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EXPERIMENTAL ARTICLES

Estimation of the Total and Active Microbial Biomasses in Buried Subkurgan Paleosoils of Different Age

T. E. Khomutova, T. S. Demkina, and V. A. Demkin

Institute of Physicochemical and Biological Problems of Soil Science, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia

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Abstract—Microorganisms that were isolated from steppe soils buried below kurgans from 5800 to 750 years ago were analyzed for the completeness of isolation, total biomass (the sum of glucose-reactivated and resting microbial cells), and active biomass (metabolically active cells). The metabolic state of microbial communities in buried and modern background soils was estimated from the proportion of active and total biomasses. The paleosoils were found to be characterized by lower total and active biomasses and a lower proportion of active microorganisms as compared to the modern background soils. The age-dependent decrease in the content of active microorganisms in the microbial communities of paleosoils was not monotonic. For instance, the 4000-year-old paleosoil was characterized by a high total biomass and a relatively low content of active microorganisms, whereas the 1950-year-old paleosoil was characterized by a relatively low total biomass and a relatively high content of active microorganisms. This could reflect the temporal dynamics of paleoclimatic conditions in the geographic region under study.

Key words: soil microorganism, total and active biomasses, paleosoils.

Microorganisms are an integral part of soils. Soil microorganisms can long withstand stressful conditions (such as an unfavorable hydrothermal regime and nutritional deficiency) by transforming to a resting state [1]. Some microorganisms produce spores, and others pass to a sporelike resting stage, which is characterized by a small size of microbial cells (less than 0.3 μm), a low level of their metabolism (respiration), and inability to grow and produce colonies under laboratory conditions [2]. It would be reasonable to suggest that most microorganisms of the modern and buried soils of arid regions (dry steppes and semideserts) occur in a resting state. An essential problem of relevant studies is the correct determination of the total biomass of microorganisms occurring at different stages of their life cycles. The current microbiological methods (such as culture methods, direct microscopic count, and physiological methods) make it possible to assess only part of soil microorganisms [3, 4].

In 1977, Faegri *et al.* [5] proposed a rapid fractionated centrifugation technique, which allowed 50 to 80% of soil bacteria to be extracted from peat soils, whereas soil fungi and the rest of the bacteria remain in the soil residue. On the whole, this method makes it possible to extract up to 88% of microorganisms from sandy soils and approximately 30% of microorganisms from loamy soils [6].

In subkurgan paleosoils ranging in age from hundreds to thousands of years, a considerable fraction of microorganisms occur in a resting state. This poses the problem of determining the total biomass of soil microorganisms, which include cells that can be readily reactivated with glucose (arbitrarily called metabolically active cells) and extensively resting cells (such as spores). The isolation of microorganisms from soil and the evaluation of the completeness of their isolation make it possible to calculate the total microbial biomass, whereas the proportion between the total biomass and the biomass of metabolically active microorganisms may be a useful characteristic of the microbial communities of paleosoils and modern background soils.

The aim of this work was to isolate microorganisms from soils, to determine the total biomass of metabolically active and resting microbial cells (including cells that are unable to produce colonies on standard microbiological media), and to estimate the content of metabolically active cells in subkurgan paleosoils of different age and in modern background soils.

MATERIALS AND METHODS

Soils. Investigations were carried out with the paleosoils of ten burial kurgans located in three different dry steppe regions of Volgograd oblast on the Volga and Yergeni uplands. The kurgans were raised 5800 to 750 years ago (in the Eneolithic Period, the Bronze Age, the Early Iron Age, and the Middle Ages). The background modern soils are chestnut and alkali (solonets) soils. Below are given the characteristics of the

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burial kurgans, which are combined according to their geographic location.

The kurgans of the Volga Upland. The Avilov burial kurgan is located on the first and second floodplain terraces of the Ilovlya River, a left tributary of the Don River. The investigated soil chronoseries of this kurgan represented chestnut soil (K) (section D-510) buried 4000 years ago and chestnut soils (K2) buried 1950 (section D-503), 1800 (section D-509), and 750 (section D-504) years ago. Section D-505 of the modern background chestnut soil was located in a virgin region with fescue–wormwood vegetation.

The Malaya Vorobtsovka burial kurgan is located on the first floodplain terrace of the Berdiya River (a left tributary of the Ilovlya River) on fallow land with grass–wormwood vegetation. Sections D-526 and D-525 corresponded to the chestnut soil that was buried 1950 years ago and modern background chestnut soil, respectively.

The kurgans of the Yergeni Upland. The Peregruznoe burial kurgan is located on an intergully watershed with virgin fescue–wormwood vegetation. One of the two studied chronoseries of the subkurgan and modern soils represented dark chestnut soil (K3) buried 5800 years ago (section D-523), chestnut-like soil buried 3900 years ago (section D-520), chestnut soils buried 1950 and 1800 years ago (sections D-518 and D-512, respectively), and modern background chestnut soil (section D-519). The second chronoseries represented solonets soil buried 1950 years ago (section D-516) and modern background solonets (section D-514).

