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Enumeration and antimicrobial activity of bacterial isolates from undisturbed and contaminated soils in North Macedonia

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Abstract

Particularly in temperate regions, seasonal change has an effect on microbial communities directly through climatic function. The microbial populations of an undisturbed and contaminated soil undergo temporal change over the course of four seasons. Eight soil samples were collected from different regions in North Macedonia. The undisturbed soil was collected from mountain Karadzica (protected area Jasen) and the contaminated soil was collected around the factory OHIS, operating in the chemical industry. The samples were collected using sterile bags along with sterile spatula. All the samples were transffered to the Microbiology laboratory, under sterile conditions. *Bacillus* spp. and Actinobacteria were isolated from the samples using a dillution method, based on the use of heat pretreatment for the isolation of *Bacillus* spp. and a selective media for the isolation of Actinobacteria. Confirmation was carried out by Gram staining method of all isolates. Soil samples corresponding to the autumnal season showed the greatest number of antimicrobial strains, 30 in total from both regions. Soil pH and moisture contents and as well as the concentration of organic carbon were determined for all soil samples. We found an inverse correlation between the organic carbon content and the number of isolates from undisturbed soil and a positive correlation between the both parameters in contaminated soil. Antimicrobial assays of the isolates were carried out against six bacteria and three fungi, using the agar well diffusion method. *Bacillus* strains from the undisturbed soil samples showed greater antimicrobial activity against Gram negative test microorganisms from the winter samples, while *Bacillus* strains from the summer contaminated soil samples demonstrated larger activity. In comparison, Actinobacteria strains demonstrated antagonistic activities only from the autumn season from both samples. The findings of this study show that the relationship between of the microbial communities and their ecosystem depend on the presence of anthropogenic contamination.

Keywords: soil microbiology, antimicrobial activity, *Bacillus*, Actinobacteria

Introduction

Pollution of the environment by various inorganic and organic substances, such as pesticides, pharmaceuticals, and metals, is a significant environmental issue primarily caused by human activity. Chemical contaminants can elicit more subtle but nevertheless important and harmful ecological impacts. The increasing production and release of chemicals means that wildlife, humans, and ecosystems are continuously exposed to chemical contaminants (Hellou et al. 2011). Moreover, chemical contamination of the environment extends beyond short-term, acute exposures (Saaristo et al. 2018). Pollutants penetrate soil pores, bind to soil particles, and undergo vertical movement driven by capillary action and gravity,

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leading to changes in the soil's physical, chemical, and biological characteristics. Soil, essential for agriculture and human survival, serves as a pivotal „universal reservoir" supporting human productivity and connecting various economic interactions (Zhang et al. 2023).

At least a quarter of the Earth's total biodiversity resides in the soil microbiome, making it the most diverse community in the biosphere. In the field of microbial ecology, ‚community structure' refers to the taxonomic diversity of a microbial assemblage and the abundance of its individual members (Sokol et al. 2022). In various biomes across the Earth, the diversity of soil microbes is positively correlated with a variety of ecosystem functions, including nutrient cycling, decomposition, and plant productivity (Sokol et al. 2020). Soil microorganisms, through the formation and decomposition of soil organic matter (SOM), influence terrestrial biogeochemistry significantly. SOM is the planet's largest terrestrial stock of organic carbon and nitrogen, providing essential macronutrients and micronutrients crucial for ecosystem health (Crowther et al. 2019). Recent research reveals that soil biogeochemistry is shaped by microbial life and death processes, including population dynamics, trophic relationships, and interactions with the soil environment. Microbial communities exhibit varied growth and death rates in response to factors like root growth and environmental changes, leading to distinct patterns of ecological succession in different soil habitats (Maynard et al. 2017; Vieira et al. 2020) Additionally, biotic interactions such as competition and predation influence microbial physiology and the chemistry of microbial necromass (Buckeridge et al. 2020) This necromass, comprising components like cell walls and proteins, contributes significantly to soil organic matter formation through decomposition processes (Sokol et al. 2022).

Soil microbes possess the ability to gauge the overall measure of soil health in a way that chemical or physical assessments cannot achieve. They respond rapidly to changes in their environment and adapt to stress conditions. Extended soil contamination can alter microbial diversity by promoting the proliferation of microorganisms that are tolerant to contaminants, while simultaneously inhibiting those that are sensitive to them. These alterations can result in changes to ecosystem functions, potentially leading to the reduction of pollutant toxicity over time (Wu et al. 2021). Therefore, soil microbes serve as exceptional indicators of soil health. Hence, it is essential to evaluate how soil microecosystems respond and to compare the differences between undisturbed and contaminated soil from a microbial ecological standpoint (Zhang et al. 2023). The aim of this study was to determine the abundance of culturabe antibiotic-producing Actinobacteria and *Bacillus* sp. in undisturbed and contaminated soils in North Macedonia.

