Microbiology, Vol. 72, No. 6, 2003, pp. 744–749. Translated from Mikrobiologiya, Vol. 72, No. 6, 2003, pp. 840–846.

EXPERIMENTAL ARTICLES

Quantitative Isolation of Microbial DNA from Different Types of Soils of Natural and Agricultural Ecosystems

E. V. Blagodatskaya*, S. A. Blagodatskii*, and T.-H. Anderson**

**Institute of Physicochemical and Biological Problems of Soil Science, Russian Academy of Sciences, Pushchino, Moscow oblast, Russia 142290*

***FAL Institute of Agroecology, Braunschweig, Germany*

Received July 30, 2002; in final form, December 23, 2002

Abstract—A novel procedure was developed for direct quantitative isolation of microbial DNA from soil. This technique was used to evaluate microbial DNA pools in soils of contrasting types (chernozems and brown forest soils) under different anthropogenic loads. A strong correlation was found between microbial biomass and DNA contents in soils of different types (R^2 = 0.799). The ratio of soil CO_2 emission rate to the amount of extractable DNA in the soil was shown to reflect the physiological state of the soil microbial community; this ratio can be used as an ecophysiological parameter similarly to the metabolic quotient qCO_2.

Key words: soil microorganisms, DNA isolation, microbial biomass.

Wide application of molecular biological and biochemical techniques in the ecology of soil microorganism communities has revived interest in the problem of efficient isolation of microbial DNA from soil samples [1, 2] for qualitative and quantitative estimates of soil microbial diversity [3, 4]. Although the content and composition of nucleic acids in soils of various types have been studied before [5–7], analyses of the isolated preparations were complicated by the abundance of contaminating humic compounds. The recently developed PicoGreen reagent (Molecular Probes, Inc.) allows spectrofluorimetric detection of double-stranded (ds) DNA in highly diluted samples, thus minimizing the interference from soil extract compounds [8]. As a result, it became possible to characterize soil microbial communities by the amounts of extractable DNA. For one particular soil type, DNA content was shown to correlate with the respiration rate of soil microorganisms upon substrate addition [9] and could serve as a proxy of their biomass under steady-state conditions [10]. However, the sensitivity of this parameter to changes in the ecophysiological state of microbial communities in other soils remained unclear. Therefore, the present study was designed to study the correlation between the amount of extractable DNA in various soils and the biomass of soil microorganisms, either actively growing or persisting under steady-state conditions.

MATERIALS AND METHODS

The samples under study came from brown forest soils of Lower Saxony (Germany) and chernozems of Voronezh Region (Russia), either primary (forest and meadow ecosystems) or under arable crops (wheat, maize, or sugar beet production). The major parameters of the soils are summarized in Table 1.

The soil samples were taken from a depth of 0–10 cm, composited, sieved (3-mm mesh size), cleaned of roots and large plant debris, and kept at 4–6°C in cotton-plugged polyethylene containers. One day before the start of an experiment, the soil samples were transferred in a 20°C incubator.

DNA isolation. On the basis of several published procedures [2, 8, 10, 11], we developed an optimized technique for isolation of DNA from soil samples. Soil suspension (1 g soil in 10 ml of Tris-EDTA (TE), pH 8) was sonicated for 2 min and a 2-ml aliquot was treated with 1 mg of aurintricarboxylic acid (ATA), a nuclease inhibitor. The sample was quickly frozen at –80°C and thawed at 60°C, and the freeze-thaw cycle was repeated. The following enzymatic lysis step was performed for 1 h at 37°C with lysozyme (5 mg/ml final concentration). Proteinase K (0.2 mg/ml), sodium dodecyl sulfate (SDS, 10 mg/ml) and sterile acid-washed glass beads (Sigma-Aldrich, Inc.) of three different sizes (710–1180, 212–300, and <106 μm) were then added, and the mixture was vigorously shaken on a Vortex homogenizer for 5 min at 2000 rpm. The samples were incubated for 30 min in a water bath at 65°C, mixed with 2.5 ml of TE and centrifuged for 10 min at 5500 g. The supernatant was aliquoted and stored at –20°C until analysis.

