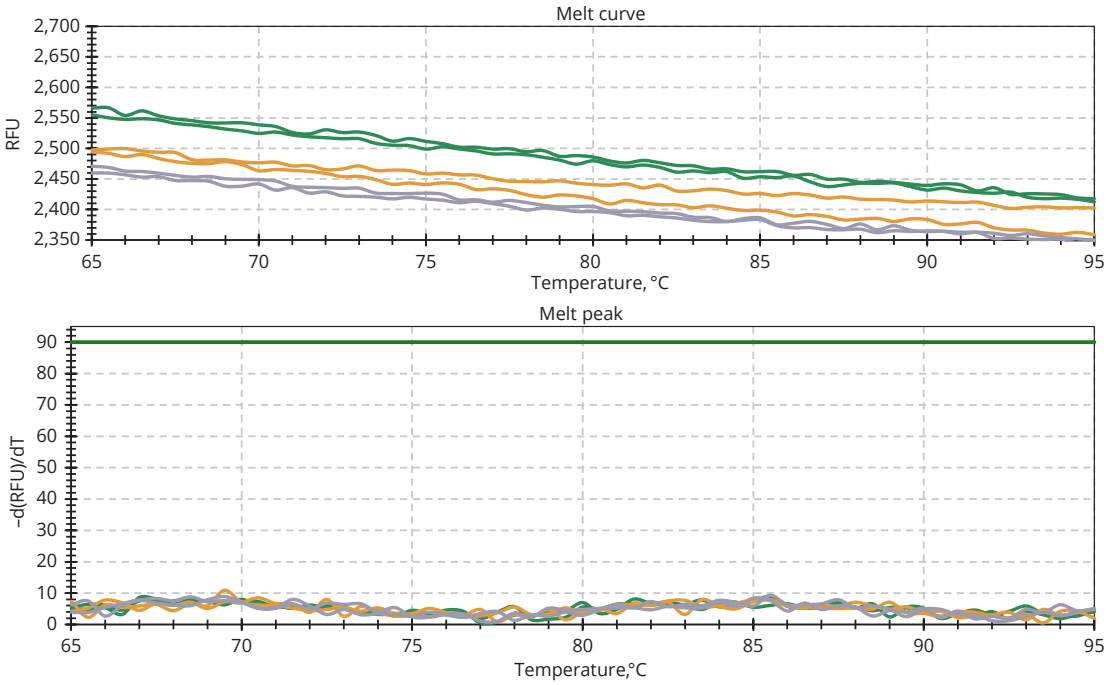

Figure 3. Melting curves and their derivatives for the surfactin biosynthesis gene (*srfA*)
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

In SYBR Green chemistry a genuine transcript, even at a high C_q, would generate a defined melting peak; its complete absence in every replicate therefore indicates that no *srfA* template was reverse-transcribed and amplified, and the concordance between the two replicates confirms th e reproducibility of this negative result. For *B. pumilus* E11 the outcome is consistent with the species-level restriction of its surfactin-family repertoire to pumilacidin, whose variant operon is not necessarily recognised by primers designed against the *srfA* sequence. For the iturin A biosynthesis gene *ituA*, the flat amplification traces that remained below the fluorescence threshold and the absence of a derivative melting peak confirmed that no specific amplicon was generated in either strain, thereby excluding the presence of a detectable iturin A transcript (Fig. 4).

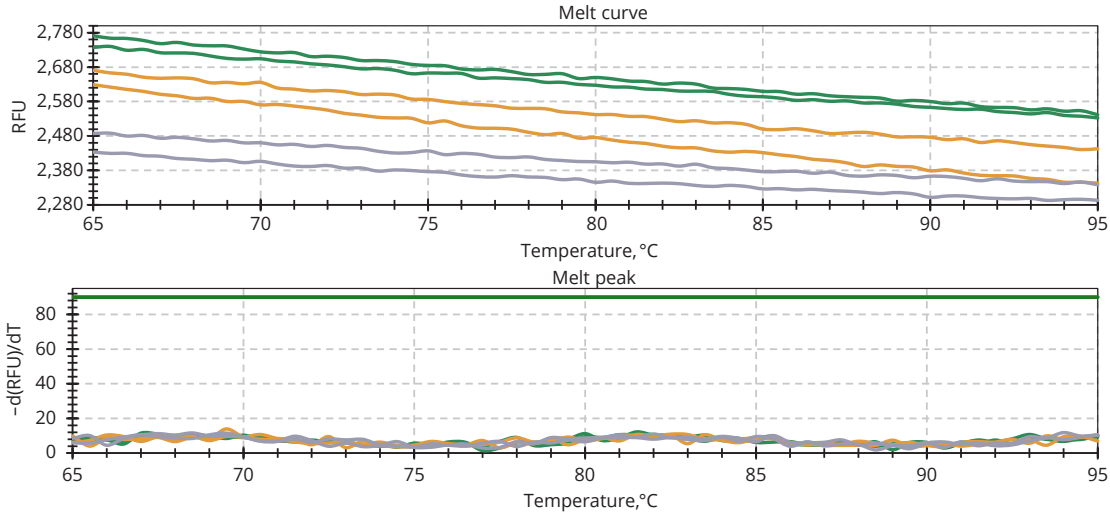

Figure 4. Melting curves and their derivatives for the iturin A biosynthesis gene (*ituA*)
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

The result is particularly informative for *B. amyloliquefaciens* E12, in which the iturin operon is generally present, and therefore points to a regulatory cause, namely insufficient activation of the *itu* promoter by the Spo0A-DegQ cascade on nutrient-rich LB medium in the absence of a fungal inducer, rather than to the absence of the gene cluster itself. For the fengycin biosynthesis gene *fenD*, both strains produced a single clear, sharp melting peak within the narrow range of 74.0-74.5°C, with no additional peaks, shoulders, or low-temperature signals indicative of non-specific amplification or primer-dimer formation, while no peak was detected in the no-template control (NTC) (Fig. 5). The near-identical peak position in *B. pumilus* E11 and *B. amyloliquefaciens* E12 confirms that the same specific product was generated regardless of the strain of origin, while the greater peak height recorded for E12 is consistent with a larger amount of accumulated amplicon and, by extension, with a higher initial transcript abundance, in agreement with its lower C_q value. The mean values of amplification C_q and melting temperatures obtained during RT-qPCR analysis for the target genes and strains under investigation are presented in Table 4.

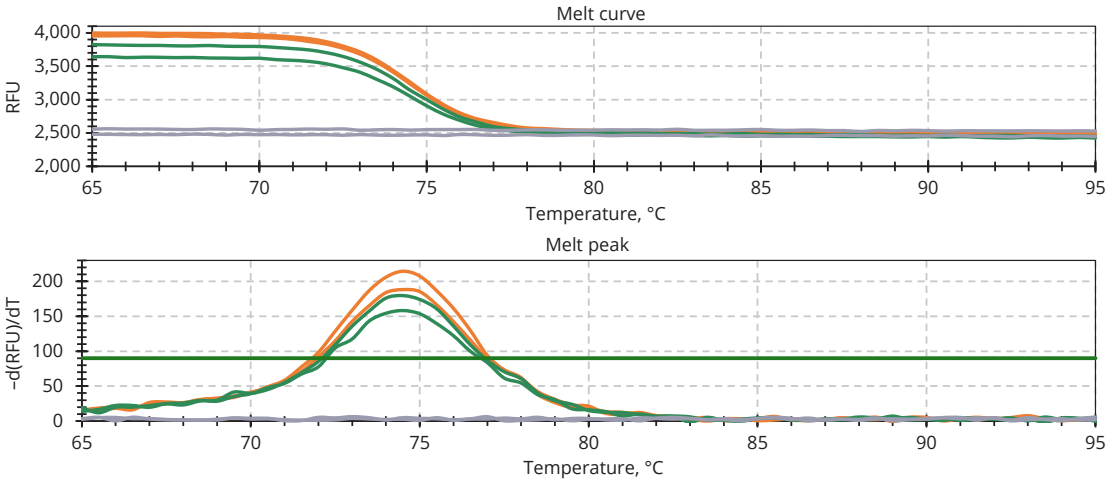

Figure 5. Melting curves and their derivatives for the fengycin biosynthesis gene (*fenD*)
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

Table 4. Results of RT-qPCR analysis:
Cq values and melting temperatures for cyclic lipopeptide synthesis genes

Target gene	B. pumilus E11, C_q	B. amyloliquefaciens E12, C_q	Melting temperature (°C)
16S rRNA (reference)	21.8	23.8	83.0-83.5
Fengycin (*fenD*)	30.3	29.8	74.0-74.5
Surfactin (*srfA*)	N/D	N/D	No peak
Iturin A (*ituA*)	N/D	N/D	No peak
NTC (negative control)	N/D	N/D	No peak

Note: N/D – not detected
Source: compiled by the authors

The fengycin operon gene (*fenD*) was detected and confirmed in both strains studied. For *B. pumilus* E11, the mean C_q value across three technical replicates was 30.3; for *B. amyloliquefaciens* E12, it was 29.8. Stable amplification was also observed for the 16S rRNA reference gene: for *B. pumilus* E11, the mean C_q value across three technical replicates was 21.8, and for *B. amyloliquefaciens* E12, it was 23.8. The surfactin (*srfA*) and iturin A (*ituA*) biosynthesis genes were not detected (Cq = 0) in any of the samples studied. The specificity of the amplification was confirmed by a single melting peak at (74.0-74.5°C), corresponding to the *fenD* gene. No melting peak was detected in the NTC. The data in Table 4 show a consistent gap of approximately 8-9 cycles between the reference gene 16S rRNA (mean C_q 21.8 and 23.8 for E11 and E12) and the target gene *fenD* (mean Cq 30.3 and 29.8). Since each cycle corresponds to an approximate doubling of template, this gap indicates that *fenD* transcripts are present at a substantially lower abundance than the constitutively expressed 16S rRNA, which is expected for a growth-phase-regulated secondary-metabolite operon rather than a housekeeping gene. It is important that the higher reference C_q of *B. amyloliquefaciens* E12 does not by itself imply lower target expression, because in the $2^{-\Delta\Delta Ct}$ method each target is normalised to its own reference; after normalisation the balance shifts in favour of E12, as shown in Table 5. The uniform absence of amplification and melting peaks for *srfA*, *ituA* and the NTC delimits the fengycin-restricted transcriptional profile common to both strains. The values of the relative transcriptional activity of the fengycin biosynthesis gene, calculated using the $2^{-\Delta\Delta Ct}$ method, are presented in Table 5.

Table 5. Calculation of the relative level of transcriptional activity of the fengycin biosynthesis gene using the $2^{-\Delta\Delta Ct}$ method

Strain	C_q		ΔC_t	$2^{-\Delta\Delta Ct}$ (relative expression)
	fenD	**16S rRNA**		
B. pumilus E11	30.3	21.8	8.5	1.0 (calibrator)
B. amyloliquefaciens E12	29.8	23.8	6.0	5.7

Source: compiled by the authors

The value of ΔC_t for *B. pumilus* E11: 30.3 – 21.8 = 8.5. The value of ΔC_t for *B. amyloliquefaciens* E12: 29.8 – 23.8 = 6.0. The value of $\Delta\Delta C_t$ for *B. amyloliquefaciens* E12 relative to *B. pumilus* E11: 6.0 – 8.5 = -2.5. Accordingly, $2^{-(-2.5)} \approx 5.7$. Thus, the relative transcriptional activity of the fengycin operon in *B. amyloliquefaciens* E12 is 5.7 times higher than in *B. pumilus* E11. This 5.7-fold difference should be interpreted at the level of relative transcript abundance rather than as a direct measure of secreted fengycin, since transcription and final peptide yield may diverge because of post-transcriptional and translational regulation. Nevertheless, a positive relationship between *fenD/fenA* transcription and fengycin output has been reported for engineered *B. amyloliquefaciens* strains, in which increased *fen*-operon transcription was accompanied by higher fengycin accumulation (Zhao *et al*., 2016). On this basis, the markedly higher *fenD* transcription in *B. amyloliquefaciens* E12 predicts a correspondingly greater fengycin-producing capacity and provides a quantitative, molecular rationale for prioritising this strain in subsequent fermentation-optimisation and LC-MS verification experiments, while *B. pumilus* E11, with a confirmed but lower level of *fenD* transcription, remains a relevant complementary producer. The surfactin (*srfA*) and iturin A (*ituA*) biosynthesis genes were not detected in any of the strains in three technical replicates (no C_q values were obtained, and no melting peaks were detected).

G. Balleux *et al*. (2025) found that microorganisms belonging to different Bacillus species possess different sets of CLP operons. It has been established that *B. pumilus* and *B. licheniformis* are typically capable of synthesising only one class of CLP – from the surfactin family; *B. subtilis* and *B. amyloliquefaciens* produce two types of CLPs, whilst *B. velezensis* and *B. siamensis* produce three types of CLPs simultaneously. These data provide a fundamentally important context for interpreting the results obtained: the absence of the *srfA* transcript in *B. pumilus* E11 can be explained either by the absence of the *srf* cluster itself or by species-specific features of its regulation. Furthermore, the characteristic lipopeptide of *B. pumilus* is pumilacidin, a structural analogue of surfactin synthesised by an *srf*-like operon with a modified amino acid composition. As emphasised by A. Saggese *et al*. (2018) and J. Dobrzynski *et al*. (2023), primers for *srfA*, developed primarily for strains of *B. subtilis* and *B. amyloliquefaciens*, may not recognise the variant pumilacidin operon. For *B. pumilus* E11, only the fengycin operon has been identified, despite the fact that the synthesis of surfactin-like cyclic lipopeptides is more characteristic of this species. However, J. Dobrzynski *et al*. (2023) reported the presence of fengycin biosynthesis genes in certain strains of *B. pumilus* and found that a mixture of *B. pumilus* SS-10.7 and *B. amyloliquefaciens* SS-12.6 and SS-38.4 produced fengycin A and inhibited the growth of *Pseudomonas syringae* pv. *aptata* on sugar beet. S. Wang *et al*. (2022) and D. Zhang *et al*. (2025) reported that the iturin operon is present in most described strains of *B. amyloliquefaciens*; therefore, the absence of the *ituA* transcript in strain E12 may be considered atypical. This may be due to the specific features of the regulation of the expression of the corresponding gene cluster. In particular, transcription of the *itu* cluster is controlled by the level of the phosphorylated regulator *Spo0A* and the involvement of *DegQ*; under conditions of cultivation on enriched LB medium, the level of *Spo0A*~P may be insufficient to activate the *itu* promoter (Wang *et al*., 2022). G. Balleux *et al*. (2025) also found that, in some endophytic strains, iturin synthesis is induced only through interaction with phytopathogenic fungi or their volatile metabolites, indicating the co-stimulated production of lipopeptides. D.J. Hazarika *et al*. (2019) found that the *fenA* gene may be present in the genome of the endophytic microorganism *B. subtilis*, although the corresponding product was not detected by LC-ESI-MS

under standard cultivation conditions. This indicates the conditionally specific nature of fengycin biosynthesis and a possible mismatch between the presence of the biosynthetic cluster and the production of the final metabolite. The data obtained are consistent with the fact that the presence of BGCs in the genome is not necessarily accompanied by their expression under standard conditions, and the use of RT-qPCR analysis allows the actual transcriptional activity of the corresponding genes to be assessed and expands the possibilities of genomic research.

The results obtained, in particular the detection of transcription of the fengycin cluster in the absence of transcriptional activity of the surfactin and iturin biosynthesis genes, differ from the typical profile described in the literature for most endophytic strains of *B. amyloliquefaciens*, in which all three classes of CLPs are detected simultaneously. S. Ma *et al*. (2025) used PCR to analyse the profile of genes involved in lipopeptide biosynthesis in three endophytic strains isolated from cereal crops – *B. velezensis* ZH179 and *B. amyloliquefaciens* ZH409 and ZH99 – which actively inhibited *Aspergillus flavus*. In all three strains, the genes *srfAA*, *srfAD*, *fenA*, *fenB*, *ituA*, *ituB*, *ituD*, *bmyA*, *bmyB*, and *bmyC* were simultaneously detected; LC-MS analysis confirmed the production of surfactin, fengycin, and iturin. Thus, *B. amyloliquefaciens* strains ZH409 and ZH99 are effective co-producers of all three classes of CLPs, whereas *B. amyloliquefaciens* E12 in the current study exhibited only a fengycin transcript. This reflects strain-specific variability in BGC expression, which is consistent with the concept of condition-specific lipopeptide synthesis (Balleux *et al*., 2025). As noted by X.H. Chen *et al*. (2007), such interspecies variability has a genomic basis: the reference strain *B. amyloliquefaciens* FZB42 allocates 8.5% of its genetic capacity to the non-ribosomal synthesis of secondary metabolites, which exceeds the corresponding value for *Streptomyces avermitilis* (6.4%), and the number and composition of biosynthetic gene clusters differ markedly between strains of the same species. T. Ahmad *et al*. (2023) investigated the lipopeptide profile of the soil-isolated *B. subtilis* Y17B against *Alternaria alternata*. PCR screening identified the genes involved in the biosynthesis of fengycin, surfactin and iturin; UPLC Q-TOF mass spectrometry confirmed the production of surfactin (m/z 994.64; 1,022.68; 1,026.62), iturin (m/z 1,043.56) and fengycin (m/z 1,491.85) in the culture extract. The presence of fengycin in *B. subtilis* Y17B correlated with the inhibition of *A. alternata* growth, which, in terms of mechanism of action, is consistent with the results obtained: the detection of the fengycin transcript *fenD* in *B. amyloliquefaciens* E12 correlates with the endophyte's antagonistic activity against *A. alternata* GTA2 (Shopinskyi & Butsenko, 2026).

However, unlike *B. subtilis* Y17B, the studied strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 showed no transcriptional activity of the surfactin and iturin synthesis genes. K. Yang *et al*. (2024) conducted a genomic analysis of the endophytic strain *B. amyloliquefaciens* MR4, which was isolated from the roots of wild medicinal plants. Using PCR, 8 of the 9 antimicrobial agent synthesis genes under investigation were detected, including *fenA*, *ituA* and *srfAA*. Compared to *B. amyloliquefaciens* MR4, the *B. amyloliquefaciens* E12 exhibited a more restricted transcriptional profile under standard cultivation conditions. The absence of the *ituA* transcript may be related to the specifics of expression regulation, in particular insufficient promoter activation in LB medium, or to the absence of the corresponding cluster or mutations in regulatory regions, which requires further verification by whole-genome sequencing. In addition, J. Zhao *et al*. (2016) used RT-PCR to investigate the transcription level of the *fenA* gene in *B. amyloliquefaciens* ES-2-4 under various conditions. Increased fengycin production (an 8.30-fold increase) in the recombinant strain *B. amyloliquefaciens* FMB72 was accompanied by a 12.77-fold increase in the *fenA* transcription level compared to the wild-type *B. amyloliquefaciens* ES-2-4. This confirms that the quantitative level of *fenA* transcription directly correlates with the efficiency of fengycin biosynthesis. Therefore, the 5.7-fold difference in relative transcriptional activity between *B. amyloliquefaciens* E12 and *B. pumilus* E11, which was identified in this study, may predict a significant difference in the actual concentrations of fengycin in the culture media of the strains under investigation, which can be confirmed by chemical methods (HPLC or mass spectrometry). D.J. Hazarika *et al*. (2019) found that the presence of the fenA gene in the B. subtilis genome was not always accompanied by fengycin production under

standard cultivation conditions, emphasising the dependence of biosynthetic gene cluster expression on environmental conditions. In this study, the use of RT-qPCR analysis allows the assessment of the actual transcriptional activity of the relevant genes and provides functional verification of the results of genomic analysis. The data obtained indicate that cultivation in the stationary phase on LB medium is sufficient to activate the fengycin cluster in the studied strains *B. pumilus* E11 and *B. amyloliquefaciens* E12.

The detected transcriptional activity of the fengycin biosynthesis gene in both strains provides direct molecular-genetic confirmation of the antifungal activity reported by V. Shopinskyi & L. Butsenko (2026), who assessed the strains using the agar diffusion method. *B. amyloliquefaciens* E12, which exhibits 5.7-fold higher transcriptional activity of the fengycin synthesis gene compared to *B. pumilus* E11, formed zones of growth inhibition against *F. oxysporum* GTF1 and *A. alternata* GTA2 with a diameter of 15-18 mm. This correlation between the level of *fenD* transcription and the antifungal phenotype is consistent with the results of other scientific studies. Using *B. subtilis* NCD-2 as a model, Q. Guo *et al*. (2014) demonstrated that fengycin is the principal antifungal agent against *Rhizoctonia solani* associated with soil-borne cotton diseases, as inactivation of the fengycin biosynthesis gene *fenC* resulted in a complete loss of antifungal activity. H. Fan *et al*. (2017) investigated the biocontrol potential of *B. subtilis* 9407 against *Botryosphaeria dothidea* and found that inactivation of the *ppsB* gene, an orthologue of *fenA*, completely suppressed the antifungal activity of the strain against this phytopathogen. These results confirm the important functional role of fengycin as the primary antifungal agent, particularly under conditions where the synthesis of iturin and surfactin is absent or limited. D. Romero *et al*. (2007) determined that iturin, surfactin and fengycin are important factors in the antagonism of *B. subtilis* against *Podosphaera fusca*: the strains studied, which produced only fengycin (without iturin), exhibited characteristic, albeit somewhat reduced, antifungal activity. Thus, the fengycin-dominated profile identified for *B. pumilus* E11 and *B. amyloliquefaciens* E12 is functionally sufficient to provide an antifungal effect.

M. Ongena *et al*. (2005) and S. Breen *et al*. (2015) also identified fengycin as a key antifungal component produced by *B. subtilis* M4, reporting that the protection of bean plants against *Pythium ultimum*, apples against *Botrytis cinerea*, cucumbers against *Colletotrichum lagenarium*, and tomatoes against *Pythium aphanidermatum* was associated with the production of lipodecapeptides, particularly fengycin. The results obtained are of significant comparative importance, as *B. cinerea* and *Alternaria* spp. are characterised by similar mechanisms of pathogenesis. Therefore, fengycin synthesised by *B. amyloliquefaciens* E12 has the potential to exhibit similar efficacy against a broad spectrum of phytopathogenic fungi. Thus, the fengycin-dominant transcriptional profile of strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 is molecularly confirmed and ensures the expression of antifungal biocontrol activity. The results obtained indicate the advisability of optimising the conditions for fengycin biosynthesis (selection of the C:N ratio, regulation via the *ComA/DegU* system and induction in the presence of phytopathogenic fungi), whilst the induction of iturin or surfactin synthesis is not a priority. Strain *B. amyloliquefaciens* E12, characterised by a higher level of transcription of the *fen* cluster genes, can be considered a priority agent for the development of a biopesticide. In contrast, for *B. pumilus* E11, whole-genome sequencing is advisable to determine the presence or absence of a complete set of CLP-cluster genes and to verify the structure and functional characteristics of the fengycin operon.

CONCLUSIONS

Using the RT-qPCR method with SYBR Green detection and verification of specificity via analysis of melting curves (peak 74.0-74.5°C), the transcriptional activity of the fengycin operon gene (*fenD*) was detected and confirmed in the endophytic microorganisms *Bacillus pumilus* E11 and *Bacillus amyloliquefaciens* E12, isolated from the seeds *of Pinus sylvestris* L. Analysis of the results obtained using the $2^{-\Delta\Delta Ct}$ method revealed that the relative transcriptional activity of the *fen* cluster in *B. amyloliquefaciens* E12 was 5.7 times higher than the corresponding value in *B. pumilus* E11 ($\Delta\Delta C_t = -2.5$). This finding confirms the effective antifungal activity of the *B. amyloliquefaciens* E12 strain against *F. oxysporum* GTF1 and *A. alternata*

GTA2, as determined in previous studies using the agar diffusion method. The surfactin (*srfA*) and iturin A (*ituA*) biosynthesis genes were not detected in any of the strains in all technical replicates under stationary-phase growth conditions on LB medium. The absence of the *srfA* transcript in *B. pumilus* E11 corresponds to the specific limitation of the set of cyclic lipopeptides in this species, where the characteristic lipopeptide is pumilacidin rather than surfactin. The absence of *ituA* in *B. amyloliquefaciens* E12 indicates the conditionally specific nature of *itu* operon transcription under the cultivation conditions studied, or the absence of this BGC in the strain's genome, which requires verification by whole-genome sequencing followed by antiSMASH analysis. Thus, fengycin proved to be the only transcriptionally confirmed antifungal CLP for both endophytic strains under the conditions studied, providing the necessary functional efficacy for the realisation of antifungal activity, which is consistent with similar results for other fengycin-producing biocontrol strains of *Bacillus* spp. The *B. amyloliquefaciens* E12 strain, which exhibits a higher level of *fen*-operon transcription compared to the *B. pumilus* E11 strain, has been identified as a promising candidate for further optimisation of fengycin biosynthesis conditions and the development of biocontrol agents for plant protection against phytopathogens. Further studies should include whole-genome sequencing and antiSMASH analysis of the BGC of both strains under investigation, LC-MS verification of fengycin production in culture filtrates, and optimisation of fermentation conditions.

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CONFLICT OF INTEREST

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Виявлення генів синтезу ліпопептидів методом RT-qPCR у ендофітних штамів *Bacillus pumilus* та *Bacillus amyloliquefaciens*

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Анотація. Ендофітні бактерії роду *Bacillus* є перспективними агентами біологічного захисту рослин завдяки широкому спектру антимікробних метаболітів, зокрема циклічних ліпопептидів (ЦЛП). Ендофітні штами *Bacillus pumilus* E11 і *Bacillus amyloliquefaciens* E12, виділені з насіння *Pinus sylvestris* L., виявляли антагоністичну активність щодо фітопатогенних мікроорганізмів, однак молекулярно-генетичні засади цієї активності залишалися невстановленими. Метою дослідження було охарактеризувати транскрипційну активність генів синтезу ЦЛП – фенгіцину, сурфактину та ітурину А – у *B. pumilus* E11 і *B. amyloliquefaciens* E12 за допомогою RT-qPCR із перевіркою специфічності методом аналізу кривих плавлення. Тотальну РНК виділяли з чистих культур у стаціонарній фазі росту із застосуванням буфера GuSCN, що містив DTT і протеїназу K; ДНК усували шляхом оброблення DNase I; зворотну транскрипцію проводили з використанням набору RevertAid First Strand cDNA Synthesis Kit, а RT-qPCR-аналіз здійснювали на ампліфікаторі CFX96 Opus (Bio-Rad) із застосуванням SYBR Green. Відносні рівні експресії

розраховували методом $2^{-\Delta\Delta Ct}$, використовуючи 16S rRNA як референсний ген. У ході дослідження в обох досліджуваних штамах було виявлено ген *fenD* оперону фенгіцину, а його специфічність підтверджено піком плавлення в діапазоні 74,0-74,5 °C. Середнє значення Cq для ампліфікації *fenD* становило 30,3 для *B. pumilus* E11 і 29,8 для *B. amyloliquefaciens* E12, тоді як середні значення Cq для 16S rRNA дорівнювали 21,8 і 23,8 відповідно. Відносна транскрипційна активність гена біосинтезу фенгіцину у *B. amyloliquefaciens* E12 у 5,7 раза перевищувала відповідний показник у *B. pumilus* E11 ($\Delta\Delta Ct = -2,5$). Гени синтезу сурфактину (*srfA*) та ітурину А (*ituA*) не було виявлено в жодному зі штамів. Отже, серед досліджених ендофітних штамів фенгіцин виявився єдиним протигрибковим ЦЛП, транскрипційну активність якого було підтверджено, що узгоджувалося з установленою в попередніх дослідженнях активністю цих штамів щодо мікроміцетів *Fusarium oxysporum* і *Alternaria alternata*. Високий рівень транскрипції оперону *fen* у *B. amyloliquefaciens* E12 підтвердив потенціал цього штаму як продуцента протигрибкових метаболітів для біоконтролю патогенів рослин

Ключові слова: мікроорганізми; фенгіцин; біосинтез; оперон; біоконтроль; протигрибкова активність; *Pinus sylvestris*

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Bioinformatic design and pilot validation of a PCR assay for species-level identification of *Candida* spp. in biological samples

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Abstract. Species-level identification of representatives of the genus *Candida* in complex biological samples of natural, agrobiological and technological origin is necessary for the accurate analysis of mixed microbial communities, the control of microbial contamination and the assessment of potential sources of fungal pathogen dissemination. This study aimed to provide bioinformatic justification, design and preliminary laboratory verification of a polymerase chain reaction system for the specieslevel differentiation of *Candida glabrata*, *Candida parapsilosis* and *Candida auris*. The study included analysis of the ribosomal cluster, selection of reference sequences, in silico design of primers and hydrolysis probes, assessment of their specificity, optimisation of the reaction mixture and testing on control samples of DNA and archived isolates of biological origin. The internal transcribed spacer region of the ribosomal cluster was selected as the main target, as it combines conserved flanking fragments with variable internal sequences. Primer-probe sets were developed for *Candida glabrata*, *Candida parapsilosis* and *Candida auris*, with amplicon sizes of 82, 91 and 118 base pairs, respectively. The amplification reaction was performed in a final volume of 25 µl. The final concentration of each primer was 0.28 µM; the concentration of the hydrolysis probe was 0.2 µM, MgC_2 3 mM, dNTPs 0.3 mM and betaine 1.5 M. Positive amplification was obtained for 40 positive control samples, including 29 *Candida* spp. samples, 7 *Candida glabrata* samples and 4 *Candida parapsilosis* samples. No cross-reactivity was detected in the negative control panel, which included 43 samples of non-target microorganisms. The proposed system confirmed its suitability for primary qualitative analytical species-level identification of selected representatives of the genus *Candida* under controlled laboratory conditions. The developed approach may serve as a basis for further sanitary and biological screening of complex matrices of soil, aquatic and plant microbiota, for biosafety control in the agro-industrial sector, and for subsequent adaptation to the early molecular detection of fungal pathogens relevant to invasive infections

Keywords: ribosomal cluster; yeast-like fungi; probes; screening; microbial contamination; microorganisms

INTRODUCTION

Species-level identification of yeast-like fungi of the genus *Candida* in biological samples of different origin remains a methodologically complex task, since these microorganisms may form part of the accompanying microbiota, act as markers of contamination and microbial transfer between different ecological niches, or represent potential infectious agents in people at increased risk. In such

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matrices, genus-level detection of *Candida* does not always provide sufficient interpretative accuracy, as individual species differ in their capacity for survival, surface colonisation, biofilm formation and interaction with other components of microbial communities. Therefore, ecological and agrobiological analysis requires not only confirmation of the presence of yeast deoxyribonucleic acid, but also clarification of the species affiliation of the detected taxon. Environmental matrices, including soil, water, plant and technologically modified samples, are examples of objects in which the presence of *Candida* may have different origins and therefore requires cautious interpretation. According to M. Sautour *et al*. (2021), target *Candida albicans* deoxyribonucleic acid was detected in only 7 of the 460 soil samples examined, that is, in 1.5% of cases, which indicates a low but confirmed possibility of detecting this species in soil. At the same time, *Candida albicans* was found to persist for up to 30 days in 80% of the soils studied. Its short-term survival was associated with specific physicochemical soil properties, particularly cation-exchange capacity and clay content. This points not to soil as the principal matrix, but rather to the possibility of temporary preservation of cells or their DNA under certain physicochemical conditions. Accordingly, soil should not be regarded merely as an incidental contamination matrix: under certain conditions, it may support the temporary persistence of yeast cells or their deoxyribonucleic acid, which increases the importance of species-level identification. The issue of environmental reservoirs of *Candida* is not limited to soil. A.B. Akinbobola *et al*. (2023) showed that representatives of this genus may be isolated from freshwater and seawater, plant substrates, soil environments and technologically modified sources. On the basis of these data, the authors concluded that non-anthropogenic and anthropogenically transformed environments may support yeast persistence and facilitate their transfer between ecological niches. For *Candida auris*, this issue has an additional dimension: according to I. Silva *et al*. (2024), the potential reservoirs of this species include seawater, sand, salt marshes, estuaries, plant materials and other environments. However, the available reports remain heterogeneous in terms of geography, sample type and confirmation methods. Accordingly, such objects require standardised molecular tools capable of providing species-level, rather than merely genus-level, detection. D. Corrêa-Moreira *et al*. (2024) confirmed the possibility of detecting *Candida* in wastewater, which accumulates microbial material from various sources and may reflect changes in the biological status of a particular territory or facility. In addition, M.M. Oliveira *et al*. (2022) established that marine isolates of *Candida parapsilosis* are capable of early adhesion to high-density polyethylene and may contribute to the formation of microbial associations on polymer substrates. These findings are important not as evidence that every case of human infection originates directly from the environment, but as a justification for the need for rapid screening of potential transmission links for fungal pathogens. Soils, water, plant materials, wastewater and technological surfaces may accumulate or transfer yeast cells and their DNA. When barrier mechanisms are disrupted, or in the context of invasive procedures, catheterisation or immunosuppression, individual *Candida* species acquire clinical significance as potential agents of invasive infections, candidaemia and septic conditions. For this reason, molecular detection in complex matrices logically connects agro-environmental monitoring with the tasks of biosafety and clinically oriented diagnostics. It is also relevant to aquatic and technological systems, where polymer surfaces may serve as stable zones of colonisation and where the presence of *Candida parapsilosis* requires species-level interpretation.

