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SOIL BIOLOGY

Specific Features of Determination of the Net Production of Nitrous Oxide by Soils

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Abstract—The rate of the net nitrous oxide (N_2O) production, the content of microbial biomass carbon (C_{mic}), and its portion in the total soil organic carbon (C_{org}) were determined in the samples from podzol, soddy-podzolic soils, gray forest soils, chernozems, burozems, and carbolithozems of natural, arable, and fallow ecosystems in Kostroma, Vladimir, Moscow, Kaluga, Voronezh oblasts, and Krasnodar region. The most sustainable N_2O production was found in the soils enriched with glucose or its mixture with ammonium sulfate at 22°C upon the preliminary incubation of the soil samples (7 days, 60% of water holding capacity). In the profiles of forest soils, a direct correlation was found between the N_2O production and the C_{mic} content ($r = 0.74$, $p \le 0.05$, $n = 18$). In the upper mineral layers (0–10 cm) of soddy-podzolic soils of the cropland, fallow, young, secondary and native forests, the inverse relationship between the N_2O production and the C_{mic} content ($r = -0.75$, $p \le 0.05$, $n = 6$) was observed. In a series of the fallowed, cultivated, and forest soils, the net N_2O production decreased (239, 69, and 38 ng $N_2O–N \times 10^{-3}$/g per h), and the C_{mic} content and $C_{mic} : C_{org}$ ratio increased (181, 569, and 1020 μg C/g; 1.4, 2.6, and 3.0%, respectively) attesting to the increasing N_2O flux in the anthropogenically transformed ecosystems. The application of cycloheximide (20–50 mg/g) to the soil lowered the N_2O production by 69–99%, which pointed to a significant contribution of fungi to this process. An approach to separate nitrification and denitrification in the soil using low concentrations of acetylene (1.8 Pa) was proposed. The conditions of preparation of the soil samples for sustainable detection of N_2O production were specified. It was shown that this process is tightly related to the soil microbial biomass and its fungal component.

Keywords: nitrous oxide, microbial biomass, denitrification, nitrification, organic carbon of soil, fallow soils, arable soils, forest soils

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INTRODUCTION

Nitrous oxide (N_2O) is one of the important greenhouse gases responsible for the recent climate changes on the Earth [37, 38, 59, 65]. Nowadays, the contribution of N_2O to the radiation activity of main greenhouse gases (CO_2, CH_4, N_2O) is only 6% [78]. However, N_2O is the most stable (long-living) gas; its residence time in the atmosphere is almost 160 years [37]. This gas participates in reactions that destroy the ozone shield of the planet, and its *global warming potential* is about 300 times higher than the CO_2 potential [72].

The N_2O concentration in the atmosphere of the Earth reached, on the average, 324.2 ± 0.1 ppb, exceeding that by more than 1 ppb in 2010. For the last ten years, the mean annual N_2O concentration in the atmosphere increased with the rate of 0.78 ppb due to anthropogenic impact and, primarily, to agricultural production [78]. Soils are the main source of N_2O entering the atmosphere (about 65%) [5, 20, 66].

Soil microorganisms are of great importance in the emission of greenhouse gases and control of the gaseous composition of the atmosphere [26]. Nitrous oxide results from the processes of nitrification, denitrification, and chemodenitrification [61, 71, 79]; the first two processes are the basic ones. The N_2O formation depends on the hydrothermal conditions (temperature, moisture), the physicochemical properties of soils, and the type of land use: application of mineral and organic fertilizers, vegetation, liming, and mechanical treatment of soils [4, 8–10, 39, 64]. The N_2O flux from agricultural soils, including also the arable ones, is stronger and more variable in time and space (complex interactions between physical, chemical, and biological parameters) as compared to that from natural ecosystems [15, 20, 25, 30, 52, 65]. The volumes of N_2O emission were noted to be closely associated with the nitrogen cycle and can characterize the degree of its disturbance [5, 54, 73].

The N_2O production from the soil resulting from its formation and absorption is called net production [5]. Net production depends on the rate of N_2O formation, its diffusion in soil layers, and absorption (reduction) by denitrifying agents [16]. The rate and scope of this process are determined in natural (uncontrolled) and laboratory (controlled) conditions. However, there are no clear guidelines for the evaluation of this process in the laboratory.

For better understanding of the regulation of N_2O emission in the atmosphere, the relative contribution of nitrification and denitrification should be discriminated. The main requirement of this differentiation is related to the selective inhibition of one process without any effect on the other. The contribution of nitrification and denitrification into the N_2O emission by different soils is still poorly known [7, 68]. One of the methods to specify these processes is associated with the use of acetylene (C_2H_2). The high partial pressure of C_2H_2 (5–10 kPa) in a test vessel inhibits N_2O-reductase of denitrifiers (N_2 is not formed), which allows estimation of the contribution of denitrification to the N_2O emission from the soil [42, 82]. However, C_2H_2 can stimulate mineralization of soil organic matter indirectly, increasing the rate of denitrification [32]. In addition, soil microorganisms can absorb acetylene [11, 81] and, hence, reduce its concentration resulting in the incomplete inhibition of denitrification. There are studies showing that, in contrast, the low partial pressure of C_2H_2 (0.1–10 Pa) can inhibit autotrophic nitrification, while not affecting the N_2O-reductase of denitrifiers [17, 35, 77]. The method proposed has a definite advantage for the estimation of the N_2O production by autotrophic nitrifying agents in the soil. Some specialists discuss the complexity of this approach mainly related to the selection of a suitable low concentration of C_2H_2 (inhibition of nitrification) for each soil studied [43].

Previously, it was believed that the formation of N_2O by soils occurred as a result of the activity only of prokaryote microorganisms (mainly of bacteria). However, the recent investigations showed that in this process mycelial fungi also played a significant role [22, 23, 46, 47, 63, 80]. Fungi make up a high share in the total microbial biomass and are capable of carrying out nitrification and denitrification [46, 47]. It is worthy of note that the assessment of the contribution of fungi and bacteria to the N_2O production is related to a number of methodological problems, including the application of selective inhibitors of respiration for these groups of microorganisms.

