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EXPERIMENTAL ARTICLES

Comparative Analysis of the Functional Activity and Composition of Hydrolytic Microbial Complexes from the Lower Volga Barrow and Modern Chestnut Soils

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Abstract— The structure and specific characteristics of the hydrolytic microbial complexes from chestnut paleosols buried under the barrows of different ages (~4500 and ~3500 years) was compared with their modern analogue in microcosm experiments. Potential activity of the hydrolytic complex of the microbial community of the barrow paleosols was found to be higher than in the modern soil complex. The share of metabolically active cells revealed by FISH after the introduction of a growth-stimulating polysaccharide into the paleosol microcosm was 50% of the whole prokaryotic cell number. The paleosol community exhibited a more pronounced response to addition of the substrate than the modern soil community. The differences in the phylogenetic taxonomic structure of the prokaryotic metabolically active hydrolytic complex in the buried and modern soils were revealed. The hydrolytic complex of modern soil was more diverse, while the dominant hydrolytic organisms revealed in paleosols were unicellular and mycelial *Actinobacteria*, as well as *Proteobacteria*.

Keywords: paleosols, modern chestnut soil, hydrolytic microbial complex, FISH, number of active cells, phylogenetic structure

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The properties of paleosols as indicators of the biosphere development in pleistocene and galien gain increasing attention of researchers for evaluation of the possible consequences of global changes of environment and climate. Additionally, there is a need to solve a number of theoretical and applied problems of investigation and rationalization of nature management. For example, the group headed by Zvyagintsev and Gilichinskii revealed that Arctic and Antarctic microbial communities of permafrost deposits of various ages were reliably preserved and maintained their original characteristics [1–3]. In recent years, studies on the state of microbial communities of paleosols at settlement or burial (kurgan) ground archeological sites gained attention [4–9]. Characterization of a microbial community of subkurgan paleosols is an important diagnostic indicator of the processes in the biosphere in the past epochs of Holocene [10, 11], while the isolated active strains of microorganisms may be used in biotechnology. Microbial hydrolytic complexes of ancient microbial communities therefore draw much attention. The fungal component in late Pleistocene and Holocene burial soils was found to be mainly represented by spore mass rather than the mycelium; no pronounced activation of fungal hydrolytic organisms was observed [12]. There is practically no information on the bacterial component of the hydrolytic complexes of buried soils.

The goal of the present work was to carry out a comparative study of the taxonomic structure of a metabolically active hydrolytic (chitinolytic and pectinolytic) bacterial complex from microbial communities of subkurgan soils of various ages and from modern chestnut soils of the Lower Volga dry steppe region.

MATERIALS AND METHODS

Region of the studies. The studies were performed in the dry steppe zone of the Lower Volga region on the territory of Volgograd oblast. The climate of the region is moderately continental. Mean annual precipitation is 380–400 mm and mean annual temperature is 6–8°C.

Subjects of the study were the subkurgan and modern chestnut soils and microbial soil communities of two key regions of burial mounds located to the southeast of the Central Russian Upland (Perekopka site)

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Table 1. Some chemical characteristics of the A1 horizon of studied soils

Section no.	Thickness and depth of the A1 horizon deposition, cm	C_{org}. %	$pH_{aq.}$	Total salts, %	$CaCO_3$, %
Subkurgan chestnut residually solonchak-like non-saline paleosol, Perekopka, middle of the third millennium BC (~4500 years ago)					
E-28	206–215	0.14	7.6	0.13	0.8
Subkurgan chestnut non-alkali non-saline paleosol, Salomatino, 16th–15th cc. BC (~3500 years ago)					
D-652	50–61	0.47	8.3	0.07	1.8
Modern chestnut residually solonchak-like deeply salted soil					
–	2–10	1.16	7.2	0.05	0.4

and south of Volga Upland (Salomatino site) in the Don river basin.

The Perekopka burial mound is located 2 km to the southwest of the village Perekopka in Kletskii region, on the border between the upland part of the interfluve and the smooth slope of southwestern exposition at 100–105 m.a.s.l. At the depth of 80 cm, the layer of clay-loamy soil is supported by sandy deposits with inclusions of gravel, crushed limestone, and sandstone. Kurgans are located in an ancient deposit zone with restored natural vegetative cover dominated by a wormwood–forb–fescue association. Projection coverage is ~80%. The kurgan construction dates back to the middle of the third millennium BC or ~4500 years ago (catacomb culture of the Middle Bronze Age). Its height is over 2 m and its diameter is ~40 m. The subkurgan paleosol belongs to chestnut residual solonchak-like non-saline soils (section E-28). Its morphological and chemical properties evidence that the climate of this region in the middle of the third millennium BC was drier than today [13].