Materials and methods

Sample Collection

Eight soil samples were collected from different regions in North Macedonia. The four samples from undisturbed soil wwere collected from mountain Karadzica (protected area Jasen) and the four samples from the contaminated soil were collected around the factory OHIS, operating in the chemical industry. The samples were collected from November 2021 to March 2022, using sterile bags along with sterile spatula. All the samples were transffered to the Microbiology laboratory and processed after 24 hours.

Soil Geochemical Parameters

After collecting the soil samples, the moisture content, pH of the soil and the organic carbon content were determined for each soil type as described before (Atanasova-Pancevska et al. 2023).

Bacterial Isolation, Enumeration and Characterization

To isolate and count *Bacillus* spp. and Actinobacteria from soil, the serial dilution method was utilized with various aqueous dilutions ranging from 10^{-1} to 10^{-5} using a 0.9% saline solution. To promote the proliferation of spore-forming bacteria, the diluted soil samples were subjected to heating at 70 °C for 30 minutes as part of the isolation procedure, while a selective media was used for the isolation of Actinobacteria. A sample from each dilution was then streaked on a nutrient agar (NA) (yeast extract 2 $g{\cdot}L^{-1}$, peptone 5 $g{\cdot}L^{-1}$, sodium chloride 5 $g{\cdot}L^{-1}$, agar 15 $g{\cdot}L^{-1}$) for *Bacillus* spp. and starch nitrate agar (SNA) (starch 20 $g{\cdot}L^{-1}$, K_2HPO_4 1 $g{\cdot}L^{-1}$, KNO_3 2 $g{\cdot}L^{-1}$, $MgSO_4$ 0.5 $g{\cdot}L^{-1}$, $CaCO_3$ 3 $g{\cdot}L^{-1}$, $FeSO_4$ 0.1 $g{\cdot}L^{-1}$, nystatin 0.05 $g{\cdot}L^{-1}$, agar 15 $g{\cdot}L^{-1}$) for Actinobacteria. Each of the dilutions was inoculated onto a nutrient agar plate (NA) using the pour plate method. The inoculated plates were incubated at 37 °C, and bacterial growth was monitored after 24 and 48 hours. For the microscopic characterization, the Gram stain method was used (Rasool et al. 2017).

Test Microorganisms

The test bacteria (*Salmonella enterica* ATCC 10708, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Listeria monocytogenes* ATCC 13393, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25922) and test fungi (*Candida albicans* ATCC 10239, *Aspergillus niger* ATCC 16404, *Penicillium* sp.) used in this study were obtained from the Culture Collection

of the Microbiology Laboratory at the Department of Microbiology and Microbial Biotechnology, Faculty of Natural Sciences and Mathematics in Skopje, North Macedonia. The test bacteria were incubated at 37 °C and all were activated by incubating for a period of 24 hours in a nutrient broth (NB), while the test fungi were incubated at 25 °C, followed by activation after a period of 72 hours in sabourad dextrose broth (SDB).

Inhibitory Effect by the Agar-well Diffusion Method

The assessment of the isolates inhibitory effects on test bacteria and fungi was conducted using the agar-well diffusion method. All bacteria were cultured on NB medium and incubated at the specified temperature for 24 hours. The test fungi were cultured on SDB medium and incubated for 72 hours (bacterial and fungal suspension turbidity was compared to the 0.5 McFarland standard). Each plate had four wells with an 8 mm diametar created using a sterile steel borer. Each sample of the overnight cell cultures (40 µL) was subsequently added to the wells of agar plates already inoculated with target strains using a sterile swab. For the antibacterial activity the plates were incubated for 24 hours at 37 °C, while for the antifungal activity the plates were incubated for 72-96 hours at 25°C and the diameter of the inhibition zone was measured.

Statistical Analyses

To assess the statistical relationship between the antimicrobial activities of the tested bacteria, a one-way analysis of variance (ANOVA) test was used. Results were considered significant if the p-values were less than 0.05.

Results

Geochemical analyses of the samples showed an expected seasonal variance of all evaluated parameters for both soil types. Moisture content, presented a grams of water per 100 grams of soil, is shown below in Table 1, along with soil pH and organic carbon content, presented as grams of organic carbon per 100 grams of soil.