The agrobiological context in this study is considered as one possible area of application, rather than as a self-contained concept of soil-based identification. In plant systems, yeasts do not always have an unequivocally negative significance, since they may participate in interactions with plants, other microorganisms and substrates. J. Kowalska *et al*. (2022) concluded that yeasts can colonise plant surfaces, compete for space and nutrients, produce biologically active compounds and contribute to the suppression of certain phytopathogens. This indicates that the detection of yeasts in plant matrices cannot automatically be interpreted as contamination, because their role depends on the species, abundance, localisation and environmental conditions. At the same time, S. Zhu *et al*. (2021) showed that rhizosphere soils of fruit agrocenoses contain diverse yeast

communities, the structure of which varies depending on the region, climatic factors and soil parameters. A relationship was established between the composition of yeast communities and precipitation, humidity, solar radiation, electrical conductivity, total phosphorus and potassium; these findings emphasise the dynamic nature of such systems. Plant microbiota are generally regarded as a multi-level system of interactions between the plant, soil, microorganisms and environmental factors. G. Berg & T. Cernava (2022) emphasised that the plant microbiome is involved in nutrition, stress resistance and the maintenance of the functional stability of the plant organism; therefore, its composition is important for sustainable agriculture. At the same time, M. Chialva *et al*. (2022) showed that microbiota is shaped by plant species, organ, soil type, environmental conditions and interactions among the microorganisms themselves. This means that the presence of *Candida* in plant or rhizosphere samples requires analysis not merely at the level of general yeast detection, but at the level of species identification within a specific biological matrix. Under such conditions, culture-based methods do not always provide rapid or sufficiently accurate differentiation of species in mixed microbial communities. Molecular approaches based on analysis of the ribosomal cluster make it possible to improve both the speed and specificity of detection. A.A. Rivera-Zizumbo *et al*. (2024) confirmed the suitability of internal transcribed spacer 1 metabarcoding for analysing soil microeukaryotic diversity, while also emphasising that the results depend on marker selection, the reference database and interpretation criteria. For *Candida*, this supports the use of multicopy regions of the ribosomal cluster, namely the internal transcribed spacers, which combine conserved flanking regions for primers with variable internal fragments for species-level discrimination.

Thus, the available data indicate that *Candida* may be present in various biological systems. However, its ecological, sanitary-biological or clinical significance depends on species affiliation, matrix type and the context of detection. This study aimed to justify the selection of molecular targets and to perform a primary analytical assessment of a primer-probe approach for differentiating selected representatives of the genus *Candida* in biological material, using control deoxyribonucleic acid matrices and archived isolates of biological origin. In a broader perspective, this approach may be useful both for monitoring potential environmental sources of contamination and for subsequent adaptation to the early molecular detection of fungal pathogens relevant to invasive infections and sepsis diagnostics.

MATERIALS AND METHODS

The research was conducted as a methodological study involving the analysis of scientific publications and bioinformatic databases, together with pilot laboratory verification of a PCR assay designed for species-level identification of selected representatives of the genus *Candida* in biological samples. *C. glabrata*, *C. parapsilosis* and *C. auris* were selected as the target taxa because these species require accurate differentiation from related yeast taxa and can be used as model objects for testing the discriminatory capacity of a multiplex PCR assay. The theoretical and analytical part of the study was based on scientific publications used to justify the choice of molecular targets and to interpret fungal diversity in biological matrices. Environmental and technological materials may serve as sources of contamination or transfer of fungal DNA, whereas, in the clinical context, specieslevel differentiation of *Candida* is important for the further interpretation of invasive infections. In particular, the study took into account the findings of C. Djemiel *et al*. (2024) concerning the spatial variability of the soil mycobiome and the influence of ecological factors on the structure of fungal communities. It also considered the conclusions of C.L. Schoch *et al*. (2012) regarding the suitability of the internal transcribed spacer region of the ribosomal cluster as a universal marker for fungal identification. The ITS1/ITS2 regions of the ribosomal cluster and the 18S rRNA and 28S rDNA sequences were considered as the main molecular targets. The latter were treated as auxiliary regions for genus-specific screening, confirmation that the amplified material belonged to fungal DNA, and potential further expansion of the test system. Reference sequence selection, *in silico* primer and probe design, specificity assessment and compatibility testing in a multiplex format were performed using bioinformatic analysis, with consideration of melting temperature, GC content, amplicon length, spatial positioning of the

hydrolysis probe and the risk of undesirable secondary structure formation.

Bioinformatic analysis was performed using open resources of the National Center for Biotechnology Information (NCBI), including nucleotide sequence databases and search tools for comparing reference fragments, which provide access to standardised genomic and taxonomic data described by E.W. Sayers *et al*. (2024a; 2024b). Nucleotide sequences of the target loci and reference genomic sequences of the relevant microorganisms were selected from the GenBank database, which operates as an open international database of nucleotide sequences and is regularly updated within the NCBI resources described by E.W. Sayers *et al*. (2024a; 2024b). For *C. glabrata*, *C. parapsilosis* and *C. auris*, the available ribosomal cluster sequences were analysed to identify regions suitable for the design of primers and TaqMan probes. The first stage involved comparing homologous regions of the ribosomal cluster and identifying fragments in which conserved regions could be used for primer binding, while internal variable regions could be used for probe-based species discrimination. The ITS1-5.8S-ITS2 fragments were prioritised because of their suitability for amplification in a real-time PCR format and the presence of sequences useful for differentiating individual taxa of the genus *Candida*. The 18S rRNA and 28S rDNA sequences were considered auxiliary loci for assessing the stability of the selected regions and for providing methodological justification for genus-level detection. Oligonucleotide design and melting temperature calculations were performed using the OligoAnalyzer tool (Integrated DNA Technologies, USA). The probability of secondary structure formation was assessed using the standard programme parameters for the specified reaction conditions. For each candidate primer pair, the following parameters were evaluated: oligonucleotide length, calculated melting temperature, GC content, expected amplicon size, primer orientation, probe position within the amplicon and the presence of potential sites of self-dimerisation, heterodimerisation or hairpin formation.

To assess the specificity of the selected primers and probes, they were checked *in silico* using the NCBI BLAST tool (USA). The assessment was based on the identification of complete and partial matches with target and non-target sequences, including closely related yeast taxa and model nontarget microorganisms. Selection was carried out with consideration of several parameters: oligonucleotide length, expected amplicon size, melting temperature, GC-pair content, spatial positioning of the probe and compatibility of all components within a single reaction mixture. This approach made it possible to compare the requirements for an individual primer pair with those for the multiplex system as a whole. Particular attention was paid to the potential complementarity of the 3′ ends of primers to non-target sequences, as such interactions may create conditions for non-specific amplification. Primer-probe combinations that met the established physicochemical criteria and showed no predicted non-specific binding to non-target sequences during the *in silico* assessment were selected for subsequent laboratory testing. The genetic targets were selected with regard to the need to combine genus-level detection of yeast-like fungi with the possibility of species-level differentiation of selected representatives of the genus *Candida*. For this purpose, the ITS1, 5.8S rRNA, ITS2, 18S rRNA and 28S rDNA regions were analysed. The ITS1-5.8S-ITS2 fragment became the main working target for constructing the speciesspecific panel, because its flanking regions were suitable for primer binding, while its internal variable sequences allowed the placement of hydrolysis probes. For each target species, the presence of regions suitable for generating short amplicons appropriate for real-time PCR was assessed. The absence of large insertions or deletions within the target sequences, which could affect amplification stability, was checked separately. The compatibility of candidate primers and probes with the other components of the future multiplex reaction was also considered during selection, since all targets had to be amplified under a single temperature profile. The study used control DNA samples of commercial or synthetic origin together with archived isolates of biological origin from the internal collection of Laboratory X, which had been deposited as reference strains. The material was considered solely as an analytical matrix for pilot verification of the PCR assay. No clinical biomaterials were used in the study, no blood samples were analysed, and no personal data were accessed.

The species affiliation of the archived isolates was established before the start of the study using culture-based and molecular methods in accordance with internal laboratory procedures. At the beginning of the experiments, the cells were washed twice with phosphate-buffered saline, standardised by concentration and used to obtain the DNA template. Positive control samples and archived isolates of biological origin were used for *C. glabrata* and *C. parapsilosis*, whereas synthetic control material was used to assess the species specificity of *C. auris*. To assess specificity and cross-reactivity, a control panel was formed, which included both positive samples of *Candida* spp., *C. glabrata* and *C. parapsilosis*, and negative control samples containing DNA from non-target microorganisms. The positive panel consisted of 29 *Candida* spp. samples, 7 *C. glabrata* samples and 4 *C. parapsilosis* samples. The negative control panel included *Staphylococcus aureus* (n = 3), *Streptococcus pneumoniae* (n = 2), *Streptococcus pyogenes* (n = 2), *Escherichia coli* (n = 5), *Klebsiella pneumoniae* (n = 2), *Pseudomonas aeruginosa* (n = 2), *Acinetobacter baumannii* (n = 2), *Mycoplasma hominis* (n = 5), *Mycoplasma genitalium* (n = 5), *Ureaplasma parvum* (n = 5), *Ureaplasma urealyticum* (n = 5) and *Gardnerella vaginalis* (n = 5). The negative samples were used as a model non-target panel to determine whether the selected primers and probes generated a non-specific fluorescent signal in the presence of accompanying microbial DNA. Real samples of soil, aquatic and plant microbiota were not examined at this stage. These matrix types were considered a potential area for further application of the developed system after completion of the primary laboratory testing on control DNA samples and archived isolates of biological origin. PCR was performed in a final volume of 25 µl in a buffer solution containing 50 mM Tris, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% non-ionic detergent and 4% glycerol. This buffer composition was selected to maintain a stable ionic environment, provide suitable conditions for Taq DNA polymerase activity and reduce the effect of potential amplification inhibitors. The concentrations of $MgCl_2$, dNTPs, primers, probes and enzyme were optimised separately, taking into account the requirements of the multiplex reaction format. The final concentration of each primer was 0.28 µM, while the concentration of the TaqMan probes was 0.2 µM. The concentrations of $MgCl_2$ and dNTPs were determined empirically and used at 3 mM and 0.3 mM, respectively. The optimal composition of the master mix was determined on the basis of the shape of the amplification curves, Ct values, fluorescent signal amplitude, stability of the internal control and absence of signal in the negative control samples. Betaine was added to the reaction at a concentration of 1.5 M. The appropriateness of its use was assessed by changes in Ct values, the shape of the amplification curve and the preservation of reaction specificity.

To monitor amplification and detect possible PCR inhibitors in samples with a complex matrix, the pUC19 plasmid vector (GenScript Biotech, China), containing a cloned heterologous control sequence, was added to the reaction. This construct was used as an internal control to monitor the course of the reaction and reduce the risk of false-negative interpretation of the results. The control target was detected using the following primers and probe: FWD: GAGAAGTCTGCCGTTACTGC; REV: GATAGCAACCTGCCCAGGG; PRB: HEX-TGGGGCAAGGTGAACGTGGA-BHQ1. The internal control served as a technical indicator of whether the reaction was suitable for result interpretation. If the control signal was present, this indicated that amplification had proceeded normally and had not been affected by inhibitors. In such cases, the absence of a signal from the target sequence could therefore be regarded as a true negative result. Nucleic acid amplification was performed using the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA) in a two-phase thermal cycling mode. The amplification protocol included initial denaturation at 94°C for 3 minutes, followed by 9 cycles consisting of denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 30 seconds. A further 38 cycles were then performed with shorter step durations: 94°C for 15 seconds, 58°C for 15 seconds and 72°C for 15 seconds. Fluorescence was measured during the elongation step. Amplification results were assessed according to the presence or absence of a specific amplification curve in the relevant detection channel, the Ct value, the amplitude of the fluorescent signal, the stability of the internal control and the absence of signal in the negative control samples. For positive control samples, the

expected result was the presence of a specific signal in the corresponding detection channel. For the negative control panel, the expected result was the absence of amplification in the channels specific to *Candida* spp. The assessment of the results was qualitative and analytical in nature and was intended to confirm the functionality of the primer–probe combinations, analytical specificity and the absence of detectable cross-reactivity within the constructed control panel. Quantitative determination of the limit of detection, construction of calibration curves, calculation of amplification efficiency as a percentage and comparison with sequencing data were not performed at this stage and are regarded as subsequent stages in the validation of the proposed PCR assay.

RESULTS

Justification of the selection of molecular targets for species-level identification of representatives of the genus *Candida*

Following the systematisation of molecular targets suitable for the identification of yeast-like fungi, the ITS1-5.8S-ITS2 fragment of the ribosomal cluster was found to be the most informative region for differentiating the selected representatives of the genus *Candida*. Its advantage lies in the combination of three features important for the construction of a primer-probe system: multicopy occurrence, the presence of relatively conserved flanking regions and sufficient interspecies variability of the internal sequences. This was also established in the study by C. Djemiel *et al*. (2024), in which the authors demonstrated the high informativeness of fungal molecular markers for analysing the spatial variability of the soil mycobiome. It is also supported by the conclusions of C.L. Schoch *et al*. (2012) regarding the suitability of the internal transcribed spacer region as a universal marker for fungal identification. Assessment of the ribosomal cluster showed that 18S and 28S ribosomal deoxyribonucleic acid are more conserved and may be useful for confirming the fungal origin of amplified material or for genus-level detection. At the same time, their variability was insufficient for reliable differentiation of closely related *Candida* species within the selected panel. These loci should therefore be regarded as auxiliary, whereas the ITS1-5.8S-ITS2 region performs the main species-specific function. For *Candida glabrata*, *Candida parapsilosis* and *Candida auris*, fragments suitable for the placement of hydrolysis probes in the internal part of the amplicon were identified within ITS1-5.8S-ITS2. This target structure provided a dual level of specificity: the flanking regions allowed primer binding, while the internal variable sequences enabled species-level discrimination by means of the probe. As a result, primer-probe combinations were generated with expected amplicons of 82 base pairs for *Candida glabrata*, 91 base pairs for *Candida parapsilosis* and 118 base pairs for *Candida auris*.

For *Candida glabrata*, the selected target proved effective in distinguishing this species within the genus *Candida* without relying solely on genus-specific conserved regions. This was important for testing the discriminatory capacity of the system, since a positive signal had to be generated only in the presence of the target species sequence. For *Candida parapsilosis*, the selected target also enabled species-level identification, which is important given the potential presence of this species in aquatic, surface-associated or technological biological matrices. For *Candida auris*, the suitability of the selected sequence for inclusion of this species in the multiplex panel was confirmed using a synthetic control, without the need for additional biological isolates. The results obtained indicate that ITS1-5.8S-ITS2 is the most suitable target for constructing a test system aimed at species-level identification of the selected representatives of the genus *Candida*. Unlike the 18S and 28S ribosomal regions, which are mainly auxiliary, the ITS region provides the necessary balance between the stability of primer binding and species-level variability in the probe-binding regions. This combination of properties provided the basis for the subsequent construction of a multiplex primer-probe system and its pilot laboratory testing. The initial *in silico* assessment using BLAST showed that correct primer selection should be based not only on complete matching with target sequences, but also on the analysis of partial complementarity to non-target organisms. After comparison of reference sequences in databases and assessment of their specificity using BLAST, regions were selected that proved to be the most suitable for designing primers and hydrolysis probes. On the basis of the *in silico* analysis, a set of primers and

probes was generated for the detection and differentiation of selected representatives of the genus *Candida*. The characteristics of the selected oligonucleotides are presented in Table 1.

Table 1. Characteristics of the selected primers and TaqMan probes for detecting selected representatives of the genus *Candida*

Species/component	Sequence	Length, nt	Target region	Amplicon size, bp	Modification
Candida parapsilosis, Fprimer	TTG GTT CTC GCA TCG ATG	18	ITS1-5.8S-ITS2	91	–
Candida parapsilosis, Rprimer	ATG TGC GTT CAA AGA TTC G	19	ITS1-5.8S-ITS2	91	–
Candida parapsilosis, TaqMan probes	CGA AAT GCG ATA CGT AAT ATG AAT TGC AGA	30	Internal region of the amplicon	91	5′-Cy-5; 3′-BHQ3
Candida glabrata, Fprimer	CCA TTT CTC CTG CCT GC	17	ITS1-5.8S-ITS2	82	–
Candida glabrata, Rprimer	CAC TCC CAG GTC TTT GTC	18	ITS1-5.8S-ITS2	82	–
Candida glabrata, TaqMan probes	TGC GCG GTT GGT GGG TGT T	19	Internal region of the amplicon	82	5′-FAM; 3′-BHQ1
Candida auris, Fprimer	GAT GAA GAA CGC AGC GAA	18	ITS15.8SITS2	118	–
Candida auris, R-primer	ATC ACG CTC AAA CAG GCA	18	ITS1-5.8S-ITS2	118	–
Candida auris, TaqMan probes	GCA CAT TGC GCC TTG GGG T	20	Internal region of the amplicon	118	5′-ROX; 3′-BHQ2

Source: compiled by the author

Analysis of the data in Table 1 shows that comparatively short amplicons were selected for the three target taxa: 82 bp for *C. glabrata*, 91 bp for *C. parapsilosis* and 118 bp for *C. auris*. This size range corresponds to the aim of developing a real-time PCR system, in which shorter amplicons are generally better suited to rapid amplification and to work with samples in which DNA may be partially fragmented. Primer length was maintained within the range of 17-19 nt, which allowed annealing conditions to be compared within a single temperature programme. The length of the TaqMan probes was set at 19-30 nt, with each probe located in the internal region of the corresponding amplicon. Signal differentiation in the multiplex format was made possible by the use of different fluorophores and quenchers for the three targets. The design criterion for the primers and probes was the ability of all targets to function within a single unified temperature profile. To achieve this, the calculated melting temperature of the primers was optimised within a narrow range of 60-62°C, ensuring similar annealing conditions for all target sequences. In a multiplex format, this parameter is methodologically important, since substantial differences in Tm between primer pairs may lead to uneven amplification efficiency, competition between reactions and preferential accumulation of individual amplicons. The melting temperature of the hydrolysis probes was designed to be higher than the Tm of the corresponding primers, mainly within the range of 68-70°C. The probes were positioned within the amplicons in regions containing discriminatory nucleotide differences, while local regions with excessive GC content or a potential tendency to form secondary structures were avoided. Within the developed system, this arrangement provided a combination of two levels of specificity: primer binding to the target region and probe-based detection of the internal fragment of the amplicon.

A two-phase thermal cycling mode was selected to allow the sequential formation of a specific product followed by signal accumulation. The extended annealing and elongation steps in the first cycles promoted stable formation of the primary amplicon, which is important for matrices with complex secondary structures or a low amount of target DNA. The shortened steps in the second cycling block ensured the accumulation of the already formed specific product without excessively increasing the duration of the reaction. Thus, the amplification mode was aligned with the logic of the primer-probe design and the multiplex format.

Experimental optimisation of the reaction mixture was aimed at aligning amplification sensitivity, specificity and reproducibility. The most significant parameters were the concentrations of $MgCl_2$, dNTPs, primers, probes and betaine. During optimisation, the shape of the amplification curves, Ct values, fluorescent signal amplitude, the presence or absence of signal in the negative control, and the stability of the internal control were assessed. This set of criteria made it possible to evaluate not only whether amplification occurred, but also the quality of the reaction under conditions of potential competition between several primer–probe systems. The multiplex PCR format imposed stricter requirements on component compatibility than a monoplex system. Therefore, during panel development, consideration was given not only to the specificity of each individual primer pair but also to the overall thermodynamic compatibility of all reaction components. The risks of self-dimer, heterodimer and hairpin formation were assessed, since such interactions could reduce primer availability for the target template and affect the intensity of the fluorescent signal. Betaine at a concentration of 1.5 M was added to improve amplification of GC-rich fragments.

Assessment of specificity and cross-reactivity

A panel of positive and negative control samples was formed for experimental assessment of specificity. The negative control samples included model non-target bacterial microorganisms and representatives of the accompanying microbiota, which were used to assess the cross-reactivity of the test system. This structure of the control panel made it possible to determine whether a fluorescent signal was generated only in the presence of target fungal DNA and to assess the behaviour of the system in the presence of DNA from organisms that do not belong to the genus *Candida*. Positive samples were used to confirm the capacity of the system to detect *Candida glabrata* and *Candida parapsilosis*. Within the methodological study, this made it possible to maintain control over the experimental component without expanding the object of investigation beyond control DNA samples and archived isolates of biological origin. The negative panel was used to exclude cross-reactivity. Testing of the negative control panel showed no amplification in the relevant detection channels. All samples containing DNA from non-target microorganisms, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma parvum*, *Ureaplasma urealyticum* and *Gardnerella vaginalis*, produced negative results. This indicates the absence of detectable cross-reactivity with the non-target microorganisms examined within the constructed test panel. The results are consistent with the preliminary *in silico* analysis, according to which the primer–probe systems were selected for variable regions of the target ITS1-5.8S-ITS2 region. The absence of amplification in the negative panel confirms that the selected oligonucleotides do not generate a significant non-specific signal in the presence of DNA from non-target microorganisms. At the same time, positive amplification in *C. glabrata* and *C. parapsilosis* samples indicates that the system retained analytical sensitivity to the target sequences (Table 2).

Table 2. Results of the assessment of the specificity and cross-reactivity of the PCR assay

Sample category	Microorganism/control	n	Amplification result
Negative control	*Staphylococcus aureus*	3	Negative
	Streptococcus pneumoniae	2	Negative
	Streptococcus pyogenes	2	Negative
	Escherichia coli	5	Negative
	Klebsiella pneumoniae	2	Negative
	Pseudomonas aeruginosa	2	Negative
	Acinetobacter baumannii	2	Negative
	Mycoplasma hominis	5	Negative
	Mycoplasma genitalium	5	Negative
	Ureaplasma parvum	5	Negative
	Ureaplasma urealyticum	5	Negative
	Gardnerella vaginalis	5	Negative

Table 2. Continued

Sample category	Microorganism/control	n	Amplification result
Positive samples	*Candida* spp.	29	Positive
	Candida glabrata	7	Positive
	Candida parapsilosis	4	Positive
Synthetic positive control	*Candida auris*	1	Positive

Source: compiled by the author

Analysis of the data in Table 2 shows that all samples in the negative control panel produced negative amplification results. This applied both to Gram-positive and Gram-negative bacteria and to microorganisms that may form part of the accompanying microbiota in complex biological matrices. Thus, within the tested panel, no signals were recorded that could indicate cross-binding of primers or probes to non-target DNA. The 29 positive *Candida* spp. samples, as well as the separate positive samples of *C. glabrata* (n = 7) and *C. parapsilosis* (n = 4), produced positive amplification results. This distribution confirms the correspondence between the presence of target fungal DNA and the generation of a fluorescent signal in the system. Taken together, the results allow the developed PCR assay to be considered a methodological basis for species-level identification of selected representatives of the genus *Candida* in biological samples. The bioinformatic stage enabled the selection of ITS1-5.8S-ITS2 regions suitable for primer-probe design, while the experimental assessment confirmed the absence of detectable crossreactivity within the control panel used. The persistence of a positive signal for target samples and a negative result for non-target microorganisms indicates consistency between the *in silico* prediction and the laboratory testing. From a methodological standpoint, the data obtained demonstrate that the proposed approach may be used for further screening of complex matrices in which *Candida* may be a component of the microbiota, a marker of contamination or a potentially clinically significant taxon. What matters is not only the detection of *Candida* spp. DNA, but also the ability to distinguish the target signal from the background DNA of other microorganisms. The results of control panel testing show that the selected combination of primers, probes, temperature profile and reaction mixture composition provide a functional basis for such differentiation under the tested conditions. Thus, the study results confirm the consistency of the methodological approach: from the selection of the ribosomal ITS region as the main target to laboratory verification of specificity using control samples. The developed system does not go beyond the tasks of primary analytical testing, but it provides a basis for further application in the monitoring of biological systems, the control of potential sources of fungal contamination, biosafety, and subsequent testing in matrices relevant to the diagnosis of invasive infections.

DISCUSSION

The results should be considered within the methodological logic of the staged development of a PCR assay for species-level identification of yeast-like fungi. At this stage, primary analytical testing was performed, including bioinformatic selection of molecular targets, design of primers and TaqMan probes, assessment of their compatibility in a multiplex format, and testing using a control panel. This approach is consistent with the principles of molecular test validation for environmental monitoring, according to which transition to real multi-component matrices should take place only after preliminary confirmation of specificity, inhibition control and reaction reproducibility. In the readiness and environmental DNA analysis scale proposed by B. Thalinger *et al*. (2021), the initial stages include laboratory testing of specificity and sensitivity, whereas application in routine monitoring requires further levels of validation. In this context, the results of the present study should not be interpreted as complete ecological validation of the PCR assay. Their methodological significance lies elsewhere: the selected oligonucleotides were shown to provide specific detection of target DNA templates and to produce no detectable cross-signal within the negative panel used. This level of evidence corresponds to primary laboratory testing and is necessary before moving to environmental, technological or clinical matrices,

where sample composition, PCR inhibitors and accompanying DNA may substantially affect the result. The selection of the ITS1-5.8S-ITS2 region as the main molecular target is justified by the balance between conservation and variability within the ribosomal cluster. In environmental studies of fungal diversity, R. Winand *et al*. (2025) showed that ITS1 and ITS2 may produce different results depending on the reference database, classification algorithm and composition of the mock community. For the interpretation of the present study, this means that the selection of the ITS region cannot be automatic; it requires prior sequence comparison and assessment of discriminatory regions. In addition, H. Orsud *et al*. (2025) compared 18S, ITS1 and ITS2 as markers for fungal profiling and demonstrated that different ribosomal regions have unequal taxonomic resolution. Therefore, inclusion of ITS1/ITS2, 18S rRNA and 28S rDNA in the bioinformatic stage made it possible to assess not only the main species-specific target, but also auxiliary regions for potential genus-level screening.

It should be emphasised that, in the present study ITS1-5.8S-ITS2 was used not as a universal marker for describing the entire mycobiota, but as a basis for targeted species-level identification of predefined *Candida* taxa. This fundamentally distinguishes the proposed approach from metabarcoding, where the outcome is a broad taxonomic profile of the fungal community. For targeted PCR, the key requirement is not maximum coverage of all fungi, but the generation of a specific signal only in the presence of target DNA. In plant samples, this requirement is particularly important, as C. Viotti *et al*. (2024) showed that primer pairs, PCR conditions and additional approaches to blocking non-target sequences can alter the profile of detected fungal diversity. Thus, the results of the present study provide a basis for further testing of the system on plant matrices, but do not replace that stage. The methodological appropriateness of using TaqMan probes is supported by current practice in the development of species-specific PCR assays for *Candida*. A. Lima *et al*. (2019) described a TaqMan-based real-time PCR approach for identifying *Candida auris* using ribosomal DNA as the target region. For the present study, the principle of dual specificity is particularly relevant: amplification is determined by the primer pair, while detection is determined by hybridisation of the probe with the internal region of the amplicon. This is consistent with the selected design, in which the probes were positioned in discriminatory fragments of ITS1-5.8S-ITS2. At the same time, even for a single target species, the characteristics of different PCR assays may differ substantially. J.B. Buil *et al*. (2025) compared several real-time PCR assays and considered analytical sensitivity, specificity and the limit of detection as separate assessment parameters. Therefore, the interpretation of the results in the present study is appropriately limited to the level of primary testing: functionality and the absence of detectable cross-reactivity within the control panel were confirmed, but full analytical validation was not claimed. Compared with approaches based on melting-curve analysis, the TaqMan format has an additional advantage in multiplex detection. E.R. Tavares *et al*. (2025) showed that melting-curve-based real-time PCR may be suitable for differentiating selected *Candida* species; however, such identification depends on the thermal characteristics of the amplicons. In the system presented here, differentiation of the target taxa is ensured not only by amplification parameters, but also by separate fluorescent channels for the corresponding probes. Interpretation of a positive signal in DNA-based PCR should be limited to the detection of the target nucleic acid. B.L. Freitas *et al*. (2022) justified the use of RT-qPCR for rapid detection of viable *C. auris* cells in environmental samples, demonstrating the difference between DNA- and RNA-based approaches. In the present study, a DNA template was used; therefore, the results confirm the presence of target DNA and the functionality of the primer–probe system, but do not allow conclusions about cell viability or the metabolic activity of *Candida* spp. in a biological environment.

The potential application of the developed system to aquatic microbiota is methodologically justified, but requires further experimental confirmation. P. Pham *et al*. (2024) considered fungal communities in agricultural aquatic systems as a dynamic component shaped by ecological and anthropogenic factors. This highlights the value of targeted methods for rapid screening of selected fungal taxa in aquatic environments. At the same time, experience with molecular monitoring of

Candida in wastewater demonstrates the connection between environmental screening and epidemiological and clinical contexts. C. Barber *et al*. (2023) described community-level wastewater surveillance for *C. auris*, in which control of sample preparation, test specificity and result reproducibility were key conditions for interpretation. K.M. Babler *et al*. (2023) also showed the possibility of detecting *C. auris* in hospital and municipal wastewater, confirming the suitability of aquatic environments for molecular detection of yeast DNA. For the present study, these data are not direct evidence that the proposed system is ready for aquatic monitoring, but rather provide a rationale for the next stage of matrix-specific testing. Further scaling of such approaches requires standardisation. A. Rossi *et al*. (2023) linked the detection of *C. auris* in wastewater with an epidemiological context, providing an example of the transition from laboratory detection to applied monitoring. A. Zulli *et al*. (2024) investigated *C. auris* nucleic acids in wastewater solids across a broad set of treatment facilities, which highlights the importance of standardised protocols, replicates and spatial coverage. For the PCR assay presented here, this means that, after primary testing, it will be necessary to determine the limit of detection, assess inter-run reproducibility, test different DNA extraction methods and examine samples with varying levels of inhibition. For plant microbiota and agrobiological systems, the key issue is not only the presence of target DNA, but also the accurate differentiation of *Candida* spp. from the broad background of endophytic, epiphytic, soil-associated and technologically associated microorganisms. A.E. Fadiji *et al*. (2025) emphasised that plant microbiome studies require standardised sampling protocols, metadata control, reproducible extraction methods and verification of results under different soil and climatic conditions. This is fully consistent with the limitations of the present study: real plant, soil and water matrices were not examined, and should therefore be regarded as the next stage rather than as an already confirmed field of application. The ecological presence of representatives of the genus *Candida* requires cautious interpretation. A.D. Valério *et al*. (2025) summarised data on the detection of *Candida albicans* outside its usual biotopes, including in water, soil and plant material, but also emphasised that the ecology of this species in natural environments remains insufficiently understood. In the broader agro-industrial context, species-level identification of yeast-like fungi may form part of a biosafety system.

M.C. Fisher *et al*. (2024) considered the problem of fungal antimicrobial resistance within a One Health framework, in which natural, agricultural, hospital, technological and anthropogenically altered environments are interconnected. J.C. Allison & E.M. Hyland (2025) additionally drew attention to the role of agricultural use of azole compounds in creating ecological conditions for the selection of resistant fungal variants. Resistance was not assessed in the present study; however, the proposed species-specific PCR assay may be used as a first-level screening tool in broader monitoring programmes, to which resistance testing, sequencing or population analysis may subsequently be added. The bioinformatic component of this study also has independent methodological value. F.J. Ambrosio III *et al*. (2023) emphasised the role of standardised workflows for fungal genomic characterisation, since the reproducibility and transparency of computational procedures determine the quality of subsequent laboratory or genomic interpretation. In the present study, the bioinformatic stage performed a similar function at the level of primer-probe design: it enabled preliminary assessment of specificity, compatibility and the risk of non-specific oligonucleotide binding. Thus, the laboratory testing was not an isolated procedure, but was based on a previously established computational framework. Overall, the results confirm that the developed PCR assay has the status of a primarily tested methodological tool for species-level identification of selected representatives of the genus *Candida*. Its strengths include the combination of *in silico* target selection, the use of TaqMan probes, a multiplex format, an internal amplification control, and testing on positive and negative control panels. At the same time, the limits of the study remain clearly defined: the limit of detection was not determined, calibration curves were not constructed, amplification efficiency was not calculated quantitatively, comparison with sequencing was not performed, and real soil, water or plant samples were not tested.

CONCLUSIONS

The study confirmed the appropriateness of developing a PCR assay for species-level identification of *Candida* spp. as a methodological tool for analysing complex biological samples, controlling potential sources of fungal contamination and further adapting the system to biosafety tasks. The transition from general genus-level detection to species-level differentiation is important for interpreting results in complex microbial consortia, where yeast-like fungi may be components of the microbiota, symbionts, accompanying colonisers, contaminants or potentially clinically significant pathogens. Under such conditions, genus-level detection does not always provide sufficient information about the structure of the fungal component, whereas a species-specific approach allows the biological significance of the detected signal to be assessed more accurately. Based on the *in silico* analysis, primers and TaqMan probes were selected for the detection of *Candida glabrata*, *Candida parapsilosis* and *Candida auris*. The selected oligonucleotides had compatible physicochemical parameters, similar melting temperatures and suitability for use within a unified temperature profile for multiplex real-time PCR. The choice of the ITS1-5.8S-ITS2 region of the ribosomal operon as the main molecular target was justified by the combination of conserved flanking regions and variable internal fragments suitable for species-level discrimination. The developed and optimised multiplex PCR approach demonstrated analytical specificity, satisfactory amplification performance and suitability for detecting fungal DNA in control samples and archived isolates of biological origin. The absence of amplification in the negative control panel indicated no detectable cross-reactivity with the non-target microorganisms examined. The use of TaqMan probes, an internal amplification control and optimised reaction conditions improves the reproducibility of result interpretation in complex biological matrices. Thus, the proposed approach is suitable for monitoring biological systems and may be integrated into sanitary and biological screening of complex matrices, biosafety control in the agroindustrial sector, assessment of microbial contamination in technological environments, and further adaptation to the early molecular detection of fungal pathogens in clinically significant materials. At the same time, a limitation of the study is the use of control DNA samples, synthetic controls and archived isolates of biological origin without extensive testing on real multi-component environmental matrices. Future research should focus on increasing the number of species-specific targets, testing the system on samples of soil, aquatic and plant microbiota, assessing its reproducibility under different laboratory conditions, and comparing PCR identification results with sequencing data and, where appropriate, with clinical diagnostic algorithms for invasive mycoses.

