Super-long anabiosis of ancient microorganisms in ice and terrestrial models for development of methods to search for life on Mars, Europa and other planetary bodies

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Abstract

Successful missions to Mars, Europe and other bodies of the Solar system have created a prerequisite to search for extraterrestrial life. The first attempts of microbial life detection on the Martian surface by the Viking landed missions gave no biological results. Microbiological investigations of the Martian subsurface ground ice layers seem to be more promising. It is well substantiated to consider the Antarctic ice sheet and the Antarctic and Arctic permafrost as terrestrial analogues of Martian habitats. The results of our long-standing microbiological studies of the Antarctic ice would provide the basis for detection of viable microbial cells on Mars. Our microbiological investigations of the deepest and thus most ancient strata of the Antarctic ice sheet for the first time gave evidence for the natural phenomenon of long-term anabiosis (preservation of viability and vitality for millennia years). A combination of classical microbiological methods, epifluorescence microscopy, SEM, TEM, molecular diagnostics, radioisotope labeling and other techniques made it possible for us to obtain convincing proof of the presence of pro- and eukaryotes in the Antarctic ice sheet. In this communication, we will review and discuss some critical issues related to the detection of viable microorganisms in cold terrestrial environments with regard to future searches for microbial life and/or its biological signatures on extraterrestrial objects.

Keywords: Super-long anabiosis; Antarctic ice sheet; Viable microorganisms; Terrestrial analogs of Martian habitats; Europa; Icy satellites; Comets

1. Introduction

As evidenced from the results of biological experiments within the frames of Viking missions, no microorganisms as even biogenic substances were present in samples of Martian ground. However, if life ever existed on Mars, it is quite possible to assume that living microorganisms might occur in subsurface layers of permafrost. Theoretically, this assumption can be made based on their protection from unfavorable external conditions. Under these conditions, microorganisms if any might be at the state of anabiosis for unlimitedly prolonged time.

Numerous calculations suggested that microorganisms may persist in super-long anabiosis, being exposed to negative temperatures for hundreds thousands and millions of years (Sneath, 1962, Imshenetsky, 1966, Weber and Greenberg, 1985, Aksyonov, 1982 and Aksyonov, 1990). Until the 70s of the past century, despite many sensational publications, there were no experimental works that could convincingly support the recovery of algal or bacterial cells after their existence in an anabiotic state for such long periods in Permian salts, ancient rocks, and animal remnants deeply buried in permafrost. Undoubtedly, along with these ancient objects it is worthwhile to regard Antarctic and Greenland ice sheets, as well as Arctic and Antarctic permafrost as good natural objects on Earth to test the hypothesis of the existence of super-long anabiosis. A distant location of the Arctic and Antarctic regions and therefore avoidance of negative anthropogenic influence and a relative stability of subsurface horizons are substantiated reasons to regard these objects as suitable models in developing various methods for detection of microorganisms that existed in super-long anabiosis under the negative temperatures.

In the biological contest of searching for subsurface life, Duxbury et al. (2004) have proposed novel methods to search for methane clathrate hydrate deposits in the Martian subsurface. Methane clathrate is not only a valuable fuel for future landed missions, but also a biosignature. Duxbury et al. (2004) have combined the upcoming sounding radar observations (by MARSIS on the currently orbiting European Mars Express and/or SHARAD on Mars Reconnaissance Orbiter 2005) with their thermal modeling used for the Earth's subsurface (Duxbury and Romanovsky, 2003; cf., Duxbury et al., 2001).
This communication has a focus on the review of the methodology that we used in microbiological studies of Central Antarctic ice sheet and on some important questions to be solved for implementation of further program to search for microbial life or its evidence in samples from extraterrestrial objects.

2. Objects of our investigations

In order to solve the task of finding the evidence for long-term existence of microbial cells in anabiotic state, we chose the Central Antarctic ice sheet at the Vostok station where boring of deep layers had been started from the 1960s within the framework of large projects. According to the data obtained by glaciologists, the Central Antarctic ice sheet, the depth of which accounts for more than 4000 m, is thermally stable and thawing of surface snow and the upper ice is excluded. The rate of sediment accumulation over past 5000 years was estimated to be about 2.4 cm per one year (Barkov, 1973). The temperature of ice varies from −57.13 °C at a depth of 48 m to −53.5 °C at 507 m (Lipenkov and Barkov, 1998). Gradually increasing at more deep horizons, the temperature of the ice sheet at the glacial base is approximately −2.4 °C.