Quantitation of double-stranded DNA. The supernatant was diluted 1 : 100, and 0.5 ml of the dilution was mixed with 0.5 ml of a 1 : 200 dilution of PicoGreen™ (Molecular Probes). After a 4-min incu-

0026-2617/03/7206-0744$25.00

Table 1. Chemical parameters of the soils under study

Soil	Ecosystem and/or crop	Variants	C_{org}, %	N_{total}, %	pH_{aq}
			Intervals		
Brown forest	Sugar beet	Rhizospheric and nonrhizospheric soil, chemical fertilizers	1.02–1.17	0.086–0.1	6.8–7.7
	Maize	Rhizospheric and nonrhizospheric soil	1.0–1.02	0.070–0.072	6.7–6.9
	Pasture meadow	Rhizospheric and nonrhizospheric soil	1.15–1.37	0.119–0.188	5.8–6.2
	Beech forest		3.3	0.43	7.6
Chernozem	Barley field; 10, 46, or 76 years of exploitation	Rhizospheric and nonrhizospheric soil	1.92–2.3	0.289–0.432	6.3–7.2
	Idle land, >100 years of age	Mowed, unmowed	2.45–2.50	0.461–0.479	6.4–7.1
	Forest strip, >100 years of age		2.65	0.521	6.6

bation, the fluorescence was measured on an SFM-25 spectrofluorimeter (Kontron, Germany) at an excitation wavelength of 480 nm and an emission wavelength of 523 nm. Bacteriophage lambda DNA was used as a standard; samples for the standard curve were prepared in TE in the same way as the experimental samples, with the addition of all reagents in appropriate concentrations.

To measure the amount of extracellular DNA, ATA and TE (or water) were added to soil samples, but mechanical disruption and enzymatic lysis steps were omitted from the isolation procedure.

To determine the rate of degradation of extracellular DNA in soil, 2000 ng of phage lambda DNA was added to 200 mg of fresh soil, and the samples were incubated for one day at 22°C. After 5 h and 24 h, the extracellular DNA was isolated as described above.

Completeness of DNA extraction was assayed as follows: pure cultures of fungi (*Mortierella ramanniana, Aspergillus niger*) and bacteria (*Pseudomonas putida, Bacillus subtilis*) obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig) were added to sterile soil samples, and the DNA isolation procedure was carried out. The completeness of DNA extraction was 103.6 ± 4% for bacteria and 111 ± 13% for fungi.

To assay DNA adsorption on soil particles, completeness of phage lambda DNA extraction was estimated for brown forest soil and chernozem. The highest extent of DNA adsorption (2.7% of total DNA added) was found in brown forest soil.

To analyze CO_2 emission rate in glucose-amended soils, 10-g soil samples, wetted to 60% of total moisture capacity, were mixed with glucose (4 mg C per g soil) and mineral salts: $(NH_4)_2SO_4$ (1.9 mg/g), K_2HPO_4 (2.25 mg/g), and $MgSO_4$ (3.8 mg/g). The CO_2 emission rate was measured using a continuous infrared gas analyzer [12].

Total biomass of soil microorganisms (C_{micr}) was determined using a physiological assay [13] from the initial rate of substrate-induced respiration, according to the following relationship:

$$C_{micr}\ (\mu g\ C\ per\ g\ soil) = 40.04 \times \mu l\ CO_2\ per\ g\ soil\ per\ hour + 0.37$$

The ratio between fungal and bacterial biomass in microbial communities was estimated after selective suppression of growth of eukaryotes and prokaryotes with, respectively, streptomycin (0.5 or 1 mg per g soil) and cycloheximide (0.75 or 1 mg per g soil), added to soil samples together with glucose and mineral salts [14]. When the change in the soil DNA content during microorganism growth was assayed the control samples were supplemented with both antibiotics at 1 mg/g, which provided for complete inhibition of microbial growth.

All determinations were performed in three replicates. The results were analyzed statistically by one-way ANOVA.

RESULTS AND DISCUSSION

Optimization of the procedure of dna isolation from soil. Figure 1 shows the fluorescence emission spectra (arbitrary units of intensity) of standard solutions of bacteriophage lambda DNA and solutions containing DNA extracted from soil samples. The fluorescence emission maximum in the standard solutions was shifted towards longer wavelengths due to addition of all reagents used in DNA isolation. As expected, this fluorescence maximum (at 525 nm) coincided with the maximum observed for soil extracts. Analysis of the efficiency of different treatment steps showed that every step of the DNA extraction procedure caused a statistically significant increase ($P < 0.001$) in the amount of isolated DNA. Losses upon omission of various treatment steps depended on the type of soil under study (Table 2). The lowest yield of DNA, as deter-

