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EXPERIMENTAL ARTICLES

Nitrogen-Fixing Activity in Peat Soils from a Raised Bog

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Abstract—The nitrogenase (acetylene reductase) activity in monolithic and minced peat samples was found to be low, no more than 0.014–0.022 mg N/(kg h). Incorporation of the $^{15}N_2$ isotope into organic compounds of peat soil was 2.71–8.13 mg N/kg over 15 days. The nitrogen-fixing activity was the highest in a 10- to 20-cm layer of soil and much lower in the upper (under green moss) and deeper (20- to 30-cm) layers. The addition of glucose to soil samples stimulated nitrogen fixation considerably after 18–26 h. The maximum nitrogenase activity (3.5–3.8 mg N/(kg h)), observed after 60–70 h, coincided with the peak of respiratory activity. A repeated addition of glucose after its exhaustion increased nitrogenase activity, without a lag period, to 8.5 mg N/(kg h). Investigation of the effect of environmental factors (temperature, pH, aeration, and light intensity) on potential nitrogen-fixing activity in peat samples revealed that nitrogen fixation could proceed in a wide range of pH values (from 3.0 to 7.5) and temperatures (from 5 to 35°C). The nitrogen-fixing bacteria belonging to different trophic groups were enumerated by using nitrogen-free media with pH values and mineralization levels close to those in situ. In samples of peat soil, diazotrophic methanol-utilizing bacteria prevailed ($2.0–2.5 \times 10^6$ cells/g); the second largest group was facultatively anaerobic bacteria of the family *Enterobacteriaceae*.

Key words: nitrogen-fixing activity, raised bog.

Wetlands and waterlogged boreal soils play a significant role in the biosphere. In recent decades, the tolerance of wetland ecosystems to anthropogenic load and their ability to resist environmental impacts have attracted considerable interest from researchers.

Wetlands and waterlogged forests occupy large territories in Russia (over 20%) and contribute much to global cycles of biogenic elements, first of all, carbon and nitrogen. At present, intensive studies of wetlands in central Russia mainly focus on their role in carbon storage [1] and emission of greenhouse gases, methane in particular, into the atmosphere [2, 3]. At the same time, little is known on the nitrogen cycle in wetlands.

Like most natural ecosystems, wetlands are nitrogen-deficient; in raised bogs, microbial nitrogen fixation is the only source of nitrogen compounds available for plants and microorganisms. The methods for the estimation of nitrogenase activity in sphagnum bogs are few, often yield contradictory results, and are unsuitable for balance calculations; the values of nitrogen-fixing activity obtained by the acetylene and isotope methods are extremely low and never exceed 0.5–1.5 kg N/ha per year [5–7]. At the same time, there are data on high rates of nitrogen fixation under sedge, which reach 40–90 kg N/ha per vegetation period [8].

A study of the effect of environmental factors on nitrogen fixation can provide valuable insight into the regulatory mechanisms of this process and the role of various groups of diazotrophs involved. Laboratory experiments on the effect of glucose, carbon dioxide, and aeration on nitrogenase activity in peat soils of England allowed Waughman to infer the involvement of nitrogen-fixing associations of aerobic and anaerobic bacteria in nitrogen fixation [5].

There are only scarce data on the composition of nitrogen-fixing communities in wetlands. Facultatively anaerobic nitrogen-fixing bacteria *Paenibacillus (Clostridium) polymyxa* [9] and aerobic bacteria of the genera *Azotobacter* [10] and *Beijerinckia* [11] have been isolated from wetlands by traditional bacteriological methods; however, their contribution to nitrogen fixation remains unclear.

The aim of this work was to study nitrogen fixation in peat soils and elucidate mechanisms involved in its regulation.

MATERIALS AND METHODS

Peat samples were taken in June 2000 from peat soil in the central part of the Sosvyatskoe raised bog (West Dvina Station, Institute of Forestry, Russian Academy of Sciences, Tver oblast) overgrown with a plant association consisting of *Sphagnum* sp., *Oxycoccus quadripetalus*, and *Carex rostrata*. The samples were taken as blocks of 200 cm^3 in size at depths of 0–10, 10–20, and 20–30 cm from the moss surface and stored at 4°C for no longer than 48 h until use.

