ISSN 1064-2293, Eurasian Soil Science, 2015, Vol. 48, No. 3, pp. 288–293. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © L.M. Polyanskaya, I.P. Pinchuk, D.G. Zvyagintsev, 2015, published in Pochvovedenie, 2015, No. 3, pp. 330–336.

SOIL BIOLOGY

Assessment of the Number, Biomass, and Cell Size of Bacteria in Different Soils Using the "Cascade" Filtration Method

L. M. Polyanskaya, I. P. Pinchuk, and D. G. Zvyagintsev

Faculty of Soil Science, Moscow State University, Leninskie gory, Moscow, 119991 Russia

e-mail: lpolyanskaya@mail.ru

Received June 4, 2014

Abstract—Soddy-podzolic, gray forest, brown forest, primitive Antarctic soils, typical chernozems, and solonchaks were studied. Many ultrafine bacterial cells, along with fine ones, were found in all the soils studied. The gray forest, brown forest, and primitive Antarctic soils were especially distinguished in this respect. Formerly, in the works on soil microbiology, the fact of the cell size reduction was insufficiently taken into account because of the absence of reliable methods. A decrease in the number and biomass of bacteria down the profile in all the soils, except for the solonchak, was shown. In the solonchak, the bacterial number and biomass increases with decreasing salinity of the soil horizons. The bacterial biomass mainly depends on the predominance of cells of definite sizes (0.38 and 0.23 μm). In the B1fungi horizon of the primitive Antarctic soil, a considerable number of large (1.85 μm) bacterial cells was recorded, and this resulted in the maximal microbial biomass in this horizon. The data on the average volume of a cell correlate with those on the number and biomass of bacteria. The largest diameters of cells were registered in the humus and B1fungi horizons of the primitive Antarctic soil.

Keywords: bacterial cells, number, size, biomass, volume

DOI: 10.1134/S1064229315030096

INTRODUCTION

The existence of a great number of bacteria called ultramicrobacteria, nanobacteria, and dwarf cells, which are found in the air, thunderclouds, minerals, soils, etc., is well known [9, 13–15, 20, 22, 23, 27]. Special attention is paid to the finest life forms on our planet—bacteria measured in nanometers with diameters of 0.05–0.2 μm. Using an epifluorescent microscope, the least diameter of a bacterial cell, 0.2 μm, was found [23]. The formation of nanobacteria as a response of cells to unfavorable environmental conditions and stress factors was studied basing on the behavior of trivial bacterial forms in laboratory experiments [10, 11]. The cultivation of nanoforms on rich nutrient media leads to a return to their initial forms [1, 26]. The majority of the available data concerns only the presence of ultrabacteria or nanobacteria in the soils and other habitats [4, 3, 17, 24], but there is no information on the number of bacteria different in size.

The aim of this work is the determination of the total number and the biomass of bacteria and cell sizes in different soils using the method of "cascade" filtration.

MATERIALS AND METHODS

The following soils and soil horizons were the objects of the study:

(1) Soddy strongly podzolic soil (Moscow oblast). The soil is cultivated, not eroded, loamy sandy–light loamy on mantle loamy clay underlain by sandy–loamy moraine under an aspen–maple forest. The horizons are as follows: A, 0–10 cm—forest litter; AE2, 10–40 cm—transitional; E, 40–50 cm—eluvial; Ebt, 50–60 cm—transitional; B1t, 60–90 cm—illuvial clayey subhorizon; B2t, 90–110 cm—illuvial clayey subhorizon.

(2) Gray forest soil (Tula oblast). The relief is a hilly plain dissected by ravines. There were no signs of soil salinity and waterlogging. The parent rock is loess-like loam. The horizons are as follows: A, 0–20 cm—humus-accumulative; AB, 20–40 cm—eluvial–illuvial; B1, 40–50 cm—illuvial; B2, 50–80 cm—illuvial; BC, 80–90 cm—transitional to the parent rock.