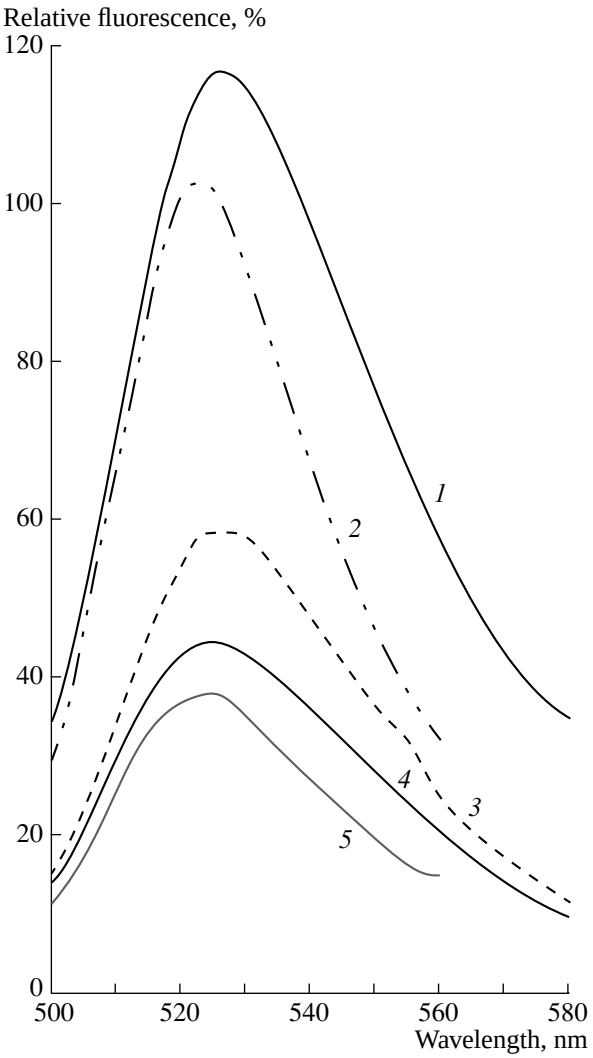

Fig. 1. PicoGreen fluorescence spectra: (*1*) standard solution of phage lambda DNA (22 ng/ml) supplemented with all reagents used in the isolation procedure; (*2*) standard solution of phage lambda DNA (22 ng/ml) in TE buffer without adding any reagent; (*3*) extract of chernozem under forest; (*4*) extract of brown forest soil under forest; (*5*) extract of brown forest soil under sugar beet.

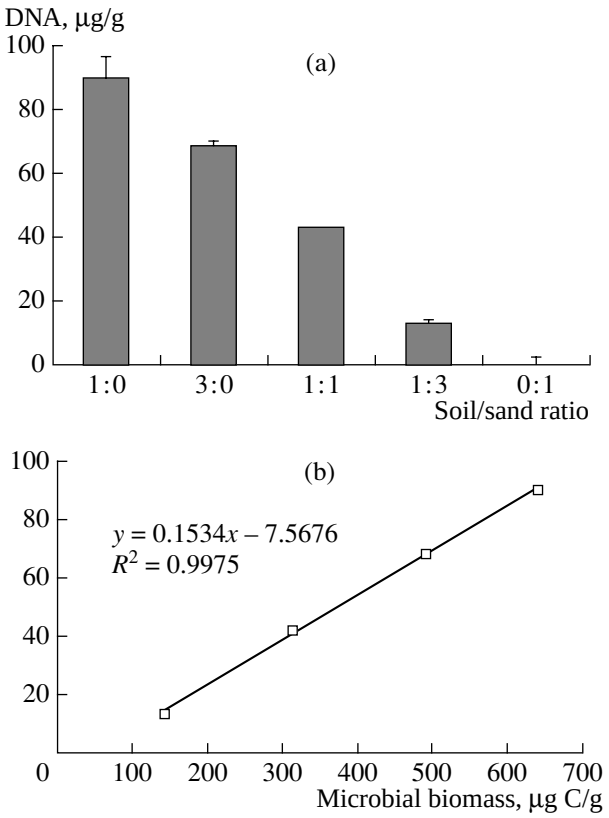

Fig. 2. Changes in the amount of extractable DNA: (a) brown forest soil under forest, dilutions with sand; (b) dependence on microbial biomass.

