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PERMAFROST MICROBIOLOGY

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PERMAFROST MICROORGANISMS IN THE OUTER SPACE: RESULTS OF THE "EXOBIOFROST" EXPERIMENT

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Results of the Exobiofrost experiment aboard the BION-M1 biosatellite showed that the impact of space flight factors (ionizing radiation, g-force, and temperature fluctuations) did not lead to complete sterilization of the permafrost samples. The post-flight analysis of samples revealed that a significant part of the bacterial community has remained viable after the space experiment. Assessment of the protist resistance showed that resting cysts of contemporary ciliates are more stable under stressful space conditions, than ancient, while *Colpoda steinii* strains are more resistant than ciliate *Exocolpoda augustini*. The greatest resistance to space flight conditions demonstrated by acanthamoeba cysts (*Acanthamoeba* sp.) allows us to view them as model organisms for both terrestrial and extraterrestrial experiments in future.

Permafrost, microorganisms, outer space

INTRODUCTION

The existence of viable organisms in the Earth's cryosphere (terrestrial permafrost) can be viewed as a conceptual permafrost model of the extremely cold extraterrestrial habitat. The interdisciplinary synthesis of geological and biological knowledge will expand our understanding of the spatial and temporal boundaries of the biosphere and of the possibility of life existence beyond the Earth [*Rivkina et al., 2018*].

Results of earlier studies showed that microorganisms preserved in permafrost for geologically significant time periods (from several thousand to million years), retain their viability [*Rivkina et al., 2018*]. During that time, they are continuously exposed to the adverse conditions of sub-zero temperatures, absence of free water, and natural radioactivity of the host rock minerals. Their retained viability makes these microorganisms a unique object for astrobiological model research. The composition of the microbial community that survived in the permafrost environment is a result of the continuous selection in the conditions of low temperatures along with low availability of free water and nutrients [*Friedmann, 1994; Morita, 2000*]. Over the past 20 years, a long list of microorganisms have been isolated from permafrost sediments and modern Arctic and Antarctic soils including aerobic and anaerobic bacteria, methanogenic archaea, cyanobacteria and green algae, yeast and mycelial fungi, and heterotrophic protists [*Gilichinsky and Rivkina, 2011*], of which many represent novel species [*Rivkina et al., 2018*]. The selective role of cryogenesis in the formation of viable microbial communities adapted to the subzero temperatures of permafrost comes into play in the form of freeze–thaw stress as the sediments experience freezing. [*Spirina and Fyodorov-Davydov, 1998; Gubin et al., 2016*].

It has been established that microorganisms from tundra soils and permafrost sediments have higher resistance to low temperatures and recurrent freezing–thawing cycles, as compared to microorganisms from permanently unfrozen sediments [*Gilichinsky et al., 1991*]. Experiments showed no qualitative or quantitative changes in microbial communities from permafrost sediments after numerous phase transitions. This attests to microbiocenoses being capable of retaining the physiological features acquired through multiple freeze-thaw cycles during their formation period. In contrast, samples from permanently unfrozen soils were shown to become almost sterile after 5–12 freeze-thaw cycles. The accumulation of osmoprotectants in cells is one of the possible mechanisms of stabilizing cellular structures of permafrost microorganisms affected by the freezing–thawing processes [*Soina and Vorobyova, 2004*]. Another important factor contributing to the preservation of microorganisms is their radiation resistance. The maximum dose of ionizing radiation that cells can survive at above zero temperatures is $3 \cdot 10^4$ Gy [*Battista, 1997*]. The radiation dose that the microbial cells were exposed to over a million years of their continuous residence in the permafrost totals to $0.6 \cdot 10^4$ Gy, which is not sufficient for complete sterilization of a sediment layer. In the experiments inves-

