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EXPERIMENTAL ARTICLES

Extractable Microbial DNA Pool and Microbial Activity in Paleosols of Southern Urals

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Abstract—An evaluation of microbial DNA pools was performed using direct quantitative isolation of DNA from contemporary soils of Southern Urals and paleosols sealed under burial mounds early in the Bronze Age more than 5000 years B.P. Significant regression dependence was found between the biomass and DNA contents in these soils ($R^2 = 0.97$). Activity and dominant ecological strategies of microbial communities of paleosols and contemporary southern black soil were compared from growth parameters obtained by analysis of respiratory curves. The ratio of maximum specific growth rates of soil microorganisms on glucose and on yeast extract was shown to provide an auxotrophy index for soil microbial communities.

Key words: quantitative DNA isolation, growth strategies, soil microbial community.

The oldest viable forms of microorganisms in permafrost and buried soils have been actively studied for the past two decades [1, 2]. It was recently shown that microbial communities of soils sealed under ancient burial mounds [3] as well as complexes of microscopic fungi in excavated ancient Russian settlements [2] differ from contemporary soil microbial communities, possibly conserving the characteristic community features at the time of burial. Unlike metabolic activity in permafrost, that of soil microbial paleoforms is not impossible under the conditions of conservation under burial mounds and in soils of ancient settlements (the soil moisture content in the buried horizons can reach 7–14% and the average annual temperature exceeds 0°C) [3]. The field of archaeological pedology is well acquainted with the necessity to take microbiological parameters into account when studying Holocene soil formation [4]. Soil microbiologists, on the other hand, hold it obvious that modern comprehensive soil studies must borrow heavily from the approaches used in microbiology, pedology, and molecular biology [5]. The problem here lies in the selection of microbiological parameters allowing meaningful comparisons of biomass and activity of microbial communities in soils buried for several millennia. The recent advances in biochemistry, particularly the development of a highly specific ultrasensitive PicoGreen reagent (Molecular Probes Inc.), have made possible quantitation of microbial double-stranded (ds) DNA in highly diluted soil extracts, a procedure that minimizes interference from humic compounds [6]. DNA content was shown to correlate with microbial biomass in both fresh soil samples and samples kept moist at 4°C— for 1–6 months; thus, the DNA content of the soil can be used to characterize the soil microbial community [7]. In addition, it was shown that prolonged storage of desiccated soil (several months to several years) cause a significant decrease in microbial biomass attributed to the partial loss of *r*-strategists [8]. It is thus reasonable to suggest that microbial communities of paleosols, having been persisting in resting state for several millennia, should be different from their contemporary analogues not only in the microbial biomass or DNA content but also in the ecological structure, which should influence the parameters of microbial growth. Nevertheless, the relationship between the amount of DNA extractable from paleosol samples and the biomass and activity of soil microorganisms remains poorly investigated.

The present study was concerned with the relationships between the amount of DNA extractable from contemporary and buried black earth soils of the Southern Urals and kinetic parameters of respiratory activity of soil microorganisms.

MATERIALS AND METHODS

The studies were performed with samples of soil sealed under two burial mounds of the pit-grave culture (3rd millennium B.C.) located on the first terrace above the flood plain of Irtek River (a tributary of the Ural River) near Shumaevo village in the southwestern part of Orenburg Region. The mounds were dated by archaeological and radiocarbon methods. Mound 3 (a part of the Shumaevo burial complex I) and a separate Shumaevo Mound 2 were chosen for analysis. This selection was stipulated by the maximal height of the

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Table 1. Chemical properties of the soils under study

Soil age	Horizon	Organic carbon, C %	Displaceable sodium, mg equiv./100 g	Carbonate CO_2, %	pH_{aq}
>5000 years	A_{diag}	0.54	0.65	0.49	7.56
>5000 years	A1	0.74	1.46	0.39	7.59
5000 years	A1	1.10	1.17	not detected	7.15
Contemporary	A1	0.75	0.04	not detected	7.04

mounds, which exceeded 200 cm in the central part and was 130–200 cm at the section sites. The groundwater level was at least 10–12 m. The average annual precipitation in the study region amounts to 350 mm, while total evaporation exceeds the precipitation 1.5-fold. A 4-cm thick white strip of former turf horizon was found to cover two of the studied buried soils; we followed the nomenclature suggested by Ivanov [9] in terming this layer A_{diag}. This horizon appears in the buried soil profile as a result of diagenetic processes of gleying and mineralization of the buried vegetation at the interface between loose mound material and dense buried soil. The studied paleosols were characterized by the lack of diagenetic carbonates in the buried A1 humus horizons, suggesting that the latter were not percolated by atmospheric precipitation.

