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SOIL MICROBIAL BIOMASS AND ENZYME ACTIVITIES RESPONSE TO COPPER AND MOLYBDENUM POLLUTION IN THE VICINITY OF ZANGEZUR COPPER AND MOLYBDENUM COMBINE, ARMENIA

K. A. GHAZARYAN [*], H. S. MOVSESYAN [**]

Chair of Ecology and Nature Protection, YSU, Armenia

The aim of this study was to define a relationship between heavy metal (Cu, Mo) pollution of soil and various extracellular enzyme activities. Six enzymatic activities involved in cycles of carbon, nitrogen, phosphorus and sulfur (β-glucosidase, chitinase, leucine-aminopeptidase, acid phosphomonoesterase, alkaline phosphomonoesterase, and arylsulphatase) as well as microbial biomass were determined in soil samples collected in the surroundings of Zangezur Copper and Molybdenum Combine. The investigations showed that pollution of soil with copper and molybdenum led to a decrease in microbial biomass and soil enzymatic activity, which in turn had a negative impact on cycles of chemical elements, in particular C, P, N and S. This gives reason to conclude that the changes in soil microbial biomass and enzymatic activity may act as indicators of soil biological activity and quality.

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Introduction. Mining, smelting and processing activities have polluted soil resources by heavy metals throughout the world [1]. Geochemical weathering processes influencing on mining wastes initiate the process of transporting heavy metals from contaminated zones and reallocating them to surrounding soils. Heavy metals can negatively influence on environment (particularly on soil resources) and expose the health of surrounding ecosystems and human populations [2].

Many studies have shown that the long-term or short-term influence of heavy metals resulted in the decrease of microbial diversity and activity in the soil [2, 3]. These two parameters are important indicators of soil quality [4]. In polluted soils, the activity of microbial community is bound with abiotic properties of the soil [5]. Processes like nitrogen cycling or cellulose degradation can be measured as indicators by using enzymatic activities of the soil [6].

Soil enzymes are the extracellular enzymes that are mainly produced by microbial communities. They show the soil potential to support biochemical processes in the ecosystem [7–10]. The study of soil function, particularly of enzymatic activity, is an increasingly significant approach to understanding of soil

[*] E-mail: kghazaryan@ysu.am
[**] E-mail: hasmikmov@ysu.am

conditions in polluted environments. Many studies have suggested that the activity of enzymes may serve as indicator of the contamination type and level in soil and of soil ecosystem health [11–17]. Study of soil microbial communities adjacent to mining areas showed lower enzymatic activity and lower community diversity with higher heavy metal concentration of soil [18, 19]. Similar results are obtained also during the studies on experimentally contaminated soils [20].

Khan et al. [21] reported that heavy metals inactivated soil enzymes. This process occurs by different ways: denaturing the protein conformation, substrate complexation, combination with protein-active groups on the enzyme, competing with metal ions that are needed to form enzyme-substrate complexes, reaction with the enzyme-substrate complex, and indirectly reducing the microbial community responsible for producing the enzymes [22–26].

The aim of this study was to define a relationship between heavy metal pollution of soil and various extracellular enzyme activities that are proxy measures for soil health and nutrient cycling. The results of this research will help to identify the effects of long-term soil pollution by heavy metals on nutrient cycling.

Materials and Methods.

Studied area. The area is situated in the southern part of Armenia, near Kajaran town in the surroundings of Zangezur Copper and Molybdenum Combine. The combine started to deliver production in 1951. It is operated by open-cut method. The combine extracts and processes the copper and molybdenum ore, further processing of which yields copper and molybdenum separate concentrates.

The soils of study region belong to mountain cambisol. This soil type in Armenia is distributed on 500–1700 *m* above sea level and on southern dry slopes it extends up to a height 2400 *m* [27]. The relief of studied area is multifarious and is characterized by many heights and trenches. The mean annual air temperature is 8–12°C, in August it can increase up to 37°C and in January decrease to –23°C. In this region the annual mean precipitation ranges to 450-560 *mm* [28].

