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Influence of selected soil parameters on cellulase and catalase activity in soils from the Botevgrad Valley, Western Bulgaria

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Abstract

Soils in the Botevgrad Valley, Western Bulgaria, have been exposed to long-term anthropogenic pressure arising from industrial emissions of the Kremikovtsi metallurgical complex, intensive agriculture, and expanding urbanisation. These drivers have contributed to the accumulation of heavy metals and the alteration of soil fertility, raising concerns about the long-term ecological stability of the valley ecosystem. Despite the significance of the region, information on the microbiological and enzymatic characteristics of these soils as sensitive bioindicators remains limited. The present study aimed to examine how key physicochemical factors influence these enzymes and to evaluate their potential for assessing soil quality and resilience under anthropogenic impact. Representative soil samples were taken following a spatial grid covering agricultural, forest, and semi-urban sites, with agricultural soil dominating. Basic soil parameters (pH, humus, moisture, nutrients) and trace elements (Pb, Mn, Cu, Fe, Zn, Cd) were determined, while microbial composition and the activities of cellulase and catalase were analysed by conventional microbiological and enzymatic methods. Correlation and multiple regression analyses were applied to identify the main dependencies and independent predictors. Lead concentrations exceeded the permissible levels at two sites, while cadmium and copper were elevated at one. Cellulase activity was generally high, indicating active microbial decomposition, and correlated positively with soil moisture, total nitrogen, potassium, and microbial abundance, whereas Pb and Mn exerted pronounced inhibitory effects. Catalase activity reached maximum values in the Eutric Fluvisols and minimum values in the Pb- and Cu-enriched Skeletic Phaeozems. It correlated positively with iron, humus, and available phosphorus, showing weaker positive relationships with microbial indicators. Regression analysis confirmed Pb as the strongest negative predictor of both enzymes, while humus, phosphorus, and pH showed significant positive effects on catalase. Iron played an indirect stimulatory role by associating with organic matter and nutrients. The integration of correlation and regression analyses identified Pb and Mn as the main inhibitors of enzymatic activity, while soil nutrients, pH, and moisture stimulated microbial processes. The persistence of relatively high catalase and cellulase activities, even under contamination, indicated the presence of resilient microbial communities and a potential for natural self-purification. Overall, the findings confirm that cellulase and catalase are reliable bioindicators for evaluating soil quality, fertility, and resilience in valley ecosystems under anthropogenic stress, providing a scientific basis for sustainable land-use management and environmental restoration strategies.

1. Introduction

The Botevgrad Valley is a valley depression in the Western Fore-Balkan region and represents part of the youngest Late Pliocene–Quaternary basin system (Dotseva and Vangelov, 2018. It is situated between the Bilo and Murgash Mountains to the south, Golema Planina to the southwest, and Rzhana Planina to the northwest, all of which belong to the Western Stara Planina (Hristov, 2020). The ecological situation in the region is mainly influenced by the activities of the Kremikovtsi metallurgical plant until 2010, with consequences for the accumulation of heavy metals in the soils, especially considering the fact that part of the territory of the Botevgrad Valley is agricultural land and pastures (Hristov, 2020; Ilieva, 2025). This fact determines

the importance of monitoring chemical indicators (mainly heavy metals) and microbiological indicators in the soils of the region as an opportunity for self-purification of the soils from heavy metals.

The activity of soil microorganisms and the enzymes they produce was widely recognized as a sensitive bioindicator of soil quality and ecosystem functioning, and could also provide valuable evidence of the soil's historical development. Enzymes are sensitive biochemical indicators of soil condition, and further analyses are required to better understand the factors controlling enzymatic activity. One reason researchers reported contradictory information was the many factors that affect enzyme activity. Thus, many factors affect enzyme activities – from soil structure to climatic factors, from the amount of organic matter to soil cultivation technologies, as well as the amount, composition and activity of soil microbiota. Soil microorganisms, such as bacteria (non-spore-forming, bacilli, and filamentous actinomycetes) and micromycetes, are key producers of extracellular enzymes involved in organic matter turnover, with basidiomycetous fungi being among the most potent cellulose degraders (Baldrian and Valášková, 2008). In agrogenic soils, many authors had found stronger development of bacteria (especially non-spore-forming) and a weaker development of mycelial microorganisms (actinomycetes and molds), mainly aerobic and mesophilic microorganisms (Naskova et al., 2016; Plamenov et al., 2016; Yankova et al., 2016; Malcheva et al., 2018; 2019; Malcheva, 2021; Frene et al., 2022; Guo et al., 2022; Mladenova et al., 2023; Mladenova et al., 2024; Koleva et al., 2024). The study of cellulase and catalase activities in soils is an important tool for assessing soil fertility and the impact of heavy metal contamination. Cellulase plays a key role in the decomposition of organic matter, providing carbon sources for the microbial community and enhancing soil nutrient availability. Catalase reflects the metabolic health of microbes and their resistance to oxidative stress, including that induced by heavy metals. The activity levels of these enzymes serve as sensitive bioindicators of soil biological health, allowing simultaneous evaluation of fertility and the toxic effects of contamination. The enzymes cellulase and catalase were major biochemical markers for processes occurring in soils (Lemanowicz, 2019; Malcheva, 2020; Wang et al., 2023). Assessing soil enzymatic functional diversity contributed to the analysis of relationships among resource content, the composition and function of soil microflora, and ecosystem processes (Caldwell, 2005; Wang et al., 2023).

Cellulose degradation in soil ecosystems is a fundamental process that plays a significant role in nutrient cycling and in the decomposition of organic matter (Datta, 2024). In addition to its plant origin, cellulose is also produced by some bacteria – *Acetobacter* sp., *Sarcina ventriculi*, and *Agrobacterium* sp. (Mishra et al., 2022). The latter have different physicochemical properties, although they share the same structure (Datta, 2024). *Rhodococcus wratislaviensis* YZ02 and *Pseudomonas xanthosomatis* YZ03 were found to contain a series of genes from the glycoside hydrolase group and other genes related to cellulose degradation (Ma et al., 2024). According to other authors, fungi secreted cellulases to a higher extent than bacteria (Mondal et al., 2021) – *Penicillium* sp. (Du et al., 2018), *Trichoderma* sp. (Dashtban et al., 2009) and *Aspergillus* sp. (Zhang et al., 2014). Cellulases are usually secreted by microorganisms living in humus-rich soils (Lakshmi and Narasimha, 2012).

Catalase is a respiratory enzyme that breaks down hydrogen peroxide, released during the decomposition of organic matter in the soil into water and oxygen, and has an important role in the transformation of substances in the soil. Catalase activity was determined by both the organic part of soils and their mineral part (Grozeva and Nustorova, 1995; Hristov, 2009). Catalase is a key enzyme reflecting microbial activity and soil aeration, a biological marker for early diagnosis and assessment of soil health (Brzezińska et al., 2005; Türkay et al., 2024).

Catalase and cellulase activities depend on a complex of factors: quantity, composition and activity of soil microflora, accumulation of microbial biomass, temperature, soil moisture, pH, soil type and mechanical composition of the soil, sampling depth, content of organic matter and nutrients in the soil, type of vegetation, research methods, applied agrotechnical measures, presence of inhibitors, soil contamination and other factors (Brzezińska et al., 2005; Miller et al., 2006; Sardar et al., 2007; Uzun and Uyanöz, 2011; Malcheva, 2012; Sethi and Gupta, 2015; Naskova et al., 2015, 2016; Malcheva et al., 2018; Haddad et al., 2019; Malcheva et al., 2019; Malcheva, 2020; Malcheva, 2021; Malcheva et al., 2023; Yeboah et al., 2021). Catalase activity was not limited in soils with a higher content of heavy metals – Pb, Cd, Cu, Zn (Malcheva, 2012, 2020), suppressed by zinc but not by lead (Sethi and Gupta, 2015) and could serve as an indicator for rapid determination of soil contamination and the course of initial stages of soil self-cleaning with the help of soil microorganisms. While cellulase is reduced in soils with higher anthropogenic heavy metal loading, especially cadmium, which also indicates the biological state of soils in these conditions (Malcheva, 2012; Sethi and Gupta, 2015; Haddad et al., 2019; Malcheva, 2020).

The selection of catalase and cellulase as enzymatic indicators in the present study was based on their ecological and biochemical relevance to soil functioning under both heavy-metal stress and agricultural impact. Both enzymes are highly sensitive to environmental disturbances, including metal contamination, fertilizer input, and the long-term application of pesticides and soil amendments. Since most of the investigated soils in the Botevgrad Valley are agricultural or have been cultivated in the past, their enzymatic and microbiological properties are expected to reflect not only the influence of industrial emissions (e.g., from Kremikovtsi) but also the cumulative effects of land management and agrochemical use. Consequently, catalase and cellulase provide complementary and integrative measures of soil health and biological functioning under complex anthropogenic pressures.

