DEVELOPMENT OF MICROBIAL PREPARATION FOR BIOREMEDIATION OF SOILS CONTAMINATED WITH ROCKET FUEL COMPONENTS

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Heptyl rocket fuel and aviation kerosene are widely used in the propulsion systems of the Proton and Soyuz spacecraft. The propellant components (RFC) enter the environment, causing strong toxic effects, when the separating first stages of rockets fall away or in case of emergencies. The study was aimed to isolate strains of microorganisms-destructors of RFC, as well as to assess their safety for bioremediation of contaminated soils. Microorganisms capable of decomposing heptyl, formalin, and aviation kerosene were isolated from natural soils. An association of two strains of bacterial destructors *Pseudomonas putida* 5G and *Rhodococcus erythropolis* 62M/3 was obtained, and a method of their use in recultivation of soil contaminated with RFC was developed. The results of laboratory and field tests showed high efficiency of the microbial destruction of pollutants, the decrease in integral toxicity and phytotoxicity of the cleaned soil to safe levels, and an increase in the soil biological activity. Thus, dehydrogenase activity increased by 2.4 times, hydrolase activity by 2.1 times, and cellulase activity by 5.1 times. Microbial association can be recommended for recultivation of soil contaminated with RFC.

Keywords: rocket fuel, heptyl, dimethylhydrazine, formaldehyde, aviation kerosene, rocket fuel components, degrading microorganisms, soil bioremediation

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Author contribution: Khaitov MR — general research management; Zharikov GA — planning and management of laboratory and field research, experimental procedure, data analysis; Krainova OA — microbiological testing (isolation and selection of bacterial destructors of rocket fuel, preparation of microbial suspensions for experiments, biodestructor collection maintenance); Marchenko AI — microbiological and biochemical testing (enzyme activity of soil), biotests for assessment of soil toxicity, statistical data processing.

Compliance with ethical standards: animals were treated in accordance with the principles of Good Laboratory Practice. Veterinary protocols № 669 and № 677 for strains 5G и 62M/3 were approved by the Bioethics Commission (protocol № 165/2019 of 19 February 2019, protocol № 169/2019 of 16 April 2019).

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РАЗРАБОТКА МИКРОБИОЛОГИЧЕСКОГО ПРЕПАРАТА ДЛЯ БИОРЕМЕДИАЦИИ ПОЧВ, ЗАГРЯЗНЕННЫХ КОМПОНЕНТАМИ РАКЕТНЫХ ТОПЛИВ

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Ракетное топливо, гептил и авиационный керосин, широко используют в двигательных установках космических кораблей «Протон» и «Союз». При падении отделяющихся первых ступеней ракет и в случае аварийных ситуаций компоненты ракетных топлив (КРТ) попадают в окружающую среду, вызывая сильные токсические эффекты. Целью исследования было выделить штаммы микроорганизмов-деструкторов КРТ и изучить их безопасность для биоремедиации загрязненных почв. Из природных почв выделены микроорганизмы, способные разлагать гептил, формалин и авиационный керосин. Получена ассоциация из двух штаммов бактерий *Pseudomonas putida* 5Г и *Rhodococcus erythropolis* 62М/3, отработана методика их применения для рекультивации загрязненной КРТ почвы. Результаты лабораторных и полевых испытаний показали высокую эффективность микробной деструкции загрязнителей, снижение интегральной токсичности и фитотоксичности очищаемой почвы до безопасных уровней, повышение ее биологической активности. Так, было отмечено повышение дегидрогеназной активности в 2,4 раза, гидролазной — в 2,1 раза, целлюлазной — в 5,1 раза. Ассоциацию микроорганизмов можно рекомендовать для рекультивации почв, загрязненных КРТ.

Ключевые слова: ракетное топливо, гептил, диметилгидразин, формальдегид, авиационный керосин, компоненты ракетных топлив, микроорганизмы-деструкторы, биоремедиация почв

Финансирование: исследования проводили в рамках государственных заказов Федерального медико-биологического агентства России (№ 26.008.02.0, № 22.009.21.800).