3. Microbiological methods

Ice core fragments from various depths were taken using the aseptic sampling method used in our previous investigations that proved the existence of super-long anabiosis of microorganisms in nature. The method of aseptic sampling from ice cores and the experimental results were

already described in previous publications and reports at the COSPAR assemblies (Abyzov et al., 1977, Abyzov et al., 1979, Abyzov, 1993 and Abyzov et al., 1995).
Aseptically taken samples of ice cores from different horizons of Central Antarctic glacial cap were inoculated into rich media (potato broth with 0.05% yeast autolysate or glucose-, yeast autolysate-, and liver extract-containing medium) or synthetic Winogradsky's medium (glucose, 20 g/l; KH_2PO_4, 1 g/l; $MgSO_4 \cdot 7H_2O$, 1 g/l; NaCl, traces; $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$, traces; $CaCO_3$, 20 g/l) to produce enrichment cultures. Aliquots of enrichment cultures were seeded on selective media to produce individual colonies. Melted samples were also plated on nutrient agar to determine the number of colony-forming units (CFU) (Abyzov, 1993).
Cells from samples taken from different glacier layers were sedimented on nucleopore filters and studies by direct microscopy in an epifluorescence microscope (LYUMAM-12) after staining by fluorescamine as previously described (Abyzov et al., 1998, Mitskevich et al., 2001 and Poglazova et al., 2001).

4. Results and discussion

Now we would like to summarize the results of long-standing microbiological investigation of glacial sheet above the Lake Vostok and to discuss methodological approaches, used both in our studies and in experiments of the other renowned specialists. In the first stages of our investigations, we have developed of aseptic boring and sampling of cores from deep Antarctic ice layers and tested under the conditions of external artificial contamination by test-microorganisms placed on the surface of ice cores.
We did not detect any contaminating test-microorganisms after the removal of external bottom parts of the cores extracted by drilling. This proved the applicability of the developed method and allowed us to exclude contamination of central the parts of the ice cores by microorganisms from outside. In our opinion, it is very important for further exobiological explorations to prove that any method for sampling of ice, rocks, and subsurface layers would ensure the purity of experiments. Using traditional microbiological methods based on inoculations into nutrient media allowed us to isolate microorganisms for the first time and to obtain pure cultures of bacterial and fungi after super-long anabiosis in the glacier for hundreds to thousands of years. In a course of these experiments, the representatives of prokaryotes (spore-forming and non-spore-forming bacteria) and eukaryotes (yeasts and mycelial fungi) were isolated, described and deposited in the collections. By the sum of phenotypic, physiological, and biochemical features, these microorganisms were identified to genera and species (Abyzov, 1993).
The recent phylogenetic analysis of some selected isolates of the bacteria and yeast from Central Antarctic glacial layers above the lake Vostok (conducted in National Institute of Polar Research, Tokyo, Japan, and School of Biosphere Sciences, Hiroshima University) has proved the correctness of previous affiliation of these microorganisms based on phenotype properties. The isolates of microorganisms that persisted under long-time action of temperatures below zero in a state of anabiosis for millennia years were characterized and deposited as unique representatives of post-cold-anabiosis life.
However, it is axiomatic now that only a small part of the total number of microorganisms present in natural habitats is detectable upon plating on solid media (Novitsky and Morita, 1976, Morita, 1988, Kjelleberg et al., 1987, Roszak and Colwell, 1987 and Amman et al., 1995). When we studied the abundance of microbial cells in the Central Antarctic glacial layers,

we found out that the number of microorganisms that are culturable on the liquid and solid media is of several orders of magnitude lower than the total number determined by direct counts (Abyzov, 1993).

According to the data of direct counting of cells on nuclear filters in epifluorescence microscope, the total cells numbers for Antarctic ice samples from the depth 1500 to 2500 m accounted for 10^2–10^3 per 1 cm^3 of ice, whereas in most samples there were non-culturable cells. Only in 6 of 40 samples, we observed the growth of microorganisms after inoculation in the liquid media or plating onto nutrient agar, but microbial cells were detected by direct counting in all of them.