Soil samples were collected aseptically in August 2000 from the A1 horizons of the buried and modern background soils and were kept at a temperature and humidity observed at the time of sampling.

The isolation of microorganisms from soil included the stages of the ultrasonic destruction of soil aggregates [7], the extraction of microorganisms with one-twentieth-strength Winogradsky solution (2 g/l $(NH_4)_2SO_4$, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4$, 0.4 g/l $FeSO_4$, and 2 g/l NaCl), and the obtaining of the aqueous phase of the extract by centrifugation [5]. Specifically, a soil sample (5 g) was suspended in 300 ml of one-twentieth-strength Winogradsky solution and treated ultrasonically for a total of 1 min in 30-s bursts with a 30-s break between the bursts (UZDN-2 ultrasonic disintegrator; 44 kHz; 0.5 mA; titanium probe 15 mm in diameter). The aqueous phase of the extract, which contained microbial cells, was separated from the soil residue by centrifugation at 2000 *g* for 30 min at 4°C and kept in the cold. The soil residue was resuspended in 250 ml of the saline solution and treated as described above. The extraction procedure was repeated three times. The three extracts, which contained not only microbial cells, but also tiny mineral particles and probably water-soluble humic substances, were pooled and centrifuged at 2000 *g* for 30 min to remove mineral particles. The supernatant was centrifuged at 7000 *g* for 90 min to harvest microbial cells. The cells were suspended in 0.1% sodium pyrophosphate, and the cell suspension was dispensed into weighed centrifuge tubes 13 mm in diameter and 75 mm in height. Microbial cells were precipitated by centrifugation at 10000 *g* for 30 min, lyophilized, and weighed. All the solutions were sterilized and cooled to 4°C before use. The extracts were stored at 4°C until further use.

Analysis of organic carbon in microbial fractions. The content of organic carbon (C_{org}) in microbial fractions was determined by the bichromate method [8]. Measurements were performed in triplicate. The standard error of these measurements did not exceed 11%.

The completeness of the extraction of microbial cells was estimated by comparing the content of microbial cells (expressed in colony-forming units, CFU) in the original soil sample and in the extracted microbial fraction. The CFU was determined by plating appropriate serial dilutions onto soil agar. The number of colonies grown on the agar plates was determined after 7 days of incubation at 25°C. The coefficient of the completeness of cell extraction (K_c) was defined as the ratio of 100% to the percent of extracted microbial cells. The analysis was replicated five times.

The total microbial biomass was calculated by the formula

$$TMB = 10\ LF \times C_{org} \times K_c,$$

where TMB is the total microbial biomass expressed in μg/g soil, LF is the mass of the isolated and lyophilized microorganisms expressed in mg/g soil; C_{org} is the organic carbon content in the microbial fraction expressed as a percent; and K_c is the coefficient of the completeness of cell extraction from soil.

The biomass of glucose-reactivated microorganisms was evaluated by the glucose-induced respiration of microbial cells. For this purpose, 15-ml flasks with 2 g of soil were incubated for 1.5–2 h and then flushed out with air. The soil samples in the flasks were moistened with a glucose solution to a soil moisture content corresponding to 60–65% of the total moisture capacity (the resulting content of glucose was 10 mg per g soil). The flasks were sealed and incubated at 22°C for 3–4 h. The amount of CO_2 liberated from the soil to the headspace was determined by using a Chrom-5 gas chromatograph (Czech Republic) equipped with a thermal conductivity detector. The mass of microbial cells with glucose-induced respiration was estimated by the formula described by Anderson and Domsch [4] and expressed in μg C/g soil. All the measurements were performed in triplicate. The results were processed by conventional statistical methods.

Table 1. The main characteristics of subkurgan paleosoils and modern background soils

Section	Soil age	Soil	Depth of A1 horizon, cm	Granulometric composition, %		pH_{aq}	C_{org} of soil, %
				Mud (<0.001 mm)	Clay (<0.01 mm)		
Avilov burial kurgan							
D-510	4000	K	74–86	22	36	8.3	0.4
D-503	1950	K2	48–61	21	43	8.1	0.8
D-509	1800	K2	26–36	21	37	8.7	1.3
D-504	750	K2	90–102	14	35	8.6	0.7
D-505	Modern	K2	0–10	9	26	7.9	1.6
Malaya Vorobtsovka burial kurgan							
D-526	1950	K2	50–61	5	17	8.8	0.3
D-525	Modern	K2	0–10	10	18	7.0	1.0
Peregruznoe burial kurgan							
D-523	5800	K3	35–45	21	35	9.3	1.0
D-520	3900	K	49–58	24	39	8.8	0.6
D-518	1950	K2	21–28	18	31	8.9	0.9
D-512	1800	K2	39–49	ND	ND	8.1	0.7
D-519	Modern	K2	0–10	10	32	8.2	1.6
D-516	1950	S	77–84	7	21	7.7	0.2
D-514	Modern	S	0–8	12	25	7.3	1.0

Note: ND stands for "not determined."