Determination of nitrogen-fixing activity by the acetylene method. Peat was minced into 3- to 5-mm pieces and a 10-g aliquot was put into a 30-ml flask. All

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analyses were performed in triplicate. The nitrogenase (acetylene reductase) activity was determined from the amount of ethylene formed from the acetylene added to the gas phase (10 vol %), as described earlier [12]. Peat samples containing no additional carbon sources were incubated with acetylene for 72 h. To estimate potential nitrogenase activity, 0.25 ml of a water solution of glucose was added to a peat sample to give a final concentration of 10 mg carbon per 1 g wet peat, and the mixture was incubated with acetylene for 2 h; nitrogenase activity was determined every day for 10–14 days (the flasks were kept in a desiccator).

The effect of environmental factors on nitrogenase activity was studied using peat samples supplemented with glucose and incubated with acetylene for 48 h. Anaerobic conditions were established by replacing air in the gas phase with argon; the flasks were then incubated at room temperature (22–23°C) in the dark or at a constant light intensity of about 6 klx. To study the effect of temperature on nitrogenase activity, peat samples were preincubated at 5, 10, 15, 20, 25, 30, 37, and 45°C for 4 h; then, glucose and acetylene were added, and the samples were incubated at an appropriate temperature in the dark. Different values of initial pH in peat samples were adjusted by the addition of chalk or orthophosphoric acid at concentrations determined in advance.

Determination of the nitrogen-fixing activity by the isotope method. Flasks with peat samples prepared as described above were incubated for 15 days in a desiccator under a gas mixture containing 30% $^{15}N_2$, 20% O_2, and 50% Ar. Mass spectrometric analyses and calculation of nitrogenase activity were performed as described earlier [12].

Enumeration of nitrogen-fixing bacteria was carried out by the acetylene method as described earlier [13]; a modification was the use of media with a low content of mineral salts (600 mg/l) and pH 5.5.

Chemical analyses. The concentration of CO_2 was determined by gas chromatography. The contents of glucose and products of its oxidation were determined in a water extract of peat (5 : 1) by the glucose oxidase method and the potassium dichromate oxidation method, respectively.

RESULTS AND DISCUSSION

Nitrogenase activity in monolithic and minced peat samples taken from a depth of 10–20 cm was low and did not exceed 1.5–2.4 nmol C_2H_2/(g h), which approximately corresponded to 0.014–0.022 μg nitrogen/(g h). In the two other peat layers (0–10 and 20–30 cm), the amount of ethylene formed was below the detection limit under all incubation conditions tested. As seen from Table 1, constant illumination caused no increase in nitrogenase activity, and incubation of peat samples under an argon atmosphere decreased nitrogenase activity. Thus, the contribution of photosynthetic and strictly anaerobic bacteria to nitrogen fixation in peat appeared to be small. Therefore, in further experiments, peat samples were incubated aerobically in the dark.

Table 1. Effect of illumination and aeration on the nitrogenase activity of microflora in the 10- to 20-cm layer of peat soil as determined by the acetylene method

Incubation conditions	Nitrogenase activity, nmol C_2H_2/(g h)
Light, aerobic	2.1 ± 0.7
Light, anaerobic	1.5 ± 0.4
Dark, aerobic	2.4 ± 0.5
Dark, anaerobic	1.5 ± 0.2

The total amount of fixed nitrogen determined by the ^{15}N isotope method in peat samples taken from depths of 0–10 and 10–20 cm was 0.17 and 0.91 mg N/kg, respectively, over 15 days (Table 2). This is in conformity with the data of other researchers on the low nitrogenase activity in peat soils determined by the isotope method [6, 7].

The addition of glucose to peat samples increased considerably the nitrogenase activity, which made it possible to follow its dynamics in the three layers of peat soil by the acetylene method. As seen from Fig. 1, in all peat samples studied, a lag period of 18–26 h was observed, which might be due to the necessity of synthesis or activation of nitrogenase in bacterial cells. Then, the rate of nitrogen fixation increased and reached its maximum values after 40–45 h in the 0- to 10-cm layer and after 60–70 h in the 10- to 20- and 20- to 30-cm layers. The process ceased completely after 110–120 h in the 0- to 10-cm layer and after 130–135 h in the underlying layers.