The aim of the study was to investigate how selected chemical and physicochemical indicators influence biological and biochemical properties that characterize and reflect ongoing changes in soils from the Botevgrad Basin, which have been exposed to anthropogenic loading with heavy metals. The study also considers the potential of these parameters for future monitoring of soil self-purification processes and for improving soil

fertility. Since no other published studies on microbiological and enzymatic indicators in the area were found, as confirmed in a dissertation on the region (Ilieva, 2025), this research can be regarded as an initial step toward long-term monitoring of soils in the Botevgrad Valley.

To achieve this goal, a set of research hypotheses and questions was formulated. It was expected that soils with higher heavy-metal concentrations would exhibit reduced microbial abundance, changes in the structure of the soil microbiome, lower enzymatic activity, and consequently decreased fertility. At the same time, it was hypothesized that soil microorganisms may adapt to stress conditions by increasing the abundance and activity of spore-forming species and by enhancing enzymatic production, thus promoting soil self-cleaning and partial restoration of fertility. The research further sought to clarify the relationship between heavy-metal contamination and soil microbiological and enzymatic activity, and to assess whether dynamic biological and biochemical markers could serve as reliable tools for monitoring soil recovery and fertility improvement in polluted areas. Future long-term monitoring of these indicators will allow for a more comprehensive evaluation and generalization of the results.

2. Materials and methods

2.1. Study area and sampling

Twenty soil samples were collected once in spring 2023 from the 0–20 cm layer of manually excavated soil pits (SP) in the Botevgrad Valley region, Western Bulgaria. Soil samples were collected as part of a preselected grid to ensure representativeness. Approximately 1 kg of soil was taken from each site. Soil samples intended for microbiological and enzymatic analyses were collected aseptically using sterile instrument and placed into sterile paper bags. The samples were stored under refrigerated conditions at 4°C and analyzed within 48 hours of collection. Before analysis, they were cleaned of stones, roots, and other debris.

The Botevgrad Valley is located in the northern part of the Stara Planina region, bordering the Rzhana and Murgash mountains, the western part of the Etropol Balkan and the Botevgrad Predbalkan (Fig. 1).

The Botevgrad Foreland has a hilly relief. According to the Landscape Zoning of the country, the territory studied in this study falls into the Balkan zone – a zone of young folded mountains, which include the Foreland and the northern slope of the western Stara Planina. In terms of geology and petrography of the Botevgrad Basin, in the middle of this land there is a huge eruptive massif, composed of diorites and various porphyrites. The Botevgrad basin is a part of the youngest Late Pliocene–Quaternary basin system (Dotseva and Vangelov, 2018). The climate is temperate continental, with temperature inversions often observed during the winter season. The total amount of precipitation is 830 mm. Considered by seasons, summer precipitation (299 mm) is more than spring (220 mm). Winter precipitation is – 132 mm, autumn – 175 mm. The largest amounts of precipitation fall in June – 117 mm, followed by May – 104 mm. The average annual temperature is 10.7°C, with the coldest month (January) being –1.9°C. In summer, the highest temperatures are in the months of July (20.8°C) and August (20.7°C). Northwesterly winds prevail, which are associated with the transfer of moist air masses from the west and northwest. About 50% of the territory of Botevgrad municipality is occupied by forest areas, and about 11% – by urbanized territories. During the period 1956–2010, the Kremikovtsi metallurgical plant operated, located about 50 km from the municipality's territory, which has affected the accumulation of heavy metals in the region's soils. The agricultural sector has a plant-livestock structure. Agricultural lands occupy 45.3% of the territory of the municipality, with 68.4% of them being arable (27.5% of them are irrigated areas), other 22% are used for mainly for forestry, settlements, industrial zones and other infrastructure occupy 9% of the valley (Hristov, 2020). The agro-ecological and soil conditions in the settlements of the region are favorable for the cultivation of fruit crops such as plum (*Prunus domestica* L.), raspberry (*Rubus idaeus* L.), apple (*Malus domestica* Borkh.), and sour cherry (*Prunus cerasus* L.); cereal crops such as wheat (*Triticum aestivum* L.), maize (*Zea mays* L.),

Fig. 1. Botevgrad Field – satellite photo and general view

Table 1
Research objects

Soil samples	Altitude, m	IUSS Working Group WRB. 2022. World Reference Base for Soil Resources. International soil classification system for naming soils and creating legends for soil maps. 4th edition. International Union of Soil Sciences (IUSS), Vienna, Austria	GPS coordinats UTM (Universal Transverse Mercator)
SP1	352	Haplic Luvisols (Gray forest soil)	N 42.909413 E 23.778998
SP2	352	Haplic Luvisols (Gray forest soil)	N 42.912086 E 23.769619
SP3	340	Eutric Fluvisols	N 42.914794 E 23.758240
SP4	350	Haplic Luvisols (Gray forest soil)	N 42.917856 E 23.744070
SP5	348	Haplic Luvisols (Gray forest soil)	N 42.930438 E 23.738205
SP8	352	Gleyic Fluvisols	N 42.950893 E 23.693881
SP9	350	Haplic Luvisols (Gray forest soil)	N 42.953993 E 23.702613
SP15	374	Haplic Luvisols (Gray forest soil)	N 42.938728 E 23.775270
SP16	360	Haplic Luvisols (Gray forest soil)	N 42.977165 E 27.746553
SP20	370	Haplic Luvisols (Gray forest soil)	N 42.983780 E 23.735981
SP25	450	Skeletic Regosols	N 42.897467 E 23.931269
SP26	445	Skeletic Regosols	N 42.907298 E 23.915824
SP28	467	Skeletic Phaeozems	N 42.917675 E 23.887925
SP29	417	Skeletic Phaeozems	N 42.918744 E 23.806011
SP31	386	Skeletic Phaeozems	N 42.928469 E 23.841824
SP33	371	Haplic Luvisols (Gray forest soil)	N 42.926624 E 23.783744
SP34	367	Haplic Luvisols (Gray forest soil)	N 42.931023 E 23.776917
SP37	612	Eutric Cambisols	N 42.585101 E 23.354240
SP38	644	Haplic Luvisols (Gray forest soil)	N 42.893574 E23.717637
SP39	644	Haplic Luvisols (Gray forest soil)	N 42.955774 E 23.709669

and barley (*Hordeum vulgare* L.); industrial crops such as sunflower (*Helianthus annuus* L.) and hop (*Humulus lupulus* L.); vegetable crops such as tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.) under irrigated conditions; as well as potato (*Solanum tuberosum* L.) (Hristov and Mitreva, 2021). The samples studied are from agricultural, arable lands (without agricultural crops at the time of sampling), with the exception of SP16 (meadow grass *Poa pratensis* L., fescue *Festuca*, yarrow *Achillea millefolium* L., eryngo *Eryngium campestre* L.), SP37 (common hornbeam *Carpinus betulus* L.) and SP38 (common hornbeam *Carpinus betulus* L.), which are from forest areas.

The studied sites, with indicated soil type, are presented in Table 1.

2.2. Soil physicochemical analyses

The following methods for soil analysis were used: Humus by the Turin method (Penkov et al., 1991), which is based on the complete oxidation of soil organic matter with potassium dichromate and sulfuric acid, followed by titration of the excess dichromate; Total Nitrogen content, by a modified version of the classic Kjeldahl method (Penkov et al., 1991) – In this approach, soil samples were digested with concentrated sulfuric acid in the presence of a catalyst mixture, and the released ammonium was determined after distillation and titration; Available forms of phosphorus (spectrophotometrically, UV/VIS Lambda 5) and potassium (flame photometry, PFP7 Flame photometer JENWAY) – P_2O_5 (mg·kg□¹) and K_2O (mg·kg□¹) (Ivanov, 1984) – A complex extraction solution of ammonium acetate, acetic acid, calcium lactate and hydrochloric acid is used (Bogdanov, 2023); Available forms of nitrogen – NH_4 and NO_3 content (Bremner and Keeney, 1966) – Soil ammonium was determined after extraction with 2 M KCl and subsequent steam distillation with MgO, while nitrate was determined from the same extract after reduction with Devarda's alloy and steam distillation; Total P and Total K content by decomposition with aqua regia (ISO 11466:1995) and ICP-OES determination (PlasmaQuant 9100 (Elite) Analytik Jena); Mn, Fe, Zn, Pb, Cu and Cd soil content were mineralized with aqua regia (HCl and HNO_3 in a ratio of 3:1). After that the concentrations of Mn, Fe, Zn, Pb, Cu and Cd were determined by atomic absorption spectrometer using Atomic absorption-spectrophotometry (Perkin-Elmer Model Analyst 5000).

The active soil reaction (pH) was determined potentiometrically in an aqueous extract.

The soil moisture was determined on a moisture balance, type DBS 60-3.

