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EXPERIMENTAL ARTICLES

Changes in the Structure and Activity of a Soil Microbial Community Caused by Inorganic Nitrogen Fertilization

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Abstract—The changes in the structure and activity of a soil microbial community caused by addition of moderate and high rates of the mineral nitrogen fertilizer (KNO_3) were studied in a laboratory incubation experiment. The structure of the microbial community was evaluated from the phospholipid fatty acid (PLFA) profile; specific growth rate of the microorganisms was determined by the method of the kinetics of substrate-induced respiration; the total pool of microbial carbon was estimated by the fumigation–extraction method. The amounts of nitrogen fertilizer applied in three treatments of the experiment were 0 (control), 100, and 2000 μg N/g soil. Even in the absence of additional sources of organic carbon, a considerable portion of the added ^{15}N (up to 74%) was immobilized. No significant increase in the amount of microbial carbon was observed during incubation. The specific growth rate of the microbial community in soil supplemented with glucose decreased twofold after addition of 2000 μg N/g soil. In this treatment, the ratio of cyclic fatty acids to their monoenoic precursors also increased, indicating the adaptation of microbial cells to extremely high amounts of nitrogen fertilizer. Moreover, considerable changes in the structure of the soil microbial community, such as an increase in the ratio of fungal- to bacterial markers and a decrease in the ratio between PLFA of gram-positive and gram-negative bacteria, were observed in the treatment with addition of 2000 μg N/g soil. Our data clearly indicate that mineral nitrogen fertilization of soil under carbon limitation has a pronounced impact on the structure and activity of soil microbial communities.

Keywords: soil microbial community, phospholipid fatty acids, nitrogen immobilization, microbial biomass, specific growth rate

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Immobilization of nitrogen by soil microorganisms is of great importance for maintaining "soil health" at the optimal level; this process prevents the nitrogen from washing out of the soil profile into lower horizons and groundwaters, as well as its loss as dinitrogen gas or nitrogen oxides. Nitrogen immobilization stimulates a prolonged positive effect of nitrogen fertilizers on plant nutrition due to the remobilization of initially immobilized nitrogen via the immobilization–mineralization cycles.

For a long time, it has been considered that microorganisms immobilize nitrogen only in the presence of carbon sources and that this process results in increased microbial growth rate [1]. However, there is information that considerable amounts of nitrogen may be immobilized even in the absence of additional carbon substrates [2, 3]. By using labeled ^{15}N, it was revealed that up to 72% of added inorganic nitrogen was immobilized by microbial biomass during the first 4–10 days after fertilization without addition of organic carbon sources [3]. Mechanisms of such intensive nitrogen immobilization remain unclear. At present, two hypotheses exist: (a) nitrogen incorporation into the low-molecular-weight organic compounds without its involvement in biosynthetic processes [4]; (b) abiotic nitrate fixation in soils (the nitrozation reaction) [5].

Rapid nitrogen immobilization resulted in extremely low C : N ratios in the biomass (less than 2) [6] without a marked increase in the soil microbial biomass. Taking into account the absence of additional carbon substrates, it may be assumed that even moderate rates of mineral nitrogen (≤200 μg N/g) can evoke deficiency of available organic carbon or even carbon starvation in soil microorganisms. Moreover, extremely high amounts of nitrogen (≥1000 μg N/g)

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can cause osmotic stress. Both effects can change the structure and functions of soil microbial communities.

The goal of the present work was to study nitrogen immobilization, changes in the growth kinetics of microorganisms, and a shift in the structure of the soil microbial community after addition of moderate or extremely high amounts of a mineral nitrogen fertilizer.

MATERIALS AND METHODS

Soil. Samples of brown soil (Eutric Cambisol) were taken 40 km northeast of Munich (Germany) from the area, which was subjected to crop rotation (potato, maize, and winter wheat) for over 20 years. The soil samples had the following composition: C, 1.4 ± 0.2%; N, 0.14 ± 0.01%; pH_{KCl} 5.9 ± 0.2. The fresh samples were sieved (2-mm mesh size), cleaned of roots, and kept wetted at 4°C for two weeks before the onset of the experiment (day 0).