The objectives of this work were the following: (1) the approbation of experimental (laboratory) conditions for the highest net production of N_2O by the soil provided by its moisture and high supply of carbon and nitrogen from additional sources; (2) the determination of interrelationship between the net production of N_2O and the microbial biomass along the profile, associated ecosystem, and spatial gradients; (3) the subdivision of the sources of N_2O production into nitrification and denitrification ones; (4) the estimation of the contribution of fungi and bacteria to the production of N_2O by soils.

OBJECTS AND METHODS

Soils (podzol, rzhavozem, soddy-podzolic soil, gray soil, leached chernozem, burozem, and carbolithozem) of natural, arable, and fallow ecosystems in Kostroma, Vladimir, Moscow, Kaluga, and Voronezh oblasts and Krasnodar region (a total of 48 localities) were the objects for studies. In August–September of 2006–2009, soil samples were taken from the upper mineral 0–10-cm layer on a flat plot (10 × 10 m) by the method of *envelope*. The litter was not collected. In Moscow (Zvenigorod), Vladimir, and Kaluga (the *Kaluzhskie zaseki* Reserve) oblasts, samples were also taken along the soil profile. The averaged soil samples (53) were prepared. They were marked, delivered to the laboratory and stored (at 8–10°C, natural moisture) in plastic bags with air exchange for 4–6 weeks before the tests. Coarse plant residues were removed from the samples. Then, the samples were sieved (2–3 mm mesh). One part of the sample was dried in the air, the other was incubated during 7 days at 22°C and 55–60% of water holding capacity.

Net production of N_2O by soil. A soil sample (2 or 3 g from the upper or lower mineral horizons, respectively) was placed in a vessel (15 mL), then 0.1 mL/g of water or solution of glucose (2 mg/ g), and solution of ammonium sulfate (0.08 mg N–$(NH_4)_2SO_4$/g) were added to the vessels together and separately. The vessels were hermetically closed and incubated for 24 h at 22°C. The rate of N_2O production was determined by gas-solid chromatography (chromatograph LKhM-2000, *Chromatograph* company, Moscow: an electron capture detector (^{63}Ni), a glass column of 2 m long with a diameter of 3 mm, and Porapak Q adsorbent). The temperature of the columns and detector was 50 and 310°C, respectively. The rate of the carrier gas (nitrogen of special purity) flux was 50 mL/min, the volume of the gas introduced was 0.5 mL. The amount of N_2O produced was calculated by the equation:

$$N_2O\text{–}N = CVves/Tm,$$

where N_2O–N is the rate of N_2O production, ng N/g of soil per h; C is the N_2O concentration in the gas sample, vol %; $Vves$ is the volume of the air in the vessel with soil, mL; T is the duration of incubation of the vessel with soil, h; and m is the weight of dry soil, g.

The net N_2O production of soil was estimated at different concentrations (partial pressure) of acetylene. In the gas of the test vessels, the partial pressure of C_2H_2 was 0.08, 0.12, 0.6, 1.8, 3.6 and 9.0 Pa. In the vessel filled with C_2H_2 by 100%, the pressure was 101.3 kPa [77]. Therefore, the C_2H_2 pressure from 0.1 to 10 Pa corresponds to its concentration in the test vessels ranging from 0.0001 to 0.01% of their volume.

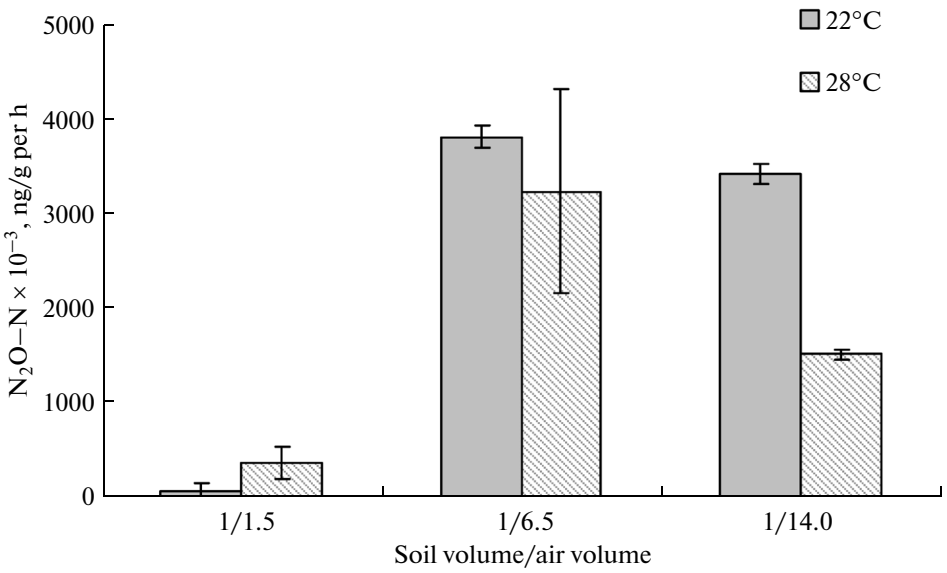

Fig. 1. Net production of N_2O by the moistened (0.6 mL H_2O) dry gray forest soil (forest, 0–10 cm, incubation for 24 h, $n = 3$) in the vessels with different volumes of soil and air.

Consequently, in order to create the pressure of C_2H_2 within the given interval, one should apply 0.00002 to 0.002 mL of the initial (100%) gas. To obtain the concentration of 10 Pa in the vessel (15 mL), 0.0015 mL C_2H_2 should be applied there (10 Pa = 10^{-4} atm, 15 mL × 10^{-4}). It is worthy of note that to take this volume of gas from a gasometer filled with 100% acetylene is very difficult. Therefore, the initial C_2H_2 (100%) acetylene is sequentially *diluted*.