mined from Duncan's multiple range test, was obtained in the shortest isolation protocol, which included two freeze-thaw cycles and incubation with SDS and ATA. Sequential incorporation of Proteinase K, glass beads, and ultrasound treatment steps in the protocol improved the yield of isolated DNA at each step (Table 2). It is worth noting that the increase in the amount of extractable DNA with additional treatment steps was not additive but depended on the nature and sequence of the treatment steps. For example, omission of a single step from the procedure (sonication, ATA treatment, or freeze-thaw) caused a decrease in the amount of extracted DNA by 25, 35, and 35%, respectively. At the same time, a shortened extraction protocol that included the same three steps (sonication, ATA treatment, and freeze-thaw) allowed a recovery of 35.5% of total extractable DNA only. Analysis of the importance of each treatment step revealed that incubation with lysozyme contributes most in the efficiency of DNA extraction from soil. Addition of ATA and quick freeze-thaw of the samples improved the yield of DNA extraction to the same extent. Similarly, omission of soil treatment with ultrasound or glass beads decreased the DNA yield with equal efficiency.

Content of microbial DNA in different soils varied from 32.2 to 147 μg/g. This value is in good agreement with the results reported for soils and bottom sediments [2, 15, 16]. The yield of DNA per weight of microbial biomass falls within the interval of DNA content in cultures of fungal and bacterial soil isolates [17], varying from 3.8 to 9.3% in different soils. Such variation may be explained by possible differences in the extent of cell lysis and by the dependence of DNA adsorption on soil particles on the physicochemical properties of the soil [16, 18].

Dependence of extractable DNA on the microbial biomass in the soil was analyzed by mixing in different ratios brown forest soil with sterile, acid-washed sand (Fig. 2a). Dilution of soil with sand allowed us to lower the microbial biomass gradually without changing the total sample mass. The amount of extractable DNA

Table 2. Yield of DNA isolated from soils of different types using different extraction protocols

Soil	Brown forest				Chernozem
Ecosystem	forest	meadow	field (maize)		meadow
			rhizospheric	nonrhizo-spheric	
	DNA content, μmg/g				
Complete DNA extraction procedure	92.4[a]* ± 3.2	80.4 ± 2.7	98.1 ± 5.9	85.2 ± 7.7	120.1 ± 1.8
Steps omitted:					
shaking with glass beads (SGB)	69.8[b] ± 9.9	47.8 ± 1.1			98.8 ± 1.2
ultrasound	69.1[b] ± 8.3				
ATA	60.2[c] ± 0.2		53.6 ± 0.3	46.6 ± 8.0	
quick freeze	59.6[c] ± 0.6				
ultrasound, lysozyme, Proteinase K	48.1[d] ± 9.2				
lysozyme	43.2[d] ± 3.2	70.2 ± 0.5			59.8 ± 3.4
ultrasound, lysozyme, SGB	28.9[e] ± 1.7				
ultrasound, lysozyme, SGB, Proteinase K	22.18[e] ± 2.3				

* Mean values falling into the same range by Duncan's test are denoted by the same letter.

decreased proportionally to the decrease in microbial biomass in the soil sample; the regression coefficient was 0.99 (Fig. 2b).

As samples of native soil differing only in the microbial biomass, we used rhizospheric and nonrhizospheric brown forest soils under agricultural crops (maize, sugar beet, wheat) and soils from a pasture meadow. In all samples studied, DNA content correlated with the microbial biomass; the DNA content in the rhizosphere was significantly higher than in the nonrhizospheric soil of the same nature (Fig. 3a). We also found a good correlation ($R^2 = 0.89$) between the content of extractable DNA and microbial biomass in several chernozems, both primary and long exploited for arable crop production (Fig. 3b). We therefore conclude that in soils of the same type with similar physicochemical properties, the amount of DNA reflects the microbial biomass.

Comparison of the amount of extractable DNA in 24 samples of various soils, both similar and different in their physicochemical properties (Fig. 3c), revealed a statistically significant correlation ($R^2 = 0.799$) between microbial biomass values and microbial DNA pool in different soils under steady-state conditions, i.e., when the microorganisms are not growing actively.

Extracellular DNA content. We found no extracellular DNA in aqueous soil extracts. When TE buffer was used for extraction, the amount of extracellular DNA was 0.46 μg/g in chernozem and 1.59 μg/g in brown forest soil, or 0.43 and 2.8% of the total DNA content in these types of soils, respectively.