Using the epifluorescence and scanning electron microscopy, we detected microorganisms belonging to different taxonomic groups and characterized by considerable morphological diversity. Prokaryotic microorganisms (bacteria of various shape and sizes) were found across the whole tested layer. They included cocci, diplococci, rods of different length and width (straight or curved), and oval cells. Actinomycetes occurred in the studied Antarctic ice samples, as well as a variety of cyanobacteria (Fig. 1).

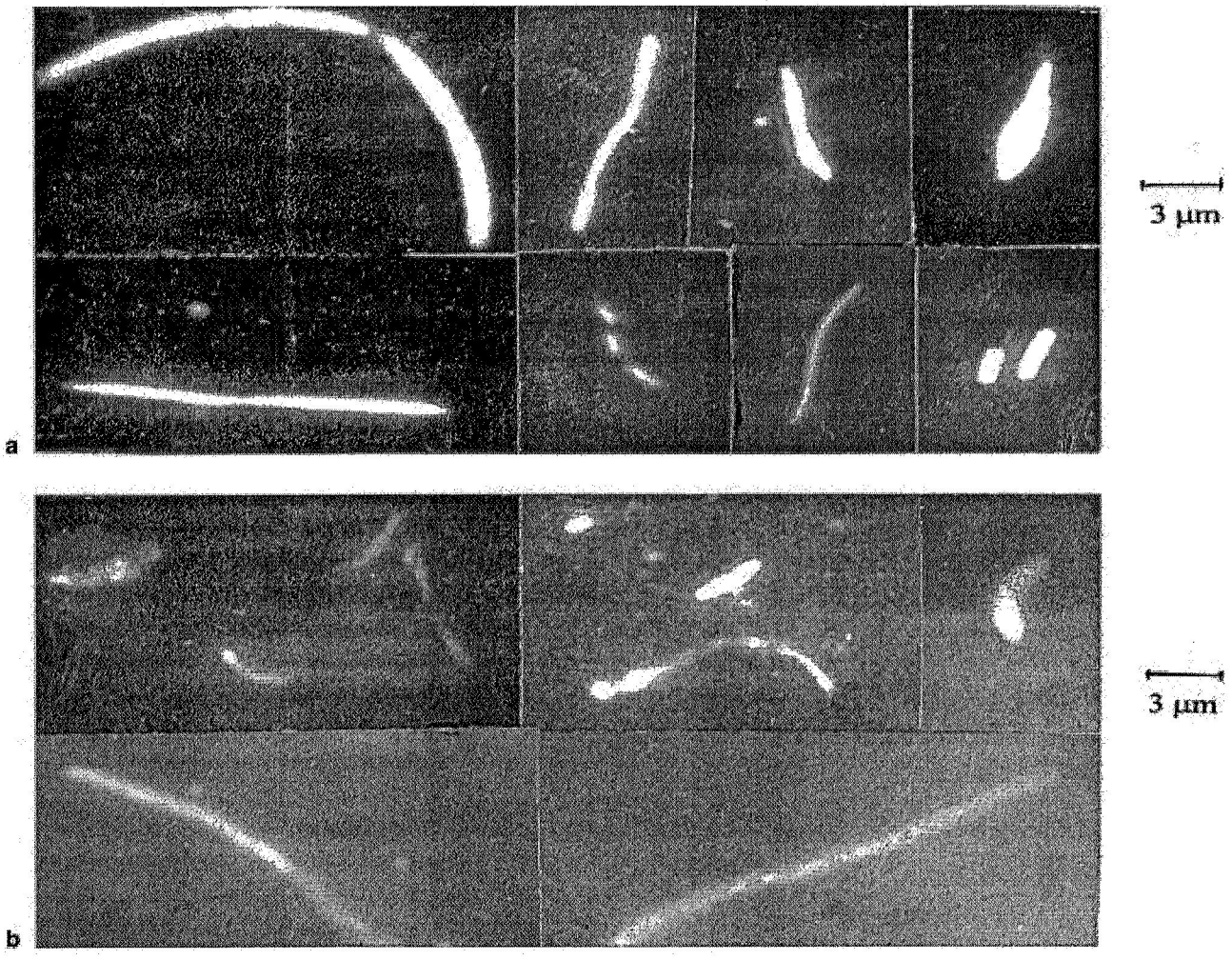

Fig. 1. Some representatives of prokaryotic microorganisms from different horizons of central Antarctic ice sheet (Fluorescamine, luminescence microscopy). (a) Bacterial cells displayed bright fluorescence typical for viable cells that contain sufficient amounts of proteins. (b) Dead bacteria cells with typically weak fluorescence and disrupted intracellular structure.