Substrate-induced respiration (SIR) of the soil was determined in preliminarily incubated samples. This respiration was assessed as the rate of initial maximum respiration of microorganisms (CO_2 emission) after the incubation (22°C, 3–5 h) of the soil (1 or 2 g from the upper and lower soil horizons, respectively) enriched with glucose (solution, 10 mg/g or 0.1 mL/g) [13, 14].

Soil microbial biomass carbon (C_{mic}) was calculated according to the following formula: C_{mic} (μg C/g soil) = SIR (μL CO_2/g soil per h) × 40.04 + 0.37 [14]. For some soil samples, the $C_{mic} : C_{org}$ ratio was calculated.

For inhibiting the N_2O production in the soil, cycloheximide ($C_{15}H_{23}NO_4$) was applied at the concentration from 20 to 50 mg/g, which also inhibited SIR to a greater extent (found in preliminary tests) [2, 3, 69]. Cycloheximide (actidione) belongs to the group of aminoglycoside antibiotics that inhibit the synthesis of proteins in organisms having 80*S* ribosomes. Eukaryote cells, including also the fungi, have 80*S* and 70*S* ribosomes, while the prokaryote ones have only 70*S* ribosomes. Cycloheximide has the neutral properties (pH > 7, 20°C); it is weakly bound with the soil; its solubility in the soil solution is 2 g/100 g (2°C). The high activity of cycloheximide in the soil is preserved for a long time.

Statistic processing of the results. The net N_2O production was measured in 3–5 replicates; for SIR, 4 replicates were used. All the calculations were made on the dry weight of soil (105°C, 8 h); the results were expressed as the average ± standard deviation (Excel). The experimental data were processed using the single-factor analysis of variance (Statistica 10.0) according to the Dunñan and Kruskal–Wallis. The data on the spatial distribution were illustrated using a *box-plot*; the values of median, lower and upper quartiles (limits) are given. The range (distance) between different parts of the *box* (median, quartiles) permits to us judge the degree of dispersion and asymmetry of the data.

RESULTS

Net production of N_2O by soil in laboratory (moisture, sample weight, incubation, preincubation, replicates of measurement, additional substrate). An initial objective of the tests was related to the selection of experimental conditions that provide the highest gas production by the soil and its sustainable detection. A sample of air-dried soil (1, 2, and 10 g) was placed in a test vessel (15 mL), moistened with water (0.6 mL/g) to activate the microbial activity, hermetically sealed, and incubated (24 h, 22°C and 28°C). The highest net N_2O production was found in the variants with a wide ratio between the volume of the soil (*Vs*) and the volume of the air (*Va*) in the vessel equal to 1 : 14 and 1 : 6.5 (Fig. 1). The incubation of the soil at 28°C did not increase the N_2O production as compared to that at 22°C.

The rate of the net N_2O production in the gray soil (*Vs* : *Va* = 1 : 6.5, the weight is 2 g) grew with the increasing time of incubation, reaching the highest

values after 24 h (Fig. 2). The longer incubation of the soils (more than 24 h) did not promote any increase in the N_2O production (the data are not presented); after 7 days, it was even reduced (almost by two orders of magnitude) compared to that in the 24-h variant. Therefore, in further tests, the weight of the soils (predominantly from the upper mineral horizons or layers) was 2 g, the *Vs* : *Va* ratio = 1 : 6.5, and the temperature and the duration of incubation were 22°C and 24 h, respectively.

The rate of the net N_2O production strongly varied (Table 1). After moistening the dry soil, it was nearly 200%; after the preincubation, its variation was lower, about 100%. The preliminary incubation of the soils significantly (by 3–5 times) lowered the net N_2O production as compared to that in the variant with the repeated moistening of the dry soil sample.

The application of additional sources of nitrogen and carbon increased the N_2O production and allowed us to avoid the problems related to the low emission of this gas [9, 33]. Therefore, in the further tests, the soil was enriched with an additional substrate. The input of glucose (2 mg/g soil) to the soil increased (by several orders of magnitude) the N_2O production (Table 2). In the upper (0–10 cm) mineral soil layer, the net production of N_2O was 1990–7167 $N_2O–N \times 10^{-3}$ ng/g per h for the deciduous and spruce forests. The lowest rate of the N_2O production (after the application of glucose) was determined in the gray soil under the aspen forest. The highest rate was registered in the soddy-podzolic soil of the cropland, fallow, and under the young forest (the difference was almost two orders of magnitude).

The data on the dynamics of N_2O production in the soddy-podzolic soil enriched with glucose under the native forest and cropland (Kostroma oblast) are presented in Table 3. With increasing the time of incubation, the N_2O production became higher and was maximal after 24-h incubation in the native soil (Fig. 2) and in the soils enriched with glucose.

The enrichment of the soil with ammonium sulfate (0.08 mg N/g) did not promote any increase of the net N_2O production as compared to the control (Table 4). The combined application of carbon (glucose) and nitrogen (ammonium sulfate) enhanced the rate of N_2O production (by 16–90%) compared to the variant with the application of only glucose. The highest increase in the rate was recorded in the soddy-podzolic soil and chernozem (by 56 and 70–90%, respectively); the lowest increase (by 16–17%) was recorded in the gray soil.

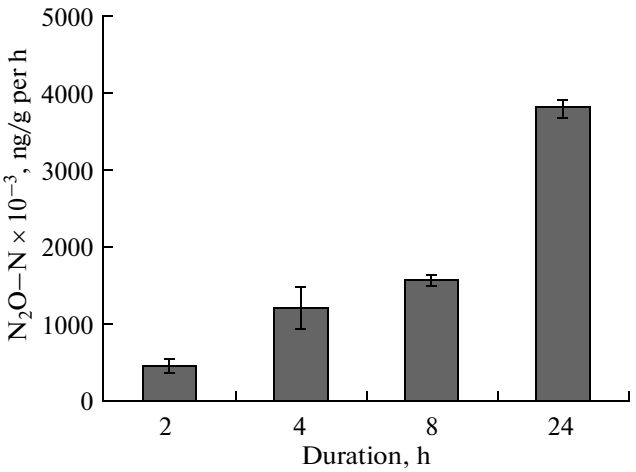

Fig. 2. Dynamics of N_2O net production by the moistened (0.6 mL H_2O) dry gray forest soil (forest, 0–10 cm, weight 2 g, incubation at 22°C, $n = 3$).