tigating the effect of radiation on the biota, permafrost samples with a known number of viable cells were exposed to radiation from 10^4 to 10^5 Gy, which was followed by another count of viable microorganisms [*Cheptsov et al., 2018; Vorobyova et al., 2018*]. A dose of radiation sufficient for complete sterilization of a thawed sample left most of the permafrost microbial community in a viable state. Microorganisms from permafrost samples demonstrated their culturability even after being exposed to the radiation dose as high as 10^5 Gy. Thus, the effect of radiation from minerals in the Arctic and Antarctic sedimentary covers proved not to be lethal for microorganisms with permafrost acting as a shield that enhances the resistance of the microbiocenoses to radiation. Numerous scientific findings support the possibility of preservation of traces of life on other planets of the Solar system [*Gilichinsky et al., 2007, 2011, 2015; Demidov et al., 2012; Rivkina et al., 2018*], and the interplanetary transfer of living matter in space (panspermia) [*Crick and Orgel, 1973; Wickramasinghe et al., 2018*]. The viable organisms transport and preservation in the composition of comets and meteorites are viewed as one of the main mechanisms for biota transfer. Meteorite material (dust grains) is putatively capable of protecting microbial cells against cosmic (ionizing) radiation and destruction. The artificial-meteorite material layer that is several micrometers thick was shown to protect bacilli spores from the ultraviolet (UV) radiation [*Rettberg et al., 2002; etc.*]. Meteorite material with a mass of 0.5 g/cm^2 is capable of shielding bacteria from scattered X-rays, whereas meteorite material with a mass of 30 g/cm^2 can protect them from solar wind particles. Besides viable microorganisms, Earth permafrost contains traces of their metabolic activity ranging from potentially active enzymes and pigments to biogenic gases and authigenic minerals. Metabolic reactions take place in permafrost at temperatures as low as –20 °C ensuring preservation of the microorganism viability over a geologically significant time (from several thousand to a million years) [*Rivkina et al., 2000, 2007*]. Thus, the mere fact of existence of viable organisms and their metabolic products in the Earth's cryosphere warrants its use for a conceptual model adapted for space objects of cryogenic type [*Cameron and Morelli, 1974; Gilichinsky et al., 1993, 1995; Vorobyova et al., 1996; Soina and Vorobyova, 2004*].

Seven of the nine planets in the Solar system, their moons, many comets and asteroids are known to be cryogenic objects. Given that they went through several formation stages, primitive forms of life may have incepted there. However, the absence of liquid water, high level of UV radiation and other adverse factors renders the detection of traces of life on a planet surface highly unlikely. Thus, identification of specific ecosystems in the near-surface layer of perennially frozen sediments has become a new strategy to search for life on Mars, with the Earth's cryosphere seen as a model of the extremely cold extraterrestrial habitat. Viable microorganisms and their metabolic products within terrestrial permafrost serve as a prototype of possible ecosystems on other planets at early stages of their evolution. In other words, if life ever existed there, then its traces, i.e. microorganisms and their metabolic products, would be best preserved within the permafrost layer.

OBJECTIVES AND EXPERIMENT DESIGN

The goal of the study was to evaluate the effect of the outer space on the biological samples. Within the framework of the *Exobiofrost project* aboard the BION-M1 biosatellite, we have studied permafrost microbial complexes of different ages and origin collected in Northeastern Siberia, Russia (Kolyma Lowland) and Antarctica (Table 1), as well as pure cultures of microorganisms isolated from these sediments. The objective was to conduct a comparative analysis of the isolated microbial complex composition in their pre- and post-flight conditions, i.e. the study of effects of the outer space and spacecraft flight factors on viability of microorganisms. Special containers required for the experiment were designed and fabricated by Special Design Bureau of the Space Instrument Manufacture, Russian Academy of Sciences (KB IKI RAN) (Fig. 1). The sample containers

Table 1. **Description of permafrost samples**

No.	Borehole	Depth, m	Age	Description
1	4/09 (Meadstream of the Chukochia Rv., Kolyma lowland, Arctic)	8.7–8.8	Late Pleistocene (Q_{III})	Ice-rich clay loam
2	3/09 (ibidem)	9.25–9.45	Pleistocene–Late Pleiocene (Q_I–N_2)	Ice-poor sandy loam
3	2/08 (Duvanny Yar, Kolyma Rv., Arctic)	7.0	Holocene (Q_{IV})	Clay-loam
4	3–4/07 (Arctic)	20.6–20.7	Late Pleistocene (Q_{III})	Sand-loam
5	LA56-Pr-04 Progress station (Antarctic)	0–0.02	Modern	Moss mat with green mass