Soil sampling and analysis. The sampling of soil was performed in 2014. Considering the peculiarities of climate, relief, vegetation cover, as well as the nature of land-use and the extent of anthropogenic impact, 68 soil samples were taken in study area from the following land plots (LP):

- LP-1 - south-facing slope near the processing plant (a total of 16 soil samples),
- LP-2 - public areas of Kajaran town (a total of 24 soil samples),
- LP-3 - cultivated lands in the vicinity of open mine (a total of 16 soil samples),
- LP-4 - forested north-facing slope between the open mine and processing plant (a total of 12 soil samples).

Soil sampling was performed from the topsoil (0–25 *cm* depth). All soil samples were air-dried at room temperature (20–22°C) for two weeks, then were ground and passed through 0.42 *mm* nylon mesh. The content of humus in the soil was determined according to the method of Tyurin [29]. For determination of copper and molybdenum total content the soil samples were digested with mixture

HNO_3 + $HClO_4$ + HF (5:1:1, v:v:v) [30]. Cu and Mo contents were measured by Atomic Absorption Spectrometer PG990 (PG Instruments LTD).

Six soil enzymatic activities involved in cycles of C, P, N and S were assayed (β-glucosidase (beta_G), chitinase (chit), leucine-aminopeptidase (leu), acid phosphomonoesterase (acP), alkaline phosphomonoesterase (alkP) and arylsulphatase (aryS)) as described by Fornasier and Margon [31] and modified by Cowie et al. [32]. For desorption of enzymes heteromolecular exchange method was used, additionally extraction was carried out through bead-beating. The enzyme activities were determined through measurement of the soil extracts absorbance at 410 *nm* by colorimetric method.

Air dried soil was passed through 0.5 *mm* sieve. For the extraction of enzymes 2 *g* of field-moist soil was put in a 2-*ml* Eppendorf tube and 1.25 *ml* of a solution containing 1% Triton X-100 solution, 4% bovine serum albumin (BSA) with glass beads was added. The contents of the tubes were bead milled at 30 strokes per second for 3 minutes and then centrifuged at 20000 *g* for 5 minutes. Subsequently 50 *mM* Tris-HCl buffer (pH 7.5) was added to the supernatant [33].

Soil enzyme activities were determined using the substrates that are conjugates of the highly fluorescent compounds 4-methylumbelliferone and 7-amino-4-methylcoumarin. The activities are expressed as nanomoles of 4-methylumbelliferone (for acid phosphomonoesterase, alkaline phosphomonoesterase, β-glucosidase, arylsulphatase, and chitinase) and 7-amino-4-methylcoumarin (for leucine-aminopeptidase) products produced per gram of soil (oven-dry weight) per hour.

For the measurement of soil microbial biomass double-stranded DNA (dsDNA) was determined according to Ventura et al. [33]. 0.5 g of soil (air-dried and passed through 0.5 *mm* sieve) was placed in 2-*ml* tubes together with glass microbeads and 1 *ml* of sodium phosphate solution was added. Subsequently the tubes were agitated for three minutes through the mill at a frequency of 30 strokes per second and then were centrifuged at 20000 *g* for three minutes. The content of dsDNA was measured by fluorimetry method in the supernatant on microplate by the usage of specific fluorophore PicoGreen reagent (Life Technologies) that selectively binds the DNA double helix. The analysis was conducted following instructions given by the producer house. All results were recorded by a microplate lecturer (Synergy HT, BioTek; software Gen 5). The analysis process was repeated twice.

Results and Discussion. During the studies it was revealed that the soils in all studied areas belong to mountain cambisol type and decalcified subtype. However, these four areas differ by microclimatic peculiarities and vegetation cover as well as by the nature and the extent of anthropogenic impact. In particular, the area LP-1 is located in the vicinity of processing plant and has a south-facing position, the slope angle is 35–38°, and the vegetation coverage constitutes 60–65% (the main vegetation is represented by herbs and shrubs). High extent of stoniness and erosion as well as heavy impact of anthropogenic factor (specifically, of mining activities) have also been observed in this area. The area LP-2 is located in Kajaran town, in the eastern direction from the processing plant. It is an area of public importance, mainly has low slopes (0–15°), the vegetation cover – 75–100%

(herbs are the main vegetation), no stoniness and erosion effects have been observed; and the extent of anthropogenic impact is lower than in the area LP-1, though it is diverse by its nature: mining activities, motor transport, etc. The area LP-3 is located in the eastern direction from the open mine and is used for agricultural purposes. The slope of this area is insignificant (0–8°), the vegetation is represented by annual crops, no stoniness and erosion effects have been observed. Here, as in the previous area, the extent of anthropogenic adverse impact, particularly, the level of pollution by heavy metals, is lower in comparison with area LP-1, and the nature of the impact is more diverse (agriculture and mining). The area LP-4 is located between processing plant and the open mine. It has north-facing position, the slope angle of 31–41°, the vegetation cover of 75–85% (trees are the main vegetation), medium stoniness and weak erosion have been observed there. In this area, as compared with foregoing three areas, the level of anthropogenic factor impact is the lowest, and the natural landscape is in a relatively better condition.