The onset of nitrogen fixation coincided with the maximum of CO_2 release; by this time, about 50% of the glucose added had been metabolized (Fig. 2). A repeated addition of glucose to peat samples taken from the 10- to 20-cm layer resulted in immediate nitrogen fixation (Fig. 2); in this case, potential nitrogenase activity reached 8.5 mg nitrogen/(kg day). The concentration of microbial biomass in the period of maximum nitrogenase activity was similar to that before the repeated addition of glucose (data not shown). This suggests that glucose (or the products of its oxidation) stimulate nitrogen fixation by inducing enzyme synthesis or by activation of existing enzymes rather than via an increase in the number of nitrogen-fixing bacteria.

The total amount of nitrogen fixed over 15 days in peat samples supplemented with glucose was 8.13 and 2.71 mg/kg for the 10- to 20- and 0- to 10-cm layers, respectively (Table 2).

Peat samples taken from the 10- to 20-cm layer and supplemented with glucose were used for the study of the effect of environmental factors on potential nitrogen fixation. Nitrogenase activity was invariant in the pH

Table 2. Nitrogen-fixing activity in peat soil as determined by the ^{15}N isotope method

Depth, cm	Total nitrogen content, mg/g	Without additives		With glucose, 10 mg/g	
		$\sigma^{15}N$, at. %	mg nitrogen/kg over 15 days	$\sigma^{15}N$, at. %	mg nitrogen/kg over 15 days
0–10	8.92	0.001	0.17	0.048	8.13
10–20	9.52	0.005	0.91	0.015	2.71

range from 4.0 to 6.0, declined to 10% of the maximum value at pH 3.0, decreased slightly (by 20%) at pH 7.0, and virtually ceased at pH 8.0 (Fig. 3a).

Like other processes occurring in natural ecosystems, nitrogen fixation is greatly affected by physicochemical factors, primarily temperature and humidity. As seen from Fig. 3b, nitrogenase activity was revealed within a wide range of positive temperatures, from 5 to 45°C, with an optimum at 25–30°C; this is in agreement with data on the effect of temperature on free-living heterotrophic diazotrophs [14]. At the same time, our findings are inconsistent with the results of field experiments, in which no correlation was observed between temperature and acetylene reductase activity in subarctic mires [15].

The nitrogen-fixing community of peat soil was more sensitive to the elevation of temperature than to its lowering; nitrogenase activity decreased more than twofold with a temperature elevation from 30 to 37°C and by only 20% with lowering of the temperature from 25 to 15°C.

We revealed that, in peat soil, the temperature coefficient characterizing the changes in nitrogenase activity with a temperature increase by 10°C (Q_{10}) was 4.1 within the temperature range from 5 to 25°C; this is in good agreement with the Q_{10} value of 3.7 determined by Canadian researchers for aerobic soils supplemented with glucose [16]. Thus, the great effect of temperature on nitrogenase activity should be taken into account when comparing values of nitrogenase activity determined at different temperatures, especially in the case of field experiments and balance calculations. The dependence of nitrogenase activity in peat soils under investigation on the temperature in the range from 5 to 30°C may be described by the following equation:

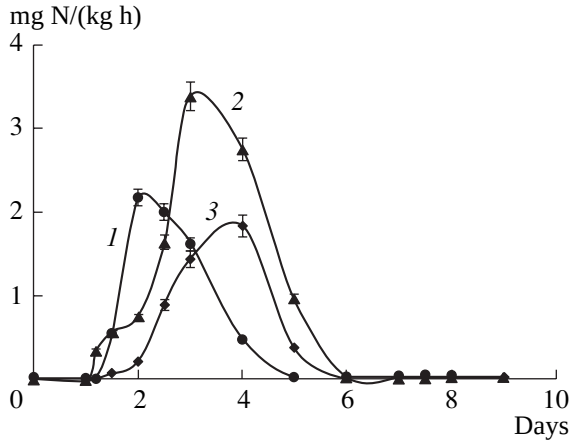

Fig. 1. Time course of potential nitrogen-fixing activity determined by the acetylene method in samples of peat soil taken from a depth of (*1*) 0–10 cm, (*2*) 10–20 cm, and (*3*) 20–30 cm.