RESULTS AND DISCUSSION

The subkurgan paleosoils under study were found to be slightly alkaline and had less organic carbon and, as a rule, more mud and clay than did the modern background soils (Table 1). The mud and clay particles present in the paleosoils are extracted with microbial cells and can hardly be separated from them [6]. For this reason, the biomass of cells in the microbial fraction extracted from soil was estimated from the content of organic carbon in the supernatant obtained by centrifugation of the fraction. In this case, humic substances were supposed to be removed with pyrophosphate (see the Materials and Methods section). The relative content of the organic carbon of the muddy fraction in chestnut soils is low (approximately 4%) [9] as compared to the error of the determination of organic carbon (11%). Consequently, almost all the organic carbon of the microbial fraction extracted from soil can be referred to microbial cells. As can be seen from Table 2, the percent of organic carbon in the microbial fractions extracted from the soils under study varied from 1.4 to 9% and the content of microbial biomass in these soils varied from 35.1 to 670 μg C/g soil.

It should be noted that the purification of microbial fractions from mineral particles leads to a considerable loss of microbial cells [6]. To estimate the completeness of the extraction of microbial cells from soil, we performed a more detailed analysis of the modern chestnut soil (section D-505). The number of CFU in this soil, which was determined by plating soil solution dilutions on soil agar, was taken to be 100%. The number of CFU in the microbial fraction extracted from this soil was found to comprise 10.6% as compared to that of the soil (Table 3). Since the extraction of microbial cells from soil with saline solutions is not selective, it would be reasonable to suggest that the total number of microbial cells (both capable and incapable of producing colonies) in the microbial fraction extracted from the soil also comprises 10.6% of the total number of microbial cells in the soil. This corresponds to a coefficient of the completeness of cell extraction from soil (K_c) equal to 9.43. This coefficient can be used for the calculation of the total biomass of soil microorganisms, including both resting (unable to produce colonies) and metabolically active microbial cells. Taking into account the fact that the soil chronoseries under study were represented by chestnut soils and genetically related solonets soils, we used the same coefficient K_c = 9.43 for all the modern and buried soils studied (Table 4). The total microbial biomass in the A1 horizon of the modern background soils was found to be 2698.9–6318.1 μg C/g soil. In the same horizon of the subkurgan paleosoils, it was 1.4 to 8.2 times lower.

Table 2. The amounts of microorganisms extracted from the subkurgan paleosoils of different age and modern background soils, as is evaluated by the weight of the lyophilized microbial fraction and by the content of organic carbon

Soil section	Soil age	Lyophilized microbial fraction, mg/g soil	Content of organic carbon, %	Microbial biomass evaluated by organic carbon, μg C/g soil
Avilov burial kurgan				
D-510	4000	3.9 ± 0.3*	4.4	171.6 ± 3.3
D-503	1950	3.5 ± 0.3	5.9	206.5 ± 9.9
D-509	1800	2.1 ± 0.2	4.4	92.4 ± 3.1
D-504	750	16.1 ± 1.2	3.0	481.0 ± 19.4
D-505	Modern	10.0 ± 0.8	6.7	670.0 ± 17.9
Malaya Vorobtsovka burial kurgan				
D-526	1950	4.3 ± 0.3	5.2	223.6 ± 6.1
D-525	Modern	7.1 ± 0.2	9.0	639.0 ± 3.1
Peregruznoe burial kurgan				
D-523	5800	1.0 ± 0.1	5.4	59.4 ± 7.1
D-520	3900	4.5 ± 0.3	1.4	63.0 ± 1.5
D-518	1950	1.3 ± 0.1	2.7	35.1 ± 0.5
D-512	1800	3.9 ± 0.3	3.3	128.7 ± 1.5
D-519	Modern	5.4 ± 0.2	5.3	286.2 ± 6.2
D-516	1950	2.2 ± 0.2	1.9	41.8 ± 0.4
D-514	Modern	5.1 ± 0.0	6.7	341.7 ± 16.0

* The data are the means ± the standard errors of the mean.

To characterize the state of microorganisms in the soils, we estimated the biomass of metabolically active cells and calculated their content in the total microbial biomass (Table 4). The biomass of metabolically active cells in the modern background soils varied from 497.2 to 779.5 μg C/g soil. In the paleosoils, this biomass decreased more steeply than did the total microbial biomass. In the 4000-year-old paleosoil, the biomass of metabolically active cells was 1645 times lower than in the modern background soil.

