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Bobryk Nadiia Yuriivna,
Assistant at the Department of Clinical Laboratory and Morphofunctional Diagnostics, Uzhhorod National University
ORCID ID: 0009-0001-8914-5390
Uzhhorod, Ukraine

Kryvtsova Maryna Valeriivna,
Doctor of Biological Sciences, Professor, Professor at the Department of Clinical Laboratory and Morphofunctional Diagnostics Uzhhorod National University
ORCID ID: 0000-0001-8454-2509
Uzhhorod, Ukraine

SANITARY AND HYGIENIC STATE OF SOIL BY MICROBIOLOGICAL INDICATORS: DATA FROM METAGENOMIC AND CULTURAL ANALYSES

Introduction. Under conditions of increased technogenic load and enterprise relocation, monitoring the sanitary and hygienic state of soils is critical for the region's environmental safety. Current regulatory gaps and the limitations of classical analytical methods necessitate the implementation of modern molecular genetic approaches for an objective assessment of territories.

Objective. The work aims to evaluate the sanitary and hygienic state of technogenically modified soils in Transcarpathia using traditional cultural and modern metagenomic methods.

Materials and Methods. The study focused on soil samples from the zones influenced by a timber chemical plant and railway transport, collected in June 2025. The methodology included classical plating on selective media and metagenomic sequencing of 16S rRNA gene regions, followed by functional prediction using the FAPROTAX database.

Results. It was established that the total microbial count in technogenic zones significantly exceeds the control, although no direct signs of recent fecal contamination were found (coli-index <1000 CFU/kg). Metagenomic analysis recorded a twofold higher content of nitrifiers in technogenic soils, indicating intensive self-purification processes, and identified functional groups of potential pathogens (*Burkholderiales*, *Aquabacterium*, *C. perfringens*) that indicate potential epidemic hazards and long-term contamination.

Conclusions. The combination of classical and metagenomic methods ensures high accuracy in hygienic assessment, revealing hidden changes in the microbial community structure of technogenic areas. The findings emphasize the need to modernize the monitoring system and include genetic indicators in the protocols for sanitary-epidemiological soil control.

Key words: soil, sanitary and hygienic state, technogenic load, coliforms, metagenomic analysis, 16S rRNA, cultural analysis, FAPROTAX.

Бобрик Надія Юріївна, асистент кафедри клініко-лабораторної та морфофункціональної діагностики, ДВНЗ «Ужгородський національний університет»; ORCID ID: 0009-0001-8914-5390**,** м. Ужгород, Україна

Кривцова Марина Валеріївна, доктор біологічний наук, професор, професор кафедри клініко-лабораторної та морфофункціональної діагностики, ДВНЗ «Ужгородський національний університет»; ORCID ID: 0000-0001-8454-2509, м. Ужгород, Україна

САНІТАРНО-ГІГІЄНІЧНИЙ СТАН ҐРУНТУ ЗА МІКРОБІОЛОГІЧНИМИ ПОКАЗНИКАМИ: ДАНІ МЕТАГЕНОМНОГО ТА КУЛЬТУРАЛЬНОГО АНАЛІЗІВ

Вступ. В умовах посиленого техногенного навантаження та релокації підприємств моніторинг санітарно-гігієнічного стану ґрунтів є критично важливим для екологічної безпеки регіону. Наявна нормативна неврегульованість та обмеженість класичних методів аналізу створюють необхідність впровадження сучасних молекулярно-генетичних підходів для об'єктивної оцінки територій.

Мета. Робота спрямована на оцінку санітарно-гігієнічного стану техногенно змінених ґрунтів Закарпаття із застосуванням традиційних культуральних та сучасних метагеномних методів.

Матеріали та методи. Об'єктами дослідження стали зразки ґрунту в зоні впливу лісохімкомбінату та залізничного транспорту, відібрані у червні 2025 року. Методологія включала класичний посів на селективні середовища та метагеномне секвенування ділянок 16S рРНК з подальшим функціональним прогнозуванням за допомогою бази FAPROTAX.