were attached onto the satellite casing (on the payload platform) shortly before the rocket launch. When the spacecraft has reached its destination orbit, the access door lid swung open, to allow the payload platform to stay exposed in open space, whereas after the flight, the door lid closed, protecting the payload platform (PLP) from overheating while descending. In addition to the biological samples placed into specialized PLP containers, similar samples were placed inside the satellite, where a constant temperature (20–22 °C) was maintained. For the temperature control during the flight, the iButton DL1922 temperature loggers (measuring error: 0.5 °C) were installed into three containers. The flight duration was one month, from April 19 to May 19, 2013. The satellite orbit was close to circular, with an altitude of 575 km. The calculated temperature difference on the vehicle surface was +125/–150 °C. According to [*Olsson-Francis and Cockell, 2010*], space ionizing radiation at the satellite altitude, can vary from 33 to 830 Gy per month which is 6–8 orders of magnitude higher than on Earth.

OBJECTS OF RESEARCH

Permafrost samples of different age and origin selected for the experiments in the space environment (Table 1), and protozoan cysts were placed in the containers that were stored at a temperature of –12 °C before their transportation to the rocket launching site and after their returning to the laboratory. During the 4-hour transportation to the Baikonur cosmodrome, they were placed in thermostatic chambers with refrigerants capable of retaining their initial temperature during 24 hours. Upon arrival to Baikonur, the containers were relocated to a stationary freezer and were kept at a temperature of –12 °C until the PLP unit was mounted onto the biosatellite. The temperature of the PLP unit installation was 26 °C, to which the samples were exposed for 3 days while waiting for the rocket launch. This unfortunately ruined the temperature regime of the samples.

The viable protist strains of the phylum *Amoebozoa* and *Ciliophora* previously isolated from Arctic permafrost belong to different genus: *Acanthamoeba*, *Flamella*, *Acramoeba*, *Vannella*, *Phalansterium*, *Cohliopodium* [*Shmakova and Rivkina, 2015*] and *Colpoda, Exocolpoda, Oxytricha, Platyophrya, Blepharisma, Chilodonella* [*Shatilovich et al., 2015*]. The strains of *Colpoda steinii* and *Exocolpoda augustini* collected from modern tundra soil and Arctic permafrost of Holocene and Late Pleistocene age were chosen to be the experimental objects. Similar to many other soil protists, these ciliates can form dormant cysts and endure long-term cryptobiosis under unfavorable conditions. A preliminary analysis [*Shatilovich et al., 2015*] revealed that cysts of ancient ciliates *Colpoda steinii* display lower tolerance to the impact of desiccation, cyclical supercooling and freezing compared to the cysts of contemporary ciliates collected from permafrost affected soils. Two strains of the genus *Acanthamoeba* isolated from Holocene and Late Pleistocene sediments were also selected for the experiment. Acanthamoeba inhabiting soil, freshwater and marine biotopes are the most widely spread amoeba on Earth. Viable acanthamoebae are quite common within permafrost. The microorganisms chosen for the experiment were isolated from Holocene and Late Pleistocene permafrost affected soils. Acanthamoeba cysts are known to be very resistant to unfavorable effects and can withstand desiccation, remaining viable for decades [*Sriram et al., 2008*].

The complete list of the microorganisms selected for the experiment includes: *Acanthamoeba* sp. cysts, strains am8, am88; cysts *Colpoda steinii,* strains 1086, 7/91, 1019, SS1; cysts *Exocolpoda augustini*, strain 1/01. Protozoan cysts desiccated on membrane filters