Thus, the conditions of measuring the net N_2O production by different soils were optimized related to the weight of sample; its preliminary incubation, temperature, and duration of incubation; enrichment with carbon and nitrogen; and replicates of measurements. The most sustainable net N_2O production was revealed when glucose (2 g; the volume of vessel is 15 mL) or a mixture of glucose and ammonium sulfate were added: the incubation lasted not less than 24 h at 22°C, the preincubation was carried out at 60% of water holding capacity and 22°C during 7 days, and the replicates were not less than 5.

Net N_2O production and soil microbial biomass. For the determination of the interrelationship between the net N_2O production and the microbiological characteristics (C_{mic} and C_{mic} : C_{org}) of the soils, experiments with soil samples reflecting the vertical (soil profile), horizontal (transect), and spatial (territory) gradients were performed. The N_2O production was significantly higher (183–4734 $N_2O–N \times 10^{-3}$ ng/g per h) in the upper horizons of the soils under different forests than in the lower horizons (0.28–69 $N_2O–N \times 10^{-3}$ ng/g per h) (Table 5). The C_{mic} content drastically and significantly ($p \leq 0.05$) decreased down the profile of the soils under different

Table 1. Net production of N_2O by the soils (0–10 cm) under different forests (*Kaluzhskie zaseki*) after preliminary treatments

Forest (Kaluga oblast)	Soil	$N_2O–N \times 10^{-3}$, ng/g per h ($n = 5$)	
		moistening of air-dry soil, 0.6 mL H_2O/g	preincubation for 7 days, 22°C, 60% of water holding capacity
Oak forest	Soddy-podzolic	6.0 ± 11.0	2.0 ± 2.0
Spruce forest		2.8 ± 1.7	0.9 ± 0.8
Aspen forest	Gray forest	10.0 ± 17.0	1.9 ± 1.8

Table 2. Net production of N_2O by the soils (preincubation during 7 days at 22°C, 60% of water holding capacity) of different forests and croplands, without and with application of glucose (incubation for 24 h)

Ecosystem (age, year)*	Oblast (locality)	Soil (layer, cm)	N_2O–N, $\times 10^{-3}$ ng/g per h ($n = 5$)	
			without glucose	glucose, 2 mg/g
Oak forest	Kaluga (Reserve *Kaluzhskie zaseki*)	Soddy-podzolic (0–10)	2.0 ± 2.0	2546 ± 2019
S_a			0.9 ± 0.8	4734 ± 842
Aspen forest		Gray forest (0–10)	1.9 ± 1.8	240 ± 94
S_{gm}	Moscow	Podzol (1.5–5)	199 ± 77	4065 ± 1569
S_{bl}	(Zvenigorod);	Rzhavozem (2–6)	149 ± 32	1990 ± 697
Cropland	Kostroma	Soddy-podzolic (0–10)	0.6 ± 0.5	22002 ± 7111
Fallow (7)	(Parfen;evo, Kologriv)		1.6 ± 0.8	16475 ± 8987
Forest (20)			2.2 ± 1.7	38347 ± 2922
Forest (45)			1.1 ± 1.1	17074 ± 9041
Forest (90)			Not det.	7167 ± 1973
Forest (450)			2.2 ± 1.0	3442 ± 2161

S_a—archangel spruce forest, S_{gm}—green moss spruce forest; S_bl— spruce–broad-leaved forest.

forests. A positive correlation ($r = 0.74$, $p \le 0.05$, $n = 18$) was found between the rate of the N_2O production by different soil loci and the C_{mic} content there. Consequently, the greater the microbial biomass in the soil is, the higher the net production of N_2O, testifying to the interrelation between this process and the abundance and activity of soil microorganisms.

In the young ecosystems (cropland, abandoned lands, young forest) with the soddy-podzolic soils (horizontal gradient), the net N_2O production was high and significant ($p \le 0.05$), while in the *mature* ones (secondary and native forests), it was low (Table 6). In the soils of *mature* ecosystems, the C_{mic} content was significantly higher than that in the young ecosystems. The correlation between the N_2O and C_{mic} contents in the soils of this succession series was close but negative ($r = -0.75$, $n = 6$). The rate of net N_2O production was found to be low ($r = -0.56$, $p \le 0.05$, $n = 6$) if the $C_{mic} : C_{org}$ ratio was high (Table 6). Consequently, the higher the share of the microbial biomass in the total organic matter content (that is characteristic of natural weakly disturbed ecosystems) was, the lower the rate of net N_2O production.

The direct interrelationship between the microbial biomass and the rate of the net N_2O production was found in the soils of natural ecosystems. An anthropogenically changed soil (cropland, fallow, young forest) can produce more N_2O than its natural analogue. In addition, the interrelation between the microbial component (C_{mic}) and the N_2O production by the soils of the disturbed ecosystems was close, but negative. This fact shows that the N_2O flux increases under the anthropogenic transformation of terrestrial ecosystems.

Table 3. Dynamics of net N_2O production by the soddy-podzolic soil (2 mg glucose/g, $n = 5$) under the native forest and cropland (Kostroma oblast), N_2O–N $\times 10^{-3}$ ng/g per h

Duration of incubation, h	Forest (6–14 cm)	Cropland (0–24 cm)
3	11.9 ± 14.4	8.9 ± 8.9
6	21.5 ± 17.4	4.4 ± 5.3
12	1761 ± 2502	136 ± 204
24	3442 ± 2161	22002 ± 7111

Spatial distribution of the rate of N_2O production was estimated in the soil (soddy-podzolic, gray, alluvial–meadow) samples from different ecosystems (forests, abandoned lands, croplands, $n = 9$, 7, and 6, respectively) of Podol'sk and Serpukhov districts (Moscow oblast). The N_2O production by the plowed soils was 15–105; the fallow and forest soils produced 126–387 and 1–107 ng N_2O–N $\times 10^{-3}$/g per h. On average, the soils of the forest, cropland, and fallow produced 38, 69, and 239 ng N_2O–N $\times 10^{-3}$/g per h. Thus, in the soils of the natural ecosystems (forest), the N_2O production was much less than in the cropland and fallow (by 1.8 and 6.3 times, respectively) (Fig. 3). The C_{mic} content and the $C_{mic} : C_{org}$ ratio in the plowed, fallow, and forest soils, on average, were 181, 569, and 1020 μg and 1.4, 2.6, and 3.0%, respectively, indicating a significant decrease of these characteristics in the anthropogenically disturbed ecosystems compared to those in the natural ones.

