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THE PLANETARY QUARANTINE PROBLEM AND ITS SUBSTANTIATION ON THE BASIS OF THE RESULTS OF EXOBIOLOGICAL EXPERIMENTS

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Abstract

The RF SRC – Institute of Biomedical Problems, Russian Academy of Sciences, has developed Biorisk hardware to study the effects of long-term exposure of dormant forms of various organisms to outer space and completed experiments on the Russian Module of the International Space Station. The first experiment was performed using spores of bacteria (Bacillus) and fungi (Penicillium, Aspergillus and Cladosporium) housed in 3 boxes that were exposed to outer space for 7, 12 or 18 months. It was for the first time demonstrated that bacterial and fungal spores could survive an exposure to outer space during the time period comparable with the duration of a return mission to Mars. Moreover, the microbial strains proved viable and highly active. The second experiment was expanded by flying, in addition to the above spores, dormant forms of higher plants, insects, lower crustaceans and vertebrates. The 31-month experiment showed that, in spite of harsher than in the first study temperatures, some specimens remained viable and capable of further multiplication. In summary, our experiments provided evidence that not only bacterial and fungal spores but also dormant forms of organisms that reached higher levels of evolutionary development had the capability to survive a long-term exposure to outer space. This observation suggests that they can be transferred on outer walls of space platforms during interplanetary missions. Our findings are of great scientific interest as well as of huge importance for the development of planetary quarantine concepts related to future space exploration missions.

Key words: *planetary quarantine, microorganisms, dormant forms of various organisms, outer space*

INTRODUCTION

In accordance with article IX of the Treaty on Principles Governing the Activities of States in the Exploration and Use of Outer Space, Including the Moon and Other Celestial Bodies of the Outer Space Treaty (London/Washington, January 27, 1967), extraterrestrial missions will require to protect the Earth and other planets from biological contamination. In view of this, the Committee on Space Research (COSPAR) has established the policy of planetary protection for all the participants of interplanetary projects

The main purpose of the measures is protection of planets from terrestrial microbiological contamination and protection of Earth from extraterrestrial samples and/or return probes (SC systems, sub-systems, etc.).

Risk of contamination of Mars and other planets with terrestrial microorganisms delivered by spacecrafts is the one of the most discussed problems of exobiology and planetary protection. As a result of these discussions it has become apparent, that developing of joint position on the given issue is impossible without conducting of detailed research focused on survival rate of microorganisms from Earth on materials, used in space techniques in space environment.

One can assume, that on the external side of both unmanned and manned space crafts there can be millions of microbial cells, many of which form spores and have an extremely high stability towards space flight factors. And low temperatures lead only to prolongation of survival periods of these microorganisms. Stability of these microorganisms towards high temperatures, high doses of ionizing radiation and ultra-violet radiation is also known (Yayanos, 1998).

It is very important, that many organisms, ranging from bacteria to vertebrates, have the capability to remain dormant which allows them to survive the environmental effects they will be unable to endure in other, non-dormant forms (Alekseev & Starobogatov, 1996). Since organisms that have often reached very different stages of evolutionary development display similar resting forms, it can be postulated that they have common molecular and genetic mechanisms (Alekseev et al, 2007). It won't be an exaggeration to postulate that essentially all living beings have the ability to slow down their metabolism in response to specific environmental challenges. It is very likely that dormancy mechanisms are associated with specific signals and/or factors of innate nature (biological clocks). Due to this, it can be assumed that they can be controlled in both individual organisms and in entire ecosystems. From the perspective of fundamental space biology and biomedical support

of manned interplanetary missions, it is crucially important to explore the possibilities of controlling adaptation processes in living beings belonging to various taxonomic groups, ranging from bacteria to mammals and higher plants. In view of this, several areas of research into responses of living systems to the effects of the space environment can be identified. Of key importance is the area related to planetary quarantine. It is understood that stable life forms can cause unsanctioned spread of terrestrial organisms to other planetary bodies as well as Earth contamination with extraterrestrial beings. It is known that organisms can survive adverse environmental effects in the state of diapause or cryptobiosis, which can be reached via different mechanisms of going into dormancy and which are responsible for different population survival rates. The range of resistance of living beings in diapause or cryptobiosis to the adverse chemical and physical effects is very wide. Such a great level of resistance of living systems to the harsh environment detected in unicellular and, moreover, in multi-cellular organisms points to a high probability of their transfer across outer space (Horneck, 1993).