(3) Typical chernozem. The samples were taken in a forest belt (Kursk oblast) composed of *Quercus robur* L., *Fráxinus excélsior*, and *Acer platanoides*. The horizons are as follows: Asod, 0–12 cm—sod; A, 12–35 cm—humus-accumulative; AB, 35–65 cm—humus-accumulative transitional to the B horizon; B1, 65–110 cm—carbonate-accumulative.

(4) Solonchak (Astrakhan oblast). The vegetation is represented by rare small Artemisia bushes. In the soil profile, some fine crystals of easily soluble salts are observed. The degree of salinity decreases with the depth. The horizons are as follows: A_{salt}, 0–20 cm—surface saline; B_{salt}, 20–50 cm—saline; B2, 50–80 cm—transitional; C, 80–100 cm—parent rock.

(5) Unsaturated loamy brown forest soil on eluvium–colluvium (Kamchatka). The vegetation is *Betula ermanii*, *Populus suaveolens*, and *Sorbus sambucifolia*. The horizons are as follows: O, 0–6 cm—friable forest litter; A, 6–13 cm—humus-accumulative; AB, 13–23 cm—humus-accumulative podzolized; B1, 23–41 cm—illuvial–metamorphic; B2, 41–64 cm—illuvial–metamorphic; BC, 64–94 cm—transitional; C, 94–125 cm—parent rock.

(6) Primitive Antarctic soil of moist valleys (Eastern Antarctic, Princess Elizabeth Land) in a moist valley of the zone subject to the influence of snowmelt water. The bottom of the valley is covered with granitoid eluvium–colluvium; the relief is polygonal with signs of frost heaving. The soil is excessively moistened due to the stagnant melt water above the frozen waterproof layer. In the B algae horizon, there are many aggregations of unicellular algae (blue-green and green). In the B1 fungi horizon, mineral grains are densely covered with fungal (or actinomycetal) mycelium so that the color of the horizon has a whitish hue. From a depth of 45 cm, water stagnates on the frozen layer; the pit is fast filled with water. The horizons are as follows: B_{algae}, 0–2 cm—moss litter; B1 fungi, 2–10 cm—mineral material, ocherous-brown coarse-grained sand covered with whitish mycelium; B2, 10–14 cm—mineral material, ocherous-brown coarse-grained sand with signs of fungal or actinomycetal mycelium; B3, 14–40 cm—sandy layer under desert pavement without signs of macrobiota; B3, 40–60 cm—coarse-grained sand without macrobiota.

The method of "cascade" filtration was used for the determination of the number and size of bacteria. The soil suspensions were filtered through filters of 1.85, 0.43, 0.38, and 0.23 μm and membrane Synpor filters (0.17 μm) using a Bunsen flask and a water jet pump. The proper luminescence of the filters was quenched by coloration with a saturated alcohol solution of Sudan black (Feinchemie K.; H. Kallies KG, Germany). For this purpose, the filters were placed into this solution for several days and, then, they were washed in sterile water, dried, and used for filtration [21].

Four layers of filter paper were placed on the metallic filter screen of a Bunsen flask on which a nuclear or membrane filter was forced with a metallic ring; there, a suspension tested was added. The suspension was filtered using filters sequentially from the filters with great pores to those with smaller ones; the filter with 1.85-μm pores was applied only for the removal of large soil particles from the suspensions.

The suspension stained with acridine orange (1 : 10000; for 2–3 min) was filtered, and the bacterial cells were counted in 30 microscope fields. The size of a cell was conventionally accepted equal to the diameter of the filter pores where they were settled. The calculations assumed that the cells had a spherical form [10].

