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EXPERIMENTAL ARTICLES

Effect of Mechanical Grinding of *Sphagnum* on the Structure and Physiological State of Bacterial Communities

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Abstract—The microcosm method was used to demonstrate an increase in bacterial numbers and drastic changes in the taxonomic structure of saprotrophic bacteria as a result of mechanical grinding of *Sphagnum* moss. Eccrisotrophic agrobacteria predominant in untreated moss were replaced by hydrolytic bacteria. Molecular biological approaches revealed such specific hydrolytic bacteria as *Janthinobacterium agaricum* and *Streptomyces purpurascens* among the dominant taxa. The application of kinetic technique for determination of the physiological state of bacteria in situ revealed higher functional diversity of hydrolytic bacteria in ground moss than in untreated samples. A considerable decrease of the C/N ratio in ground samples of living *Sphagnum* incubated using the microcosm technique indicated decomposition of this substrate.

Keywords: *Sphagnum*, mechanical grinding, abundance and taxonomic structure of bacterial complexes, physiological activity of bacteria, *Sphagnum* decomposition

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High-moor peatlands, which contain large reserves of viable microbial biomass, are characterized by low rates of peat mineralization. Most researchers consider low oxygen concentration, acidic pH, lack of nutrients, low temperatures, specific composition of *Sphagnum* polysaccharides, and the presence of phenols as the factors hampering decomposition of high-moor peat [1–6]. In our monograph *Functioning of Microbial Complexes in High-Moor Peatlands: Analysis of the Reasons for Slow Peat Decomposition* [7], all the above factors were examined. It was concluded that the specific structure of *Sphagnum* polysaccharides, which are poorly degraded even by fungi, is the main limiting factor at the early stages of *Sphagnum* decomposition. Long-term preservation of *Sphagnum* stems is also favored by the low abundance and diversity of soil animals, which may homogenize the plant tissues.

The effect of artificial mechanical grinding of *Sphagnum* moss on its chemical composition was studied by chemical researchers of peat from Tomsk [8]. It was found that grinding of *Sphagnum* moss in mills favors the partial enzymatic disintegration of strong glycoside bonds in polysaccharides typical for undisturbed moss and decreases its antioxidant properties. Thus, *Sphagnum* should become more available for decomposition by hydrolytic microorganisms. However, microbiologists have not yet verified this fact.

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The goal of the present work was to assess the effect of mechanical grinding of *Sphagnum* on the ecological and taxonomical structure and physiological activity of bacterial communities inhabiting the living moss and litter in model experiments.

MATERIALS AND METHODS

The subject of study was the high-moor peatland on a stationary test plot of the Western Dvina Forest–Bog Research Station of the Institute of Forestry of the Russian Academy of Sciences (56°09′ N, 32°10′ E and 56°09′ N, 32°08′ E) in the Tver oblast. This peatland is a part of a bog area, formation of which began (according to the radiocarbon data and spore–pollen analysis) about 9000 years ago, which corresponds to the beginning of the boreal period. This is a classical bog area of central-oligotrophic development, which occurs on domed bogs with afforested slopes. The studied peatland is located under a cotton grass–*Sphagnum* pine forest in the upper part of a slope. Magellan's *Sphagnum, S. magellanicum* and brown peatmoss *S. fuscum* are the dominants of the plant cover. The peatland includes the following layers of high-moor peat: to 1.5 m in depth, slightly decomposed peat (3 to 20%); from 1.5 to 4 m, medium-decomposed peat (25 to 35%); from 4 to 4.5 m, transitional peat; and from 4.5 to 5 m, low-moor peat, which is underlain by organo-mineral deposits. The pH value varies throughout the profile from 2.8 to 4.5.

For determination of the effect of mechanical milling of *Sphagnum* on the abundance, structure, and physiological activity of bacterial complexes, a monolith 50 × 50 × 30 cm was taken from the peatland under study. *Sphagnum* stems were thoroughly separated from the monolith and divided into two parts: the upper green parts about 5 cm long (living *Sphagnum*) and the lower yellowish-brown parts about 10 cm long (*Sphagnum* litter). Living *Sphagnum* and its litter were placed into separate pots to be used as the controls. Experimental treatments included the samples of living *Sphagnum* and its litter mechanically chopped with scissors to fragments 5–7 mm in length (coarsely dispersed samples), as well as the samples of living *Sphagnum* and its litter mechanically ground in a propeller mixer (an RT-2 tissue microgrinder, 5000 rpm) to particles 0.5–0.7 mm in size (finely dispersed samples). The mean weight of the samples in the pots was about 1 kg. Selection and analysis of the samples for microbiological analysis were performed on the 0th, 3rd, 7th, 14th, 30th, 60th, 90th, 120th, 150th, and 180th days. The water content was controlled at all stages of model experiments.