Hardware and a research program have been developed at the Institute for Biomedical Problems with the goal of carrying out a space experiment “Biorisk”. The first stage of this experiment provided a unique opportunity to study the most interesting species of bacteria and fungi, exposed to the open space for a long period of time and to evaluate adaptation and variability limits of these organisms.

The purpose of the present "Biorisk" experiment is to obtain new data on possible limits of phenotypic adaptation and genotypic changes in bacterial and fungal associations, shaping a standard microbiota of construction materials, used in space techniques. Task of the experiment – is definition of basic capability of preservation of life capacity of test-cultures of microorganisms during the long (comparable with time of flight from Earth to Mars) modelling in space the "microorganisms - construction materials" systems.

Since the first stage of this experiment addressed the survival of dormant forms of bacteria and fungi only, it was important to investigate the viability of a larger number of various organisms, i.e., bacteria, fungi, animals and plants, and to concentrate on the deleterious effects of the space environment upon living systems at different stages of evolutionary development. That was the objective of the second Biorisk experiment performed on the on the Russian Module of the International Space Station (RM ISS).

MATERIALS AND METHODS

To study the capability of dormant forms of various biological specimens to survive an exposure to the space environment, the Russian Federation State Research Center – Institute of Biomedical Problems of the Russian Academy of Sciences (IMBP) developed a Biorisk-MSO (MSO stands for Microbe-Substrate-Outside) container that can be flown on the outer wall of the RM ISS. The Biorisk container (Fig. 1) consists of 3 units, each containing 24 Petri dishes of 65 mm in diameter with systems "microorganisms – structural materials". On the end of each container there is the hole for air access to dishes. During transportation the hole is closed hermetically and during exposition in outer space it is opened. Every plastic dish is equipped with a filter permeable to air and impermeable to microbes. In addition, each Petri dish was equipped with a thermoluminescent dosimeter to measure total radiation dose accumulated throughout the exposure. The Biorisk-MSO container was placed on a support platform and attached to the outer wall of the Pirs docking node (Fig. 1). Prior to the placement, lids of each unit were opened. According to the experimental protocol, approximately every 6 months a crewmember removed one box, brought it into the ISS and then delivered to Earth for postflight analysis, which included bacterial and fungal count and identification, as well as a detailed study of biological, phenotypical and genotypical characteristics.

Samples of materials were contaminated with spores of bacteria – Bacillus (4 strains) and fungi - Aspergillus, Penicillium, Cladosporium (3 strains) known to be common residents of various environments on Earth and resistant to multiple alternation of high and low temperatures. Materials used in the construction of external spacecraft surfaces such as steel, aluminium alloy and heat-insulating coating were chosen as test samples for the experiment. The surface of each material sample was 16 cm^2.

In January 2005 three containers with materials and test microorganisms were placed on the external side of the Russian segment of the ISS. In one container was returned to Earth through 7, 12 and 18 months exposure.

The second Biorisk experiment was performed on dormant forms of taxonomically different specimens ranging from bacteria to vertebrates and higher plants listed in Table 1.