The soils were classified by texture according to the FAO (2006) Guidelines for Soil Description (https://www.fao.org/4/a0541e/a0541e.pdf).

Measurement uncertainty for the determined chemical parameters was estimated based on method verification protocols and is reported as expanded uncertainty (U, k = 2) in the corresponding tables.

2.3. Soil microbiological and enzymatic analyses

Microbiological soil analyses were carried out by the method of limiting dilutions and subsequent inoculations on solid nutrient media: meal peptone agar (non-spore-forming bacteria and bacilli), Chapek-Dox agar (mold fungi), Actinomycete isolation agar (actinomycetes and bacteria that absorb mineral nitrogen) (Mishustin and Emtsev, 1989; Gushterov et al., 1977). For each soil sample, inoculations on every medium were performed in three replicates. The samples were cultivated in a thermostat (at 27°C – 48 hours for bacilli and non-spore-forming bacteria, and 7 days for actinomycetes and molds) and the colony-forming units (CFU) were subsequently counted and converted to 1 g of absolutely dry soil (CFU·g^{-1} dry soil). A dilution of soil with sterile water 1:1000 was used and the amount of inoculation from it was 0.05 mL. The total culturable microbial community (TCMC) (CFU·g^{-1} dry soil) was calculated.

Cellulase activity was assessed using two methods: an incubation-based method and a spectrophotometric assay. A laboratory incubation method was used to determine the cellulase activity in dynamics (Khaziev, 1976), in which soil was poured into a Petri dish with d=90 mm, a layer of 7 mm was periodically moistened and 3 strips of sterile cellulose filter paper with dimensions 10 x 50 mm were placed. The percentage of degraded cellulose area was recorded with a grid standard every 15 days. Cellulase activity was determined spectrophotometrically (UV-21 ONDA) according to the method of Gradova et al. (2004) by incubating microcrystalline cellulose ($C_6H_{10}O_5$)n with culture liquid at 50°C for 20 min. The reaction was stopped with potassium ferrocyanide ($C_6FeK_4N_6$), and the absorbance was measured at 420 nm against a glucose calibration curve. Cellulase activity was calculated as the amount of glucose released per unit volume of culture liquid during the hydrolysis time. Catalase activity was determined by titration manganometric method using 2 g of soil, as hydrogen peroxide was added to the soil sample for the experiment, and sulfuric acid for the control. Titration was performed with a DLAB dTrite digital burette. After 15 minutes of shaking in a shuttle apparatus, the reagents were exchanged, followed by another 15 minutes of shaking, filtration and titration with potassium permanganate until pink coloration. The result was calculated by the formula: A = Vc – Ve, where A – catalase activity (mL O_2·30 min^{-1}), Vc – mL of potassium permanganate used for titration of the control, and Ve – mL of potassium permanganate used for titration of the experiment (Khaziev, 1976).

The expanded uncertainty (U) of the measurements was calculated based on method verification protocols by multiplying the combined standard uncertainty by a coverage factor of k = 2, corresponding to a confidence level of approximately 95%. For enzymatic activities, scatter plots with vertical error bars representing U (k = 2) were applied to visualize the measured values together with their associated uncertainty.

2.4. Statistical analysis

Data preprocessing and descriptive statistics were performed in Microsoft Excel. Stepwise multiple regression analysis was conducted using IBM SPSS Statistics, version 25.0 (IBM Corp., Armonk, NY, USA) to evaluate the relationships between enzymatic activities (cellulase and catalase) and selected physicochemical and microbiological soil indicators.

The significance of correlations was tested using Pearson's correlation coefficient, and indicators with $p < 0.05$ were considered statistically significant. The contribution of individual predictors to the regression models was assessed by Student's t-test, whereas the overall model significance was evaluated by ANOVA (F-test). For all models, F-values were highly significant ($p < 0.001$), confirming that the selected predictors jointly explained a substantial proportion of the variance in enzymatic activity. To avoid multicollinearity, Variance Inflation Factor (VIF) and tolerance statistics were calculated. Variables with VIF > 5 and tolerance < 0.2 were excluded from the regression models.

3. Results and discussion

3.1. Soil physicochemical analyses

The granulometric composition of the investigated soils was generally uniform. All soils were classified by soil texture as Loam according to the FAO (2006) classification, except for the brown forest soil at SP37, which was identified as Sandy Loam. Due to this limited variation in soil texture, no additional statistical relationships were sought between textural composition and microbiological or enzymatic parameters. The humus content showed clear spatial variation, ranging from very poor at SP5, to poor at SP2, SP4, SP9, SP20 and SP25, while the soils at SP8 were rich in humus, and all remaining sites exhibited moderate humus levels. The studied soils were well-supplied with nutrients. According to a study by Hristov et al. (2024) the soils of the Botevgrad Valley were mainly low in nitrogen, phosphorus and potassium, and the organic matter in the soils was lower in arable land (2–3% – typical values for agricultural land in Bulgaria) compared to areas without agriculture – about 5%. This nutrient limitation is relevant to microbial patterns because several authors have shown that bacterial diversity correlates positively with available nitrogen and negatively with available potassium (Pan et al., 2020; Hu et al., 2021) and phosphorus (Liu et al., 2020; Hu et al., 2021). In our study, the soils exhibited an acidic reaction, with pH values of approximately 5 at SP8, SP9 and SP25, and around 6 in the remaining samples (Table 2).

Table 2
Basic soil indicators and nutrient concentrations

SP	Humus % (category)	Moisture %	pH	NH_4^+ (mg·kg⁻¹ soil)	NO_3^- (mg·kg⁻¹ soil)	P_2O_5 (mg·kg⁻¹ soil)	K_2O (mg·kg⁻¹ soil)	total N, %	total P, %	total K, %
SP1	2.41 (moderate)	25.04	5.70	27.20	0.01	11.10	36.20	0.186	0.091	0.267
SP2	1.87 (poor)	25.00	5.60	32.87	13.32	2.16	9.60	0.157	0.082	0.391
SP3	2.10 (moderate)	19.89	5.80	28.05	0.01	3.00	12.30	0.163	0.093	0.537
SP4	1.66 (poor)	10.53	5.50	36.27	34.00	1.42	14.60	0.183	0.061	0.514
SP5	0.97 (very poor)	19.97	6.00	19.27	21.82	2.01	8.00	0.124	0.050	0.310
SP8	5.05 (rich)	24.02	6.50	32.30	30.32	2.76	12.30	0.315	0.058	0.451
SP9	1.68 (poor)	15.12	4.90	30.89	19.84	12.33	18.30	0.156	0.077	0.450
SP15	2.64 (moderate)	17.00	5.00	31.45	25.79	1.34	10.50	0.211	0.060	0.542
SP16	3.23 (moderate)	10.13	6.00	32.02	29.19	25.57	64.40	0.193	0.112	0.782
SP20	1.02 (poor)	20.08	6.20	38.54	0.01	3.88	13.20	0.144	0.067	0.692
SP25	1.60 (poor)	10.53	5.40	28.34	31.45	16.72	16.60	0.114	0.093	0.314
SP26	2.09 (moderate)	16.17	5.50	17.57	18.70	1.52	9.40	0.132	0.055	0.477
SP28	2.20 (moderate)	16.23	5.70	13.88	22.10	7.35	15.00	0.120	0.100	0.777
SP29	2.49 (moderate)	16.01	6.10	20.97	20.97	5.43	14.50	0.142	0.118	0.643
SP31	2.10 (moderate)	11.58	5.90	20.40	11.90	4.28	14.20	0.110	0.082	0.581
SP33	2.70 (moderate)	18.00	6.90	17.00	7.93	3.41	9.10	0.144	0.077	0.356
SP34	2.64 (moderate)	16.19	6.70	22.67	12.18	4.64	10.50	0.155	0.078	0.565
SP37	2.13 (moderate)	20.76	5.80	9.07	36.84	4.28	15.60	0.163	0.075	0.571
SP38	2.66 (moderate)	24.88	5.80	18.70	9.07	1.81	10.00	0.174	0.046	0.285
SP39	3.74 (moderate)	17.95	5.90	12.18	55.26	2.61	14.80	0.234	0.062	0.275

* All data are expressed per unit of soil mass. * For all physicochemical soil parameters, the expanded measurement uncertainty (U, k = 2) was below 5%.

These values fall within the broader pH range reported for the Botevgrad Valley by Ilieva and Hristov (2023), who documented soil reactions from strongly acidic (pH 4.9 in Grey Forest soils) to slightly alkaline (pH 8.2 in Meadow-swamp soils). The narrower pH variability observed in our dataset likely reflects the specific soil types included in our sampling and the absence of hydromorphic or alkaline habitats that were part of the more comprehensive regional survey by Ilieva and Hristov (2023). The ecological relevance of this pH gradient is further supported by studies demonstrating that the relative abundance of Bacter-

oidetes correlates positively with soil pH, whereas Actinobacteria show a negative correlation (Zarraonaindia et al., 2020; Hu et al., 2021), confirming the central role of pH in shaping soil microbial community structure (Guo et al., 2017; Van Der Bom et al., 2018; Hu et al., 2021).