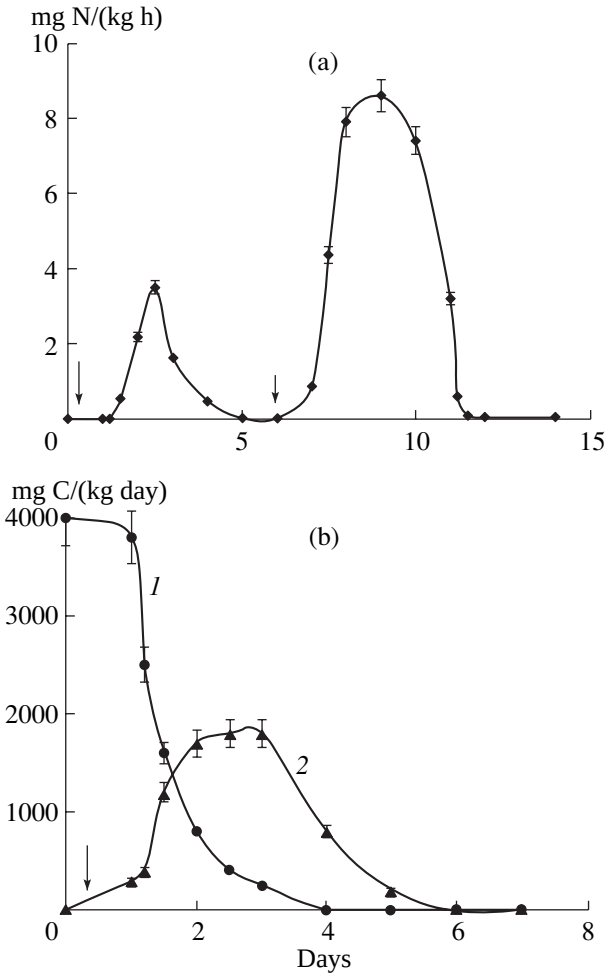

Fig. 2. Time courses (a) of potential nitrogen-fixing activity and (b) of (*1*) glucose consumption and (*2*) accumulation of fermentation products in samples of peat soil taken from a depth of 10–20 cm. Arrows indicate the moments of glucose addition to the samples.

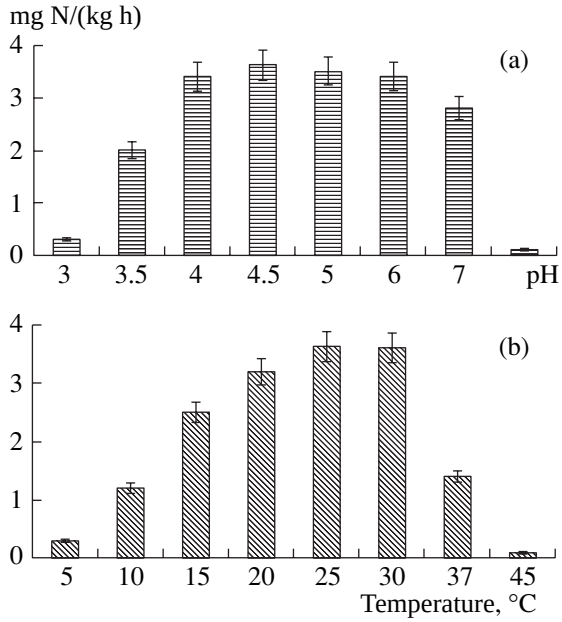

Fig. 3. Effect of (a) pH and (b) temperature on potential nitrogen-fixing activity in samples of peat soil taken from a depth of 10–20 cm.