The results shown above demonstrate clear seasonal variation in the evaluated parameters. Soil samples from the undisturbed soil of Mount Karadzica showed the greatest range of variation of moisture content as a function of seasonal climate change, with a sample variance of ± 1.98%. Interestingly, the industrial contaminated soil samples of OHIS also demonstrated that water content was the single most variable geochemical parameter of those assayed in this study, however they presented a sample variance of only ± 0.68 %, which may be attributed to the soil sample's heavy metal content or to the presence of other contaminant factors which may impact the soil's water retention capacity. Furthermore, the variance in pH of the soil samples also showed a markedly greater seasonal variation among the soil samples from the undisturbed soil of Mount Karadzica, with a variance of ± 0.06 pH unit, while the samples of OHIS showed a seasonal sample variance of ± 0.02 pH units. Similarly, the sample variance for the organic carbon content of the samples from Mount Karadzica demonstrated a sample variance of ± 0.03%, while the samples from OHIS demonstrated a sample variance of ± 0.01%.

ANOVA analysis indicated statistically significant differences between the three evaluated geochemical parameters of moisture content, pH and organic carbon content ($p < 0.05 \times 10^{-23}$). Linear regression analysis showed a minor linear inverse correlation between the organic carbon content of the soils and the pH value ($R = -0.845$), however In-group linear regression analysis for each soil type separate showed a positive correlation

Table 1. Results for moisture content, pH and organic carbon content of the soil samples

Location	Season	Moisture content, %	pH	Organic carbon content, %
Mt. Karadzica	Winter	43.90	7.7	23.30
Mt. Karadzica	Spring	29.15	7.18	23.56
Mt. Karadzica	Summer	9.65	7.13	23.28
Mt. Karadzica	Autumn	25.70	7.39	20.22
OHIS	Winter	15.15	8.15	3.21
OHIS	Spring	7.00	7.87	2.28
OHIS	Summer	4.30	7.76	4.21
OHIS	Autumn	22.50	7.97	4.14

for the pH value and moisture content of the Mount Karadzica soil samples (R = 0.85), which warrants further analysis with a larger sample size so as to generate a conclusion. Both soil samples demonstrated a considerable presence of both *Bacillus* spp. and bacteria belonging to the group of Actinobacteria. Out of the total number of *Bacillus* spp. and Actinobacteria colonies, colonies that demonstrated identical cultural and morphological properties as well as microscopic properties were considered unique strains from that specific sample. Figure 1 and 2 show the fluctuation of the number of these communities among the respective soil samples as a function of seasonal change. Figure 3 shows the fluctuation of the total number of unique strains.

The data signifies that the evaluated bacterial groups have different population dynamics within the same soil samples, fluctuating as a function of seasonal change and soil type. The number of viable colony forming units per gram soil of *Bacillus* spp. reaches a peak during the autumnal season for both soil types, however the same cannot be said for the number of viable colony forming units for the bacteria belonging to the group of Actinobacteria Notably, the undisturbed soil samples showed a peak value of Actinobacteria during spring, while the summer sample held the highest number of viable bacteria among the samples from contaminated soil.

All soil samples demonstrated a greater abundance of Actinobacteria strains, with the exception of the soil sample in the Autumn for the undisturbed soil of Mount Karadzica, where the ratio of viable count of *Bacillus* spp. to viable count of Actinobacteria reaches a peak of 3.36, allowing us to infer that *Bacillus* spp. communities may tend to occupy a more dominant role during this season. Further evaluation and comparison may yield greater insights into this phenomenon. The sole other sample that showed a greater contribution of *Bacillus* spp. count in comparison to Actinobacteria was observed to be the soil sample corresponding to spring for the undisturbed soil of Mount Karadzica, with a ratio of 0.97.

Sample variance analyses showed that while both soils demonstrate differing seasonal sample variance for the viable count of *Bacillus* spp. and Actinobacteria respectively, the sample variance of the ratio of *Bacillus* spp. $CFUsg^{-1}$ over Actinobacteria $CFUsg^{-1}$ remained a stable variance of 2.56 ± 0.18 for both soils. Individual soil sample variance discrepancy was observed once again in the favor of soil samples of OHIS showing a reduced degree of seasonal variance (0.11 variance for the decimal logarithm of viable count of *Bacillus* spp. and