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Біоінформатичний дизайн та пробна апробація ПЛР-системи для видової ідентифікації *Candida* spp. у біологічних зразках

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Аспірант

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Анотація. Видова ідентифікація представників роду *Candida* у складних біологічних зразках природного, агробіологічного та технологічного походження є необхідною для коректного аналізу змішаних мікробних угруповань і контролю мікробної контамінації та оцінювання потенційних джерел поширення грибкових патогенів. Метою дослідження було біоінформатичне обґрунтування, конструювання та пробна лабораторна верифікація системи полімеразної ланцюгової реакції для видової диференціації *Candida glabrata*, *Candida parapsilosis* і *Candida auris*. Дослідження передбачало аналіз рибосомного кластера, добір референтних послідовностей, комп'ютерне конструювання праймерів і гідролізних зондів, перевірку їх специфічності, оптимізацію реакційної суміші та тестування на контрольних зразках ДНК й архівних ізолятах біологічного походження. Як основну мішень було визначено ділянку внутрішніх транскрибованих спейсерів рибосомного кластера, що поєднувала консервативні фланкувальні фрагменти та варіабельні внутрішні послідовності. Для *Candida glabrata*, *Candida parapsilosis* і *Candida auris* було сформовано праймерно-зондові набори з розмірами ампліконів 82, 91 і 118 пар основ відповідно. Ампліфікаційну реакцію проводили в кінцевому об'ємі 25 мкл. Кінцева концентрація кожного праймера становила 0,28 мкМ, гідролізного зонда – 0,2 мкМ, $MgCl_2$ – 3 мМ, дНТФ – 0,3 мМ, бетаїну – 1,5 М. Позитивна ампліфікація була отримана для 40 позитивних контрольних зразків, серед яких було 29 зразків *Candida* spp., 7 зразків *Candida glabrata* і 4 зразки *Candida parapsilosis*. У негативній контрольній панелі, що включала 43 зразки нецільових мікроорганізмів, перехресної реактивності не виявлено. Запропонована система підтвердила придатність для первинної якісно-аналітичної видової ідентифікації окремих представників роду *Candida* у контрольованих лабораторних умовах. Розроблений підхід може бути використаний як основа для подальшого санітарно-біологічного скринінгу складних матриць ґрунтової, водної та рослинної мікробіоти, контролю біологічної безпеки в агропромисловості та подальшої адаптації до задач ранньої молекулярної детекції грибкових патогенів, важливих для інвазивних інфекцій

Ключові слова: рибосомний кластер; дріжджоподібні гриби; зонди; скринінг; мікробна контамінація; мікроорганізми

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Conceptual ecobiological screening of municipal plastic waste pressure on riverine ecosystems

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Abstract. Municipal plastic waste represents not only a management and technological challenge but also a potential source of ecobiological pressure on riverine and riparian ecosystems. Inadequate separate collection, uncontrolled accumulation and fragmentation of plastic materials may facilitate the release of macroplastics and microplastics into freshwater ecosystems, where they can interact with macroinvertebrates, fish, microbial communities, bottom sediments, soils and riparian habitats. The aim of this study was to identify and characterise the potential pathways through which municipal plastic waste may enter riverine ecosystems, synthesise the possible mechanisms of its interaction with biota, and develop a preliminary conceptual framework for identifying priorities for ecobiological monitoring. A theoretical and analytical approach was applied, combining a targeted narrative review of recent scientific literature, a "pressure – pathway – receptor – response" framework, and an illustrative application of the developed by the authors plastic waste collection efficiency index to two hypothetical scenarios of municipal waste collection. The synthesis of the scientific literature showed that municipal plastic waste may interact with biota through physical habitat contamination, particle ingestion, transport of associated pollutants, formation of the plastisphere, and alterations in microbial processes in water, bottom sediments and soils. The illustrative application of the index to the two hypothetical scenarios yielded values of 0.67 and 0.64 and demonstrated how different combinations of service coverage and infrastructure accessibility affected the overall assessment. These scenario-based values do not characterise specific municipalities and do not quantify actual plastic leakage, concentrations in the aquatic environment, biological toxicity or biodiversity degradation. The study proposes a conceptual ecobiological framework for prioritising separate collection, local microplastic monitoring, assessment of bottom sediments, bioindication, and subsequent field verification in river basins affected by pressure from municipal plastic waste

Keywords: macroinvertebrates; plastisphere; bottom sediments; bioindication; floodplains

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INTRODUCTION

The environmental consequences of municipal solid waste have traditionally been discussed through the language of infrastructure, costs, recycling rates, waste generation trends, and institutional capacity (Kaza *et al*., 2018). Such a perspective is necessary, but it is incomplete when plastic waste escapes municipal collection systems and enters riverine landscapes. In that case, waste management failure becomes a biological pressure: plastic items occupy habitats, fragment into smaller particles, become colonised by microorganisms, transport additives and sorbed pollutants, and create exposure pathways for aquatic and riparian organisms. Therefore, the assessment of environmentally safe waste management should not be limited to the amount of material recovered or diverted from landfills; it should also include the capacity of a system to prevent adverse effects on living components of ecosystems. Municipal plastic waste is especially relevant for river basins because plastics are light, persistent, mobile, and frequently generated in spatially dispersed household and commercial activities. When collection coverage is incomplete or when container accessibility is poor, plastics can accumulate in streets, ravines, drainage channels, riverbanks, floodplains, and informal dumps. High-flow events then mobilise a fraction of this material into watercourses. T.H.M. van Emmerik *et al*. (2023) showed that flood episodes could sharply intensify river plastic transport and deposition, demonstrating that rivers acted not as passive conduits but as dynamic reservoirs and remobilisation corridors for plastic debris. The Carpathian and Upper Tisza context is particularly important because mountain and foothill rivers combine high ecological value with strong hydrological variability and infrastructural constraints. A. Balla *et al*. (2024) used the Tisza River as a case study to examine the spatial distribution of microplastics in fluvial sediments and confirmed that plastic contamination was measurable in transboundary river systems and required basin-scale interpretation.

The biological relevance of this problem is increasingly supported by studies on freshwater and terrestrial receptors. A.K. Mora-Teddy *et al*. (2024) found that macroinvertebrates could ingest microplastics and modify their drift and benthic behaviour under particle exposure. Fish may experience physical, physiological, biochemical, immunological, and reproductive effects under microplastic exposure, although the magnitude of effects depends on polymer type, particle size, concentration, exposure duration, and associated chemicals (Banaee *et al*., 2025). V.K. Aralappanavar *et al*. (2024) showed that microplastics in soils and riparian zones could influence microbial diversity, nutrient cycling, and carbon-related processes. At the ecosystem scale, V. Nava *et al*. (2024) found that biofouled plastics could alter community structure and functional traits, indicating that the plastisphere played a role in ecological processes rather than functioning as an inert surface. For this reason, the circular economy should be interpreted not merely as an economic model of resource recovery, but as a preventive ecological safety approach. When separate collection, reuse, recycling, extended producer responsibility, and local logistics work effectively, they reduce the probability that plastic waste will enter rivers and interact with biota. Conversely, when circular strategies are evaluated only through resource efficiency indicators, the biological endpoint may remain invisible. This gap is especially problematic for journals and disciplines focused on ecology, biodiversity, and biological systems.

The aim of this study was to identify and characterise the main pathways through which municipal plastic waste may enter riverine ecosystems, synthesise information on biologically relevant receptors and their potential interactions with plastic pollution, and develop a preliminary framework for prioritising ecobiological monitoring. The specific objectives were: (1) to systematise the main potential pathways connecting municipal plastic waste with freshwater and riparian environments; (2) to identify receptor groups and biological endpoints relevant to macroplastic and microplastic exposure; and (3) to demonstrate the calculation and interpretation of the authors-developed Plastic Collection Efficiency Index (PCEI) using two hypothetical municipal collection scenarios and to define monitoring priorities for subsequent field verification of plastic occurrence and possible biological responses.

MATERIALS AND METHODS

The study used a theoretical-analytical design combining a targeted narrative review with an illustrative index-modelling component. It was not designed as a laboratory toxicity experiment or a direct field assessment of biological effects. Instead, the study developed a conceptual ecobiological framework linking municipal plastic-flow control with potential exposure pathways, biologically relevant receptors, and priorities for subsequent field monitoring in riverine and riparian ecosystems. The targeted narrative review drew on publicly accessible peer-reviewed articles, official methodological documents, regulatory acts, and institutional reports identified through targeted online searches and backward reference checking. The search focused primarily on studies published between 2023 and 2025; earlier sources were retained when they provided foundational concepts, established methods, or relevant legal and policy context. Because the review was narrative rather than systematic, no exhaustive database-specific search protocol or quantitative record of identified, screened, and excluded publications was maintained. The principal search terms and their combinations included "river plastic pollution", "riverine macroplastics", "freshwater microplastics", "microplastic transport", "plastisphere", "microplastic macroinvertebrates", "microplastic fish", "soil microorganisms microplastics", "plastic waste circularity", and "plastic waste leakage". Sources were included when they: (1) addressed freshwater, riverine, riparian, sediment, or floodplain environments; (2) examined transport pathways, biological receptors, ecosystem processes, or municipal plastic-waste control; (3) contained a sufficiently described methodology or represented an official methodological or policy document; and (4) were relevant to the pressure – pathway – receptor – response framework. Sources focused exclusively on marine environments without a transferable methodological or conceptual contribution, duplicate publications, non-scientific opinion materials, and publications without sufficient methodological information were excluded. Titles and abstracts were screened first, followed by full-text assessment of potentially relevant sources. The selected evidence was grouped into four thematic categories: environmental pressures, migration pathways, biological receptors, and potential biological or ecosystem responses. The analytical structure followed a pressure – pathway – receptor – response logic adapted to municipal plastic waste in riverine environments. The framework was developed by integrating evidence on riverine transport and remobilisation of plastics, environmental factors controlling microplastic movement, biological quality elements used in freshwater assessment, and documented interactions of plastic particles with freshwater organisms and microbial communities, including studies by E.K. Owowenu *et al*. (2023), European Environment Agency (2024), and A.K. Mora-Teddy *et al*. (2024).

In this framework, pressure is represented by municipal plastic waste generation, incomplete source separation, insufficient collection accessibility, informal dumping, and residual losses from controlled waste systems. Pathways include transport from urban surfaces and drainage channels, riverbank and floodplain remobilisation, fragmentation of macroplastics, sedimentation of microplastics, ingestion, trophic transfer, and colonisation of plastic surfaces by microbial biofilms. Receptors include benthic macroinvertebrates, fish, microbial communities in water and sediments, soil microorganisms in riparian zones, and aquatic or riparian habitats affected by physical obstruction or substrate modification. Responses include organism-level exposure, changes in behaviour or physiological condition, modification of microbial processes, and alteration of habitat structure. These responses are treated as potential endpoints identified from the literature and were not measured directly in any specific Upper Tisza municipality within the present study. To demonstrate the calculation and interpretation of the PCEI without presenting the results as an empirical assessment of named municipalities, two hypothetical municipal collection scenarios were constructed. Scenario A represents a relatively compact territorial community with broad collection coverage but spatially fragmented infrastructure specifically intended for separate plastic-waste collection. Scenario B represents a more territorially dispersed community with lower overall collection coverage but relatively better infrastructure availability in the administrative centre

and principal settlements. The component scores were assigned solely for methodological demonstration. They were not derived from municipal records, operator statistics, household surveys, field measurements, or observations from any specific municipality. No calendar-year reference period was assigned to the scenarios. Their purpose is to show how contrasting combinations of collection coverage and infrastructure availability can produce numerically similar composite PCEI values and why such values require direct empirical verification before application to real communities. The illustrative modelling component applied the authors-developed PCEI to two hypothetical municipal collection scenarios. The index was designed to demonstrate an integrated assessment of selected operational characteristics that may influence the capacity of a local collection system to retain plastic waste within organised collection flows. In the present study, the calculated values are methodological examples rather than empirical estimates of actual municipal performance. The index does not measure the quantity of plastic entering watercourses or the biological effects of plastic pollution. The PCEI was calculated using the following weighted additive formula:

$$PCEI = 0.25C + 0.20F + 0.20A + 0.20Y + 0.15I, \quad (1)$$

where C is plastic collection coverage; F is collection service frequency; A is transport accessibility; Y is the per-capita yield of separately collected plastic waste; and I is the availability of collection infrastructure, including containers, stationary collection points, and mobile collection arrangements. Each component was converted to a standardised score of 1.0, 0.7, 0.4, or 0.1, where 1.0 represented the most favourable condition for organised plastic collection and 0.1 represented the weakest condition.

The weighting coefficients were determined using an authors-defined rank-normalisation procedure. Collection coverage received the highest initial rank of 5 because the absence of territorial service coverage directly excludes residents and settlements from organised collection. Service frequency, transport accessibility, and per-capita collected yield each received a rank of 4 because they jointly characterise the operational continuity, logistical feasibility, and practical output of the collection system. Infrastructure availability received a rank of 3 because the presence of containers or collection points alone does not ensure effective collection without regular servicing and transport capacity. The initial ranks of 5, 4, 4, 4, and 3 were divided by their total of 20, producing normalised weights of 0.25, 0.20, 0.20, 0.20, and 0.15, respectively. The weights sum to 1.00. The operational criteria used to assign standardised scores to the five PCEI components are presented in Table 1. The same scoring matrix was applied to both illustrative scenarios.

Table 1. Operational scoring criteria for the PCEI components

Indicator	Score 1.0	Score 0.7	Score 0.4	Score 0.1
Collection coverage (C)	At least 80% of the resident population or settlements covered by organised plastic-waste collection	From 50% to less than 80% covered	From 20% to less than 50% covered	Less than 20% covered or no regular organised service
Service frequency (F)	Weekly or more frequent collection	Two to four collections per month	Monthly or seasonal collection	Irregular or on-demand collection
Transport accessibility (A)	Main serviced settlements accessible by all-season roads and located no more than 15 km from the collection route or transfer point	Moderate accessibility; typical distance of more than 15 km but no more than 30 km	Difficult accessibility; typical distance of more than 30 km but no more than 50 km, or seasonal road limitations	Very difficult accessibility; typical distance of more than 50 km or unstable road access
Per-capita collected plastic yield (Y)	At least 10.0 kg per person per year	From 6.0 to less than 10.0 kg per person per year	From 3.0 to less than 6.0 kg per person per year	Less than 3.0 kg per person per year

Table 1. Continued

Indicator	Score 1.0	Score 0.7	Score 0.4	Score 0.1
Infrastructure availability (I)	Permanent containers or collection points available in most settlements	Infrastructure available in the administrative centre and main settlements	Limited or spatially fragmented collection infrastructure	No permanent plastic-waste collection infrastructure

Note: the thresholds and numerical scores are authors-defined preliminary operational categories developed solely for illustrative PCEI modelling; they are neither statutory service standards nor validated environmental-risk thresholds. No categorical thresholds of low, moderate, or high environmental leakage pressure were applied in this study. Empirical calibration is required before these categories are used for actual municipal assessment
Source: developed by the authors

The four-level scoring scale was selected to distinguish favourable, relatively favourable, constrained, and critically limited collection conditions without implying unsupported numerical precision. The scores of 1.0, 0.7, 0.4, and 0.1 form an equally spaced ordinal scale with increments of 0.30. The upper and lower anchors represent the most and least favourable operational categories, respectively; the lower value of 0.1 denotes critically limited rather than completely absent collection capacity. The percentage, frequency, distance, yield, and infrastructure boundaries presented in Table 1 were defined as provisional operational cut-offs for differentiating contrasting collection scenarios. They were not derived from statutory requirements, measured biological responses, or empirical calibration. These component-level cut-offs should not be interpreted as thresholds of low, moderate, or high environmental leakage pressure. Consequently, they require validation and, where necessary, recalibration before the PCEI is applied to actual municipalities. The component scores were assigned ex ante as hypothetical combinations of collection-system characteristics using the common operational matrix presented in Table 1. Their purpose was not to reproduce actual municipal conditions but to demonstrate the behaviour of the weighted index when collection coverage and infrastructure availability differ between otherwise comparable scenarios. Scenario A was assigned the scores C = 1.0, F = 0.7, A = 0.7, Y = 0.4, and I = 0.4. This combination represents broad hypothetical collection coverage accompanied by spatially fragmented dedicated infrastructure. Scenario B was assigned the scores C = 0.7, F = 0.7, A = 0.7, Y = 0.4, and I = 0.7. This combination represents lower hypothetical coverage but more favourable infrastructure availability in central and principal settlements. Identical scores for service frequency, transport accessibility, and per-capita collected yield were used to isolate the influence of the contrasting C and I components. The component scores and weighted calculations are presented in Table 2.

Table 2. Illustrative component scores and calculation of the PCEI

Illustrative scenario	C	F	A	Y	I	Weighted calculation	PCEI
Scenario A: broader coverage and fragmented infrastructure	1.0	0.7	0.7	0.4	0.4	$(0.25 \times 1.0) + (0.20 \times 0.7) + (0.20 \times 0.7) + + (0.20 \times 0.4) + (0.15 \times 0.4)$	0.67
Scenario B: lower coverage and more favourable central infrastructure	0.7	0.7	0.7	0.4	0.7	$(0.25 \times 0.7) + (0.20 \times 0.7) + (0.20 \times 0.7) + + (0.20 \times 0.4) + (0.15 \times 0.7)$	0.64

Note: C – collection coverage; F – collection service frequency; A – transport accessibility; Y – per-capita collected plastic yield; I – infrastructure availability. All component scores are hypothetical and were assigned solely to demonstrate the calculation and interpretation of the index. They do not describe any actual municipality and should not be used as municipal performance estimates without verification using documented operational data
Source: developed by the authors

The difference of 0.03 between the illustrative scenarios results solely from the balance between the higher weight assigned to collection coverage and the lower weight assigned to infrastructure availability. Scenario A has a higher C score, whereas Scenario B has a higher I score. Under equal weighting of all five components, both scenarios would produce the same value of

0.64. This sensitivity check demonstrates that the observed difference is a consequence of the selected weighting structure rather than evidence of different environmental leakage, biological risk, or actual municipal performance. No categorical thresholds for low, moderate, or high environmental leakage pressure were applied in the present study. The PCEI was interpreted as a continuous screening indicator of the relative capacity of a municipal collection system to retain plastic waste within organised collection flows. Values closer to 1 indicate more favourable collection conditions according to the five components included in the index, whereas values closer to 0 indicate greater operational limitations. However, a higher or lower PCEI value does not directly quantify the amount of plastic released into the environment, the concentration of plastics in water or sediments, or the magnitude of biological effects. Consequently, the index values were interpreted descriptively and comparatively, without assigning them to environmental-risk classes. Direct field measurements are required to determine whether differences in PCEI correspond to differences in actual plastic leakage or biological exposure.

The biological assessment component was developed as a receptor-and-endpoint matrix through thematic synthesis of the reviewed literature. Benthic macroinvertebrates were included because they are established biological quality elements in freshwater ecological-status assessment and may interact with microplastics through sediment contact, ingestion, and behavioural responses (European Environment Agency, 2024; Mora-Teddy *et al.*, 2024). Fish were included because they integrate direct ingestion, trophic exposure, and organism-level responses reported in microplastic toxicology studies (Banaee *et al.*, 2025). Microbial communities in water and sediments were included because plastic surfaces can support distinct biofilms and influence microbial community structure and ecosystem processes (Nava *et al.*, 2024). Riparian soil microorganisms were included because floodplain and riverbank soils may act as sinks and secondary sources of microplastics, while microbial processes regulate nutrient and carbon cycling (Aralappanavar *et al.*, 2024). Riparian and aquatic habitats were included because macroplastics may modify substrate availability, sediment dynamics, and the physical structure of riverine microhabitats (Russell *et al.*, 2023). The resulting matrix identifies candidate receptors and endpoints for subsequent monitoring; it does not represent biological effects directly measured in any specific municipality. The methodology was based on a conceptual ecobiological synthesis with illustrative index modelling, without laboratory, field, or statistical testing.

RESULTS AND DISCUSSION

Municipal plastic waste as a potential ecobiological pressure

Municipal plastic waste may become a source of ecobiological pressure when it escapes controlled collection and treatment and subsequently enters riverine or riparian environments. This transition is determined not only by waste generation but also by the occurrence of environmental leakage and the exposure of biological receptors. Once released into the environment, plastic materials may persist in habitats, move between environmental compartments, and undergo physical, chemical, and biological transformations. Macroplastic items can entangle organisms, obstruct or cover benthic substrates, accumulate in riparian vegetation, and modify the physical structure of local microhabitats. Fragmentation, abrasion, ultraviolet degradation, and mechanical stress may subsequently generate smaller particles capable of entering the microplastic size range. These mechanisms represent potential exposure pathways identified from previous studies and were not measured directly in any municipality within the present study. The transition from macroplastic items to smaller particles is potentially ecologically relevant because particle size influences mobility, retention, bioavailability, and the probability of interaction with organisms. Large items are generally more visible and may be removed through clean-up operations, whereas smaller particles can be retained in sediments, transported during high-flow events, or ingested by organisms. Experimental research by C.E. Russell *et al*. (2023) has also shown that plastics present in riverbeds can modify natural sediment-transport processes, demonstrating a possible interaction between plastic pollution and riverbed dynamics. These findings provide a conceptual basis for including sediment condition and benthic habitats among the proposed monitoring priorities.

In mountainous and foothill river systems, hydrological variability may increase the mobility and redistribution of improperly managed plastic waste. Periods of low discharge can favour local accumulation on banks, bars, and floodplains, whereas storm events and floods may remobilise deposited material. Evidence that extreme floods amplify riverine plastic transport and deposition supports the inclusion of hydrological conditions in plastic-waste monitoring frameworks (van Emmerik *et al.*, 2023). For municipalities located near rivers, particularly in narrow valleys and transboundary basins, incomplete control of plastic flows may therefore create a potential basin-scale environmental concern. However, the presence and magnitude of such leakage in any specific municipality can only be established through direct field verification. Because the present article is a theoretical-analytical screening study, the receptor matrix presented in Table 3 should be interpreted as a literature-based conceptual synthesis rather than as a statistically tested dataset. Its function is to organise biologically relevant receptors, potential exposure pathways, and candidate endpoints that can guide subsequent field monitoring. The matrix does not document biological effects measured in any specific municipality.

Table 3. Biological receptors and candidate endpoints for monitoring municipal plastic waste pressure

Receptor group	Main exposure pathway	Relevant endpoints	Interpretation for monitoring
Benthic macroinvertebrates	Contact with contaminated sediments; ingestion of particles; habitat obstruction by macroplastics	Taxonomic richness, EPT share, drift response, ingestion frequency, functional feeding groups	Priority group for river bioindication because it links sediment pressure with ecological status
Fish	Ingestion of particles and prey; contact with suspended microplastics and sorbed pollutants	Gut content, condition factor, growth, oxidative stress biomarkers, reproductive indicators	Useful for trophic and organism-level effects, but requires ethical and standardised sampling
Microbial communities in water and sediments	Colonisation of plastic surfaces; changes in organic matter degradation and biofilm structure	Microbial diversity, respiration, extracellular enzyme activity, plastisphere composition	Sensitive to functional ecosystem changes, especially where plastic surfaces become microbial habitats
Riparian soil microorganisms	Deposition of litter and microplastics on floodplains; incorporation into soils	Microbial biomass, nutrient cycling, carbon transformation, community composition	Important for floodplain and riverbank ecosystems that act as secondary sinks of plastics
Riparian and aquatic habitats	Accumulation of macroplastics, sediment modification, shading or physical coverage	Habitat heterogeneity, bank vegetation condition, substrate availability	Links waste leakage with biodiversity-supporting habitat quality

Note: the listed receptors and endpoints represent literature-derived monitoring candidates; their responses were not measured directly in any specific Upper Tisza municipality within the present study and require field validation.
EPT – ephemeroptera, plecoptera, trichoptera
Source: developed by the authors based on the freshwater biological-quality assessment framework and studies by C.E. Russell *et al*. (2023), V.K. Aralappanavar *et al*. (2024), A.K. Mora-Teddy *et al*. (2024), V. Nava *et al*. (2024), European Environment Agency (2024), M. Banaee *et al*. (2025)

The pathway from municipal plastic waste to biota can be divided into five linked stages, consistent with the general plastic waste management framework described by N.L. Bhandari *et al*. (2021) and with recent reviews of macroplastic and microplastic transport in riverine systems by L. Gallitelli & M. Scalici (2022) and E.K. Owowenu *et al*. (2023). First, plastic is generated as packaging, household waste, consumer goods, disposable items, and mixed residual waste. Second, if separate collection is incomplete or inconvenient, plastic enters mixed waste containers, informal storage points, street litter, or illegal dumping sites. Third, wind, runoff, drainage systems, and direct human disposal move this material towards rivers and floodplains. Fourth, hydrological processes transport, retain, bury, or remobilise plastic particles. Fifth, biological receptors interact with plastics through physical contact, ingestion, biofilm colonisation, or habitat modification. This pathway-based interpretation is essential because it clarifies why circular

economy measures can have biological significance. Deposit-return schemes, accessible collection points, source separation, local sorting, and extended producer responsibility reduce the quantity of plastic available for leakage. Their ecological value is therefore not limited to resource recovery; they operate as upstream controls for biological exposure. This is especially relevant for mountain and border communities where waste can be exported downstream by rivers even when the source is local. Freshwater microplastic governance studies emphasise that monitoring, source reduction, recycling, substitution, and policy coordination should be considered together (Shi *et al*., 2024). In an ecobiological framework, these instruments can be ranked by their capacity to interrupt exposure pathways. Prevention and reuse reduce the initial pressure; separate collection and recycling reduce leakage probability; clean-up activities remove already leaked macroplastics but do not fully address microplastic residues; and biological monitoring detects whether residual pressure is associated with ecosystem response. The biological interactions discussed below are derived from the reviewed literature and represent mechanisms that may be relevant to future monitoring. They should not be interpreted as effects demonstrated in any municipality of the Upper Tisza Basin within the present study.

Potential effects on macroinvertebrates, fish and microbial communities

Macroinvertebrates are among the most relevant organisms for assessing riverine effects of plastic pollution because many taxa live in close contact with sediments and organic detritus. Microplastics may be ingested accidentally during feeding, especially by deposit feeders, filter feeders, and shredders. Experimental and field-oriented research by A.K. Mora-Teddy *et al*. (2024) suggests that microplastics may influence drift behaviour and ingestion patterns in lotic invertebrates, although these effects may be weaker or more context-dependent than those associated with fine sediment. This nuance is important: plastics should not be presented as a universal cause of biodiversity loss in every setting, but as an emerging stressor that may interact with sedimentation, hydromorphology, nutrients, temperature, and chemical pollution. Fish integrate several pathways of exposure. They may ingest particles directly, consume contaminated prey, or experience indirect effects through changes in benthic food resources and habitat structure. M. Banaee *et al*. (2025), in their review of microplastic toxicology in fish, described potential physical damage, inflammatory responses, oxidative stress, immune effects, genetic damage, and reduced growth or reproductive performance under certain exposure conditions.

For river basin management, this means that fish monitoring should not be limited to visual observation of abundance; it may require gut content analysis, condition metrics, and biochemical endpoints where resources and ethical approval permit. Microbial communities represent a less visible but ecologically crucial receptor group. Plastic surfaces in water and sediments become colonised by biofilms, forming the plastisphere. These biofilms can differ from surrounding microbial communities and may influence organic matter processing, nutrient cycling, and microbial dispersal. Large-river research by V. Nava *et al*. (2024) indicates that plastic pollution can affect ecosystem processes, including community structure and functional traits, suggesting that plastics may alter biological functioning beyond their physical presence. Riparian soils and floodplain deposits also require attention. Municipal plastic waste deposited during floods or informal dumping can fragment and enter soils. V.K. Arulappanavar *et al*. (2024) reported that microplastics in soil can alter microbial diversity and functions associated with nutrient and carbon cycling. For river valleys, this creates a link between waste leakage, floodplain ecology, soil processes, and downstream aquatic exposure. Therefore, a complete ecobiological assessment should combine water, sediment, biota, and riparian soil compartments. The illustrative PCEI application produced a value of 0.67 for Scenario A and 0.64 for Scenario B. These values are mathematical outputs of predefined hypothetical component combinations. They do not characterise any named municipality and should not be interpreted as evidence of actual collection performance, environmental leakage, biological exposure, or ecosystem effects. The numerical proximity of the two outputs demonstrates that different operational profiles may produce similar composite values (Table 4).

Table 4. Illustrative interpretation of PCEI scenario outputs and required empirical verification

Illustrative scenario	PCEI value	Methodological interpretation	Environmental meaning	Required empirical verification
Scenario A	0.67	Hypothetical composite value generated from predefined component scores	No direct environmental or biological meaning without verified municipal and field data	Collection coverage, service frequency, collected plastic yield, infrastructure inventory, riverbank litter, and sediment microplastics
Scenario B	0.64	Hypothetical composite value generated from predefined component scores	No direct environmental or biological meaning without verified municipal and field data	Collection coverage, service frequency, collected plastic yield, infrastructure inventory, riverbank litter, and sediment microplastics

Note: the PCEI values are illustrative mathematical outputs and not estimates of actual municipal collection efficiency, plastic leakage, or biological impact. The common verification requirements reflect the methodological purpose of the scenarios and the absence of site-specific operational and environmental data
Source: developed by the authors

Scenario A combines broader hypothetical collection coverage with fragmented infrastructure, whereas Scenario B combines lower coverage with more favourable infrastructure availability in central settlements. Because the two scenarios differ only in the C and I components, they support the same general requirement: composite index values should be disaggregated and verified using actual service records, collected-mass data, infrastructure inventories, and direct environmental measurements before management or monitoring decisions are made. Table 4 presents an illustrative interpretation of the two scenario outputs. Its purpose is not to establish site-specific monitoring priorities but to demonstrate the type of empirical verification required before PCEI can be applied to real municipalities. Because the scenarios are hypothetical and no biological, environmental, or operational municipal measurements were performed, no statistical comparison or environmental-risk classification was applied. Table 4 should therefore be interpreted as a methodological guide for future data collection rather than as an assessment of conditions in the Upper Tisza municipalities.

The potential relevance of municipal plastic waste to biodiversity depends on the occurrence and intensity of environmental leakage, exposure duration, particle characteristics, hydrological connectivity, and the ecological sensitivity of receiving habitats. Plastic waste may represent one of several interacting stressors in urban and modified river channels. In mountain streams, tributaries, floodplain wetlands, and riparian zones, its ecological relevance may vary according to habitat condition, flow regime, substrate structure, and the presence of other pressures. Therefore, the same quantity of plastic waste may have different ecological implications in different receiving environments. The present study does not quantify these relationships but identifies factors that should be considered during subsequent field verification. Benthic macroinvertebrates may be considered a priority receptor group for subsequent bioindication because they are widely used in freshwater ecological-status assessment and respond to changes in substrate, oxygen conditions, organic matter, sediment quality, and hydromorphology. Microplastic sampling may be combined with conventional macroinvertebrate assessment by collecting sediment and biological samples at the same sites. However, any observed association should be interpreted cautiously. Changes in macroinvertebrate communities near waste-accumulation sites may also be related to wastewater discharge, nutrient enrichment, channel modification, fine-sediment deposition, temperature, or hydrological stress. Consequently, a field study should include reference sites and measurements of major co-occurring pressures before plastic waste is considered a plausible explanatory factor.

Fish may provide supplementary information on organism-level and trophic exposure, but their inclusion in the initial monitoring programme should be carefully justified. Where fish assessment is feasible, non-lethal approaches, opportunistic examination of legally obtained specimens, or integration with existing fisheries-monitoring programmes may reduce ethical

and logistical constraints. Gut-content screening can be combined with species identification, body length, body mass, and condition metrics. Biochemical or reproductive biomarkers should be included only where adequate laboratory capacity, quality assurance, sample size, and ethical or legal permissions are available. Such indicators are proposed for future validation and were not assessed in the present study. Microbial indicators may complement organism- and habitat-based monitoring by characterising biological processes associated with plastic surfaces, sediments, and riparian soils. Plastisphere composition, microbial respiration, extracellular enzyme activity, and community structure are potential indicators of whether plastic particles function as biologically active substrates. In riparian soils, microbial measurements may help assess changes in nutrient and carbon cycling in areas where plastics accumulate or fragment. However, microbial responses are influenced by numerous environmental factors and cannot be attributed to plastic pressure without suitable reference samples, contamination controls, and measurements of relevant physicochemical variables. Overall, the proposed bioindication priorities represent a tiered set of candidate measurements rather than a confirmed causal assessment scheme. Macroinvertebrates and habitat condition may form the initial monitoring tier, while fish and advanced microbial endpoints may be added where preliminary environmental measurements confirm the presence of plastic contamination and sufficient analytical capacity is available.

Circular economy measures as potential upstream controls

The ecobiological interpretation proposed in this study does not replace conventional circular economy analysis but extends it by considering the potential environmental consequences of uncontrolled plastic flows. Circular economy policy is commonly structured around prevention, reuse, separate collection, recycling, resource efficiency, and reduction of residual waste streams (Geissdoerfer *et al.*, 2017; European Commission, 2020). Within the proposed framework, these measures are treated as potential upstream controls because they may reduce the quantity of plastic available for environmental leakage. However, the present study did not measure changes in riverine plastic abundance or biological responses following the implementation of such measures. Their ecological effectiveness therefore requires verification through comparative monitoring before and after intervention or between appropriately selected reference and intervention sites. Extended producer responsibility may support improved control of plastic flows when it finances collection, sorting, public communication, and take-back systems (OECD, 2016; Tumu *et al.*, 2023).