Результати. Встановлено, що загальне мікробне число у техногенних зонах суттєво перевищує контроль, проте прямих ознак свіжого фекального забруднення не виявлено (колі-індекс <1000 КУО/кг). Метагеномний аналіз зафіксував у техногенних ґрунтах удвічі вищий вміст нітрифікаторів, що свідчить про інтенсивні процеси самоочищення, а також виявив функціональні групи потенційних патогенів (*Burkholderiales*, *Aquabacterium, C. perfringens*), які вказують на потенційну епідемічну небезпеку та давнє забруднення.

Висновки. Поєднання класичних і метагеномних методів забезпечує високу точність гігієнічної оцінки, виявляючи приховані зміни в структурі мікробних спільнот техногенних територій. Отримані дані підкреслюють необхідність модернізації системи моніторингу та включення генетичних показників до протоколів санітарно-епідеміологічного контролю ґрунтів.

Ключові слова: ґрунт, санітарно-гігієнічний стан, техногенне навантаження, БКГП, метагеномний аналіз, 16S рРНК, культуральний аналіз, FAPROTAX.

Introduction. The state of martial law, resulting from Russia's full-scale invasion of Ukraine, has significantly affected the Zakarpattia region, despite the absence of direct destruction from military operations. Over the past few years, there has been a notable relocation of enterprises, including industrial facilities, to the region. Soil contamination compromises the quality of food, water, and air, thereby posing a threat to human health and environmental integrity. The majority of pollutants originate from anthropogenic activities, such as industrial processes and mining, inadequate waste management, unsustainable agricultural practices, and accidents ranging from minor chemical spills to disasters at nuclear power plants, as well as the extensive consequences of armed conflicts [1].

Under conditions of increased anthropogenic pressure, it is crucial to monitor the sanitary state of soils, as it directly impacts human health, environmental safety, and the sustainable development of the region. Regulation in the field of land protection aims to ensure the environmental and sanitary-hygienic safety of citizens by defining requirements for land quality, soil fertility, permissible anthropogenic loads, and the economic development of land resources [2].

Although soil-transmitted infectious and parasitic diseases account for only 1–3% of cases, the persistence of pathogenic microorganisms within the soil substrate leads to the prolonged existence of anthropogenic infection foci. This poses a continuous threat to public health in areas characterized by high technogenic pressure. Alongside the insufficient study of this issue, there is a notable regulatory inconsistency in the field of sanitary-bacteriological soil control. This is primarily due to the expiration of several outdated regulatory and technical documents that previously governed the procedures for such research. Currently, the State Sanitary Rules DSP 173-96 remain the only valid document that partially addresses this area [3]. Specifically, Annex №14 classifies only Escherichia coli (coliforms), enterococci, and pathogenic enterobacteria as sanitary-indicator microorganisms. In turn, DSTU 4288:2004 [4], which serves as the fundamental standard for assessing fertility indicators and protecting all soil types from degradation, contains references to revoked or outdated standards. This situation creates methodological difficulties in assessing the sanitary and epidemiological state of anthropogenically modified territories.

Microbiological analysis of environmental samples is traditionally based on classical culture-based methods. However, the vast majority of microbial species (exceeding 90% by current estimates), including various bacteria, remain unculturable. Consequently, culture-dependent methods may underestimate the actual number of viable organisms present in a sample [5]. Molecular methods are utilized for pathogens that are unculturable (or difficult to culture) and are sometimes preferred over cultivation or visual identification due to their high specificity and sensitivity. Modern metagenomic analysis (16S rRNA sequencing) is becoming increasingly relevant in environmental microbiology [6].

To date, the number of publications dedicated to the comprehensive assessment of the sanitary state of soils remains limited. The majority of existing studies focus on examining specific territories, such as the impact zones of livestock complexes or protected natural areas. At the same time, the dynamics of sanitary-microbiological indicators in anthropogenically transformed soils remain insufficiently studied, necessitating in-depth research to develop criteria for their environmental safety.