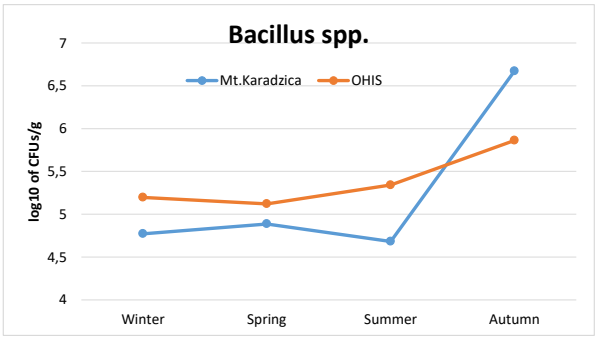

Figure 1. Comparison of the change of the number of viable *Bacillus* spp. ($log_{10}(CFUsg^{-1})$) as a function of seasonal change

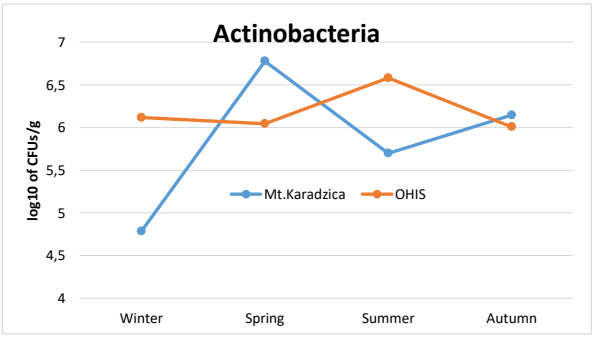

Figure 2. Comparison of the change of the number of viable Actinobacteria ($log_{10}(CFUsg^{-1})$)as a function of seasonal change

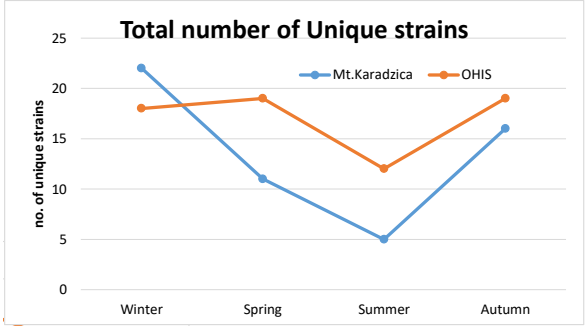

Figure 3. Comparison of the change of the number of total unique strains as a function of seasonal change

0.07 variance for the decimal logarithm of viable count of Actinobacteria). Comparatively, Mount Karadzica soil samples demonstrated a sample variance of the same parameter in the value of 9 to 10 times greater (0.9 for *Bacillus* spp. and 0.7 for Actinobacteria). ANOVA analyses showed a statistically significant difference ($p<0.05$) between the decimal logarithm values for the viable count of *Bacillus* spp. and Actinobacteria, however no linear correlation could be inferred. The number of unique strains was shown to be statistically significant as well ($p<0.05x10^{-4}$), however with no linear correlation to either of the two assayed enumeration parameters.

Conversely, correlation analysis of enumeration and geochemical parameters showed once again a discrepancy between the subgroups, with the viable number of colony forming units per gram soil of *Bacillus* spp. showing a strong inverse correlation with the organic carbon content of the soil in the case of the soil samples from the undisturbed soil of Mount Karadzica ($R = -0.99$). The same parameters showed no correlation when it came to the samples from the contaminated soils of OHIS, instead the viable count of *Bacillus* spp. demonstrated a minor positive correlation with the moisture content of these samples ($R = 0.79$).

Out of the total 122 unique strains (culturally, morphologically and microscopically unique) observed during the enumeration assays, 58 strains demonstrated preliminary antimicrobial activity, visible during the enumeration assay. Gram staining confirmed them to be Gram positive rod shaped bacteria, signifying that they belong to the group of *Bacillus*. Since these isolates stem from the enumeration plates for the heat treated samples, they are viable spore forming bacteria and since these plates were cultivated under aerobic conditions, this eliminates the possibility of mistaking them for another gram positive spore forming bacterium other than *Bacillus*.

Out of the total 58 assayed strains, 50 demonstrated antimicrobial activity against bacterial test cultures (Table 2, Figure 4) *in vitro*. All strains isolated from the soil samples of the contaminated soil of OHIS demonstrated antimicrobial activity

The number of multipotent antibacterial strains, i.e. strains exhibiting antibacterial effect against two or more test cultures, was observed to correlate positively with the total number of antibacterial strains ($R=0.93$) for all samples, which could be considered inappropriate due to small sample size and left for confirmation during a follow up study. However, subgrouping the samples based on soil type revealed an interesting positive correlation between the number of multipotent strains and the total number of viable colony forming units per gram soil of *Bacillus* spp. in the case of soil samples from OHIS ($R=0.95$) and the total number of viable colony forming units per gram soil of Actinobacteria in the case of soil samples from Mount Karadzica ($R=0.94$).