Net N_2O production by soil in applying cycloheximide (antibiotic, fungicide). The net N_2O production in the soddy-podzolic, gray, and chernozemic soils was inhibited by cycloheximide, the concentration of which corresponded to the highest inhibition of SIR (determined in preliminary experiments). The inhibition of the production by the fungicide was significant and amounted to 69–99% of that in the corresponding control variant (Table 7). Taking into account that cycloheximide can inhibit synthesis of protein only by the eukaryote microorganisms (soil fungi belong to them), one can suggest that the latter actively participate in the net N_2O production in soils.

Separation of nitrification and denitrification contributions to net N_2O production. The tests showed that the C_2H_2 concentration equaling only 1.8 Pa (0.0020% of the volume) caused a decrease in the N_2O production

Table 4. Net production of N_2O ($N_2O–N \times 10^{-3}$ ng/g per h) by different soils when applying glucose and ammonium sulfate $(NH_4)_2SO_4$)

Soil	Oblast (locality)	Ecosystem (layer, cm)	Control	Glucose, 2 mg/g	$(NH_4)_2SO_4$, 0.08 mg N/g	Glucose + $(NH_4)_2SO_4$
Soddy-podzolic	Vladimir (Safonovo)	Forest (0–5)	8.3 ± 10.5	123 ± 45	4.8 ± 5.5	280 ± 18
Leached chernozem	Voronezh (Voronezh)	Forest belt (0–10)	0.5 ± 0.5	13 ± 6	0.4 ± 0.4	142 ± 48
		Meadow (0–10)	0.7 ± 0.07	9 ± 5	8.5 ± 0.2	93 ± 5
		Cropland (0–10)	0.1 ± 0.03	17 ± 5	0.3 ± 0.2	57 ± 15
Gray forest	Moscow (Pushchino)	Forest (0–10)	1.0 ± 0.5	82 ± 38	0.7 ± 0.3	99 ± 29
		Cropland (0–10)	1.2 ± 0.7	97 ± 32	2.0 ± 0.8	114 ± 18
Burozem Carbolithozem	Krasnodar (Severnaya Ozereika)	Forest (4–14)	1.1 ± 0.4	86 ± 16	2.6 ± 0.8	106 ± 27
		Forest 1 (0–9)	5.6 ± 0.3	53 ± 21	0.8 ± 0.6	69 ± 56
		Forest 2 (0–9)	1.2 ± 0.6	34 ± 22	0.6 ± 0.4	65 ± 63
		Forest3 (0–9)	0.8 ± 0.6	75 ± 27	0.3 ± 0.06	79 ± 43

Table 5. Net production of nitrous oxide (N_2O, 2 mg glucose/g of soil) and soil microbial biomass carbon (C_{mic}) in different horizons of forest soils (figures with different letters differ significantly, $p \le 0.05$, Duncan criterion, for each parameter and forest separately)

Forest* (soil)	Oblast (locality)	Horizon (depth, cm)	$N_2O–N \times 10^{-3}$, ng/g per h	C_{mic}, μg C/g
Mixed (soddy-podzolic)	Vladimir (Safonovo)	O (0–5)	123 ± 45 *c*	462 ± 92 *c*
		AY (5–10)	183 ± 68 *c*	365 ± 34 *c*
		ELBM (10–30)	25 ± 17 *b*	195 ± 9 *b*
		AM (30–58)	0.4 ± 0.3 *a*	22 ± 4 *a*
Oak forest (soddy-podzolic)	Kaluga (Reserve *Kaluzhskie zaseki*)	AU (0–10)	2546 ± 2019 *c*	1816 ± 107 *c*
		EL (10–20)	14 ± 4 *b*	184 ± 58 *ab*
		BT1 (40–50)	0.3 ± 0.4 *a*	238 ± 57 *b*
		BT2 (80–90)	0.3 ± 0.2 *a*	110 ± 22 *a*
S_a (soddy-podzolic)		AY (0–10)	4734 ± 842 *b*	755 ± 50 *b*
		EL (20–30)	21 ± 4 *a*	110 ± 54 *a*
Aspen forest (gray forest)		AY (0–10)	240 ± 94 *b*	1351 ± 14 *c*
		EL (40–50)	1.3 ± 1.2 *a*	161 ± 18 *a*
		BT (70–80)	0.5 ± 0.3 *a*	299 ± 52 *b*
S_{gm} (podzol)	Moscow (Zvenigorod)	O (1.5–5)	4065 ± 1569 *c*	2545 ± 71 *c*
		EL (5–16)	218 ± 32 *b*	195 ± 13 *b*
		BF (16–38)	1.1 ± 0.2 *a*	27 ± 5 *a*
S_{bl} rzhavozem)		AY (2–6)	1990 ± 697 *b*	1568 ± 156 *b*
		BFM1 (11–39)	69 ± 14 *a*	75 ± 24 *a*

* S_a—archangel spruce forest; S_{bl}—spruce–broad-leaved forest.

by the soils studied (Fig. 4). Since this decrease was associated with the inhibition of nitrification in the soils [43], there is a reason to calculate a contribution of nitrification and denitrification separately to the total net N_2O production. Thus, in the soddy-podzolic and gray soils of natural ecosystems, the contribution of nitrification was 13–23%, while in the plowed ones it was 6–16% (Table 8); in the chernozem, the contribution of nitrification was not revealed. Therefore, denitrification in the soils studied was more significant, and its contribution was 77–100%.