The calculation of the number of cells per 1 g of soil was performed using the formula

$$N = \frac{S_1 an}{VS_2 c},$$

where N is the number of cells per 1 g of soil; S_1 is the area of the filter, μm^2; a is the number of cells in one microscope field (average of all the fields), n is the index for the dilution of the soil suspension, mL; V is the volume of the filtered suspension, mL; S_2 is the area of the microscopic field, μm^2; and c is the soil weight, g. Knowing the filter area and the area of the microscopic field, the formula for the calculation of the number of bacteria has the following form:

$$Nb = a \times 1.13 \times 10^7.$$

When calculating the weight of the bacteria using the traditional method, the dry weight of a bacterial cell with a volume of 0.1 μm^3 was assumed to be 2×10^{-14} [6]. In this work, the dry biomass was calculated taking into account the size of the bacteria. The biomass of a bacterial cell was found on each filter, and the biomass of all the cells was obtained according to the formula

$$B_b = \frac{3}{4}\pi r^3 \times 2 \times 10^{-14}/0.1 N_b,$$

where b is the size of each fraction, r is the radius, N is the number of cells, and B is the biomass.

The mean volume of one cell was calculated by the formula

$$V = \frac{B_{\text{total}}}{N_{\text{total}} a},$$

where B_{total} and N_{total} are the biomass and the number of all the fractions, respectively; a is the density of one cell, where a is 1×10^{-12} g/cm^3; and V is the average volume of one cell.

The statistical processing of the results was performed using the STATGRAPHICS and STATISTICA programs. The average standard deviation (δ_{n-1}) for the bacterial number did not exceed 5–10%.

RESULTS AND DISCUSSION

In the soddy-podzolic soil, the highest number of bacteria was observed in the AE2 and B2t horizons ($57–76 \times 10^7$ bacterial cell/g of soil; Fig. 1). With depth, their number decreased, and, in the illuvial horizon, it equaled 50×10^7 cell/g of soil. Bacteria with a diameter of 0.23 μm predominated, and their maximum was observed in the transitional B2t horizon. The bacterial number was maximal (34×10^7 cell/g) on the filter (0.38 μm) when analyzing the suspension from the eluvial horizon.

In the A humus horizon of the gray forest soil, the number of bacteria was 88×10^7 cell/g; in the B2 horizon, it sharply increased to 121×10^7 cell/g of soil. In