Pattern of experiment. The soil samples were divided into two portions. The first one was incubated with the labeled $K^{15}NO_3$ in order to determine nitrogen immunization, dynamics of the total microbial carbon pool (C_{mic}), and the changes in kinetics of microbial growth which were caused by nitrogen fertilization of the soil. The moist soil samples (10 g, calculated per dry soil) were placed into 100-mL glass vessels into which the solution of $K^{15}NO_3$ was added with ^{15}N enrichment of 29.3 at %; all the soil samples were wetted to 70% of water holding capacity (WHC) and incubated at 22°C. The rates of introduced nitrogen were 0, 100, and 2000 μg N/g in the treatments "0", "100", and "2000", respectively. The amounts of inorganic and total nitrogen, water-soluble organic compounds, the total carbon of microbial biomass (C_{mic}), and a ratio of $^{15}N : {}^{14}N$ in the nitrogen pools were analyzed on days 0, 4, and 30 of soil incubation. Parameters of microbial kinetics were determined on the 30th day of the experiment.

In the second portion of the soil samples, the structure of the soil microbial community was determined. Moist soil samples (50 g, calculated per dry soil) were put into 500-mL vials with rubber stoppers, wetted to 70% WHC, and incubated as described above. The structure of microbial communities was determined on the 30th day of experiment. All analyses were performed in four replicates.

Determination of soil microbial biomass C, growth rate of microbial communities, and ^{15}N immobilization. The microbial biomass C was analyzed by the method of fumigation–extraction (FE) [7]. The samples of biomass were incubated under chloroform vapors for 24 h in order to kill soil microorganisms, then organic compounds were extracted from the treated biomass with 0.5 M K_2SO_4 solution in a soil/solution ratio of 1 : 4; the extracts were filtered through a dense paper filter, frozen, and stored until the analyses of organic carbon, nitrogen, and a $^{15}N : {}^{14}N$ ratio were performed. The extracts of the control soil samples (without fumigation) were filtered, dried, homogenized, and used for analysis of the $^{15}N : {}^{14}N$ ratio.

The pool of microbial carbon was calculated from the value of C flush, which was determined as a difference between carbon content of fumigated soil samples and that of untreated control samples with the use of the conversion factor k_{EC} equal to 0.45 (the extraction coefficient for fumigated microbial biomass) [7]:

$$C_{mic} - FE = C\ flush/k_{EC}. \quad (1)$$

Specific growth rate of the soil microbial community was determined by the method of the kinetics of substrate-induced response [9]. Kinetics of the carbon dioxide release was measured after soil supplementation with N-, P-, and K-containing mineral salts under excess of a carbon substrate (usually glucose). Specific growth rate of the microbial community was calculated from the equation of exponential growth induced by addition of a carbon substrate and mineral salts [8, 9]:

$$v(t) = v_u + v_c \exp(\mu_{max} t), \quad (2)$$

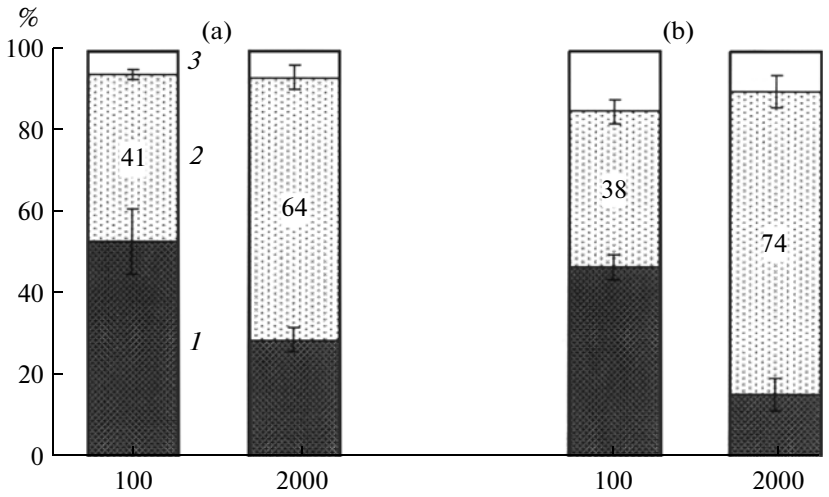
where $v(t)$ is the rate of CO_2 production, μg $C\text{-}CO_2$/(g h); t is time, h; v_c is the rate of coupled, μg $C\text{-}CO_2$/(g h); v_u is the rate of uncoupled respiration, μg $C\text{-}CO_2$/(g h); and μ_{max} is the maximal specific growth rate of microorganisms, h^{-1}. The gas samples were withdrawn every hour during the first 27 h after glucose addition.

We did not calculate the ^{15}N incorporation into microbial biomass by the FE method since the value of the conversion factor k_{EN} was uncertain under extremely high nitrogen concentrations in soil [6]. Instead, we calculated the gross immobilization of ^{15}N incorporated into both microbial biomass and other pools of organic substances according to the following equation:

$$\text{Immobilized } {}^{15}N = {}^{15}N_s - {}^{15}N_{WSN}, \quad (3)$$

where $^{15}N_s$ is labeled nitrogen remained in soil after incubation and $^{15}N_{WSN}$ is water-soluble nitrogen (WSN) incorporated into the soluble nitrogen-containing compounds, which were extracted from the control (unfumigated) soil samples. The $^{15}N_{WSN}$ pool was composed mainly by inorganic compounds; the data on the concentrations of organic nitrogen compounds in this pool were statistically insignificant.

Analytical methods. The organic carbon concentration in soil extracts was determined on a TOC 5050 automatic analyzer (Shimadzu, Japan); nitrogen content of soil extracts was assayed on a continuous flow analyzer (Skalar Analytical, Germany); a ratio of $^{15}N : {}^{14}N$ in soil was determined on an IRMS MAT 253 mass spectrometer (Thermo Finnigan, Germany) coupled with an Euro EA elemental analyzer (Eurovector, Italy). The proportions of nitrogen iso-



Distribution of ^{15}N (%) on days 4 (a) and 30 (b) after addition of $K^{15}NO_3$: inorganic nitrogen pool (*1*); immobilized nitrogen pool (*2*); the loss of gaseous nitrogen calculated as a difference between the amount of labeled nitrogen added to soil and that determined after incubation (*3*). Bars indicate the values of standard deviation (±*SD*).

topes in soil extracts were measured on an NOI-6PC emission spectrometer (FAN Fischer Analyseninstrumente, Germany) coupled with a NA 1500 elemental analyzer (Carlo Erba, Italy). A ratio of ^{13}C : ^{12}C and CO_2 concentration in the gas samples were determined on a Delta plus mass spectrometer (Finnigan MAT, Germany).