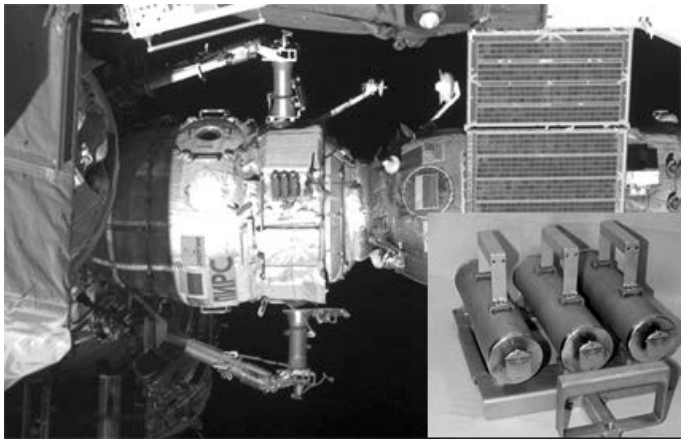


Fig. 1. Biorisk-MSO units and their location on the outer wall of the Pirs docking node

Table 1. Test-Organisms Used in the Second Biorisk-MSO Experiment

Taxonomic group	State	Common name	Species	Strain, line, variety
HIGHER PLANTS	Air-dry seeds	Mustard	*Brassica rapa*	7 lines
		-	*Arabidopsis thaliana*	2 lines
		-	*Nicandra physaloides*	1 line
		Tomato	*Lycopersicum esculentium*	1 line
		Radish	*Raphanus sativus*	1 lines
		Barley	*Hordeum vulgare*	2 lines
		Rice	*Oryza sativa*	2 lines
VERTEBRATES	Dry eggs	Fish	*Nothobranchius guenteri*	-
INSECTS	Cryptobiotic larvae	Chironamid	*Polypedilum vanderplankii*	-
LOWER CRUSTACEANS	Dormant eggs	Daphnia	*Daphnia magna*	-
		Daphnia	*Daphnia pullicaria*	-
		Artemia	*Artemia salina*	-
		Triops	*Triops cancriformis*	-
			Chirocephalus sp.	-
			Streptocephalus torvicornis	-
		Ostracod	*Eucypris ornate*	-
FUNGI	Spores	Aspergillus	*A. sedowii, A. versicolor*	# 9-6, 4-3-4
		Penicillium	*P. aurantiogresium, P. expansum*	# 9-9, 4-3-3
BACTERIA	Spores	Bacillus	*B.pumilus, B.subtilis B. licheniformis*	# 25, 20, 2335/105, 24

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On February 16, 2007, IMBP loaded test-organisms into three Biorisk-MSO-2 containers, with the test-organisms and their quantities being identical in each of them. Bacterial and fungal spores were placed onto metal plates made of construction materials similar to those in the exterior wall of the ISS RM. Plant seeds, dormant eggs of lower crustaceans, mosquito larvae and fish dry eggs were kept in cotton bags housed in plastic Petri dishes (Fig. 2).

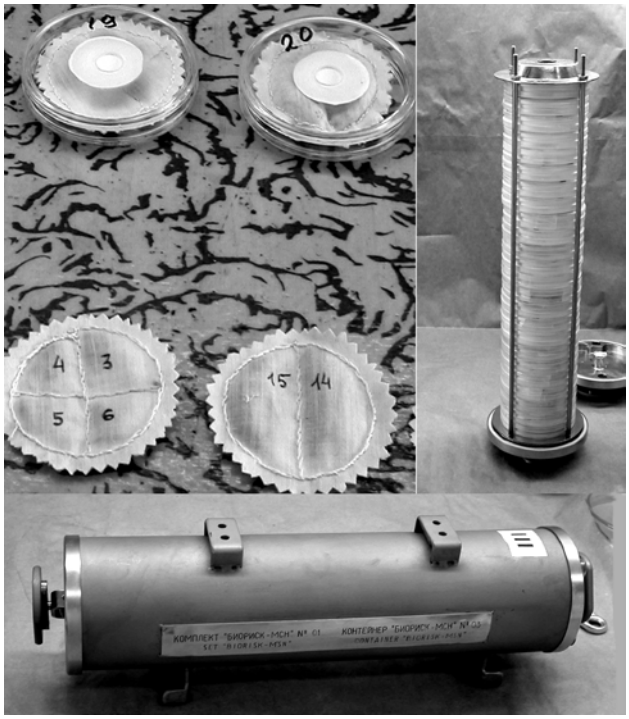


Fig. 2. Petri dishes and cotton bags containing test-organisms and Biorisk-MSO container