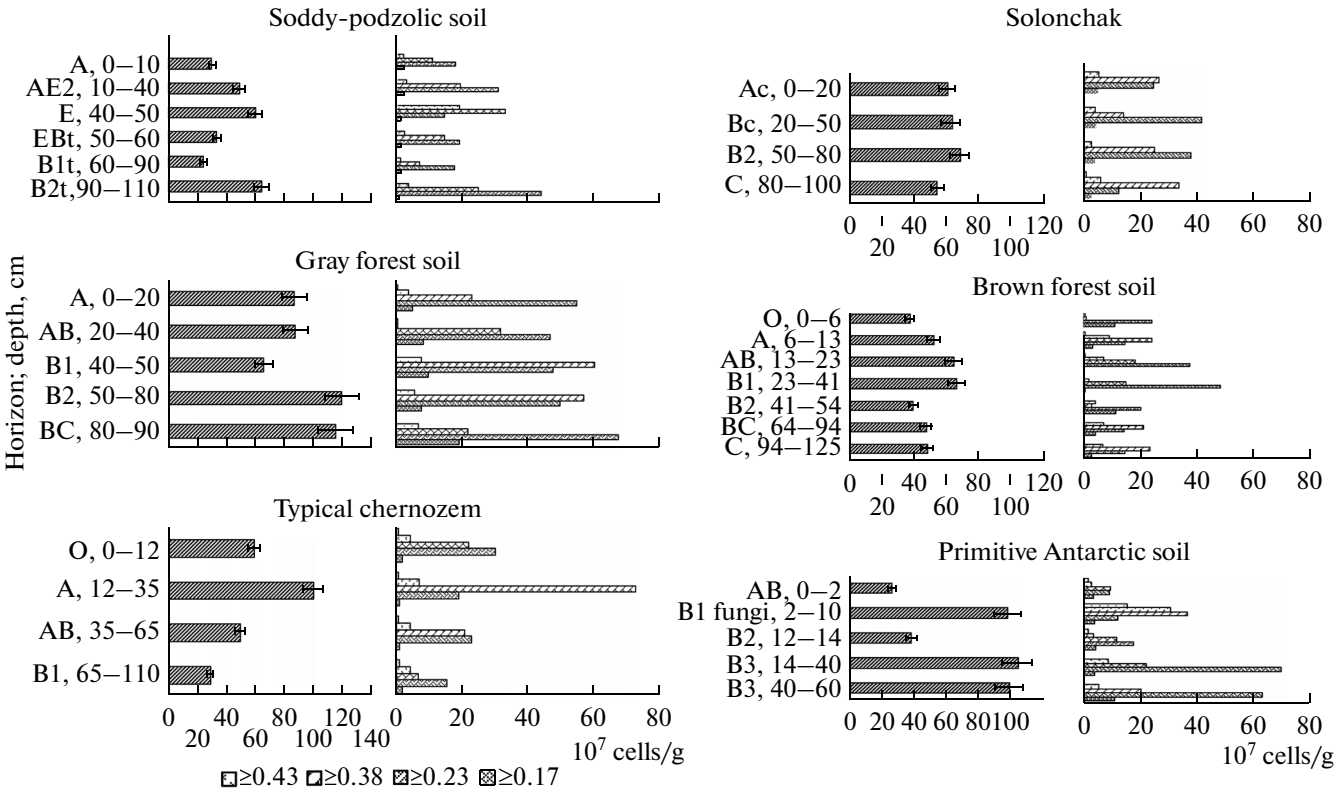

Fig. 1. Number of bacterial cells determined by the method of cascade filtration and their size distribution in the studied soils.

the BC horizon, it amounted to 116×10^7 cell/g due to the predominance of bacteria with diameters of 0.23 and 0.38 μm ($50–68 \times 10^7$ cell/g). The number of 0.38-μm bacteria was maximal in the B1 horizon (61×10^7 cell/g of soil). The number of 0.43-μm bacteria was highest in the B1 horizon and minimal in the AB horizon (0.50×10^7 cell/g). The number of bacteria with a cell diameter of 0.17 μm increased in the deeper soil layers; in the BC horizon, it reached the maximum—19×10^7 cell/g of soil.

In the typical chernozem, the bacterial number determined by the method of cascade filtration was 100×10^7 cell/g in the humus horizon and 49×10^7 in the transitional AB horizon. In the B1 horizon, their number was minimal (29×10^7 cell/g) corresponding to the earlier obtained data on the maximal number of bacteria in the humus horizons of chernozems [12]. The number of 0.23-μm bacteria was maximal in the O, A, and AB horizons ($(19–30) \times 10^7$ cell/g) and that of bacteria with a diameter of 0.38 μm, in the A horizon (73×10^7). Our results well agree with the previous data [11]. In all the horizons, the number of the largest (1.85 μm) and smallest (0.17 μm) bacteria was low and did not exceed 2×10^7 cell/g of soil.

In the profile of the solonchak, the total number of bacteria decreased downward (Fig. 1). Their maximal number was observed in the B2 horizon (51×10^7 cell/g of soil). The maximal number of bacteria settled on the 0.38-μm filter was observed in the parent rock (33×10^7 cell/g; in the surface saline A_{salt} horizon, it amounted to 26×10^7 cell/g). It may be related to a decrease in the soil salinity down the profile, i.e., to the more favorable environmental conditions there. The number of large bacteria (1.85 μm) was very low in all the horizons; their number did not exceed 0.50×10^7 cell/g and was minimal in the B_{salt} horizon.

The total number of bacteria in the brown forest soil was maximal in the illuvial–metamorphic B1 horizon (66×10^7 cell/g of soil); in the deeper horizons, their number decreased; and, in the parent rock, it was 48×10^7. The number of bacteria (0.43 and 0.38 μm) was maximal in the A humus-accumulative horizon, and that of the 0.23-μm bacteria was maximal in the illuvial–metamorphic B1 horizon (48×10^7 cell/g). The number of large (1.85 μm) bacteria was not so considerable and did not exceed 0.70×10^7 cell/g of soil.