Observing the frequency of multipotent antibacterial strains, the data points towards a local maximum for the autumnal season for the OHIS samples (14 strains, 28% of the total antibacterial strains isolated), and a local maximum for spring for the Mount Karadzica samples (9 strains, 18% of the total antibacterial strains isolated). If we subgroup this frequency of occurance, the data points towards a maximum value for the autumnal season (18 strains across both soils) with a greater number of strains present across all season for the OHIS samples (total of 22 multipotent strains) compared to the Mount Karadzica samples (total of 15 multipotent strains).

Out of the total 58 assayed strains, 31 demonstrated antimicrobial activity against fungal test cultures (Table 3). Antifungal activity was more common among the strains isolated from the soil samples of the undisturbed soil of Mount Karadzica, with only 7 strains failing to demonstrate any antifungal activity. Across all assayed strains, only three strains failed to show any antimicrobial activity, and all three were isolated from the undisturbed soil samples of Mount Karadzica.

Among the strains that demonstrated both antibacterial and antifungal activity (37 strains), the vast majority (75%) demonstrated larger antifungal inhibitory zones, while only 24% of assayed strains showed greater antibacterial inhibitory zones. ANOVA

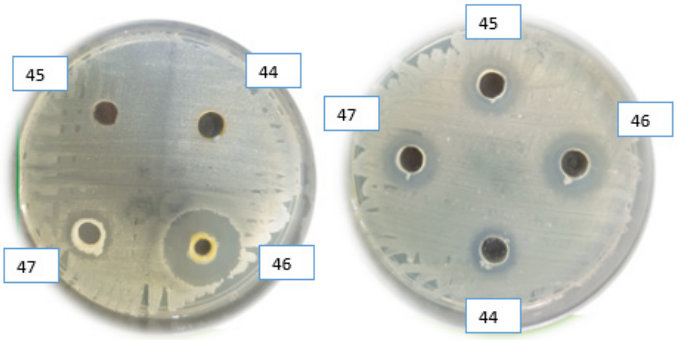

Figure 4. Well diffusion assay with A) *Bacillus subtillis* ATCC 6633 and B) *Staphylococcus aureus* ATCC 25922, with inhibition

Table 2. Zones of inhibition (mm) of the test bacteria caused by bacterial isolates

Isolate no.	Origin	Season	*Salmonella enterica* ATCC 10708	*Escherichia coli* ATCC 8739	*Pseudomonas aeruginosa* ATCC 9027	*Listeria monocytogenes* ATCC 13393	*Bacillus subtillis* ATCC 6633	*Staphylococcus aureus* ATCC 25922
1	Mt. Karadzica	Winter	13.21	17.26	9.09	8.76		18.14
2	Mt. Karadzica	Winter	15.31	14.16	9.88	14.83		14.98
3	Mt. Karadzica	Spring	14.97	15.25				
4	Mt. Karadzica	Spring	15.55	14.48		10.18		14.95
5	Mt. Karadzica	Spring	18.02	14.82		9.00		13.7
6	Mt. Karadzica	Spring	15.67					16.21
7	Mt. Karadzica	Spring	19.84	16.96		8.03		
8	Mt. Karadzica	Spring				12.1		15.92
9	Mt. Karadzica	Spring		13.5		11.8		17.3
10	Mt. Karadzica	Spring						16.51
11	Mt. Karadzica	Spring						
12	Mt. Karadzica	Spring	16.36					14.51
13	Mt. Karadzica	Spring	18.21		10.15			18.75
14	Mt. Karadzica	Autumn						
15	Mt. Karadzica	Autumn						
16	Mt. Karadzica	Autumn	17.22			13.39	10.51	
17	Mt. Karadzica	Autumn	14.14			11.46	8.18	20.09
18	Mt. Karadzica	Autumn				15.24	10.17	19.17
19	Mt. Karadzica	Autumn						
20	Mt. Karadzica	Autumn						
21	Mt. Karadzica	Autumn						
22	Mt. Karadzica	Autumn						
23	Mt. Karadzica	Autumn						
24	Mt. Karadzica	Autumn		11.97		17.07		
25	OHIS	Spring	14.22			17.42	14.35	18.02
26	OHIS	Spring	12.24			17.75		16.96
27	OHIS	Spring	13.05					16.25
28	OHIS	Spring	13.7	15.12		16.75		17.2
29	OHIS	Summer				13.1	15.52	
30	OHIS	Summer	14.83			18.37		
31	OHIS	Summer	12.08					24.09
32	OHIS	Summer					10.8	
33	OHIS	Summer					12.54	
34	OHIS	Summer					11.44	
35	OHIS	Summer		25.23				
36	OHIS	Summer		23.2				
37	OHIS	Summer						12.3
38	OHIS	Summer		12.26				
39	OHIS	Summer		11.7				14.41
40	OHIS	Autumn				14.19	12.46	
41	OHIS	Autumn						20.82
42	OHIS	Autumn						17.13
43	OHIS	Autumn					11.98	16.18
44	OHIS	Autumn			15.19		14.85	