The loaded containers were transferred to RKK Energia and delivered to the ISS onboard Soyuz-TMA-10 on April 15, 2007. On June 6, 2007, the Russian cosmonauts Fyodor Yurchikhin and Oleg Kotov performed EVA, which included Biorisk-MSO hardware installation. Due to changes in overall EVA schedules, the first container was removed 13 months later (rather than 6 months, as expected). The manipulation was performed by the Expedition-17 crewmembers Sergei Volkov and Oleg Kononenko on July 15, 2008. The container was returned to Earth on October 24, 2008. The second container was removed by the Russian cosmonaut Yuri Lonchakov on December 23, 2008, i.e., 18 months after its installation and returned to Earth on April 9, 2009. The third container was removed on Febriary 14, 2010, i.e., 31 months after its installation and returned to Earth on April 16, 2010.

The first Biorisk container was delivered to IMBP at 5:00 PM on October 24, 2008. Immediately after that Petri dishes were removed from the metal container and examined visually. They looked deformed. When the dishes were opened, it was found that the metal plates that supported bacterial and fungal spores melted and penetrated the plastic whereas cotton bags seemed intact. The second Biorisk container was received by IMBP on April 9, 2009. Visual examination of Petri dishes after their removal from the metal container did not show any deformities, thus suggesting that the container was exposed to lower temperatures than the first one. Third Biorisk container was received by IMBP on April 16, 2010. Visual examination of Petri dishes after their removal from the metal container did not show any deformities. Petri dishes with the flown test-organisms were handed over to participating laboratories for further study.

RESULTS AND DISCUSSION

Microorganisms

Examination of microorganism – construction material systems from the three Biorisk-MSO containers demonstrated that bacteria and fungi remained viable after an 18-month exposure to outer space (Table 2).

Table 2. Survival Rate of Bacterial and Fungal Spores after 7, 13 and 18- Exposure to Outer Space (M + m, n = 6)

Species	Control (before exposure) CFU/ 16 cm^2 M±m, n=6		After 7-month exposure CFU/ 16 cm^2 M±m, n=6		After 12-month exposure CFU/ 16 cm^2 M±m, n=6		After 18 month exposure CFU/16$см^2$ M±m, n=6	
	*abs	** %	*abs	** %	*abs	** %	*abs	** %
B.pumilus-25	$5,6±0,8x10^5$	100	$5,0±2,6x10^3$	0,89	$2,6±0,1x10^3$	0,46	$4,1±1,2x10^2$	0,07
B.licheniformis-24	$5,0±0,1x10^5$	100	$5,6±1,1x10^4$	11,2	$2,5±0,7x10^4$	5	$2,7±0,6x10^3$	0,54
B.subtilis-2335/105	$4,4±0,5x10^5$	100	$1,4±0,5x10^4$	3,18	$8,5±3,1x10^3$	1,93	$3,1±1,2x10^3$	0,71
B.licheniformis-ВКМ-1711Д	$5,4±0,1x10^5$	100	$2,9±0,8x10^4$	5,37	$2,3±0,7x10^4$	4,26	$5,7±0,9x10^3$	1,05
Aspergillus versicolor	$2,6±0,01x10^4$	100	$2,0±0,8x10^2$	0,77	$8,7±2,6x10^1$	0,22	extinct	0
Penicillium expansum	$4,0±0,01x10^4$	100	$9,3±4,3x10^1$	0,23	$4,5±2,1x10^1$	0,11	$1,6±0,6x10^1$	0,04
Cladosporium cladosporioides	$4,9±0,01x10^4$	100	$1,5±0,9x10^1$	0,03	$1,0±0,6x10^1$	0,02	extinct	0

Note: abs - shows the total number of viable bacterial and fungal spores

% - shows the number of viable bacterial and fungal spores as a percentage of the pre-exposure values.

n – number of Petri dishes in experiments

It can be seen that the number of spores that survived the exposure decreased significantly, particularly after the initial exposure (Table 2). It should also be mentioned that bacteria, which are prokaryotic, i.e. more ancient, organisms displayed higher resistance to the harsh environmental effects than eukaryotic, i.e. younger from the evolutionary point of view, fungi. The number of surviving microbes on polymers was greater than on metals, indicating the potential effects of the structure and chemical composition of the construction materials.

