ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 6, pp. 841–846. © Pleiades Publishing, Ltd., 2013.
Original Russian Text © S.A. Kharin, A.V. Kurakov, 2014, published in Mikrobiologiya, 2014, Vol. 83, No. 1, pp. 83–89.

EXPERIMENTAL ARTICLES

Characterization of the Physiological State of Fungi by Dynamics of Colony Emergence on Solid Media

S. A. Kharin[a] and A. V. Kurakov[b, 1]

[a] *Institute of Medico-Biological Problems, Russian Academy of Sciences, Khoroshevskoe sh. 76a, Moscow, 123007 Russia*
[b] *Faculty of Biology, Moscow State University, Russia*

Received April 17, 2013

Abstract—Poisson distribution was shown to be applicable to the dynamics of emergence of fungal colonies on plates inoculated with pure cultures or environmental samples, indicating the possibility for application of Hattori approach for assessment of the physiological state of fungi. The differences in physiological activity of different fungal species and genera, between spores and mycelia, or between the fungal populations from different environments, were revealed using the t_r (delay time for colony emergence) and λ (potential capacity for growth) parameters.

Keywords: fungi, plating, physiological state, lag phase, probability of division, Poisson distribution, Hattori equation, soil, earthworms

DOI: 10.1134/S0026261714010056

Plating has been the experimental technique that allowed microbiologists to conduct various investigations for over one hundred years. This method is used to assess the relative abundance and isolate pure microbial cultures from natural and technogenic environments, and to study the spectra of utilized compounds and the effect of physicochemical factors on microbial growth. The plating method may be used to determine the physiological state of microbial populations in nature. The activity of microbial populations and communities is unequal in the habitats with diverse conditions. This is obviously connected with such external factors as humidity, temperature, pH, availability of nutrients and oxygen, or the presence of toxicants; it also depends on the stage in the life cycle of an organism and on the species composition of the community. The Japanese microbiologist Tsutomu Hattori pioneered research in this field [1, 2]. As a result of the comparative observations of plating of various bacterial species on solid media, he established that their colonies, given equal other conditions (temperature, the composition of a nutrient medium, etc.), differed in dynamics of their development. A detailed study of the time of emergence of visually detectable colonies showed that the dynamics of their appearance on solid nutrient media obeyed the Poisson distribution and was described by the following equation:

$$N(t) = N_\infty(1 - e^{-\lambda(t - tr)}) \ (t > t_r), \qquad (1)$$

where $N(t)$ is the number of colonies at a given moment t; N_∞ is the final number of colonies; λ is the probability of colony formation by a cell in a unit time; and t_r is the delay time (including the lag period and the time before the formation of a colony visible with a naked eye). $N(t)$ and t are variables, and N_∞, λ, and t_r are the constant parameters characterizing the physiological state of bacterial populations [3, 4].

Counting of the colonies formed on a solid nutrient medium after certain time intervals makes it possible to determine the timetable of of colony development and to calculate the parameters λ and t_r. According to Poisson distribution, a certain event triggering the process of cell division occurs in the probability per unit time λ, resulting in formation of a colony after the time t_r. The parameter λ characterizes the potential capacity of the cells for growth. Note that there is no direct relationship between λ and the offspring growth rate. Therefore, the probability of cell division must change at different stages of the life cycle, depending on stresses, the content of intracellular storage compounds, etc. [3, 5].

This was the method used by Hattori et al. [2–4, 6] to determine the physiological state for the pure cultures of bacteria of the oligotrophic and copiotrophic species. The λ values for active bacterial cells were above 0.042 h^{-1}; for the dormant cells, $\lambda < 0.021$ h^{-1}; the cells with $0.042\ h^{-1} < \lambda < 0.021\ h^{-1}$ were in the intermediate phase of the cycle of development. This approach made it possible to carry out quantitative assessment of the differences in the activity of bacterial populations in various ecological niches, soils of various types, as well as in the rhizosphere and water bodies [2–6]. Despite the effectiveness of this approach for bacterial ecology, no attempts were made to show

[1] Corresponding author; e-mail: kurakov57@mail.ru

the possibility of its use and its prospects for characterization of the physiological state of fungi under different conditions of their development.

The goal of the present work was to determine the applicability of Hattori's approach to assessment of the physiological state of fungi in pure cultures and of fungal populations in natural environments.