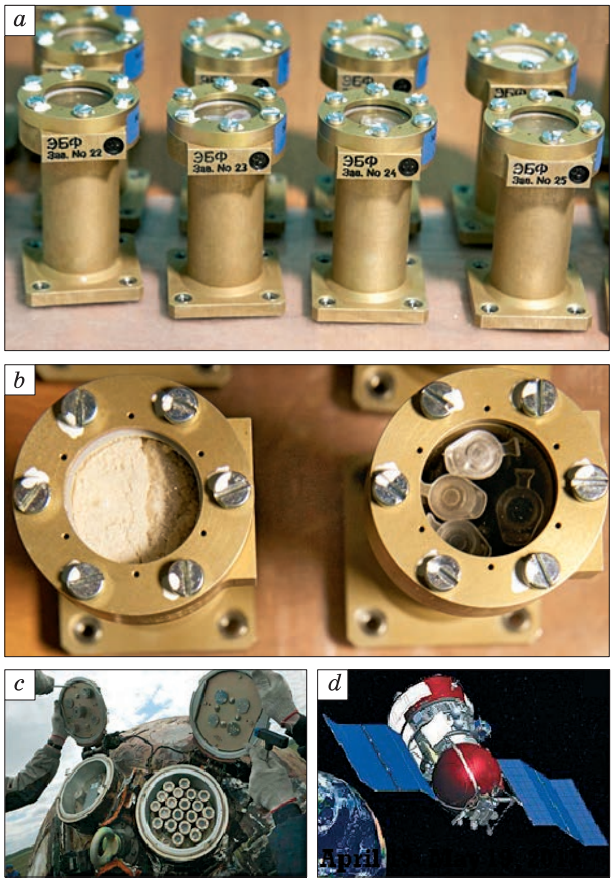

Fig. 1. Containers with samples for the Exobiofrost experiment.

a – containers exterior design; *b* – placement of containers on the surface of the BION-M1 satellite playload platform; *c* – top view of containers with samples; *d* – BION-M1 satellite during the flight (diagram).

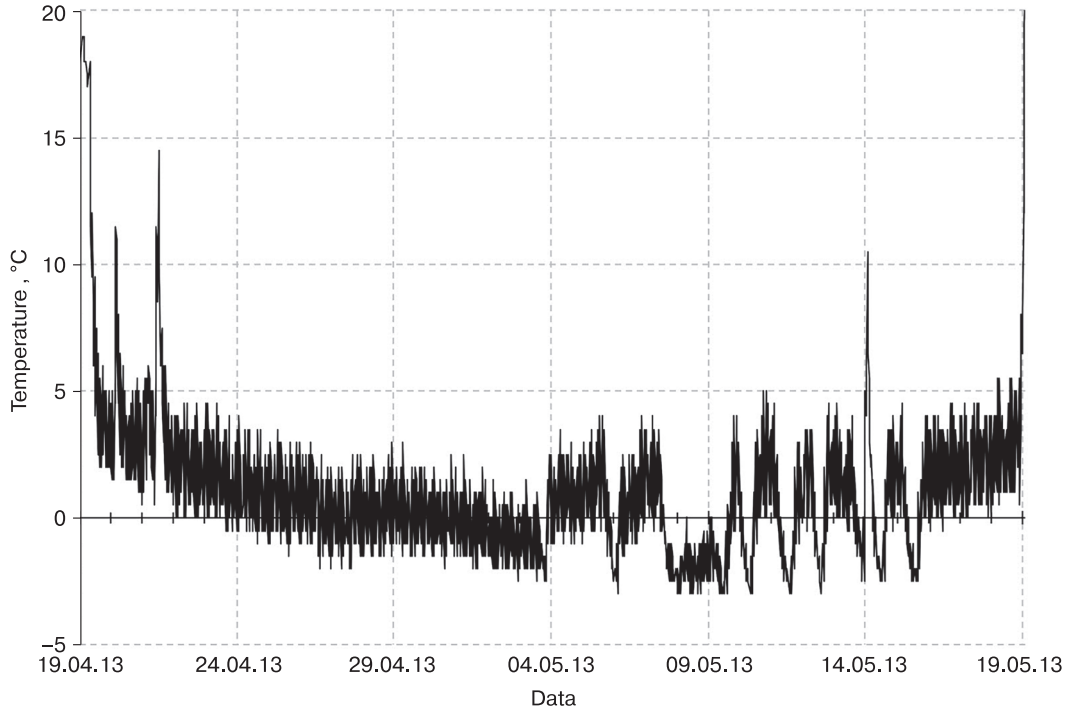

Fig. 2. The temperature curve derived from the iButton loggers measurements (placed inside containers with samples).

and in sterile kaolinite, and also cysts in 1.5 mL eppendorf tubes with culture medium were placed into containers. The temperature was monitored with iButton loggers in 30-minute increments during the entire flight, as well as in the pre-flight and post-flight periods (Fig. 2). All of the microbiological analyses were repeated three times.