Objective. To assess the sanitary and hygienic state of technogenic soils using both traditional culture-based and modern genetic methods, and to compare the effectiveness of these two approaches.

Materials and Methods. Soils were studied in the impact zone of the Perechyn Timber and Chemical Plant and railway transport in the town of Perechyn, Uzhhorod Rayon, Zakarpatska Oblast. Soil samples were collected in June 2025 from the depth of 0–10 cm in accordance with standard methodologies [7, 8]. The studied samples included: S1 – soils in the Perechyn Timber and Chemical Plant and railway lines (at a distance of 50 m) impact zone; S2 – soils in the Perechyn Timber and Chemical Plant impact zone (50 m); S3 – control site characterized by the absence of technogenic pollution sources and transport load within a radius of 250 m, ensuring representativeness of background conditions. Each sample consisted of four individual subsamples.

Inoculation was performed according to DSTU 7847:2015 «Soil Quality: Determination of the Number of Microorganisms in Soil by the Method of Inoculation on a Solid (Agarized) Nutrient Medium». The enumeration of bacteria from the *Enterobacteriaceae* family was conducted using CHROMagar Enterobacteria, while enterococci were assessed on Enterococcus Agar. The differentiation of coliform bacteria from accompanying oxidase-positive microflora was carried out using the oxidase test and confirmatory inoculation on lactose-containing media (in accordance with DSTU 4777.1:2007). Metagenomic sequencing of the variable regions (V3–V4) of the 16S rRNA gene from total DNA extracted from enrich-

ment cultures was performed on the Illumina platform. Bioinformatic processing of raw data was carried out using Uparse v7.0.1001 and the Python programming language (v3.6.13). The taxonomic assignment of operational taxonomic units (OTUs) was determined using the Silva 138.2 database. Functional potential prediction of the microbiome was performed using the FAPROTAX database and the *microeco* package within the R environment (v4.x).

Results and Discussion. Understanding the composition and significance of soil microflora for humans, as well as the impact of various environmental conditions, is essential for an accurate sanitary-microbiological assessment of soils (in terms of their epidemic hazard). For an adequate soil assessment, the selection of indicator microorganisms is of particular importance. The most commonly used sanitary-indicator microorganisms of the soil include coliforms (BCGP), *Clostridium perfringens*, thermophilic bacteria, and *Proteus vulgaris*. Additional indicators include the total microbial count (TMC), the percentage of spore-forming microorganisms, aerobic cellulose-decomposing bacteria, actinomycetes, and ammonifiers [9].

We have determined several indicator sanitary-bacteriological parameters using both culture-based and genetic methods. The summarized research results are presented in Table 1.

Microbial contamination is assessed based on the Total Microbial Count (TMC), which reflects the total number of microorganisms per unit mass of the object under study. This criterion is generally based on the assumption that the more an object is contaminated with organic matter, the higher the TMC, and consequently, the more likely the presence of pathogens. In sanitary microbiology, TMC serves as a key indicator of cleanliness [9]. It has been established that the highest TMC (Total Microbial Count) is characteristic of soils within the impact zone of both the industrial enterprise and the railway (35.53±1.92 million CFU/g). In contrast, for soils influenced only by the plant, this indicator decreases by half. The TMC in the control group is the lowest compared to all other sampling points.

CHROMagar Enterobacteria is a chromogenic medium for enterobacteria used for the rapid enumeration and primary differentiation of bacteria within the *Enterobacteriaceae* family. Upon inoculation of soil suspensions from the test samples and incubation for 24 hours at 37°C, the growth of colonies with various morphotypes – ranging in color from milky to orange-pink – was observed (Fig. 1).