The data of statistical analysis of investigation results of studied four areas are presented in Table 1. Copper and molybdenum are chosen as polluting heavy metals as they are the main soil pollutants in this region [34–36]. According to the data of statistical analysis, of studied four areas, the highest mean value of copper content while the lowest mean values of humus and dsDNA contents, as well as of six enzymatic activities were observed in soils of area LP-1, and the lowest mean values of copper and molybdenum contents, but the highest mean values of dsDNA content and of the activities of enzymes arylsulphatase, chitinase and acid phosphomonoesterase were noted in soils of area LP-4. The highest mean values of humus and molybdenum contents as well as of leucine-aminopeptidase and alkaline phosphomonoesterase activities were observed in soils of area LP-2, and the highest mean value of β-glucosidase activity was observed in soils of area LP-3.

According to various researchers, heavy metal pollution of soils results in a decrease of soil microbial biomass and enzymatic activity. Kandeler et al. [20] during the studies on soils experimentally polluted by copper, zinc, cadmium, nickel, and vanadium found out that the pollution led to a decrease in urease, alkaline phosphatase, and xylanase activities. Similar results have been observed in Maryland (USA), where field studies have shown a decrease of enzymatic activities in soils polluted by zinc, copper and lead [37]. D'Ascoli et al. [25] found out that the pollution of soils by Cu and Cr(III) resulted in a decrease of fluorescein diacetate hydrolase, dehydrogenase, β-glucosidase, urease, arylsulfatase, and acid phosphatase activities. Wyszkowska et al. [38] observed that in case of simultaneous pollution of soil by some heavy metals also the synergism may take place and the enzymatic activities may decrease more sharply.

Considering the described above, correlation analysis of obtained data has been performed for the determination of the dependence of double-stranded DNA content as well as of the changes of six enzymatic activities on level of soil pollution by copper and molybdenum. As can be seen from Table 1 the soils of LP-1 are the most polluted by copper, the soils of LP-2 are the most polluted by molybdenum, and the soils of LP-4 are the less polluted by both heavy metals.

Table 1

Descriptive statistics of soil some chemical properties, enzyme activities (nM 4-methylumbelliferone or 7-amino-4-methylcoumarin g^{-1} soil h^{-1}) and dsDNA (μg dsDNA g^{-1} dry soil) of studied four areas

Area	Statistical data	*dsDNA*	*aryS*	*beta_G*	*chit*	*leu*	*acP*	*alkP*	*humus, %*	*Cu, mg/kg*	*Mo, mg/kg*
LP-1	Mean	23.75	4.25	5	4.5	15.25	12.75	134.3	3.34	1750	362.5
	Median	22.5	4	5	4	15.5	12.5	134.5	3.26	1550	295
	Standard Deviation	4.35	1.50	0.82	1.73	4.27	4.35	20.32	0.59	506.62	231.86
	Minimum	20	3	4	3	10	9	111	2.74	1400	180
	Maximum	30	6	6	7	20	17	157	4.09	2500	680
LP-2	Mean	38.83	7.33	8.33	7	31.67	20.17	231.33	4.87	821.67	530
	Median	35	5	7	8	28	17	215.5	4.78	795	390
	Standard Deviation	17.10	5.61	3.20	1.55	9.93	15.16	68.43	1.25	288.33	332.39
	Minimum	23	3	6	5	22	7	173	3.10	390	260
	Maximum	71	17	14	8	46	48	336	6.72	1230	1100
LP-3	Mean	30.75	6.25	8.5	8.5	30	20.25	204.8	4.49	1320	193.75
	Median	31	6.5	8	8	28	21.5	215	4.65	955	200
	Standard Deviation	13.07	3.20	2.65	2.52	10.86	10.53	148.12	1.01	1077.93	27.50
	Minimum	18	3	6	6	20	8	48	3.10	470	155
	Maximum	43	9	12	12	44	30	341	5.53	2900	220
LP-4	Mean	39.67	37	5	11.33	29.67	47.33	180	3.55	486.67	126.67
	Median	28	18	4	5	16	31	164	3.00	500	120
	Standard Deviation	38.84	47.44	2.65	12.74	31.79	56.31	150.64	1.57	100.66	50.33
	Minimum	8	2	3	3	7	1	38	2.33	380	80
	Maximum	83	91	8	26	66	110	338	5.33	580	180