Hereafter, cycloheximide (an inhibitor of SIR for fungi) was applied to the soddy-podzolic (under the forest) and gray (the cropland) soils, and the soils were incubated with acetylene (1.8 Pa) (Table 9). The application of cycloheximide and the following incubation in the presence of C_2H_2 decreased the N_2O production by 93–96% of the control. Thus, nitrification in the soddy-podzolic and gray forest soils was performed due to the bacterial activity by 54% (7 × 100/13) and 25% (4 × 100/16), respectively.

The application of cycloheximide to the soils significantly (by 69–99%) inhibited the net N_2O production, testifying to the strong contribution of the eukaryote component of the microbial biomass to this process. An approach for the separation of nitrification and denitrification in the N_2O production in the soils using acetylene in low concentration is proposed.

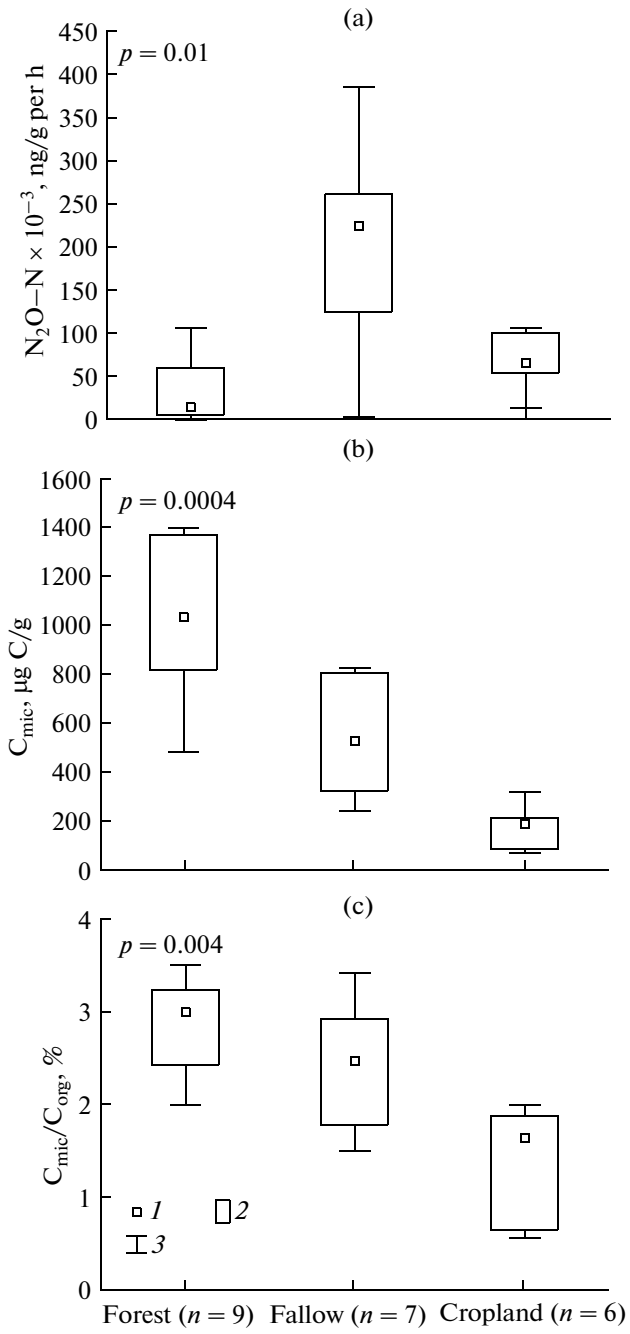

Fig. 3. Distribution of net production of N_2O (a), microbial biomass carbon (b) and the C_{mic}: C_{org} (c) ratio in the forest, fallow, and plowed soils (0–10 cm, Podol'sk and Serpukhovsk districts, Moscow oblast): *1*—median, *2*—25–75%, *3*—interquartile distance.

DISCUSSION

Soil moisture and additional substrate. The experiments showed that after moistening of the air-dry soils, the net N_2O production increased, and their preliminary incubation (60% of water holding capacity, 22°C, 7 days) sharply (by 3–5 times) lowered the intensity of this process. Some authors also noted that moistening of air-dry soils increased the rate of N_2O production [29, 50, 70]. In our tests, the variation of N_2O production in the repeatedly moistened and preliminarily incubated soils reached nearly 200 and ≤100%, respectively. The high variability (60–225%) of this process, including its spatial variation, was also noted in [12, 40, 74]. Therefore, the preliminary treatment (preincubation) of soil is recommended before the performance of microbiological analyses for some microbial components [24, 57] and production of carbon dioxide [13, 27]. This procedure permits mitigation of the high variability of the parameters measured due to different moisture and disturbance of soils (sampling, mixing of samples, sieving, drying, and storage).

To avoid problems related to low emissions of N_2O from the soils, mineral ammonium and nitrate nitrogen are added [9, 10]. Different forms of nitrogen fertilizers (NH_4NO_3, $NH_4)_2SO_4$, $CO(NH_2)_2$, KNO_3) promote increasing N_2O emission by 3–5 times compared to the control variant with the cultivated soil [33]. The increase of N_2O emission from soils is associated with higher content of organic carbon and total nitrogen [44, 48, 60]. In our experiments, the enrichment of the soil with glucose (carbon source) drastically increased the net N_2O production. The application of nitrogen with ammonium sulfate and glucose increased or did not affect this process. Some authors stated that the application of glucose (1 mg/g) intensified (by 20 times) the N_2O production as compared to the emission from the non-enriched soil [51].

Contribution of nitrification and denitrification to N_2O production by the soils is still a subject of wide discussion [5]. According to the data of different authors, the contribution of nitrification to the N_2O flux from steppe soils is 61–98 [56] and 60–80% [58], while from the forest soils it is 3–50 [61] and about 50% [28]. The contribution of heterotrophic microorganisms to nitrification of the soddy-podzolic soil under the wood sorrel spruce forest was found to be 94%, in the gray forest soil under the birch forest it was 44%, and in the plowed soils it was 1–16% [6].