In the primitive Antarctic soil, the highest bacterial number was recorded in the B1 fungi horizon (98×10^7 cell/g) due to the active vital activity of fungi and the input of nutrients necessary for the normal functioning of bacteria. In this soil, bacteria with diameters of 0.23 and 0.38 μm predominated. The number of 0.43-μm bacteria was maximal (31×10^7 cell/g) in the B2 horizon. It is worth noting that only in the Antarctic soil the number of bacteria settled on the 1.85-μm

filter was maximal (15×10^7 cells/g soil). The B1 fungi horizon of this soil, as compared to the other horizons and soils, has the most favorable medium for the growth and development of bacteria.

Thus, one can say that the chernozem, gray forest soil, and brown forest soils are the most favorable for the growth and development of bacteria, and the least favorable is the solonchak. The humus horizons are the most suitable for the development of bacteria, and, in the primitive Antarctic soil, it was the B1 fungi horizon with a great number of large bacteria.

The bacterial biomass in 1 g of soil was calculated in each soil horizon (Fig. 2).

In the soddy-podzolic soil, the highest biomass was determined in the AE2 and E horizons (57–75 μg/g); down the profiles, the bacterial biomass decreased. In the gray forest soil, the maximal biomass of bacteria (70–75 μg/g) was recorded in the A and B1 horizons; it decreased in the deeper horizons to 45 μg/g. In the typical chernozem, the bacterial biomass was maximal (85 μg/g) in the A horizon; in the B1 horizon, it decreased to 65 μg/g. In the solonchak, the bacterial biomass in the Bc horizon was lower than that in the Ac horizon. However, it increased in the lowermost C horizon to a maximum of 65 μg/g. In the brown forest soil, the highest biomass (60–70 μg/g) was found in the A and AB horizons, in the deeper ones, it decreased to 30 μg/g. In the primitive Antarctic soil, in the B1 fungi horizon, the bacterial biomass increased to 1060 μg/g and drastically decreased to 54 μg/g in the deeper soil horizons.

For each horizon in all the soils, the mean average diameters of one cell were calculated (table).

In the humus-accumulative (A) and eluvial (E) horizons of the soddy-podzolic soil, the mean diameter of the cells was 0.64–0.58 μm; in the B2t horizon, it decreased down the profile to 0.50 μm.

In the A horizon of the gray forest soil, the mean diameter of a cell was 0.54 μm; it gradually decreased downward; and, in the BC horizon, it was 0.42 μm. Although the biomass in all the horizons of the chernozem was approximately the same, in the B1 horizon, the maximal diameter (0.78 μm) of the cells was observed, while the bacterial number in this horizon was minimal. In the A horizon, the diameter of one cell was 0.56 μm; it corresponded to the earlier obtained data [12].

In the solonchak, the greatest mean diameter of a cell was observed in the parent rock (1.32 μm), and, in the saline horizons, it was 0.5 μm. These fluctuations in the size may be explained by the stress under strong salinity.

In the A and AB horizons rich in organic matter, the greatest mean diameter of the cells was observed in the brown forest soil (0.64 and 0.56 μm, respectively).

In the primitive Antarctic soil, a drastic increase in the bacterial volume (1.10 μm^3) and diameter (1.28 μm) was observed in the B1fungi horizon, where