Deposit-return systems may similarly reduce the probability that beverage containers remain outside organised collection, although their local environmental effect depends on system coverage, return rates, accessibility, and enforcement. Pay-as-you-throw and other charging schemes may encourage waste prevention and source separation, but their design should account for possible unintended consequences, including illegal dumping or unequal access to services, as noted by G. Di Foggia & M. Beccarello (2023). In the conceptual framework developed here, these instruments are regarded as potential source-control measures rather than interventions whose biological effectiveness has already been demonstrated in the Upper Tisza Basin. In Ukraine, the waste hierarchy established by the Law of Ukraine No. 2320-IX (2022) and the Order of the Cabinet of Ministers of Ukraine No. 1353-r (2024) provide a policy basis for strengthening prevention, separate collection, recovery, and monitoring. For river-basin communities, implementation may be complemented by indicators that characterise the degree of plastic-flow control, the presence of waste outside organised systems, and the condition of receiving environments. This is particularly relevant in the Upper Tisza Basin, where hydrological connectivity creates the possibility that locally released plastic waste may be transported beyond municipal and national boundaries. The present study does not quantify such transboundary transport but identifies it as a priority for subsequent field investigation and coordinated monitoring. A practical implication of the proposed framework is that municipal waste planning may be complemented by environmental verification indicators. These may include the location of waste-accumulation points relative to watercourses, standardised riverbank-litter density, the condition and

servicing frequency of collection infrastructure, microplastic abundance in sediments, habitat characteristics, and selected biological indicators. Such measurements would make it possible to test whether limitations identified through PCEI correspond to observable plastic contamination or biological exposure. Until these relationships are empirically verified, municipal collection indicators and ecobiological indicators should be interpreted as connected but methodologically distinct evidence streams.

A staged monitoring framework is recommended for municipalities where direct biological data are not yet available. Stage 1 should map pressure sources: container coverage, informal dumping points, drainage outlets, riverbank litter, floodplain deposits, and high-risk zones near markets, bridges, schools, transport nodes, and recreational areas. Stage 2 should quantify environmental compartments: macroplastic item density on riverbanks, microplastic abundance in sediments, and, where feasible, particles in surface water. Stage 3 should add biological receptors: macroinvertebrate community composition, selected fish indicators, microbial activity in sediments and soils, and riparian habitat condition. Quality assurance is essential because microplastic sampling is vulnerable to contamination and methodological inconsistency. Laboratory procedures should use procedural blanks, contamination control, particle-size definitions, and polymer verification where possible. Established methodological guidance, including laboratory of the National Oceanic and Atmospheric Administration recommendations, emerging standardised methods for microplastic analysis, and sample-specific protocols for freshwater, sediment, and fish matrices, can help improve comparability (Masura *et al*., 2015; Vural *et al*., 2025). For initial local studies, even a limited but well-documented protocol is preferable to broad claims based on unverified observations. The minimum dataset for a future empirical article should include: sampling coordinates; date and hydrological conditions; number of sediment or water samples; macroplastic density per unit area of riverbank; microplastic abundance per kilogram of dry sediment or per cubic metre of water; macroinvertebrate metrics; and at least one habitat-quality descriptor. If fish are included, the dataset should specify species, sample size, body length, weight, gut-content procedure, and ethical or legal basis for sampling. These data would allow statistical comparison among sites with contrasting observed plastic occurrence, collection-system conditions, and environmental characteristics and would provide the empirical basis required to examine possible relationships between municipal plastic-flow control and biological indicators.

Ecological mechanisms linking plastic leakage and biodiversity change

Plastic waste may influence biodiversity through several mechanisms that operate at different spatial and biological scales. At the habitat scale, macroplastics can cover stones, gravel, coarse particulate organic matter, and marginal vegetation. Such physical coverage may reduce habitat availability for benthic invertebrates and alter the distribution of refugia used by juvenile fish. In small mountain streams, where habitat mosaics are spatially limited, even local accumulation can modify the microhabitat structure that supports sensitive taxa. At the organism level, ingestion is the most direct pathway. Microplastic particles may be consumed because they resemble food particles, are attached to biofilms, or are trapped in organic matter. The ecological significance of ingestion depends on particle size, shape, polymer, concentration, and residence time in the digestive tract. Fibres, fragments, films, and foams can differ in their physical behaviour. Therefore, future field studies should report particle morphology and polymer type rather than only total particle counts. At the community level, plastics can act as artificial substrates. The plastisphere may support microbial assemblages that differ from natural biofilms on stones, wood, or sediment. These assemblages can influence nutrient cycling, organic matter processing, and microbial dispersal. The concern is not only the presence of bacteria on plastics, but the possibility that persistent anthropogenic substrates alter the spatial structure of microbial habitats in rivers and floodplains. At the ecosystem level, plastic pollution may interact with existing stressors. Nutrient enrichment can increase biofilm growth on plastics; hydromorphological alteration can enhance retention zones; floods can remobilise buried litter; and chemical contaminants can sorb to polymer surfaces. As a

result, biodiversity responses to plastic waste are unlikely to be linear. The same observed plastic load may have different biological relevance in a sensitive tributary and a heavily modified channel because ecological vulnerability and co-occurring pressures differ among habitats.

The PCEI describes selected operational characteristics of municipal plastic-waste collection, including service coverage, collection frequency, transport accessibility, separately collected yield, and infrastructure availability. It does not evaluate the full circularity of plastic materials and does not directly measure environmental leakage, ecological status, or biological effects. Its role in the present framework is limited to identifying potential weaknesses in organised plastic-flow control that may justify targeted environmental verification. Accordingly, PCEI should be interpreted together with direct environmental and biological measurements rather than as a substitute for them.

A staged verification chain is proposed. First, municipal collection indicators, including PCEI and its individual components, characterise the capacity of the organised system to collect and retain plastic waste. Second, direct environmental indicators document whether plastic is present outside the controlled system, for example through standardised riverbank-litter counts, macroplastic density, or microplastic abundance in water and sediments. Third, exposure and biological indicators may include particles detected in organisms, macroinvertebrate community metrics, fish condition or gut-content data, microbial functional measurements, and habitat-condition descriptors. Fourth, the interpretation stage compares these evidence levels while accounting for wastewater discharge, nutrients, hydromorphological modification, sedimentation, land use, and hydrological variability. Only this combined approach can test whether limitations in municipal collection are associated with observable environmental contamination or biological responses.

This staged approach helps avoid two methodological errors. The first is to assume that favourable municipal collection indicators automatically demonstrate low environmental contamination or improved biodiversity status. Collection efficiency may reduce the probability of uncontrolled waste accumulation, but its environmental effect must be tested directly. The second error is to attribute biological degradation observed near waste-accumulation sites exclusively to plastic pollution. Riverine ecosystems are exposed to multiple interacting pressures, and causal interpretation requires reference sites, measurements of co-occurring stressors, adequate replication, and appropriate statistical analysis. In the proposed framework, PCEI is therefore an initial screening tool for directing field investigation, not evidence of ecological condition or causality. The proposed framework therefore separates three analytically distinct levels: municipal collection performance, environmental occurrence of plastic, and biological exposure or response. Relationships between these levels remain hypotheses until they are tested using spatially and temporally matched field data. Maintaining this distinction is essential for preventing collection-system characteristics from being interpreted as direct evidence of environmental or biological condition.

Implications for mountainous and transboundary river basins

Mountainous and transboundary river basins require a specific interpretation because plastic released into watercourses may produce both local and downstream consequences. In narrow valleys, settlements, roads, drainage systems, and river channels are often spatially connected, reducing the distance between municipal plastic-waste sources and aquatic environments. During heavy rainfall, snowmelt, and flood events, plastic accumulated on banks, slopes, floodplains, or informal disposal sites may be mobilised and transported downstream. The probability and distance of transport depend on waste characteristics, topography, hydrological connectivity, discharge conditions, and retention within channel and floodplain environments (Owowenu *et al.*, 2023; van Emmerik *et al.*, 2023). The Upper Tisza is a transboundary river system in which plastic waste transported from upstream areas may cross administrative and national borders and accumulate in downstream river reaches, sediments, floodplains, and riparian zones. A. Balla *et al.* (2024) documented plastic contamination in the Tisza River and mismanaged plastic waste along Carpathian watercourses, respectively, supporting the relevance of basin-scale and internationally coordinated monitoring.

The present study did not quantify the contribution of any specific municipality to transboundary plastic transport. Nevertheless, the hydrological connectivity of settlements within the Upper Tisza River network makes the prevention of local plastic leakage relevant to downstream and cross-border environmental safety. For small municipalities with limited laboratory resources, the first practical step should be a low-cost monitoring package: mapping of visible plastic accumulation points, standardised photographic documentation, item counting by riverbank segment, and basic habitat description. The second step should add sediment microplastic screening in cooperation with a university laboratory. The third step should include macroinvertebrate sampling using nationally or internationally compatible bioindication methods. This staged approach is more realistic than immediate implementation of a full laboratory programme in every community. Transboundary cooperation is necessary for distinguishing locally generated plastic pollution from material transported from upstream parts of the basin. Comparable protocols applied across upstream and downstream sites can support the identification of accumulation zones, temporal changes, and possible source areas. Monitoring should account for hydrological conditions, particularly flood events, because plastic deposited at one location may subsequently be remobilised and transported across municipal or national boundaries. Accordingly, prevention of plastic leakage in upstream communities should be regarded as a component of downstream and transboundary environmental protection, while source attribution requires coordinated spatial sampling and hydrological interpretation.

For field validation of the proposed framework, a comparative design should include three to five river or stream sites representing contrasting municipal collection conditions and directly observed plastic occurrence. The site network should include at least one reference site with no visible waste accumulation and limited nearby municipal pressure, one or more sites near settlements or collection infrastructure, and at least one site near a documented accumulation zone, drainage outlet, or informal dumping location. Site classification should be based on preliminary field reconnaissance and standardised observations rather than on PCEI values alone. Sampling should be conducted during the same hydrological season and, where possible, repeated under comparable low-flow and post-flood conditions to reduce seasonal and hydrological bias. For each site, the minimum environmental dataset should include coordinates, date, river width, approximate flow conditions, substrate type, bank condition, nearby waste sources, and macroplastic item density along a standardised bank transect. Sediment samples should be collected from comparable depositional zones and analysed for microplastic abundance using contamination-control procedures. Results should be reported as particles per kilogram of dry sediment, with size classes and particle morphology where possible. The biological dataset should include benthic macroinvertebrate taxa identified at the lowest feasible taxonomic level. At a minimum, the study should report total richness, abundance, EPT taxa or an equivalent sensitivity indicator, and the relative share of tolerant groups. If resources allow, selected specimens can be examined for ingested particles, but this should be done using a transparent digestion and visual or spectroscopic identification protocol. Fish data are useful but not essential for the first field paper; if included, they should follow ethical and legal requirements. For riparian soils, a small number of composite samples near accumulation zones and reference zones could provide valuable information on the terrestrial component of leakage. Microbial biomass, respiration, or enzyme activity would strengthen the biological argument, but even a documented soil microplastic screening would improve the empirical basis. Such a dataset would transform the proposed risk-screening synthesis into a direct ecobiological field assessment. Until such field sampling is completed, the framework should be interpreted as a preliminary prioritisation tool. Adding a pilot dataset on plastic occurrence and biological receptors would allow future research to examine relationships between municipal collection conditions, measured environmental plastic occurrence, biodiversity indicators, and ecosystem functioning.

CONCLUSIONS

This study developed a conceptual ecobiological screening framework that links municipal plastic-waste collection conditions with potential environmental pathways, biologically relevant

receptors, and priorities for subsequent field monitoring in riverine and riparian ecosystems. The literature synthesis indicates that improperly managed plastic waste may be transported into watercourses, retained in sediments and floodplains, fragmented into smaller particles, and interact with organisms and microbial communities. These mechanisms were identified from previous studies and were not measured directly in any specific Upper Tisza municipality within the present study. The authors-developed PCEI integrates collection coverage, service frequency, transport accessibility, per-capita collected plastic yield, and infrastructure availability. Its illustrative application produced values of 0.67 for Scenario A and 0.64 for Scenario B. These mathematical outputs show that different hypothetical scenarios can produce similar composite scores and should not be interpreted as empirical evidence of environmental conditions. The small difference reflects the selected weighting structure, while both scenarios require empirical verification through collection data, litter surveys, sediment microplastic analysis, habitat assessment, and benthic macroinvertebrate monitoring. Fish, microbial, and riparian-soil indicators may be added where direct evidence of plastic occurrence and sufficient analytical capacity are available. Circular economy and waste-management instruments, including prevention, separate collection, accessible infrastructure, extended producer responsibility, deposit-return systems, and local sorting, may function as upstream controls by reducing the amount of plastic available for environmental release. However, their environmental and biological effectiveness in the Upper Tisza Basin requires empirical verification. This study has several limitations. The PCEI values are hypothetical and do not represent empirical municipal estimates, while the authors-defined weights and scoring categories remain unvalidated. The index does not measure actual plastic leakage, environmental concentrations, biological exposure, toxicity, or biodiversity degradation, and no direct field sampling was conducted. The receptor-and-endpoint matrix also requires validation under Upper Tisza conditions, particularly given the influence of multiple co-occurring riverine stressors. Future research should therefore integrate municipal collection data with direct plastic measurements, biological monitoring, hydrological conditions, reference sites, and appropriate spatial, temporal, and statistical analyses. The framework should be viewed as a tool for prioritising field investigation rather than evidence of causal relationships.

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Концептуальний екобіологічний скринінг тиску муніципальних пластикових відходів на річкові екосистеми

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Анотація. Муніципальні пластикові відходи не лише становлять управлінську або технологічну проблему, а й є потенційним джерелом екобіологічного тиску на річкові та прибережні екосистеми. Недостатнє роздільне збирання, неконтрольоване накопичення та фрагментація пластикових матеріалів здатні сприяти надходженню макро- та мікропластику до прісноводних екосистем, де вони можуть взаємодіяти з макробезхребетними, рибами, мікробними угрупованнями, донними відкладами, ґрунтами та прибережними оселищами. Метою роботи було систематизувати потенційні шляхи надходження муніципальних пластикових відходів до річкових екосистем, узагальнити можливі механізми їх взаємодії з біотою та розробити попередню концептуальну рамку для визначення пріоритетів екобіологічного моніторингу. Застосовано теоретико-аналітичний підхід, який поєднував цільовий наративний огляд сучасних наукових джерел, рамку «тиск – шлях міграції – рецептор – відповідь» та ілюстративне застосування розробленого авторами індексу ефективності збирання пластикових відходів для двох умовних сценаріїв організації муніципального збирання. Узагальнення наукових джерел показало, що муніципальні пластикові відходи можуть взаємодіяти з біотою через фізичне засмічення оселищ, ковтання частинок, перенесення пов'язаних із ними забруднювачів, формування пластисфери та зміну мікробних процесів у воді, донних відкладах і ґрунтах. Ілюстративне застосування індексу до двох умовних сценаріїв дало значення 0,67 і 0,64 та продемонструвало, як різні поєднання охоплення послугою й доступності інфраструктури впливають на інтегральну оцінку. Ці сценарні значення не характеризують конкретні громади та не вимірюють фактичні витоки пластику, його концентрації у водному середовищі, біологічну токсичність або деградацію біорізноманіття. У роботі запропоновано концептуальну екобіологічну рамку для визначення пріоритетів роздільного збирання, локального моніторингу мікропластику, оцінювання донних відкладів, біоіндикації та подальшої польової верифікації в річкових басейнах, що зазнають тиску муніципальних пластикових відходів

Ключові слова: макробезхребетні; пластисфера; донні відклади; біоіндикація; заплави

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Biotechnological aspects of digestate production during methane fermentation and utilisation

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Abstract. The relevance of the study is conditioned by the rapid development of the bioenergy industry, namely, the biogas sector in Ukraine, which leads to the generation of significant volumes of one of the by-products of anaerobic digestion – digestate, which is valuable in many aspects. The purpose of this study was to investigate the main stages of methane fermentation and factors affecting the composition and quality of digestate, and to review conventional and innovative methods of its utilisation. Analysis of the available literature has shown that current methods of storing the produced digestate are ineffective and act as a temporary way of its accumulation, given the growing scale of its generation every year and in the future. Each of the four stages of anaerobic digestion (hydrolysis, acidogenesis, acetogenesis, methanogenesis) was considered in detail in the paper and specific groups of microorganisms involved in these processes were characterised. The relationship between the composition of the raw material and the final physical and chemical characteristics of digestate was determined. The influence of the initial feedstock on the composition and key characteristics of the resulting digestate were presented. The paper analysed the results of contemporary research in the field of digestate processing and utilisation using biotechnological methods to ensure the main goals of sustainable development and compliance with the principles of the circular economy. Special attention was paid to the key characteristics of the resulting digestate, which have a predominant influence on the choice of further methods and technologies for its utilisation. Based on the analysis, the information was systematised and presented in the form of generalising tables. The results of this review paper are of practical value and can be used for the selection and implementation of digestate processing technologies in existing enterprises

Keywords: fermented residue; anaerobic digestion; processing; biogas; circular economy; sustainable development

INTRODUCTION

The transition to sustainable development models is one of the key strategies of the global economy amid the depletion of natural resources and the accumulation of substantial amounts of waste. Of particular importance is the concept of a circular economy, which aims to minimise waste by

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including it in new production cycles or reusing it. Thus, one of the stages on the way to sustainable development and a circular economy is the introduction of renewable energy sources, which have been increasingly developed over the past decades. The production of biogas and biomethane from organic waste of various origins is a relatively widespread technology that combines energy production with waste management and contributes to the achievement of the Sustainable Development Goals. The introduction of circular economy concepts is promising, which would allow obtaining new valuable products and reducing the accumulation of waste that remains as a result of the technological process of obtaining biogas and biomethane.

As noted by M.-N. Dincă *et al*. (2025), circular economy involves extending the life cycle of products through actions such as reuse, recovery, and recycling. In analytical notes, the non-governmental organisation UABIO (n.d.) noted that the total share of biomethane and biogas produced in Europe was more than 22 billion m^3 in 2024. Biomethane production remains the fastest growing segment (5.2 billion cubic metres, of which 4.3 billion cubic metres are produced in the EU-27), supported by an installed capacity of 7 billion m^3 per year in Europe at the beginning of 2025. In addition, as noted by K. Chojnacka & K. Moustakas (2024), the latest report of the Intergovernmental Panel on Climate Change indicates that global greenhouse gas emissions reached 59.1 gigatonnes of CO_2 equivalent in 2022. Appropriate waste management and utilisation will help to reduce greenhouse gas emissions, conserve resources, and produce valuable new products.

An important by-product of biogas production is digestate, a mixture of non-fermented organic residues. D. Kovačić *et al.* (2022) reported that approximately 180 million tonnes of digestate are produced annually in the EU. About 120 million tonnes are of agricultural origin (usually a mixture of manure and plants, in particular energy crops), 46 million tonnes are produced from the organic fraction of mixed municipal solid waste, at least 7 million tonnes from organic waste, while a smaller amount (about 1.7 million tonnes each) comes from the fermentation of sewage sludge and by-products of the agro- and food industries. According to M.-N. Dincă *et al*. (2025), the European Biogas Organisation estimates that 75 million tonnes of dry matter digestate will be produced in Europe by 2030, and the total potential of digestate produced in Europe by 2050 will be 177 million tonnes of dry matter.

M. Nowak & W. Czekała (2024) noted that the vast majority of digestate is used as fertiliser in liquid form in private enterprises or stored in special non-sealed tanks – lagoons. When digestate is stored for a long time in lagoons, solid particles settle and accumulate at the bottom. In addition, V. Polishchuk *et al*. (2020) indicate that due to non-sealed tanks, greenhouse gas emissions – methane, carbon dioxide, etc. – increase and unpleasant odours spread.

Accordingly, lagoons are only one of the ways to accumulate and temporarily store digestate, but not a solution to the problem of its utilisation, and given the growing scale of biogas production, the amount of digestate generated will continue to increase. Thus, it is important to find new ways to dispose of it and process it in order to obtain valuable products and comply with the principles of a circular economy. It is also important to consider the main characteristics and properties of digestate to choose the most optimal method of its processing. The purpose of this study was a comprehensive analysis of biotechnological aspects of digestate generation as a product of anaerobic digestion and analysis of current methods of its processing to ensure the principles of circular economics.

MATERIALS AND METHODS

To achieve the stated objective and ensure objectivity, a systematic analysis of contemporary scientific literature, primarily by foreign and Ukrainian authors, was conducted. This was a narrative review with elements of a systematic approach to literature search and analysis. The methodology for selecting relevant research was based on determining the search query, forming keywords for its implementation, analysing relevant information sources, and including or discarding them as relevant. Databases such as Scopus, Web of Science, Google Scholar, PubMed, and Science Direct were used for the search. In total, about 200 papers were analysed, the search for which was carried out by keywords mainly in English: biogas technology, substrate for biogas production, digestate characteristics or utilisation, physico-chemical methods

or biotechnology of digestate utilisation, composting and vermicomposting, microalgae. The time frame for searching for the main sources was the period during 2022-2026, which was explained by the rapid development of this segment and the development of new technologies. In accordance with this, the results of studies in the specified time range were used and processed to obtain up-to-date information.

The key selection criteria were publications from peer-reviewed scientific journals, research focused on the biological and biochemical features of digestate generation in the process of biogas production, the influence of the main factors on this process, and the features of its utilisation, studies that included the experimental part of the real implementation of these technologies, and the presence of certain statistically significant results. The main exclusion criteria were non-peer-reviewed articles and conference abstracts; studies that duplicated previously published results by the same researchers without providing additional findings; and papers focusing primarily on the economic and logistical aspects of the process under study. The analysis of the selected literature was based on methods of systematisation, identification of key results of publications, comparison, and structuring of the selected information in text format and using summary tables. As a result of the analysis and systematisation according to the above criteria, 35 publications were selected, including review papers and experimental studies on the main stages of methane fermentation and the biological agents involved in each of them, the main characteristics of digestate obtained as a result of anaerobic digestion and their influence on processing methods.

RESULTS AND DISCUSSION

Anaerobic digestion is a complex biochemical process of converting organic waste into biogas and a liquid fraction – digestate with the participation of a whole consortium of microorganisms. In their publication, D. Kovačić *et al*. (2022) indicated that this technology is widely used for the treatment of agricultural and food waste, organic fraction of municipal solid waste, and sediments generated as a result of wastewater treatment. As noted by K. Pilarski & A. Pilarska (2025), anaerobic digestion was carried out in hermetically sealed reactors, which ensured stable process conditions and control over operating parameters. The resulting biogas is sent to storage tanks and then most often used in combined heat and electricity generation systems. It can also be burned directly for heat generation or converted to biomethane for injection into the grid or used as a transport fuel, depending on local energy demand profiles and the availability of appropriate infrastructure.

According to M. Jameel *et al*. (2024) biogas is a mixture of gases consisting of 50-70% methane (CH_4), 30-50% of carbon dioxide (CO_2), and residues of other gases, such as water vapour (H_2O) and hydrogen sulphide (H_2S). Depending on further use, the impurities present in the biogas are removed. As indicated by H.G. Katariya & H.P. Patolia (2023), various methods are being developed that various methods are being developed for upgrading biogas to biomethane by removing CO_2, H_2O and other residual gases that may cause equipment and engine damage and reduce their service life. In addition, some of them, namely hydrogen sulphide, are toxic and, if released into the atmosphere, can pose a threat to human health and the state of ecosystems. As mentioned above, in addition to biogas, as a result of anaerobic fermentation of organic waste, a by-product is formed – digestate, consisting of a mixture of solid and liquid fractions that do not decompose during fermentation. Digestate is rich in organic substances and macro- and microelements, and that is why it is quite a valuable product.

To better understand the composition and characteristics of digestate, it is necessary to consider the key stages and conditions of fermentation. D. Kovačić *et al*. (2022) noted that the anaerobic fermentation process is carried out due to the joint action of groups of microorganisms: hydrolytic and acidogenic bacteria, syntrophic acetogenic bacteria, and methanogenic archaea, and is generally divided into 4 stages – hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The syntrophic nature of this process makes each stage important, as indicated by T. Induchoodan *et al*. (2022). For example, acidogenesis is a prerequisite for methanogenesis, but if a large amount of acids is formed during the acidogenesis stage, this will affect the methanogens and interfere with the further process. As noted by K. Pilarski & A. Pilarska (2025), during the first phase of anaerobic

digestion, polymers such as starch, proteins, and fats are hydrolysed to monomers – simple sugars, amino acids, glycerol, and fatty acids – using hydrolytic exoenzymes such as cellulases, hemicellulases, and proteases, which are mainly secreted hydrolytic and fermentative bacteria of the genera microorganisms belonging to the phyla *Firmicutes* and *Bacteroidetes* and the genera *Ruminococcus* and *Clostridium*.

Hydrolysis is a relatively slow step, and it can limit the speed of the overall anaerobic digestion process, especially when using large amounts of solid waste as a substrate. According to P. Bajpai (2017), the next step is acidogenesis, during which simple organic substances are converted to higher organic acids (propionic and butyric acids), and acetic acid, hydrogen, and carbon dioxide. This phase is managed by a broad consortium of enzymatic and acidogenic bacteria, as noted by K. Pilarski & A. Pilarska (2025), including representatives of *Clostridium*, *Lactobacillus*, *Enterobacter*, and *Bacteroides* which convert sugars, amino acids, and other hydrolysis products into volatile fatty acids (VFA), alcohols, CO_2, and H_2. In the third phase of acetogenesis, acid-forming bacteria produce carbon dioxide, hydrogen, acetate, hydrogen sulphide, ethanol, and very small amounts of methane, nitrogen, and ammonia (Jameel *et al*., 2024). The key participants at this stage are syntrophic acetogenic bacteria, such as *Syntrophomonas* and *Syntrophobacter*, which oxidise VFA (e.g. propionate and butyrate) to acetate and H_2, as indicated in the publication by K. Pilarski & A. Pilarska (2025). However, according to P. Bajpai (2017), it is difficult to clearly draw the line between the acidogenic and acetogenic stages. Acidogenic and acetogenic bacteria belong to a large and diverse group that includes both facultative and obligate anaerobes. Species isolated from anaerobic reactors include *Clostridium*, *Peptococcus*, *Bifidobacterium*, *Desulfovibrio*, *Corynebacterium*, *Lactobacillus*, *Actinomyces*, *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Bacillus*, *Pseudomonas*, *Selemonas*, *Veillonella*, *Sarcina*, *Desulfobacter*, *Desulfomonas*, and *Escherichia coli.*

The next stage is methanogenesis – a complex phase carried out due to the metabolic action of a complex of methanogenic archaea. They grow slowly, are very sensitive to changing environmental conditions, and are strict anaerobes, as indicated by M. Jameel *et al.* (2024). As noted by K. Pilarski & A. Pilarska (2025), there are several pathways of methane generation by methanogens: acetoclastic and hydrogenotrophic. Acetoclastic methanogenesis is carried out mainly by archaea of genera *Methanosaeta* and *Methanosarcina*, which convert acetate to CH_4 and CO_2, whereas hydrogenotrophic methanogenesis is carried out by genera such as *Methanobacterium*, *Methanospirillum* and *Methanococcus*, which reduce CO_2 to methane using H_2. In addition, there is also a pathway for methane production from methanol and methylated compounds, but it is less common. According to P. Bajpai (2017), some of the species that have been isolated and classified include *Methanobacterium formicicum*, *M. bryantic* and *M. Thermoautotrophicum*; *Methanobrevibacter ruminantium*, *M. arboriphilus*, and *M. smithii*; *Methanococcus vannielli* and *M. voltae*; *Methanomicrobium mobile*; *Methanogenium cariaci* and *M. marinsnigri*, *Methanospirilum hungatei*, and *Methanosarcina barkei.*

In general, according to V. Polishchuk *et al.* (2020), digestate is a product of bioconversion of organic materials during methane fermentation, causing complex organic matter to break down into simpler organic compounds, inorganic substances, microbial biomass, and biogas. The amount of digestate obtained is usually 70-90% of the volume of incoming raw materials, as indicated by M.-N. Dincă *et al.* (2025). It is biologically stable and rich in nitrogen, phosphorus, and potassium. Due to the decomposition of organic matter, mineralisation processes and the generation of biogas, in comparison with the input raw materials, the content of dry matter in the resulting digestate decreases, and moisture content increases accordingly (it can be in the range of 92-99%, but, as a rule, it is 94-96%) and viscosity decreases. Digestate has a high content of easily accessible ammonium nitrogen for plants (about 60-80% of the total nitrogen content); has a balanced C/N ratio (20-30), and a pH value close to neutral (6.5-8.0); almost does not contain viable weed seeds and pathogenic microflora (provided that the required duration and temperature of the process is observed, which mainly provides a thermophilic fermentation regime), which was indicated by V. Polishchuk *et al.* (2020). For these reasons, digestate is often used in agriculture as a substitute for synthetic fertilisers.

According to R. Feiz *et al.* (2022), the most common method of further digestate treatment is separation into two fractions, solid and liquid, with distinct nutrient profiles, i.e., different N:P:K concentrations and ratios. The liquid fraction can be used as fertiliser, and the solid fraction can be used as a soil improver. The solid fraction consists of a certain amount of organic matter, but contains a large amount of cellulose and lignin, which affects its sorption properties. Its use in agriculture allows increasing the moisture capacity of the soil and content of organic matter. In addition, the vast majority of phosphorus passes from digestate to the solid fraction. The liquid fraction contains more soluble forms of nitrogen, phosphorus, and potassium, which have a higher level of assimilation by plants, compared to the solid form. The liquid phase is characterised by a high content of nitrogen and potassium, with 80% nitrogen in the form of ammonia ions, as noted by M. Nowak & W. Czekała (2024). It was also proved, as indicated by M.-N. Dincă *et al*. (2025), that digestate contains beneficial microorganisms such as nitrogen-fixing, denitrifying, and nitrifying bacteria, saprophytic fungi, and soil methanogens. In addition, digestate-derived microorganisms can also be used in the soil bioremediation process due to their ability to decompose aliphatic hydrocarbons from oil-contaminated soils (Table 1).

Table 1. Characteristics of the biogas generation process

Stage	Process	Substrate	Reaction products	Microorganisms
Hydrolysis	Hydrolysis of biopolymers to monomers	Blend of protein, fat, and carbohydrates	Amino acids, fatty acids, mono- and oligosaccharides	Cellulolytic fungi, hydrolytic microorganisms: *Bacillus subtilis*, *Pseudomonas putida*, *Aspergillus niger*
Acidogenesis	Organic compounds obtained as a result of hydrolysis are converted into organic acids with the participation of bacteria	Amino acids, fatty acids, mono- and oligosaccharides	Short-chain organic acids (butyric, propionic, etc.)	Acidogenic bacteria: *Clostridium acetobutylicum*, *Bacteroides fragilis*, *Enterobacter aerogenes*
Acetogenesis	Acetate is formed in two ways: from the acids of the previous stage, with the release of hydrogen, and by reduction of CO_2 using hydrogen	Short chain organic acids (butyric, propionic, etc.)	Hydrogen, carbon dioxide, acetic acid	Homoacetogenic bacteria: *Butyribacterium methylotrophicum*, *Acetobacterium woodii*, *Syntrophomonas wolfei*
Methanogenesis	Methane is formed in two ways: by splitting acetate and carbon dioxide reduction	Acetic acid, carbon dioxide	Biogas, digestate	Hydrogenotrophic and acetotrophic methanogens: *Methanobrevibacter smithii*, *Methanosarcina barkeri*, *Methanogenum thermautotrophicus*, *Methanosarcina thermophila*
Sulphate reduction	Generation of hydrogen sulphide and carbon dioxide	Substrates from previous stages	Hydrogen sulphide, carbon dioxide	*Desulfovibrio vulgaris*, *Desulfobacter postgatei*

Source: created by the authors based on the analysis performed

As mentioned above, the composition of the initial substrate used for anaerobic digestion significantly affects the further composition and physico-chemical characteristics of the digestate. In addition, the features and parameters of fermentation are important, including the operating conditions of the biogas plant and the physico-chemical parameters of the fermentation process (temperature, pH, etc.). For consideration, it is customary to take such a division of types of

input substrates into 5 categories: agricultural waste and by-products (animal manure, silage), crop residues (plant residues, straw), sewage sludge (municipal and household), organic fraction of municipal solid waste, and food production waste.

Thus, according to, P. Ragályi *et al.* (2025), animal manure is one of the most common substrates used for biogas production. During anaerobic fermentation, approximately 70% of the total nitrogen content in raw animal manure is mineralised to form ammonium ions (NH_4^+) or free ammonia (NH_3). Digestate obtained from animal manure usually contains 0.8-5.0 g/L of ammonium nitrogen. Phosphorus (P) comes partly from feed and partly from inorganic additives, and its concentration can reach 1.2 g/L in the resulting digestate. Dissolved P can be detected mainly in the form of orthophosphate (PO_4^{3-}), and its presence strongly depends on the pH indicator. Approximately 90% of the phosphorus content in the digestate forms a precipitate in the form of compounds with metals, while the dissolved fraction is only about 10%. In addition, poultry manure and cattle manure contain a higher proportion of solid components and lead to slower decomposition than in the case of pig manure. Compared to others, poultry manure has a higher content of ammonium nitrogen and total nitrogen, which accordingly affects the nitrogen content in the digestate formed from it. Digestate obtained from pig manure contains approximately 80% of the total nitrogen content in the form of NH_4^+.