MATERIALS AND METHODS

Micromycetes. The physiological state of the fungi was assessed for the cultures of *Aspergillus niger* 3P42 and *Alternaria alternata* 1C321 (the spore and mycelial forms), as well as for the strain *Penicillium aurantiogriseum* 5P623 inoculated into sterile sod-podzol soil, the complexes (populations) of microscopic fungi of the native sod-podzol soil, and of the digestive tract, its content and the coprolites of the earthworms *Aporrectodea caliginosa* inhabiting this soil.

In order to obtain mycelial suspensions, *Asp. niger* and *Alt. alternata* cultures were grown on solid Czapek medium for 24 h at 27°C. Such duration of incubation resulted in formation of the fungal mycelium without conidia. The mycelial biomass was transferred to a test tube with sterile water, homogenized by vortexing for 5 min, and diluted with sterile water to the concentration not exceeding 50 CFU per 100 μL of the suspension. The spores of *Asp. niger* and *Alt. alternata* were obtained after seven-day cultivation on Czapek medium at 27°C. The initial spore suspension was prepared by washing the conidia off the colony surface with sterile water and then diluted to the concentration of 10–50 spores per 100 μL of suspension.

Soil. Fresh samples of cultured sod-podzol soil under legume-grass vegetation from many years' experiment of the Agrochemistry Department of Moscow State University (Moscow oblast, Chashnikovo Moscow State University Soil Ecology Center) were used. The contents of total carbon and nitrogen were 1.72 and 0.13%, respectively; pH of the aqueous extract was 5.7. In 1989, lime was used at doses sufficient to neutralize the hydrolytic activity. In 1990–1999, the soil received 340 t/ha of organic fertilizers and mineral fertilizers $N_{100}P_{500}K_{500}$ per year; in 1994–1999, an average of $N_{100}P_{40}K_{100}$ per year was introduced.

Earthworms. The earthworms *Aporrectodea caliginosa* were collected from the 0–20-cm horizon of the soil studied. The earthworms were maintained in containers with soil at 12–15°C.

To obtain the content of the earthworm stomach and its purified digestive tract, an earthworm was immersed in water at 100°C for 1 s and then placed on a freezing table (Peltier element) where it was cooled to –16°C in 20–30 s [7]. The earthworm was then dissected, and the gut content and the digestive tract were removed at the time of defrosting. Purification of the digestive system from soil and plant residues was performed in 100-mL glasses with sterile sand for 5–7 days (3–5 earthworms per glass).

The samples of fresh coprolites were obtained by maintaining the earthworms in petri dishes on moistened sterile filter paper at 4–5°C for several hours. Three-day coprolites were also used after incubating the earthworms on the soil surface for three days.

In order to assess the physiological state of the fungi, the schedule of emergence of the fungal colonies on solid medium was determined, and its correspondence to the Poisson distribution was evaluated in accordance with Hattori's approach. Equality of the mean and variance, which is the main feature of the Poisson distribution and may be used as its proof, was the criterion of correspondence [8]. Glucose–peptone–yeast agar (GPYA) of the following composition (g/L): glucose, 1.0; peptone, 2.0; yeast extract, 1.0; casein hydrolysate, 1.0; KH_2PO_4 – 0.5; K_2HPO_4, 0.5; agar, 15.0; tap water, 1 L; pH 7.2 was used. Bacterial growth was inhibited by addition of streptomycin (30 mg/L) to the medium. Fungus desorption from the soil particles, the gut content, the digestive tract, and the coprolites of *A. caliginosa* was carried out in a DIAX 9000 homogenizer (Heidolph). Inoculated plates were incubated in the thermostat at 27°C, and after certain time intervals, the numbers of the newly formed colonies were determined by marking them with a marker on the reverse side of the petri dish. The experiments were carried out in 10 replicates with a periodicity of colony count every 3–8 h for 3–14 days from the moment of emergence of the first colony. If their development corresponded to the Poisson distribution, i.e., in the case of the applicability of equation (1), the physiological state of the fungal populations was assessed using the parameters of the multiplication probability λ and the duration of the lag phase t_r from equation (1). These parameters were calculated by the linear mode of the least-squares method using the available experimental data on $N(t)$ and t according to the schedule of colony formation.

RESULTS AND DISCUSSION

Confirmation of applicability of the Poisson distribution for description of the dynamics of emergence of fungal colonies on solid medium. The correspondence of the schedule of emergence of microbial colonies to the Poisson distribution serves as the condition of applicability of equation (1) for determination of the lag phase duration t_r and the probability of cell division λ, which characterize their physiological state:

$$P(x) = x_{av}^{x} e^{-x_{av}} / x!, \quad (2)$$

where x is a discrete value; $x!$ is its factorial; $P(x)$ is the probability of the incidence of this value; x_{av} is the average of the discrete value (distribution parameter); and e is the base of natural logarithms ~2.7182.