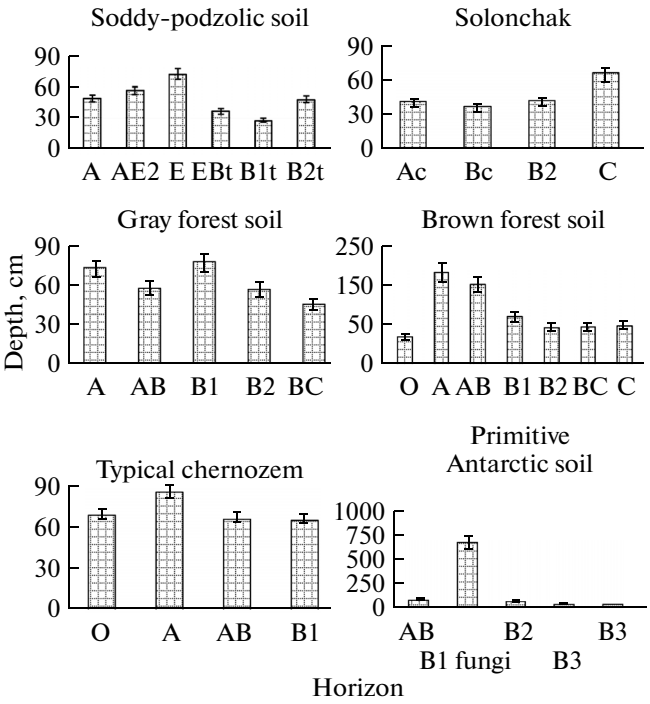

Fig. 2. Biomass of bacterial cells in the soils studied.

the activity of fungi was high providing favorable conditions for the development of bacteria (Fig. 3). Down the profile, as the degree of freezing increased and the conditions for life became poorer, the volume of the bacterial cells decreased to 0.01 μm^3. The diameter of cells in the deeper soil horizons was 0.46 μm.

The table presents the mean diameters and volumes of the bacterial cells in the soils studied. As one can see, the mean diameter of a cell in the B1 horizon of the typical chernozem and in the B algae, B1 fungi, and B2 horizons of the primitive Antarctic soil, as well as in the C horizon of the solonchak, was 1.5–2.0 times greater than that in all the horizons of the other soils. Except for the soils mentioned above, the trends of the changes in the diameter of the bacterial cells were similar; their sizes were greater in the humus-accumulative horizons and decreased down the profiles. The mean bacterial volumes calculated reflect the trends revealed for the diameters of the bacteria. According to the literature data, bacteria with diameters of 0.38 [5], 0.58 [6], 0.76–0.84 [8, 19, 25], and 1.1–1.24 μm [2, 16] occur in soils. The values obtained in the course of our work coincide with those described in the literature.

CONCLUSIONS

The humus-accumulative horizons were shown to be the most favorable for the growth and development of bacteria. In the primitive Antarctic soil, it was the B1 fungi (colonized by fungal mycelium) horizon and in the solonchak, the less saline horizons. In all the

Mean diameter (above the line) and mean volume (under the line) of one cell in the soils studied

Horizon	d, μm/V, μm^3	Horizon	d, μm/V, μm^3
Soddy-podzolic soil		Solonchak	
A	0.64/0.14	Ac	0.52/0.07
AE2	0.48/0.06	Bc	0.50/0.07
E	0.58/0.10	B2	0.50/0.07
Ebt	0.56/0.09	C	1.32/1.21
B1 bt	0.58/0.10		
B2bt	0.50/0.07		
Gray forest soil		Brown forest soil	
A	0.54/0.08	O	0.46/0.05
AB	0.52/0.07	A	0.64/0.1
B1	0.50/0.07	AB	0.56/0.09
B2	0.46/0.05	B1	0.46/0.05
BC	0.42/0.04	B2	0.52/0.07
		BC	0.50/0.07
		C	0.50/0.07
Typical chernozem		Primitive Antarctic soil	
O	0.60/0.11	AB	0.92/0.41
A	0.56/0.09	B1 fungi	1.28/1.10
AB	0.64/0.14	B2	0.80/0.27
B1	0.78/0.25	B3	0.46/0.05
		B3	0.46/0.05

soils, 0.38- and 0.23-μm bacteria predominated. In the primitive Antarctic soil, in the B1fungi, the number of large bacteria (1.85 μm) significantly increased. In all the soils, except for the solonchak and chernozem, the maximal diameters were observed in the horizons rich in organic matter. In the B1fungi of the primitive Antarctic soil, due to the presence of fungi, the diameter of the bacterial cells was much greater. In the solonchak, the diameter increased as the soil salinity reduced down the profile; in the chernozem, it increased as the number of bacteria decreased.