Eukaryotic organisms were represented by diverse yeast species (budding or dividing cells), hyphae of various fungi and their conidia and also by microalgae, including diatomic (Fig. 2).

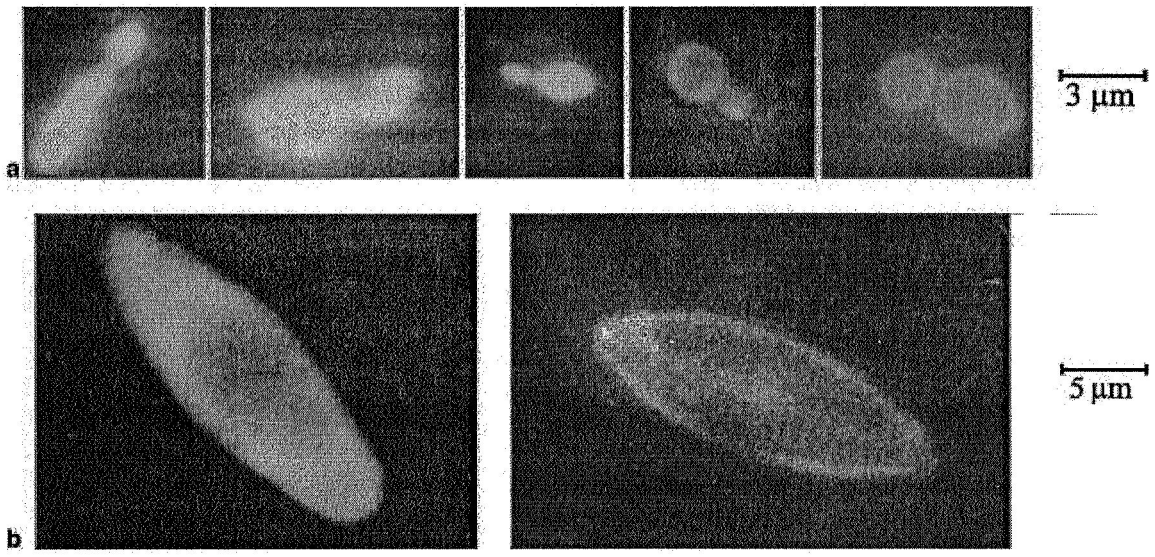

Fig. 2. Representatives of eucaryotic microorganisms from different horizons of central Antarctic ice sheet (Fluorescamine, luminescence microscopy). (a) To the left: brightly fluoresced viable budding yeasts. To the right: weakly fluoresced dead cells. (b) To the left: a diatomic alga cell with the signs of a viable organism (sufficient amount of proteinous compounds and a vacuole in the central part of the cell). To the right: a lyzed dead cell of the same type of algae that reveal only the siliceous skeleton with remains of proteinous substances.

The micrographs of these organisms found in ice depth were given in the previous works (Abyzov et al., 1998, Abyzov et al., 2004a, Abyzov et al., 2004b and Poglazova et al., 2001). The presence of luminescent chlorophyll in the algae cells, which were stored for at least 400000 years in the glacier, is a very interesting fact (Abyzov et al., 2004a and Abyzov et al., 2004b). Using fluorescamine for microscopy examinations of microorganisms from the melted Antarctic ice allowed us to discriminate between brightly and weakly fluorescent microbial cells, which respectively differed in the intracellular content of proteinous substances (Fig. 1 and Fig. 2).