Table 1. Comparison between the dynamics of emergence of fungal colonies and the Poisson distribution

Asp. niger mycelial suspension										
x_{i*}	0	1	2	3	4	5	6	7	8	$x_{av} = \sum p_i x_i / \sum p_i = 3.2$***
p_{i**}	8	20	25	36	27	22	8	3	1	$\sigma^2 = \sum p (x_i - x_{av})^2 / (n-1) = 3.0$
$p_i x_i$	0	20	50	108	108	110	48	21	8	$F = \sigma^2 / x_{av} = 0.94 \quad F < F_{(P=0.95)}$
Asp. niger spore suspension										
x_i	0	1	2	3	4	5	6	7	8	$x_{av} = 2.7$
p_i	12	23	38	37	19	15	5	1	0	$\sigma^2 = 2.4$
$p_i x_i$	0	23	76	111	76	75	30	7	0	$F = 0.89 \quad F < F_{(P=0.95)}$
Alt. alternata mycelial suspension										
x_i	0	1	2	3	4	5	6	7	8	$x_{av} = 1.8$
p_i	29	41	37	27	8	6	2	0	0	$\sigma^2 = 2.0$
$p_i x_i$	0	41	74	81	32	30	12	0	0	$F = 1.11 \quad F < F_{(P=0.95)}$
Alt. alternata spore suspension										
x_i	0	1	2	3	4	5	6	7	8	$x_{av} = 2.1$
p_i	21	35	43	26	19	3	1	2	0	$\sigma^2 = 2.1$
$p_i x_i$	0	35	86	52	76	15	6	14	0	$F = 1.00 \quad F < F_{(P=0.95)}$
Sterile soil inoculated with *P. aurantiogriseum*										
x_i	0	1	2	3	4	5	6	7	8	$x_{av} = 1.3$
p_i	132	170	110	47	15	6	1	0	0	$\sigma^2 = 1.4$
$p_i x_i$	0	170	220	141	60	30	6	0	0	$F = 1.10 \quad F < F_{(P=0.95)}$
Sod-podzol soil										
x_i	0	1	2	3	4	5	6	7	8	$x_{av} = 0.8$
p_i	174	114	51	15	4	2	0	0	0	$\sigma^2 = 0.9$
$p_i x_i$	0	114	102	45	16	10	0	0	0	$F = 1.13 \quad F < F_{(P=0.95)}$

* The number of fungal colonies formed on one plate at intervals between the measurements.

** The occurrence rate of the x_i values.

*** Correspondence to the Poisson distribution was analyzed on the basis of comparison between the average number of colonies formed and the variance of the mean using the *F*-test.

One of the proofs that colonies developed on solid media at a rate corresponding to the Poisson distribution was reliable equality of the average number of colonies formed in unit time on one petri dish and the variance of this average [5]:

$$x_{av} = \sigma^2, \quad (3)$$

where x_{av} is a the average number of colonies formed in unit time and σ^2 is variance of the average.

The absence of significant differences between x_{av} and σ^2 may be considered an indication that the probabilistic distribution of the colony emergence rate obeys the Poisson's law. Analysis of emergence rates of the fungal colonies and comparison between the colony emergence mean and the variance of the mean with the *F*-test are presented in Table 1.

The experiments with laboratory cultures of both spore and mycelial suspensions of *Asp. niger* and *Alt. alternata*, as well as with soil containing the spores and mycelium of *P. aurantiogriseum*, established that the dynamics of formation of visible fungal colonies on the nutrient medium was described by the Poisson distribution. Plating of of *Asp. niger* mycelial suspension is used below to illustrate this equivalence. The *Asp. niger* colonies were counted on 10 petri dishes after every 5 h for 72 h. Thus, after 150 measurements ($n = 150$), the values of the number of colonies forming on one dish after every 5 h (x_i) and the incidence of each value of this number (p_i) were determined (Table 1). Using these data, it is possible to find the average number (x_{av}) of colonies formed on one dish

Table 2. The values of λ and t_r parameters for pure cultures of fungi and fungal populations from different habitats