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Соблюдение этических стандартов: работы с животными выполняли в соответствии с принципами надлежащей лабораторной практики. Ветеринарные протоколы № 669 и № 677 по штаммам 5Г и 62М/3 утверждены комиссией по биоэтике (протокол № 165/2019 от 19 февраля 2019 г., протокол № 169/2019 от 16 апреля 2019 г.).

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Heptyl (unsymmetrical dimethylhydrazine, UDMH) is used as a liquid rocket fuel to launch into orbit the Proton, Cyclon, Kosmos, Rokot, Strela spacecraft and unmanned satellites of the Earth. Extensive use of UDMH in the rocket and space technology is due to its unique operational properties. It can hardly be replaced by any other fuel in the near future [1]. Because of its physical and chemical properties (high volatility and solubility in aqueous solutions), dimethylhydrazine migrates in the natural environment, is decomposed into a number of highly toxic products, and persists for a long time in soil. UDMH is assigned hazard class 1, it shows carcinogenic, mutagenic, embryotoxic (yellow children), and teratogenic (abnormal development of an embryo) effects, and causes cancer in people engaged in working with UDMH of living in contaminated areas [1, 2]. Aviation kerosene, that is used in the manned spacecraft of the Soyuz type, also shows high toxicity [1].

To date, no effective, environment-friendly and cheap techniques for cleaning of soils contaminated with heptyl and aviation kerosene have been developed. All the existing techniques can be conditionally divided into three groups: thermal methods (burning); methods of the UDMH deep oxidation that involve the use of aqueous solutions containing active substances capable of interacting with UDMH – these substances form insoluble or poorly soluble complexes in one case, and promote decomposition into simpler compounds in another. Chemical agents (hydrogen peroxide, potassium permanganate solutions, quicklime) are mainly used, which is expensive, environmentally harmful and results in the reduced fertility of recultivated soil. Methods of the other type involve the use of aqueous solutions containing active substances, such as meta-nitrobenzoic acid that forms a complex with UDMH at certain pH values in the form of solid phase. Then, in accordance with the proposed method, solutions contaminated with UDMH undergo thermal deactivation in the specialized furnace. Fixation of UDMH in soil with the formilations containing humic acids, turf, schungite is also used. However, this method does not provide soil decontamination to the level of PEL (0.1 mg/kg) [3].

Calcium peroxide, decomposition of which results in the release of atomic oxygen that is involved in degradation of UDMH, is used for detoxication of soil and deactivation of UDMH on the surface of metal constructions, walls of shelters, etc. Poor decontamination of contaminated areas and long process of detoxication are the disadvantages of this method [3].

Biological techniques for recultivation of contaminated areas are the most preferable due to environmental safety, low cost and relatively high efficiency, which were demonstrated repeatedly when coping with various ecological issues. There are numerous known environmentally friendly biological preparations containing aerobic bacterial strains that are targeted on biochemical destruction and utilization of pollutants, mostly hydrocarbons (pertoleum and its industial derivatives) [4–7]. Biological methods based on the controlled bio-composting are known. Furthermore, biological preparations have been created that contain microoorganisms, for which hazardous waste is a source of nuitrition. This method of soil detoxication does not involve the use of toxic chemicals; microorganisms-destructors die after destruction of UDMH due to the lack of nuitrition, and the soil treated retains biological activity and fertility [8].