Bacterial abundance was determined by the direct epifluorescence microscopy [9]. A 1-g sample was placed in a flask with 100 mL of sterile water. For desorption of the cells, the obtained suspension was treated on a Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Germany) at 50% power for 2 min. The suspension (0.01 mL) was applied with a micropipette onto a glass slide and uniformly distributed with a loop over an area of 4 cm^2. Six preparations per sample were used; 20 microscope fields were analyzed for each preparation. Acridine orange-stained preparations were examined under a LUMAM-IZ fluorescence microscope (Russia) (filters ZhS-19 and ZhS-18, objective ×90 L, eyepieces ×4 or ×5).

The number of bacterial cells in 1 g of fresh sample was calculated from the formula $N = S_1an/vS_2c$, where N is the number of bacterial cells per 1 g of fresh sample; S_1 is the preparation area (μm^2); a is the mean number of bacteria in the microscope field; n is the suspension dilution parameter (mL); v is the volume of the drop applied onto the glass (mL); S_2 is the microscope field area (μm^2); and c is the sample weight (g). The density of microorganisms was accepted as 1 g/cm^3; the content of water in the cells was accepted as 80%.

The content of bacterial cells was recalculated per 1 g of dry sample. The content of water was determined by drying the samples at 105°C for 6 h.

Abundance and taxonomic composition of saprotrophic bacteria were determined using glucose–peptone–yeast extract agar (GPYA). To inhibit fungal growth, 50 mg of nystatin per 0.5 L of medium was used. The suspension remained after the preparation of samples for fluorescence microscopy was used for inoculation, which was performed in five replicates from experimentally determined tenfold dilutions. The inoculated samples were incubated at room temperature for two to three weeks. The total number of bacteria was expressed in colony-forming units (CFUs) per 1 g of the sample. The bacterial colonies of different taxonomic groups were counted separately. The main bacterial representatives of the most numerous colonies were isolated into pure culture. The isolated strains were identified up to the genus level on the basis of their morphological, cultural, and chemotaxonomic characteristics [10, 11]. Identification of the dominating bacterial taxa based on the 16S rDNA nucleotide sequences was carried out using the BLAST software [12].

Physiological activity of bacteria was determined by the kinetic method [13, 14]. Bacteria were desorbed from the sample by shaking a 1 : 5 suspension in a Vortex (2000 rpm, 20 min) and centrifuging it (3075 *g*, 5 min). The supernatant used as the inoculum was dispensed into 96-well plates, 100 μL per well. Each of these wells contained the Czapek mineral medium (100 μL), nystatin (2 mg/L) to inhibit the growth of fungi, and 10 g/L of a biopolymer (carboxymethyl cellulose (CMC), starch, pectin, xylan, chitin, casein, or dextran-500). The plate was then incubated at 25°C in a Sunrise immune-enzymatic analyzer under automatic shaking during 150–170 h, and the growth of bacteria was determined from the optical density of the suspension at $\lambda = 620$ nm.

An aliquot of the inoculum was plated onto the GPYA medium (to determine the initial bacterial concentration). The content of the wells was also plated after the end of inoculation in order to (1) confirm the correlation between the optical density and the concentration of cells in the wells and (2) reveal the physiological groups of bacteria grown on specific polymers.

From the correlation equation between the optical densities of the populations grown in the wells and their cell concentrations, kinetic parameters were obtained to describe the growth of bacteria by the equation $x(t) = x_0(1 - r_0 + r_0e^{\mu_m t})$, where $x(t)$ is the concentration of cells (CFU/mL) in time moment t; x_0 is the initial concentration of the cells; r_0 is the initial value of the variable characterizing the physiological state of microorganisms; and μ_m is the maximum specific rate of microbial growth. The activity was determined as $-\ln(r_0)$: the higher the ln value, the lower the activity.

Efficiency of assimilation of the substrate was determined from the ratio between the increase in the