Fornasier et al. [39] found out that the content of dsDNA in the soil can be a simple reliable index of microbial biomass. dsDNA content may change depending on both different physico-chemical properties of soil (e.g., humus content) and extent of soil pollution [40]. The data presented in Tables 2–5 show that there is a very strong positive correlation relationship ($r > 0,9$) between contents of humus and dsDNA in the soil of area LP-4 and a strong positive correlation relationship ($r = 0,7–0,9$) between mentioned parameters in the soils of areas LP-1, LP-2 and LP-3. This indicates that the high content of humus facilitates the increase of microbial biomass. The increase of soil microbial biomass in turn resulted in a growth of microbiological activity that is manifested in intensification of plant residue decomposition in soil and increase of nutrients availability for plants. A

positive correlation was observed in almost all of the study areas between dsDNA and studied six enzymes, only in LP-3 a weak negative correlation was revealed with enzymes β-glucosidase (r = –0,477) and chitinase (r = –0,319). Different levels of soil pollution by copper were observed in four areas studied, which, as was demonstrated by correlation analysis results, had different degrees of negative impact on microbial biomass leading to a decrease in dsDNA content. In the case of molybdenum, the situation is slightly different: in the areas where molybdenum high content was observed, a negative correlation was revealed with dsDNA content (r = –0,554 in LP-1 and r = –0,291 in LP-2), and in the case of relatively low molybdenum content, on the contrary, a positive correlation was observed (r = 0,722 in LP-3 and r = 0,989 in LP-4). This indicates that in case of relatively low content of molybdenum in the soil its increase leads to an increase in dsDNA content. Conversely, in the case of relatively high content of molybdenum in the soil its increase leads to a decrease in dsDNA content.

Table 2

Pearson correlation coefficients between contents of some heavy metals and biochemical properties of soil of area LP-1

	dsDNA	arylS	beta_G	chit	leu	acP	alkP	humus, %	Cu, *mg/kg*	Mo, *mg/kg*
dsDNA	1									
arylS	0.524	1								
beta_G	0.657	–0.272	1							
chit	0.863	0.064	0.943	1						
leu	0.901	0.767	0.287	0.563	1					
acP	0.665	0.984	–0.094	0.243	0.848	1				
alkP	0.555	0.960	–0.261	0.062	0.844	0.944	1			
humus, %	0.883	0.184	0.745	0.836	0.770	0.330	0.326	1		
Cu, *mg/kg*	–0.688	–0.636	–0.081	–0.304	–0.901	–0.673	–0.814	–0.721	1	
Mo, *mg/kg*	–0.554	–0.817	–0.035	–0.320	–0.570	–0.852	–0.640	–0.099	0.220	1

Table 3

Pearson correlation coefficients between contents of some heavy metals and biochemical properties of soil of area LP-2

	dsDNA	arylS	beta_G	chit	leu	acP	alkP	humus, %	Cu, *mg/kg*	Mo, *mg/kg*
dsDNA	1									
arylS	0.945	1								
beta_G	0.191	0.215	1							
chit	0.174	0.391	0.564	1						
leu	0.673	0.595	0.821	0.403	1					
acP	0.984	0.973	0.287	0.315	0.715	1				
alkP	0.928	0.907	0.010	0.089	0.493	0.930	1			
humus, %	0.832	0.711	–0.357	–0.267	0.224	0.746	0.852	1		
Cu, *mg/kg*	–0.459	–0.406	–0.512	–0.493	–0.641	–0.449	–0.119	–0.150	1	
Mo, *mg/kg*	–0.291	–0.268	–0.327	–0.501	–0.483	–0.373	–0.345	–0.101	0.219	1