The Salomatino burial kurgan is located 5 km to the north of Salomatino village in Kamyshinskii region, at the edge of the flat part of interfluve of the Ilovlya and Bol'shaya Kazanka rivers. Surface deposits down to 50–60 cm are represented by induviate sandy loess loam, which are supported by deluvial iron gleyed sandy clay material with inclusions of alluvium and broken stone. Surface height is 170–175 m.a.s.l. The area is virgin. The vegetative cover is dominated by a forb–fescue–stipa association with projection coverage of 90%. The studied kurgan was built in the 16th–15th centuries BC or ~3500 years ago (block house culture of the Late Bronze Age). Its height is approximately 50 cm and its diameter is 30 m. The subkurgan paleosol is classified as a chestnut non-alkali non-saline soil (section D-652). Its morphological and chemical characteristics and properties of the microbial community provide evidence that in the first half of the second millennium B.C. a change from extremely dry to more humid climate occurred, and in the 16th–15th centuries BC and the following 100–200 years mean annual precipitation exceeded the modern values by 40–50 mm [14].

Studies of the modern background chestnut's residually alkalinized deeply salinized soil were performed on an old deposit fragment at the top of flat interfluve of the rivers Sakarka and Tishanka (left-side tributaries of Don) 5 km to the east of Kachalino station of the Ilovlinskii district (southern Volga Upland). The soil-forming materials are loess-like carbonate clay loams. Vegetative cover is represented by forb–wormwood–fescue association. Projection coverage is 60–70%.

Microbiological studies were performed with samples (5 g) of the A1 horizon from subkurgan and modern chestnut soils; some characteristics of the soils are presented in Table 1.

The structure of the hydrolytic microbial complex in the samples under study was determined by the microcosm method with initiation of microbial succession by humidification and introduction of purified polysaccharides: chitin (ICN Biomedicals, Germany), pectin, and starch (Sigma, Germany) at concentrations of 0.2% (10 mg/5 g soil). Soil humidified with water (1 mL/5 g soil) without a substrate was used as a control.

Carbon dioxide emission from soil samples under study was determined using a gas chromatograph equipped with a heat conductance detector and a 3-m column filled with Polysorb-1 at the carrier gas (He) flow rate of 25 mL/min. During the microcosm incubation at room temperature, carbon dioxide accumulation in the gaseous phase over the experimental samples and the control was determined daily. The experiment lasted for 10 days.

Bacterial cell numbers, mycelium length, and biomass of actinomycetes and fungi were determined by the fluorescence microscopy (Axioskop 2 plus, Zeiss, Germany) on days 0 (immediately after the sample humidification), 3, and 10 [15].

The number of microbial cells contained in 1 g soil was calculated according to the formula: $N =$

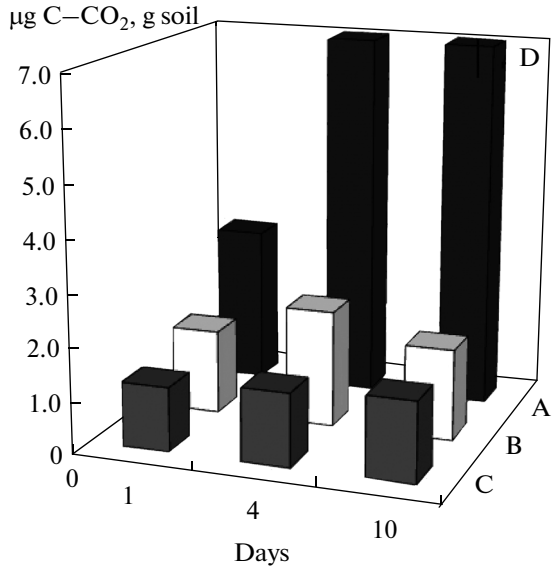

Fig. 1. Dynamics of CO_2 emission by samples of soil in the control variants: modern chestnut soil (A); soil buried 3500 years ago (B); and soil buried 4500 years ago (C).

S_1an/vS_2c, where N is the number of cells (mycelium length, μm) per 1 g soil; S_1, the area of the preparation (μm^2); a, the number of cells (mycelium length, μm) in a single field of view (averaged over all preparations); n, soil suspension dilution factor, mL; v, volume of the drop applied to a slide, mL; S_2, area of the microscope fields of view, μm^2; and c, weighed amount of soil, g; specific mass of microorganisms was accepted as 1 g/cm^3; water content in the cells was accepted as 80%. The biomass of microbial cells was calculated using the following parameters of dry biomass for: (1) mass of a single bacterial cell with the volume of 0.1 μm^3 is 2×10^{-14} g; (2) 1 m of actinomycete mycelium 0.5 μm in diameter weighs 3.9×10^{-8} g; and (3) 1 m of the fungal mycelium with arbitrary diameter of 5 μm weighs 3.9×10^{-6} g [16]. Taking into account the measured diameter of the fungal mycelium, the biomass was calculated according to the following formula: $0.628r^2 \times 10^{-6}$ g [15].

To elucidate the patterns of microbial transformation of carbohydrate polymers in buried soils, the coefficients of microbial response to introduction of chitin (CMRC) and pectin (CMRP) were calculated: CMRC (CMRP) = b/b_0, where b is the biomass of a certain group of microorganisms in the case of chitin (pectin) introduction and b_0 is the biomass of this group of microorganisms in the control variant [17].