The processes of nitrification and denitrification responsible for the N_2O flux are separated using selective inhibitors; for the determination of nitrification, these are acetylene, nitrapyrin, dicyandiamide, etc. The low concentration of acetylene (C_2H_2) in the test vessel (0.1–10 Pa) inhibits the ammonium monooxidase activity (by covalent fixation) and nitrification [21, 35, 53, 75]. Therefore, after the treatment with C_2H_2, the total N_2O flux will be only due to denitrification. Some authors consider that the C_2H_2 concentration should be not more than 1 Pa [35]; others suggest that it is 0.1–10.0 [17, 21, 53, 75] or 2.5–5.0 Pa [43]. For field experiments, the concentration of 5–10 Pa C_2H_2 were used [55]. In this connection, it is important to determine the C_2H_2 concentration for each soil. The C_2H_2 concentration to inhibit nitrification was shown to depend on the content of nitrates in the soil [31]. The high concentration of acetylene (5–10 kPa) efficiently inhibits

Table 6. Net production of nitrous oxide (N_2O, 2 mg glucose/g of soil) by the soddy-podzolic soils (0–10 cm) of different ecosystems, soil microbial biomass carbon (C_{mic}) and its portion of the total soil organic carbon (C_{org}) (figures with different letters differ significantly, $p \le 0.05$, Duncan criterion, for each parameter and forest separately)

Ecosystem (age, year)	$N_2O–N \times 10^{-3}$, ng/g per h	C_{mic}, μg C/g	C_{mic}/C_{org}
Cropland	22002 ± 7111 *b*	149 ± 12 *ab*	2.2 ± 0.2 *abc*
Fallow (7)	16475 ± 8987 *b*	187 ± 22 *bc*	2.1 ± 0.3 *abc*
Young forest (20)	38347 ± 2922 *c*	245 ± 23 *c*	2.0 ± 0.2 *abc*
Young forest (45)	17074 ± 9041 *b*	502 ± 25 *e*	2.1 ± 0.1 *abc*
Secondary forest (90)	7167 ± 1973 *a*	759 ± 135 *f*	5.7 ± 1.0 *d*
Native forest (450)	3442 ± 2161 *a*	755 ± 34 *f*	3.0 ± 0.1 *bc*

Table 7. Net production of nitrous oxide ($N_2O–N \times 10^{-3}$, ng/g per h) by different soils (preincubation; incubation: 2 g of soil, 24 h, 22°C, $n = 5$; glucose 2 mg/g)

Soil (locality)	Ecosystem (layer, cm)	N_2O (glucose, I)	CH*, mg/g	N_2O (CH + glucose, II)	Inhibition, % of I
Soddy-podzolic (Safonovo)	Forest (0–5)	123 ± 45	40	2.8 ± 3.2	98
(Efremovo)	Forest (5–15)	7 ± 4	30	0.7 ± 0.2	91
Gray forest (Pushchino)	Forest (0–10)	82 ± 38	20	8.6 ± 6.1	90
	Cropland (0–10)	97 ± 32	20	27.3 ± 55.4	72
Leached chernozem (Voronezh)	Forest belt (0–10)	13 ± 6	50	0.4 ± 0.2	97
	Meadow (0–10)	9 ± 5	50	3.8 ± 1.8	69
	Cropland (0–10)	17 ± 5	50	2.6 ± 0.2	85
	Cropland (0–10)/ mc**	11 ± 7	50	2.3 ± 1.9	79
	Cropland (0–10)/rot 120	312 ± 66	50	1.6 ± 1.0	99

* CH—cycloheximide, corresponded to the strongest inhibition of SIR (preliminary tests);

** mc—monoculture of corn (without application of mineral fertilizers); rot 120—rotation with annual application of nitrogen fertilizers at the rate of 120 kg/ha.

the reduction of N_2O to N_2 by denitrifying bacteria that permits estimation of the denitrification [42, 82]. Consequently, acetylene in different concentrations can inhibit both nitrification and denitrification. However, acetylene may stimulate the mineralization of soil organic matter (formation of excessive CO_2), indirectly intensifying denitrification [32]. In addition, soil microorganisms can absorb C_2H_2 [11, 76] and