RESULTS AND DISCUSSION

The temperature curve behavior over the entire experimental period (Fig. 2) indicates that the temperature inside the containers installed on the outside surface of the mainframe during the entire flight varied between +5 and –2 °C with frequent passes through zero, conditioned by the satellite rapidly rotating around its axis. This very factor prevented experimental samples from either cooling down to –150 °C or heating up to +125 °C, as had been anticipated before the experiment. The analysis of the actual data shows that the temperature regime was not extreme (the absolute minimum was –3 °C, maximum +14.5 °C) despite several hundred passes over 0 °C during the flight. As noted above, the temperatures measured during the flight differed considerably from the calculated flight regime parameters (–150...+125 °C). However, the frequent passes of temperature through zero proved not to be critical for the experimental microorganisms. It is important to note that the numerous passes through zero degree was not an extreme factor for microorganisms in the containers with desiccated cultures, inasmuch as in the absence of water, there are no phase transitions. Results of the post-flight experiments were compared with the pre-flight data analyses.

Count of the culturable bacteria

Plate inoculations from the post-flight (inside and outside the biosatellite) and control (native) permafrost samples were carried out on the standard culture media R2A (Difco, USA) and 2-fold diluted culture medium TSA (Difco, USA), in three-fold repetition. Their incubation was performed at 4 and 20 °C in aerobic conditions. Combinations of the culture media and incubation temperatures (Fig. 3, 4) correspond to the groups of aerobic members of the cultivated portion of microbial community: oligotrophs, psychrophiles/psychrotolerant bacteria (R2A medium; culture temperature: 4 °C); oligotrophs, mesophiles (R2A, 20 °C); heterotrophs, psychrophiles/psychrotolerans (1/2 TSA, 4 °C); and heterotrophs, mesophiles (1/2 TSA, 20 °C).

Analysis of plate inoculations showed the differences (both in number of colony-forming units per gram of frozen soil (CFU/g) and in diversity of colony morphotypes in microbial communities) between the control and post-flight samples, regardless of the incubation temperature. Specifically, a considerable amount of the pigmented forms that appeared in the