Diversity and abundance of metabolically active cells representing individual phylogenetic groups of chitinolytic microorganisms in soil samples were determined using fluorescence in situ hybridization (FISH).

An array of probes specific to the representatives of the *Archaea* and *Bacteria* domains, as well as to individual phylogenetic groups of bacteria [17], was used in the work. Hybridization of the preparations with fluorochrome-labeled probes (Cy3) was performed as reported in [18] at 46°C. Hybridization conditions used for various probes, formamide concentrations in hybridization buffer, and concentration of NaCl in the washing buffer are reported in Table 2. Total cell numbers, including the quiescent forms, were counted in the samples stained with acridine orange under an Axioskop 2 plus (Zeiss, Germany) microscope equipped with Filter set 15 for the probes and Filter set 09 for acridine orange staining.

RESULTS AND DISCUSSION

The parameters characterizing initiation and succession of microbial communities in buried and modern soils were compared in the course of microcosm development. Analysis of the absolute values of carbon dioxide emission (Fig. 1) and biomass (especially for actinomycetes and fungi) in the control soil samples (without addition of polysaccharides) revealed a gradual decrease of the values with increasing age and depth of deposition of the soil samples (Table 3). In modern soils, the determined activities were significantly higher than in the buried ones. This feature may probably be explained by the paleoclimate conditions described previously [14], with the extreme dry climate (the third millennium BC) having changed to a more humid one (the second millennium BC) [13, 14]. These tendencies did not change upon addition of polysaccharide substrates (experimental samples). The values of CO_2 emission by buried soil samples with polysaccharides were higher than in the control samples. Notably, in the sample from 2-m depth (no. 50, 206–215 cm) incubated with pectin, CO_2 emission reached 5 μg $C–CO_2$/g soil per day after 4 days of the experiment, which was five times higher than in control variants (1 μg $C–CO_2$/g soil per day). This was probably the result of certain specific features of the ancient microbial community, where the organisms capable of pectin hydrolysis predominated.

Analysis of the dynamics of biomass accumulation in the control and experimental samples (Table 3) confirmed the data on carbon dioxide emission (Fig. 1); the samples of buried soils responded much better to introduction of organic substrates (biomass increased 5–7-fold if compared to control) than the samples of modern soils. Actinomycete biomass in soils under study increased considerably upon addition of the substrates: four- to eightfold and up to 50-fold for addition of pectin and chitin, respectively. Upon succession on chitin, the length of actinomycete mycelium reached 600 m in the sample of soil buried 3500 years ago.

The characteristics of microbial transformation of carbohydrate polymers in buried soils were confirmed by analysis of the coefficients of microbial response to introduction of chitin (CMRC) and pectin (CMRP)

Table 2. rRNA-specific oligonucleotide probes used in the study notes: a, formamide concentration in hybridization buffer; b, concentration of NaCl in washing buffer; c, Y = C or T, W = A or R, R = A or G; d, target molecule is the 23S rRNA; e, f, probe is used in combination with an unlabeled oligonucleotide 5'-ATAGTTACGGCCGCCGT-3'; g, equimolar mixture of three labeled oligonucleotides

Probe	Target group of organisms	Target fragment of 16S rRNA	Nucleotide sequence of the probe (5'–3')	Formamide, %[a]	NaCl, mM[b]	Reference
EUB338 I EUB338 II EUB338 III	*Bacteria* *Bacteria* (*Planctotomycetales*) *Bacteria* (*Verrucomicrobiales*)	338–355	GCT GCC TCC CGT AGG AGT GCA GCC ACC CGT AGG TGT GCT GCC ACC CGT AGG TGT	20	225	[18]
ARCH915	*Archaea*	915–934	GTG CTC CCC CGC CAA TTC CT TCG	30	112	[19]
ALFlb ALF968	*Alphaproteobacteria*	19–35 968–986	CGT TCG YTC TGA GCC AG[B] GGT AAG GTT CTG CGC GTT	20	225	[20, 25]
BET42a	*Betaproteobacteria*	1027–1043[d]	GCC TTC CCA CTT CGT TT	35	80	[20]
GAM42a	*Gammaproteobacteria*	1027–1043[d]	GCC TTC CCA CAT CGT TT	35	80	[20]
SRB385Db	*Deltaproteobacteria*	385–402	CGG CGT TGC TGC GTC AGG	20	225	[22]
CF319a CFB560	*Bacteroidetes*	319–336 560–575	TGG TCC GTG TCT CAG TAC WCC CTT TAA ACC CAR T	35 30	80 112	[20]
HGC69a	*Actinobacteria*	1901–1918[d]	TAT AGT TAC CAC CGC CGT[e]	25	159	[17, 25]
LGC354A, LGC354B, LGC354C[e]	*Firmicutes*	354–371	TGG GAA GAT TCC CTA CTG C, CGG GAA GAT TCC CTA CTG C, CCG GAA GAT TCC CTA CTG C	35	80	[20]
HoAc1402	*Acidobacteria*	1401–1420	CTT TCG TGA TGT GAC GGG[d]	10	450	[17, 25]
Verl38 Verl455	*Verrucomicrobia*	138–155 1455–1472	CGA GCT ATT CCC CTC TTG CCA TCC ATA CCT TCG GCA	10	450	[25]
PLA46 PLA886	*Planctomycetes*	46–63 886–906	GAC TTG CAT GCC TAA TCC GCC TTG CGA CCA TAC TCC C	30 30	112 112	[21]