The rate of degradation of standard phage lambda DNA solution in the soil depended on the level of microbial biomass (250 μg C/g in brown forest soil and 600 μg/g in chernozem). After a 5-h incubation, 100% of added standard DNA remained in brown forest soil, whereas only 64.8% of DNA remained in chernozem. After a one-day incubation, 39% of added DNA was degraded in brown forest soil, and 73.5%, in chernozem. Until now, existence of extracellular DNA in soil still remained questionable because of its easy availability for microbial degradation. On the other hand, there is a theoretical possibility that DNA-like biopolymers could resist biodegradation as complexes with clay minerals and humic soil compounds [19]. In our experiments, the amount of extracellular DNA in soil did not exceed 5% of the error of measurement, and the time of existence of added DNA depended on the biomass and activity of the soil microbial community.

Changes in the amounts of microbial DNA during the glucose-induced growth of soil microorganisms were followed in samples of arable brown forest soil exploited for sugar beet production (Fig. 4a). The content of fungal and bacterial biomass in this soil, as determined from substrate-induced respiration, was 69.9 and 30.1%, respectively. In the exponential growth phase the DNA content correlated with the rate of CO_2 emission from the soil. After 25 h, when the added substrate had been exhausted, the microbial respiration rate slowed sharply but the DNA content remained high for at least ten more hours. Changes in respiration rate and DNA content in this experimental setup reflects the total activity of soil microorganisms, with fungal and bacterial parts of the microbial community competing for the substrate. Addition of specific inhibitors relaxes this competition and selectively influences the activity of soil eukaryotes and prokaryotes. After the addition of cycloheximide and streptomycin, the amount of

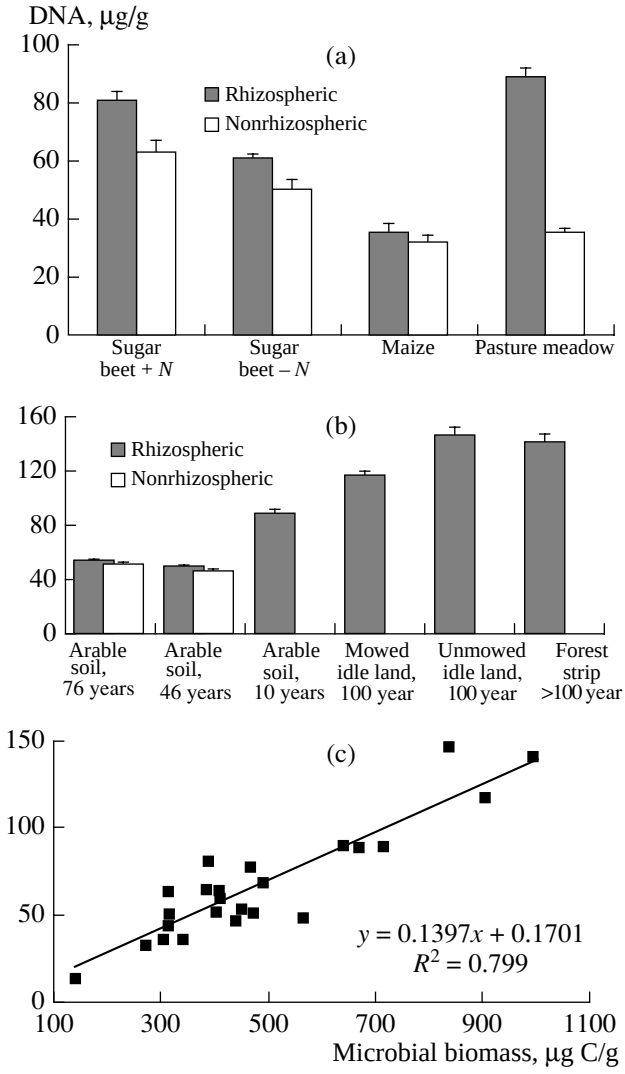

Fig. 3. Amount of DNA extractable from (a) brown forest soil and (b) chernozem under various ecosystems and (c) regression dependence of DNA content on the microbial biomass in all soils studied.