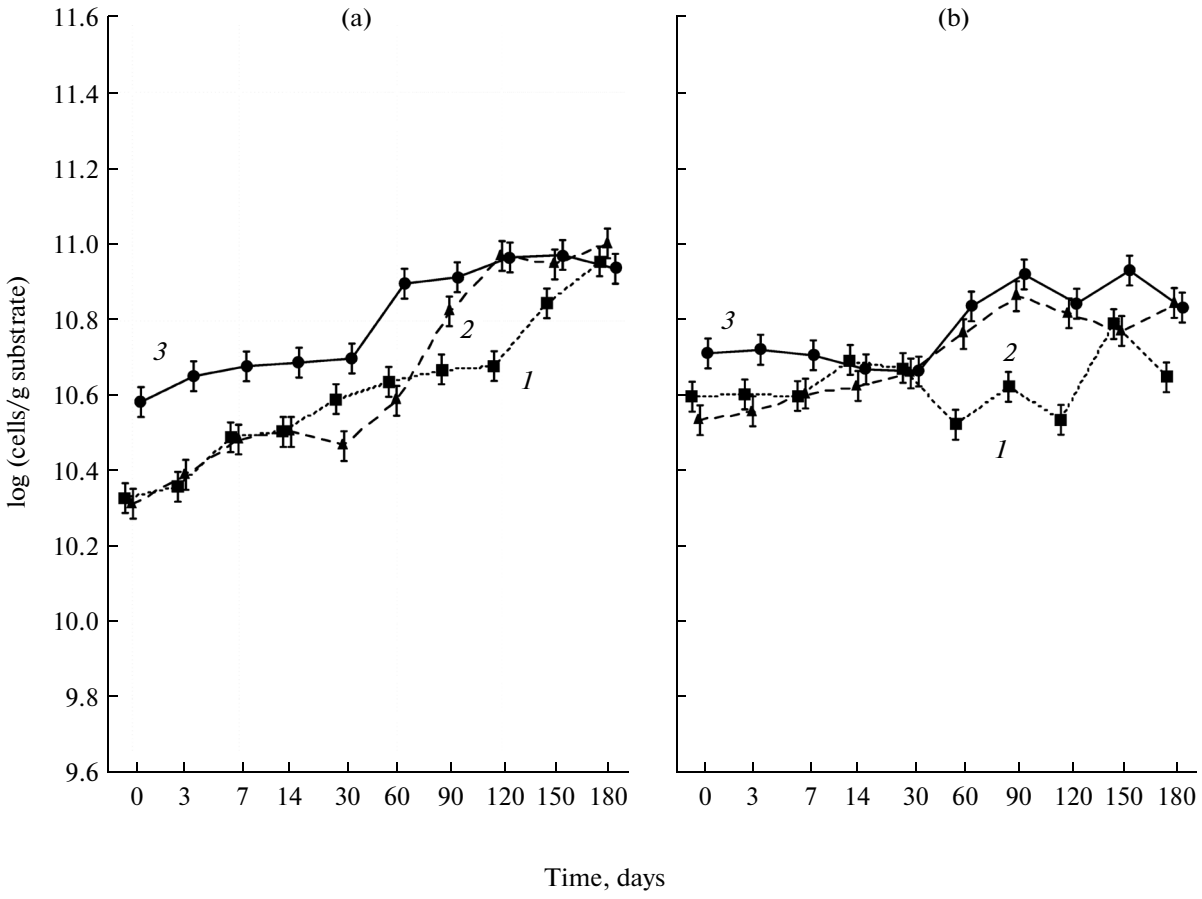

Fig. 1. Dynamics of bacterial abundance (direct count) at different succession stages in the control samples and after the mechanical grinding of living *Sphagnum* (a) and its litter (b): control samples (*1*), coarsely dispersed samples (*2*), and finely dispersed samples (*3*).

number of bacteria in liquid media (CFU) and the initial concentration of the polymer.

The percentages of carbon and nitrogen in different experimental treatments were determined on an Elementar Vario EL III elemental analyzer (Germany) in an oxygen stream at 1150°C. The samples were analyzed in triplicate.

RESULTS AND DISCUSSION

The number of bacteria determined by direct count varied from 2×10^{10} to 1×10^{11} cells/g *Sphagnum* depending on the degree of dispersion of the sample, the succession stage, and the nature of the substrate analyzed. Throughout the experiment, the number of bacteria in the mechanically ground samples was on average two times higher (Fig. 1). The maximum numbers of bacteria in the finely dispersed samples exceeded those in the coarsely dispersed samples; peak values were reached on the 60th day in the living *Sphagnum* and on the 90th day in the litter. A tendency of increasing the number of bacteria was also observed by the end of the experiment; it was significantly more pronounced for the living *Sphagnum* than for the litter (Fig. 1). The products of lysis of the fungal mycelium could also be additional nutrient sources for bacteria at these stages.

Homogenization of *Sphagnum* into finely dispersed particles also increased the number of saprotrophic bacteria by one to two orders of magnitude (from 10^6 to 10^8 CFU/g) compared to the control and coarsely dispersed samples, which were characterized by insignificant variations of bacterial abundance with time.

Let us consider the dynamics of the taxonomic structure of bacterial communities developed in different experimental variants in the living *Sphagnum* and its litter.