To verify the sanitary-indicator microorganisms isolated on the chromogenic medium, additional tests for lactose fermentation and oxidase activity were performed. The results showed that while the cultures exhibited varying lactose fermentation properties, they were oxidase-positive, which allows for their exclusion from the Coliform Group Bacteria (CGB).

The obtained data are consistent with the results of the metagenomic analysis: the proportion of representatives from the order Enterobacterales is very low; the percentage of sequences identified as the genus *Aeromonas* was 0.0055%, and the genus *Vibrio* was 0.00138%.

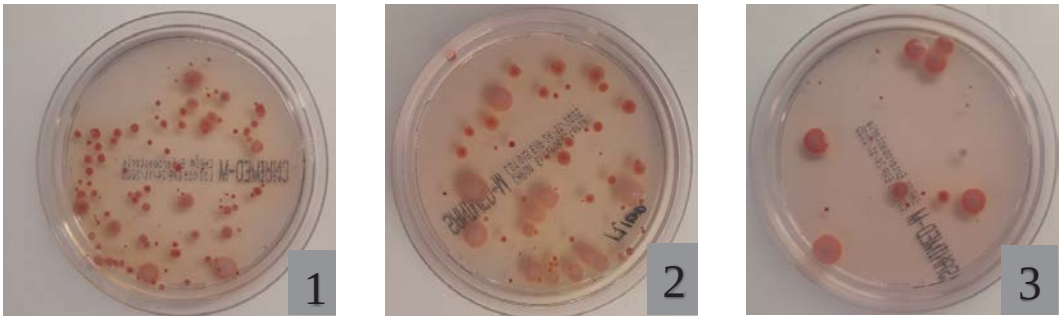

Fig. 1. Bacterial growth on CHROMagar Enterobacteria medium

Table 1

Sanitary-bacteriological indicators of soils in the zone of technogenic impact

№	Indicator	S1	S2	S3	Method
1	Total Microbial Count (TMC), million CFU/g	35,53±1,92	16,00±1,15	12,63±0,91	Cultural
2	Coli-index, CFU/kg	<1000	<1000	<1000	Cultural
3	CGB (Coliforms): *Escherichia* *Klebsiella Enterobacter* *Citrobacter*	n/d n/d n/d n/d	n/d n/d n/d n/d	n/d n/d n/d n/d	Cultural Genetic
4	Total Enterobacteriaceae count, million CFU/g	1,23±0,03	0,90±0,06	0,32±0,05	Cultural
5	Enterococci	n/d	n/d	n/d	Cultural Genetic
6	*Clostridium perfringens*	0,039%	0,0055%	n/d	Genetic (relative abundance among all 16S rRNA sequences)

Notes: n/d – not detected.

The presence of bacteria from the genera *Escherichia*, *Klebsiella*, and *Citrobacter* was not established by either cultural or genetic methods; therefore, the coli-index for all sampling points is < 1000 CFU/kg. Upon inoculation of soil suspensions onto Enterococcus agar (dilutions 10^{-1}–10^{-6}), no microbial growth was observed. The results indicate the absence of fecal contamination in all studied samples.

In assessing the total Enterobacteriaceae count, it was found that in technogenic soils, this indicator exceeds the control by three times, amounting to 1.23 ±0.03 and 0.90 ±0.06 million CFU/g, respectively.

FAPROTAX, which stands for Functional Annotation of Prokaryotic Taxa, is a database based on current scientific literature regarding the functional significance of bacteria. The database contains over 7,600 functional annotations gathered from more than 4,600 prokaryotic microorganisms across more than 80 functional subgroups [10]. It should be noted that FAPROTAX is based on annotations of cultured representatives; therefore, the resulting functional profiles are predictive and reflect the potential capabilities of the microbial community.

In the context of our study, we selected for further analysis those functional groups of bacteria that can serve as indicators of the soil's potential epidemic hazard (Table 2).