Microorganisms are increasingly being used for decontamination of soil contaminated with RFC and water in Russia and abroad. Thus, studies are conducted at the Baikonur Cosmodrome (Kazakhstan) that are focused on using indigenous soil microorganisms for degradation of dimethylhydrazine. Microorganisms are isolated from soil and grown in the fermentation devices to be introduced into the contaminated soil [9]. Currently, there are sporadic reports of the microbial species and associations of microorganisms capable of utilizing UDMH. That the method for biodestruction of heptyl has been developed that involves the use of the association of microorganisms *Acinetobacter sp*. H-1, *Rhodococcus sp*. H-2, *Arthrobacter sp*. H-3 [10]. The method has been proposed for biological decontamination of water and soil contaminated with petroleum and oil products using the Centrum-MMS ecobiopreparation [11], also capable of the UDMH biodestruction in the aqueous solutions. Ecobiopreparation contains microorganisms *Pseudomonas fluorescens* BKM B-6847 and *Rhodococcus erythropolis* AC-1769. However, according to the authors of this invention, Centrum-MMS ecobiopreparation is not capable of cleaning up soil contaminated with heptyl [11].

The development of effective techniques for bioremediation of soils contaminated with highly toxic RFC and their introduction into practice are extremely relevant. Currently, there are no ready-to-use microbial preparations and highly efficient industrial bacterial strains for bioremediation of soil contaminated with UDMH and aviation kerosene, which provides grounds for the study.

The study was aimed to isolate strains of microorganisms-destructors of RFC, as well as to assess their toxicological and environmental safety together with the possibility of application for bioremediation of contaminated soils.

METHODS

The culture collection of microorganisms, that degrade various toxic chemicals (petroleum products, polycyclic aromatic hydrocarbons, mineral oils, phenols, polychlorinated biphenyls, ethylene glycol, heptyl, aviation kerosene, pesticides, mustard gas, lewisite, organochlorines, and organophosphate compounds), was created at the Research Center for Toxicology and Hygienic Regulation of Biopreparations after many years of expeditionary works on the contaminated soil sampling and further laboratory testing. Soil samples collected from the territories contaminated with pesticides and petroleum products for a long time, as well as from the areas of heptyl spill emergencies and the site of the Proton-M launch vehicle crash (Site 81 at the Baikonur Cosmodrome, Kazakhstan), were used to isolate microbial strains capable of degrading heptyl and aviation kerosene.

The method of enrichment culture with subsequent inoculation of minimal medium, containing formaldehyde (primary product of heptyl degradation) or primary degradation product of heptyl as the only source of carbon, was used to isolate microorganisms-destructors of RFC [12].

Pure cultures of isolated microorganisms were identified by MALDI in the All-Russian Collection of Microorganisms (IBPM RAS, Pushchino; Russia).

The biomass of microorganisms-destructors of RFC for the laboratory and field tests was built up in the Certomats-BS1 incubation shaker (Sartorius; Sweden) at a temperature of 28 °C and speed of 180 rpm until the culture entered stationary phase (24–48 h depending on the strain).

Strain 19F of *Rhodococcus globerulus*, the UDMH (heptyl) biodestructor [13], was used as a reference strain.

The sod-podzol soil was used for both laboratory and field experiments.

Bacterial suspension was treated with polyurea microcapsules (BNT LLC; Russia).

Integral (overall) toxicity of water and soil samples was assessed in the laboratory culture of *Daphnia magna* grown

Table 1. Changes in microbial cell counts of microorganisms-destructors and saprophytic microflora during the microbial remediation of soil performed in laboratory environment, CFU/g of soil