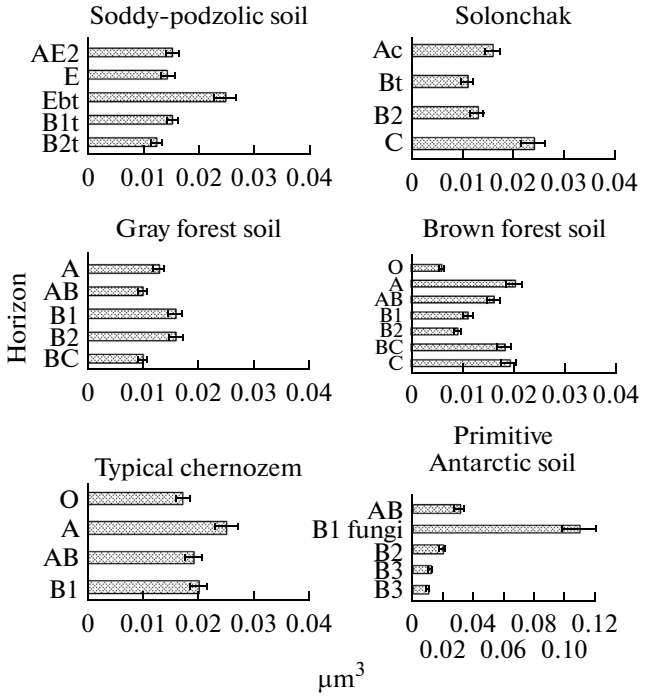

Fig. 3. Mean volume of one cell in the soils studied.

ACKNOWLEDGMENTS

The authors thank Z.N. Tyugai and O.B. Tsvetnova (Faculty of Soil Science of Moscow State University) and N.S. Mergelov (Institute of Geography, Russian Academy of Sciences) for the soil samples presented for the analysis.

REFERENCES

1. M. Vainshtein, N. Suzina, and T. Abashina, "Nanobacteria," Nauka Rossii, No. 3(159), 10–14 (2007).
2. V. S. Guzev and D. G. Zvyagintsev, "The biometric analysis of bacterial cells in soil," Microbiology (Moscow) **72** (2), 187–192 (2003).
3. V. V. Dmitriev, N. E. Suzina, T. G. Rusakova, R. R. Oleinikov, T. Z. Esikova, V. P. Kholodenko, V. I. Duda, A. M. Boronin, and P. Yu. Petrov, "Electron microscopic detection and in situ characterization of bacterial nanoforms in extreme biotopes," Microbiology (Moscow) **77** (1), 39–46 (2008).
4. V. I. Duda, N. E. Suzina, V. N. Akimov, M. B. Vainshtein, V. V. Dmitriev, R. R. Oleynikov, T. Z. Esikova, A. M. Boronin, E. S. Barinova, and T. N. Abashina, "Ultrastructural organization and development cycle of soil ultramicrobacteria belonging to the class Alphapro-

teobacteria," Microbiology (Moscow) **76** (5), 575–584 (2007).