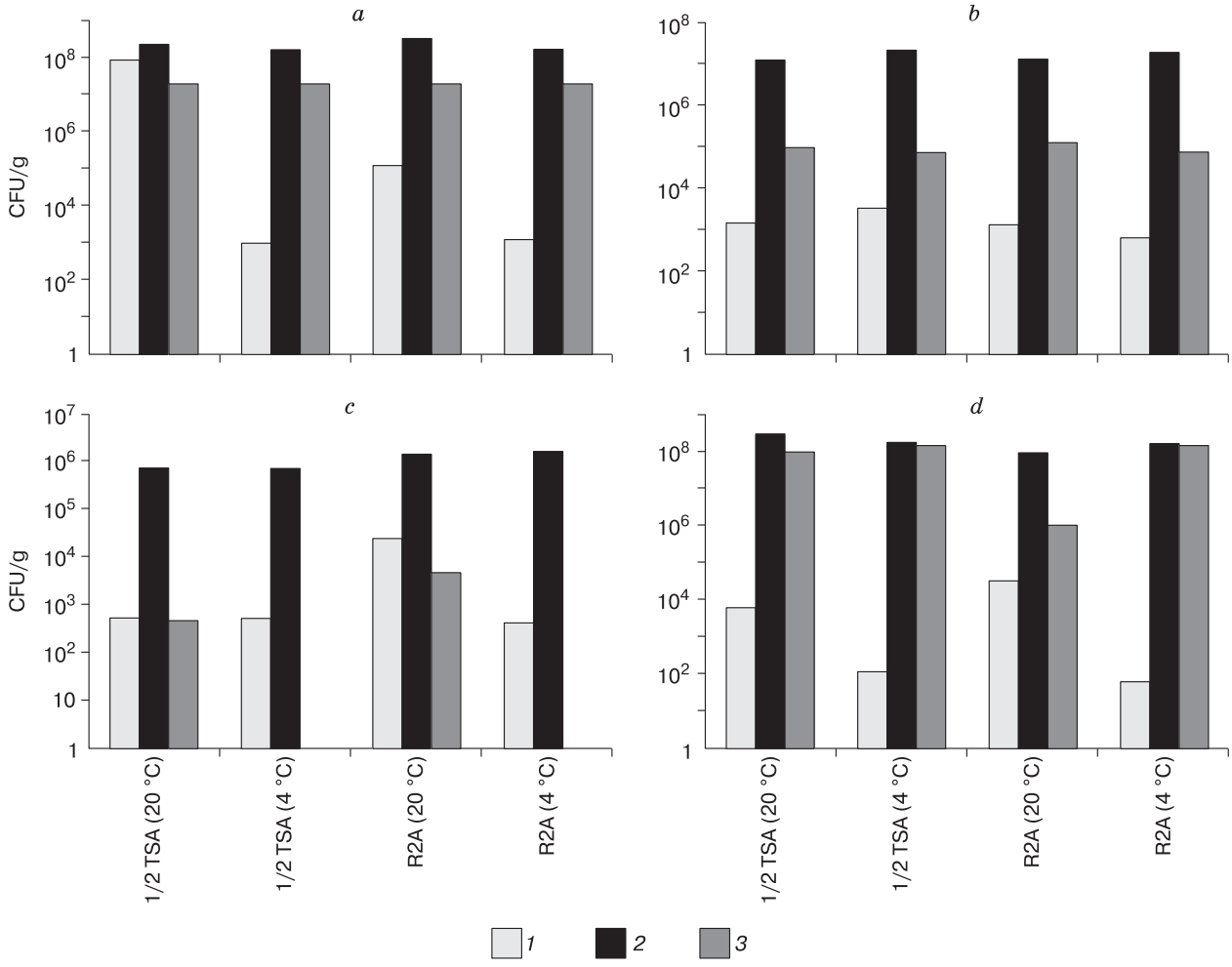

Fig. 3. Changes in the microorganisms count in the Arctic permafrost samples resulted from the outer space experiment.

a – BH 2/08; *b* – BH 3–4/07; *c* – BH 3/09; *d* – BH 4/09. The abscissa axis shows the media and temperature at which the cultivation proceeded; *1* – control, *2* – inside the satellite, *3* – on the outer shell of the satellite.

post-flight samples located outside the biosatellite may indicate a significant impact of the space flight conditions on the stability of individual microbial species. Analysis of the CFU/g number in Arctic permafrost samples (post-flight) inside- and outside the satellite showed a considerable increase in the number of microorganisms (up to 2–4 orders) compared to the control (pre-flight) sample (Fig. 3). At the same time, the maximum values of CFU/g were detected solely in the post-flight samples located inside the biosatellite in all of the Arctic samples, irrespec-

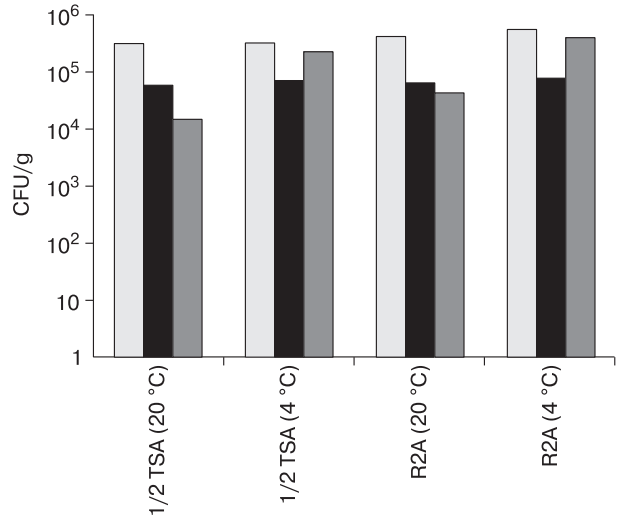

Fig. 4. Changes in the microorganisms count in modern Antarctic soil permafrost samples (Progress station) resulted from the outer space experiment.

For notations see Fig. 3.

tive of their culture medium type and incubation temperature. It is very likely that a comfortable temperature regime and initial moisture content of the sample created favorable microbial growth conditions. Heterotroph and oligotroph members of the psychrophilic-psychrotolerant species of the microbial community from borehole (BH) 4/09 were the only exception (Fig. 3, *d*). Taking into account the initially low count ($10–10^2$ CFU/g) in the control samples, the values of the CFU/g number after incubation at 4 °C were high (10^8 CFU/g) for both post-flight samples (inside and outside the biosatellite) indicating either a high resistance of these microorganisms to the temperature fluctuations in the outer space, or favorable cryopreservation conditions during permafrost formation.