Determination of the phospholipid fatty acid (PLFA) profiles. Analysis of PLFA was performed according to the previously described methods [10, 11]. Lipids were extracted from fresh soil samples (50 g, calculated per dry soil). The resulting lipid material was fractionated into neutral lipids, glycolipids, and phospholipids (polar lipids) on a silica-bonded phase column (SPE-SI Bond Elut, Varian, Palo Alto, United States) by elution with chloroform, acetone, and methanol, respectively. Four fractions of ester-linked PLFA (L-PLFA) and two fractions of non-ester-linked PLFA (NEL-PLFA) were obtained by using various methods of extraction, separation, hydrolysis, and esterification. Fatty acid methyl esters (FAME) in these fractions were analyzed on a GC/MS Hewlett Packard 5971 mass spectrometer combined with a 5890 series II gas chromatograph. The FAME identification was performed by comparing retention times of the substances on a chromatographic column and the results of mass spectrometry with those parameters of standard FAME [12, 13] using the HP ChemStation, SOVLVIT software package (Switzerland). The analyses of quantitative and qualitative composition of PLFA as markers of specific groups of microorganisms made it possible to determine comparative diversity of microorganisms in soil. Saturated PLFA (i14:0, n14:0, i15:0, a15:0, n15:0, i16:0, i17:0, a17:0, n17:0, cy17:0, and cy19:0) and monounsaturated PLFA (16:1ω7, 16:1ω9, and 18:1ω7) were used for identification of bacteria. Among the above-mentioned PLFA, fatty acids i15:0, a15:0, i16:0, i17:0, a17:0, and n17:0 are specific markers of gram-positive bacteria, whereas fatty acids 16:1ω7, 18:1ω7, cy17:0, and cy19:0 are the markers of gram-negative bacteria [12, 14]. Specific markers of the other groups of microorganisms were the following: 18:2ω6,9 and 18:1ω9 (for fungi) [15]; 20:2ω6,9c; 20:3ω6,9,12c; 20:4ω6,9,12,15c (for microeukaryotes) [12]; 10-methylated PLFA (for actinomycetes) [12]; and PLFA containing no ester bonds (for anaerobic prokaryotes) [11]. In order to characterize a shift in the structure of soil microbial communities, we used the following ratios: cyclopropyl PLFA to their monoenoic precursors [16], fungi to bacteria [17], and gram-positive to gram-negative bacteria [18].

RESULTS AND DISCUSSION

Nitrogen immobilization and growth dynamics of microbial biomass. The immobilized nitrogen on the 4th day of incubation amounted to 41 and 64% of the added ^{15}N in the "100" and "2000" treatments, respectively. Further nitrogen immobilization during days 4–30 was insignificant and comprised 38 and 74% in the treatments "100" and "2000", respectively (figure). Therefore, immobilization of ^{15}N generally occurred within a few days after the introduction of nitrogen fertilizers. These results agree well with the literature data on rapid immobilization of inorganic (ammonium and nitrate) nitrogen in soil. For example, Bristow et al. revealed that 37% of inorganic nitrogen was immobilized in soil two days after its addition [19]; Okereke and Meints reported that up to 10% of ammonium was immobilized during the first 12 h after its introduction [20]; according to our previous data, the level of immobilized ^{15}N reached 80 and 72% of the added amount in field experiments [3] and in lab-

Table 1. The carbon content of microbial biomass (C_{mic}) and maximal growth rate of the microorganisms (μ_{max}) determined on the 30th day after soil fertilization with 0, 100, and 2000 μg N-NO_3/g. Letters "a" and "b" stand for significant differences between the treatments at $p < 0.05$ estimated with the use of the Tukey test

Treatment (dose of N-NO_3, μg/g	C_{mic}, μg/g	μ_{max}, h^{-1}
0	235_a	0.33_a
100	303_a	0.35_a
2000	327_a	0.17_b

oratory incubation experiments [6], respectively. Results of the present study on the immobilization of up to 74% of the added labeled nitrogen indicate that the immobilization potential of the soil was not completely realized, even under high amounts of mineral nitrogen fertilizer.

The gaseous nitrogen loss calculated from the difference between ^{15}N content of soil before and after incubation were 4–7 and 10–18% on the 4th and 30th days, respectively. This nitrogen loss was most likely due to the formation of N_2O and N_2 in the course of denitrification. The obtained results on the gaseous nitrogen loss in soil are in good agreement both with our previous data (0–28% during 5–6 months after mineral nitrogen fertilization) [6] and with literature data (20–50% for 1–6 months) obtained for soils under different field crops [21]. The major portion of added nitrate nitrogen was apparently immobilized by microorganisms so rapidly that it was not involved in the denitrification process.