[a], Formamide concentration in hybridization buffer; [b], NaCl concentration in washing buffer; [c], Y = C or T, W =A and T, R = A G; [d], Target molecule, 23S rRNA; [e], The probe was used with the unlabeled oligonucleotide; [f], Equimolar mixture of three labeled oligonucleotides; [g], The probe was used with the unlabeled oligonucleotide 5'-CTTTCGTGACGTGACGGG -3'.

Table 3. Biomass of bacteria, actinomycetes, and fungi in control and experimental samples of studied soils on day 10 of succession

Microbial group	Substrates	Biomass, mg/g soil		
		modern soil	soil buried 3500 years ago	soil buried 4500 years ago
Bacteria	Control	0.019 ± 0.002	0.033 ± 0.009	0.016 ± 0.003
	Pectin	0.025 ± 0.003	0.039 ± 0.005	0.024 ± 0.006
	Chitin	0.025 ± 0.003	0.042 ± 0.001	0.043 ± 0.011
Actinomycetes	Control	0.004 ± 0.002	0.002 ± 0.002	0.000 ± 0.000
	Pectin	0.009 ± 0.005	0.008 ± 0.003	0.003 ± 0.002
	Chitin	0.009 ± 0.005	0.024 ± 0.003	0.019 ± 0.004
Fungi	Control	0.102 ± 0.060	0.062 ± 0.050	0.000 ± 0.000
	Pectin	0.138 ± 0.070	0.094 ± 0.053	0.296 ± 0.198
	Chitin	0.117 ± 0.060	0.066 ± 0.019	0.014 ± 0.009

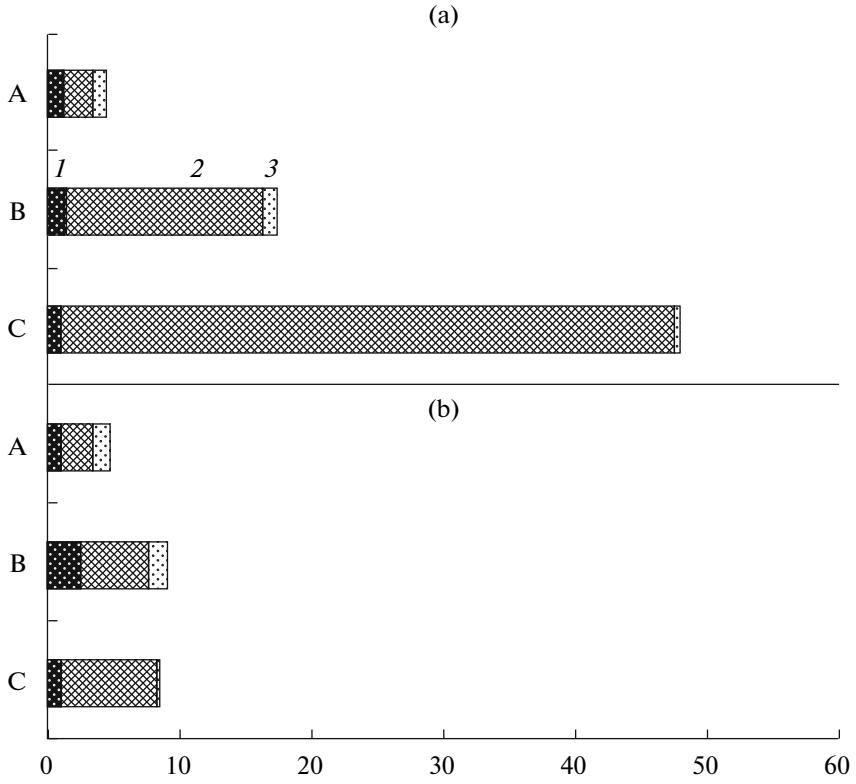

Fig. 2. Total (throughout the whole period of succession) response of the biomass of bacteria (*1*), actinomycetes (*2*), and fungi (*3*) to the introduction of chitin (a) and pectin (b) to the samples of studied soils. A, modern chestnut soil; B, soil buried 3500 years ago; and C, soil buried 4500 years ago. The response was calculated as a value b/b_0, where b is the biomass of a certain group of microorganisms in the variant with chitin (pectin) and b_0 is the biomass of this group of microorganisms in control variant.