The proportion of active cells in the total microbial biomass seems to be an informative characteristic of the state of soil microorganisms (Table 4). For instance, in the modern background soils, the biomass of metabolically active cells comprised from 9.2 to 24.2% of the total microbial biomass. This suggests that most of the microorganisms of chestnut soils occur in an extensively resting or nonculturable state, which is in agreement with the finding of Roszak and Colwell that 70% of soil microorganisms are unable to produce colonies [2]. In general, the fraction of active microbial cells in the paleosoils was one to three orders lower than in the modern background soils and tended to decrease with the increasing age of the paleosoil. It should, however, be noted that this fraction was relatively high in the paleosoils that were buried 5800 and 1950 years ago.

We attempted to analyze the relationship between the depth of buried paleosoils and the fraction of active microbial biomass and found that the depth of the upper boundary of the A1 horizon of the paleosoils varied from 21 to 90 cm, which implies that this horizon of the paleosoils is involved in modern biogeochemical processes. Pairwise comparisons of the contents of active microbial biomass in the A1 horizons of the paleosoils that lie at approximately the same depths (the A1 horizons of soils D-509 and D-518 buried 1800 and 1950 years ago and now occurring at depths of 26 and 21 cm, respectively; the A1 horizons of soils D-523 and D-512 buried 5800 and 1800 years ago and now occurring at depths of 35 and 39 cm, respectively; and the A1 horizons of soils D-510 and D-516 buried 4000 and 1950 years ago and now occurring at depths of 74 and

Table 3. The number of CFU in modern chestnut soil (section D-505) and in the microbial fraction extracted from the soil

Object	Million CFU/g soil	%
Original soil	80.3 ± 1.1*	100
Microbial fraction	8.5 ± 0.1	10.6

* The data are the means ± the standard errors of the mean.

Table 4. The total and active microbial biomasses in the subkurgan paleosoils of different age and in modern background soils

Soil section	Soil age	Microbial biomass, μg C/g soil		Fraction of active microbial biomass, %
		total	active	
Avilov burial kurgan				
D-510	4000	1618.2 ± 31.0*	0.4 ± 0.0	0.02
D-503	1950	1947.3 ± 93.6	45.9 ± 3.5	2.4
D-509	1800	871.3 ± 29.6	6.5 ± 1.3	0.8
D-504	750	4554.6 ± 182.7	13.0 ± 6.4	0.3
D-505	Modern	6318.1 ± 169.2	657.9 ± 18.3	10.4
Malaya Vorobtsovka burial kurgan				
D-526	1950	2108.5 ± 57.6	33.7 ± 14.3	1.6
D-525	Modern	6025.8 ± 29.6	552.0 ± 23.4	9.2
Peregruznoe burial kurgan				
D-523	5800	560.1 ± 66.5	10.7 ± 8.3	1.9
D-520	3900	594.1 ± 13.9	0.4 ± 0.0	0.1
D-518	1950	331.0 ± 4.5	63.2 ± 24.9	19.1
D-512	1800	1213.6 ± 13.7	2.0 ± 1.6	0.2
D-519	Modern	2698.9 ± 58.3	497.2 ± 22.2	18.4
D-516	1950	394.2 ± 3.4	6.1 ± 3.7	1.6
D-514	Modern	3222.2 ± 150.7	779.5 ± 72.3	24.2

* The data are the means ± the standard errors of the mean.

77 cm, respectively) showed they differed by 10 to 60 times. Consequently, the observed differences in the active microbial biomasses of the paleosoils are not due to the different depths of these paleosoils. Nor can these differences be explained by modern seasonal variations, since all the soil samples were collected in August 2000 and analyzed simultaneously under the same conditions. Thus, it can be anticipated that the temporal dynamics of the content of active microbial cells in the paleosoils is related to the intense dynamics of paleoclimatic conditions during the last 6000 years in the geographic region under study [10]. Noteworthy is the fact that the subkurgan paleosoils, which have been deprived of plant debris for a long time (for thousands of years), still retain a pool of microbial cells that can be reactivated by glucose (Table 4).

To conclude, the method of the mechanical disintegration of soil aggregates and the extraction of microbial cells from soil, combined with the conventional microbiological method of sample plating onto soil agar, allows the total biomass of soil microorganisms to be determined. The isolation of microbial cells and the evaluation of the completeness of their extraction from one modern background chestnut soil make it possible to determine the total microbial biomass in several chronoseries of chestnut and solonets soils in one geographic region. In order to perform relevant studies in another region, it is necessary to determine the coefficient of the completeness of cell extraction from modern background soils in this region.

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