No statistically significant difference was revealed between the C_{mic} pools determined by the FE method in treatments "0", "100", and "2000", which amounted to about 300 μg C/g in all treatments (Table 1). Therefore, immobilization of the mineral nitrogen fertilizer in soil was not accompanied by intensive growth of microbial biomass.

Specific growth rate of microorganisms and the structure of the soil microbial community. Unlike the total pool of microbial carbon, the growth characteristics of the microbial community varied in dependence on the dose of mineral nitrogen added; the value of μ_{max} in the treatment with 2000 μg N-NO_3/g decreased almost twofold compared to the treatments "0" and "100" (from 0.33–0.35 to 0.17 h^{-1}) (Table 1). This may be due to osmotic stress caused by the extremely high concentrations of added nitrogen, as well as to the adaptation of soil microorganisms to unfavorable environments, which may either be associated with the changes in the structure of the microbial community or not. In order to confirm or reject this hypothesis, we compared the PLFA profiles in the treatments with addition of mineral nitrogen fertilizer and in the control. The total pool of PLFA did not differ markedly in all three treatments (Table 2), similar to the total pool of C_{mic} determined by the FE method (Table 1). However, in the treatment with the maximum dose of nitrogen fertilizer, the ratio of cyclopropyle PLFA to their monoenoic precursors was the largest (0.92) as compared with the treatments "0" (0.71) and "100" (0.75). This finding confirms our assumption on the adaptation of microbial cells to extremely high rates of nitrogen by changing the fatty acid composition of cellular membranes and, therefore, their structure and functions, including the transport functions regulating nitrogen delivery into the cells. A similar phenomenon has been earlier observed under stress conditions in soil which were not associated with nitrogen fertilization [18, 22, 23].

No statistically significant effect of nitrogen fertilization on the content of the PLFA markers of microeukaryotes was observed (Table 2). The level of fungal markers (18:1ω9 and 18:2ω6,9) in PLFA was higher in the treatments with nitrogen addition ("100" and "2000") than in the control (Table 2). At the same time, the content of specific markers of actinomycetes and gram-positive and gram-negative bacteria increased in the treatment "100" as compared with the control and decreased in the treatment "2000" in comparison with the treatment "100" (Table 2). The ratio between fungal and bacterial PLFA markers in the treatment "2000" (0.089) was therefore higher than in the control and the treatment "100" (0.073 and 0.082, respectively) (Table 2). A decrease in specific growth rate of the microbial community (Table 1) was probably due, in addition to the adaptive rearrangement of the membranes, to a shift in the fungi to bacteria ratio, since under stress impact, a change in the structure of a microbial community occurs usually in the direction of slow-growing microorganisms (K-strategists) [24], which, in our case, were represented by soil micromycetes.

Similar shifts in the structure of soil microbial community were described by Bargett and McAlister [25]; the ratio between fungi and bacteria increased upon addition of nitrogen and other mineral fertilizers. However, according to other authors, nitrogen fertilization of soil either had no effect on the structure of microbial community [26] or even decreased the fungi to bacteria ratio [27]. We think that this inconsistency of the literature data indicates that nitrogen impact caused osmotic shock in the presence of high salt concentrations in the soil solution that disguised the microbial response to the additional amount of such an important nutrient as nitrogen. It should be noted that a shift in the fungi to a bacteria ratio in favor of fungi was observed under stresses imposed by other factors, e.g., allelopathic impact of root exudates on rhizosphere microorganisms [28]; increased acidity [17], or soil flooding [29]. Thus, it may be assumed that when nitrogen fertilization results in a stress impact on the growth and survival of soil microorganisms, the structure of a microbial community changes

Table 2. Composition and characteristics of the microbial community determined from the phospholipid fatty acid (PLFA) profiles on the 30th day after soil fertilization with 0, 100, and 2000 μg $N\text{-}NO_3$/g. Letters "a" and "b" stand for significant difference between the treatments at $p < 0.05$ estimated with the use of the Tukey test. The number of replicates $n = 4$