Variant	Test duration, days				
	0	7	14	21	30
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	$(3.2 \pm 0.62) \times 10^4$ $< 10^3$ $(1.2 \pm 0.17) \times 10^5$	$(1.1 \pm 0.34) \times 10^5$ $< 10^3$ $(2.7 \pm 0.31) \times 10^5$	$(1.5 \pm 0.21) \times 10^4$ $< 10^3$ $(3.1 \pm 0.24) \times 10^5$	$(1.0 \pm 0.4) \times 10^4$ $< 10^3$ $(6.5 \pm 0.24) \times 10^5$	$(1.0 \pm 0.24) \times 10^4$ $< 10^3$ $(6.5 \pm 0.24) \times 10^5$
Soil + formalin + aviation kerosene + microencapsulated microorganisms	$(3.3 \pm 0.54) \times 10^4$ $< 10^3$ $(1.0 \pm 0.12) \times 10^5$	$(1.8 \pm 0.41) \times 10^5$ $< 10^3$ $(1.6 \pm 0.45) \times 10^5$	$(1.7 \pm 0.38) \times 10^4$ $< 10^3$ $(4.5 \pm 0.34) \times 10^5$	$(1.2 \pm 0.24) \times 10^4$ $< 10^3$ $(7.2 \pm 0.54) \times 10^5$	$(1.2 \pm 0.24) \times 10^4$ $< 10^3$ $(7.2 \pm 0.48) \times 10^5$
Soil + formalin + aviation kerosene + strain 19F (reference strain)	$< 10^3$ – $(1.6 \pm 0.48) \times 10^5$	$(1.5 \pm 0.44) \times 10^4$ – $(4.2 \pm 0.61) \times 10^5$	$(1.2 \pm 0.41) \times 10^5$ – $(1.4 \pm 0.37) \times 10^5$	$(2.4 \pm 0.45) \times 10^4$ – $(6.5 \pm 0.59) \times 10^5$	$(1.1 \pm 0.44) \times 10^4$ – $(8.5 \pm 0.24) \times 10^5$
Soil + formalin + aviation kerosene (control)	– – $(1.5 \pm 0.24) \times 10^5$	– – $(4.5 \pm 0.54) \times 10^5$	– – $(3.8 \pm 0.24) \cdot 10^5$	– – $(2.5 \pm 0.45) \times 10^6$	– – $(6.5 \pm 0.44) \times 10^6$
Clean soil (control)	– – $(2.7 \pm 1.2) \times 10^6$	– – $(3.2 \pm 0.26) \times 10^6$	– – $(4.3 \pm 0.18) \times 10^6$	– – $(5.4 \pm 0.24) \times 10^6$	– – $(3.4 \pm 0.24) \times 10^6$

Note: the columns show indicators for strain 5G, strain 62M/3, soil saprophytes.

in the climatostat at the Research Center for Toxicology and Hygienic Regulation of Biopreparations. Biotests were performed in accordance with the methods [14, 15].

Integral toxicity of soil was assessed by the bioluminescence method using the Ecolum bacterial test system (MSU; Russia) in the Biotox-10M test device (MSU; Russia) in accordance with the guidelines [16, 17].

The colorless 2,3,5-triphenyltetrazolium chloride was used as a substrate for assessment of the soil dehydrogenase activity. Total hydrolase activity of the soil was assessed using the fluorescein diacetate hydrolysis assay. The application method was used to assess the soil cellulose-destroying capacity [18].

Phytotoxicity of soil samples was tested using the oat seeds by the method introduced by O.A. Berestetsky [19].

The content of aviation kerosene was defined with the petroleum product analyzer, the KN-2 infrared spectrometer (Novolab; Russia) in accordance with the method [20].

Pathogenicity (safety) of microorganisms-destructors of RFC was evaluated in accordance with the guidelines of the Ministry of Health of the USSR № 2620-82, № 4263-87 taking into account the guidelines issued by the World Health Organization [21–23]. Evaluation included asessment of virulence, toxicity, toxigenicity, and dissemination in the internal organs of white mice and rats.

Statistical processing of the experimental data was performed with the Excel 7.0 (Microsoft; USA) and Statistica 10.0 (StatSoft; USA) software packages. Experimental data were represented as mean values and 95% confidence intervals.