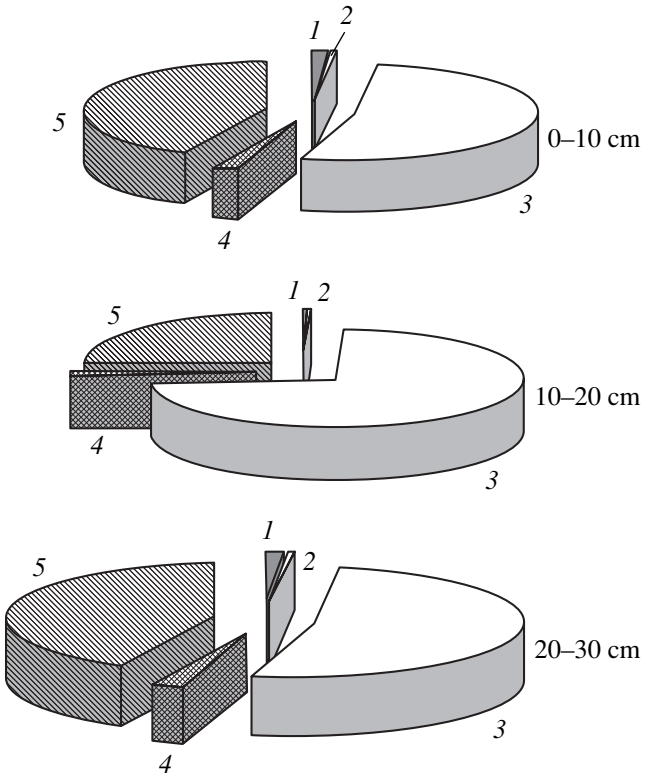

Fig. 4. Composition of nitrogen-fixing bacterial complexes in samples of peat soil as determined by inoculation of selective media: (*1*) bacteria grown on glucose–autolysate medium; (*2*) bacteria grown on sodium malate; (*3*) methanol-utilizing bacteria; (*4*) strictly anaerobic sugar-utilizing bacteria; (*5*) facultatively anaerobic sugar-utilizing bacteria. The total number of diazotrophic bacteria grown on five media was taken as 100%.

$$V_t = 1.7072\ln(t) - 2.3864,$$

where V_t is the nitrogenase activity (nmol C_2H_4/(g h)) at a given temperature t (°C). It should be noted that the dependence pattern may differ considerably in different peat soils. For example, the temperature dependence of the nitrogen-fixing activity in a rheotrophic peat in England was described by the following equation [5]:

$$V_{t2} = V_{t1}K^{(t2-t1)},$$

where V_{t1} and V_{t2} are the nitrogenase activities (nmol C_2H_4/(ml h) at temperatures t_1 and t_2 and K is a coefficient (equal to 0.4) that characterizes the change in nitrogenase activity with a temperature increase by 1°C.

Figure 4 demonstrates the most probable numbers of nitrogen-fixing bacteria in the three layers of peat soil. Aerobic methanol-utilizing diazotrophs and facultatively anaerobic bacteria of the family *Enterobacteriaceae* were of greatest abundance. The largest number of nitrogen-fixing bacteria was found in the upper (0- to 10-cm) layer of peat soil, whereas the maximum nitrogenase activity was revealed in the subjacent (10- to 20-cm) layer.

Thus, nitrogen fixation in wetlands proceeds in wide ranges of temperatures and pH; its rate is mainly determined by the availability of carbon and energy sources. An important problem of modern soil ecology is the relationship between the activity of the processes occurring in soils and the structure of the microbial communities involved. Our approach to the study of a nitrogen-fixing community revealed the important role of methylotrophs and facultatively anaerobic sugar-utilizing bacteria in nitrogen fixation but could not be used to assess the possible role of bacteria unable to grow on the media we used, such as methanotrophs; at the same time, their contribution to nitrogen fixation may be significant in waterlogged soils [17]. To solve this problem, we are going to undertake a study of the nitrogen-fixing community of peat soil by the methods of molecular ecology. We have proposed a system of oligonucleotide primers [18] that makes it possible to obtain PCR fragments of the *nifH* genes of nitrogen-fixing eubacteria and archaea and created a voluminous database on the primary structure of nitrogenase genes of methanotrophic bacteria [19]. Our future research will be concerned with investigation of the biodiversity of nitrogen-fixing methanotrophs as components of the integrated microbial community of peat soils.

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