As noted by J. Ries *et al*. (2023), digestate produced by the fermentation of food waste is usually rich in nitrogen, chlorine, and sodium, along with lower concentrations of phosphorus, sulphur, boron, magnesium, manganese, and molybdenum. The total percentage of nitrogen is usually between 4-5%, but there is evidence that it can be 1.1-9.6%. Common elements found in excess include Na and Cl, which can be explained by the high salt content of food waste. Recorded Na^+ concentration ranges from 36-319 mg/L, while the Cl^- concentration is in the range of 87-2,100 mg/L. The pH value is mostly alkaline. Due to the fermentation of raw materials with a high protein content, which prevails in the composition of food waste, the resulting digestate is characterised by an increased content of ammonium nitrogen, as noted by F. Rivera *et al*. (2022).

P. Ragályi *et al.* (2025) indicated that digestate formed from the organic fraction of municipal solid waste usually has a slightly alkaline pH and contains both macro- (N, P, K, Ca, S, and Mg) and microelements (B, Cl, Mn, Fe, Zn, Cu, Mo, and Ni). In some cases, the N, P, and K content may be high, but in general, the nutrient concentration is usually lower compared to digestate from manure or sewage sludge. High (about 30-50%) total solid content, high C:N ratio and increased content of non-fermented components (plastic, metal, rubber, glass, ceramics, sand, and stones) in the organic SHW fraction can significantly complicate the anaerobic digestion process and, accordingly, affect the composition of digestate. Crop residues (plant residues left after harvesting: roots, straw, etc.) are difficult to decompose due to the suboptimal C:N ratio, so they are usually used as a co-substrate in combination with animal waste. Plant tissues have a high content of lignocellulose, and therefore, a complex structure that makes them resistant to decomposition, so the resulting digestate is characterised by a high content of cellulose. A higher yield of digestate is formed from plants with a high fibre content, and, conversely, with a lower fibre content – less digestate is formed. In addition, this digestate does not contain pathogenic microorganisms and heavy metals.

Classical wastewater treatment plants form sediments that differ in organic matter content and humidity. Primary sludge is formed as a result of the process of precipitation of wastewater in primary settling tanks and consists mainly of organic substances, with a solid content in the range of 2-8% and with a moisture content of 96%. Sediment from secondary sedimentation tanks contains mainly microbial biomass after biological treatment facilities (excess activated sludge from aeration tanks, biofilm from biofilters, etc.). It has a lower dry matter content than primary silt, about 1-2%, and a humidity of about 98-99%. A mixture of such sediments, according to A. Elgarahy *et al.* (2024), contains about 50% organic residue, 1-6% total nitrogen and other macro- (mainly phosphorus, sulphur) and trace elements. The possible presence of surfactants, pharmaceuticals and antibiotics, cosmetics, heavy metals (Ni, Pb, Cr, and Zn) and pathogenic bacteria is dangerous. In general, according to D. Yang *et al*. (2022), anaerobic digestate from various sources has the

common characteristics of high ammonium nitrogen content, alkaline pH (6.5-8.0), low C/N ratio, and different content of valuable macronutrients. Thus, digestate from food waste usually contains 1 kg N/t and 0.25 kg P/t, from sewage sludge – 2 kg N/t and 0.5 kg P/t, and cattle and chicken manure – 5-15 kg N/t and 0.1-1 kg P/t.

In addition, during anaerobic digestion, about 70% of the total nitrogen in organic waste is converted to its ammonium form, which leads to its high content in digestate. This factor is one of the obstacles in the processing of digestate, especially by biological methods. As mentioned at the beginning, the operating parameters and operating mode of the biogas plant during the fermentation process are important. Operating parameters include temperature, fermentation duration, C/N ratio, and pH. Thus, the operation of a thermophilic (~55°C) plant requires greater energy input, but the amount of biogas produced will be higher, and the digestate will be of high quality and free of pathogens. However, the decomposition process is often unstable, and plants operating in a higher temperature range are more expensive to maintain than mesophilic (~35°C) systems, so most commercial biogas plants operate in mesophilic mode.

P. Ragályi *et al*. (2025) indicate that the fermentation time and organic load factor should be sufficient to form biogas from the organic substrate available in the raw material. Insufficient duration along with a high organic load can lead to a high content of unprocessed organic substances in the digestate and, accordingly, vice versa. The optimal C/N ratio for most anaerobic digestion processes is between 15 and 30, depending on the characteristics of the substrate. A low C/N ratio means a higher concentration of N, which will eventually turn into excess ammonia. Excess ammonia increases the alkalinity of the reactor and slows down the fermentation process, which leads to a decrease in the yield of biogas and an increased content of ammonia in the digestate. On the other hand, a high C/N ratio means a lack of N in the substrate. Microorganisms quickly consume N, and it is not enough for their metabolism.

According to M. Uddin & M. Wright (2023), most microorganisms prefer a neutral pH value. Methanogens are very sensitive to pH and require a pH of 7 for maximum performance. Hydrolytic and acid-forming bacteria work better at pH values between 5.5 and 6.5. Acid-forming bacteria, on the other hand, are more tolerant of a larger pH range. Maintaining an optimal pH for all microorganisms in a single reactor is a challenge, especially for substrates with uneven composition, such as sewage sludge or organic SHW residues. Therefore, Table 2 summarises the data on the effect of substrate type and conditions on the fermentation process and digestate characteristics.

Table 2. Influence of the input substrate composition on the fermentation process and digestate characteristics

Substrate	Features	Influence on the fermentation process and digestate composition
Animal excreta	High content of ammonium nitrogen and total nitrogen in poultry manure; presence of methanogens in cattle manure	Gradual accumulation of nitrogen compounds causes an increase in pH and cessation of fermentation
Crop residues (plant remains)	High fibre content, high C:N ratio, no methanogens; no heavy metals	Fermentation without mixing with other types of substrates is ineffective due to the high C:N ratio and the absence of methanogens
Organic part of SHW (food scraps, food waste, organic waste)	High total solid content; unstable composition throughout the year; presence of mechanical impurities and hazardous chemicals: lower nutrient content compared to other substrates	Mechanical impurities can cause disruption of the fermentation process and damage to equipment; instability of the composition requires frequent formulation changes and affects the final composition of digestate
Sediments formed as a result of wastewater treatment in cities or private farms	High content of phosphorus and trace elements; possible exposure to surfactants, medicines, antibiotics, cosmetics, and other chemicals; low fibre content	Negative impact of chemicals on the fermentation process and the composition of digestate; the need to combine with other types of substrates in order to increase the fibre content

Table 2. Continued

Substrate	Features	Influence on the fermentation process and digestate composition
Waste from food production and catering establishments	Increased content of Na^+ and Cl^-; variable composition depending on the type of enterprise	A higher proportion of animal-derived products results in a higher nitrogen content in digestate; a higher proportion of plant-derived products results in a lower nitrogen content

Source: created by the authors based on the analysis performed

The use of mechanical methods for the processing and utilisation of digestate is more widespread today. The purpose of mechanical processing is to separate digestate into 2 fractions: liquid and solid, which is one of the most common methods of processing digestate. For this purpose, as noted by A. Cathcart *et al.* (2023), the screw press and decanter centrifuge are the most used. Screw presses separate the particles by size by pressing the digestate against the filter material with a screw, which allows liquid and solid particles smaller than the filter pores to pass through them and form a liquid fraction. The dehydrated solid fraction moves along the screw, the diameter of which gradually increases, which leads to an increase in pressure. The final stage of the process is the removal of digestate to the outside, as indicated by M. Nowak & W. Czekała (2024).

A. Cathcart *et al*. (2023) wrote that decanter centrifuges are divided by density; the digestate rotates in a rotating bowl, and an internal auger pushes the solids stuck in the bowl into the solid release hole. With centrifuges, a higher separation quality is achieved, since the process is not limited by the pore size. The predominant application of the two resulting digestate fractions is in agriculture, where they are used as fertilisers, soil amendments, and growing media, providing a relatively simple and economically viable means of digestate utilisation. The dry matter, nitrogen, phosphorus and potassium contents, and pH, are the most important parameters determining the suitability of digestate as a fertiliser, according to W. Czekała *et al.* (2020). The dry matter content, which usually does not exceed 8%, makes the application of digestate similar to manure. After preliminary separation of digestate by mechanical means, its solid fraction consists of a certain amount of organic matter, contains a large amount of cellulose and lignin, about 20-30% of dry substances, and a higher phosphorus content. The solid fraction is used for composting and subsequent application to the soil. In their publications, M. Nowak & W. Czekała (2024), described that the liquid fraction contains more soluble forms of nitrogen, phosphorus and potassium, which are better absorbed by plants. One of the ways to use it is liquid fertiliser, due to the high content of more readily assimilable forms of nitrogen, potassium, and phosphorus. The second method is to partially return the part from the filtered digestate back to the reactor to replenish the liquid part level and reduce water use. However, this pathway requires strict control of the nitrogen content, since it can return together with the digestate and accumulate in the reactor, which leads to inhibition of the fermentation process. However, despite the common processing method, digestate does not meet several requirements and standards that allow it to be used as fertiliser without pretreatment and careful analysis. Digestate, depending on the composition of the substrate used for fermentation, has a rather unstable composition and nutrient ratio. This, in turn, was indicated by M. Zielińska & K. Bułkowska (2024). Accordingly, the application of such fertiliser may have an unknown effect on the state of the soil and the ecosystem as a whole. Excess nutrients can lead to their leaching and contamination of surface and ground water.

The next reason, according to M. Malhotra *et al*. (2022), are pathogens, toxic substances, and heavy metals that can accumulate in the field where fertilisers are applied. Biogas production mainly uses waste that may be biologically contaminated and contain heavy metals, including Cd, Cr, Cu, Pb, Ni, Zn and As, or other toxic substances. In addition, some of the microorganisms involved in fermentation are dangerous for humans and animals. When they enter the soil, they can pass through the food chain to humans, causing several diseases. Such microorganisms, according to D. Kovačić *et al*. (2022) include

Escherichia coli, *Salmonella* spp., *Listeria monocytogenes*, *Clostridium perfringens*, *Campylobacter jejuni*, *Cryptosporidium parvum*, *Giardia intestinalis*, *Clostridium botulinum*.

The economic component is also important: the cost of transporting fertilisers from digestate is often quite high and unprofitable. In addition, many farmers refuse to use such fertilisers, since synthetic fertilisers are cheaper and give a more predictable result. There are also a number of legal restrictions that prevent the use of digestate as fertiliser. This industry is at the stage of development and requires the establishment of clear norms and legislative documents. One of the reasons may also be the lack of agricultural land due to weather and climatic conditions. There are a number of countries that have almost no developed agricultural sector. Accordingly, the processing of digestate for fertilisers is economically impractical due to the rather high cost of transportation and the lack of places for direct use. Thus, the above list of reasons that prevent the processing of digestate into fertilisers, and the rapid development of the bioenergy industry, proves the need to find new methods of its utilisation to obtain valuable products and achieve the sustainable development goals.

A more promising way, according to M. Zielińska & K. Bułkowska (2024) the recovery and reuse of valuable elements such as N, P, and K, which are essential for plant growth, can reduce dependence on synthetic fertilisers and the impact associated with direct application of digestate to the soil. Restoring nutrients and macro- and microelements from digestate instead of direct application to the soil helps to control the dosage of nutrients, prevents water pollution and soil depletion, and reduces the risk of infection with pathogens or contamination with other toxic compounds. However, this technology requires further research to find effective methods of isolation.

One of the promising ways to process the liquid fraction of digestate is to extract valuable macro- and microelements using physical methods. The recovery of nutrients from digestate follows the principles of a circular economy by converting waste into valuable products such as fertilisers and water. These methods include micro- and ultrafiltration, reverse osmosis, and membrane technologies. As indicated by V. Proskynitopoulou *et al*. (2024), micro- and ultrafiltration allowed minimising the solid content of liquid digestate, and the use of reverse osmosis gave almost deionised water. Solids trapped by filters can be dried and used as soil improvers. However, M. Zielińska & K. Bułkowska (2025) noted that the disadvantages of using these technologies are significant economic costs, scale-up limitations, and gradual contamination of membranes and filters, which as a result require constant cleaning or even disposal.

According to M. Lee *et al*. (2021), biochar is a renewable soil improver that is produced by pyrolysis of organic matter under anaerobic conditions, resulting in a substance that preserves nutrients in soil ecosystems and stabilises soil structure. After treatment, biochar has a large surface area, which increases microbial activity and water absorption, and reduces nutrient leaching and ultimately improves plant growth when used as an additive to substrates, as indicated by J. Ries *et al*. (2023). Due to its special properties, biochar is suitable for various agricultural applications. It helps to improve the physical properties of the soil and increases the availability of nutrients. When added to compost, biochar not only improves the composting process, but also improves the quality of the resulting product. Despite these advantages, M. Zielińska & K. Bułkowska (2024), reported that the biochar production process faces difficulties, including the initial cost of pyrolysis equipment, the need for specialised technical knowledge, and the unstable quality of the resulting finished product due to the unstable composition of digestate.

Composting is one of the ways to process the solid fraction of digestate. In general, this is the biodegradation of biomass under controlled aerobic conditions, with the production of the final product – compost, which is stable enough for safe storage and processing, and mature enough for safe use in agriculture (Tambone *et al*., 2015). As indicated by D. Kovačić *et al*. (2022) during composting, organic matter from the feedstock is converted by microorganisms into a stable humus-like material with the release of carbon dioxide and thermal energy. The composting process includes several phases: mesophilic, thermophilic, cooling, and maturation phases. In the beginning, biomass has a slightly acidic pH and room temperature. Further, microorganisms use monosaccharides, starch and lipids to form organic acids, which reduces the pH. In the next step, microorganisms

begin to break down proteins, and then more complex compounds such as cellulose, hemicellulose, and lignin are partially converted to humus, which is the product of the humification process.

According to A. Aguilar-Paredes *et al*. (2023), the dominant phyla of bacteria in the composting process are: *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi*. Members of the phyla Firmicutes and Actinobacteria play an important role in lignocellulose decomposition, while members of the phylum Bacteroidetes are involved in the degradation of a wide range of complex carbohydrates. As a result of composting, a valuable soil amendment is obtained, rich in humic and fulvic acids, which contribute to increased soil fertility. In addition, the application of such compost improves the structure of the soil and reduces dependence on synthetic fertilisers. M. Zielińska & K. Bułkowska, (2024), noted that during composting, moisture evaporates from the digestate, reducing its volume and facilitating transportation. Due to the increase in temperature as a result of microbial activity to almost 60°C, neutralisation of pathogenic microorganisms that may be present in the digestate is ensured. However, D. Kovačić *et al.* (2022) reported that, because digestate is characterised by a high ammonium content, NH_4^+ can be converted into toxic and volatile NH_3 during composting through a series of reactions, potentially entering the atmosphere and contributing to acid rain and subsequent eutrophication of local ecosystems. In addition, raw digestate is quite moist and dense, which can reduce air access to the compost system. Therefore, in order to prevent this, it is often necessary to add fillers (usually lignocellulose waste), which increases operating costs.

According to A. Vuković *et al.* (2021), vermicomposting is the process of composting a wide range of organic waste, which involves certain types of earthworms that improve the process of converting waste into a high-quality final product, known as vermicompost. The principle of the process is similar to composting, but it is carried out with the help of earthworms and a whole association of microorganisms. M. Sani *et al*. (2025) reported that earthworms provide substrate grinding, improve aeration through constant movement, and secrete specific mucus, which is a source of nutrients for microorganisms. The following types of earthworms are mainly used for vermicomposting: *Eisenia fetida*, *Eisenia andrei*, and *Perionyx excavatus*. The association of microorganisms consists mainly of genera *Bacillus* (*Firmicutes*), *Streptomyces* (*Actinobacteria*), *Pseudomonas* sp. (*Proteobacteria*) and *Azotobacter* (*Proteobacteria*). Microorganisms provide biochemical conversion of nutrients into a form accessible to plants and secrete specific phytohormones – auxins, gibberellins, etc.

Compost formed as a result of vermicomposting is a balanced granular organic fertiliser containing approximately 30% humus, 0.8-3.0% nitrogen, 0.8-5% phosphorus, 1-2% potassium, 2-5% calcium (in terms of absolutely dry matter). For one development cycle (180 days) 0.5 kg of worms per 1 m^2 produce 400-500 kg of humus with a moisture content of 50% from 1 tonne of compost mass and increase their biomass to 8 kg. Using digestate as a substrate for vermicomposting has the same advantages and disadvantages as conventional composting. This includes increasing the content of nutrients in compost and increasing their availability to plants. One of the main disadvantages, in addition to those listed above, is the high content of ammonia in the digestate, which can lead to the death of worms.

Nowadays, considerable attention is being paid to the use of microalgae as a source of biomass, valuable products and energy, and a way to recover nutrients from waste. One of the ways to process digestate and produce useful products is to cultivate microalgae in a liquid fraction, as noted by R. Rasheed *et al*. (2023). Microalgae are photosynthetic microorganisms that use atmospheric carbon dioxide and solar energy to produce a variety of proteins, carbohydrates, lipids, minerals, vitamins, polyphenols, flavonoids, and carotenoids. Products based on them can be used in various industries, including food processing, agriculture (as animal feed and feed additives), fisheries, cosmetics, bioenergy, etc. as noted by A. Ahmad *et al*. (2022).

Microalgae occur as individual cells or in groups of several cells and can vary considerably in shape and size. As indicated by E. Thoré *et al*. (2023), more than 30,000 microalgae species have already been described, many of which have found industrial applications. For example, *Dunaliella salina* can grow in very salty water and

is grown as a source of beta-carotene; *Haematococcus pluvialis* accumulates high concentrations of the carotenoid astaxanthin, which is used as an antioxidant; the genus *Arthrospira* accumulates the blue pigment phycocyanin; *Phaeodactylum tricornutum* synthesises the orange-brown pigment fucoxanthin. In addition, many microalgae are high in omega-3 fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid. J. de Carvalho *et al*. (2022) noted that microalgae can grow on fairly simple sources of carbon and other nutrients, including digestate and wastewater. Algae grown on digestate can absorb dissolved carbon dioxide and nutrients such as ammonia, nitrates, nitrites, and phosphates. These characteristics make them very effective for processing liquid digestate for the purpose of accumulating algae biomass and further use for other purposes.

In addition, according to G. Geletukha *et al*. (2024), the possibility of creating a closed cycle of growing microalgae on digestate with the return of unseparated microalgae suspensions to the bioreactor for renewed biogas production is promising. The idea is to effectively grow algae on a nutrient medium from digestate, which contains a large amount of nutrients, macro- and microelements necessary for their growth, and with the addition of carbon dioxide obtained as a result of biogas enrichment. The cultured algae biomass can be returned to the reactor for fermentation or used to produce biodiesel or other valuable products. An example of this technology is a pilot plant for growing microalgae on a liquid fraction of digestate at a wastewater treatment plant in Milan, Italy. Digestate is obtained as a result of anaerobic digestion of sediments formed after urban wastewater treatment. The dominant algae species during cultivation were *Chlorella* and *Scenedesmus* spp. with an average growth rate of 9.5 ± 6.4 g dry matter/m^2·day during the entire cultivation period. The plant saved about 20% of nitrification energy, while producing valuable microalgae biomass that can be used in many areas, as indicated by S. Rossi *et al.* (2023). A generalised table of biological and physico-chemical methods of digestate processing is shown in Table 3.

Table 3. Comparative characteristics of digestate processing methods

Method	Method principle	Advantages	Disadvantages
Biological methods			
Composting	Composting of the solid fraction of digestate to obtain a valuable fertiliser	Low cost; high content of nutrients, macro- and microelements; partial destruction of pathogens due to increased temperature during composting	Need to add fillers; unknown composition; risk of pathogens and heavy metals entering the soil; insufficient regulation by legislation
Vermicomposting	Composting of the solid fraction of digestate to obtain valuable fertiliser with the help of worms of the genus *Eisenia* and others	Low cost; high content of nutrients, macro- and microelements	Need to add fillers; the risk of worm death due to the high content of nutrients and unknown composition; the risk of pathogens and heavy metals entering the soil
Cultivation of microalgae	Cultivation of microalgae on a liquid fraction of digestate to obtain valuable food additives	Obtaining a valuable food additive or fertiliser from microalgae and purified liquid digestate fraction; the possibility of using the resulting biomass for re-fermentation	Inhibition of microalgae growth due to the high content of ammonium and other nutrients in the digestate; possible crop death due to the presence of pathogens and heavy metals in the digestate; risks of use as a food additive
Physical methods			
Mechanical separation	Separation of digestate into 2 fractions: solid and liquid, mainly for use as fertilisers	Simplicity and relatively low cost; reduction of the volume of digestate generated; low cost; high content of nutrients, macro- and microelements	The need for further processing of the formed 2 fractions; uneven distribution of nutrients; the risk of ingress of increased volumes of methane and ammonia into the atmosphere

Table 3. Continued

Method	Method principle	Advantages	Disadvantages
Nutrient recovery	Use of ultra- and microfiltration methods, reverse osmosis, membrane technologies for concentrating nutrients from the liquid fraction and obtaining purified water	Obtaining a valuable concentrate of nutrients; obtaining purified water that can be reused; reducing the volume of digestate formed	Expensive equipment; the need for constant cleaning of filter materials and membranes; difficulty in scaling; unknown composition of the resulting concentrate
Biochar	Pyrolysis of the solid fraction of digestate to produce biochar as a soil improver	Reduced digestate volumes; lower transportation costs; high content of valuable macro- and microelements; destruction of pathogens	Expensive equipment and its maintenance; risk of equipment damage due to excessive ash accumulation; risk of heavy metals entering the soil

Note: unknown composition – the need for constant thorough analysis of each batch of digestate to determine the specific content of nutrients and other macro- and microelements, heavy metals, and the presence of pathogenic microorganisms

Source: created by the authors as a summary of the above information

The analysis of the literature showed that biological methods of digestate processing are the most environmentally safe and promising technologies that ensure compliance with the principles of a circular economy with the possibility of simultaneously obtaining valuable by-products.

CONCLUSIONS

Due to the growing sector of biogas and biomethane production in Europe and Ukraine, there is a problem in processing large volumes of the resulting digestate to meet the Sustainable Development Goals and maintain a circular economy. Digestate is a by-product of anaerobic digestion and can serve as a valuable source of nutrients, and micro- and macronutrients. It was established that its composition and properties are significantly influenced by the raw material and key parameters of anaerobic digestion, namely, the temperature regime, pH, process duration, organic load coefficient, and C/N ratio. In order to choose the most effective method of its processing, it is necessary to consider the above factors. The choice of processing methods was determined by the physical and chemical characteristics of digestate, the scale of production, and economic requirements. Physical processing methods are the most common, but biological methods are quite promising and require further research. Physical methods include mechanical dewatering with division into 2 fractions for use mainly as fertilisers in agriculture, biochar production, and extraction of nutrients from digestate. The main obstacle to the implementation of these methods is the unstable composition of digestate, its uncontrolled application as fertiliser, and more expensive and more difficult-to-operate equipment for the two extreme methods.

Biological processing methods include microalgae cultivation on liquid digestate fraction, composting, and vermicomposting. These methods make it possible to obtain valuable by-products such as fertilisers, food and feed additives, and co-substrates for re-fermentation. In addition, as a result of biological processing, the composition of digestate becomes more stable. The disadvantage of such methods is mainly the instability of the composition of digestate and the content of compounds in it in high concentrations, which become toxic to living organisms, complicating the process or completely inhibiting the processing. It is worth noting the combination of several technologies at the same time in order to create a closed production cycle with minimising the resulting waste. Thus, efficient processing of digestate is an important component of circular bioeconomics, contributes to the rational use of resources and reduces the environmental burden. Based on the analysis, further scientific research should be aimed at gradually integrating biological methods of digestate processing with a simultaneous combination of several technologies. The development of legislative regulatory mechanisms and legal instruments aimed at controlling the methods of digestate utilisation and processing is also important.

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Біотехнологічні аспекти утворення дигестату при метановому зброджуванні та його утилізації

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Анотація. Актуальність дослідження зумовлена стрімким розвитком біоенергетичної галузі, а саме біогазової сфери в Україні, що зумовлює утворення значних обсягів одного з побічних продуктів анаеробного зброджування – дигестату, що є цінним в багатьох аспектах. Метою статті було дослідження основних етапів метанового бродіння та чинників, що впливають на склад та якість дигестату, а також огляд традиційних та новітніх методів його утилізації. Аналіз наявної літератури показав, що сучасні способи зберігання утвореного дигестату є неефективними та виступають як тимчасовий спосіб його накопичення, враховуючи зростаючі з кожним роком та в подальшому майбутньому масштаби його утворення. У статті детально розглянуто кожен із чотирьох етапів анаеробного розщеплення (гідроліз, ацидогенез, ацетогенез, метаногенез) та охарактеризовано специфічні групи мікроорганізмів, які беруть участь у цих процесах. Визначено взаємозв'язок між складом вихідної сировини та кінцевими фізико-хімічними характеристиками утвореного дигестату. Представлено вплив складу вихідної сировини на особливості складу та основних характеристик утвореного дигестату. В роботі проаналізовано результати сучасних досліджень в галузі переробки та утилізації дигестату за допомогою біотехнологічних методів з метою забезпечення основних цілей сталого розвитку та дотримання принципів циркулярної економіки. Особлива увага приділялася ключовим характеристикам утвореного дигестату, що чинять переважний вплив на вибір подальших методів та технологій його утилізації. На основі проведеного аналізу інформацію було систематизовано та представлено у вигляді узагальнюючих таблиць. Результати цієї оглядової статті мають практичну цінність і можуть бути використані для підбору та впровадження технологій переробки дигестату на існуючих підприємствах

Ключові слова: зброджений залишок; анаеробне зброджування; переробка; біогаз; циркулярна економіка; сталий розвиток

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Effect of heavy metal ions on early development of *Sinapis alba* L.

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Abstract. Soil contamination with heavy metal ions can limit land use and reduce the effectiveness of phytoremediation. *Sinapis alba* L. is considered as a promising test culture and a potential phytoremediant, but its response to various metals in the soil system needs to be clarified. The purpose of this study was to evaluate the effect of Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+} ions on the viability of seeds, the length of shoots and roots of *S. alba*. The experiment was performed in a model soil experiment with three repetitions for each variant. On Day 5, a significant decrease in viability was detected only under the action of Cu^{2+} at a concentration of 300 mg/dm^3, where this indicator was 77.9 ± 1.2%. On Day 10, the strongest effect was observed for Fe^{3+}: viability decreased to 34.9 ± 1.2%, 27.7 ± 1.2% and 20.5 ± 1.2% at concentrations of 600, 800, and 1,000 mg/dm^3, respectively. For Cu^{2+} this indicator was 74.7-56.6%, Zn^{2+} – 80.7-67.5%, and Pb^{2+} – 81.9% at the highest concentration. The length of shoots and roots decreased under the action of all the metals under study. Fe^{3+} caused the greatest growth suppression: the length of the shoots decreased from 54.62 to 9.94 mm, and the roots – from 35.96 to 13.09 mm. Therefore, morphometric parameters were found to be more sensitive to metal stress than to viability, and should be used for a comprehensive assessment of soil phytotoxicity. The results can be used by specialists in the field of environmental monitoring, soil science, and phytoremediation for a comprehensive assessment of the toxicity of soils contaminated with heavy metals

Keywords: phytotoxicity; biotest of phytotoxicity; grey forest soils; phytoremediation; metal-specific stress

INTRODUCTION

Soil contamination with heavy metals is an urgent problem for areas where the effects of hostilities, industrial stress, and intensive land use are combined. Ingress of Zn^{2+}, Cu^{2+}, Pb^{2+} and Fe^{3+} ions in the soil environment can disrupt seed germination, root system development, and the development of aboveground biomass. For phytoremediation, it is important to evaluate not only the plant's ability to accumulate pollutants, but also the preservation of viability and growth in

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their presence. This is particularly important for gray forest soils, where the acidity, organic matter content, sorption capacity, and metal mobility can change the actual toxicity of the same reference concentrations.

C. Luo *et al*. (2024) summarised the effects of various hazardous substances in the soil on seed germination and showed that the effect is determined not only by the concentration, but also by the chemical form of the pollutant, the type of plant, and the duration of exposure. The researchers identified impaired water uptake, oxidative damage, and destabilisation of cellular structures as the main mechanisms of germination inhibition. However, this study was a review and examined a wide range of pollutants, so it did not provide an experimental answer regarding the comparative effects of Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+} for one test crop in the same soil environment. B. Krasnodębska-Ostręga *et al*. (2022) reviewed *Sinapis alba* as a promising plant for phytoremediation and summarised data on the accumulation of As, Tl, and platinum group elements. G.-G. Vasile *et al*. (2021) and N. Vasilache *et al*. (2023) confirmed this prospect for soils contaminated with As, Cd, Ni, and Pb, but showed a metal-specific plant response: As mostly stabilised in the roots, Cd and Ni accumulated more actively, and Pb was largely retained by the soil. The availability of metals depended on the properties of the soil and mobile fractions. However, these papers did not cover a comparative investigation of Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+} in the model grey forest soil in the early stages of development of *S. alba*.

P. Singh *et al*. (2024) examined five varieties of *Brassica juncea* in soils irrigated with wastewater and borehole water. The researchers found that contaminated irrigation reduced the morphological, physiological, and productive characteristics of plants, and the genotype determined the level of metal accumulation and the suitability of the variety for phytoremediation. These results highlighted the importance of species, genotype, and soil-water regime. However, the study concerned *B. juncea*, longer cultivation, and complex contamination, rather than an early reaction of *S. alba* on separate concentration series of metals. Y. Peng & I. Grigorieva (2024) analysed the effect of heavy metals and soil properties on plant germination in the system of *Brassica napus* and sorghum. The researchers showed that roots may be more sensitive to soil factors than shoots, and toxicity assessment should consider the combined effects of pollution and the physicochemical characteristics of the soil. However, the multivariate model of this study did not replace the controlled comparison of specific ions within a single plant species. The paper did not consider *S. alba* and grey forest soils of northern Ukraine. A. Díaz *et al*. (2024) and D. Kramski *et al*. (2026) reported that soil additives can simultaneously alter the availability of metals and support growth of *Sinapis alba*. For As, compost increased accumulation, while the combination of dunite, compost, and nanoscale nulvalent iron reduced mobilisation; for Cd, sawdust improved growth, and spruce sawdust increased the bioaccumulation rate by more than 30%.

Thus, these studies confirmed the prospects of *S. alba* for phytoremediation, but a specific combination of Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+} in the soil system, the experiment requires further investigation. Current studies confirmed the importance of soil conditions, species specificity, and simultaneous analysis of germination and growth, but do not provide an answer regarding the early metal-specific reaction of*S. alba* on Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+} in the grey forest soil. The purpose of this study was to determine how individual concentrations of these ions affect seed viability on Day 5 and Day 10 and the length of roots and shoots on Day 10, and to determine which indicators are most sensitive to metal-specific stress.

MATERIALS AND METHODS

The study was conducted under the conditions of a model soil experiment using *Sinapis alba* L. as a test crop. The substrate was soil collected from the outskirts of Kyiv, in the forest zone of Desnianskyi district. Working soil moisture was 60.0 ± 0.5%, the proportion of organic matter – 3.0 ± 0.5%. The studies were conducted on plant seeds and did not involve the involvement of humans, vertebrates, or invertebrates, nor the use of protected species. Therefore, bioethical approval procedures were not applied for these categories. Work with solutions of metal salts and contaminated substrate was carried out in compliance with laboratory chemical safety requirements; materials after the experiment were collected separately for disposal as metal-containing laboratory waste. The study was

conducted in accordance with the ethical standards of Convention on Biological Diversity (1992) and Convention on International Trade in Endangered Species of Wild Fauna and Flora (1973).

The experiment was performed in Petri dishes. Each Petri dish was filled with 25 cm^3 of soil, after which a solution of the respective metal was added. Zn^{2+}, Cu^{2+} and Pb^{2+} were introduced in the form of nitrates, Fe^{3+} – in the form of chloride. Three concentrations expressed in mg/dm^3 of soil were used for each metal: Fe^{3+} – 600, 800, and 1,000 mg/dm^3; Cu^{2+} – 100, 200, and 300 mg/dm^3; Zn^{2+} – 200, 300, and 400 mg/dm^3; Pb^{2+} - 50, 100, and 150 mg/dm^3. For each variant of the experiment, 3 repetitions were performed. 30 seeds of *S. alba* were sown in each Petri dish. The control version contained the same volume of soil, water, and the corresponding amount of nitrate ions, but without the introduction of metals. Since Zn^{2+}, Cu^{2+}, and Pb^{2+} were introduced in the form of nitrates, and calcium nitrate was additionally introduced to equalise the effect of nitrate ions in experimental and control versions. Its amount was calculated so that the total intake of nitrates corresponded to 400 mg/dm^3 of soil. After applying the solutions, all Petri dishes were supplemented with water to the same initial volume of liquid – 20 cm^3. By Day 5, germination was carried out in the dark in closed Petri dishes. After Day 5, the dishes were opened and transferred to 200 lux lighting conditions. In the future, the plants were grown with sufficient watering, preventing the soil substrate from drying out. The phytotoxic effect was evaluated based on seed viability, shoot length, and root length. Viability was determined by the number of seeds that formed seedlings. The length of shoots and roots was measured on Day 10 of the experiment. These indicators were used as indicators of early response of *S. alba* to the action of Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+} in model soil conditions.