Groups of organisms, PLFA	Treatment ($N\text{-}NO_3$, μg/g)		
	"0"	"100"	"2000"
	PLFA concentration, nmol/g		
Protozoa			
20:4ω6,9,12,15c	0.12_a	0.11_a	0.12_a
Fungi			
18:2ω6,9	0.86_a	0.98_{ab}	1.06_b
18:1ω9	2.80_a	3.57_b	3.43_b
Total:	3.66_a	4.55_b	4.49_b
Actinomycetes			
Total 10ME17:0; 10ME18:0; 10ME19:0	5.59_a	6.44_b	5.66_a
Gram-positive bacteria			
a15:0	2.60_a	3.48_b	2.79_a
a17:0	1.56_a	1.51_a	1.36_a
i15:0	3.73_a	4.73_a	4.04_a
i16:0	2.00_a	1.68_a	1.65_a
i17:0	2.06_a	1.61_a	1.57_a
n15:0	0.28_a	0.34_a	0.31_a
n17:0	0.40	n.d.*	n.d.*
Total:	12.64_a	13.34_a	11.71_a
Gram-negative bacteria			
cy17:0	1.72_a	2.08_a	1.73_a
cy19:0	2.98_a	3.44_{ab}	4.06_b
16:1ω7	1.66_b	1.85_b	1.08_a
18:1ω7	4.98_a	5.52_a	5.21_a
Total:	11.34_a	12.89_b	12.08_b
All bacteria, the total of all specific and nonspecific bacterial PLFA	50.17_a	55.67_a	50.34_a
Cyclic PLFA	4.70_a	5.52_{ab}	5.79_b
Monoenoic precursors of cyclic PLFA	6.64_a	7.37_b	6.29_a
Indices of shifts in the microbial community (ratios of PLFA abundance)			
Cyclic PLFA : monoenoic precursors	0.71_a	0.75_{ab}	0.93_b
Fungi : bacteria	0.073_a	0.082_{ab}	0.089_b
Gram-positive : gram-negative	1.12_a	1.03_a	0.97_a

Note: "n.d." stands for "not detected".

in favor of fungi; however, when nitrogen fertilizers have no stress impact, the ratio of fungi to bacteria changes in favor of bacteria due to increasing bacterial growth at the expense of additional nitrogen.

The addition of nitrogen fertilizer in a moderate dose (treatment "100") increased the level of PLFA markers of both gram-negative and gram-positive bacteria, whereas under extremely high rates of nitrogen (treatment "2000"), the content of the gram-positive bacterial markers decreased compared with that in treatment "100" to a higher extent than variations in the markers of gram-negative bacteria (Table 2); therefore, a ratio between gram-positive and gram-negative bacteria decreased in the following descend-

ing order: "0" > "100" > "2000" (from 1.12 to 0.97) (Table 2). Although variations in the proportions of gram-positive and gram-negative bacteria were statistically insignificant, we assume that this was an indication of stress impact on the development of the soil microbial community. According to the literature data, a shift in the ratio of gram-positive and gram-negative bacteria in favor of the latter was observed in the lower horizons of soil profiles under increased stress impact (carbon starvation), which was not associated with nitrogen fertilization [18]. A similar phenomenon of decreasing biomass of gram-positive bacteria which was not accompanied by statistically significant changes in the biomass of gram-negative bacteria was revealed under phosphate starvation of a microbial community [30]. Thus, it can be assumed that in the treatment "2000", stress impact was caused both by osmotic shock and by rapid exhaustion of available carbon in the presence of excessive nitrogen.

Thus, this study revealed the ability of the soil microbial community to immobilize a bulk of inorganic nitrogen and to retain it in immobilized state for a month. This nitrogen immobilization was not associated with increased microbial growth. The data on the growth kinetics of microorganisms and the soil PLFA profile indicate that extremely high rates of the mineral nitrogen fertilizer induced a number of adaptive processes in the microbial community, which promoted its survival under stress conditions evoked by strong carbon limitation and high nitrogen concentrations in soil.

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