Another important parameter characterizing adaptive capabilities of microorganisms is their susceptibility to antimicrobial substances. Every B. subtilis strain isolated from the Biorisk unit developed greater resistance to ampycilline and carbenycilline and a slightly lower resistance to ristomycine. Although the factor(s) responsible remains unclear, potential risks associated with reduced antibiotic susceptibility of pathogenic strains should not be ignored.

It is important to note that after exposure most bacterial strains showed greater biological and biochemical activity as evidenced by increased DNase and RNase. They also displayed changes in the stability of species-related biochemical parameters. For instance, 10 out of 30 biochemical parameters examined in Bacillus subtilis showed changes associated with their loss or gain of the ability for utilizing and decomposing sugars and other substrates.

Results of the second experiment confirmed the results of the first experiment. All bacteria and one fungus (Penicillium expansum) survived after 18 months of exposure in space environment. Only one bacterium (Bacillus subtilis) survived after 31 months.

After exposing in conditions of outer space a change of microorganism's characteristics took place. The bacteria Bacillus subtilis cell fission was observed, particularly, the appearance of multiple partitions (fig.3). This is an indirect sign showing that after being in outer space the malfunctioning of cell fission genetic group occurs.

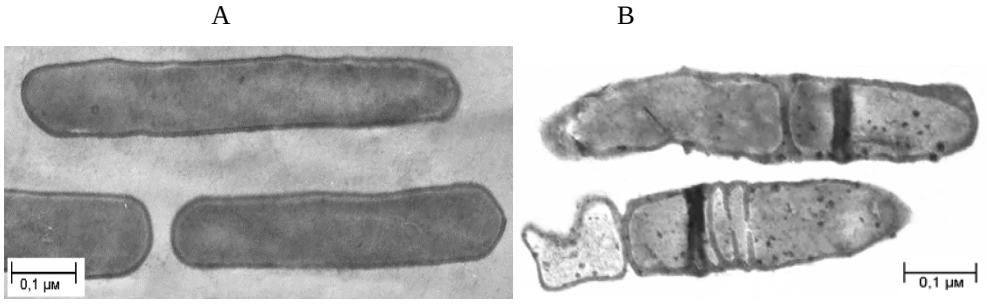


Fig. 3. Cells of Bacillus subtilis (A – before flight, B – after flight)

Higher plants seeds

Preliminary analysis of seeds of higher plants showed that except for tomato seeds they all survived. Rice and barley seeds from Container # 1 were germinated and later grown in the laboratory and in the field of the Agrobiology Institute, Okayama University for 9 months (from November 2008). The germination rate of Haruno Ninjo barley seeds was 82% (41/50) and that of Akashinriki barley seeds was 98% (49/50). After germination 41 Haruno Ninjo and 49 Akashinriki plants developed normally and did not show any significant alterations in their morphology and physiology compared to the plants grown from control seeds. The germination rate of rice seeds was 44% (22/50) in the case of SAS and 44% (23/50) in the case of KAS cultivars. However, only 9 SAS seedlings proved viable and produced normal plants, whereas all others as well as all KAS seedlings died. As was the case with barley plants, the 9 rice plants that survived did not show any significant changes in their morphological and physiological characteristics compared to the plants grown from control seeds. The survived SAS seedlings were examined with the purpose of detecting polymorphic sites in the genome that could be caused by DNA damaging factors. The analysis was performed using the AFLP (amplified fragment length polymorphism) technique. The AFLP analysis with respect to 4 primer pairs did not reveal any differences in the genome structure of the seeds exposed to the space environment for 13 months and ground controls. The deleterious effects of the exposure could produce changes in DNA structure, i.e., changes in genetic information *per se*, as well as in its interpretation and, consequently, in plant proteomics. The final stage of this research project included proteomics analysis of barley plants grown from flown seeds performed using 2-D gel-electrophoresis. The analysis revealed no differences between the proteomics of flown and control plants.