In the samples of living moss, *Agrobacterium radiobacter* was the single dominant species at the initial stages in all of the experimental treatments (Fig. 2). These are typical epiphytic bacteria feeding on the exoosmosis products of plants. After 14 days of succession, along with *Agr. radiobacter*, the chitinolytic spe-

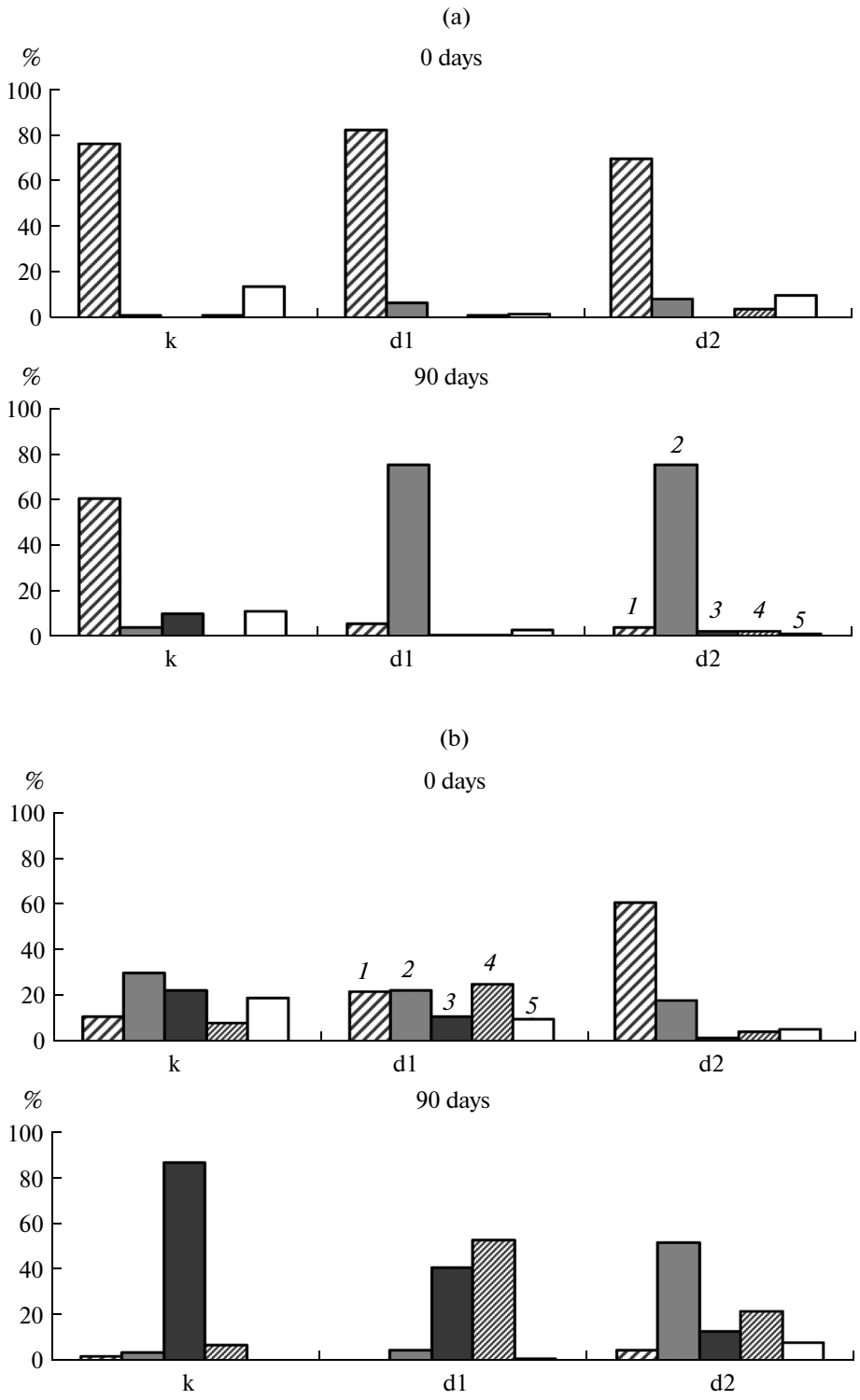

Fig. 2. Taxonomic structure of the saprotrophic bacterial complex at different succession stages in the control and experimental samples at the mechanical milling of living *Sphagnum* (a) and its litter (b): control samples (k); coarsely dispersed samples (d1); and finely dispersed samples (d2). The percentages of taxonomic groups to the total number of bacteria (CFU/g sample) are given on the Y axis: *Agrobacterium* (*1*), *Janthinobacterium agaricum* (*2*), *Bacillus* (*3*), *Streptomyces purpurascens* (*4*), and *Cytophaga* (*5*).

cies *Janthinobacterium agaricum* became dominant in the coarsely dispersed samples; these bacteria were first found on agaric fungi and then in the hydromorphic soils of the northern regions [15]. Bacilli were revealed as subdominants (24%) at this succession stage. In the finely dispersed samples, monodominance of the above chitinolytic bacteria was simultaneously noted; their share in the bacterial community increased to 79%. The share of agrobacteria, on the contrary, decreased to 16%. After 90 days, the share of *Jant. agaricum* in the ground samples of living moss continued to increase and reached 87%. Other bacterial genera were isolated in minor amounts (Fig. 2). On the 120th day of the experiment, streptomycetes—which were identified as *Streptomyces purpurascens* by molecular biological techniques—became a dominant species (26%) in the ground *Sphagnum*, along with chitinolytics. The change of the *Agrobacterium* species dominant in the control samples of the living *Sphagnum* should also be noted: *Agr. radiobacter* was replaced by *Agr. rhizogenes* (94%).