Table 4

Pearson correlation coefficients between contents of some heavy metals and biochemical properties of soil of area LP-3

	dsDNA	aryS	beta_G	chit	leu	acP	alkP	humus, %	Cu, *mg/kg*	Mo, *mg/kg*
dsDNA	1									
aryS	0.974	1								
beta_G	–0.477	–0.334	1							
chit	–0.319	–0.103	0.851	1						
leu	0.934	0.882	–0.290	–0.317	1					
acP	0.974	0.917	–0.664	–0.484	0.865	1				
alkP	0.990	0.955	–0.585	–0.389	0.885	0.994	1			
humus, %	0.770	0.608	–0.622	–0.769	0.844	0.808	0.769	1		
Cu, *mg/kg*	–0.302	–0.505	–0.534	–0.806	–0.245	–0.126	–0.231	0.309	1	
Mo, *mg/kg*	0.722	0.592	–0.951	–0.807	0.569	0.859	0.800	0.787	0.345	1

Table 5

Pearson correlation coefficients between contents of some heavy metals and biochemical properties of soil of area LP-4

	dsDNA	aryS	beta_G	chit	leu	acP	alkP	humus, %	Cu, *mg/kg*	Mo, *mg/kg*
dsDNA	1									
aryS	0.996	1								
beta_G	0.900	0.936	1							
chit	0.943	0.969	0.994	1						
leu	0.993	1.000	0.945	0.976	1					
acP	1.000	0.995	0.896	0.940	0.992	1				
alkP	0.985	0.966	0.813	0.873	0.958	0.987	1			
humus, %	0.999	0.999	0.919	0.957	0.997	0.999	0.977	1		
Cu, *mg/kg*	–0.784	–0.838	–0.976	–0.946	–0.852	–0.779	–0.667	–0.812	1	
Mo, *mg/kg*	0.989	0.972	0.826	0.884	0.965	0.990	1.000	0.981	–0.684	1

Almost the same pattern was observed for the changes in the activities of six enzymes studied in the soils of four different areas. In particular, in the case of copper, a negative correlation with soil enzymes was revealed in all studied areas. In the case of molybdenum, in areas LP-1 and LP-2, where the soil pollution by this metal was relatively high, a negative correlation was observed with all soil enzymes. In area LP-3 a negative correlation with β-glucosidase and chitinase as well as a positive correlation with enzymes arylsulfatase, leucine-aminopeptidase, acid phosphomonoesterase and alkaline phosphomonoesterase was revealed. In the area LP-4, where the average value of molybdenum content was the lowest, a strong ($r = 0,7–0,9$) or a very strong ($r>0,9$) positive correlation was observed with all enzymes studied. In the case of copper in the areas LP-1 and LP-2 a comparatively well-defined negative correlation ($r = –0,901$ and $r = –0,641$, respectively) was observed for leucine-aminopeptidase, that releases leucine and other hydrophobic aminoacids from amino-terminus of polypeptide chains. That is, as a result of soil pollution by copper a suppressive effect on nitrogen cycle took place in areas mentioned. In areas LP-3 and LP-4 the activities of chitinase

(r = –0,806) and β-glucosidase (r = –0,976), respectively were suppressed the most due to soil pollution by copper. Chitinase is a key enzyme to degrade chitine, and β-glucosidase activity is considered as soil quality indicator. It was found out that the latter is sensitive to changes in soil and residue management [41]. In the case of molybdenum, the most pronounced negative correlation was observed in area LP-1 with acid phosphomonoesterase (r = –0,852), in area LP-2 – with chitinase (r = –0,501), and in area LP-3 – with β-glucosidase (r = –0,951). It should be noted that acid phosphomonoesterase is one of the enzymes that play key roles in P cycle.

Conclusion. Summarizing the results of the research, it can be concluded that the content of double-stranded DNA as well as the activities of acid phosphomonoesterase, β-glucosidase, arylsulphatase, chitinase, alkaline phosphomonoesterase, and leucine aminopeptidase in the soil are the indicators of soil biological activity that respond to changes in soil chemical composition. Of the four areas studied, the lowest levels of double-stranded DNA content and six enzymatic activities were registered in the area LP-1 which was exposed to the negative effects of anthropogenic factor the most. Pollution of soil with copper and molybdenum leads to a decrease in microbial biomass and soil enzymatic activity, which in turn has a negative impact on cycles of chemical elements, in particular carbon, nitrogen, phosphorus and sulfur.