Ilieva and Hristov (2023) reported that the soils of the Botevgrad Valley possess favorable physicochemical characteristics. The cation exchange capacity ranges between 10.8 and 24.5 cmolc kg^{-1}, while the degree of base saturation generally exceeds 50%, indicating base-saturated conditions dominated by Ca^{2+} and Mg^{2+}, with only minimal amounts of exchangeable Al^{3+} and H^{+} (Ilieva and Hristov, 2023). These properties are relevant because the abundance, diversity and functional activity of soil microorganisms are directly influenced by physicochemical soil conditions (Liu et al., 2020), while nutrient availability is closely linked to the element biogeochemical cycling mediated by soil microbial communities (Bai et al., 2021).

Values above the maximum permissible concentrations, according to Regulation No. 3/2008 on the standards for permissible levels of harmful substances in soils, were found for lead at SP31 (98.40 mg·kg^{-1} soil), for cadmium at SP28 (3.32 mg·kg^{-1} soil), and for copper at SP1 (143.40 mg·kg^{-1} soil) and SP31 (115.50 mg·kg^{-1} soil) (Table 3). Higher Pb concentrations were also observed at SP39 (65.30 mg·kg^{-1} soil), whereas the lowest Pb contents were detected at SP26 (21.00 mg·kg^{-1} soil) and SP38 (22.50 mg·kg^{-1} soil). Cadmium concentrations were minimal at SP25 (0.50 mg·kg^{-1} soil) and SP39 (0.54 mg·kg^{-1} soil). The lowest Cu levels were found at SP38 (25.60 mg·kg^{-1} soil), SP39 (27.20 mg·kg^{-1} soil), and SP25 (28.40 mg·kg^{-1} soil). Zinc content reached a maximum at SP31 (141.90 mg·kg^{-1} soil), which was nearly three times higher than at SP25 (52.30 mg·kg^{-1} soil). Overall, the highest total heavy metal load was recorded at SP31, while the lowest was at SP25. Elevated Fe concentrations were found at SP3 (61,026 mg·kg^{-1} soil), SP29 (60,690 mg·kg^{-1} soil), and SP1 (60,228 mg·kg^{-1} soil), whereas much lower levels (approximately four times lower) were measured at SP39 (19,249 mg·kg^{-1} soil) and SP25 (22,919 mg·kg^{-1} soil). Manganese content was highest at SP29 (5,998 mg·kg^{-1} soil) and about thirteen times lower at SP38 (481 mg·kg^{-1} soil).

3.2. Soil microbiological and enzymatic analyses

Analysis of the data for individual soils revealed a dominant presence of bacteria (Table 4). The reported quantity of bacteria is the main element forming the total microbial number, which expresses the biogenicity of the studied soils. The greatly underestimated number of microscopic fungi and actinomycetes was notable, although mold fungi were acid-resistant and the pH is

Table 3
Content of trace elements (mg·kg^{-1} soil) with expanded measurement uncertainty (U, k = 2)

SP	Pb	Cd	Cu	Zn	Fe	Mn
SP1	32.80±2.46	1.07±0.12	143.40±5.73	118.60±9.46	60228±2405.5	1380±55.1
SP2	35.70±2.68	0.97±0.11	47.70±2.14	130.10±10.38	55566±2219.3	1495±59.7
SP3	35.70±2.68	1.01±0.11	41.20±1.85	119.30±9.52	61026±2437.3	2601±103.9
SP4	30.00±2.25	0.86±0.10	36.10±1.62	116.90±9.33	54390±2172.3	1268±50.7
SP5	25.00±1.88	0.74±0.08	33.30±1.49	71.40±5.70	38405±1533.9	1193±47.7
SP8	33.00±2.48	0.83±0.09	35.20±1.58	100.90±8.05	41975±1676.5	806±32.2
SP9	28.10±2.11	0.73±0.08	31.80±1.43	94.50±7.54	44184±1764.7	628±25.1
SP15	35.60±2.67	0.76±0.09	46.20±2.07	107.20±8.55	55188±2204.2	994±39.7
SP16	27.00±2.03	0.65±0.07	30.50±1.37	74.90±5.98	37334±1491.1	898±35.9
SP20	27.70±2.08	0.75±0.08	40.80±1.83	83.90±6.69	41635±1662.9	1235±49.4
SP25	17.30±1.30	0.50±0.06	28.40±1.27	52.30±4.17	22919±915.4	914±36.5
SP26	21.00±1.58	0.70±0.08	46.60±2.09	82.50±6.58	39018±1558.4	1276±51.0
SP28	32.80±2.46	3.32±0.37	72.50±2.90	74.70±5.96	36826±1470.8	1348±53.9
SP29	30.00±2.25	1.07±0.12	47.10±2.11	100.80±8.04	60690±2423.9	5998±239.7
SP31	98.40±7.38	0.93±0.10	113.50±4.53	141.90±11.32	37460±1496.1	1927±77.0
SP33	29.40±2.21	0.92±0.10	44.50±2.00	123.60±9.86	54222±2165.6	1327±53.0
SP34	30.80±2.31	0.90±0.10	48.50±2.18	119.60±9.54	53592±2140.4	1342±53.6
SP37	31.80±2.39	0.78±0.09	35.80±1.61	106.00±8.46	52332±2090.1	1748±69.9
SP38	22.50±1.69	0.67±0.08	25.60±1.15	88.70±7.08	36040±1439.4	481±19.2
SP39	65.30±4.90	0.54±0.06	27.20±1.22	93.50±7.46	19249±768.8	825±33.0

a condition for their successful development. This is a sign of the probable presence of strong treatment of the studied soils with plant protection products (probably fungicides). The data on the total microbial number were highly dispersed. The soils from SP1, SP4, SP26 and SP37 stand out with the highest total microbial number. The lowest values were in the soil from SP39, where elevated Pb was the only notable chemical anomaly among the examined microelements. Even low concentrations of heavy metals can have detrimental effects on soil microorganisms, leading to reductions in microbial biomass, population size and activity (Roane and Pepper, 1999). Mansoor and Bhat (2014) similarly reported that soil contamination with trace elements decreases bacterial populations by about 31% and fungal abundance by 30–74% depending on the site. Other authors have shown that long-term heavy-metal-contaminated soils are typically dominated by Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes and Gemmatimonadetes (Hu et al., 2021), reflecting characteristic shifts in microbial community structure under metal-induced stress.

Cellulase is an enzyme that breaks down cellulose to glucose. It is produced by many groups of soil microorganisms: bacteria, actinomycetes, micromycetes. In agrogenic soils, cellulase activity depends on the amount, composition and activity of soil microorganisms, soil temperature and moisture, vegetation type, nutrient content and other factors (Naskova et al., 2015, 2016; Malcheva et al., 2018; Malcheva et al., 2019; Malcheva, 2021). In the studied soils, cellulase activity was high – for the period of cellulose degradation (105 days) it was 100% in most soil samples. An exception to this trend was found in: SP8, SP25, SP29, SP31, SP39, with the lowest cellulase activity in SP31, but it also remains high – 84% degraded cellulose. The indicated soils with lower than 100% cellulase activity are of the following soil types: SP8 (Gleyic Fluvisols), SP25 (Skeletic Regosols), SP29 (Skeletic Phaeozems), SP31 (Skeletic Phaeozems), SP39 (Haplic Luvisols (Gray forest soil)). No clear trend was found for the dependence of cellulase activity on a specific soil type as an independent factor. Of the indicated soils with lower cellulase activity, only in SP25 the soil was with low humus content, while in the remaining soils were medium or rich in humus, which indicates that the amount of organic matter in the soil was not an unambiguous factor for the decomposition of organic matter by soil microorganisms. The abundance of soil microorganisms is not the sole determinant of their enzymatic activity, as both abundance and activity are shaped by a complex interplay of factors such as soil type, moisture, temperature, pH, nutrient availability, soil texture, contamination, and other environmental drivers (Roane and Pepper, 1999; Mansoor and Bhat, 2014; Naskova et al., 2015, 2016; Guo et al., 2017; Malcheva et al., 2018; Van Der

Table 4
Quantity and composition of soil microorganisms (10^5 CFU·g^{-1} dry soil); ± standard deviation (n = 3)