However, the general tendency for the microorganisms in the post-flight samples located outside the spacecraft and exposed to the open space conditions to increase in number compared to the control ones (Fig. 3, *a*, *b*, *d*), is not supported in a sample from BH 3/09 (Fig. 3, *c*). For the post-flight sample located inside the satellite, the CFU/g values were shown to be either equal to those in the control sample or decreased by one order for mesophilic microorganisms. At the same time, we did not find any visible growth in bacteria with the optimum in the low positive temperatures. Even small temperature fluctuations and the open-space exposure were probably lethal for psychrophilic-psychrotolerant microorganisms from these sediments. A slight decrease (within one order) in the count of cultivated microorganisms (Fig. 4) was observed for Antarctic soil samples located both outside- and inside the satellite. Despite the observed instability of the Antarctic soil microbial community to the space flight conditions, heterotrophs and oligotrophs were shown to have the highest cell viability with growth temperature corresponding to the psychrophilic temperature range.

DNA analysis

A commercial PowerSoil DNA Isolation Kit manufactured by MO BIO Laboratories, Inc. (Carlsbad, USA) was used for isolation of common genomic DNA. The original DNA extraction protocol was modified to be applied to permafrost samples. The concentration of isolated DNA was measured using a NanoDrop ND 2000 spectrophotometer. Table 2 shows DNA concentrations in the pre-and post-flight samples. The data analysis demonstrated that, considering generally low DNA content in the Arctic permafrost samples, there were only minimal variations in the post-flight experimental samples. The modern Antarctic soil has the highest DNA concentration; its considerable decrease in the post-flight samples follows the general trend of reduced CFU/g number compared to the control sample (Fig. 4). Apart from the loss of viability by the part of the microbial population, this also indicates the partial destruction of the total DNA in the post-flight experiments.

Table 2. **The extracted DNA concentration in the post-flight and control samples**

No.	Borehole	Genomic DNA concentration, μg/g soil		
		Control, permafrost sample	Post-flight sample	
			inside the biosatellite	outside the biosatellite
1	4/09	0.85	0.69	0.97
2	3/09	0.69	1.20	0.69
3	2/08	0.46	1.25	0.36
4	3–4/07	0.94	0.61	1.21
5	LA56-Pr-04	34.09	7.67	11.07

Protists

The experiment results showed that the soil ciliate cysts are capable of maintaining viability in space conditions. Analysis of the post-flight and control samples of cysts was carried out by the methods of light, fluorescence and scanning electron microscopy. The ability of cysts to excystation, changes in membrane permeability and morphological signs of cell necrosis served as the diagnostic criteria for distinguishing between living and dead cells. The *in vivo* fluorescent LIVE-DEAD cell staining (Fig. 5) using acridine orange (AO) and propidium iodide (PI) revealed a high proportion of dead cysts in the post-flight samples (70–97 % compared to the control sample).

Cysts cultivation from the post-flight and control samples was performed on a PJ liquid mineral cultural medium with the addition of *E. coli* at 22 °C for a week. The growth curve analysis showed an increase in the lag phase, a decrease in the maximum growth rate and in the total number of ciliates in the cultures obtained from the cysts samples that remained desiccated during the flight (on membrane filters and in kaolinite) compared to the control sample. At the same time the proportion of living cysts was higher in cultures incubated during the flight in a liquid culture medium, which indirectly points to the presence of DNA repair processes in resting cells. The study of cyst morphology using the SEM methods revealed that some cysts from the post-flight samples have considerably damaged walls, the cause still remains unclear.

The post-flight analysis of acanthamoeba *Acanthamoeba* sp. (am8 and am88 strains) from Late Pleistocene perennially frozen sediments of the Kolyma Lowland showed the preservation of viable cysts both when incubated on liquid culture medium (PJ), as well as when dry-incubated in kaolinite and on filters. All variants of the experiment (dry cysts on filters and on kaolinite, cysts on wet kaolinite and cysts in a liquid culture medium PJ) had viable cysts capable of