Soil samples were taken from the A_{diag} and A1 buried horizons (Mound 3), the A1 horizon (Mound 2), and from the A1 horizon of contemporary southern chernozem. Since the humus of the A1 horizon from the separate Shumaevo Mound 2 was dated at 5030 ± 120 years B.P. by radiocarbon methods (2391 RAS Institute of Geology), we henceforth designate these samples as "5000 years old." Mound 3 of the Shumaevo burial complex I was built 200–300 years earlier; soil samples from this location are thus referred to as ">5000 years old." General features of the studied soils are listed in Table 1; organic carbon content in paleosols is corrected for the post-burial mineralization, which can reach 50% of the initial carbon content [9].

Optimized procedure for direct quantitative DNA isolation with mechanic and enzymatic disruption of the microbial cell wall involved sonication of soil suspension (in Tris-EDTA buffer, pH 8) treatment with aurintricarboxylic acid (a nuclease inhibitor) and sodium dodecyl sulfate, two cycles of quick freeze at –80°C and thaw at +65°C, enzymatic digestion with lysozyme and Proteinase K, and shaking with sterile acid-washed glass beads (Sigma–Aldrich, Inc.) of three different sizes (710–1180, 212–300, and <106 μm) on a Vortex homogenizer at 2000 rpm. The samples were diluted with an equal volume of Tris–EDTA buffer and centrifuged for 10 min at 5500 g. Aliquots of the supernatant were stored at –20°C until analysis.

Quantitation of double-stranded DNA. The supernatant was diluted 1 : 100, and 0.5 ml of the dilution was mixed with 0.5 ml of a 1 : 200 dilution of PicoGreen™ (Molecular Probes). After 4-min incubation, the fluorescence was measured on an SFM-25 spectrofluorimeter (Kontron, Germany) at an excitation wavelength of 480 nm and an emission wavelength of 523 nm. Bacteriophage lambda DNA was used as a standard; samples for the standard curve were prepared in TE in the same way as the experimental samples, with the addition of all reagents in appropriate concentrations.

Kinetic parameters of microbial growth were determined from the dynamics of the rate of CO_2 emission from soil amended with glucose or yeast extract. 10-g soil samples wetted to 60% of total moisture capacity were mixed with glucose (4 mg C per g soil) or yeast extract (40 mg per g soil) and mineral salts: $(NH_4)_2SO_4$ (1.9 mg/g), K_2HPO_4 (2.25 mg/g), and $MgSO_4 \cdot 7H_2O$ (3.8 mg/g). The CO_2 emission rate was measured using a continuous gas analyzer [10]. Preliminary experiments were conducted to determine the optimal concentrations of glucose, yeast extract, and mineral salts for unlimited microbial growth in the soil. To avoid microbial contamination, the soil was not preincubated wet. Acid-washed sand supplemented with all organic and mineral substrates listed above was used as a control. The highest specific rate of microbial growth (μ_m) was determined from CO_2 emission rate values (v) by fitting the data to the equation

$$v = v_0^{pr} e^{(\mu_m t)} + v_0^{w}, \quad (1)$$

where v_0^{pr} pr is the initial rate of productive substrate oxidation, v_0^{w} w is the initial rate of waste oxidation of the substrate to CO_2, and t is time.

The lag phase duration (t_{lag}) was defined as the time from the moment of adding glucose to the time point when the increasing rate of growth-associated respiration (v^{pr}) equals the rate of waste respiration (v^{w}). The lag was calculated from the above-described parameters of the fitted respiration rate curve according to the equation

$$t_{lag} = \ln(v_0^{w}/v_0^{pr})/\mu_m. \quad (2)$$

Biomass of active soil microorganisms (X_0') was calculated from the parameters found in Eq. (1), according to the equation [11]