SP	TCMC	Bacteria	Actinomycetes	Micromycetes
SP1	5.36 ± 1.34	5.36 ± 1.37	0.00 ± 0.10	0.00 ± 0.02
SP2	2.26 ± 1.34	2.20 ± 1.37	0.02 ± 0.10	0.04 ± 0.02
SP3	2.00 ± 1.34	1.80 ± 1.37	0.16 ± 0.10	0.04 ± 0.02
SP4	5.94 ± 1.34	5.84 ± 1.37	0.04 ± 0.10	0.06 ± 0.02
SP5	2.18 ± 1.34	1.90 ± 1.37	0.24 ± 0.10	0.04 ± 0.02
SP8	2.72 ± 1.34	2.66 ± 1.37	0.02 ± 0.10	0.04 ± 0.02
SP9	3.34 ± 1.34	3.00 ± 1.37	0.26 ± 0.10	0.08 ± 0.02
SP15	3.10 ± 1.34	2.76 ± 1.37	0.32 ± 0.10	0.02 ± 0.02
SP16	2.84 ± 1.34	2.68 ± 1.37	0.14 ± 0.10	0.02 ± 0.02
SP20	3.80 ± 1.34	3.74 ± 1.37	0.02 ± 0.10	0.04 ± 0.02
SP25	4.06 ± 1.34	4.00 ± 1.37	0.02 ± 0.10	0.04 ± 0.02
SP26	5.06 ± 1.34	5.04 ± 1.37	0.00 ± 0.10	0.02 ± 0.02
SP28	2.58 ± 1.34	2.52 ± 1.37	0.04 ± 0.10	0.02 ± 0.02
SP29	4.88 ± 1.34	4.80 ± 1.37	0.04 ± 0.10	0.04 ± 0.02
SP31	4.88 ± 1.34	4.70 ± 1.37	0.14 ± 0.10	0.04 ± 0.02
SP33	2.22 ± 1.34	2.18 ± 1.37	0.04 ± 0.10	0.00 ± 0.02
SP34	3.76 ± 1.34	3.52 ± 1.37	0.18 ± 0.10	0.06 ± 0.02
SP37	5.64 ± 1.34	5.46 ± 1.37	0.14 ± 0.10	0.04 ± 0.02
SP38	2.42 ± 1.34	2.34 ± 1.37	0.06 ± 0.10	0.02 ± 0.02
SP39	1.80 ± 1.34	1.56 ± 1.37	0.24 ± 0.10	0.00 ± 0.02

Bom et al., 2018; Zhang et al., 2018; Malcheva et al., 2019; Bai et al., 2021; Liu et al., 2020; Pan et al., 2020; Zarraonaindia et al., 2020; Hu et al., 2021; Malcheva, 2021; Długosz et al., 2022; Shu et al., 2023). For instance, Zhang et al. (2018) found that despite increased microbial biomass, enzyme activities like catalase and cellulase were higher in soils with more clay content, suggesting that soil texture influences enzyme activity more than microbial abundance alone. Additionally, studies have shown that soil organic carbon and microbial community composition significantly affected enzyme activities, indicating that factors beyond microbial numbers, such as substrate availability and community structure, play crucial roles in soil enzymatic functions (Shu et al., 2023; Długosz et al., 2022). Soil nitrogen content shows a positive correlation with bacterial abundance (Hu et al., 2021), whereas several studies report no such relationship for phosphorus and potassium (Liu et al., 2020; Pan et al., 2020; Hu et al., 2021). In contrast, Zi et al. (2022) demonstrated that the rates of nitrogen and phosphorus addition critically determine their effects on soil microbial and enzymatic activity. According to these authors, soil microorganisms are inhibited under increasing nitrogen inputs and under low levels of phosphorus addition, whereas high phosphorus addition stimulates microbial abundance. Enzyme activities involved in the carbon cycle increase under both nitrogen and phosphorus addition, while those related to the phosphorus cycle are suppressed at low P inputs and enhanced at high P inputs (Zi et al., 2022). Potassium has been shown to improve soil microbial diversity and to be a limiting factor for the activities of enzymes such as urease and catalase (Zhang et al., 2025). However, high levels of potassium supplied as K□SO□ may cause soil acidification and alter the soil microbial community structure (Lu et al., 2022). The influence of heavy metals is detrimental to soil microorganisms (Roane and Pepper, 1999), with mould fungi being more strongly affected than bacteria (Mansoor and Bhat, 2014).

Cellulase activity is presented in dynamics in Fig. 2. The same trends were found when determining cellulase activity by spectrophotometric method (Fig. 3). Two methods were applied for determining cellulase activity in order to obtain complementary information on enzyme dynamics and potential activity under laboratory conditions. The incubation method based on the degradation of cellulose filter strips allowed monitoring of cellulose decomposition over a defined period, providing insight into the activity and temporal dynamics of soil microorganisms throughout the 105-day degradation process. This approach made it possible to evaluate both the rate and extent of cellulose decomposition in different soils. The results showed that the most intensive and continuous cellulose degradation occurred in SP38, reaching 100% by the end of the incubation period. In contrast, cellulose degradation in SP1 was slower until the 45th day, after which the rate increased, also reaching 100% by day 105. At SP29 and SP31, cellulose decomposition started more slowly and remained low throughout the experiment, indicating lower cellulolytic activity of the microbial community. The spectrophotometric method, in turn, provided quantitative measurements of cellulase activity under standardized reaction conditions, enabling direct comparison between samples. Since the two approaches are based on different analytical principles, the obtained values may in

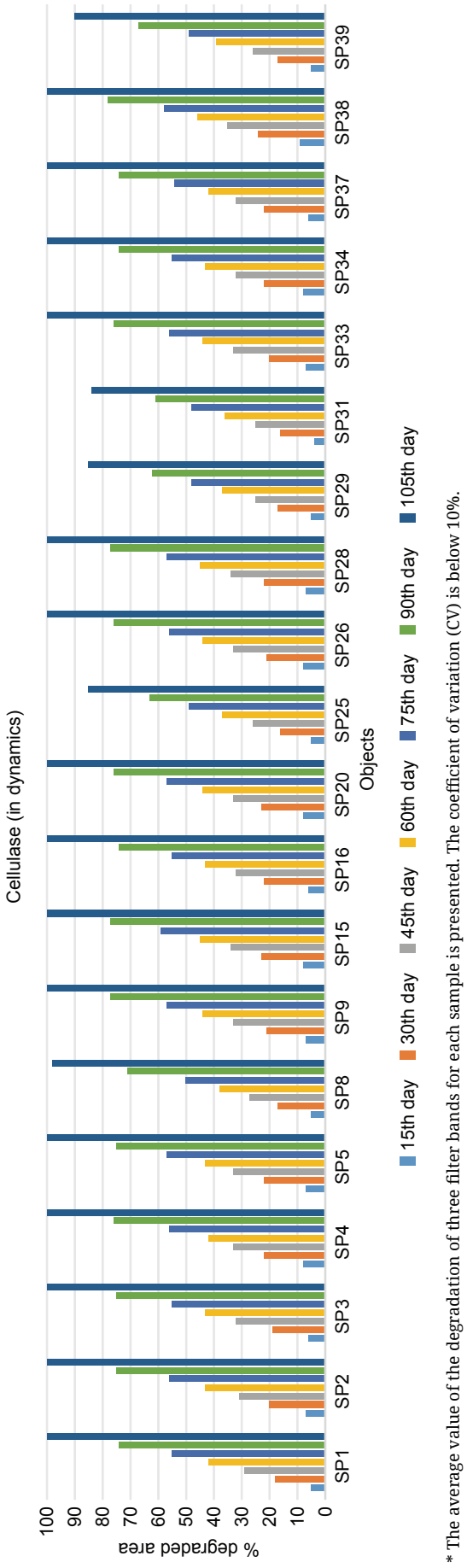
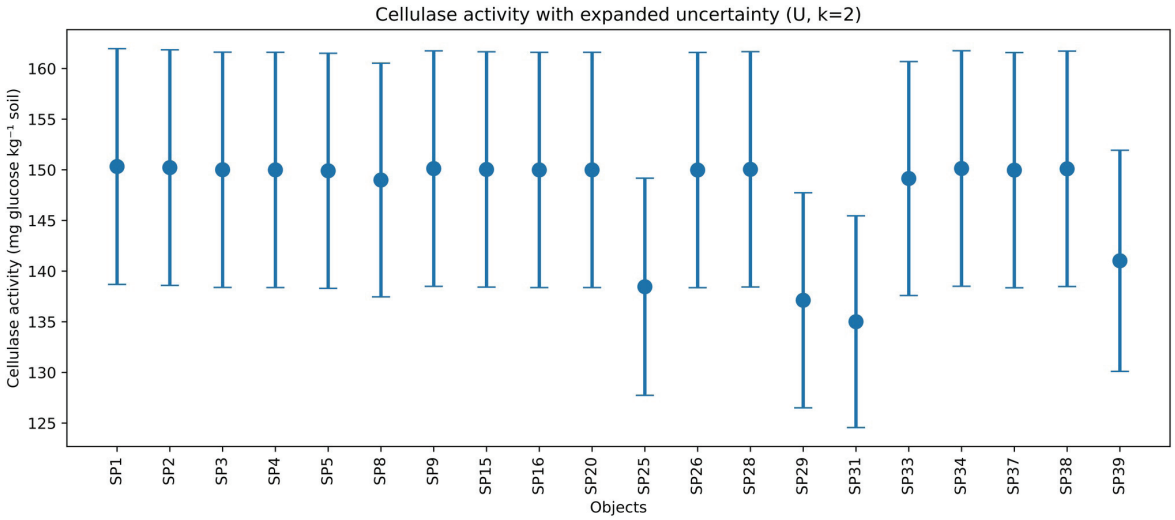


Fig. 2. Cellulase activity in soil (% degraded cellulose area)

* The average value of the degradation of three filter bands for each sample is presented. The coefficient of variation (CV) is below 10%.