At the beginning of the experiment, the taxonomic structure of the studied community in the samples of *Sphagnum* litter was not monodominant, unlike that of the living moss. It included hydrolytic bacteria *Janthinobacterium*, *Bacillus*, and *Cytophaga*. In the coarsely dispersed samples, along with the hydrolytics *Janthinobacterium* spp. and *Streptomyces* spp., the dominant group included agrobacteria, which became monodominants in the finely dispersed samples and in the control samples of the living *Sphagnum* (Fig. 2). On the 90th day, the ratio of hydrolytic bacteria in the experimental treatments changed. The spore-forming *Bacillus* spp. became dominant (87%) in the control samples; the streptomycetes *S. purpurascens* and the chitinolytic *Jant. agaricum* predominated (53 and 52%) in the coarsely and finely dispersed samples, respectively (Fig. 2).

Thus, mechanical grinding of *Sphagnum* resulted in an abrupt structural reorganization of bacterial communities in both the living moss and its litter. Eccrisotrophic agrobacteria were replaced by hydrolytic bacteria chitinolytic *Jant. agaricum* and streptomycete *S. purpurascens*. In the course of grinding, homogenization of fungal hyphae probably also occurred, making chitin and other fungal biopolymers accessible to hydrolytic bacteria.

The dominant taxa determined by the molecular biological methods are briefly characterized below. The genus *Agrobacterium* belongs to the family *Rhizobiaceae*, order *Rhizobiales*, class *Alphaproteobacteria*. *Agrobacterium* species are typical inhabitants of the rhizosphere and phyllosphere of different plant species. Many of them are plant pathogens. However, bacteria of the two species isolated in the model experiment were not pathogenic. Moreover, *Agr. radiobacter* have growth-stimulating properties. Peat biopreparations (Rizoagrin) were based *Agr. radiobacter* strain 204 [16]. Detection of this bacterial species as an endophyte in the mycorrhiza of the fungus *Piriformospora indica* is notable. This symbiosis provides for the stimulation of plant growth [17].

Another agrobacterial species, *Agr. rhizogenes*, is frequently revealed as a typical inhabitant of plant rhizosphere and soil. Bacteria of this species were also isolated from the root nodules of legume plants [18]. It is most interesting that bacteria of this species can grow at pH 4, i.e., under the acidic conditions typical for high-moor bogs. It should be noted that bacteria of the other species (*Agr. radiobacter*) detected in the *Sphagnum* as the dominant can grow within a wide pH range from 5 to 9.

The genus *Agrobacterium* and the species *Agr. radiobacter* and *Agr. rhizogenes* were revised in 1993 [19] and 2001 [20]. In the latter publication, it was proposed to transfer these and many other *Agrobacterium* species to the genus *Rhizobium*.

In the samples of *Sphagnum* litter ground to the coarsely dispersed fraction, actinomycetes *S. purpurascens* were isolated first as minor components and then as dominants. The streptomycetes of this species have been isolated from various plant and moss species [12]. It follows from the dendrograms of phylogenetic relationships presented below that the accuracy of identification of bacterial cultures up to the species level is beyond doubt, since the similarity with the sequences available in the species databases is 99% (Fig. 3).

Physiological activity of bacteria in the microbial communities of living *Sphagnum* (control and coarsely dispersed samples) was assessed from their growth in liquid media with a specific polymer. A linear relationship was assumed between the optical density (OD_{620}) and the concentration of bacterial cells in the suspension ($C_{CFU/mL} = 2 \times 10^9 \times OD$).

Growth of bacterial communities in the studied samples was shown on starch, CMC, pectin, xylan, casein, and dextran-500. Growth on chitin was observed only for the ground samples (Fig. 4), which agrees with the data obtained by plating on Petri dishes: different strains of chitinolytic bacteria were isolated.