5. D. G. Zvyagintsev, *Interaction of Microorganisms with Solid Surfaces* (Moscow State University, Moscow, 1973) [in Russian].
6. P. A. Kozhevin, *Microbial Populations in Nature* (Moscow State University, Moscow, 1989) [in Russian].
7. P. A. Kozhevin, L. M. Polyanskaya, and D. G. Zvyagintsev, "Growth rate of different microorganisms in soil," Mikrobiologiya **48** (4), 490–494 (1979).
8. N. B. Naumova and P. Kuikman, "Bacterial biomass and DNA diversity in an alluvial meadow soil upon long-term fertilization," Eurasian Soil Sci. **34** (66), 621–627 (2001).
9. D. I. Nikitin, "Implementation of electron microscopy for analysis of soil successions," Pochvovedenie, No. 6, 642–656 (1964).
10. L. V. Polyanskaya and M. A. Gobacheva, "Bacterial size in anaerobic and aerobic conditions in chernozem," Mezhd. Sb. Nauchn. Tr. Posvyashchennyi Godu Germanii Rossii, 180–186 (2012).
11. L. V. Polyanskaya, R. B. Gorodnichev, and D. G. Zvyagintsev, "Sizes of bacterial cells in soils determined by cascade filtration technique," Biol. Bull. **40** (2), 130–137 (2013).
12. L. V. Polyanskaya, N. I. Sukhanova, K. V. Chakmazyan, and D. G. Zvyagintsev, "Specific features of the structure of microbial biomass in soils of annular mesodepressions in Lipetsk and Volgograd oblasts," Eurasian Soil Sci. **47** (9), 904–909 (2014).
13. H. C. Bae, E. H. Cota-Robles, and L. E. Casida, "Microflora of soil as viewed by transmission electron microscopy," Appl. Microbiol. **23**, 637–648 (1972).
14. L. E. Casida, "Microorganisms in unamended soil as observed by various forms of microscopy and staining," Appl. Microbiol. **21**, 1040–1045 (1971).
15. J. O. Cisar, De-Qi Xu, J. Thompson, W. Swaim, Lan Hu and D. J. Kopecko, "An alternative interpretation of nanobacteria-induced biomineralization," Proc. Natl. Acad. Sci. U.S.A. **97** (21), 11511–11515 (2000).
16. M. Diaz-Ravina, I. Carballas, and M. J. Acea, "Microbial biomass and metabolic activity in four acid soils," Soil Biol. Biochem. **20** (6), 817–823 (1988).
17. R. L. Folk, "SEM imaging of bacteria and nanobacteria in carbonate sediments and rocks," J. Sediment. Res., No. 63, 990–999 (1993).
18. R. L. Folk and F. L. Lynch, "The possible role of nanobacteria (dwarf bacteria) in clay mineral diagenesis and the importance of careful sample preparation in high-magnification SEM study," J. Sediment. Res., No. 67, 583–589 (1998).
19. S. D. Frey, E. T. Elliot, and K. Paustian, "Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems among two climatic gradients," Soil Biol. Biochem. **31** (4), 573–585 (1999).
20. R. D. Jones, R. Y. Morita, H. P. Koops, and S. W. Watson, "A new marine ammonium–oxidizing bacterium, *Nitrosomonas cryotolerans* sp. nov.," Can. J. Microbiol., No. 34(10), 1122–1128 (1988).
21. J. E. Hobbie, R. J. Daley, and S. Jasper, "Use of nuclepore filters for counting bacteria by fluorescence microscopy," Appl. Environ. Microbiol. **33** (5), 1225–1228 (1977).
22. O. Kajander, "Nanobacteria," Proc. Natl. Acad. Sci. U.S.A., 8270–8274 (1998).
23. J. Maniloff, "Nanobacteria: size limits and evidence (letter)," Science, No. 276, 1776–1777 (1997).
24. N. S. Panicov, "Contribution of nanosized bacteria to the total biomass and activity of a soil microbial community," Adv. Appl. Microbiol. **57**, 245–289 (2005).
25. S. Scheu and D. Parkinson, "Changes in bacterial and fungal biomass C, bacterial and fungal biovolume end ergosterol content after drying, remoistening and incubation of different layers cool temperature forest soils," Soil Biol. Biochem. **26** (11), 1515–1525 (1994).
26. M. Vainshtein, E. Kudryashova, N. Siizina, et al., "Formation of bacterial nanocells," Proc. SPIE **3441**, 95–104 (1998).
27. P. J. R. Uwins, R. A. Webb, and A. P. Taylor, "Novel nanorganisms from Australian sandstones," Am. Mineral. **83**, 1541–1550 (1998).

Translated by L. Kholopova