Statistical analysis was performed separately for morphometric parameters and viability. For shoot and root lengths, ANOVA analysis of variance was used, followed by post hoc comparison between variants. The χ^2 test was used to analyse viability with subsequent post hoc correction of multiple comparisons. The viability of the control was considered to be 100%. The differences were considered statistically significant at the accepted level of significance after adjusting for multiple comparisons. The choice of statistical approaches was determined by the nature of the data obtained. The length of shoots and roots were continuous quantitative indicators, so it was advisable to compare the average values between the experiment variants using variance analysis. Instead, the viability in each Petri dish was actually a ratio of two categories – seeds that formed seedlings and seeds that did not form viable seedlings. That is why χ^2 test was applied for this indicator. The control variant was taken as 100%, and all experimental variants were interpreted as deviations from it. This allowed assessing not the absolute similarity of the crop, but the loss of viability associated with the introduction of metal. This approach was consistent with the purpose of the study, since it was not aimed at pairwise ranking of all metals and concentrations against one another, but at determining which variants actually alter normal early development of *S. alba* compared to control.

RESULTS AND DISCUSSION

On Day 5, seed viability of *Sinapis alba* significantly differed from the control only in the variant with Cu^{2+} 300 mg/dm^3 of soil and was 77.9 ± 1.2%, $p = 0.002$. In other variants, viability did not significantly differ from the control on Day 5 (Table 1). On Day 10, the most pronounced decrease in viability was observed for Fe^{3+}: 34.9 ± 1.2%, 27.7 ± 1.2%, and 20.5 ± 1.2% at 600, 800, and 1,000 mg/dm^3, respectively, $p < 0.001$. For Cu^{2+}, viability was significantly reduced at all concentrations and was 74.7 ± 1.2%, 66.3 ± 1.2%, and 56.6 ± 1.2% at 100, 200, and 300 mg/dm^3, respectively. For Zn^{2+}, a significant decrease was also found at all concentrations: 80.7 ± 1.2%, 74.7 ± 1.2%, and 67.5 ± 1.2% at 200, 300, and 400 mg/dm^3, respectively. For Pb^{2+}, viability was significantly reduced at just 150 mg/dm^3 and was 81.9 ± 1.2%, $p = 0.014$; for 50 and 100 mg/dm^3 it was not different from control.

On Day 10, the average length of shoots and roots of *Sinapis alba* decreased in all variants of metal application compared to the control (Fig. 1). The strongest growth inhibition was observed for Fe^{3+}: shoot length decreased from 54.62 ± 0.79 mm in the control to 16.14 ± 0.57 mm, 13.14 ± 0.74 mm, and 9.94 ± 0.69 mm at 600, 800, and 1,000 mg/dm^3, respectively; root length – from 35.96 ± 0.38 mm to 18.35 ± 0.56 mm, 15.92 ± 0.52 mm, and 13.09 ± 0.41 mm. All differences in shoot and root

length were statistically significant, $p < 0.001$. For Cu^{2+}, Zn^{2+}, and Pb^{2+} there was also a decrease in the length of shoots and roots with an increase in the metal content in the soil. For Cu^{2+}, the length of shoots decreased from 35.16 ± 0.88 mm at 100 mg/dm³ up to 23.11 ± 0.74 mm at 300 mg/dm³, and the length of the roots – from 28.24 ± 0.43 mm to 20.20 ± 0.42 mm. For Zn^{2+}, the length of shoots decreased from 44.61 ± 0.90 mm to 29.51 ± 0.69 mm, the length of roots – from 30.53 ± 0.49 mm to 24.09 ± 0.43 mm. For Pb^{2+}, the length of shoots decreased from 47.79 ± 0.82 mm to 32.62 ± 0.70 mm, the length of roots – from 32.96 ± 0.34 mm to 25.42 ± 0.39 mm. For all variants, differences from the control for shoot and root length were statistically significant, $p < 0.001$.

Table 1. Seed viability of *Sinapis alba* on Day 5 and Day 10 of the experiment

Metal	Concentration, mg/dm³ of soil	Viability, Day 5, % ± SE	p χ^2, Day 5	Viability, Day 10, % ± SE	p χ^2, Day 10
Control	–	100.0	–	100.0	–
Fe^{3+}	600	96.5 ± 1.2	1.000	34.9 ± 1.2	<0.001
Fe^{3+}	800	91.9 ± 1.2	0.740	27.7 ± 1.2	<0.001
Fe^{3+}	1,000	84.9 ± 1.2	0.059	20.5 ± 1.2	<0.001
Cu^{2+}	100	88.4 ± 1.2	0.203	74.7 ± 1.2	0.001
Cu^{2+}	200	84.9 ± 1.2	0.059	66.3 ± 1.2	<0.001
Cu^{2+}	300	77.9 ± 1.2	0.002	56.6 ± 1.2	<0.001
Zn^{2+}	200	95.3 ± 1.2	1.000	80.7 ± 1.2	0.011
Zn^{2+}	300	91.9 ± 1.2	0.740	74.7 ± 1.2	0.001
Zn^{2+}	400	84.9 ± 1.2	0.059	67.5 ± 1.2	<0.001
Pb^{2+}	50	100.0 ± 1.2	1.000	92.8 ± 1.2	0.236
Pb^{2+}	100	96.5 ± 1.2	1.000	88.0 ± 1.2	0.097
Pb^{2+}	150	93.0 ± 1.2	0.820	81.9 ± 1.2	0.014

Note: values are given as the mean ± SE for three repetitions; 30 seeds were sown in each repetition. p χ^2 – adjusted p-value post hoc comparison with the control; $p < 0.05$ – highlighted in red

Source: compiled by the authors

The overall picture of the results indicates that the reaction of *S. alba* had not only a concentration but also a time dependence. On Day 5, most of the variants were still indistinguishable from the viability control, but on Day 10, the differences between the metals became much more pronounced. This was especially noticeable for Fe^{3+} and Cu^{2+}, where a decrease in viability was combined with a sharp suppression of the length of roots and shoots. For Zn^{2+} and Pb^{2+}, viability changed less, but morphometric parameters still decreased, which indicates a sublethal nature of the action. Therefore, it is impossible to fully assess the early phytotoxicity of the studied metals based on the proportion of viable seedlings alone.

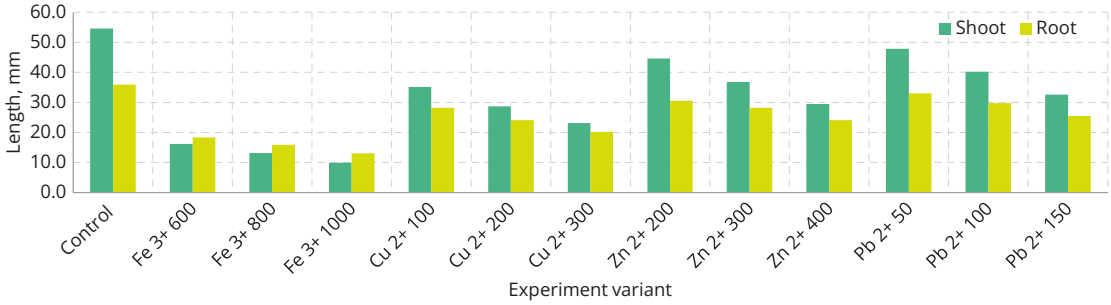

Figure 1. Length of roots and shoots of *Sinapis alba* with different content of heavy metal ions in the soil

Source: compiled by the authors

The results show that root and shoot length was a more sensitive indicator of phytotoxicity than viability. Viability decreased statistically significantly mainly under the action of Fe^{3+} and high concentrations of Cu^{2+}, while the length of roots and shoots decreased in all variants of metal application. M. Gupta *et al*. (2024) linked the Corene-centric response of *Brassicaceae* with the primary interaction of metals in the rhizosphere, apoplexy, cell wall, and root meristem. S. Busoms *et al*. (2021) also showed that Pb tolerance depends on metal accumulation processes and the root system response. This is consistent with the authors' data, in which morphometric parameters reacted earlier and more stable than viability.

The difference between viability and morphometric parameters is of great biological importance. The fact of the appearance of a seedling does not mean that the plant is able to develop effectively in the future in a contaminated substrate. In the first stages, the seed partially uses internal reserves, so the short-term viability index can remain relatively high even in conditions when the root meristem is already under stress. Root length in this case is a more sensitive sign of sublethal toxicity, since it reflects cell division and stretching in the growth zone, and the ability of the seedling to maintain water absorption and contact with the soil solution. The length of the shoot, in turn, shows whether early development passes into the development of biomass. That is why variants in which the viability did not differ statistically from the control, but the roots and shoots were shorter, cannot be considered completely safe for using *S. alba* as a phytoremediant.

The experimental conditions could enhance the root component of phytotoxicity. Under such conditions, the root system developed in a limited volume of substrate with high contact with the pore solution. Therefore, to interpret the results, it is important to consider the pH, organic matter content, cation exchange capacity, and availability of metals in the soil. J. Liu *et al*. (2022) noted that changes in soil properties can affect acidity, nutrient availability, structure, and microbial activity, and therefore change the plant's response to pollution.

The strongest phytotoxic effect was observed for Fe^{3+}. All its concentrations dramatically reduced viability, root length, and shoot length. In this experiment, Fe^{3+} was introduced in the $FeCl_3$ form and in the highest range among all metals – 600-1,000 mg/dm³ of soil. H. Li *et al*. (2022) showed that $FeCl_3$ can change the bioavailability of metals in the soil during remediation washing, but this procedure differs from direct application of Fe^{3+} to the substrate for germination. J. Clúa *et al*. (2024) link Fe^- homeostasis regulation with processes in the root meristems. C. Xue *et al*. (2023) and F. Fodor (2024) reported that excess Fe can be associated with ROS/RNS stress, violation of K^+- homeostasis, damage to the root apex, and inhibition of meristem activity. Excess iron causes the accumulation of NO at the root tip; this NO can increase the leakage of potassium from the cells of the apical zone, which leads to a decrease in cell viability and stops root growth.

Delayed intensification of the Fe^{3+} effect may also be conditioned by the fact that the soil system gradually changes the availability of nutrients in the root zone. On Day 5, some of the seedlings could still support development at the expense of seed reserves, while on Day 10, the dependence on the work of the root and the availability of mineral elements increased. The high content of Fe^{3+} allows simultaneously increasing oxidative stress, local damage to meristem cells, and reducing the availability of phosphates due to their binding in a slightly acidic soil environment. This explains why Fe^{3+}- variants showed not just a decrease in the length of roots and shoots, but a sharp drop in viability at a later stage of observation.

Phosphorus was not additionally introduced in the experiment, so Fe^- series may partially reflect more than just the direct toxicity of Fe^{3+}, but also changes in the availability of phosphates. In slightly acidic soil with a high concentration of Fe^{3+}, part of the available phosphorus could pass into a less accessible state, and under irrigation conditions – partially shift from the active zone of contact with the root. J. Clúa *et al*. (2024) showed that primary root suppression in phosphate deficiency is largely associated with Fe^-dependent processes in the root meristem. R. Maniero *et al*. (2024) clarified that ferritreduction in root meristems is part of the response to changes in phosphorus availability. The lack of additional introduction of phosphorus is unlikely in itself to explain such a sharp suppression of *S. alba* on Day 10. In early development, the plant can still partially use the internal reserves of seeds, while the consequences of P

deficiency are more pronounced in later stages of growth due to limited energy metabolism, biomass and root system development. N. Shen *et al*. (2021) showed that phosphate deficiency rearranged the root architecture of *Arabidopsis* by regulating Fe homeostasis and WRKY33 activity. Therefore, in this experiment, it is advisable to consider the phosphorous factor as a modifier of Fe^{3+}- toxicity, and not as an independent cause of a sharp drop in viability and growth. C. Xue *et al*. (2023), J. Clúa *et al*. (2024) and F. Fodor (2024) gave grounds to consider Fe-specific action as a combination of metallic stress, violation of Fe-P balance, and damage to the root meristem.

It is advisable to discard the chloride factor as the main cause of the Fe^{3+} effect. In the experiment, there was a calcium-nitrate background, since nitrates were equalised by applying $Ca(NO_3)_2$ up to a nitrate ion concentration of 400 mg/dm^3 of soil; this background reduces the likelihood of a sharp pH- or salt shift as the only explanation for the results. Q. Huang *et al*. (2022) in experiments with *Brassica napus* delimited the effects of NaCl, Na_2SO_4, KCl, and K_2SO_4 and found that the main contribution to growth suppression was made by Na^+, and the additional effect of Cl^- and SO_4^{2-} depended on the dose and genotype. M. Rezayian & F. Zarinkamar (2023) also showed that calcium and signalling components can alter the salt response of plants. Therefore, in the presence of calcium chloride in this system, it is not the most likely factor of sharp growth inhibition; the effect of Fe variants is more logically associated with Fe^{3+}-specific toxicity and Fe-P interactions.

Cu^{2+} also significantly suppressed development of *Sinapis alba*, especially at concentrations of 200 and 300 mg/dm^3. G. Chen *et al*. (2022) described copper as an essential but redox active element, an excess of which causes the generation of reactive oxygen species, oxidative damage to membranes, and disruption of electron transport chains. X. Zheng *et al*. (2024) summarised the role of Cu in the plant-soil system. B. Rather *et al*. (2022) showed on mustard that Cu-stress is accompanied by oxidative stress, impaired nitrogen metabolism, and reduced photosynthetic efficiency, and optimisation of nitrogen and ethylene signalling can mitigate these effects. In this experiment, the nitrate background was levelled, so the decrease in viability at high copper concentrations and stable inhibition of root and shoot length were more likely to reflect the actual toxic effect of Cu^{2+}, not the difference in nitrate intake.

Zn^{2+} did not cause a statistically significant decrease in viability, although at concentrations of 300 and 400 mg/dm^3 the p values approached 0.05. The length of roots and shoots decreased in all Zn^{2+} variants. H. Kaur & N. Garg (2021) reported that excess zinc causes erroneous incorporation of metals into metalloproteins, Fe-deficiency-like signals, disruption of membrane and enzyme homeostasis, and secondary redox stress. S. Sadr *et al*. (2024) showed that Zn toxicity in rapeseed is associated with changes in cellular redox status and can be modulated by calcium and melatonin. P. Singh *et al*. (2024) demonstrated that the effectiveness of mustard phytoremediation depends on varietal characteristics and soil conditions. In grey forest soils, this additionally depends on pH, organic matter, cation exchange capacity, and Zn^{2+} mobility. This correlation of effects means that the use of white mustard for phytoremediation can be difficult on heavily contaminated Cu^{2+} and Zn^{2+} land plots due to loss of viability and biomass. However, at lower levels of contamination, plants retained sufficient development, which makes *S. alba* more suitable for moderately polluted soils, where the expected extraction of metals is not accompanied by a sharp inhibition of growth. In practice, this means that for preliminary selection of sites or metal concentrations, it is necessary to consider not only the fact of a statistically significant decrease in viability, but also the constancy of growth inhibition. If the roots and shoots are significantly shorter than the control even with relatively high viability, this option can give a weak plant cover and less biomass, and therefore – lower practical efficiency of phytoremediation in real soil conditions of long-term cultivation, especially with unstable water regime of the substrate.

Pb^{2+} did not have a statistically significant effect on viability, but it significantly reduced the length of roots and shoots. S. Busoms *et al*. (2021) showed that accumulation and tolerance to Pb in *Arabidopsis* depend on the genotype and processes associated with the root system. Z. Tan *et al*. (2022) and H. Manne *et al*. (2024) described the ability of Pb to disrupt antioxidant systems, destabilise membranes, and trigger secondary oxidative stress. At the studied concentrations

of 50-150 mg/dm^3, this could manifest mainly as growth inhibition rather than as a decrease in viability. Low impact of Pb^{2+} on viability can also be associated with its binding to the soil and a corresponding decrease in the fraction available to the plant. N. Vasilache *et al*. (2023) showed that the availability of metals for *S. alba* was determined by the properties of the soil and the mobile fraction, and G.-G. Vasile *et al*. (2021) recorded a blockage of Pb in the soil. Since the availability of Pb was not measured in this experiment, this explanation should be considered as a well-founded hypothesis that requires separate testing. Y. Peng & I. Grigorieva (2024) also showed that in the soil system, the roots *Brassica* may be more sensitive to pollution factors than shoots, which supports this interpretation.

Overall, the results are better explained not by a single factor, but by a combination of metal-specific toxicity with soil system conditions. Grey forest soil with moderate organic matter without additional application of phosphorus created conditions in which root effects were most sensitive. For Fe^{3+}, this implies the need for further separate investigation of the role of available phosphorus: Fe+P variants, control of P without Fe, pH measurement, mobile Fe, mobile P, and Fe-P ratios in pore solution are necessary to distinguish between direct Fe^{3+}- toxicity and phosphate-modulated effect.

CONCLUSIONS

Metal ions of PB^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{3+} had different effects on early development of *Sinapis alba*. The most sensitive indicators were the length of shoots and roots, while viability varied less steadily and depended on the metal and duration of exposure. On Day 5, a significant decrease in viability was observed only under the action of Cu^{2+} at 300 mg/dm^3 – 77.9 ± 1.2%, p = 0.002. On Day 10, the strongest and most delayed effect was found for Fe^{3+}: 34.9 ± 1.2%, 27.7 ± 1.2% and 20.5 ± 1.2% at 600, 800, and 1,000 mg/dm^3, respectively, p < 0.001; Cu^{2+} reduced viability to 74.7 ± 1.2%, 66.3 ± 1.2%, and 56.6 ± 1.2% at 100, 200, and 300 mg/dm^3; Zn^{2+} – to 80.7 ± 1.2%, 74.7 ± 1.2%, and 67.5 ± 1.2% at 200, 300, and 400 mg/dm^3; Pb^{2+} – to 81.9 ± 1.2% at 150 mg/dm^3, p = 0.014. The length of roots and shoots decreased under the action of all metals under study: according to Fe^{3+}, the length of shoots decreased from 54.62 ± 0.79 mm in the control to 9.94 ± 0.69 mm, and roots – from 35.96 ± 0.38 mm to 13.09 ± 0.41 mm. Cu^{2+}, Zn^{2+}, and Pb^{2+} also reduced this indicator. All differences in shoot and root length were statistically significant, p < 0.001. The use of *Sinapis alba* for phytoremediation of grey soils under high metal load is limited due to inhibition of early development. With moderate contamination, the crop may be suitable for further evaluation if viability and sufficient development of root and shoot biomass are maintained. Further research should focus on distinguishing the direct toxicity of Fe^{3+} and the phosphate-modulated effect by comparing variants with and without phosphorus addition, and measuring pH, mobile forms of Fe and P, and their ratios in the basal zone.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Вплив іонів важких металів на ранній розвиток *Sinapis alba* L.

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Анотація. Забруднення ґрунтів іонами важких металів може обмежувати використання земель і знижувати ефективність фіторемедіації. *Sinapis alba* L. розглядають як перспективну тест-культуру та потенційний фіторемедіант, однак її реакція на різні метали в ґрунтовій системі потребує уточнення. Метою роботи було оцінити вплив іонів Zn^{2+}, Cu^{2+}, Pb^{2+} і Fe^{3+} на життєздатність насіння, довжину пагонів і коренів *S. alba*. Дослід проводили в модельному ґрунтовому експерименті з трьома повтореннями для кожного варіанта. На 5-ту добу значуще зниження життєздатності виявлено лише за дії Cu^{2+} у концентрації 300 мг/дм^3, де цей показник становив 77,9 ± 1,2 %. На 10-ту добу найсильніший ефект спостерігали для Fe^{3+}: життєздатність знижувалася до 34,9 ± 1,2 %, 27,7 ± 1,2 % і 20,5 ± 1,2 % за концентрацій 600, 800 і 1000 мг/дм^3 відповідно. За дії Cu^{2+} цей показник становив 74,7-56,6 %, Zn^{2+} – 80,7-67,5 %, а Pb^{2+} – 81,9 % за найвищої концентрації. Довжина пагонів і коренів зменшувалася за дії всіх досліджених металів. Найбільше пригнічення росту спричиняв Fe^{3+}: довжина пагонів зменшувалася з 54,62 до 9,94 мм, а коренів – з 35,96 до 13,09 мм. Отже, морфометричні показники виявилися чутливішими до металевого стресу, ніж життєздатність, і мають використовуватися для комплексного оцінювання фітотоксичності ґрунтів. Результати можуть бути використані фахівцями у сфері екологічного моніторингу, ґрунтознавства та фіторемедіації для комплексної оцінки токсичності ґрунтів, забруднених важкими металами

Ключові слова: фітотоксичність; біотестування фітотоксичності; сірі лісові ґрунти; фіторемедіація; метал-специфічний стрес

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Chronic effect of low urea concentration on the content of carotenoid pigments in *Lymnaea stagnalis*

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Abstract. Urea is one of the most common nitrogen-containing pollutants in freshwater ecosystems and, if it enters water bodies for a long time, can disrupt the course of metabolic processes in aquatic organisms. Since carotenoids serve as non-enzymatic antioxidants, changes in their content reflect the development of oxidative stress and serve as sensitive biochemical markers of toxic effects. The purpose of the study was to find out the features of the effect of low urea concentration on the content of β-carotene and xanthophylls in the haemolymph, hepatopancreas, mantle, and foot of *Lymnaea stagnalis*. The study was conducted on sexually mature uninfected mollusks that were kept in an environment with urea at a concentration equivalent to twice the maximum permissible concentration for water bodies used for fisheries purposes (2 $MPC_{fisheries}$), for 2, 7, 14, and 21 days. The carotenoid content was determined spectrophotometrically, and the results were processed using two-factor analysis of variance and correlation. It was found that with short-term urea exposure (2 days), the content of β-carotene in the mantle and foot of *L. stagnalis* increased by 1.93-2.88 times ($p < 0.01$-0.05). The content of xanthophylls in these organs significantly decreased by 50.73-56.68%, while no significant differences were found in hepatopancreas. An increase in the duration of exposure to 14-21 days was accompanied by a systemic decrease in the content of β-carotene (by 25.94-69.24%; $p < 0.001$-0.05) and xanthophylls (by 38.89-88.41%) in the hepatopancreas, mantle and foot of *L. stagnalis*, which is probably conditioned by increased pro-oxidant processes and the use of carotenoids in free radical neutralisation reactions. Correlation analysis revealed a statistically significant negative association between urea exposure and β-carotene and xanthophyll content, which indicates depletion of the carotenoid reserve under toxic load. Consequently, chronic exposure to urea in the studied concentration causes tissue-specific changes in the carotenoid profile of *L. stagnalis*, which reflect the transition from compensatory-adaptive reactions to depletion of the non-enzymatic link of the antioxidant system. The results obtained can be used by environmental and fisheries organisations for ecotoxicological assessment of the impact of nitrogen-containing pollutants on freshwater ecosystems, and the content of β-carotene and xanthophylls – as an informative biochemical marker of such effects

Keywords: freshwater shellfish; β-carotene; xanthophylls; oxidative stress; nitrogen-containing compounds

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INTRODUCTION

One of the most pressing environmental issues is anthropogenic pollution of aquatic ecosystems with nitrogen-containing compounds entering reservoirs with industrial and household wastewater, and due to the widespread use of mineral fertilisers in agriculture and fish farming. The accumulation of these substances in the aquatic environment is accompanied by a change in its physico-chemical properties, deterioration of the conditions of existence of aquatic organisms, which leads to the restructuring of physiological and biochemical processes in the body, changes in the limits of their tolerance, and, in general, can negatively affect the functioning of aquatic ecosystems. Under such conditions, the search for informative biochemical parameters that can reflect the early compensatory and adaptive reactions of the body to the action of toxicants even before the appearance of pronounced morphofunctional disorders becomes particularly relevant.

The most common nitrogen-containing fertilisers include urea, which is widely used in agriculture and fisheries both in its pure form and as part of complex mineral fertilisers for fertilising water bodies and stimulating the development of a natural feed base. At natural concentrations, urea plays an important role in the functioning of hydroecosystems, participating in the processes of the nitrogen cycle, and as indicated by N. Arandia-Gorostidi *et al*. (2024), serves as an accessible source of this element for microorganisms and is involved in the processes of its transformation and assimilation. However, excessive intake of this compound in water bodies is accompanied by changes in the physical and chemical properties of the aquatic environment, disruption of biological processes and the development of toxic effects in aquatic organisms of various systematic groups. Due to its high solubility in water and ability to easily penetrate biological membranes, urea quickly enters the tissues of aquatic organisms and is involved in physiological and biochemical processes in the body.

According to A. Ojha *et al*. (2025), urea toxicity may be enhanced by the action of the urease enzyme, which, due to its high affinity for the substrate, catalyses its hydrolysis to form ammonia (NH_3) and carbon dioxide (CO_2) even at low concentrations in the medium. In conditions of excessive urea intake, its hydrolysis is accompanied by increased synthesis of ammonia, which increases the toxic load on aquatic organisms – both due to its accumulation in the aquatic environment and through its synthesis directly in the body. Summarising current data, S. Yun *et al*. (2026) concluded that the toxic effects of ammonia on aquatic organisms are accompanied by impaired energy metabolism, osmoregulation, immune response, and the development of oxidative stress. In this case, the severity of toxic effects is determined by the concentration of the toxicant, the duration of exposure, the parameters of the aquatic environment, and the specific features of aquatic organisms. Experimental studies by Y. Zou *et al*. (2023) showed that the action of NH_3 in fish is accompanied by inhibition of growth rates, impaired energy metabolism, ion homeostasis, and induction of oxidative stress due to excessive development of reactive oxygen species, in particular, superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\bullet}$), and hydroxyl ($^{\bullet}OH$) radicals. The consequence of this is changes in the antioxidant status, activation of inflammatory processes, and violation of physiological homeostasis. Similar patterns have been established for mollusks, in which, according to M. Cong *et al*. (2021) and H. Ge *et al*. (2021), the effect of ammonia is accompanied by a violation of the functioning of the antioxidant system, changes in nitrogen detoxification processes, the development of oxidative stress and structural tissue damage, and the intensity of these changes depends on the level of toxic load.

One of the first compensatory and adaptive responses of the cell to excessive development of reactive oxygen species is the activation of the antioxidant system, which is represented by enzymatic and non-enzymatic links and functions as a single integrated protection system, ensuring the maintenance of pro-oxidant-antioxidant balance, neutralisation of free radicals and protection of the cell from oxidative damage. An important place among the components of the non-enzymatic antioxidant system is occupied by carotenoids, which, as indicated by C. Zhuang *et al*. (2022) and K. Lim *et al*. (2023), effectively neutralise reactive oxygen species, inhibit the processes of lipid peroxidation, maintain the antioxidant status of the body, and promote the adaptation of aquatic

organisms to the action of stress factors. Of particular interest are β-carotene and xanthophylls, which, according to X. Hu *et al*. (2025), differ in physical and chemical properties and features of interaction with cellular structures. M. Zbyradowski *et al*. (2022) showed that β-carotene performs a dual function, because it is a precursor of vitamin A and is involved in the antioxidant protection of cells. M. Pasenkiewicz-Gierula *et al*. (2024) noted that xanthophylls, in addition to their antioxidant properties, are characterised by greater polarity and interaction features with biological membranes due to the presence of oxygen-containing functional groups.

Despite a significant number of studies on the enzymatic antioxidant system of hydrobionts, the role of its non-enzymatic link, in particular carotenoids, remains insufficiently elucidated. This is especially true for freshwater mollusks, for which information on tissue-specific changes in the content of β-carotene and xanthophylls with prolonged exposure to nitrogen-containing toxicants is limited. In previous studies, G. Kyrychuk (2022) found that exposure to high concentrations of urea causes tissue-and time-dependent changes in the content of xanthophylls in the body of freshwater mollusks *L. stagnalis*. However, the characteristics of the response of the carotenoid component of the antioxidant system to exposure to urea at a concentration of 2 $MPC_{fisheries}$, corresponding to a moderate level of toxic load, remain insufficiently investigated, which prompted the present study. The purpose of this study was to investigate the features of the effect of urea at a concentration of 2 $MPC_{fisheries}$ (exposure – 2, 7, 14, and 21 days) on the content of β-carotene and xanthophylls in *Lymnaea stagnalis*.

MATERIALS AND METHODS

The object of the study was sexually mature individuals of the common pond snail *Lymnaea stagnalis* (Linnaeus, 1758). Before starting the experiment, the mollusks were acclimated to laboratory conditions for 14 days in aquariums with aerated tap water settled during the day at stable hydrochemical parameters (t = 18-20°C, pH = 7.3-7.7, dissolved oxygen content = 8.5-8.9 mg/dm^3). Urea at a concentration of 2 $MPC_{fisheries}$ (160 mg/dm^3; $MPC_{fisheries}$ = 80 mg/dm^3) was used to model the chronic toxic load. Solutions were replaced daily with freshly prepared ones with the restoration of the initial toxicant concentration, which ensured the constancy of experimental conditions and minimised urea hydrolysis under the action of bacterial microflora. The control group was kept in dechlorinated water without the addition of urea. The duration of exposure to the experiment was 2, 7, 14, and 21 days. The total sample size was 80 individuals of *Lymnaea stagnalis* (10 individuals in the control and experimental groups for each exposure period; n = 10).

For biochemical analysis, haemolymph was selected using a modified targeting method, after which the hepatopancreas, mantle, and foot were prepared. To exclude the influence of trematode infection on the parameters studied, only uninfected individuals were selected for the study. To determine the content of β-carotene and xanthophylls, tissue samples were pre-homogenised, lipid saponification was performed, and then carotenoids were extracted with hexane in a ratio of 1:4. The quantitative content of pigments was determined spectrophotometrically. The content of β-carotene and xanthophylls was expressed in mg/g of raw tissue. A total of 640 detections of β-carotene and xanthophyll were performed. All measurements were conducted in three analytical repetitions, the average value of which was used for further statistical analysis.

Statistical processing of the obtained results was performed using variational statistics methods using Statistica 10.0 software suite. The results are presented as an average (M) with a 95% confidence interval (95% CI). To assess the effect of "toxic effects" and "duration of exposure" factors on the content of β-carotene and xanthophylls in the tissues of *Lymnaea stagnalis*, two-way analysis of variance (Two-way ANOVA) was used to assess the effect of factor interaction. The Tukey HSD test was used for multiple comparison of mean values between groups. Correlation analysis with calculation of the Pearson correlation coefficient (r) was used to assess the strength and direction of the relationship between the toxic effect factor, the duration of exposure, and the content of β-carotene and xanthophylls in the studied tissues (organs). Differences in $p < 0.05$ were considered statistically significant. All studies with experimental animals were performed in accordance with generally accepted international and national bioethical

principles for handling aquatic organisms and objects of biological research. The study was conducted in accordance with the ethical standards of Convention on Biological Diversity (1992) and Convention on the Trade in Endangered Species of Wild Fauna and Flora (1973).

RESULTS AND DISCUSSION

It was found that the effect of urea at a concentration of 2 $MPC_{fisheries}$ caused a restructuring of the carotenoid profile in *L. stagnalis*, which reflects a consistent transition from compensatory and adaptive responses to depletion of the antioxidant defence system. Two-way analysis of variance revealed a statistically significant interaction of the factors "toxic effect" and "duration of exposure" for all the studied tissues, which indicates the complex nature of the mollusk body's response to the toxicant. Presumably, the detected changes in the carotenoid profile are conditioned by the combined action of the studied factors, when with an increase in the duration of exposure, even a moderate concentration of the toxicant gradually leads to a restructuring of metabolism in the body of *L. stagnalis*. Such a response is characteristic of chronic toxic effects, in which the biological effect is determined not only by the concentration of the toxicant, but also by the duration of its action.

In the control group of mollusks, a gradual and stable increase in the content of β-carotene was established during the entire experimental period (2, 7, 14, and 21 days). The most intense accumulation of pigment was recorded in the hepatopancreas and foot (5.8-6.4 times), which may indicate the development of an effective antioxidant reserve under the conditions of the physiological norm. A similar tendency to increase the content was observed for xanthophylls, but the intensity of their accumulation was lower compared to the indicators obtained for β-carotene. The highest values were observed in the hepatopancreas, mantle, and foot of *L. stagnalis*, while in their haemolymph, the values remained relatively low, which is consistent with the transport function of the tissue and the absence of pronounced carotenoid deposition. The highest levels of β-carotene and xanthophylls in hepatopancreas may be associated with the leading role of the organ in metabolism, nutrient deposition, and maintenance of antioxidant homeostasis, while the growth of their content in the foot may be conditioned by the high energy costs of the organ that provides motor activity.

Such accumulation of carotenoids in the control group may be associated not only with the adaptation of shellfish to laboratory conditions, but also with the stabilisation of metabolic processes in the absence of an additional toxic load. As shown by L. Zhang *et al*. (2023) and Y. Liao *et al*. (2025), carotenoids are not synthesised by most animals *de novo*, and come with feed and accumulate mainly in tissues that perform deposition functions or are characterised by high metabolic activity. Given this, a gradual increase in their content in the hepatopancreas and foot of mollusks can be considered as a physiological process of forming an antioxidant reserve, which, according to R. Jia (2025), is an important component of maintaining the antioxidant status of the body and the normal course of redox processes. It was found that for the action of urea in a concentration that was responsible for 2 $MPC_{fisheries}$, the nature of changes in both groups of carotenoids acquired pronounced tissue and temporal specificity. Thus, with the short-term action of urea (2 days), the content of β-carotene increased in the mantle and foot of mollusks by 1.93-2.88 times ($p < 0.01$-0.05), which may indicate early activation of the non-enzymatic antioxidant system in response to pro-oxidant exposure (Fig. 1). The rapid accumulation of β-carotene in the mantle and foot of *L. stagnalis* with short-term exposure (2 days) cannot be explained by its synthesis *de novo* because shellfish lack this metabolic ability. There is a stress-induced redistribution of the endogenous pigment pool. It is likely that the primary toxic stress stimulates the mobilisation of carotenoid reserves from the main storage organ – the hepatopancreas – and their directed transport via the haemolymph to barrier tissues (the mantle), which are in direct contact with the toxic environment, and to metabolically active locomotor tissues (the foot), thereby maintaining their functional integrity under oxidative stress. The probability of such a mechanism is consistent with current ideas about the depository function of mollusk hepatopancreas and the transport role of their haemolymph (Song *et al.*, 2025).