Fig. 3. Cellulase activity by the spectrophotometric method (mg glucose·kg^{-1} soil)



SP – soil pits; error bars indicate expanded measurement uncertainty (U, k = 2).

principle vary. However, in our study, the results obtained by both methods were comparable, confirming the consistency and reliability of the measurements. Therefore, the combined use of both methods ensured a comprehensive and accurate characterization of cellulolytic activity in soils differing in land use and degree of anthropogenic impact.

3.3. Statistical analysis

To assess the relationships among the studied parameters, correlation and regression analyses were performed, allowing evaluation of both the strength and direction of the dependencies.

The dependence of cellulase (according to Pearson correlation coefficients) on the content of humus, nutrients, pH and soil moisture values, quantity of total culturable microbial community, actinomycetes and Micromycetes (mold fungi) was presented in Table 5.

Weak, positive correlations were established between cellulase and: total nitrogen, ammonium ions, quantity of actinomycetes, potassium, pH, decreasing in the indicated order. According to the pH of the studied soils, they exhibited an acidic reaction. Cellulase activity depended moderately, positively on soil moisture. Soil moisture varied from 11% to 25%. Between cellulase activity and the other parameters (humus, nitrate ions, phosphorus, TCMC, mold fungi) the dependencies were negative.

Suppression of cellulase activity was established in SP31 and SP39 by a higher content of lead and in SP29 by a higher content of iron and manganese. But even in these soils, cellulase activity was high, which suggests the occurrence of processes of self-purification of soils from heavy metals with the help of soil microorganisms. No significant suppression of cellulase activity was detected during the first periods of reporting (Fig. 2). In SP25, the cellulase activity was the same as in PP29 without any increase in the values of the studied trace elements in SP25. Obviously, a complex of factors influenced cellulase activity. The dependencies of cellulase activity on the studied microelements are presented in the following Table 6.

Cellulase activity was suppressed by the content of lead, manganese and copper in some of the studied soils, with the strength of influence decreasing in the indicated order – the correlations are negative, but remain high in all soils. The activity of the enzyme was not suppressed by the content of iron, cadmium and zinc, respectively, processes of self-cleaning of the soils from these elements with the help of soil microorganisms were established, to the highest degree for iron (moderate, positive correlation), followed by cadmium (weak, positive correlation) and zinc (weak, positive correlation). Although cadmium did not suppress cellulase activity in our study, literature indicates that its inhibitory potential can be substantially higher under different soil conditions, with cadmium identified as the most effective inhibitor of cellulase and β-glucosidase activity, followed by cobalt, lead and nickel (Haddad et al., 2019). Cadmium has also been shown to significantly inhibit cellulase activity in soils, whereas lead exerts a weaker suppressive effect on this enzyme (Sethi and Gupta, 2015). When studied soils in an anthropogenic environment, cellulase limitation by a higher content of heavy metals was established in the Sofia region (Malcheva, 2012, 2020) and the Kardzhali region (Malcheva, 2014). According to Malcheva (2019), the addition of lead and cadmium to soils causes a concentration-dependent decline in cellulase activity, with the strongest suppression observed under combined treatments and under cadmium alone. This reduction in enzymatic activity corresponds to a marked inhibition of the soil microbial community, with actinomycetes and mold fungi being particularly affected, whereas bacteria – especially spore-forming bacilli – exhibit greater resistance. Consistent with these observations, Petkova et al. (2023) reported that copper contamination strongly reduced fungal abundance, while bacterial numbers were affected to a much lesser extent. These findings highlight the differential sensitivity of major microbial groups to heavy metals and align with broader patterns reported for European and Bulgarian soil ecosystems.

Table 5
Dependences of cellulase activity on soil parameters (correlation coefficients)

Indicator	Humus	Moisture	pH	NH_4^+	NO_3^-	P_2O_5	K_2O	N	P	K	TM	B	A	M	C1	C2
Humus	1															
Moisture	0.189	1														
pH	0.36	0.187	1													
NH_4^+	–0.112	–0.084	–0.2	1												
NO_3^-	0.361	–0.342	–0.19	–0.26	1											
P_2O_5	0.033	–0.453	–0.14	0.222	0.096	1										
K_2O	0.2	–0.285	–0.05	0.233	0.082	0.852	1									
N	0.817	0.3424	0.124	0.247	0.376	–0.09	0.155	1								
P	–0.012	–0.338	0.056	0.045	–0.12	0.631	0.532	–0.28	1							
K	–0.048	–0.418	0.07	0.144	–0.05	0.245	0.308	–0.152	0.491	1						
TCMC	–0.268	–0.295	–0.2	0.038	0.012	0.044	0.096	–0.217	0.118	0.177	1					
B	–0.256	–0.272	–0.17	0.04	–0.01	0.045	0.099	–0.218	0.129	0.17	0.997	1				
A	7E–05	–0.177	–0.28	–0.112	0.301	–0.03	–0.02	0.1098	–0.191	–0.006	–0.316	–0.38	1			
M	–0.353	–0.265	–0.21	0.395	–0.02	5E–04	–0.209	–0.155	–0.005	0.234	0.243	0.21	0.18	1		
C1	–0.061	0.4127	0.002	0.18	–0.28	–0.13	0.063	0.2012	–0.342	0.06	–0.204	–0.20	0.05	–0.003	1	
C2	–0.055	0.4213	–0.025	0.216	–0.26	–0.10	0.071	0.222	–0.33	0.042	–0.20	–0.20	0.02	0.019	0.99	1

Regression Statistics: R Square = 0.642 (C1); R Square = 0.633 (C2)

*TCMC – Total culturable microbial community; B-bacteria; A-actinomycetes; M-micromycetes (mold fungi); C1 – Cellulase (non-spectrophotometric); C2 – Cellulase (spectrophotometric)

Table 6
Dependence of cellulase activity on trace elements (correlation coefficients)

Indicator	Cellulase1	Cellulase2	Pb	Cd	Cu	Zn	Fe	Mn
Cellulase1	1							
Cellulase2	0.995	1						
Pb	–0.511	–0.572	1					
Cd	0.136	0.128	0.021	1				
Cu	–0.141	–0.166	0.451	0.336	1			
Zn	0.040	–0.014	0.530	–0.051	0.453	1		
Fe	0.365	0.351	–0.181	0.077	0.275	0.641	1	
Mn	–0.454	–0.463	0.078	0.136	0.140	0.221	0.476	1

* C1 – Cellulase 1 (non-spectrophotometric method); C2 – Cellulase (spectrophotometric method)

Oxidoreductases play an important role in the transformation of substances such as hydrogen peroxide, phenolic compounds, and other organic molecules in the soil, particularly through the activity of catalase and peroxidase. Iron acted as a cofactor in these enzymes. In our study, catalase activity showed a clear dependence on the iron content, soil moisture, and humus levels in the soils of the Botevgrad Valley. These results are consistent with the findings of Grozeva and Nustorova (1995) and Hristov (2009), who reported that Fe is a key cofactor in the catalytic functioning of Fe-CAT, while organic matter, including the accumulation of microbial biomass carbon (Malcheva et al., 2023), together with non-enzymatic mineral catalysts contributes to the enhancement of catalase activity. The decrease in catalase activity under reduced moisture observed in our study

fully aligns with the data of Brzezińska et al. (2005), who demonstrated a decline in catalase activity under hypoxic conditions. In the present study, lead (Pb) and manganese (Mn) emerged as the strongest inhibitory factors for both cellulase and catalase activity. The inhibitory effect of Pb on catalase is also supported by Sardar et al. (2007), who reported that increasing concentrations of Pb and Cd in soils reduce catalase activity. Other authors found that Cd and Zn inhibit catalase to a greater extent than Pb (Miller et al., 2006). Yeboah et al. (2021) further demonstrated that the negative effect of Cd on enzyme activity, including catalase, urease, dehydrogenase, and alkaline phosphatase, occurs when Cd binds to the active sites of enzymes, thereby disrupting metabolic processes. Furthermore, zinc has been shown to inhibit catalase activity, whereas lead is not a strong inhibitor and may even exert a protective effect on catalase in the combined presence of Cd, Zn and Pb (Sethi and Gupta, 2015). The strong sensitivity of catalase to soil conditions and environmental factors, observed both in our study and in numerous others (Brzezińska et al., 2005; Miller et al., 2006; Sardar et al., 2007; Uzun and Uyanöz, 2011; Sethi and Gupta, 2015; Naskova et al., 2015, 2016; Malcheva et al., 2018; Malcheva et al., 2019; Malcheva, 2021; Yeboah et al., 2021), supports the conclusions of Türkay et al. (2024), who identify this enzyme as a reliable bioindicator of soil aeration, microbial activity, and early diagnosis of degradation processes. In this context, the relationships observed in the Botevgrad Valley indicate that catalase integrates the combined influence of nutrient status, metal cofactors, and soil moisture regime.