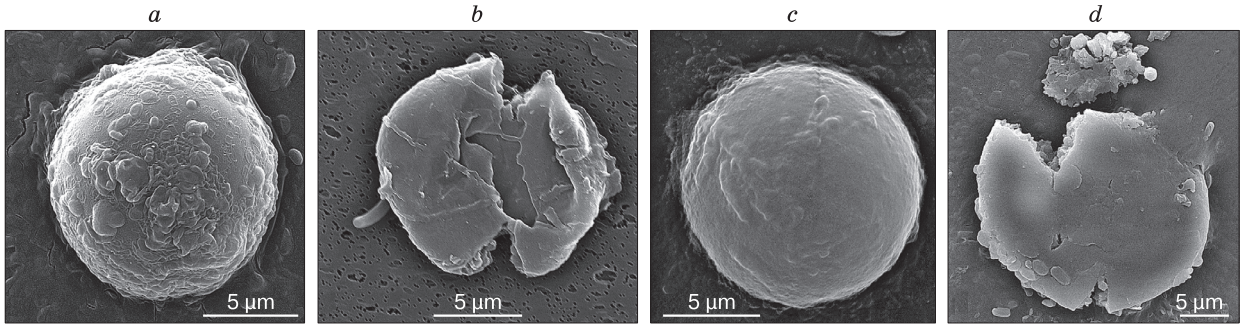

Fig. 5. Resting cysts of *Exocolpoda augustini* (*a*, *b*) and *Colpoda steinii* (*c*, *d*):
a, *c* – control samples; *b*, *d* – damaged cysts from post-flight samples. Scanning electron microscope.

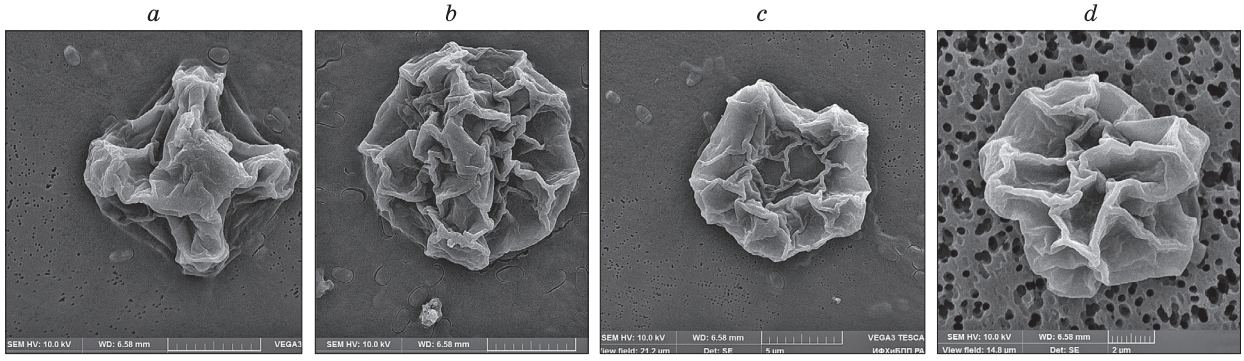

Fig. 6. Acanthamoeba cysts, the am8 strain (scanning electron microscope).
a–*c* – after space flight; *d* – control (mature cyst).

excystation after the flight. The cysts were excystated on the second day in all variants of the laboratory conditions, both on liquid media PJ and CPJ, and on agarized media APJ and ACPJ at 20 and 30 °C. on the SEM micrographs, Acanthamoebus cysts subjected to the outer space exposure (Fig. 6, *a*–*c*) looked intact and typical of mature cysts (Fig. 6, *d*). The cysts preservation in the space experiment was generally consistent with the previous data obtained in the similar experiments on the preservation of microbial endospores [*Rivkina et al., 2007; Horneck et al., 2012*].

CONCLUSION

The Exobiofrost experiment on the BION-M1 biosatellite has shown that a considerable part of permafrost microorganisms retained their viability after an orbital space flight. Neither the effects of temperature variation, nor the ionizing radiation and overload resulted in complete sterilization of the samples. The fact that almost all permafrost samples showed an increase in CFU/g in the post-flight samples, as compared to the control sample, can be explained by culturability of microorganisms in the pre- and post-flight periods, when the samples were exposed to positive temperatures. The microbial viability after the flight is certain. Analysis of the protists cysts viability behavior revealed generally low resistance of ancient permafrost ciliates to the flight conditions, although cysts of *Colpoda steinii* were significantly more flight-resistant, than those of the *Exocolpoda augustini*. At the same time, acanthamoeba *Acanthamoeba* sp. has demonstrated resistance to the flight conditions and can be considered a promising model-organism for both terrestrial and extraterrestrial experiments in the future.

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