Variant		λ, h^{-1}	t_r, h
Asp. niger mycelium		0.073	10.3
Asp. niger spores		0.067	11.3
Alt. alternata mycelium		0.054	16.8
Alt. alternata spores		0.048	18.8
Sterile sod-podzol soil inoculated with *P. aurantiogriseum*		0.029	22.8
Sod-podzol soil		0.014	23.3
Habitats associated with the earthworm *A. caliginosa*	Purified gut	0.008	40.1
	Gut content	0.009	33.7
	Fresh coprolites	0.011	32.6
	72-h coprolites	0.021	14.4

during 5 h, as well as the variance (σ^2) of this average for $f = n - 1$ degrees of freedom:

$$x_{av} = \sum p_i x_i \Big/ \sum p_i = 473/150 = 3.2,$$

$$\sigma^2 = \sum p_i (x_i - x_{av})^2 \Big/ (n-1) = 448/149 = 3.0.$$

Reliability of equality of the average number of emerging colonies and the variance of the average was established using the Fisher's test [8]:

$$F = \sigma^2 / x_{av} = 3.2/3.0 = 1.06.$$

Comparison between the F value thus obtained and the tabulated value for $P = 0.95$ ($F < F_{P=0.95}$) proves the absence of significant differences between the distribution of the rate of *Asp. niger* colony formation and the Poisson distribution. The significance of equality of the average number of colonies formed and the variance of the mean was shown for all other cases: for plating of *Asp. niger* spores, *Alt. alternata* spores and mycelium, soil with *P. aurantiogriseum*, and fungal complexes of native soil (Table 1).

Graphic comparison between the experimental rates of fungal colony formation and the theoretical curve of the Poisson distribution for the corresponding x_{av} also confirmed that fungal colonies were formed on solid nutrient media at a rate close to Poisson law (Fig. 1).

Assessment of the physiological state of microscopic fungi. According to Hattori, the parameters λ and t_r reflect the potential capacity of the cells for growth and the duration of the lag period. Their values depend much on the stages of the life cycle, the action of stresses, and the content of intracellular storage compounds; therefore, they serve as an indicator of the physiological state of microbial populations. The values of the λ and t_r parameters may be found schedule of colony formation (the t dependence of $N(t)$) using the least-squares method [9] (Fig. 2).

Plating and enumeration of the developing fungal colonies made it possible to establish the schedule of their emergence and to determine the parameters λ and t_r for both individual fungal populations and for the complexes of microscopic fungi from different habitats (Table 2).

It was established that the fungi in the mycelial form had a higher physiological activity compared to spores. The λ values for *Asp. niger* and *Alt. alternata* in the mycelial form exceeded by 10–20% those for the spores of these species, while the lag phase was noticeably shorter. Earlier, Hattori showed that the physiological activity of resting bacterial forms was lower than that of the active cells [1].

Comparison of the two fungal species revealed that *Asp. niger*, a quickly growing, abundantly sporiferous *r*-strategist, was characterized by higher values of physiological activity than *Alt. alternata*, which forms large thick-walled mycelium and multicellular spores. *P. aurantiogriseum* inoculated into sterilized soil and the fungal complex of sod-podzol soil exhibited substantially lower physiological activities (a long lag period and a lower λ value) than pure fungal cultures on nutrient media.

Using these values, we compared the physiological state of fungal populations from different ecological niches. It was shown that the passage of soil through the digestive tract of *A. caliginosa* significantly decreased the physiological activity of the fungal populations inhabiting it. The physiological activity was lower in the mycobiota of the gut content, the purified digestive tract, and fresh coprolites than in the soil. This showed up in lengthening of the lag phase and a decrease in the probability of fungal growth λ. However, the populations of fungi from fresh coprolites activated rapidly, so that their physiological state in 72-h coprolites was higher (Table 2). It is quite natural that in the earthworm guts, where the fungi are influenced by the digestive fluid, their physiological activity decreases compared to soil. Earlier, we established that