The growth parameters in the studied samples at the moment of measurement (on the 90th day after homogenization) were relatively similar. The share of rapidly growing populations ($\mu_m > 0.4$) on the media with different polymers was 32% in the control sample and 46% in the coarsely dispersed sample; this indicated a large share of rapidly growing *r*-strategists, which gained a growth advantage after mechanical grinding of moss samples. In the native substrate, the activity ($-\ln(r_0)$) and ecological growth strategy (μ_m)

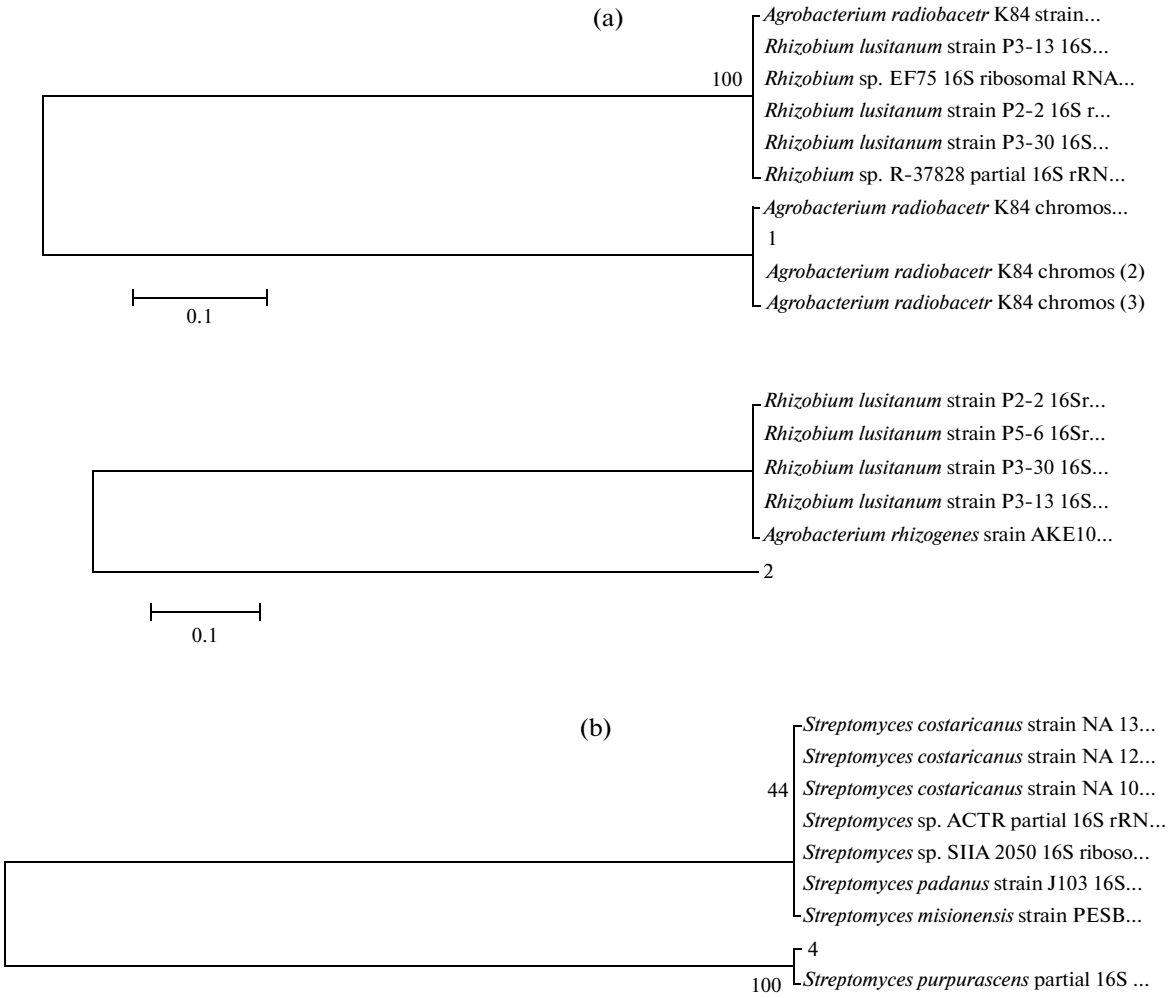

Fig. 3. Phylogenetic positions of the dominant strains in the phylogenetic group: (a) *Proteobacteria* (*Alphaproteobacteria*) *Rhizobiales*; *Rhizobiaceae*; *Rhizobium*/*Agrobacterium* group; *Rhizobium*; (b) *Actinobacteria*, mycelial actinobacteria: *Streptomyces purpurascens*. The scale indicates the evolutionary distance corresponding to 1 nucleotide replacement per 10 nucleotides. The numerals indicate the reliability of branching order revealed by bootstrap analysis of 100 alternative trees.

of the bacterial populations developed on xylan were different. Rapidly growing but inactive bacteria were isolated from the ground moss samples, and slowly growing but more active bacteria were isolated from the native moss (control samples) (Fig. 5). For the bacteria isolated from the ground moss samples, high efficiency of pectin assimilation was observed, which was evident from the yield of bacteria in the medium per 1 g of pectin (Fig. 6). This indicated the different compositions of bacteria in the pectin degradation complex in the studied samples.