The examinations of native bacterial populations in permafrost samples of different genesis and age showed the presence of both Gram-negative and Gram-positive bacteria after long-term exposure (7 thousands to 2 million years) to subzero temperatures (Soina et al., 2004). According to observations using the transmission electron microscope most of bacterial cells preserved the integrity. They had no signs of lysis or structural damage that might be expected

after thawing of the samples. Often bacteria were encased in a relatively vast amorphous electron-transparent layer that might protect cells from mechanical destruction by ice. The in situ cyst-like cells and those obtained in cultures of the permafrost isolates under the specific conditions resemble resting forms of the type strains *Micrococcus luteus* and *Arthrobacter globiformis*, which maintained the viability for prolonged periods and possessed experimentally undetectable respiration, and the elevated resistance to adverse conditions (Mulyukin et al., 1996 and Demkina et al., 2000). Based on morphological similarity of some of the above-described in situ cells, it is reasonable to consider the acquirement of the resting state by microorganisms in permafrost. Dormant cyst-like cells, being resistant to adverse external factors, are responsible for survival of the non-spore-formers under prolonged exposure to subzero temperatures and can be a target to search for living microorganisms in natural environments both on the Earth and extraterrestrial bodies.

Therefore, the data obtained upon studies of from the Antarctic ice sheet as well as the Earth permafrost give the evidence for the presence of viable bacterial cells preserved during long-term anabiosis. It was shown that the percentage of culturable microorganisms in Antarctic glacier comprises for 0.1% (or less) of total cell number. Similar estimates of cultivated cells were true for Arctic and Antarctic permafrost (Vorobyova et al., 1997 and Gilichinsky et al., 1995) as well as in many terrestrial habitats (Morita, 1988 and Roszak and Colwell, 1987). The observed discrepancy between the colony-forming and total bacterial cell numbers can be explained by bacteria entering the viable-but-not-culturable state. In this state the cells lost the ability to form colonies but possessed some attributes of metabolic activity, firstly described by Colwell et al. (1985). Later, many works were published regarding this bacterial state. Different procedures of resuscitation (from simple to very sophisticated) were described for non-culturable bacterial cells (see review by Kell et al., 1998), but it is difficult to forecast what resuscitation methods could be suitable for non-culturable cells in microbial populations in long-term frozen Antarctic glaciers.

The other substantiated explanation of why only a small part of microorganisms can be cultured is based on that the most optimal conditions favoring recovery of the cells from dormant state or non-culturable state are often unknown for experimenters. With the above in mind, we tried to select the temperature and pre-incubation conditions for growth recovery of microorganisms in melted samples from the Antarctic ice sheet.

In special experiments, we found that the use of poor liquid media is favorable for the reactivation of microbial cells preserved in ice samples. The melted samples of ice core from various horizons, incubated for 1 day at temperatures 15, 20, or 25 °C, were examined after passing through filters and staining with fluorescamine. Alive cells differed from dead cells by bright fluorescence due to high content of amino compounds in them (Fig. 1).

The growth of bacteria was observed at all temperatures. As seen from Table 1, the incubation of the samples from the ice sheet from the depths 952 to 1203 m at 15 °C and the samples from the depths 746 to 1051 m at 20 °C over 24 h resulted in a 3 to 6-fold increase in both the total cell number and that of viable cells. However, when the melted ice samples from the 2802 to 2974-m deep horizons were incubated at 25 °C, the increase of total and viable cell numbers was much greater (about by 16–24 times), despite the facts that the bacteria were at low temperatures and were preserved in the anabiotic state for much longer time than cells from upper horizons. Hence, the temperature of 25 °C was mostly optimal for the growth recovery of bacteria in melted ice samples.

Table 1.

The total number of cells and number of viable cells in melted ice samples from various depths of the Antarctic glacier after 24-h incubation at various temperatures

Dep (m)	Temperatu (°C)	Total bacterial number ($\times 10^2$			Viable cell number ($\times 10^2$ ml^{-1}		
		St	After 24-h incubation	Increment times	St	After 24-h incubation	Increment times
952	15	1.	7.2	4.5	0.	2.7	5.6
120		2.	6.0	2.7	1.	4.0	3.3
746	20	3.	15.0	4.6	1.	8.7	5.1
105		2.	9.6	3.8	1.	5.4	4.5
280	25	1.	21.0	21.0	0.	10.7	17.8
297		1.	18.0	16.4	0.	9.4	18.8

Since a few microorganisms present in Antarctic glacier can be cultivated, as evident from our experiments, it is very promising to use the advanced method based on the analysis of RNA extracted from the environmental samples, as best reviewed by Amman et al. (1995). Thus, Priscu et al. (1999) used this method and revealed the presence of representatives of the Alfa- and Beta-Proteobacteria and the actinomycetes in samples of accretion ice from Lake Vostok. Among terrestrial models considered to develop a methodology for exobiological investigations in subglacial oceans on Europa, a special attention is devoted to the Lake Vostok.

