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SOIL BIOLOGY

Absolute Number of Bacteria and Archaea in Soil

O. A. Andreeva*, N. A. Manucharova, A. L. Stepanov***, and P. A. Kozhevin******

Department of Soil Science, Moscow State University, Moscow, 119991 Russia

*e-mail: *elvi.23@mail.ru; **manucharova@mail.ru; ***stepanov_aleksey@mail.ru; ****kozhevinpa@mail.ru*

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Abstract—The possibility of using the method of exhaustion (removal sampling) applied to determine the absolute number of Bacteria and Archaea populations in soil based on accounting data for quantitative fluorescence in situ hybridization has been shown.

Key words: fluorescence in situ hybridization (FISH), evaluation of microbial abundance, soil, absolute population density, Archaea, Bacteria

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INTRODUCTION

Microbial ecology pays special attention to development methods that enable one to determine the abundance of microorganisms in natural habitats, including soil. All classical methods of quantifying microorganisms are known to be relative, and their application allows one to detect and account for only some of the objects. Special sample preparation for cell desorption and the disaggregation of microcolonies could improve the quantifying process, but these solutions are not universal and involve the need to optimize specific sites and habitats.

A fundamentally different solution to the problem of quantifying microorganisms was suggested previously [2] based on the approaches developed in general ecology. In this case, a series of standard procedures for sequentially removing objects from the same soil sample and taking into account remote cells in each trapping. During every subsequent cycle, new objects are added to those previously detected, which allows one to evaluate the level of absolute quantity. The effectiveness of the method has been proved for standard tasks of general quantitative accounting, as well as accounting for specific species and strains populations.

This paper presents the possibility of determining the absolute number of objects in the quantitative modern molecular methods based on the example of fluorescent in situ hybridization (FISH) with rRNA-specific fluorescently labeled oligonucleotide probes.

MATERIALS AND METHODS

A sample of air-dry deep calcareous heavy clay loam chernozem on loess (Belgorodskaya oblast, hor. A) was studied. The microbial succession in soil was initiated by humidification (60% of the field capacity), and moisturizing by applying readily available carbon (glucose) and nitrogen ($NaNO_3$) compounds [1]. Sampling for analysis was performed on the seventh day of succession.

In both cases, the soil suspension was treated by an ultrasonic device UZBN-2T at a frequency 22 kHz and a current intensity of 0.4 A for 2 min. A single application of the ultrasound was used to determine the relative abundance of archaea and bacteria. The absolute number was evaluated by cyclic pre-treatment, which included multiple ultrasound treatments of soil suspensions with the separation of desorbed cells from the soil. After each ultrasonic treatment, cells were removed by centrifugation (centrifuge CM-6M, 2000 rpm, 10 min). Supernatant was only used to quantify microorganisms for this particular processing cycle after concentrating cells by further centrifugation (10000 rpm, 5 min). Soil sediment with the remaining microbial cells was treated again by this scheme. A total of three treatment cycles were done. In each cycle cells of archaea and bacteria were registered. Cells of microorganisms were fixed with paraformaldehyde. Fixed material was collected by centrifugation, washed twice with phosphate buffer, and resuspended in ethanol and phosphate buffer (1 : 1). Fixed samples were stored at –20°C prior to analysis.

Fixed sample suspension was applied to glass slides. Specimen hybridization (the set of rRNA-specific oligonucleotide probes, fluorescently labeled,

Absolute and relative number of archaea and bacteria

Object	Succession options	Number by the standard method, cells number/g soil	Absolute number, cells number/g soil
Archaea	Control	4.7×10^7	9.7×10^7
	Additional resources application	4.1×10^7	9.9×10^7
Bacteria	Control	2.3×10^7	4.7×10^7
	Additional resources application	1.1×10^8	1.6×10^8

was used) was performed at a temperature of 46°C [4]. Specimens were analyzed by fluorescent microscope ZEISS Axioskop 2 plus Microscope (Germany) with Filter Set 15 light filters [3].

RESULTS AND DISCUSSION

The method of sequential exhaustion based on the assumption that the number of objects decreases in the course of sequential counts. Indeed, in all variants of the experiment, the dependence of the number of cells seized and taken into account after each cycle is linear to the total number of previously deleted cells, which indicates an increase in the completeness of accounting. In general, the relationship can be described by an appropriate regression equation: $Y = a - pX$, where Y is the cells outtake in a particular cycle; and X is the total number of previously extracted cells. Regression slope p reflects the proportion of the extracted population in one cycle.

It is possible to identify levels of absolute numbers from these equations. It should be given to the condition limiting the extraction of cells: at $Y = 0$, we obtain $X = -a/p$. For example, for archaea in control succession, without resources application, the regression equation is $Y = -0.52X + 5 \times 10^7$ (correlation coefficient $r = 0.99$). In this example, $p = 0.52$, i.e., the proportion of the population extracted and accounted for every cycle is approximately 52% of the population in the soil prior to the particular cycle. In other words, during each subsequent stage, the number of archaea cells number extracted from the soil is about two times less than the previous cycle. Thus, the estimated absolute abundance index in this example is about 9.6×10^7 cells.

During the experiments, it was found that, in different accounts of the same cell population, depending on the conditions, the extraction efficiency index p can vary. However, in the first approximation, under the given conditions, for bacteria and archaea, this index varied within a narrow range of 0.4–0.6. These results were obtained after three cycles of treatment, but it is possible to reduce the number of cycles to two. The calculations are performed according to the formula $X = C_1^2/(C_1 - C_2)$, where C_1 is number of extracted cells during the first cycle and C_2 is number of cells removed during the second cycle. The verification showed that, in this case, the abundance indices are nearly identical to those for three cycles (85–97%).

A comparison of the absolute numbers to similar numbers with standard ultrasonic pretreatment confirms the conclusion that the suggested approach allows one to increase the completeness of accounting for the objects by 1.5–2.5 times (table). This is of interest in problems of matter and energy flows in soil. Some features of the enumerated objects were identified according to the relative and absolute abundance indices. In particular, it was found that the application of readily available resources did not significantly affect the number of archaea during the investigated succession. However, relative and absolute abundance indices for bacteria significantly increased under the same conditions (at 4.6 and 3.4 times, respectively). There is also a trend of bacteria domination in the prokaryotic community during succession with the application of additional resources.

These features correspond to the hypothesis of archaea as relative *K*-selective organisms adapted to living under conditions of chronic energy stress [5]. This stress is most evident in habitats with extreme characteristics (salinity, temperature, pH); however, in general, this is typical of a variety of conditions with a chronic lack of resources, including key ecological processes, e.g., nitrification, and expressed energy restrictions of catabolism, i.e., methanogenesis, anaerobic methane oxidation. The scarcity of resources is also created on climax stages of microbial succession, which in this case allows archaea survive on a relatively high, stable level of abundance. In the presence of readily available resources quick response of *r*-selective organisms provides the aforementioned dominance of Bacteria population.

Thus, the present study demonstrated the possibility of determining the absolute number of Bacteria and Archaea populations by quantitatively accounting for objects in the soil using FISH, which improves the completeness of the registration of objects by applying modern molecular techniques in microbial ecology.

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