Isolate no.	Origin	Season	*Salmonella enterica* ATCC 10708	*Escherichia coli* ATCC 8739	*Pseudomonas aeruginosa* ATCC 9027	*Listeria monocytogenes* ATCC 13393	*Bacillus subtillis* ATCC 6633	*Staphylococcus aureus* ATCC 25922
45	OHIS	Autumn						12.34
46	OHIS	Autumn				9.83	23.77	10.67
47	OHIS	Autumn					14.62	10.51
48	OHIS	Autumn						17.12
49	OHIS	Autumn	12.23		13.24		16.71	11.2
50	OHIS	Autumn	11.29				18.79	
51	OHIS	Autumn	7.91					14.71
52	OHIS	Autumn	9.55					16.36
53	OHIS	Autumn	7.97				11.25	
54	OHIS	Autumn	7.79	18.88			10.86	15.98
55	OHIS	Autumn					10.93	
56	OHIS	Autumn	15.18				12.83	16.5
57	OHIS	Autumn	10.62					16.25
58	OHIS	Autumn					13.51	20.64

Table3. Zones of inhibition (mm) of the test fungi caused by bacterial isolates

Isolate no.	Origin	Season	*Penicillium* sp.	*Candida albicans* ATCC 10231	*Aspergillus niger* ATCC 16404
1	Mt. Karadzica	Winter	18	34.68	
2	Mt. Karadzica	Winter		42.64	
3	Mt. Karadzica	Spring		30.42	17.2
4	Mt. Karadzica	Spring			
5	Mt. Karadzica	Spring		42.53	
6	Mt. Karadzica	Spring	16.9		
7	Mt. Karadzica	Spring	16.8		
8	Mt. Karadzica	Spring			
9	Mt. Karadzica	Spring	14.52		
10	Mt. Karadzica	Spring	16.26	37.52	
11	Mt. Karadzica	Spring	15.51		
12	Mt. Karadzica	Spring		35.03	
13	Mt. Karadzica	Spring		39.93	
14	Mt. Karadzica	Autumn		31.28	
15	Mt. Karadzica	Autumn	16.4	19.64	16.38
16	Mt. Karadzica	Autumn		31.21	18.05
17	Mt. Karadzica	Autumn		40.79	12.67
18	Mt. Karadzica	Autumn			
19	Mt. Karadzica	Autumn			
20	Mt. Karadzica	Autumn	20.81		14.57
21	Mt. Karadzica	Autumn			

Isolate no.	Origin	Season	*Penicillium* sp.	*Candida albicans* ATCC 10231	*Aspergillus niger* ATCC 16404
22	Mt. Karadzica	Autumn	18.73		18.77
23	Mt. Karadzica	Autumn			
24	Mt. Karadzica	Autumn			
25	OHIS	Spring			
26	OHIS	Spring			
27	OHIS	Spring			
28	OHIS	Spring	15.31		
29	OHIS	Summer		31.41	
30	OHIS	Summer		26.8	
31	OHIS	Summer	15.09	31.71	16.63
32	OHIS	Summer			
33	OHIS	Summer	17.8	32.34	17.08
34	OHIS	Summer	19.61	33.22	17.67
35	OHIS	Summer			
36	OHIS	Summer			
37	OHIS	Summer			
38	OHIS	Summer			
39	OHIS	Summer	15.85	32.37	17.55
40	OHIS	Autumn	15.83		
41	OHIS	Autumn			
42	OHIS	Autumn			
43	OHIS	Autumn			
44	OHIS	Autumn			
45	OHIS	Autumn	17.21	43.95	
46	OHIS	Autumn			
47	OHIS	Autumn			
48	OHIS	Autumn			
49	OHIS	Autumn	17.72		
50	OHIS	Autumn			
51	OHIS	Autumn			
52	OHIS	Autumn	22.48		
53	OHIS	Autumn			
54	OHIS	Autumn	23.49		
55	OHIS	Autumn			
56	OHIS	Autumn			
57	OHIS	Autumn	18.33		
58	OHIS	Autumn	20.82		

analysis of antimicrobial zones indicated a statistically significant difference between the observed inhibitory zones of different test cultures as well as statistically significant differences between evaluated isolates ($p<0.05x10^{-40}$).