RESULTS

Isolation of microorganisms-destructors of RFC from contaminated soil

Microorganisms-destructors of heptyl were isolated from the samples of soil contaminated with UDMH. A total of 100 bacterial isolates were obtained. Then microorganisms were subcultured in the minimal medium containing formaldehyde (concentration gradient 0–500 mg/L) and incubated for a long time (up to 10 days) to monitor bacterial growth. Strains 2G, 4G, 5G, G8/2, Y-21 were capable of growing at the formaldehyde level of 200 mg/L; the reference strain 19F and strain G-803 grew at a concentration of 100 mg/L; strains 19 S/1 и 37M/1 remained viable at a concentration of 80 mg/L.

Microorganisms-destructors of aviation kerosene were isolated from the samples of soil contaminated with petroleum products collected from airports. petroleum storage depots,

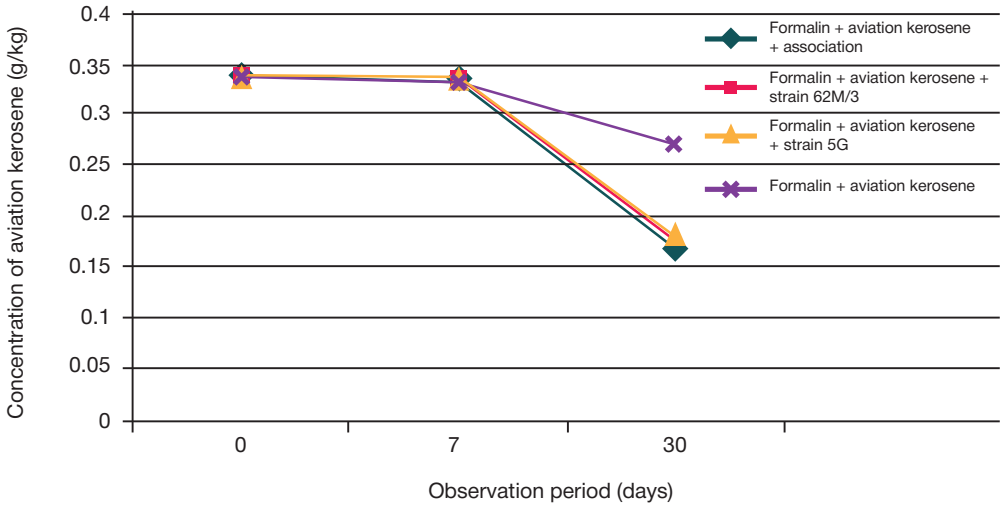

Fig. 1. The process of aviation kerosene degradation by microorganisms-destructors in laboratory environment, g/kg of soil

Table 2. Acute toxicity of the soil to Daphnia assessed during microbial degradation of formalin and aviation kerosene performed in laboratory environment

Experiment variant	Number of surviving Daphnia, *n*		Daphnia mortality rate, %	Acute toxic effects/no acute toxic effects
	control	experiment		
Soil (control)	30	30	0	No acute toxic effects
Soil + formalin + aviation kerosene	30	0	100	Acute toxic effects
After 7 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	13	57	Acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	18	40	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	8	74	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 14 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	20	33	No acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	18	40	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	8	74	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 21 day				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	18	40	No acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	15	50	Acute toxic effects
Soil + formalin + aviation kerosene (control)	30	8	74	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 30 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	30	0	No acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	29	3	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	15	50	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects

and filling stations. Six most active bacterial strains capable of growing on the minimal medium containing 5% of diesel fuel were selected among 34 isolates obtained. Three most active strains-destructors of aviation kerosene, 12R, 37M/1 and 62M/3, were selected based on the laboratory test results.

The strains *Pseudomonas putida* 5G (UDMH) and *Rhodococcus erythropolis* 62M/3 (aviation kerosene) were selected for further experiments on the creation of the RFC destructor association based on the laboratory tests of contaminated soil and assessment of the parameters of bacterial growth in the culture medium. The association of microorganisms-destructors reproduced quickly in the soil contaminated with RFC, destroying heptyl and aviation kerosene. Integral toxicity of the soil contaminated with these toxicants gradually decreased after being treated with the association of microorganisms-destructors of RFC to reach a safe level, and the soil enzyme activity increased.