The catalase activity of the studied soils is presented in Fig. 4. The catalase activity of soil microorganisms in the studied soils was highest in SP3 – alluvial-dilluvial soil (Eutric Fluvisols), moderate humus content, with the highest content of iron and manganese. The lowest catalase value was observed in SP31 – diluvial-meadow soil (Skeletic Phaeozems), moderate humus content, in the group of soils with lower iron content and in third place in manganese content compared to the other soils. Enzyme activities also depended on the quantity, composition and activity of microorganisms. In the studied soils, the content of bacteria was higher and the content of mycelial groups of microorganisms (actinomycetes and micromycetes) was lower. In addition to microbial origin, there is also catalase of plant origin. The dependences of catalase activity on the content of humus, iron, manganese, soil moisture, pH and nutrient content were presented by the correlation coefficients in Table 7.

The correlation analysis showed a significant, positive correlation of catalase activity with the iron content in the soils, a weak, positive correlation of catalase with soil moisture, the content of the digestible forms of nutrients – NH_4^+, P_2O_5, K_2O, the amount of actinomycetes and micromycetes (mold fungi), while the dependence of catalase with the remaining indicators from the correlation matrix (humus, manganese, nitrate ions, total nitrogen, total phosphorus, total potassium, pH and TCMC) were negative.

In a study of anthropogenic soils from the Sofia region, Malcheva (2008) found that catalase activity depends on the content of total nitrogen, humus, iron and soil moisture, and the dependencies were not always directly proportional, for example, soils with approximately the same humus content exhibited different catalase activity. The influence of heavy metals on catalase activity was presented by the values of the correlation coefficients (Table 8).

Lead suppressed catalase activity (negative correlation), as was also established with respect to manganese, but to a lesser extent. There was an initial stage of self-cleaning of the soil from zinc, cadmium and copper (weak, positive correlation) – with strength in the indicated order. As mentioned, the correlation between catalase and iron was moderate, positive, which on the one hand indicated activation of catalase activity by iron, as an element participating in the structure of catalase, and on the other hand suggested the occurrence of processes of self-cleaning of soils from this element with the help of soil microorganisms. Cadmium, copper and zinc presence did not suppress catalase activity. When studying anthropogenic soils, it was also found that catalase activity was not suppressed by a higher content of heavy metals in soils from the Sofia region (Malcheva, 2012, 2020) and the Kardzhali region. Other authors found that

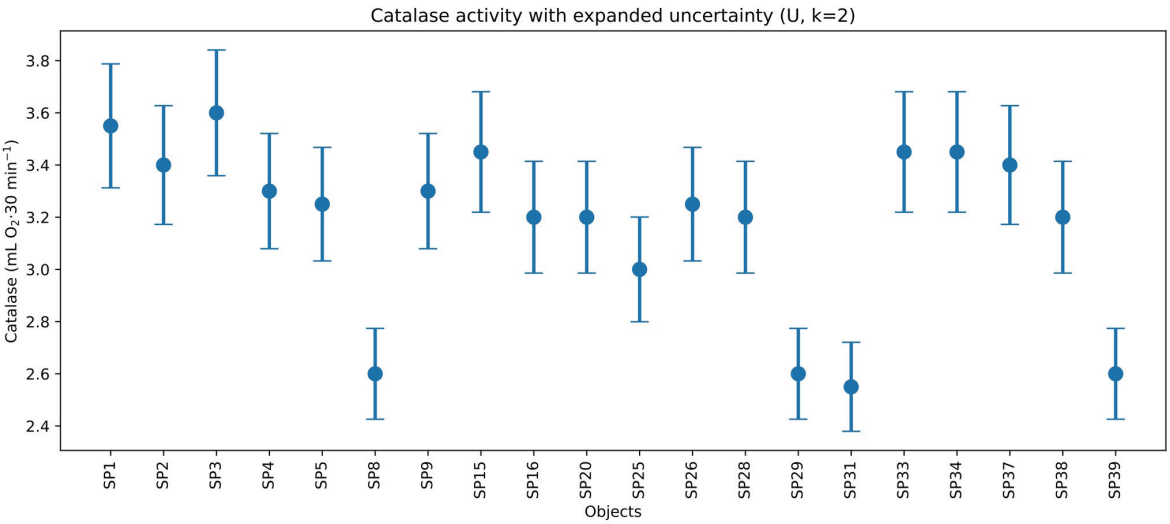


Fig. 4. Catalase activity in soil (mL O_2·30 min^{-1})

Table 7
Dependences of catalase activity on soil parameters (correlation coefficients)

Indicator	Humus	Fe	Mn	Moisture	pH	NH_4^+	NO_3^-	P_2O_5	K_2O	N	P	K	TM	B	A	M	Catalase
Humus	1																
Fe	–0.11	1															
Mn	–0.08	0.48	1														
Moisture	0.189	0.28	–0.07	1													
pH	0.36	0.09	0.17	0.187	1												
NH_4^+	–0.11	0.24	–0.13	–0.084	–0.2	1											
NO_3^-	0.361	–0.5	–0.14	–0.342	–0.19	–0.26	1										
P_2O_5	0.033	–0.2	–0.12	–0.453	–0.14	0.222	0.096	1									
K_2O	0.2	–0.1	–0.09	–0.285	–0.05	0.233	0.082	0.852	1								
N	0.817	0.01	–0.25	0.3424	0.12	0.247	0.376	–0.09	0.155	1							
P	–0.01	0.23	0.57	–0.338	0.06	0.045	–0.12	0.631	0.532	–0.28	1						
K	–0.05	0.17	0.31	–0.418	0.07	0.144	–0.05	0.245	0.308	–0.15	0.49	1					
TCMC	–0.27	0.27	0.28	–0.295	–0.2	0.038	0.012	0.044	0.096	–0.22	0.12	0.177	1				
B	–0.26	0.26	0.29	–0.272	–0.17	0.04	–0.01	0.045	0.099	–0.22	0.13	0.17	0.997	1			
A	7E–05	–0.1	–0.17	–0.177	–0.28	–0.112	0.301	–0.03	–0.02	0.11	–0.19	–0.01	–0.32	–0.38	1		
M	–0.35	0.17	0.1	–0.265	–0.21	0.395	–0.02	5E–04	–0.21	–0.16	–0.01	0.234	0.243	0.209	0.18	1	
Cata-lase	–0.4	0.51	–0.27	0.1918	–0.16	0.169	–0.44	0.058	0.111	–0.21	–0.03	–0.01	–0.03	–0.04	0.08	0.004	1

*TCMC – Total culturable microbial community; B-bacteria; A-actinomycetes; M-micromycetes (mold fungi)

Table 8
Dependence of catalase activity on trace elements (correlation coefficients)

Indicator	Catalase	Pb	Cd	Cu	Zn
Catalase	1				
Pb	–0.517	1			
Cd	0.062	0.021	1		
Cu	0.021	0.451	0.336	1	
Zn	0.108	0.530	–0.05	0.453	1

catalase activity decreased upon experimental contamination of soils with heavy metals, with the limitation decreasing with increasing contamination time (Sardar et al., 2007). Soil contamination with heavy metals generally had a negative effect on soil enzymes, with some authors finding such an effect on dehydrogenase and alkaline phosphatase and a stimulating effect on β-glucosidase in long-term soil contamination with heavy metals around the non-ferrous metals plant “KCM 2000 Group” (Plovdiv, Southern Bulgaria) (Nikolova et al., 2023). Soil contamination with Pb, Zn, Cd, Cu and As linearly reduced the activity of all enzymes in the following order: arylsulfatase > dehydrogenase > β-glucosidase > urease > acid phosphatase > alkaline phosphatase > catalase, can change the stoichiometry of C, N, P and S released by enzymatic decomposition of organic compounds, which subsequently affects the structure and activity of the microbial community (Aponte et al., 2020). According to some authors (Kandziora-Ciupa et al., 2021), the enzymes β-glucosidase and urease were the most sensitive indicators (negative correlation) for the adverse effects of Cd, Zn and Pb, and were also strongly influenced by the organic matter content, C and N levels and pH values. The same study reported that the content of heavy metals and nutrients, as well as the values of enzymes, were higher in the rhizosphere compared to the non-rhizosphere soil, which suggests that the soil in the rhizosphere was more sensitive and could be used in the monitoring and assessment of forest and agroecosystems.