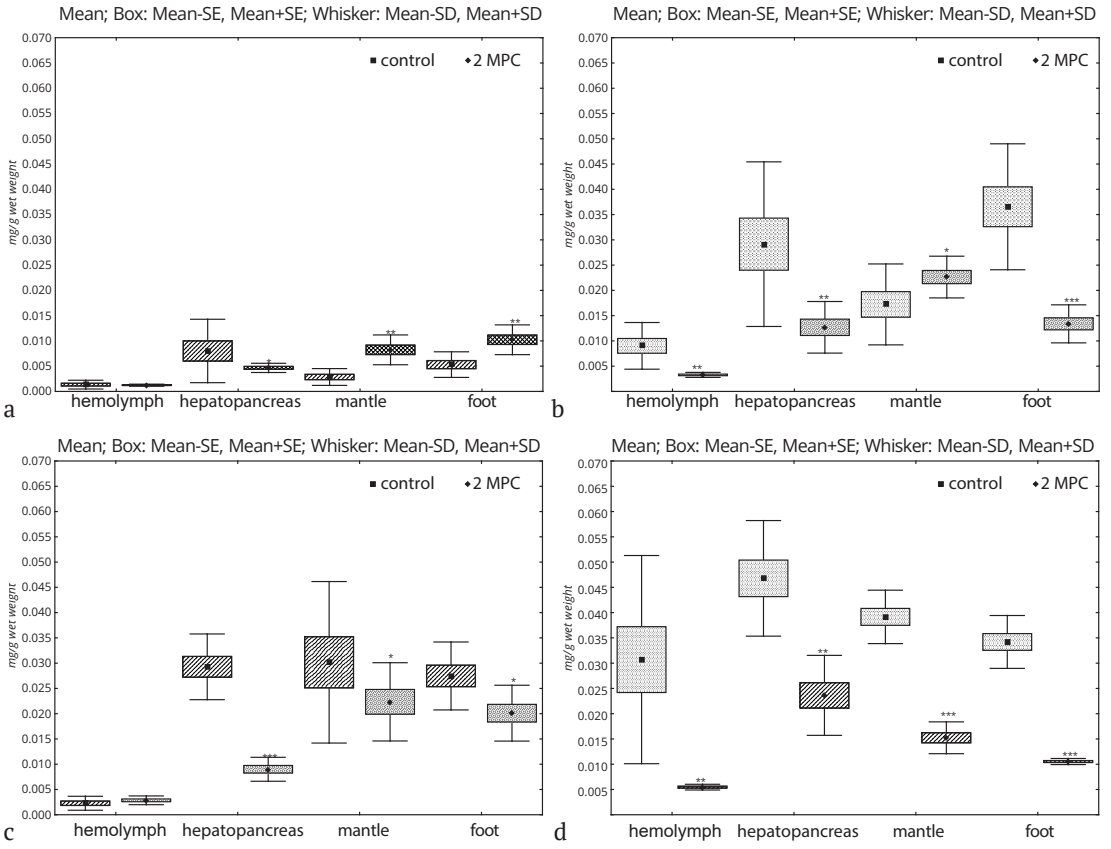

Figure 1. Effect of urea on the content of β-carotene in *L. stagnalis*
Note: A – exposure of 2 days; B – exposure of 7 days; C – exposure of 14 days; D – exposure of 21 days); n = 10 for each experiment variant; *p < 0.05; **p < 0.01; ***p < 0.001 (Tukey test)
Source: compiled by the authors

Prolonging the action of urea for up to 7 days increased its inhibitory effect on *L. stagnalis*, which was accompanied by a decrease in the content of β-carotene in the haemolymph, hepatopancreas, and foot by 56.45-63.71% compared to the control. The mantle showed an increase in pigment levels by 31.43%, which probably reflects the preservation of the local compensatory response of the tissue directly in contact with the environment. In general, the results obtained indicate a gradual transition in the body of *L. stagnalis* from the compensation stage to the depletion of antioxidant protection, which is probably due to the intensive use of β-carotene to neutralise reactive oxygen species and maintain the redox balance of the cell. This interpretation is consistent with current ideas about the functional role of carotenoids under oxidative stress. In particular, C. Zhuang *et al*. (2022) showed their active involvement in the neutralisation of reactive oxygen species, whereas K. Lim *et al*. (2023) and X. Hu *et al*. (2025) consider these compounds as an important component of the non-enzymatic antioxidant system that ensures the maintenance of cellular redox homeostasis.

With an increase in the duration of exposure to 14 days, there was a decrease in the content of β-carotene in the hepatopancreas, mantle, and foot of mollusks by 25.94-69.24% (p < 0.001-0.05) and a simultaneous increase in its level in the haemolymph by 25.69%. This multidirectional dynamics probably reflects the mobilisation of carotenoids from depot tissues and their entry into the haemolymph for further redistribution between organs in order to maintain systemic antioxidant protection under prolonged toxic stress.

Furthermore, a decrease in the content of β-carotene in hepatopancreas may indicate an increased functional load on this organ, which, as noted by T. Zhang *et al*. (2024), plays a leading role in the metabolism, detoxification, and antioxidant protection of shellfish. According to Y. Liao *et al*. (2025), carotenoids accumulated in the body of aquatic animals can be redistributed between tissues according to their metabolic needs and functional state. Therefore, an increase in the content of β-carotene in the haemolymph on the day 14 of exposure can be considered as a manifestation of activation of inter-organ transport of β-carotene, aimed at maintaining the circulating pigment pool at the compensation stage.

Under conditions of prolonged exposure to urea (21 days), a systemic decrease in the content of β-carotene in all studied tissues and organs was established at 49.49-82.19% ($p < 0.001$-0.05). Such dynamics probably indicate the depletion of the carotenoid link of the antioxidant system and a decrease in the compensatory and adaptive capabilities of mollusks, because, as noted by K. Lim *et al*. (2023) and X. Hu *et al*. (2025), with prolonged stress exposure, intense involvement of carotenoids in maintaining homeostasis may be accompanied by a gradual depletion of their tissue reserves. The smallest deviation from the control was recorded in the hepatopancreas, and the largest – in the haemolymph, which may reflect the relative preservation of the depository function of the hepatopancreas and depletion of the circulating pool of β-carotene. According to the severity of changes, the studied tissues were arranged in the following sequence: hepatopancreas → mantle → foot → haemolymph.

As for xanthophylls, with the short-term action of urea (2 days), their content increased by 73.09% ($p < 0.05$) in the haemolymph, while no statistically significant differences were found in hepatopancreas compared to the control (Fig. 2). A significant decrease in the indicator by 50.73-56.68%, respectively ($p < 0.01$-0.05), was recorded in the foot and mantle. This nature of changes indicates that even at an early stage of toxic effects, xanthophylls are intensively used in peripheral tissues that are directly in contact with the environment or are characterised by high functional activity. A similar role of xanthophylls as membrane antioxidants, which are the first to be involved in protecting cells from lipid peroxidation, was noted by K. Lim *et al*. (2023) and X. Hu *et al*. (2025). Prolongation of urea action for up to 7 days was accompanied by a subsequent violation of carotenoid metabolism and a decrease in the content of xanthophylls in most of the studied tissues. In the haemolymph of mollusks, there was a tendency to decrease by 20.06% ($p > 0.05$), while in the hepatopancreas, mantle and foot, the content of xanthophylls statistically significantly decreased by 49.55-57.28% ($p < 0.001$-0.05) compared to the control. An increase in the duration of stay of mollusks in the toxic environment up to 14 days was accompanied by a sharp inhibition of carotenoid metabolism, which was manifested by a decrease in the content of xanthophylls in their hepatopancreas, mantle, and foot by 81.28-88.41% ($p < 0.001$). This nature of changes indicates a deep depletion of the non-enzymatic link of the antioxidant system, when the rate of use of carotenoids probably exceeds the possibility of their accumulation. Similar dynamics under long-term toxic load conditions were described by K. Lim *et al*. (2023), linking reduced tissue carotenoid content to their intensive use to maintain antioxidant protection. The largest deviation from the control was registered in hepatopancreas of *L. stagnalis*, and in their haemolymph, no statistically significant differences were found from the control group ($p > 0.05$).

With the longest exposure (21 days), a pronounced decrease in the content of xanthophylls in all studied tissues and organs was recorded by 38.89-90.49% ($p < 0.001$-0.05). The largest deviation from the control was recorded in the haemolymph, and the smallest – in the hepatopancreas. Presumably, such changes indicate a decrease in the compensatory capabilities of the non-enzymatic antioxidant system with prolonged toxic effects of urea. Under these conditions, a marked decrease in the content of xanthophylls in the haemolymph may indicate depletion of the transport pool of carotenoids, while their deficiency in tissues reflects the intensive use of these pigments in the processes of neutralising reactive oxygen species and maintaining the structural and functional integrity of cells. In addition, differences in the dynamics of changes in the content of β-carotene and xanthophylls established in the tissues of *L. stagnalis* was probably conditioned by the different physical and

chemical properties of these compounds and the specifics of their functional role in the cell. As noted by M. Zbyradowski *et al*. (2022), β-carotene is a non-polar carotenoid that is localised mainly in the deep hydrophobic part of biological membranes and, in addition to its direct antioxidant function, acts as a precursor of vitamin A. However, according to W. Gruszecki & K. Strzałka (2005) and M. Pasenkiewicz-Gierula *et al*. (2024), xanthophylls, due to the presence of oxygen-containing functional groups, are characterised by greater polarity, are localised transmembranously, and are oriented perpendicular to the plane of the lipid bilayer. This spatial organisation ensures that they are located at the interface of the "lipids/water" phases, where they more effectively inhibit the processes of lipid peroxidation. It is these features that may explain the faster depletion of the xanthophyll pool compared to β-carotene in conditions of chronic urea intoxication.

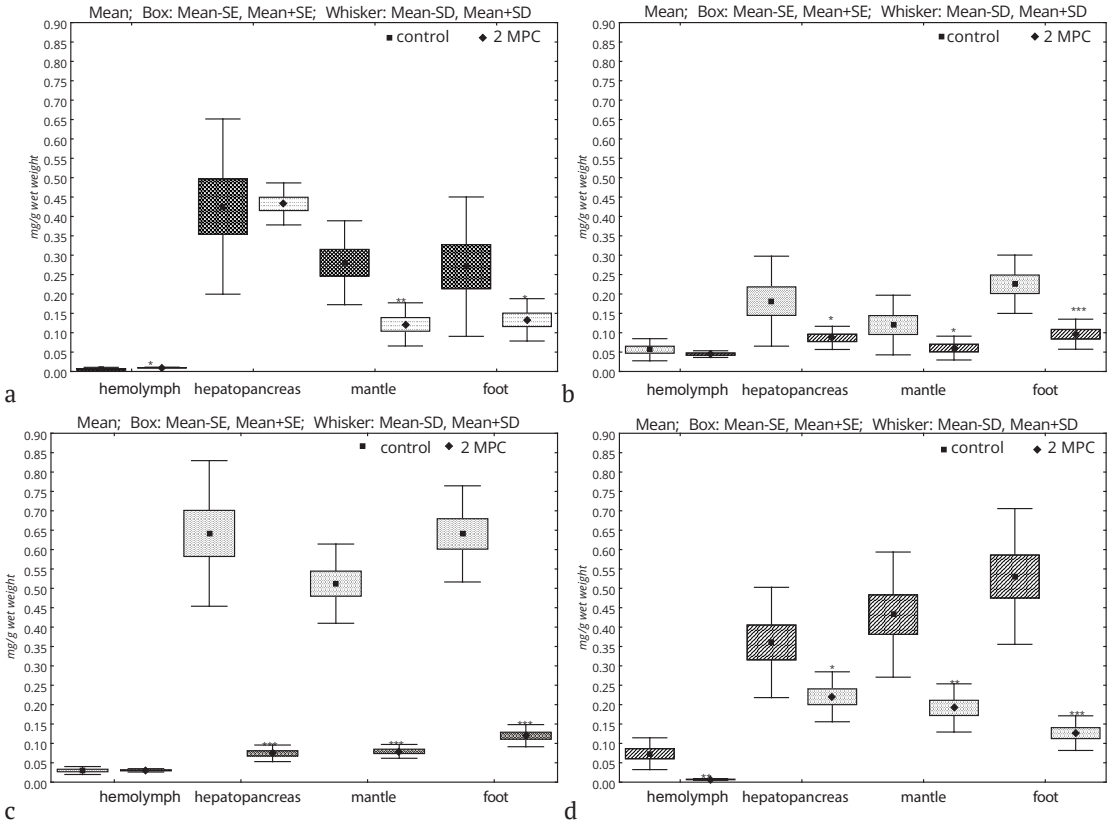

Figure 2. Effect of urea on the content of xanthophylls in *L. stagnalis*

Note: A – exposure of 2 days; B – exposure of 7 days; C – exposure of 14 days; D – exposure of 21 days); n = 10 for each experiment variant; *p < 0.05; **p < 0.01; ***p < 0.001 (Tukey test)

Source: compiled by the authors

An additional factor in a faster decrease in the content of xanthophylls in pond snails due to the action of urea may be a violation of synergistic interactions in the antioxidant protection system. R. Jia (2025) and F. Shah *et al*. (2026) showed that under normal physiological conditions, xanthophylls can regenerate by reducing their radical cations with water-soluble cytoplasmic antioxidants, in particular ascorbic acid. However, the long-term toxic effect of urea is probably accompanied by a violation of nitrogen utilisation processes and the accumulation of ammonium ions in the mollusk's body. As noted by W. Gruszecki & K. Strzałka (2005) and T. Zhang *et al*. (2024), under conditions

of ammonia-induced osmotic and oxidative stress, membrane depolarisation, and changes in intracellular pH, xanthophylls are likely involved in maintaining the structural stability of the lipid bilayer of barrier tissues (mantle) and locomotor organ (foot), which may contribute to reducing membrane permeability and maintaining their structural and functional integrity. Thus, unlike β-carotene, xanthophylls were more sensitive to the chronic effects of urea, which was manifested by their earlier and deeper depletion in most of the tissues studied. This suggests that xanthophylls are the first to be involved in neutralising reactive oxygen species and maintaining the structural and functional stability of cell membranes under long-term toxic stress, which is generally consistent with current ideas about the course of oxidative stress in aquatic animals (Ma *et al*., 2025).

Comparative analysis of the dynamics of β-carotene and xanthophylls showed that tissues (organs) of *L. stagnalis* differ significantly in the nature of the response to the action of urea, and the detected changes have a pronounced tissue-specific and time-dependent character, which may indicate an unequal degree of involvement of different tissues in maintaining homeostasis under toxic conditions. It was found that in the haemolymph of the studied mollusks, the short-term effect of urea (2 days) did not cause statistically significant differences in the content of β-carotene, but an increase in the time of contact of animals with the toxic medium to 7 and 21 days led to a decrease in the level of this carotenoid in the tissue by 63.71 and 82.19%, respectively ($p < 0.01$). Xanthophylls were characterised by a different dynamic, in which their content increased (by 73.09%) after two days of exposure, while at the end of the experiment the indicator decreased by 90.49% ($p < 0.01$) relative to the control. The results obtained may indicate a violation of the transport and intertissue redistribution of carotenoids, accompanied by a gradual depletion of their transport pool.

In the *hepatopancreas* of *L. stagnalis*, the β-carotene content, regardless of the duration of exposure, significantly decreased by 41.77-69.24% compared to the control ($p < 0.05$-0.01). A similar pattern was established for xanthophylls, the content of which decreased by 38.89-88.41% during 7, 14, and 21 day exposure, while no statistically significant differences from the control were found during 48-hour exposure. A special nature of changes was observed in the mantle of pond snail, in which the short-term effect of urea (2-7 days) caused an increase in the content of β-carotene (1.32-2.88 times), and the prolongation of toxicant exposure to 14 and 21 days was accompanied by a decrease in indicators by 25.94-61.09% ($p < 0.001$-0.05) relative to the control. As for xanthophylls, regardless of the duration of exposure, their content significantly decreased by 49.55-84.49% ($p < 0.05$-0.001), which may be due to the barrier function of the mantle and its constant contact with a toxic environment. One of the most sensitive organs to the action of urea was the foot of *L. stagnalis* in which, on the second day of the experiment, the β-carotene content increased by 92.58% ($p < 0.01$), but the prolongation of exposure was accompanied by a decrease in indicators by 26.81-69.20% relative to the control. For xanthophylls, a statistically significant decrease in the content by 50.73-81.28% ($p < 0.05$-0.001) was recorded throughout the experiment. The smallest deviation was registered after two days of exposure, and the largest – on the day 14 of the experiment.

The revealed tissue-specific features of changes in the content of β-carotene and xanthophylls probably reflect the functional specialisation of tissues (organs) of *L. stagnalis* and their unequal contribution to maintaining cellular homeostasis under toxic stress conditions. The intensity of redox processes, the rate of development of reactive oxygen species, and the effectiveness of antioxidant protection differ significantly between individual tissues. In this regard, the different dynamics of the studied carotenoids can be conditioned not only by the intensity of their use in reactions of neutralisation of reactive oxygen species, but also to the specifics of their deposition, transport, and intertissue redistribution. Similar tissue specificity in the distribution and functioning of carotenoids in aquatic animals was described by Y. Liao *et al*. (2025), who noted that the accumulation and redistribution of these pigments depends on the physiological role of individual tissues and their metabolic needs.

Analysis of tissue-specific changes in the content of β-carotene and xanthophylls at the final stage of the experiment (21 days) allowed

establishing the same metabolic series according to the degree of deviation from the control: hepatopancreas → mantle → foot → haemolymph. This pattern indicates the preservation of the overall sequence of tissue response regardless of the carotenoid group studied, although xanthophylls were characterised by an earlier and more pronounced deficiency. The results confirm that both groups of carotenoids are involved in a single metabolic response, but differ in the rate of depletion and functional role in adaptive processes. To generalise the identified patterns, a correlation analysis of the relationship between the toxic exposure factor, exposure duration, and the content of β-carotene and xanthophylls in tissues (organs) of *Lymnaea stagnalis* was performed (Table 1).

Table 1. Correlation analysis of the effects of urea and exposure duration on carotenoid content in *Lymnaea stagnalis*

Tissue (organ)	Urea effect		Exposure (2 MPC)	
	β-carotene	xanthophylls	β-carotene	xanthophylls
Haemolymph	-0.33*	-0.32*	0.85*	-0.15
Hepatopancreas	-0.50*	-0.45*	0.71*	-0.48*
Mantle	-0.20	-0.62*	0.31	0.39*
Foot	-0.50*	-0.68*	0.16	0.02

Note: * – statistically significant correlation coefficient ($p < 0.05$)
Source: compiled by the authors

To assess overall urea exposure, correlation analysis was performed for a set of control and investigate values for all exposure times, while the relationship between exposure duration and carotenoid content was evaluated only in the study group. For most tissues, a statistically significant negative correlation was established between the toxic factor and the content of β-carotene and xanthophylls, which indicates a general tendency to reduce the content of carotenoid pigments under the action of urea. For β-carotene, the closest relationship was found in the hepatopancreas and foot ($r = -0.50$; $p < 0.05$), while for xanthophylls it was more pronounced, especially in the foot ($r = -0.68$; $p < 0.05$) and mantle ($r = -0.62$; $p < 0.05$). This suggests that xanthophylls are characterised by a higher sensitivity to the toxic effects of urea compared to β-carotene.

In the experimental group of mollusks, a strong positive correlation was established between the duration of exposure and the content of β-carotene in the haemolymph ($r = 0.85$; $p < 0.05$) and hepatopancreas ($r = 0.71$; $p < 0.05$), and no statistically significant relationship was found in the mantle and foot. For xanthophylls, a significant negative association was established only in hepatopancreas ($r = -0.48$; $p < 0.05$), while a moderate positive association was observed in the mantle, and in the haemolymph and foot, the correlation between exposure duration and xanthophyll content was not statistically significant. The established positive correlation for β-carotene reflects the overall temporal trend of changes in its content in shellfish under the action of urea at a concentration of 2 $MPC_{fisheries}$ and it does not contradict the phase nature of the response established during the analysis of individual exposure times. At the initial stages of the experiment, a compensatory increase in the content of β-carotene was observed in individual tissues, while with an increase in the duration of exposure, a tendency to decrease prevailed.

Thus, the combination of the results obtained allows considering the carotenoid system as a dynamic component of the non-enzymatic antioxidant system, sensitive to changes in environmental factors of the aquatic environment. The revealed features of tissue-specific rearrangement of the content of β-carotene and xanthophylls indicate that the dynamics of individual classes of carotenoids reflects different stages of development of the compensatory and adaptive response of the body, which expands ideas about the mechanisms of antioxidant protection of freshwater pulmonate mollusks and confirms the prospects of using carotenoids as biochemical markers of ecotoxicological monitoring.

CONCLUSIONS

It was found that chronic exposure to urea at a concentration of 2 $MPC_{fisheries}$ causes significant

tissue-specific and time-dependent changes in the content of β-carotene and xanthophylls in *Lymnaea stagnalis*. Two-factor analysis of variance confirmed a statistically significant interaction of the factors "toxic effect" and "duration of exposure" on the carotenoid content, which indicates the complex nature of the body's response to toxicant action. At the initial stage of exposure (2 days), multidirectional changes in the carotenoid profile in *L. stagnalis* were established, which were accompanied by an increase in the content of β-carotene and a decrease in xanthophyll counts in the mantle and foot, indicating different sensitivity of individual groups of carotenoids to the toxic effects of urea. An increase in the duration of exposure to 7-21 days led to a progressive decrease in the content of β-carotene and xanthophylls in most of the studied tissues, which may indicate a gradual depletion of the non-enzymatic link of the antioxidant system under conditions of prolonged toxic exposure. Comparative analysis of tissue-specific features of the dynamics of β-carotene and xanthophyll content in *L. stagnalis* showed the greatest deviations from the control values in the foot and mantle, while with the longest exposure (21 days) for both groups of carotenoids, the greatest deviation was recorded in the haemolymph.

Correlation analysis showed a statistically significant negative association between urea exposure and the content of both carotenoid groups. A stronger negative correlation with the toxic factor was established for xanthophylls (up to $r = -0.68$), while a positive correlation was found between the duration of exposure and the content of β-carotene in the experimental group in the haemolymph and hepatopancreas. The obtained results expand the current understanding of the features of the functioning of the non-enzymatic antioxidant system of freshwater lung mollusks under chronic urea exposure and confirm the prospects of using β-carotene and xanthophylls as sensitive biochemical markers of ecotoxicological monitoring of nitrogen contamination of freshwater ecosystems. A promising area of further research is to investigate the relationship between changes in the carotenoid content and the activity of the enzymatic link of the antioxidant system of *Lymnaea stagnalis* by the action of nitrogen-containing toxicants of various nature and concentrations.

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Хронічний вплив низької концентрації сечовини на вміст каротиноїдних пігментів в організмі *Lymnaea stagnalis*

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Анотація. Сечовина є одним із поширених азотовмісних забруднювачів прісноводних екосистем і за тривалого надходження у водойми може порушувати перебіг метаболічних процесів в організмі гідробіонтів. Оскільки каротиноїди виконують функцію неферментативних антиоксидантів, зміни їхнього вмісту відображають розвиток оксидативного стресу та слугують чутливими біохімічними маркерами токсичного впливу. Метою роботи було з'ясувати особливості впливу низької концентрації сечовини на вміст β-каротину та ксантофілів у гемолімфі, гепатопанкреасі, мантії та нозі *Lymnaea stagnalis*. Дослідження проводили на статевозрілих неінвазованих молюсках, яких утримували у середовищі із сечовиною у концентрації, що відповідала двом гранично допустимим концентраціям для водойм рибогосподарського призначення (2 ГДК$_{рибогосп.}$), протягом 2, 7, 14 і 21 доби. Вміст каротиноїдів визначали спектрофотометрично, а результати опрацьовували із застосуванням двофакторного дисперсійного та кореляційного аналізів. Встановлено, що за короткострокової експозиції сечовини (2 доби) вміст β-каротину у мантії та нозі *L. stagnalis* збільшувався у 1,93-2,88 раза ($p<0,01$-$0,05$). Водночас вміст ксантофілів у цих органах достовірно знижувався на 50,73-56,68 %, тоді як у гепатопанкреасі значущих відмінностей не встановлено. Збільшення тривалості експозиції до 14-21 доби супроводжувалося системним зниженням вмісту β-каротину (на 25,94-69,24 %; $p < 0,001$-$0,05$) та ксантофілів (на 38,89-88,41 %) у гепатопанкреасі, мантії та нозі *L. stagnalis*, що, ймовірно, пов'язано з посиленням прооксидантних процесів і використанням каротиноїдів у реакціях нейтралізації вільних радикалів. Кореляційний аналіз виявив статистично значущий негативний зв'язок між впливом сечовини та вмістом β-каротину і ксантофілів, що свідчить про виснаження каротиноїдного резерву за токсичного навантаження. Отже, хронічний вплив сечовини у досліджуваній концентрації спричиняє тканинно-специфічні зміни каротиноїдного профілю *L. stagnalis*, які відображають перехід від компенсаторно-адаптаційних реакцій до виснаження неферментативної ланки антиоксидантної системи. Отримані результати можуть бути використані природоохоронними та рибогосподарськими організаціями для екотоксикологічної оцінки впливу азотовмісних забруднювачів на прісноводні екосистеми, а вміст β-каротину та ксантофілів – як інформативний біохімічний маркер такого впливу

Ключові слова: прісноводні молюски; β-каротин; ксантофіли; оксидативний стрес; азотовмісні сполуки

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The formation of the microbial community in acidic sod-podzolic soil within the maize rhizosphere under the integrated application of fertilisers and soil amendments

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Abstract. This study investigated the effect of the combined application of soil amendments and fertilisers on the formation and functioning of the microbial community in the maize rhizosphere under sod-podzolic soil conditions. The aim was to determine how the integrated application of fertilisers and soil amendments affects the structural and functional characteristics of the microbiocenosis of sod-podzolic soil in the maize rhizosphere and to identify microbiological indicators for the ecological bioindication of the agrocenosis. Field studies were conducted in 2025 in a long-term experiment at the Institute of Agriculture of Western Polissia, NAAS of Ukraine. The experiment comprised eight treatments combining the residual effect of liming with dolomite flour or limestone and differentiated

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mineral fertilisation with two foliar applications of a micronutrient fertiliser. The abundance of ecological-trophic groups of microorganisms was determined by plating on selective agar media, microbial biomass by the rehydration method, and CO_2 emission by the adsorption method. Soil pH increased from 4.09 to 5.48-6.20, while hydrolytic acidity decreased 2.1- and 2.9-fold. The abundance of ammonifiers increased 2.9- and 3.8-fold and reached 27.61 million CFU/g soil. When dolomite flour was combined with fertilisers, the abundance of micromycetes decreased 2.1-fold to 28.80 thousand CFU/g soil. The greatest change was recorded for oligonitrophiles, whose abundance decreased 7.7-fold. Microbial biomass increased to 128.95 μg C/g soil, CO_2 emission fell to 14.2 mg/kg per day, and the metabolic quotient decreased from 183.8 to 111.1 mg CO_2 per 1 g of biomass carbon. The microbial community shifted from a fungal-oligotrophic to a bacterial-eutrophic type, with an oligotrophy coefficient of 0.08-0.15 and a mineralisation coefficient of 0.14-0.24. Hydrolytic acidity was the leading regulator of this transition (r = 0.92 and 0.96). The practical value of the work lies in substantiating the abundance of oligonitrophiles as a bioindicator of nitrogen saturation in maize agrocenoses and in demonstrating the advantage of a magnesium-containing amendment for controlling the fungal microbiota of acidic Polissia soils

Keywords: soil microbiome; taxonomic structure; bioindication; microbial biomass; oligonitrophiles

INTRODUCTION

Soil is a living system, not merely a substrate for plant growth. Its functioning is determined by the activity of the microbial community, which ensures the cycling of carbon, nitrogen, phosphorus and sulphur, shapes soil structure, regulates the availability of nutrients and controls the population of plant pathogens. This is precisely why the study of microbiological indicators is of particular relevance: they enable the rapid assessment of soil condition, the detection of degradation processes at an early stage, and the justification of measures to restore soil health in the context of intensive agriculture and climate change.

J. Lehmann *et al*. (2020) define the concept of soil health as the soil's ability to function as a living ecosystem that supports plants, animals and humans. M. Hartmann & J. Six (2023) consider soil structure and the functions of the microbiome in agroecosystems as a single, interconnected system. N. Fierer *et al*. (2021) emphasise a substantial advantage of microbiological indicators over physical and chemical ones: they respond to changing conditions much more rapidly. Changes in soil organic carbon become detectable only after decades, whereas the abundance and activity of microorganisms can shift within a single growing season, which makes the microbiota a convenient tool for early diagnosis. O.S. Demyanyuk *et al*. (2020) substantiate this approach specifically for Ukrainian agroecosystems in the context of adapting agriculture to climate change. At the same time, A. Levishko *et al*. (2026) caution that microbiological indicators should be interpreted carefully because of the high functional redundancy of soil communities. The relevance of this topic stems from the ecological condition of the sod-podzolic soils of the Western Polissia region of Ukraine, where acidity acts as a powerful ecological filter and exerts selective pressure on microbial communities. At low pH values, acidophilic microfungi predominate, whilst the bacterial component is suppressed. Y. Duan *et al*. (2025) demonstrate that soil acidification destabilises terrestrial ecosystems by disrupting interactions within the microbiome. G. Agegnehu *et al*. (2021) further highlight mobile forms of aluminium and manganese as significant stress factors, the effects of which result in a reduction in functional diversity and a weakening of the soil's natural regulatory capacity. Q. Liu *et al*. (2025) found that it is precisely at pH values below 5.5 in soils that phytopathogenic fungi become dominant, whilst saprotrophs predominate under slightly acidic conditions.

Maize is a suitable model organism for studying such processes, as its root system forms a robust rhizosphere characterised by the intensive secretion of root exudates, which determine the composition of rhizosphere communities and nourish copiotrophic bacteria. S. Dlamini *et al*. (2023) found that the maize rhizosphere is capable of actively reshaping the diversity and structure

of the microbiome to maintain plant health. The influence of environmental, edaphic and biotic factors depends on plant growth and development processes; however, hybrids and cultivation techniques play a dominant role. Unlike legumes, maize lacks its own symbiotic source of nitrogen and is therefore grown using high doses of mineral nitrogen. M. Tosi *et al.* (2021) found that prolonged nitrogen loading alters microbial communities more significantly than a single fertiliser application during the study year. T. Meng *et al*. (2024) found that as nitrogen rates increase, bacterial diversity initially rises and then declines. D. Bebber & V. Richards (2022) demonstrated that elevated nitrogen rates inhibit biological nitrogen fixation and disrupt the stability of microbial networks. Liming is an effective measure for the ecological restoration of acidic soils, as it reduces acid stress, alters the ratio of bacterial and fungal communities, and influences the course of biogeochemical processes. At the same time, its effectiveness may depend on the soil solution's pH, which largely determines the ability of microorganisms to utilise carbon. Y. Yu *et al*. (2024) note that the integration of soil improvement and mineral nutrition creates a multi-component system of interactions that requires targeted research. For the acidic soils of Polissia, quantitative characteristics of the microbiological response of the maize rhizosphere remain poorly studied. V. Polovyi *et al*. (2023) focused mainly on the carbon balance of the agrocenosis, whereas A. Levishko *et al*. (2026) analysed the microbiocenosis of leguminous crops. K. Shemetun *et al*. (2022) reported selected microbiological assessments of sod-podzolic soils under elements of farming biologisation. The methodological basis of the present study was the ecological-trophic group approach proposed by K.I. Andreyuk *et al*. (2001). As noted by A. Levishko *et al*. (2026), this approach makes it possible to describe the trophic structure of the community and the direction of organic matter transformation.

Thus, the aim of this study was to assess the impact of the integrated application of fertilisers and soil amendments on the structure and functional activity of the microbiocenosis of sod-podzolic soil in the maize rhizosphere, and to identify microbiological indicators for the ecological bioindication of this agrocenosis.

MATERIALS AND METHODS

The study was conducted in a long-term field experiment at the Institute of Agriculture of Western Polissia of the National Academy of Agrarian Sciences of Ukraine (Rivne Region, Rivne District, Shubkiv village). The experiment was established in 2020 on a sod-podzolic loamy-sandy soil. The initial soil solution pH was 4.09, and hydrolytic acidity was 2.29 mmol per 100 g of soil. The preceding crop was sunflower. The experimental design comprised eight treatments with three replicates (24 experimental plots in total). The sown area of each plot was 99 m^2, with a harvested area of 50 m^2. The treatments were arranged systematically. The general study design is presented in Figure 1.