(Fig. 2). Analysis of these coefficients demonstrated that (1) the intensity of response of microbial hydrolytic complexes increased with changes in paleoclimate conditions from dry to more humid ones and with the increase in the depth of deposition of soil samples, from smaller (Fig. 2a) to greater (Fig. 2b) burial age; (2) the greatest response in buried soils was produced by mycelial prokaryotes (Fig. 2, position *2*); (3) the role of fungi increased upon succession on pectin (Fig. 2b, position *3*); and (4) the prokaryotic complex was involved in hydrolysis of both substrates more actively (Fig. 2, positions *1* + *2*) than the eukaryotic one (Fig. 2, position *3*).

The level of metabolic activity of the prokaryotic constituent of microbial communities in studied soils was analyzed by the method of fluorescence in situ hybridization (FISH) with rRNA-specific fluorescently labeled oligonucleotide probes. FISH is widely used for studies of microbial communities of marine and freshwater ecosystems, activated sludge and bioreactors, peats and plant rhizosphere, as well as other natural and anthropogenic habitats [19–22, 27]. The technique allows for simultaneous molecular identification, enumeration, and localization of physiologically active bacterial cells in natural samples in situ, which is an advantage over fingerprinting techniques, such as T-RFLP, DGGE, and analysis of fatty acid profiles [18–26].

Total numbers of metabolically active unicellular organisms (Fig. 3, positions *1* and *2*) in samples of modern soil (3.5×10^9 cells/g soil) (Fig. 3a) was much higher than in the buried soil ($0.5–1 \times 10^9$ cells/g) (Figs. 3b and 3c). However, the fraction of cells identified as metabolically active was comparable in all soil samples (30–40% of the total cell number). Numbers of the representatives of the *Bacteria* domain varied within the range form 1.5×10^8 to 1.7×10^9 cells/g soil and increased upon addition of polysaccharides (Fig. 3). The numbers of the representatives of the *Archaea* domain hybridized with probes ARCH915 and ARC344 were an order of magnitude lower and varied within the range from 3.0×10^7 to 1.0×10^8 cells/g soil (Fig. 3). Importantly, the value of metabolically active prokaryotes shown on Fig. 3 includes only the numbers of unicellular bacteria and does not include mycelial actinobacteria (due to the differences in measurement units of the analyzed parameters: mycelium length for actinomycetes and the cell number unicellular bacteria).

Total biomass of metabolically active prokaryotes in the control samples, which is formed by the biomass of unicellular prokaryotes (according to FISH data)

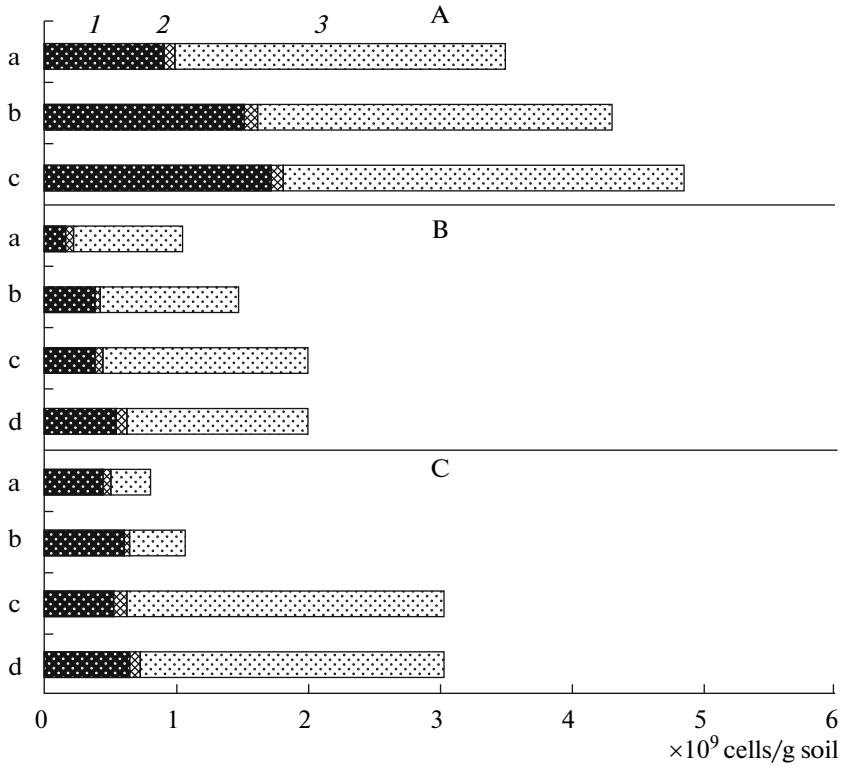

Fig. 3. Counts of active unicellular prokaryotic representatives of *Eubacteria* (*1*), *Archaea* (*2*), and unidentified organisms (*3*) after soil humidification and introduction of the substrate (on day 10 of the experiment). A, modern chestnut soil; B, soil buried 3500 years ago; and C, soil buried 4500 years ago. Humidified samples (control) (a); samples with pectin (b); samples with chitin (c); and samples with starch (d).