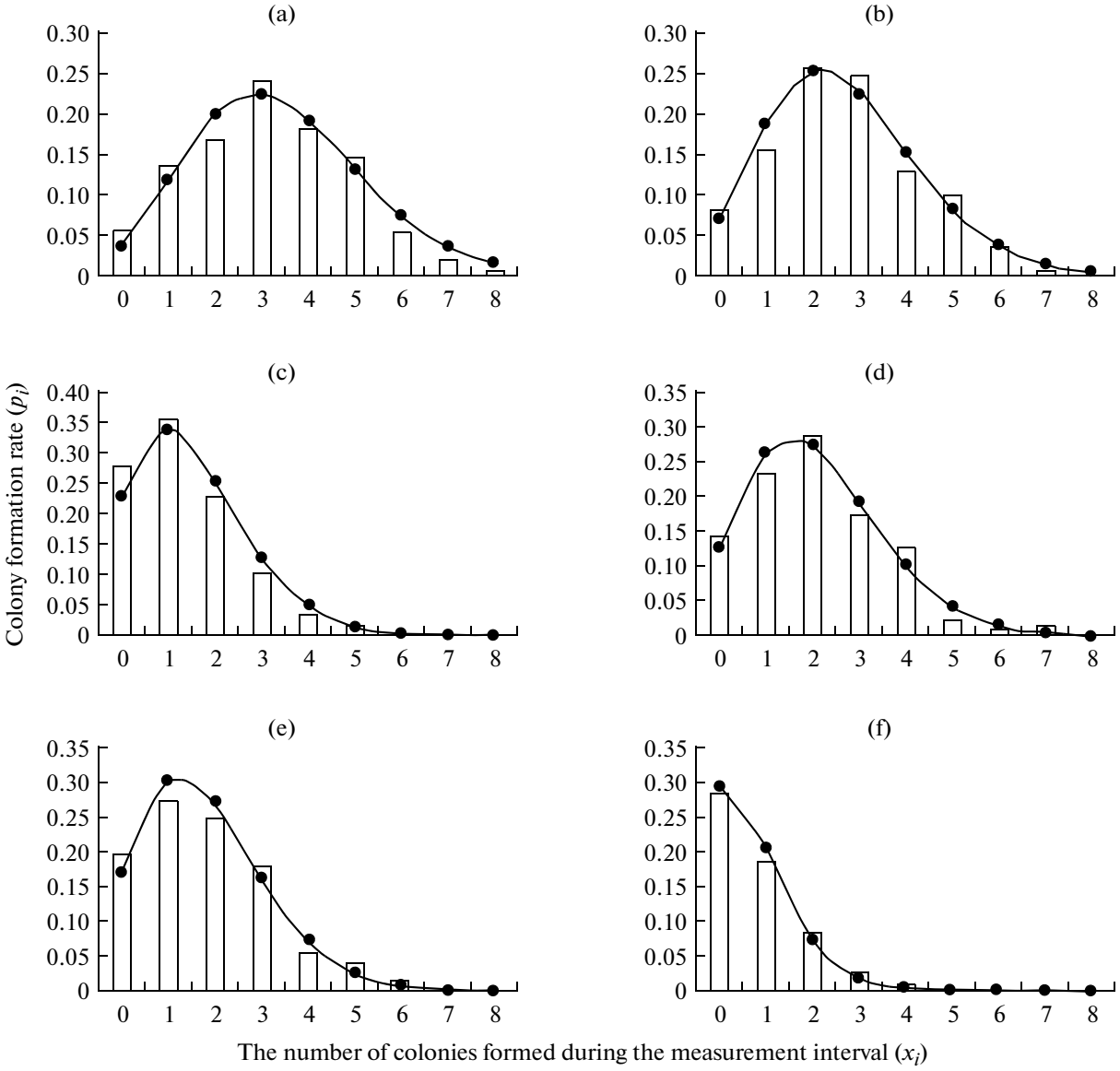

Fig. 1. Distribution of the rates of development of fungal colonies. The columns show the experimental data. The curve shows Poisson distribution for the corresponding x_{av}: *Asp. niger* mycelial suspension (a); *Asp. niger* spore suspension (b); *Alt. alternata* mycelial suspension (c); *Alt. alternata* spore suspension (d); sterile soil inoculated with *P. aurantiogriseum* (e); and sod-podzol soil (f).

the digestive fluid exerted an inhibitory effect on fungal growth [7].