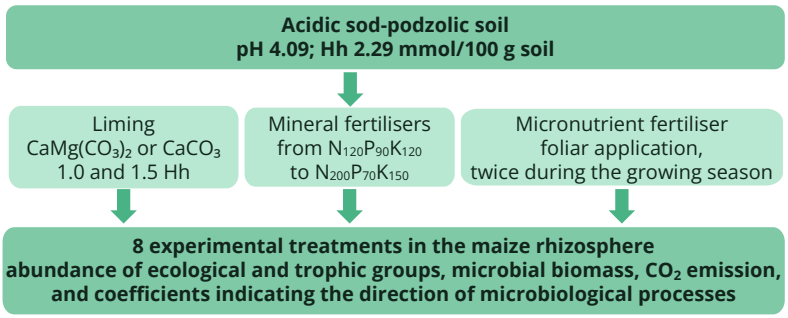

Figure 1. General scheme of the study

Source: developed by the authors

Liming was carried out in autumn 2020 prior to the first crop in the crop rotation by spreading soil amendments followed by incorporation into the soil. The after-effects of the soil amendments were assessed in 2025. The rates of soil amendments were calculated based on hydrolytic

acidity (Ng): $CaMg(CO_3)_2$ at rates of 1.0 and 1.5 Nh, and $CaCO_3$ at a rate of 1.0 Ng. Mineral fertilisers were applied in spring 2025 during pre-sowing cultivation in accordance with the experimental design (Table 1). The microfertiliser was applied foliar (by spraying) twice during the growing season during the active growth phase of maize.

Table 1. Field experiment design

Treatment	Soil amendment	Mineral fertilisers	Micronutrient fertiliser
1	Not applied	Not applied	Not applied
2	$CaMg(CO_3)_2$, 1.0 Nh	Not applied	Not applied
3	$CaMg(CO_3)_2$, 1.0 Nh	$N_{120}P_{90}K_{120}$	Twice during the growing season
4	$CaMg(CO_3)_2$, 1.0 Nh	$N_{165}P_{30}K_{50}$	Twice during the growing season
5	$CaMg(CO_3)_2$, 1.0 Nh	$N_{200}P_{70}K_{150}$	Twice during the growing season
6	$CaMg(CO_3)_2$, 1.0 Nh	N_{165}	Twice during the growing season
7	$CaMg(CO_3)_2$, 1.5 Nh	$N_{120}P_{90}K_{120}$	Twice during the growing season
8	$CaCO_3$, 1.0 Nh	$N_{120}P_{90}K_{120}$	Twice during the growing season

Source: compiled by the authors

Soil samples were collected in 2025 from the maize rhizosphere at the 7-9-leaf stage from the 0-20 cm soil layer in accordance with DSTU 4287:2004 (2004). From each experimental plot (replicate), a composite sample was prepared from five sampling points arranged diagonally across the plot. Thus, three independent composite samples were obtained for each experimental treatment, one from each replicate. The abundance of microorganisms was determined by the serial dilution plating method, using soil suspensions plated onto selective agar media in accordance with DSTU 7847:2015 (2015). Laboratory determinations of microbial abundance were performed in five replicates. Ammonifiers were enumerated on meat-peptone agar (MPA). Micromycetes were enumerated on Czapek agar (CZA). Bacteria capable of assimilating mineral forms of nitrogen and streptomycetes were enumerated on starch-ammonia agar (SAA). Oligotrophs were enumerated on nutrient-poor agar. Pedotrophs were enumerated on soil agar (SA). Oligonitrophilic diazotrophs were enumerated on Ashby's medium. The direction of microbiological processes was assessed using the oligotrophy, nitrogen mineralisation and immobilisation, and pedotrophy coefficients (Andreyuk *et al.*, 2001). The mineralisation coefficient was calculated using the formula (1):

$$C_m = C_{SAA} / C_{MPA}, \quad (1)$$

where C_m – the mineralisation coefficient, calculated as the ratio of the number of microorganisms that grew on starch-ammonia agar (C_{SAA}), to the number of microorganisms that grew on meat-peptone agar (C_{MPA}).

The oligotrophy coefficient was calculated using the formula (2):

$$C_{ol} = C_{NPA} / (C_{SAA} + C_{MPA}), \quad (2)$$

where C_{ol} – the oligotrophy coefficient, calculated as the ratio of the number of microorganisms that grew on nutrient-poor agar (C_{NPA}), to the sum of the numbers of microorganisms that grew on starch-ammonia agar (C_{SAA}) and meat-peptone agar (C_{MPA}).

The pedotrophy coefficient was calculated using the formula (3):

$$C_{ped} = C_{SA} / C_{MPA}, \quad (3)$$

where C_{ped} – the pedotrophy coefficient, calculated as the ratio of the number of microorganisms that grew on soil agar (C_{SA}), to the number of microorganisms that grew on meat-peptone agar (C_{MPA}).

Microbial biomass was determined using the rehydration method in accordance with DSTU ISO 14240-1:2003. CO_2 emission rates were determined using Shtatnov's adsorption method in accordance with DSTU ISO 16072:2005 (2005). Soil solution reaction and hydrolytic acidity were determined in accordance with DSTU ISO 10390:2022 (2022) and DSTU 7537:2014 (2014), respectively. In addition, two derived ecological

indices were calculated. The specific metabolic rate (qCO_2) was calculated as the ratio of CO_2 emissions to microbial biomass carbon. This indicator characterises the energy efficiency of the community and its level of stress (Clayton *et al*., 2021). The efficiency of carbon utilisation by microorganisms is considered a key factor in the accumulation of organic carbon in the soil (Tao *et al*., 2023). The bacterial-fungal ratio was calculated as the ratio of the abundance of ammonifiers to that of microfungi (Wang *et al*., 2019).

Statistical analysis was applied at all stages of processing the experimental data. Microbiological studies were conducted in quintuplicate; the abundance of microorganisms belonging to the main ecological-trophic groups was reported as the arithmetic mean with standard error. Based on the mean abundance values of the groups, coefficients indicating the direction of microbiological processes – mineralisation – immobilisation, oligotrophy and pedotrophy – were calculated, as well as indicators of total microbial biomass and CO_2 emission intensity. Mathematical and statistical processing of the experimental data was carried out using Microsoft Excel 2019 and Minitab 19. Statistical analysis was performed using one-way analysis of variance (One-way ANOVA). The significance of differences between the mean values of the experimental treatments was assessed using Fisher's exact test at a statistical significance level of $p \leqslant 0.05$. Field studies were conducted in accordance with the principles of biodiversity conservation as set out in the Convention on Biological Diversity (1992), as well as bioethical principles and environmental safety standards. Sampling was carried out with minimal impact on the plants and their habitats.

RESULTS AND DISCUSSION

Experimental studies were carried out in the Western Polissia region on sod-podzolic loamy-sandy soil, which is characterised by a natural tendency towards acidification and requires systematic optimisation of its physico-chemical properties. The assessment was carried out as part of a long-term field experiment, studying the long-term after-effects of chemical soil amendments applied in 2020. Particular attention was paid to a comprehensive analysis of the impact of variable maize mineral nutrition systems on the acid-base balance of the soil solution. Given the key role of the microbiome in ensuring the availability of nutrients, the structural and functional reorganisation of the rhizosphere microbial community, the potential for metabolic activity of the local microbiota, and the overall direction and dynamics of biochemical and microbiological processes were studied in detail. The after-effects of soil conditioners, in combination with mineral fertilisation, altered the ecological conditions of the microbiota (Table 2).

Table 2. Acid-base status and nutrient availability in the soil of the maize rhizosphere

Treatment	Soil pH	Hydrolytic acidity, mmol/100 g soil	Easily hydrolysable N, mg/kg	Available P_2O_5, mg/kg	Available K_2O, mg/kg
1	4.09	2.29	47.8	193.5	63.9
2	5.60	0.79	57.6	257.0	94.2
3	5.71	0.79	65.8	284.1	125.4
4	5.91	0.90	63.5	117.5	108.1
5	5.48	1.11	72.9	290.4	143.2
6	5.53	0.96	59.5	216.6	106.3
7	6.20	0.84	67.4	311.1	139.5
8	6.18	0.83	66.8	261.6	130.2

Source: compiled by the authors

The soil solution pH increased from 4.09 in the control to a range of 5.48-6.20. Hydrolytic acidity decreased 2.1- and 2.9-fold. Acid stress was alleviated most effectively in treatments 7 and 8. The soil's supply of nutrients also increased. The exception was treatment 4, where the content of available phosphorus was the lowest in the experiment. The abundance of the main ecological and trophic groups is presented in Table 3. Changes relative to the control are shown in Figure 2.

Table 3. Abundance of the main ecological and trophic groups of microorganisms in the maize rhizosphere

No.	Ammonifiers, million CFU/g	Micromycetes, thousand CFU/g	Mineral-nitrogen bacteria, million CFU/g	Streptomycetes, million CFU/g	Oligotrophs, million CFU/g	Pedotrophs, million CFU/g	Oligonitrophiles, million CFU/g
1	7.20 ± 0.16	61.20 ± 4.68	4.93 ± 0.21	4.18 ± 0.26	4.03 ± 0.08	4.75 ± 0.63	3.60 ± 0.33
2	24.12 ± 0.72	43.20 ± 5.21	5.69 ± 0.17	4.82 ± 0.21	3.49 ± 0.11	3.49 ± 0.18	3.31 ± 0.19
3	27.61 ± 0.87	43.60 ± 8.15	4.25 ± 0.12	3.45 ± 0.18	2.76 ± 0.15	3.09 ± 0.12	2.54 ± 0.27
4	20.52 ± 0.29	50.40 ± 4.46	4.25 ± 0.23	3.74 ± 0.32	3.60 ± 0.17	4.21 ± 0.52	3.20 ± 0.12
5	24.71 ± 0.99	39.97 ± 3.75	5.78 ± 0.54	4.58 ± 0.38	2.47 ± 0.25	3.12 ± 0.87	1.45 ± 0.16
6	24.34 ± 0.49	47.23 ± 6.48	3.74 ± 0.23	3.20 ± 0.17	2.62 ± 0.32	1.27 ± 0.36	1.02 ± 0.08
7	27.00 ± 0.65	28.80 ± 4.54	3.78 ± 0.15	2.92 ± 0.11	3.85 ± 0.22	3.82 ± 0.62	1.04 ± 0.16
8	24.71 ± 0.62	87.20 ± 3.61	5.27 ± 0.64	4.61 ± 0.31	3.60 ± 0.18	3.49 ± 0.31	0.47 ± 0.07

Source: compiled by the authors

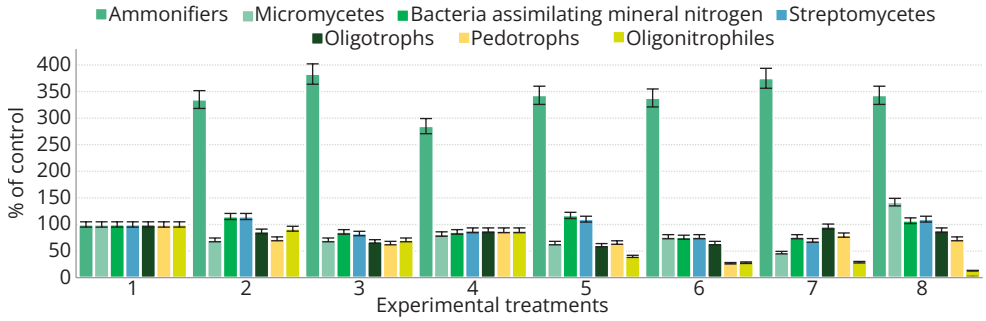

Figure 2. Abundance of key ecological and trophic groups of microorganisms in the maize rhizosphere, % of control

Source: compiled by the authors

In the control treatment, micromycetes and pedotrophs predominated. The abundance of ammonifiers was low, at 7.20 million CFU/g soil. This ratio corresponds to a fungal-oligotrophic type of organic matter decomposition, which is characteristic of uncultivated acidic soils. Ammonifiers showed the most pronounced response to agromelioration. The residual effect of dolomite flour increased their abundance 3.3-fold. The application of the full mineral fertiliser combination increased their abundance 3.8-fold, to 27.61 million CFU/g soil. This group showed the strongest relationship with soil solution pH. The correlation coefficient was +0.87, while the correlation with hydrolytic acidity reached -0.94. Thus, acidity, rather than the fertiliser rate, was the limiting factor for the ammonifying microbiota.

The abundance of micromycetes decreased in all treatments with dolomite flour. The minimum was recorded in treatment 7, where it was 28.80 thousand CFU/g soil, 2.1-fold lower than in the control. In contrast, in treatment 8 with the calcium-based soil amendment, fungal abundance increased to 87.20 thousand CFU/g soil. The soil solution pH in these two treatments was practically identical, at 6.20 and 6.18, respectively. Thus, the threefold difference in fungal abundance cannot be explained by pH. The most likely reason is the absence of magnesium in the composition of calcite. An imbalance in the calcium-to-magnesium ratio in the soil exchange complex reduces the competitive position of the bacterial component. The greatest change in abundance was observed for oligonitrophiles.

This group is adapted to a deficiency of available nitrogen. Its abundance decreased progressively from 3.60 million CFU/g soil in the control to 0.47 million CFU/g soil in treatment 8, i.e. 7.7-fold. In treatments 5-7, it ranged from 1.02 to 1.45 million CFU/g soil. This result directly reflects the alleviation of nitrogen deficiency under high nitrogen rates. In the presence of available mineral nitrogen, oligonitrophiles lose their competitive advantage. Within the same long-term experiment, the soybean rhizosphere was previously examined. At nitrogen rates of 45-65 kg/ha, the abundance of oligonitrophiles did not decrease but increased. Under maize, at rates of 120-200 kg/ha, this group almost disappeared. The comparison was made on the same soil, in the same crop rotation and by the same method. This provides a basis for proposing oligonitrophile abundance as a bioindicator of nitrogen saturation in the agrocenosis.

The abundance of pedotrophs decreased moderately in most treatments. A sharp decline to 1.27 million CFU/g soil, i.e. 3.7-fold, was recorded only in treatment 6. This treatment involved the application of nitrogen alone. The value obtained illustrates the suppression of soil-specific microbial forms under unbalanced nutrition. Complete mineral nutrition maintained pedotroph abundance at 3.09-3.82 million CFU/g soil. The abundance of mineral-nitrogen bacteria and streptomycetes changed only slightly. No significant relationship between these groups and soil solution pH was detected. The absolute values of the correlation coefficients did not exceed 0.22. The results of the assessment of the functional activity of the microbial community are presented in Table 4 and Figure 3.

Table 4. Microbial biomass, CO_2 emissions and derived ecological indices

Treatment	Biomass, μg C/g soil	CO_2 emissions, mg/kg per day	Specific metabolic rate, mg CO_2 per 1 g C of biomass	Bacteria-to-fungi ratio
1	99.55 ± 6.5	18.3 ± 1.2	183.8	118
2	114.78 ± 8.4	20.0 ± 1.3	174.2	558
3	102.81 ± 10.2	17.6 ± 1.5	171.2	633
4	97.19 ± 9.6	15.4 ± 1.7	158.5	407
5	103.99 ± 7.5	17.1 ± 2.1	164.4	618
6	128.95 ± 9.3	16.6 ± 3.1	128.7	515
7	127.77 ± 8.4	14.2 ± 1.8	111.1	938
8	117.08 ± 10.5	15.6 ± 2.4	133.2	283

Source: compiled by the authors

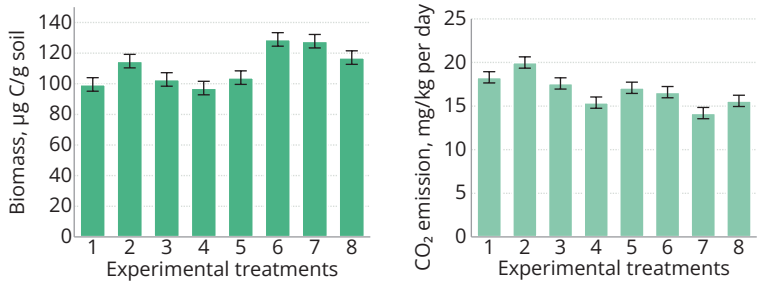

Figure 3. Microbial biomass (a) and CO_2 emission rate (b) in the maize rhizosphere
Note: the dotted line indicates the control level
Source: compiled by the authors

Microbial biomass in the control treatment was 99.55 μg C/g soil. Integrated agromelioration increased it to an average of 112.97 μg C/g soil. The maximum values were recorded in treatments 6 and 7, at 128.95 and 127.77 μg C/g soil, respectively. The increase in biomass in treatment 6 had a different underlying cause: it was driven by the supply of readily available

nitrogen without phosphorus or potassium. Under these conditions, heterotrophic bacteria accelerate the decomposition of available organic matter to balance the carbon-to-nitrogen ratio. Indirect confirmation of this is provided by the minimum abundance of pedotrophs observed specifically in this treatment. In contrast, the increase in biomass in treatment 7 was supported by balanced nutrition and was therefore more environmentally sustainable. The CO_2 emission rate in the control treatment was 18.3 mg/kg per day. The maximum was recorded in treatment 2, where it reached 20.0 mg/kg. In treatments receiving complete mineral nutrition, emissions decreased to a range of 14.2-17.6 mg/kg per day. In treatment 7, the reduction relative to the control was 22%.

The combination of increased biomass and reduced respiration is a key ecological finding of the study. The specific metabolic quotient decreased from 183.8 in the control to 111.1 mg CO_2 per 1 g biomass carbon in treatment 7, representing a 1.65-fold decrease. According to the study by R. Dang *et al*. (2024), such a reduction in this indicator may indicate reduced stress on the microbial community and increased carbon-use efficiency. The practical consequence is a reduction in unproductive carbon losses from the agrocenosis. The bacterial-fungal ratio increased from 118 to 938, confirming a shift in the type of organic matter decomposition. The values of the coefficients indicating the direction of microbiological processes are presented in Table 5 and Figure 4.

Table 5. Direction of microbiological processes in the maize rhizosphere

Treatment	Oligotrophy coefficient	Nitrogen mineralisation and immobilisation coefficient	Pedotrophy coefficient	Type of process direction
1	0.35	0.69	0.66	coprotrophic-mineralising
2	0.12	0.24	0.14	transitional
3	0.09	0.15	0.11	eutrophic-immobilising
4	0.15	0.21	0.21	transitional-immobilising
5	0.08	0.23	0.13	eutrophic-immobilising
6	0.09	0.15	0.05	eutrophic-immobilising
7	0.13	0.14	0.14	eutrophic-immobilising
8	0.12	0.21	0.14	eutrophic-immobilising

Source: compiled by the authors

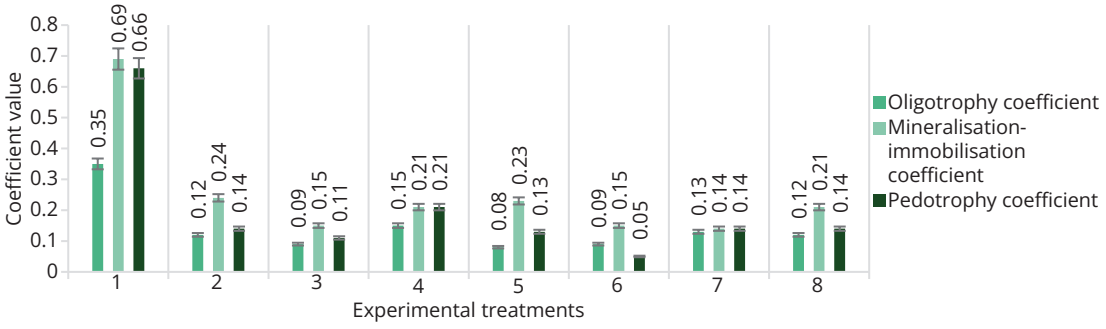

Figure 4. Directionality coefficients of microbiological processes in the maize rhizosphere
Source: compiled by the authors

The control treatment was clearly differentiated from the other experimental treatments, with an oligotrophy coefficient of 0.35, a mineralisation and immobilisation coefficient of 0.69, and a pedotrophy coefficient of 0.66. This combination corresponds to a coprotrophic-mineralising regime. Under these conditions, the microbial community intensively decomposes its own reserves of soil organic matter. For the agrocenosis, this means a gradual depletion of the humus pool. Integrated agromelioration radically altered this pattern. In the fertilised treatments, the oligotrophy coefficient

decreased to a range of 0.08-0.15. The mineralisation and immobilisation coefficient ranged from 0.14 to 0.24. This corresponds to a eutrophic-immobilising type. The microbial community is supplied with readily available substances and actively immobilises nitrogen in its biomass. This regime promotes the accumulation of organic matter and reduces nitrogen losses. Both coefficients showed a very strong relationship specifically with hydrolytic acidity. The correlation coefficients were +0.92 and +0.96, respectively (Fig. 5). This provides grounds for considering hydrolytic acidity to be the principal regulator of the direction of microbiological processes in sod-podzolic soils.

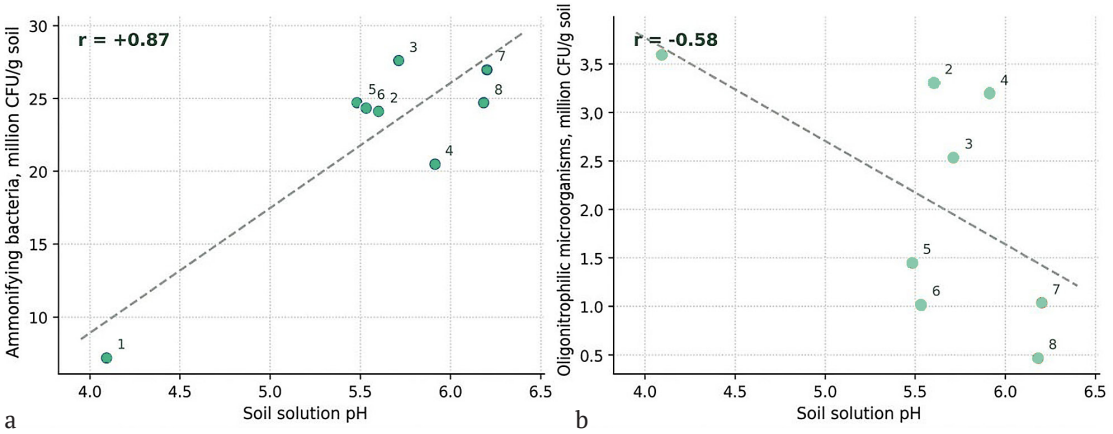

Figure 5. Relationship between the abundance of ammonifiers (a) and oligonitrophiles (b) and the reaction of the soil solution

Note: the numbers indicate the option numbers

Source: compiled by the authors

The data obtained describe a sequential ecological transition of the microbiocenosis. This transition occurs from a fungal-oligotrophic system, characterised by intensive mineralisation of its own organic matter reserves, to a bacterial-eutrophic system with an immobilisation orientation (Fig. 6).

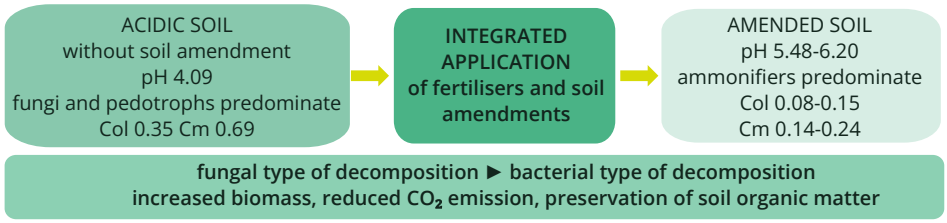

Figure 6. Diagram illustrating the ecological transformation of the maize rhizosphere microbiocenosis following the integrated application of fertilisers and soil amendments

Source: compiled by the authors

This pattern of changes is consistent with current understanding of the role of soil acidity. Y. Duan *et al.* (2025) demonstrated that soil acidification destabilises terrestrial ecosystems by disrupting interactions within the microbiome. Meanwhile, research by P. Wenyika *et al.* (2025) summarised the impact of liming on soil biota and confirmed that soil improvement can significantly alter the structure of microbial communities. The most striking finding of the study was the behaviour of oligonitrophiles. A 7.7-fold reduction in their abundance represents the greatest change

among all the groups studied. Within the same long-term experiment, oligonitrophile abundance increased under soybean at N 45-65 kg/ha and almost disappeared under maize at N 120-200 kg/ha. This comparison, made on the same soil, in the same crop rotation and by the same method, supports the use of this group as a bioindicator of nitrogen saturation. A study by M. Tosi *et al*. (2021) showed that it is the cumulative nitrogen load, rather than current application, that determines the structure of the community. A group of researchers led by J. Liu *et al*. (2024) established that excessive doses of nitrogen make the microbial networks of the maize rhizosphere the most complex, yet at the same time the least stable. T. Meng *et al*. (2024) identified a non-linear response of bacterial diversity to increasing nitrogen doses.

The study highlighted the differing ecological effects of soil amendments with varying chemical compositions. Dolomite flour and calcite produced virtually identical responses in the soil solution. However, the abundance of microfungi in these treatments differed by a factor of three. This indicates that the phytosanitary effect of liming is determined not only by the pH level. P. Wenyika *et al*. (2025) also point out that, under certain conditions, liming can increase the abundance of specific groups of fungi. Furthermore, I. Beznosko *et al*. (2022) demonstrated that the quantitative composition of microfungi under cereal crops depends significantly on soil conditions and the cultivation system. Thus, the choice of a soil conditioner for acidic soils should not be based solely on its neutralising potential.

A simultaneous increase in biomass and a decrease in respiration indicate reduced stress on the community. This is how a decline in the metabolic quotient is interpreted by T.-H. Anderson & K.H. Domsch (1993). At the same time, D.A. Wardle & A. Ghani (1995) caution that qCO_2 is not a universal index and should be interpreted carefully, because it may increase both under greater stress and when recalcitrant substrates predominate. In the present study, the decrease in qCO_2 was accompanied by an increase in biomass and a reduction in the fungal fraction; therefore, its interpretation as reduced stress is justified. R. Dang *et al*. (2024) found that the external supply of nutrients generally increases the efficiency of carbon utilisation by microorganisms. F. Tao *et al*. (2023) regard this efficiency as the leading factor in the accumulation of organic carbon in the soil. For the same stationary experiment, V. Polovyi *et al.* (2023) had previously established a positive organic carbon balance at the calculated fertiliser rates against a background of dolomite flour. The authors' microbiological data explain the biological mechanism behind this phenomenon.

The observed stimulatory effect of balanced mineral nutrition on beneficial bacterial groups is consistent with the results obtained by A. Srour *et al.* (2020) for maize and soya bean rotation on neutral soils. A similar pattern of changes was documented by S. Reznik (2021) for typical chernozems of the Left-Bank Forest-Steppe of Ukraine. The consistency of results across different soil types indicates the universality of the mechanism, which involves the displacement of acidophilic and oligotrophic forms by copiotrophic bacteria as trophic conditions improve. A similar restructuring of the rhizosphere community under different fertilisation regimes was described by S. Hudz & L. Skivka (2020) and V. Polishchuk *et al*. (2025) for cereal crops. The dependence of the direction of soil microbiological processes on the fertilisation regime was also confirmed by I. Malynovska *et al.* (2021) for grassland phytocenoses.

The practical significance of this work lies in the possibility of targeted management of the microbiocenosis. The favourable microbiological background established in variants 3 and 7 is a prerequisite for the subsequent introduction of microbial preparations, as noted by A. Levishko & P. Mamenko (2025). Y. Yu *et al.* (2024) emphasise that the effectiveness of introduced microorganisms depends on the state of the indigenous community and the trophic conditions of the soil. It has been shown that the level of nitrogen supply directly shapes the bacterial diversity of the maize rhizosphere. At the same time, variants 4 and 6 demonstrate the ecological risks of nutrient imbalance. They are accompanied by the depletion of phosphorus reserves and the suppression of soil-specific forms of microorganisms. A limitation of this study is the use of culture-based methods. These cover only a small fraction of the soil microbiome. N. Fierer *et al.* (2021) emphasise that this is a widely recognised problem in microbiological soil diagnostics. However, for a

comparative assessment of field trial options, the ecological-trophic group approach remains informative and accessible, as demonstrated in the work by A. Levishko *et al*. (2026). The results obtained provide grounds for considering the targeted management of the microbiological state of the soil as a promising direction for optimising the fertilisation system and enhancing the ecological stability of the agrocenosis.

CONCLUSIONS

The study demonstrated that the integrated application of fertilisers and soil amendments eliminates acid stress as the principal limiting factor for the development of the microbiota of acidic sod-podzolic soil. The soil solution pH increased from 4.09 to a range of 5.48-6.20, while hydrolytic acidity decreased 2.1- and 2.9-fold. Removal of this limitation initiated a consistent restructuring of the microbial community in the maize rhizosphere from a fungal-oligotrophic to a bacterial-eutrophic type. The abundance of ammonifiers increased 2.9- and 3.8-fold, while the bacterial-fungal ratio increased from 118 to 938. This demonstrates a shift in the pathway of organic matter decomposition from fungal to a more energy-efficient bacterial type. The structural restructuring of the community was accompanied by an increase in its functional efficiency. Microbial biomass increased to 128.95 µg C/g soil, while CO_2 emission simultaneously decreased to 14.2 mg/kg per day. The specific metabolic quotient decreased from 183.8 to 111.1 mg CO_2 per 1 g biomass carbon. This combination indicates reduced stress on the microbial community and a decrease in unproductive carbon losses from the agrocenosis. Accordingly, the direction of microbiological processes shifted from a coprotrophic-mineralising to a eutrophic-immobilising regime. Hydrolytic acidity was identified as the principal regulator of this transition, with correlation coefficients of +0.92 and +0.96 for the oligotrophy and mineralisation indicators, respectively. Thus, the acid-base status of the soil should be regarded as a key regulator of the biological regime of the maize agrocenosis.

The results obtained have independent diagnostic significance. The abundance of oligonitrophiles decreased 7.7-fold and proved to be the most sensitive of the indicators studied. Within the same long-term experiment, this group increased under soybean at N 45-65 kg/ha and almost disappeared under maize at N 120-200 kg/ha; therefore, its abundance was proposed as a bioindicator of nitrogen saturation in the agrocenosis. Furthermore, the ecological effects of soil amendments of different chemical compositions proved to be inconsistent. Whilst the soil solution reacted in virtually the same way, the abundance of microfungi in the dolomite flour treatment was 3.0 times lower than in the calcite treatment. This implies that the phytosanitary effect of liming is not limited solely to pH levels and depends on the chemical composition of the soil amendment.

The most favourable ecological conditions for the microbial community were established through a combination of the after-effect of dolomite flour with the recommended dose of mineral fertilisers and two applications of foliar feeding with a microfertiliser. In contrast, unbalanced fertilisation was accompanied by the suppression of soil-specific forms of microorganisms and the depletion of the soil's phosphorus reserves. The results obtained justify the need for microbiological monitoring as an essential element of maize cultivation technology on the acidic soils of Polissia. The favourable biological background that has been established is a prerequisite for the further introduction of microbial preparations into such agrocenoses.

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Формування мікробіоценозу кислого дерново-підзолистого ґрунту в ризосфері кукурудзи за інтегрованого застосування добрив та меліорантів

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Анотація. У роботі вивчався вплив сумісного застосування меліорантів та добрив на формування та функціонування мікробіоценозу ризосфери кукурудзи за умов дерново-підзолистого ґрунту. Метою роботи було визначити вплив комплексного застосування добрив і меліорантів на структурно-функціональні характеристики мікробіоценозу дерново-підзолистого ґрунту в ризосфері кукурудзи та обґрунтувати мікробіологічні показники для екологічної біоіндикації стану агроценозу. Польові дослідження було проведено упродовж 2025 року у стаціонарному досліді Інституту сільського господарства Західного Полісся Національної академії аграрних наук України. Дослід включав 8 варіантів, що поєднували залишковий вплив вапнування доломітовим борошном або вапняком із диференційованим внесенням мінеральних добрив та дворазовим позакореневим підживленням мікродобривом. Чисельність еколого-трофічних груп мікроорганізмів визначали методом посіву на селективні агаризовані середовища, мікробну біомасу регідратаційним методом, емісію CO_2 адсорбційним методом. Встановлено, що реакція ґрунтового розчину зросла від 4,09 до

діапазону від 5,48 до 6,20, а гідролітична кислотність знизилася у 2,1 та 2,9 раза. Чисельність амоніфікаторів зросла у 2,9 та 3,8 раза і досягла 27,61 млн КУО/г ґрунту. Чисельність мікроміцетів за поєднання доломітового борошна з добривами знизилася у 2,1 раза до 28,80 тис. КУО/г ґрунту. Найбільшу амплітуду змін виявлено для олігонітрофілів, чисельність яких зменшилася у 7,7 раза. Мікробна біомаса зросла до 128,95 мкг С/г ґрунту за одночасного зниження емісії CO_2 до 14,2 мг/кг за добу, а питомий метаболічний коефіцієнт зменшився від 183,8 до 111,1 мг CO_2 на 1 г вуглецю біомаси. Мікробіоценоз перейшов від грибно-оліготрофного до бактеріально-евтрофного типу з коефіцієнтом оліготрофності від 0,08 до 0,15 та коефіцієнтом мінералізації від 0,14 до 0,24. Провідним регулятором цього переходу виявилася гідролітична кислотність ґрунту, коефіцієнти кореляції з якою становили 0,92 та 0,96. Практична цінність роботи полягає в обґрунтуванні чисельності олігонітрофілів як біоіндикатора азотного насичення агроценозу кукурудзи, а також у виявленні переваги магнієвмісного меліоранту для контролю грибної мікробіоти кислих ґрунтів Полісся

Ключові слова: ґрунтовий мікробіом; таксономічна структура; біоіндикація; мікробна біомаса; оліготрофи

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