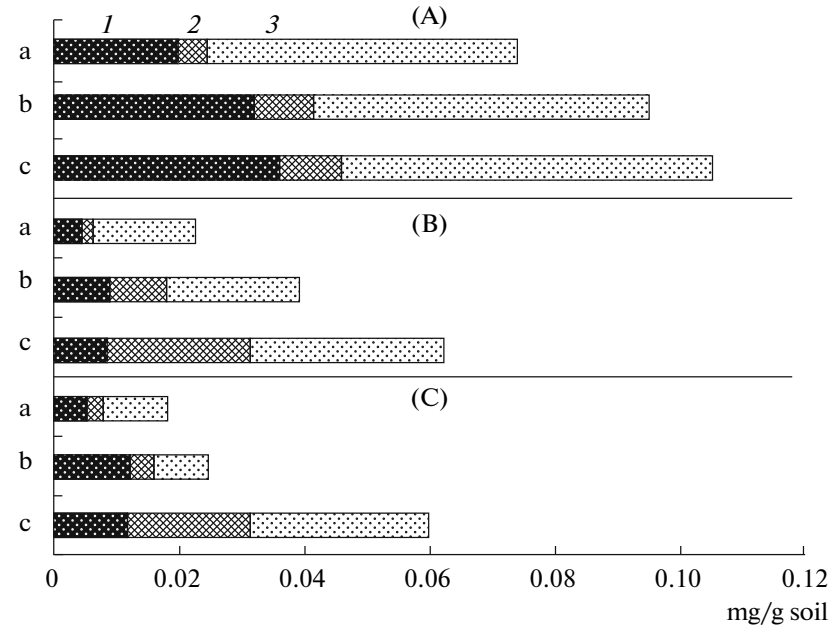

Fig. 4. Amount of active biomass of unicellular (*1*) and mycelial (*2*) prokaryotes and of unidentified cells (*3*) after soil humidification and introduction of substrate (on day 10 of the experiment). A, modern chestnut soil; B, soil buried 3500 years ago; and C, soil buried 4500 years ago. a, Humidified samples (control); b, samples with pectin; and c, samples with chitin.