Table 2

Functional groups of bacteria (FAPROTAX) characterizing the sanitary state of soils

Група	Відносний вміст, %		
	S1	S2	S3
Nitrification	1,414	1,665	0,633
Human_pathogens_pneumonia	0,003	0,013	0
Human_ gut	0,016	0,002	0
Hammal_ gut	0,016	0,002	0
Human_pathogens_septicemia	0	0	0
Human_pathogens_nosocomia	0	0	0
Human_pathogens_meningitis	0	0	0
Human_pathogens_gastroenteritis	0	0	0
Human_pathogens_diarrhea	0	0	0

The content of nitrifiers in technogenic soils (S1, S2) is twice as high as in the control (S3). This may indicate active nitrogen transformation processes (self-purification), which is frequently observed when soils are contaminated with ammonia compounds or organic matter in industrial zones. The group «human_pathogens_pneumonia» included *Burkholderiales bacterium Beta 02* and *Aquabacterium*, while «human_gut» and «mammal_ gut» included *Clostridium perfringens*. The presence of these spore-forming bacteria may indicate past (historical) fecal contamination or specific environmental loading, as spores persist in the soil significantly longer than *Escherichia coli.*

The use of the FAPROTAX database is also documented in publications by other authors. In a study of bacterial functions within various sludge samples from municipal wastewater treatment plants in China, it was established that the primary ecological groups of bacteria included nitrifiers, nitrite-oxidizing bacteria, human pathogens, and phototrophs [11].

The presence of coliform bacteria, including those belonging to the species *Escherichia coli*, is a universal indicator of the sanitary conditions of various environmental matrices and products [12, 13]. Their presence in soil indicates relatively recent contamination by human and animal feces, sewage, or sewage sludge [14].

In assessing the sanitary state of urban beach sand (based on total microbial count, coliform count, *E. coli*, and *Salmonella*), the microbiological indicators of the sand differed significantly depending on the site's usage patterns. In samples collected in the autumn, following the peak beach season, both coliform bacteria and *Escherichia coli* were detected, with the highest frequency of positive samples recorded in public areas [15].

Only isolated studies have been identified regarding the sanitary-bacteriological state of soils in the Zakarpattia region. In particular, an analysis of statistical data from the State Institution «Laboratory Center of the Ministry of Health of Ukraine in the Zakarpattia Region» was conducted to assess soil contamination in settlements within the region from 2013 to 2017. During this period, 307 soil samples were collected across the region for sanitary-chemical analysis from the sanitary protection zones of industrial enterprises. Bacteriological indicators in 2013 were within hygienic standards; however, samples showing bacterial contamination accounted for 10.7% in 2014, 47.56% in 2015, and 17.39% in 2016.

Slightly different trends were observed for soils in residential areas: in 2013, samples exceeding sanitary-bacteriological standards accounted for 2.20%; in 2016, this figure was 1.4%; and in 2017, it rose to 25%. Thus, there is a clear trend toward an increasing number of samples that exceed established sanitary and bacteriological safety levels [16].

Conclusions. The combination of methods for calculating fecal contamination indicators ensures high precision in hygienic assessment. A comprehensive analysis revealed that despite the absence of recent fecal contamination (coli-index < 1000 CFU/kg), soils within the impact zone of the wood chemical plant (S1, S2) are characterized by a restructuring of the microbial community. This is confirmed by the accumulation of nitrifiers and the emergence of functional groups associated with potential human pathogens (*Burkholderiales*, *Aquabacterium*, *C. perfringens*), as established using the FAPROTAX database.

Perspectives for Further Research. To establish a comprehensive picture of the sanitary state of technogenic soils, further investigation and determination of sanitary-physical and sanitary-chemical indicators are required. Future research will focus on identifying the structure and functional groups of the mycobiota (ITS sequencing). By integrating classical and modern analytical methods, it is advisable to develop recommendations for improving soil sanitary assessment frameworks, which could serve as the foundation for a new monitoring system. Such an approach will enable more accurate forecasting of the epidemic and environmental safety of territories under technogenic load.

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