Correlation analysis also demonstrated interesting correlations between antimicrobial and antifungal activity which can further be explored in follow up studies. Notably, the antimicrobial activity against *Penicillium* sp. was found to be negatively correlated with the antimicrobial activity against *Pseudomonas aeruginosa* ATCC 9027 (R = -0.98) and showed a minor positive correlation with the activity against *Escherichia coli* ATCC 8739 (R = 0.77). Conversely, the antimicrobial activity against *Candida albicans* ATCC 10231 showed a minor positive correlation with the antimicrobial activity against *Pseudomonas aeruginosa* ATCC 9027 (R = 0.83). Further subverting expectations, the antimicrobial activity against *Escherichia coli* ATCC 8739 was shown to be negatively correlated with the antimicrobial activity against *Pseudomonas aeruginosa* ATCC 9027 (R = -0.98). Similar correlation was discovered between the activity against *Pseudomonas aeruginosa* ATCC 9027 and *Bacilus subtillis* ATCC 6633 (R = -0.97) and *Staphylococcus aureus* ATCC 25922 (R = -0.866).

Maximum inhibitory zones across all soils showed a positive correlation between the antifungal and antibacterial zones with a correlation factor of 0.87. Subgrouping the soils based on type indicated a minor positive correlation between the antifungal and antibacterial zones with moisture content for the soils from Mount Karadzica (R=0.77 and R=0.84 respectively); while the samples from OHIS showed a negative correlation between the pH of the sample and the maximum antibacterial zone (R=-0.85). The maximum antifungal zone for the isolates from the samples collected from OHIS showed a positive correlation with the number of multipotent antibacterial strains (R=0.86), however further data is required before drawing a conclusion.

Discussion

Shifting population dynamics of *Bacillus* spp. and Actinobacteria are intimately connected with fluctuating environmental parameters. The statistically significant differences between the geochemical parameters and the microbiological parameters evaluated in this study along with the few linear correlations confirm that this interconnectedness is of a multi-factorial nature that requires further elucidation and analysis. One factor which could be located at the crux of the microbial-environment relationship is the phytocoenological factor. Other studies have pointed towards key differences of soil microbial profiles and enzymatic activity (Luo et al. 2020) that can be attributed to soil parameters as well as microbe-vegetation relationships (Solanki et al. 2024). Since the soil samples from Mount Karadzica stem from a mixed forest, this can account in part for the observed differences in population dynamics as well as geochemical fluctuation. The discrepancy in seasonal variation between the OHIS and Mount Karadzica sample extends to all three evaluated geochemical parameters, as well as to the viable count of *Bacillus* spp. and Actinobacteria. The data points singularly to a greater variability present in the Mount Karadzica sample, indicating a potential inhibitory role of soil contaminants to soil variability (Dobler et al. 2001). Weather the discrepancy in variance depends more on the degree and type of contaminants or on the degree and type of vegetation remains an interesting subject for further analysis by expanding the scope of the study. One key point observed between the two soil types subject to this study is the stable variance of bacterial populations for both soil types. This points conclusively that the microbial populations of both soil types are stable and well adapted to the standard seasonal fluctuations of their environment, as opposed to reacting to a newly introduced factor. The overall lowered variability of evaluated parameters at the level of the contaminated soil samples from OHIS may also be attributed to the urban industrial landscape as well as the heavy metal content and presence of other contaminants (Dobler et al. 2001), which makes for an optimal opportunity to further explore these factors in a follow up study.

Antimicrobial assays point uniformly to a greater potency of antifungal tactics employed by these communities in comparison to antibacterial adaptations, hinting either towards the impact fungi have on these evaluated ecosystems or perhaps towards the maladaptive response of the test fungal strains used in this study (Sarika et al. 2021). In the case of the former, a reasonable approach for a follow up study would be to evaluate the fungal biodiversity and abundance indexes in these ecosystems, in order to elucidate whether the number and type of present fungi impact the antifungal activity of the bacterial communities, or if their antifungal adaptations represent an evolutionary spandrel (Ban 2006). In the case of the latter hypothesis, evaluating the antifungal potency of these isolates against fungi present in their original environment would also shed light if perhaps the fungal test cultures used are simply too sensitive to the particular antimicrobial agent employed by the bacterial strains assayed. Both hypotheses may serve as future fodder for evaluating the potential role and potential correlation of fungal communities within the greater scope the soil microbiome. The antimicrobial potency as well as the frequency of antimicrobial adaptations among the isolates from the OHIS samples is an interesting feature that ties in on similar findings that link greater competitiveness amongst microbial populations with perturbed, contaminated and resource scarce environments as opposed to their resource rich