The IMBP Biological Life Support Systems laboratory investigated radishes (Raphanus sativus) and mustard (Brassica rapa, RG var.) plants grown from the seeds exposed for 13, 18 and 31 months. The study included phylogenetic monitoring of the flown and control plants. Mustard plants were harvested on Day 49 and radishes plants on Day 52. It was demonstrated that the growth rate and phylogenetic stages of flown and control plants were very similar. Morphological parameters of flown and control plants from Container # 1, Container # 2 and Container # 3 did not show any significant differences. For example radishes plants on Day 52 (Table 3).

Study of photosynthetic pigments in the leaves of flown and control plants did not reveal any differences in the content of individual pigments or in their ratios. Moreover, the exposure duration did not impact the photosynthetic apparatus of the plants grown from the flown seeds.

Animal dormant forms

Dormant eggs of several invertebrate species belonging to 3 crustacean orders, cryptobiotic larvae of chironamid, and dry eggs of Nothobranchius guenteri (Table 1) were investigated. Reactivation after diapause and the ability to grow and reproduce of lower crustacean and fish eggs were investigated by the Hydrobiology and Experimental Entomology Laboratory of the Institute of Zoology using the equipment simulating illumination and temperature ranges optimal for each species. Trehalose in dormant eggs of lower crustaceans was measured by Dr. Okuda and coworkers from the National Agrobiology Institute (Japan).

Dormant forms of lower crustaceans from every order survived long-term exposure to outer space (Table 4). However, representatives of some species failed to survive, which can be ascribed to the following:

- Water content in resting eggs. After 13-month exposure no hydrated embryos were capable to reactivate whereas 14 to 42% of dehydrated embryos showed the capability.

Table 3. Morphometric Characteristics of 52-Day Old Radishes Plants Exposed to Outer Space for 13 Months (Container # 1), 18 Months (Container # 2) and 31 Months (Container # 3)

(S+m, n = 12)

Container	Plant height (cm)	Number of leaves	1 plant mass, (g)	Wet/dry mass of the root (g)	Wet/dry mass of leaves (g)	Root length/ width (cm)
#1	19,0	8	10,59	4,42 0,18	6,17 0,75	2,1 2,1
#2	17,6	7-8	12,76	6,75 0,28	6,01 1,02	2,3 2,5
#3	17,1	8	12,58	6,87 0,24	5,71 1, 02 0,007	2,6 2,3
Ground control	17,5	7	9,00	4,37 0,18	4,63 0,70	2,1 2,1

Note: ± =

- Diapause level. Daphnia magna were in deep winter diapause due to a high population density, short light day (12 hours), and inadequate nutrition. In contrast, Daphnia pulicaria were in light summer diapause induced by inadequate nutrition during a long light day (16 hours), with their population density being low. Reactivation was seen only in embryos in deep (winter) diapause whereas none of the embryos in light diapause showed any signs of life (Table 4).
- Trehalose content. Trehalose was measured in three crustacean species, including two species of different size, which affects their survival rate (Table 4). Trehalose content in various organisms proved to be related to their survival. From our perspective, the results obtained from specimens belonging to the same species are more representative (Table 4). It appears that high trehalose in Daphnia magna and Streptocephalus torvicornis led to a greater survival rate (reactivation level).

Cryptobiotic larvae of African chironamid resumed metabolic activity within 50-70 min after they were placed in water. Although some larvae were deformed, most of them (>80%) remained viable. However, few larvae showed capability for further metamorphosis. Moreover, none of them displayed signs of multiplication, at least at the time when this paper was prepared. Many larvae displayed signs of heat burns, which were most likely the greatest stress factor during this experiment.