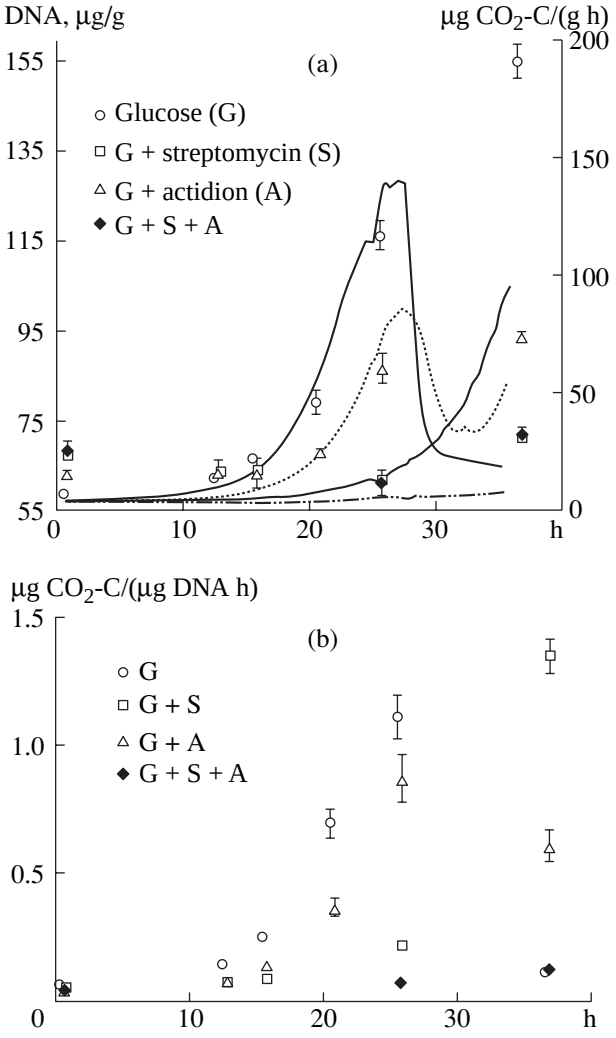

Fig. 4. (a) Dynamics of CO_2 emission rate (lines) and microbial DNA content in the soil (symbols) and (b) ratio of these two parameters during the growth of microorganisms on unadulterated glucose and on glucose with inhibitors of activity of eukaryotes and prokaryotes.

DNA remained unchanged for the initial 20 h of incubation. This can be explained by the fact that the antibiotics used in our experiments are inhibitors of protein synthesis in eukaryotes and prokaryotes, respectively, and do not degrade cellular structures (such as DNA) that already exist [13]. The same explanation can be given to the observation that during the course of the experiment the sum of DNA contents determined individually after the addition of cycloheximide or streptomycin significantly exceeded the total soil DNA content (found in the experiments with glucose but without inhibitors). Only near the end of the incubation, 35 h after the addition of glucose, the total DNA content became comparable with the sum of eukaryotic and prokaryotic DNA. After the beginning of the exponential growth of microorganisms, the amount of bacterial DNA increased along with the increase in bacterial respiration rate (Fig. 4a). Enhancement of the respiratory activity of eukaryotes became statistically significant much later, 28 h after the addition of glucose and streptomycin, and was accompanied by a small increase in fungal DNA content. Most likely, the maximum of respiratory activity and DNA content in soil fungal biomass could not be detected properly in our experiments, since after 35-h incubation the CO_2 emission rate started to increase even if both antibiotics were present, making longer incubation times unreliable. Higher concentrations of antibiotics could not be used due to their unspecific activity.

The ratio of microbial respiration rate to the amount of DNA extracted from the soil was not constant during microbial growth on glucose (Fig. 4b). This ratio was minimal at the beginning and at the end of the experiment, when the soil microbial community existed under

steady-state conditions. During the active microbial growth, the (respiration rate)/(DNA content) ratio increased and peaked after 25-h incubation of soil with glucose. Therefore, the ratio of CO_2 emission from the soil to its microbial DNA content changes along with the physiological state of the soil microbial community and, together with the metabolic quotient qCO_2, can serve as a valuable ecophysiological parameter.

ACKNOWLEDGMENTS

The authors would like to thank the German Academic Exchange Service (DAAD) and the German Ministry of Agriculture (BMVEL) for supporting the research at the Institute of Agroecology (FAL, Braunschweig).

This work was supported by the Russian Foundation for Basic Research, project no. 01-04-48533.