According to very interesting implications of the Duxbury et al. (2001)'s model, live organisms and/or their remnants in Vostok waters, could be much older than the age of the basal ice, and even older than the age of the Antarctic ice sheet, i.e., older than 5–30 Myrs. In terms of evolution of life on Earth, this time period is rather recent, and assuming that this lake was a productive ecosystem, remnants of its biota must certainly be preserved in its sediments. At the present stage of investigation, it is not yet possible to obtain samples from the lake water and especially from the bottom deposits because intensive preliminary work is required to develop a method of aseptic penetration through the ice-water interface and to construct equipment which will operate in such a way that the ecological environment of the lake remains intact.

The very interesting approach to analyze microbial cell number in a sample of the accreted ice of Like Vostok was applied in the work by Karl et al. (1999) and included cell enumeration by epifluorescence microscopy with staining by SYBR Green I, scanning electron microscopy and dual laser flow cytometry with the use of Hoechst 33342, a DNA-specific dye.

The other advanced method for direct visualization of microorganisms in various microbial communities is based on fluorescence in situ hybridization (FISH), reviewed by Moter and Göbel (2000). However, low intensity of the signal arising upon binding of oligonucleotide probe to target rRNA can be the consequence of insufficient probe penetration into the bacterial

cell, which depends on the structure of the bacterial cell wall. Another possible obstacle in using FISH is that the low physiological activity can result in low signal intensity, since the contents of rRNA (as target for the probe) varies considerably in cells of one strain in different physiological states, and non-growing cells contain low rRNA (Moter and Göbel, 2000). Partially for these reasons (but not only), dormant cells may not be directed by FISH until special procedures are used, for example, to recover these cells from dormancy. In our opinion, the serious difficulty for application of FISH method can be due to a typically low content of microorganisms in Antarctic ice sheet and accreted ice as demonstrated by us and other authors (Abyzov et al., 1999, Karl et al., 1999 and Priscu et al., 1999).

In situ identification of viable microorganisms by PCR and FISH gives no information about the state in which they exist in cold habitats, such as Antarctic and Arctic permafrost or Antarctic glacier. Thus, there is the viewpoint that microorganisms may actually function under low temperature, since the lowest temperature limit for activity of some microorganisms can be below zero, at −5 to −8 °C and even −12 °C (Gounot, 1991, Russel, 1992, Gilichinsky et al., 1995 and Rivkina et al., 2000).

In our opinion, at least some part of microorganisms can survive for prolonged periods in dormant state (Mulyukin et al., 2001). Thus, examination of thin-sections of permafrost samples revealed the presence of bacteria having the structural organization typical of resting forms (Dmitriev et al., 2001 and Soina et al., 2004). As to some bacteria revealed in situ, in Arctic permafrost their dormant forms were very similar to resting cyst-like cells described for some non-spore-formers (Mulyukin et al., 1996, Demkina et al., 2000, Soina et al., 2004 and Suzina et al., 2004). Not only electron microscopy studies, but also EDS/ESEM with X-ray microanalysis can give a key to make preliminary conclusions on the state of microorganisms in Antarctic and Arctic permafrost. Based on the data obtained by SEM with an X-ray microanalysis, Mulyukin et al. (2002) have proposed an approach to discriminate abiogenic particles and possible microbial cells among cell-like or biomorphic objects present in a sample of the Antarctic Dry Valley permafrost. Among microbial cells, dormant bacteria can be detected by reduced K^+ level low P/S and increased Ca/K ratio.

5. Conclusion

In the summary, we conclude that future exobiological investigations that aim at the search for microbial life on Mars and other celestial bodies should rely on the experience gained upon studying terrestrial Arctic and Antarctic permafrost and glaciers. In our opinion, the difficulties in using of each microbiological method should be considered before the extraterrestrial search for microbial life. A complex methodology should undoubtedly be used, based on the results of trials on the Earth's models.

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