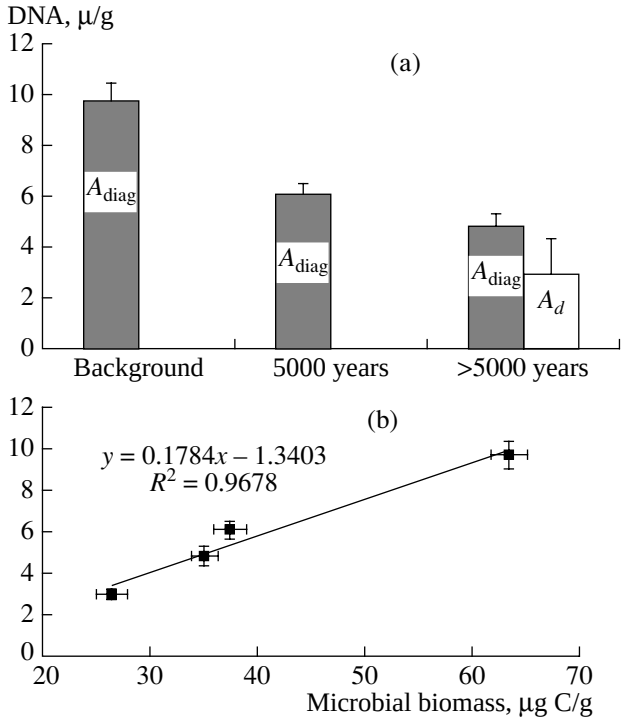

Fig. 1. (a) Microbial DNA content in paleosols and corresponding contemporary soil and (b) the regression dependence of the amount of extractable DNA on the total microbial biomass in the soil.

$$X_0' \approx v_0^{pr}/\mu_m. \quad (3)$$

The coefficient of the physiological state of microorganisms (r_0) was calculated from the rates of waste and productive respiration [12]:

$$r_0 = v_0^{pr}0.1/(v_0^{w} + v_0^{pr}0.1). \quad (4)$$

The total microbial biomass was calculated from the equation

$$X_0 = X_0'/r_0[11]. \quad (5)$$

The coefficients of Eq. (1) were determined by least-square fitting of the experimental data using Model Maker v2.0C software (SB Technology Ltd.). The approximation was restricted to the part of the curve that yielded the highest values for the *Q* and *r* statistical criteria. DNA content was analyzed in four independent experiments, and kinetic parameters of microbial growth in two independent experiments.

RESULTS AND DISCUSSION

Microbial DNA content. Figure 1a illustrates the content of extractable DNA pools in contemporary and buried soils. The DNA amount varied from 9.8 (contemporary soil) to 2.9 μg/g. The lowest DNA content was found in a diagenetic (formerly turf) horizon of the longest-buried soil; DNA content correlated strongly with the total microbial biomass of the soils studied ($R^2 = 0.97$, Fig. 1b) and decreased with a decrease in the total carbon content in the soil (Fig. 1a, Table 1). DNA content in paleosols, calculated from the slope of the regression line (0.172, Fig. 1b), was 7.74% of the dry microbial biomass (taking the carbon content of microbial cells as 45% [13]). This calculated DNA content in the microbial biomass of paleosols is comparable with the 9% value reported in the literature [14] and falls within the interval of DNA content in soil bacteria, 2.2 to 13% [15]. Values for the total microbial biomass determined by the kinetic method were very low (Table 2). Since the microorganisms in buried soils persisted under conditions of stress for several millennia, it is likely that the microbial biomass of the paleosols was much higher at the time of burial than it is now. It was shown that resting forms of microorganisms after prolonged conservation under unfavorable conditions can lose their viability while keeping intact cell morphology [15]. The presence of such "mummified" microbial forms in paleosols could be the reason for the observed high DNA content coupled with a relatively low microbial biomass of microorganisms retaining their viability.

Kinetic parameters of microbial growth. Two different organic/mineral mixtures were used to study parameters of growth activity and metabolic diversity of microorganisms. Glucose substrate supplemented

Table 2. Parameters of activity and growth of microorganisms on glucose and yeast extract in contemporary and buried soils

Soil age and horizon	μ_m, h^{-1}		Active biomass, μg C/g		Total biomass, μg C/g		Lag phase, h	
	glucose	yeast extract	glucose	yeast extract	glucose	yeast extract	glucose	yeast extract
Contemporary, A1	0.29	0.57	0.137	0.014	63.4	24.8	13.1	9.1
5000 years, A1	0.09	0.10	0.110	1.063	37.5	33.3	38.9	11
>5000 years, A1	0.18	0.31	0.042	0.044	35.1	18.7	24.6	12.3
>5000 years, A_{diag}	0.22	0.59	0.088	0.003	26.5	14.1	15.7	10.3