unperturbed counterparts (Sarika et al. 2021). The antimicrobial assay data indicates an expected inverse correlation between the antimicrobial activity of isolates against *Pseudomonas aeuriginosa* and those against *Bacillus* and *Staphylococcus* test cultures. The general assumption points towards selective adaptability of these isolates to favor either Gram positive or Gram negative antagonism, and not both simultaneously, as seen by other similar studies (Amaning Danquah et al. 2022). This adaptive trend may stem from the presence of a singular wide spectrum compound which either impacts Gram positive or Gram negative metabolism and reproduction (Lewis 2020). Conversely, there exists a possibility that these observations are made possible through the utilization of several specific compounds, which may not exhibit the same degree of cross-reactivity between phylogenic cousins (Brochado et al. 2018). Further separation, purification and evaluation of the antimicrobial compounds employed by the assayed strains should yield better insight into the cause of Gram specificity shown by the results. The same specialization of antimicrobial adaptations does not seem to be present when comparing the antimicrobial potency against fungi and against bacteria, which showed a positive correlation between the two parameters. However, the inverse correlation between the antimicrobial activity against *Pseudomonas* and *Escherichia* proves a counterargument to the primary hypothesis. It may stand to reason that among the Gram negative antagonistic portfolio of adaptations there is greater specificity to be expected (Masschelein et al. 2017), which may stem from the composition of the wider microbial community.

Finally, the correlation discovered between the number of antimicrobial strains and multipotent strains with the viable number of *Bacillus* spp. in the case of OHIS and the viable number of Actinobacteria in the case of Mount Karadzica may represent the key finding of this study, indicating that different microbial communities may take up the dominant antagonistic role in different environments. This dominance reflects key features of these communities, with *Bacillus* spp. strains showing greater fitness in resource scarce and contaminant heavy soils by routing their secondary metabolism towards producing readily available antimicrobials. Contrary to this, the relatively resource abundant soils of Mount Karadzica instead favor the dominance of bacteria belonging to the group of Actinobacteria. This environment furthermore afford a higher glass ceiling for population variance and therefore, a greater potential for adaptability, as shown in similar studies (Pettersson & Baath 2004). Overall this study presents several novel avenues of further research into the subject material, with promising results and potential for expansion of scope to further elucidate the many nuances of microbial dynamics in function of environmental change. In the case of all three geochemical parameters, the sample variance of the OHIS soil samples was shown to be 30% to 40% of the variance present in the Mount Karadzica samples. This discrepancy presents an interesting phenomenon which can be further elucidated by deeper evaluation of the geochemical composition of both soils and observing any potential correlation with heavy metal content and presence of other contaminants. Additionally, these environmental factors can be experimentally reproduced in order to establish causal connectivity between the ability to produce antimicrobial compounds, the multipotency of such compounds as well as their efficacy, and how each relates to the heavy metal content of the environment. The observed discrepancy between Gram positive and Gram negative antimicrobial adaptations can also present an interesting question for a follow up study, focusing on separating the individual antimicrobial components and determining whether the reason for such a discrepancy stems from wide spectrum antimicrobial adaptations or several specific adaptations.

Conclusion

This study reveals the intricate relationship between environmental factors, geochemical parameters, microbial dynamics, and antimicrobial activities in contrasting soil types. Significant seasonal variations were observed in moisture content, pH, and organic carbon across Mount Karadzica‘s undisturbed soil and OHIS‘s contaminated soil. The microbial populations, dominated by *Bacillus* spp. and Actinobacteria exhibited distinct seasonal dynamics and adaptations influenced by environmental conditions. Antimicrobial assays revealed a notable potency against fungal test cultures, suggesting ecological roles or selective pressures within the soil microbiome. Correlations between microbial populations and antimicrobial activities underscored the specificity and adaptability of microbial communities to environmental changes and contaminant exposures. The study‘s findings highlight the intricate relationship between microbial populations and environmental conditions, while also indicating reduced variability in contaminated soils, possibly due to urban industrial influences and heavy metal contamination. These insights provide a foundation for further research into microbial ecology, emphasizing the need for comprehensive studies to understand the broader implications of microbial dynamics on soil health and ecosystem functioning. Overall, this research provides valuable insights into microbial community dynamics under varying environmental conditions, paving the way for future investigations into the ecological roles and potential applications of soil microbiota in environmental management and sustainability efforts.

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