Toxicity testing of the isolated strains of microorganisms-destructors of RFC in animals

Evaluation of pathogenicity of microorganisms-destructors of heptyl (*Ps. putida* 5G) and aviation kerosene (*Rh. erythropolis* 62M/3) involved asessment of virulence, toxicity, toxigenicity, and dissemination in the internal organs of the outbred white mice and rats. The irritating effect of these bacteria on the ocular mucous membrane of rabbits was also defined.

Assessment of virulence involved single intragastric and intraperitoneal administration of bacteria to white mice and rats. All experimental animals were still alive by the end of the observation period. The clinical status of animals, as well as food and water consumption were normal. It was found that in case of intragastric administration of *Ps. putida* 5G and *Rh. erythropolis* 62M/3 to rats and mice, LD_{50} exceeded 109 bacterial cells, and in case of intraperitoneal administration LD_{50} exceeded 10^8 bacterial cells.

Toxicity testing involved intragastric administration of the suspensions of the studied strains 24-hour cultures to white mice. All experimental animals were still alive by the end of the observation period. The clinical status of animals, as well as food and water consumption were normal. It was found that the tested strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 showed no toxicity in warm-blooded animals.

Assessment of toxigenicity involved intraperitoneal and intragastric administration of filtrates of the 3-day and 7-day broth cultures of the studied strains to white mice. All experimental animals

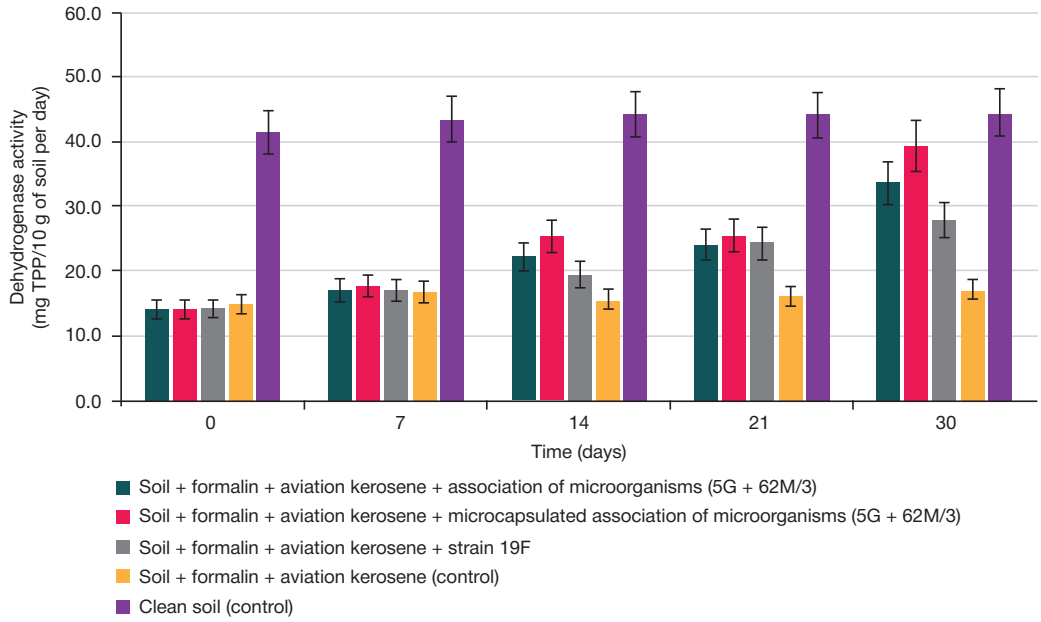

Fig. 2. Dehydrogenase activity in the soil contaminated with formalin (0.05%) and aviation kerosene (0.1%) during microbial remediation in laboratory experiments

were still alive by the end of the observation period. The clinical status of animals, as well as food and water consumption were normal, no toxic effects were observed. Thus, the tested strains of microorganisms-destructors *Ps. putida* 5G and *Rh. erythropolis* 62M/3 showed no toxigenicity in warm-blooded animals.