with mineral salts is customarily used as an inducer of respiratory activity of the bulk of soil heterotrophic microorganisms [16], whereas yeast extract, in addition to easily available carbon and basic mineral nutrients, contains readily available vitamins and growth factors required for some species. In contemporary soils the respiratory curves of microbial growth on glucose and yeast extract were quite different (Fig. 2). The growth on yeast extract became exponential much earlier and the increase in respiration rate was sharper than on glucose. The maximal specific growth rate, calculated according to Eq. (1), was almost twofold higher with yeast extract than with glucose (Table 2), indicating that the community of soil microorganisms was predominantly exploiting *K*-strategy when growing on glucose but shifted towards *r*-strategy when feeding on yeast extract. The values for total and active biomass, and the fraction of active microorganisms in total biomass, either were higher when growing on glucose than (except for the "5000 years A1" source) or were not significantly different from the corresponding values for growth on yeast extract (Table 2). We conclude that amending the soil with yeast extract, a readily available substrate rich in trace elements and growth factors, does not lead to the detection of high microbial biomass but rather establishes the conditions to take advantage of alternative metabolic pathways and provides selective advantage to microorganisms that are capable of fast growth on rich media but require growth factors. This, the ratio of maximal specific growth rates on glucose and on yeast extract, μ_{gl}/μ_{ye}, may serve as an auxotrophy index of the soil microbial community (Fig. 2b). With this index increasing and approaching unity, the fraction of a microbial community requiring additional growth factors for metabolic activation decreases, and the community becomes dominated by K-strategy microorganisms. A low auxotrophy index is indicative of large differences in growth rates of a microbial community feeding on glucose and on yeast extract, which means that a larger fraction of the microbial community is capable of fast growth on rich media and that the community is dominated by r-strategists.

Figure 3 shows the dynamics of the CO_2 emission rate in contemporary and buried soils after the addition of glucose (Fig. 3a) and yeast extract (Fig. 3b). The differences in the shape of the curves observed for contemporary soil were also found for all paleosols studied. However, the respiration rate curves of microorganisms from buried soils were more gently sloping with either substrate than curves for the contemporary soil. Interestingly, whereas the kinetics of microbial growth was similar in the most ancient soil samples and in the contemporary soil, kinetic parameters of the microbial community from the 5000 year old soil were in sharp contrast with both the contemporary soil and the older soils. Unlike soils in other samples, the 5000-year-old soils displayed an insignificant increase in the maximal specific growth rate on transition from glucose to yeast extract. Therefore, the auxotrophy

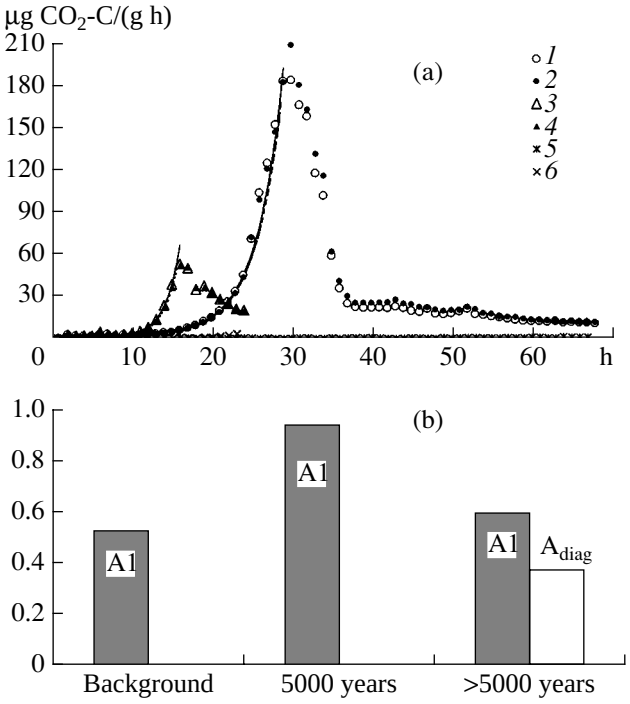

Fig. 2. (a) Changes in the rate of CO_2 emission by microorganisms from contemporary soil during growth on (*1*, *2*) glucose and (*3*, *4*) yeast extract. Controls were sand supplemented with (*5*) glucose or (*6*) yeast extract. Symbols denote experimental data; curves are plotted according to Eq. (1) with parameters obtained by fitting. (b) The values of auxotrophy index in contemporary and buried soils.

index in these soils was close to unity (Fig. 2b), suggesting that their microbial communities are unable to grow fast on rich substrates and maintain a low diversity of metabolic pathways. The total microbial biomass of the 5000-year-old soils was lower than for the contemporary soils (Table 2) but exceeded the biomass of the same soil horizon >5000 years of age, either slightly when growing on glucose or nearly twofold when growing on yeast extract. The active biomass value of the 5000-year-old soil was especially high with yeast extract (Table 2). Thus, addition of an enriched substrate did not change the growth strategy of the microbial community in the 5000-year-old soil, i.e., although yeast extract increased the number of actively growing microorganisms, this additionally activated fraction of the microbial community had the same growth parameters as the microorganisms growing on glucose.