After 12 months of the experiment, the parameter indicative of the degree of peat decomposition (the carbon-to-nitrogen ratio in the organic matter) did not change significantly in the samples of living *Sphagnum* and decreased in the ground samples, which indicated decomposition of biopolymers in the substrate analyzed (table).

Thus, the following conclusion may be drawn from our study. It was found that mechanical grinding of *Sphagnum* resulted in an increase in the number of total and saprotrophic bacteria. Abrupt changes in the taxonomic structure of the bacterial complex occurred in the saprotrophic block, which were manifested by hydrolytic bacteria displacing the dominating eccrisotrophic agrobacteria in the native moss. The use of molecular biological methods made it possible to identify such specific hydrolytic bacteria as *Jant. agaricum* and *S. purpurascens* as the taxa dominating in the *Sphagnum*. Representatives of the species *Agr. radiobacter* and *Agr. rhizogenes* have not been previously isolated from peat soils. The kinetic method showed high functional diversity of hydrolytic bacteria and a large share of *r*-strategists in the community of the ground moss. The largest differences in the growth parameters of the moss bacterial community were

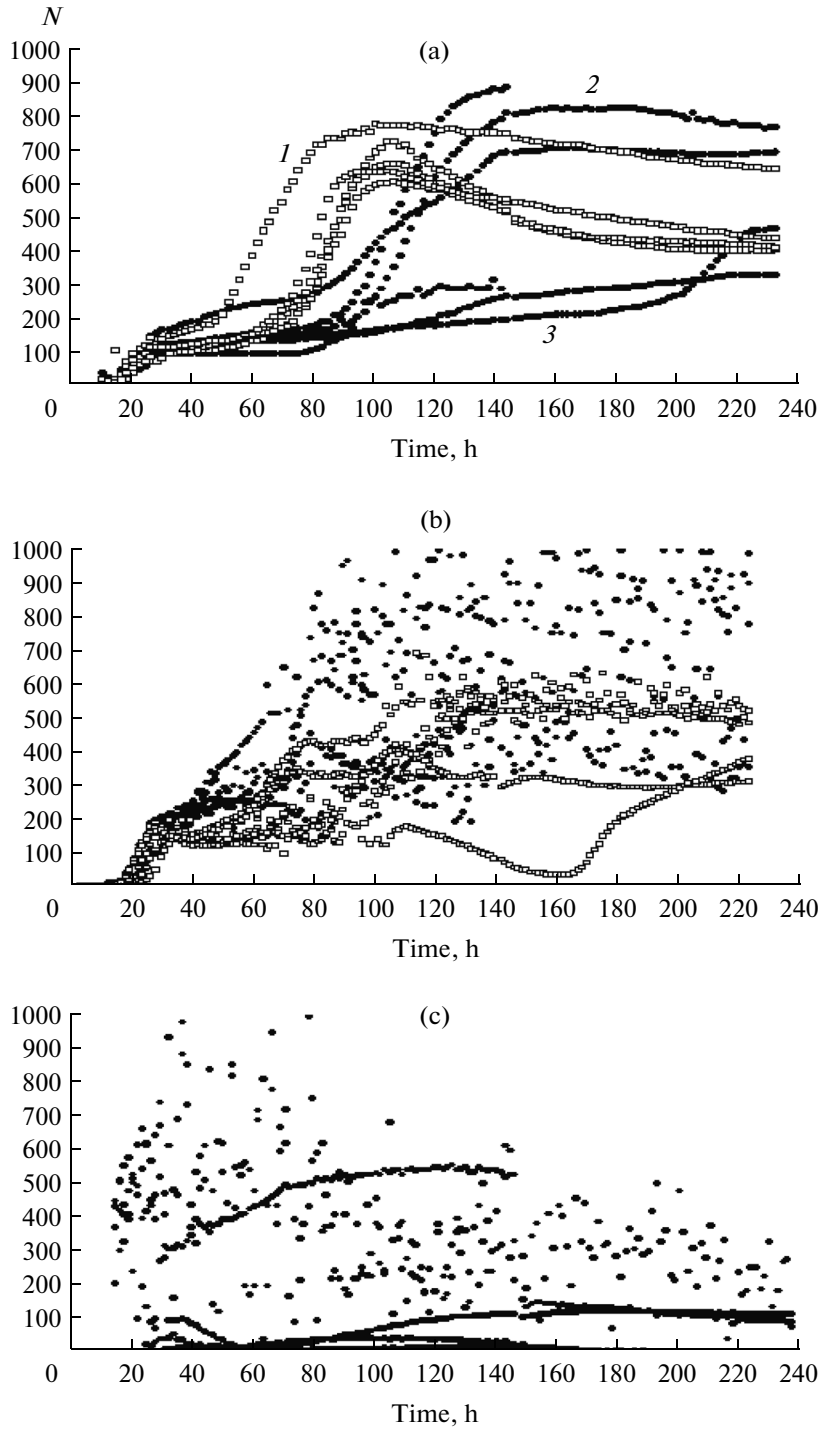

Fig. 4. Curves of batch growth of mixed bacterial populations on xylan (a), pectin (b), and chitin (c) in the media inoculated with bacterial suspensions from the control (*1*) and coarsely dispersed samples of living *Sphagnum* (*2*); sterility control (*3*).