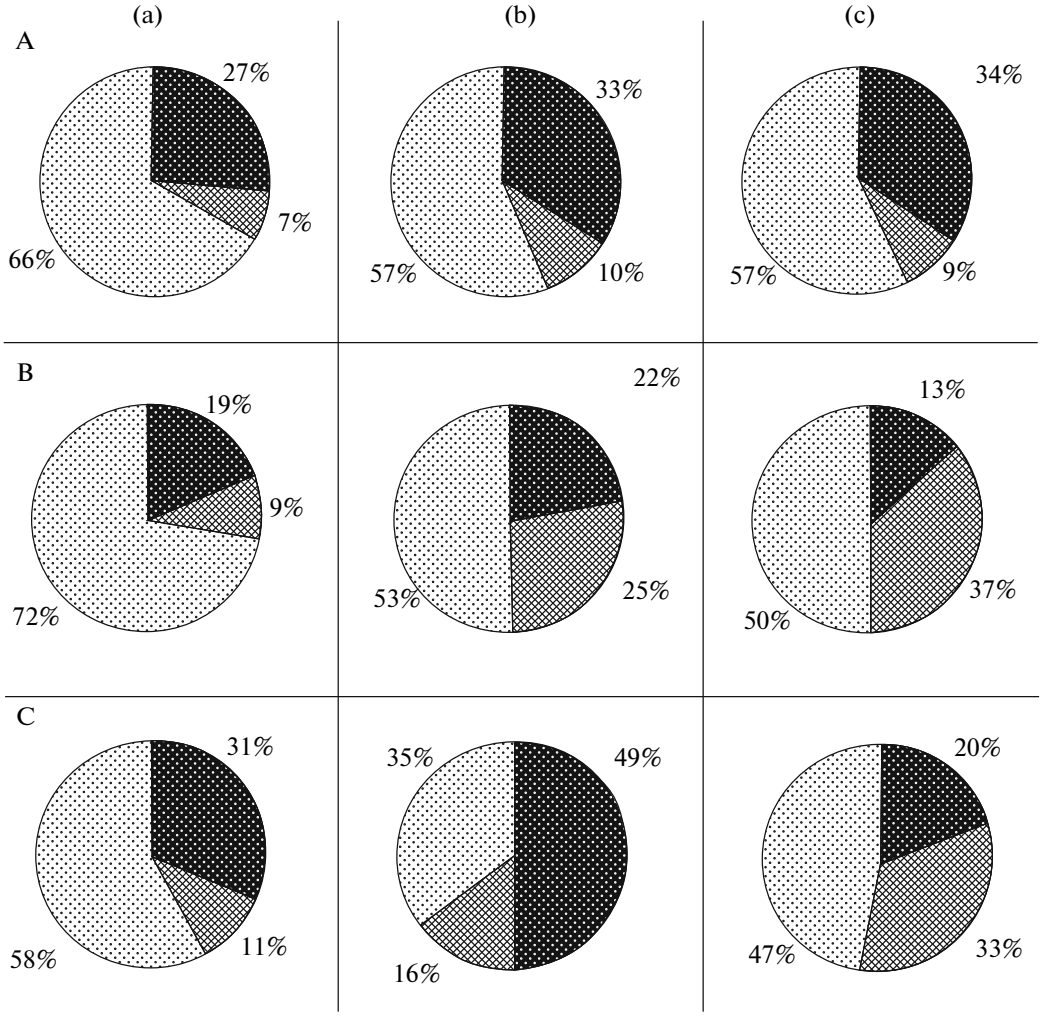

Fig. 5. Share (%) of biomass of the metabolically active (FISH) unicellular (*1*), mycelial (*2*), and unidentified (*3*) prokaryotes to the total biomass of microorganisms (acridine orange) in studied soils. A, modern chestnut soil; B, soil buried 3500 years ago; and C, soil buried 4500 years ago. Humidified samples (control) (a); samples with pectin (b); and samples with chitin (c).

and germinated actinomycete mycelium, was the largest in modern chestnut soils (Fig. 4). However, the response of prokaryotes to introduction of the substrates (increase in biomass) was much higher in buried soils than in the modern one. Introduction of the polysaccharides to buried soils resulted in an almost twofold increase in the fraction of metabolically active cells, if compared to the control sample (without the substrate), mostly due to mycelial forms, i.e. actinomycetes (compare Fig. 3 and Fig. 4, position *2*).

The rate of increase of the share of active microorganisms in both samples of buried soil was much higher than in the modern soil (Fig. 5). One may suppose that during the initial period after burial, microorganisms survived using the residual water and formed quiescent forms, which would explain the lower level of substrates in paleosol samples (Table 1). Introduction of a certain polymeric substrate after reactivation of the microbial community initiates the development of the corresponding hydrolytic complex, which is detected as a sharp increase in biomass upon the change of external conditions to favorable ones. In other words, the response of the community indicates that there should have been an active hydrolytic community dominated by spore-forming prokaryotes in the ancient soil system containing the polymer substrates under study.

Our previous studies by FISH investigation of microbial communities of various soils demonstrated that in modern soils the fraction of metabolically active microorganisms in both control and polysaccharide-treated samples usually does not exceed 30–40% to the total biomass and does not increase considerably upon the introduction of a substrate [17]. The data obtained upon studying the buried soils evidences an up to 1.7-fold increase of the number of metabolically active microorganisms upon the introduction of a substrate, which confirms the above considerations