Of all soils studied, the diagenetic turf horizon of more than 5000 years of age harbored the microbial community that was the most active metabolically and the most dependent on the presence of growth factors in the substrate, having the lowest auxotrophy index (Fig. 2b). This could possibly be explained by higher abundance of various nutrients of both plant (plant debris, root secretion) and microbial origin in this hori-

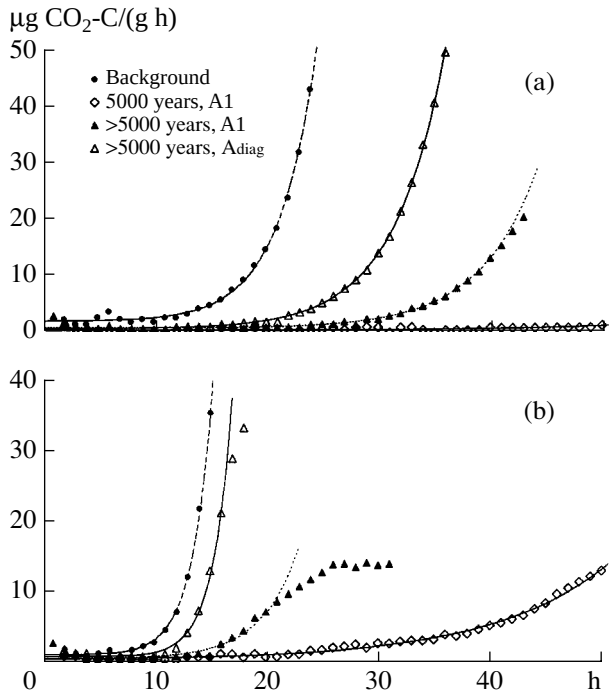

Fig. 3. Respiration curves in contemporary and buried soils during microbial growth on glucose (a) and yeast extract (b). Symbols denote experimental data; curves are plotted according to Eq. (1).

zon, as compared with a predominantly mineral A1 horizon.

In all samples studied, duration of the lag phase of the microbial growth on yeast extract was not significantly different from the contemporary samples, and was much shorter than for the growth on glucose (Table 2). The shortest lag phase was observed in contemporary soils and the longest one in the A1 horizon of buried soils. Interestingly, the time required for a metabolic shift to the active growth state was the longest in the soil with the highest auxotrophy index.

The values for microbial carbon content obtained in our experiments are comparable with the results from other laboratories for the corresponding horizons of buried light chestnut soils and solonetz soils [3]. Although the total microbial biomass was significantly lower in paleosols than in the contemporary soil (Table 2), this difference cannot be assumed a characteristic feature of ancient soils, since a fraction of microbial biomass is irreversibly lost during prolonged conservation under conditions unfavorable for growth [15].

The values of μ_m during the growth on glucose were much lower in paleosols than in the contemporary soil (Table 2), suggesting the relative predominance of *K*-strategists in the buried soils. These differences could be explained by irreversible loss of viability by a fraction of fast-growing microorganisms after several millennia of conservation. This, however, does not explain why the microbial growth in the 5000-year-old paleosols is much lower than in soils >5000 years of age. Similar conditions of conservation of microorganisms buried in paleosols with a time difference of 200-300 years can be inferred from similar location of the burial mounds, their similar height, and the presence of distinct A_{diag} horizons. Thus, the observed differences in the values of μ_m in the soils 5000 and >5000 years of age likely stem from differences in their microbial communities at the moment of burial. The morphological features of soil profiles suggest that the climatic conditions at the time of burial ca. 5000 years ago were more humid than before that or at present. This is evident from the highest humus content in the A1 horizon of the 5000-years-old soil (Table 1), a decrease in readily displaceable sodium ions in comparison with the same horizon of earlier-buried soil (Table 1), a better-leached carbonate profile, and a total lack of newly formed carbonate particles in the A1 horizon along with their presence in the same horizons of other soils studied by us. These observations support the idea of a leaching water regime existing before soil burial 5000 years ago, suggesting that the microbial community that formed in this soil turned out not to be capable of fast growth on the available substrates after prolonged conservation. To reveal the characteristic features of such a community, more detailed studies on the influence of climatic conditions on the stability of soil microbial communities are required.

We conclude that the amount of extractable DNA strongly correlates with the total microbial biomass and reflects the size of genetic material pools of microorganisms preserved in paleosols. However, the DNA content does not reflect the ability of these microorganisms to be activated physiologically and metabolically. The study of kinetic parameters of microbial growth on glucose and yeast extract provides additional information on the activity and dominant ecological strategy of microbial communities in buried soils.

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