The second Biorisk experiment was performed using Nothobranchius guenteri eggs resistant to extended dehydration. The eggs were obtained in aquariums used to mimic the environment in which fish reproduce in African savanna. Neither flown nor ground eggs survived 13-month exposure, which could be explained by an inadequate diapause or by a longer than usual diapause that exceeded fish adaptive potentials.

DISCUSSION

Our analysis of the environmental parameters, to which Biorisk containers were exposed, shows that during the second experiment they were subjected to higher temperatures than during the first one. Since they were not equipped with a temperature recorder, we could rely on indirect evidence suggesting that plastic dishes were exposed to 95 ^{0}C: this is the temperature at which they were deformed in the ground control study. Heat transfer in vacuum is realized via contact of materials of different conductivity. In our case it can be assumed that cotton

bags proved to provide good thermal insulation, due to which the test-specimens they contained were not subjected to exceedingly high temperatures. On the ground cryptobiotic larvae were able to tolerate 90-100 ^{0}C for a few hours only. The fact that in outer space they were remained viable for a longer time could be attributed to the heat insulation effect of space vacuum combined with lack of oxygen as a source of oxidase stress. In other words, larval tissues and cells were not destroyed by free radicals, though the trehalose crystalline structure could be damaged by high temperatures.

Table 4. Reactivation of Dormant Forms of Animals After 13-Month Exposure to Outer Space

Size	Species	State	Trehalose, %	Reactivation, %, compared to controls
Large	*Daphnia magna*	Winter diapause	19.9	11
Small			25.0	35
Mixture	*Daphnia pulicaria*	Summer diapause	-	-
Large	*Streptocephalus torvicornis*	Dehydrated	25.1	42
Small			8.5	14
Mixture	*Chirocephalus sp.*	Hydrated	-	-
Mixture	*Eucycpris ornata*	Diapause	17.4	7
Mixture	*Artemia salina*	Diapause	15.0	60
Mixture	*Triops cancriformis*	Diapause	N/A	14
Mixture	*Polypedium vanderplankii*	Diapause	20	80
Mixture	*Nothobranchius guenteri*	Diapause	-	0

Unlike other test-organisms, bacterial and fungal spores were probably exposed to the temperatures to which Petri dishes were subjected because they were placed on metal plates contacting the plastic dishes. Due to this, the survival rate of bacterial spores was significantly low (for Bacillus subtilis the rate amounted to 0.002% - 0.02% of the baseline). In Container # 1, all fungal spores exposed to higher temperatures perished and in Container # 2 only Penicillium expansum spores survived. This was in contrast with the results of the first experiment. We obtained data suggesting that Bacillus bacteria that survived their exposure to the space environment developed higher enzymatic activity. This can be interpreted as enhancement of adaptive mechanisms responsible for their viability.

The radiation dose to which test-organisms were exposed was recorded by thermoluminescent detectors. It was about 30 cGy.

CONCLUSION

In summary, the Biorisk experiments allow the following conclusions to be made:

1. A long-term experiments (one of them - to 31 months) in which microorganism – construction material systems were exposed to the space environment (on the outside wall of the ISS) demonstrated that bacterial and fungal spores could survive the harsh effects of outer space.

2. After exposure to the outer space environment seeds of higher plants remained viable. In spite of high temperatures and other adverse effects, the seeds germinated and produced plants that did not differ from ground controls in their genetic, biochemical, physiological and morphological characteristics.

3. Dehydrated embryos of lower crustaceans in the state of deep diapause proved capable to survive the exposure.

4. The survival rate of dormant forms of animals was correlated with trehalose concentrations. For instance, increased trehalose in Daphnia magna and Streptocephalus torvicornis of certain sizes led to their higher survival rate (reactivation level) after exposure.

Thus, the fact of long-term survival of terrestrial microorganisms in the outer space environment have been established for the first time, which suggests the existing possibility of interplanetary transfer of microorganisms on external spacecraft surfaces.

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