observed on the media with chitin, xylan, and pectin. Chitin enters in the composition of ground fungal cell walls, the content of which increases at the grinding of the moss. Xylan and pectin are polymers of the membranes of the moss cells. Interpretation of these facts favors development of the conditions accelerating decomposition of moss under mechanical grinding; this conclusion is confirmed by the parameters of organic matter decomposition, which significantly decreased after 12 months of the experiment in the

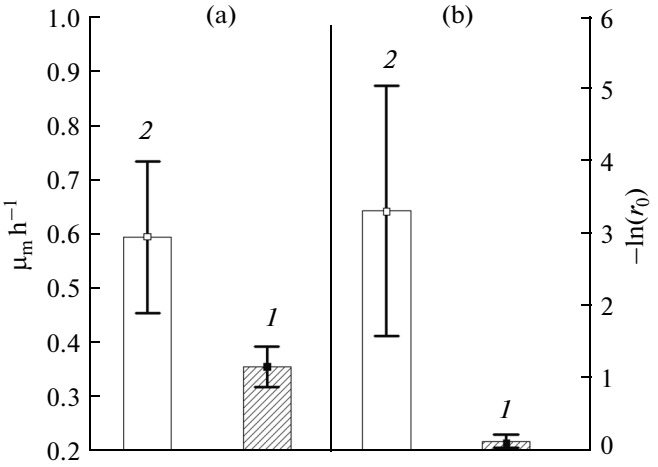

Fig. 5. Maximum specific growth rate (μ_m) (a) and metabolic capacity of bacteria for growth ($\ln(r_0)$) (b) in the mixed bacterial populations developing in xylan-containing nutrient media inoculated with suspensions from the control (*1*) and coarsely dispersed samples of living *Sphagnum* (*2*).

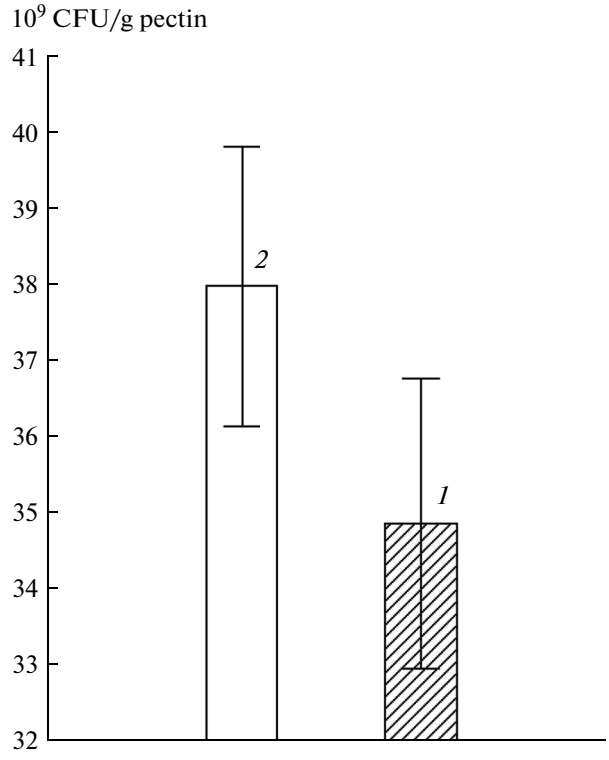

Fig. 6. Efficiency of substrate assimilation (bacterial yield, 10^9 CFU/g pectin) by bacterial associations developing in pectin-containing nutrient media inoculated with suspensions from the control (*1*) and coarsely dispersed samples of living *Sphagnum* (*2*).

The C/N ratios at different succession stages in the control samples and after mechanical treatment of living *Sphagnum*

Experimental treatment	C/N ratio at the succession stages		
	beginning of the experiment C/N	after 6 months C/N	after a year C/N
1	62	61	59
2	61	75	50
3	71	74	42

Control samples (1), coarsely dispersed samples (2), and finely dispersed samples (3).

Sphagnum samples ground to the finely dispersed state. The absence of mechanical homogenization of *Sphagnum* under natural conditions, which is due to the low number and diversity of soil saprophages in oligotrophic bogs, favors the preservation of the intact *Sphagnum* tissues. Thus, it is indirectly proved that the mechanical and chemical intactness of *Sphagnum* tissues is one of the decisive factors limiting the degradation of this moss.

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