REFERENCES

1. Zvyagintsev, D.G., Dobrovol'skaya, T.G., Bab'eva, I.P., and Chernov, I.M., Development of the Concepts of the Structure of Soil Microbial Communities, *Pochvovedenie*, 1999, no. 1, pp. 134–144.
2. Ogram, A., Sayler, G.S., and Barkay, T., The Extraction and Purification of Microbial DNA from sediments, *J. Microbiol. Methods*, 1987, vol. 7, pp. 57–66.
3. Dobrovol'skaya, T.G., Lysak, L.V., Zenova, G.M., and Zvyagintsev, D.G., Analysis of Soil Bacterial Diversity: Methods, Potentiality, and Prospects, *Mikrobiologiya*, 2001, vol. 70, no. 2, pp. 149–167.
4. Naumova, N.B. and Kaikman, P., Diversity of Bacterial DNA and Bacterial Biomass in Alluvial Meadow Soil Fertilized for a Long Time, *Pochvovedenie*, no. 6, pp. 700–707.
5. Aseeva, I.V., Panikov, N.S., and Chursina, O.T., Content and Composition of Nucleic Acids in Soddy-Podzolic Soils, *Vestn. Mosk. Univ., Ser. 17. Pochvovedenie*, 1977, no. 1, pp. 85–91.
6. Markov, G.G. and Ivanov, I.G., Hydroxyapatite Column Chromatography in Procedures for Isolation of Purified DNA, *Anal. Biochem.*, 1974, vol. 59, pp. 555–563.
7. Torsvik, V.L. and Goksoyr, J., Determination of Bacterial DNA in Soil, *Soil Biol. Biochem.*, 1978, vol. 10, pp. 7–12.
8. Sandaa, R.-A., Enger, O., and Torsvik, V., Rapid Method for Fluorometric Quantification of DNA in Soil, *Soil Biol. Biochem.*, 1997, vol. 30, no. 2, pp. 265–268.
9. Marstrop, H. and Witter, E., Extractable dsDNA and Product Formation as Measures of Microbial Growth in Soil upon Substrate Addition, *Soil Biol. Biochem.*, 1999, vol. 31, pp. 1443–1453.
10. Marstrop, H., Guan, X., and Gong, P., Relationship between dsDNA, Chloroform Labile C and Ergosterol in Soils of Different Organic Matter Contents and pH, *Soil Biol. Biochem.*, vol. 32, pp. 879–882.
11. Clegg, C.D., Ritz, K., and Griffiths, B.S., Direct Extraction of Microbial Community DNA from Humified Upland Soils, *Appl. Microbiol. Lett.*, 1997, vol. 25, pp. 30–33.
12. Heinemeyer, O., Insam, H., Kaiser, E.A., and Walenzik, G., Soil Microbial Biomass Measurements: An Automated Technique Based on Infra-Red Gas Analysis, *Plant Soil*, 1989, vol. 116, pp. 191–195.
13. Anderson, J.P.E. and Domsch, K.H., A Physiological Method for the Quantitative Measurement of Microbial Biomass in Soils, *Soil. Biol. Biochem*, 1978, vol. 20, pp. 107–114.
14. Anderson, J.P.E. and Domsch, K.H., Quantification of Bacterial and Fungal Contributions to Soil Respiration, *Arch. Mikrobiol.*, 1973, vol. 93, pp. 113–127.
15. More, M.I., Herrick, J.B., Silva, M.C., Ghorse, W.C., and Madsen, E.L., Quantitative Cell Lysis of Indigenous Microorganisms and Rapid Extraction of Microbial DNA from Sediments, *Appl. Environ. Microbiol.*, 1994, vol. 60, no. 5, pp. 1572–1580.
16. Zhou, J., Bruns, M.A., and Tiedje, J.M., DNA Recovery from Soils of Diverse Composition, *Appl. Environ. Microbiol.*, 1996, vol. 62, no. 2, pp. 316–322.
17. Christensen, H., Olsen, R.A., and Bakken, L., Flow Cytometric Measurements of Cell Volumes and DNA Contents during Culture of Indigenous Soil Bacteria, *Microb. Ecol.*, 1995, vol. 29, pp. 49–62.
18. Roose-Amsaleg, C.L., Garnier-Sillam, E., and Harry, M., Extraction and Purification of Microbial DNA from Soil and Sediment Samples, *Appl. Soil Ecol.*, 2001, vol. 18, pp. 47–60.
19. Stotzky, G.J., Persistence and Biological Activity in Soil of Insecticidal Proteins from *Bacillus thuringiensis* and of Bacterial DNA Bound on Clays and Humic Acids, *J. Environ. Qual.*, 2000, vol. 29, pp. 691–705.