To assess dissemination in the internal organs, white mice and rats were infected with the isolated strains by intragastric and intraperitoneal injection. No animals died by the end of the observation period. Autopsy revealed no differences between the organs of experimental animals and controls. Normal positioning and macrostructure of organs in the thoracic and abdominal cavities were observed, no pathological changes were revealed at the macro level. No growth was observed in the bacterial cultures isolated from animal organs. Thus, based on the bacterial cultures isolated from the internal organs, the studied strains of microorganisms-destructors of RFC are not capable of dissemination and never cause bacterial contamination of organs in warm-blooded animals.

When studying the irritating effect on the ocular mucous membrane, no irritating effect was observed both 4 h after the injection of the bacterial suspension containing *Ps. putida* 5G or *Rh. erythropolis* 62M/3 into the rabbit's conjunctival sac and throughout the observation period. There were no differences between the eyes injected with bacteria and control eyes in all animals. Research has shown that bacteria *Ps. putida* 5G and *Rh. erythropolis* 62M/3 do not irritate the ocular mucous membrane of warm-blooded animals.

Thus, toxicity testing, that involved asessment of virulence, toxicity, and toxigenicity, showed that microorganisms-destructors of formaldehyde (heptyl) (*Ps. putida*, strain 5G) and aviation kerosene (*Rh. erythropolis*, strain 62M/3), were not pathogenic (harmless) to warm-blooded animals. Microorganisms are harmless and can be used for bioremediation of contaminated soils without limitation.

Defining the main parameters and conditions for cultivation of microorganisms-destructors in the fermentation device

The selected strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 belong to different genera. That is why we assessed the possibility of co-growth in the agar Petri dishes. Co-culture of the strains showed no growth suppression.