This is the first demonstration of the applicability of the Poisson distribution equation for describing the dynamics of emergence of fungal colonies on solid nutrient media, indicating the possibility of using Hattori's approach to assessment of the physiological state of the fungi. As in the case of bacteria, this approach makes it possible to quantify the differences in activity of the fungal populations of various ecological niches, the fungi of different species and at different stages of development (spores or mycelium). For example, it is known that the time of development of fungal colonies is substantially shorter for the rhizoplane samples than for soil [8]. This confirms a more active state of fungi on the root surface than in the surrounding soil, and, evidently, the quantitative criteria are required for describing such phenomena as it has been done for bacteria. Experimental determination of the λ values characterizing the state of fungal propagules under different conditions and in different habitats may eliminate this gap. It is possible to get an idea of the physiological state of pure fungal cultures, as well as of the fungal complexes in natural environments and habitats subject to various technogenic stresses. This approach is of interest for comparison of the physiological state of fungal strains, which are stored for a

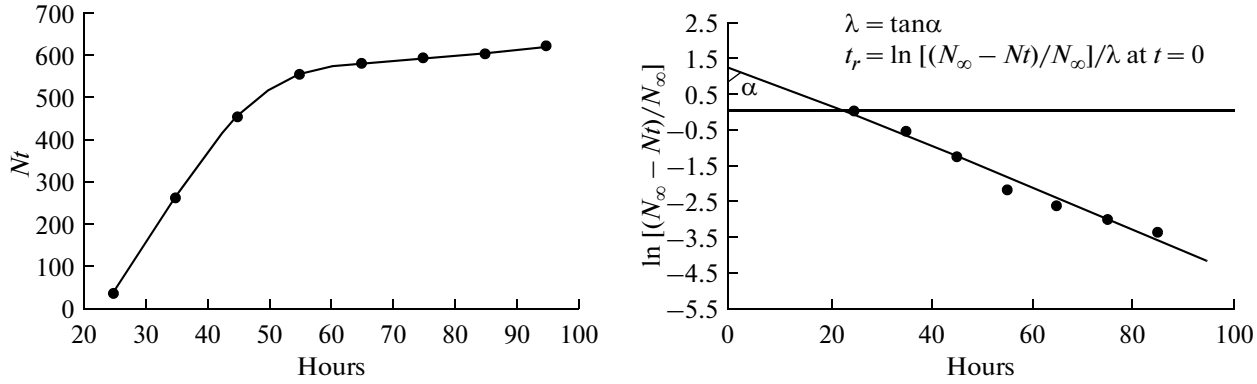

Fig. 2. Schedule of emergence of *P. auratiogriseum* colonies on GPYA medium and the calculation of the λ and t_r parameters for this species using the least-squares method.

long time in the collections of producers for biotechnological industries.

REFERENCES

1. Ishikuri, S. and Hattori, T., Formation of bacterial colonies in successive time intervals, *Appl. Environ. Microbiol.*, 1985, vol. 49, no. 4, pp. 870–873.
2. Hattori, T., Hisayuki, M., Haga, H., Wakao, N., Shikano, S., Gorlach, K., Kasahara, Y., El-Beltagy, A., and Hattori, R., Advances in soil microbial ecology and the biodiversity, *Antonie van Leeuwenhoek*, 1997, vol. 72, pp. 21–28.
3. Hattori, T., Analysis of plate count data of bacteria in natural environments, *J. Gen. Appl. Microbiol.*, 1982, vol. 28, no. 6, pp. 13–22.
4. Hattori, T., Mathematical equations describing the behaviour of soil bacteria, *Soil Biol. Biochem.*, 1982, vol. 14, pp. 523–527.
5. Kozhevin, P.A., *Mikrobnye populyatsii v prirode* (Microbial Populations in Nature), Moscow: Mos. Gos. Univ., 1989.
6. Hashimoto, T. and Hattori, T., Grouping of soil bacteria by analysis of colony formation on agar plates, *Biol. Fertil. Soils*, 1989, vol. 7, pp. 198–201.
7. Khomyakov, N.V., Kharin, S.A., Nechitailo, T.Yu., Golyshin, P.N., Kurakov, A.V., Byzov, B.A., and Zvyagintsev, D.G., Reaction of microorganisms to the digestive fluid of earthworms, *Microbiology* (Moscow), 2007, vol. 76, no. 1, pp. 45–55.
8. Gmurman, V.E., *Teoriya veroyatnostei i matematicheskaya statistika* (Probability Theory and Mathematical Statistics), Moscow: Vyssh. shkola, 2003.
9. Ishikuri, S., Suwa, Y., and Hattori, T., Method for mathematical analysis of bacterial count data, *Soil Sci. Plant Nutr.*, 1984, vol. 30, no. 2, pp. 249–253.
10. Kurakov, A.V. and Kostina, N.V., Spatial peculiarities in the colonization of the plant rhizosphere by microscopic fungi, *Microbiology* (Moscow), 2001, vol. 70, no. 2, pp. 165–175.

Translated by E. Babchenko