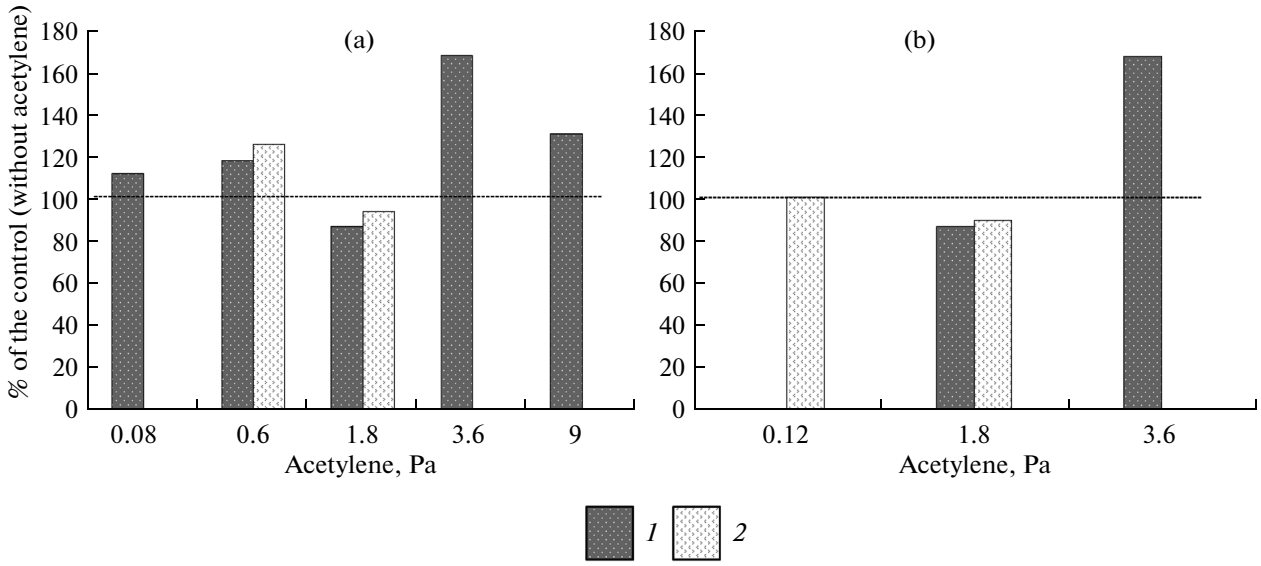

Fig. 4. Net production of N_2O by the soddy-podzolic (a) and gray forest (b) soils (forest (*1*) and cropland (*2*)) at different concentrations of acetylene (Pa).

Table 8. The contribution of nitrification and denitrification to the net production of N_2O by different soils (0–10 cm) (acetylene, 1.8 Pa)

Soil (locality)	Ecosystem	Portion, %	
		nitrification	denitrification
Soddy-podzolic (Pod-ol'sk)	Forest	13	87
	Cropland	6	94
Gray forest (Pushchino)	Forest	23	77
	Cropland	16	84
Chernozem (Voronezh)	Forest belt	0	100
	Cropland	0	100

reduce its concentration (inhibition is incomplete). This is especially important in short-term tests [43].

Contribution of fungi and bacteria to net N_2O production by soil Bacteria are shown to form about 20% of the total N_2O production by soils, while fungi (fumigation with chloropicrin) are responsible for 70% [67]. To separate the contribution of bacteria (prokaryotes) and microscopic fungi (eukaryotes) to this process, antibiotics inhibiting the synthesis of proteins of bacteria (streptomycin) and fungi (cycloheximide) are often used [6, 18, 23]. Denitrification with the formation of N_2O is mainly performed by bacteria, and, under certain conditions, by fungi as well [63]. The real denitrification by fungi was not found. Nitrite reductase of eukaryotes, unlike that of prokaryotes, has one active center. It is unrelated to cellular membrane and not inhibited by acetylene [26]. Soil fungi of the *Fusarium* genus were shown to reduce nitrites and produced N_2O at the low oxygen content [19]. Therefore, the formation of N_2O is considered an adaptation of mycelial fungi to detoxication of nitrites that accumulate under heterotrophic nitrification. Denitrification performed by fungi predominates in the soils of forests and pastures [46], as well as in the soils of semiarid territories [51]. Nitrification by fungi (application of cycloheximide) was revealed significantly in forest soils [62], particularly in the soils under coniferous forests [41]. The application of cycloheximide to soils lowered the N_2O emission by 81% [80], 89% [46], and 63% [51], demonstrating a considerable contribution of fungi to this process. Our experiments also revealed the inhibition of N_2O production by cycloheximide. For the maximal inhibition of fungal respiration by cycloheximide, its concentration should be detected in preliminary tests [36, 45]. However, the substantiation of the cycloheximide concentration applied to soil is rather contradictory. Some researchers believe that fungicide must not have a biocidal effect (for the first 48 h) on the microbial biomass [23]; others, on the contrary, believe that it must affect them [46, 80]. As shown in [46], the inhibition of SIR in the soil by two antibiotics (cycloheximide and streptomycin) was accompanied by the calculation of a coefficient of overlapping the antibiotics action [49]; in [80] this fact was not noted. In the soils studied, cycloheximide was applied in the concentration (from 20 to 50 mg/g of soil) providing the maximal inhibition of SIR, and the value of overlapping the effects of antibiotics (fungicide and bactericide) was 100 ± 5% [1–3].

CONCLUSIONS

In order to optimize the measurement of net N_2O production in laboratory, the preincubation of a soil sample (22°C, 60%of total water capacity, 7 days) is recommended. For the main incubation, (22°C, 24 h), the sample was placed into a test vessel, where the proportion between the volumes of soil and air was not less than 1 : 6.5. To obtain sustainable N_2O production, it is expedient to introduce additional sources of carbon and energy (glucose) and of mineral nitrogen (preferably, in ammonium form). Cycloheximide (an antibiotic inhibiting respiration of fungi) was used to estimate the contribution of eukaryotes and prokaryotes to the N_2O production. This fungicide significantly affected (69–99%) the N_2O production by the soil, testifying to a great participation of fungi in this process. For the subdivision of the N_2O production process into denitrification and nitrification, acetylene as an inhibitor of nitrification is proposed. The concentration of this gas in the test vessel should be 1.8 Pa (0.002% C_2H_2).

The experimental results indicate the predominant contribution of denitrification to the total net production of N_2O by the soils.

The net N_2O production by the soils in the presence of both inhibitors (C_2H_2 and cycloheximide) amounted to 4–7% of the control, pointing to a signif-

Table 9. Net production of nitrous oxide ($N_2O–N \times 10^{-3}$, ng/g per h) by the soddy-podzolic and gray forest soils (0–10 cm; 2 mg of glucose + 0.08 mg $(NH_4)_2SO_4–N$/g); the control sample, with the application of acetylene and cycloheximide

Variant	Soddy-podzolic (forest)	Inhibition, % of the control	Gray forest (cropland)	Inhibition, % of the control
Control (without C_2H_2)	146 ± 27	0	77 ± 5	0
C_2H_2, 1.8 Pa	127 ± 12	13	65 ± 7	16
Cycloheximide, 20 mg/g	17 ± 12	88	5 ± 3	93
Cycloheximide + C_2H_2	11 ± 1	93	3 ± 4	96

icant role of the fungal component of the microbial biomass in this process. The relationship between the net production of N_2O and microbiological characteristics of the soils (C_{mic}, C_{mic}: C_{org}) is shown along the vertical, horizontal, and spatial gradients.

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