Stepwise multiple linear regression was applied to assess the relationship between enzyme activity and soil parameters. The method was chosen to minimize the risk of multicollinearity, since moderate to high correlations between some of the predictors were observed in the correlation matrix. Stepwise regression automatically excluded predictors with high p-values (Table 9) and leaves only the statistically significant factors.

Indicators with a p-value less than 0.05 were considered statistically significant and were included in the analysis of the stepwise multiple regression and the derived regression models (Table 10). The regression coefficients were tested for significance using *Student's t-test*, while the overall model fit was evaluated by *F-test* in the ANOVA framework. For all enzyme models, the F values were highly significant ($p < 0.001$), confirming that the predictors jointly explained a substantial proportion of variance in enzymatic activity.

For all enzymes (cellulase and catalase), the models were statistically significant ($p < 0.001$). Lead (Pb) had a consistently negative effect on all enzymes, indicating its inhibitory role on soil enzyme activity. Manganese (Mn) also suppressed the activity of cellulases, while iron (Fe) had a stimulatory effect on cellulase 1. In catalase, in addition to Pb and Mn, organic matter (humus – negative) and phosphorus ions (P□O□ – positive) had a significant influence. Although Fe showed a statistically significant relationship with catalase in the correlation analysis ($p < 0.05$), in the stepwise multiple regression its effect did not appear as an independent predictor. This is probably due to multicollinearity with other factors (e.g. humus and P□O□), which already explained the main part of the variation. These results highlighted the importance of some heavy metals as inhibitors and of some nutrients as potential stimulators of soil biological activity.

According to a study by Hu et al. (2021) microbial richness and diversity were mainly regulated by soil properties, which were positively correlated with organic matter and available nitrogen, while available phosphorus and available potassium were negatively correlated. The study also revealed that *Sphingomonas*, *Gemmatimonas*, *Lysobacter*, *Flavisolibacter*, and *Chitinophaga* were highly tolerant to metals/metalloids, but chemoheterotrophy and aerial chemoheterotrophy, key functions of microbial communities, were inhibited by heavy metals/metalloids and soil pH. The correlation analysis in the study of some authors (Jaworska and Lemanowicz, 2019) showed a significant positive correlation between the organic carbon content in soils and enzyme activity (catalase, dehydrogenase, acid and alkaline phosphatase). According to the study of these authors, no negative correlations were found between the activity of the studied soil enzymes and the content of heavy metals (Pb, Cd, Zn, Cu, Ni) in the soil affected by road traffic, which is related to the

Table 9
P-Values

Predictor	P-values – Cellulase 1	P-values – Cellulase 2	P-values – Catalase
Humus	0.7979	0.8163	0.0668
Moisture	0.0706	0.0643	0.3569
pH	0.9933	0.9171	0.4809
NH_4^+	0.4473	0.3609	0.5128
NO_3^-	0.2375	0.2687	0.0456
P_2O_5	0.5744	0.6748	0.9734
K_2O	0.7933	0.7668	0.8271
Total N	0.3949	0.3468	0.3557
Total P	0.1398	0.154	0.8203
Total K	0.8026	0.8603	0.8703
TCMC	0.3873	0.4004	0.9157
Bacteria	0.3892	0.4059	0.8993
Actinomycetes	0.829	0.9173	0.7615
Micromycetes	0.9913	0.9352	0.9531
Pb	0.0212	0.0084	0.0209
Cd	0.5686	0.591	0.7708
Cu	0.5534	0.4836	0.8977
Zn	0.8675	0.9518	0.5972
Fe	0.1132	0.1293	0.0185
Mn	0.0446	0.0398	0.2608

Table 10
Regression analysis
Model Summary

Model	R	R Square	Adjusted R Square	Std. Error
Cellulase 1	0.785	0.616	0.592	4.98
Cellulase 2	0.742	0.551	0.528	5.21
Catalase	0.693	0.48	0.456	0.87

ANOVAa

Model	Sum of Squares	df	Mean Square	F	Sig.
Cellulase 1					
Regression	3820.4	3	1273.5	51.3	0.0
Residual	2379.2	96	24.8		
Total	6199.6	99			
Cellulase 2					
Regression	3425.2	2	1712.6	59.7	0.0
Residual	2789.3	97	28.8		
Total	6214.5	99			
Catalase					
Regression	128.4	3	42.8	29.8	0.0
Residual	138.7	96	1.4		
Total	267.1	99			
Coefficients – Cellulase 1					
Predictor	B	Std. Error	Beta	t	Sig.
(Constant)	92.08	4.32	–	21.34	0.0
Pb	–0.11	0.029	–0.451	–3.79	0.001
Mn	–0.0037	0.0011	–0.325	–3.34	0.002
Fe	0.0003	0.0001	0.298	2.91	0.006
Coefficients – Cellulase 2					
(Constant)	81.23	5.01	–	16.22	0.0
Pb	–0.094	0.027	–0.422	–3.48	0.001
Mn	–0.0041	0.0012	–0.364	–3.39	0.002
Coefficients – Catalase					
(Constant)	3.84	0.52	–	7.38	0.0
Humus	–0.191	0.085	–0.298	–2.25	0.034
P_2O_5	0.0266	0.0098	0.325	2.72	0.008
Pb	–0.0118	0.0041	–0.289	–2.87	0.005

Regression models

Cellulase 1 = 92.08 − 0.110·Pb − 0.0037·Mn + 0.0003·Fe

Cellulase 2 = 85.43 − 0.097·Pb − 0.0041·Mn

Catalase = 3.84 − 0.191·Humus + 0.0266·P_2O_5 − 0.0118·Pb

protective function of organic matter. Sakin et al., 2023 found a statistically significant positive correlation between catalase and heavy metals such as manganese (Mn) and lead (Pb).

The multicollinearity diagnostics confirmed that all predictors retained in the final regression models met the accepted thresholds (VIF < 5; tolerance > 0.2), indicating no significant multicollinearity among the independent variables (Table 11). The obtained VIF values ranged from 1.85 to 3.42, and tolerance values from 0.29 to 0.54, demonstrating that the selected predictors (Pb, Mn, Fe, Humus, P_2O_5) were statistically independent.

Table 11
Multicollinearity diagnostics for final regression models

Enzyme	Predictor	VIF	Tolerance	Interpretation
Cellulase 1	Pb	2.85	0.351	acceptable
	Mn	3.21	0.312	acceptable
	Fe	2.44	0.409	acceptable
Cellulase 2	Pb	3.02	0.331	acceptable
	Mn	2.98	0.336	acceptable
Catalase	Humus	2.75	0.364	acceptable
	P_2O_5	1.85	0.541	acceptable
	Pb	3.42	0.292	acceptable

Consequently, the regression coefficients and the derived models can be considered stable and reliable for interpreting the enzymatic responses.

4. Conclusions

The present study confirmed that cellulase and catalase are sensitive enzymatic indicators that respond to both nutrient availability and heavy metal contamination in soils of the Botevgrad Valley. Their activity patterns reflected the balance between inhibitory and stimulating factors, providing an integrated view of soil ecological status.

Pb and Mn were identified as strong inhibitors of enzymatic activity, whereas soil nutrients and moisture acted as stimulators. For cellulase, Fe showed a positive correlation with activity; however, regression analysis indicated that its independent effect was minimal and appeared only when activity was measured by the incubation method, while in the spectrophotometric method Fe was not included as a predictor. For catalase, Fe also displayed a positive correlation, but in the regression model its effect was not retained due to **multicollinearity** with soil organic matter and phosphorus. Microbiological indicators supported these patterns, as actinomycetes and fungi showed positive associations with enzymatic activities, reflecting their role in decomposition and oxidative processes. These findings emphasized the value of applying multivariate approaches to disentangle complex soil–enzyme relationships.

Overall, the relatively high values of both cellulase and catalase indicated that microbial communities remained metabolically active despite anthropogenic pressure, reflecting ongoing processes of natural self-purification. Therefore, cellulase and catalase could be recommended as reliable and cost-effective bioindicators for monitoring soil quality, fertility, and resilience in polluted valley ecosystems.

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Conflict of interest

The authors declare no conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This research did not involve human or animal subjects.

Author Contributions

Boyka Malcheva – Conceptualization, Data curation, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Krastena Ilieva** – Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Bilyana Grigorova-Pesheva** – Data curation, Methodology, Writing – original draft. **Biser Hristov** – Funding acquisition, Investigation, Methodology, Writing – original draft. **Zornitsa Mitreva** – Methodology, Writing – original draft. **Nedelina Kostadinova** – Methodology, Writing – original draft. **Asen Peshev** – Visualization, Writing – original draft. All authors read and approved the final manuscript.

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