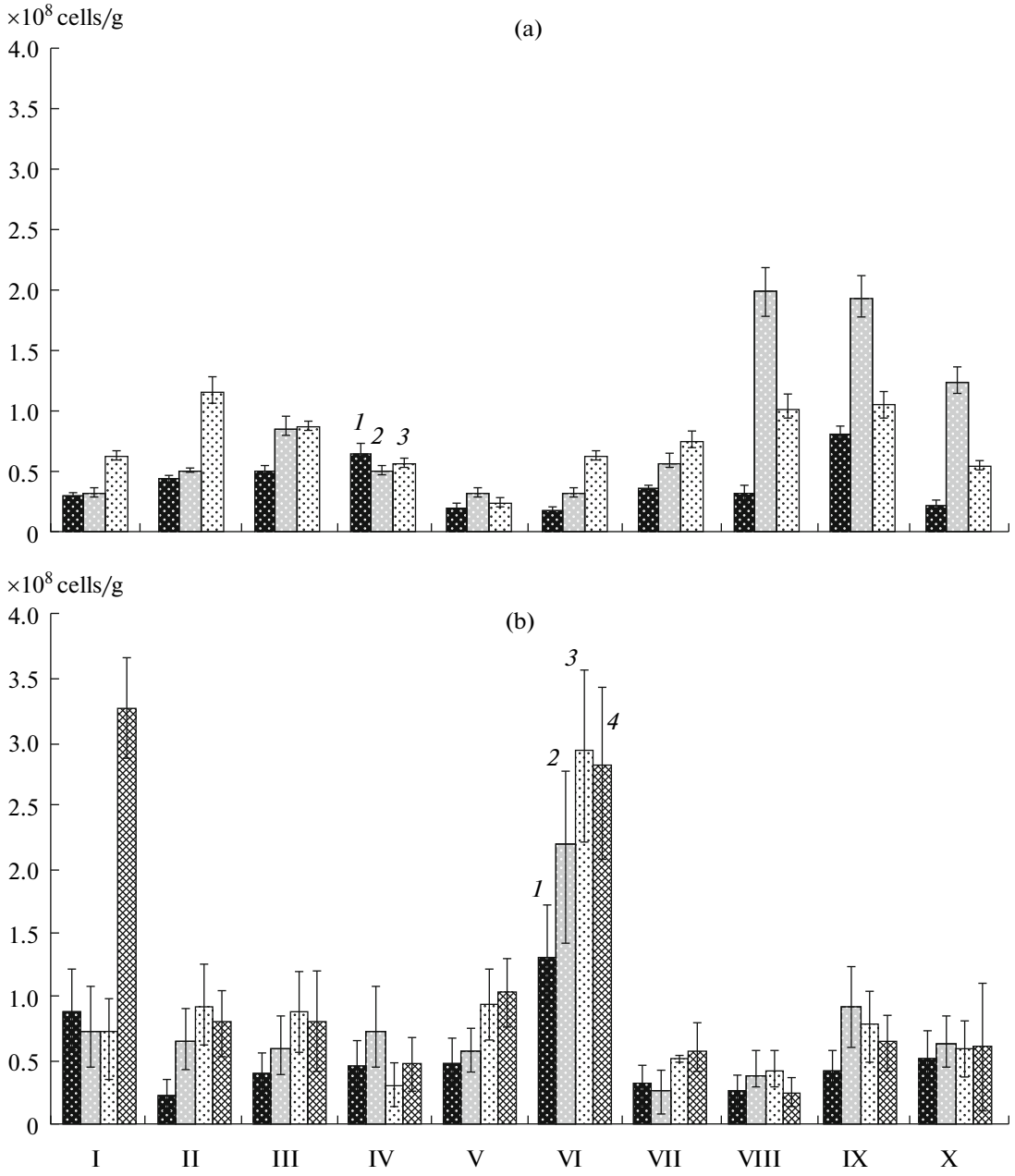

Fig. 6. Abundance of phylogenetic groups of the *Bacteria* domain in control variants (*1*) and upon introduction of pectin (*2*), chitin (*3*), and starch (*4*) to samples of modern chestnut soil (a) and soil buried 3500 years ago (b). I, *Firmicutes*; II, *Alphaproteobacteria*; III, *Betaproteobacteria*; IV, *Gammaproteobacteria*; V, *Deltaproteobacteria*; VI, *Actinobacteria*; VII, *Verrucomicrobia*; VIII, *Acidobacteria*; IX, *Bacteroidetes*; and X, *Planctomycetes*.

on greater potential hydrolytic activity of buried soils if compared to the modern ones.

The bacterial hydrolytic complex of modern soils is rather diverse and is characterized by the predomination of the phyla *Bacteroidetes, Firmicutes*, and *Proteobacteria* (Fig. 6). Introduction of pectin caused an active response in the representatives of the phyla *Bacteroidetes, Acidobacteria*, and *Planctomycetes*, and introduction of chitin resulted in a response in the representatives of the phylum *Firmicutes* and classes *Alphaproteobacteria* and *Betaproteobacteria* of the *Proteobacteria* phylum. In general, the bacterial complex of microorganisms of modern soils is characterized by a more uniform response to introduction of a substrate by all phyla of the domain.

In buried soils, the highest counts were revealed for the representatives of the gram-positive *Actinobacteria* and *Firmicutes*. Counts of the representatives of the phylum *Firmicutes* increased significantly on starch, and the value for *Actinobacteria*, upon growth on

chitin. However, in the work on isolation of chitinolytic bacteria on solid nutrient media, followed by their molecular genetic identification, Das et al. [28] demonstrated that the bacterial community of soils enriched with chitin was characterized by the absolute domination of the representatives of the class *Gammaproteobacteria*, while gram-positive *Bacillus* were detected only as one of the common genera. In our studies, members of the *Alphaproteobacteria* accumulated biomass actively upon addition of chitin to the samples of buried soils of either age; in samples of younger paleosol, they accumulated biomass in response to pectin and chitin; neither of the samples reacted significantly to starch. Moreover, in younger samples of buried soils, an increase in the number of *Betaproteobacteria* was noted. These findings may be explained by the fact that a number of representatives of these groups are active hydorlytics capable of degradation of difficult-to-consume polymers. These groups of organisms are most frequently mentioned as major agents of organic matter decomposition in natural ecosystems [29, 30].

Therefore, the performed studies made it possible to elucidate the specific features of the functional hydrolytic microbial complex in buried chestnut soils. It was found that the potential activity of a microbial hydrolytic complex in buried chestnut soils was higher if compared with that of the complex from modern chestnut soils. For the first time, FISH method was used to evaluate the fraction of metabolically active prokaryotic cells upon introduction of a polysaccharide into buried soils, which reached 50% to the total revealed prokaryotic community; the latter one reacts to the introduction of a substrate more actively than in the modern soils. The differences in the phylogenetic structure of prokaryotic metabolically active hydrolytic complexes of buried and modern chestnut soils were established. In modern soils, the hydrolytic complex was more diverse than that of the buried soils, where the groups *Actinobacteria* (both unicellular and mycelial ones) and *Proteobacteria* were the dominant hydrolytics.

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