Thus, pollution of soils by heavy metals, particularly by copper and molybdenum has a negative effect on nutrient cycling, and the changes in double-stranded DNA content and enzymatic activities may act as biological indicator of soil health.

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Կ. Ա. ՂԱԶԱՐՅԱՆ, Հ. Ս. ՄՈՎՍԵՍՅԱՆ

ՀՈՂԻ ՄԱՆՐԷՆԵՐԻ ԿԵՆՍԱԶԱՆԳՎԱԾԻ ԵՎ ՖԵՐՄԵՆՏԱՅԻՆ ԱԿՏԻՎՈՒԹՅԱՆ ՊԱՏԱՍԽԱՆԸ ՊՂՆՁՈՎ ԵՎ ՄՈԼԻԲԴԵՆՈՎ ԱՂՏՈՏՄԱՆԸ ՋԱՆԳԵԶՈՒՐԻ ՊՂՆՁԱՄՈԼԻԲԴԵՆԱՅԻՆ ԿՈՄԲԻՆԱՏԻ ՇՐՋԱԿԱՅՔՈՒՄ, ՀԱՅԱՍՏԱՆ

Այս ուսումնասիրության նպատակն է եղել բացահայտել կապը ծանր մետաղներով (Cu, Mo) հողի աղտոտման և արտաբջջային տարբեր ֆերմենտների ակտիվությունների միջև: Զանգեզուրի պղնձամոլիբդենային կոմբինատի շրջակայքից վերցված հողի նմուշներում որոշվել են ածխածնի, ֆոսֆորի, ազոտի և ծծմբի շրջապտույտների մեջ ներգրավված վեց ֆերմենտների ակտիվությունները (β-գլյուկոզիդազ, խիտինազ, լեյցին-ամինապեպտիդազ, թթվային և հիմնային ֆոսֆոմոնոէսթերազներ, արիլսուլֆատազ), ինչպես նաև՝ մանրէների կենսազանգվածը: Հետազոտությունները ցույց տվեցին, որ հողի աղտոտումը պղնձով և մոլիբդենով հանգեցնում է մանրէների կենսազանվածի և հողի ֆերմենտների ակտիվության նվազման, որն իր հերթին բացասաբար է ազդում քիմիական տարրերի, մասնավորապես՝ C-ի, P-ի, N-ի և S-ի շրջապտույտների վրա: Սա հիմք է տալիս եզրակացնել, որ հողի մանրէների կենսազանգվածի և ֆերմենտային ակտիվության փոփոխությունները կարող են ծառայել որպես հողի կենսաբանական ակտիվության և որակի ցուցանիշներ:

К. А. КАЗАРЯН, А. С. МОВСЕСЯН

РЕАКЦИЯ МИКРОБИАЛЬНОЙ БИОМАССЫ И ФЕРМЕНТАТИВНОЙ АКТИВНОСТИ ПОЧВЫ НА ЗАГРЯЗНЕНИЕ МЕДЬЮ И МОЛИБДЕНОМ В ОКРЕСТНОСТЯХ ЗАНГЕЗУРСКОГО МЕДНО-МОЛИБДЕНОВОГО КОМБИНАТА, АРМЕНИЯ

Целью данного исследования было определение взаимосвязи между загрязнением почвы тяжелыми металлами (Cu, Mo) и активностями различных внеклеточных ферментов. В образцах почвы, взятых в окрестностях Зангезурского медно-молибденового комбината, были определены активность шести ферментов, участвующих в круговороте углерода, фосфора, азота и серы (β-глюкозидаза, хитиназа, лейцин-аминопептидаза, кислая фосфомоноэстераза, щелочная фосфомоноэстераза и арилсульфатаза), а также микробиальная биомасса. Исследования показали, что загрязнение почвы медью и молибденом приводит к уменьшению микробиальной биомассы и активности ферментов почвы, что, в свою очередь, оказывает отрицательное воздействие на круговорот химических элементов, в частности, C, P, N и S. Это дает основание заключить, что изменение микробиальной биомассы и ферментативной активности почвы может служить индикатором биологической активности и качества почвы.