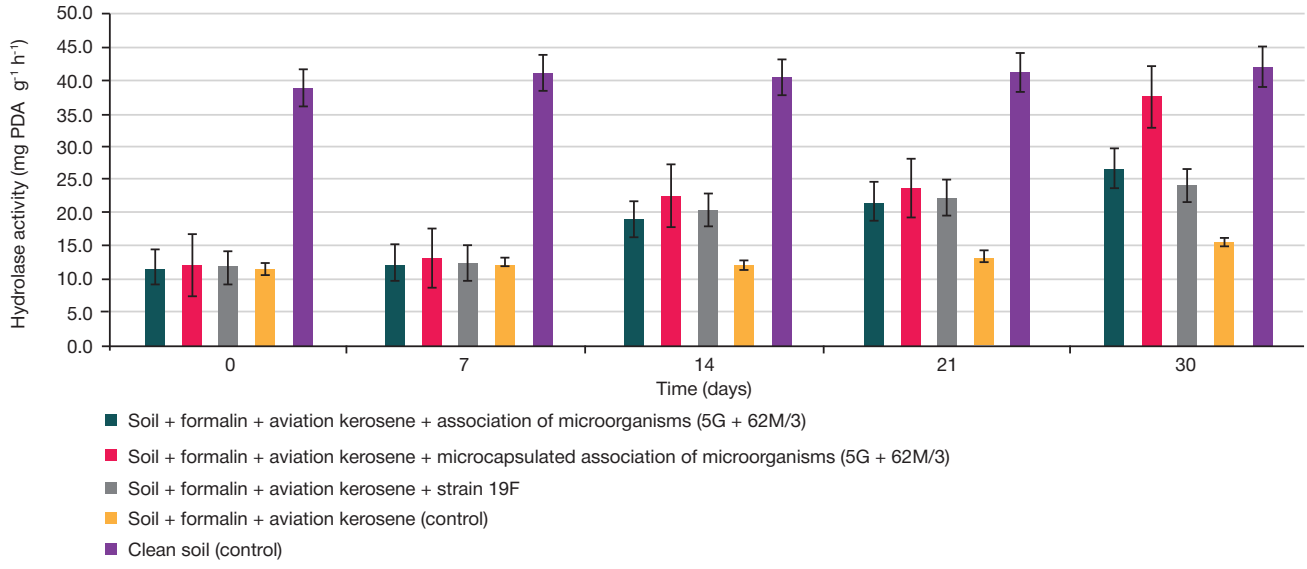

Fig. 3. Hydrolase activity in the soil contaminated with formalin and aviation kerosene during microbial remediation in laboratory experiments

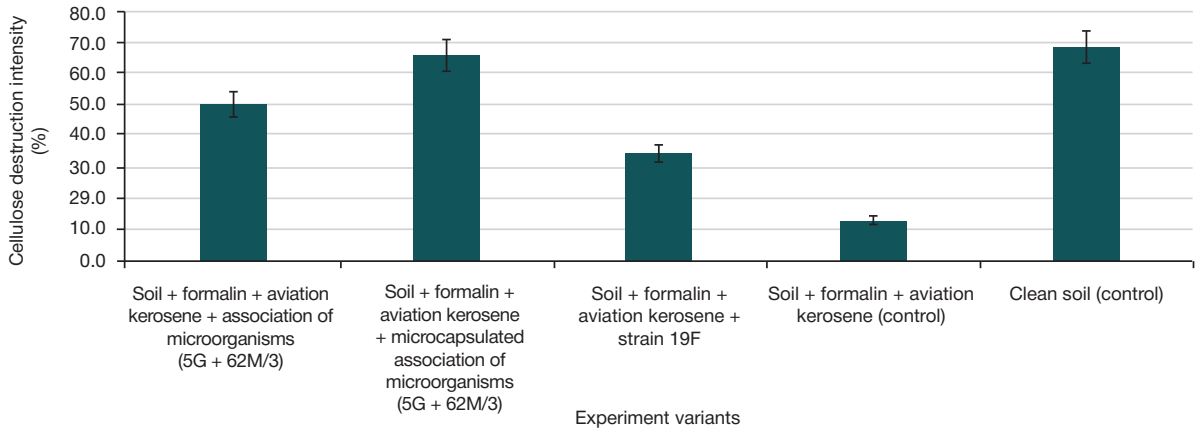

Fig. 4. Cellulase activity (%) in the soil contaminated with formalin and aviation kerosene after 30 days of microbial remediation in laboratory experiments

We defined the main parameters and conditions for cultivation of the RFC destructor strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 on various culture media. The optimum regime was cultivation at 28 °C for 24 h.

Efficiency of the 0.05% formalin and 0.1% aviation kerosene destruction in soil by the association of microorganisms-destructors of RFC in the laboratory environment

Microbial remediation of soil contaminated with RFC was performed in the laboratory settings in the 0.5 L plastic glasses. The working concentrations of pollutants in the soil were achieved by adding the following solutions: 0.05% (500 mg/кг) formalin (simulator of heptyl being the primary product of heptyl degradation) and 0.1% (1000 mg/kg) aviation kerosene.

Soil samples for chemical analysis and toxicity testing were collected before adding microorganisms-destructors, after 7, 14, 21 and 30 days (after the end of experiment).

The experimental procedure included a whole range of studies, such as determination of the aviation kerosene concentration, integral toxicity of soil to Daphnia, concentrations of microorganisms-destructors and soil microphlora, dehydrogenase, hydrolase, and cellulose-destroying activity, phytotoxicity to oat seeds.

Assessment of the soil microbial contamination showed that microbial cell counts of microorganisms-destructors of heptyl and aviation kerosene was stable throughout the period of bioremediation; these microorganisms were suppressed by saprophytic microflora (Table 1). Microbial cell counts of saprophytic soil bacteria remained almost the same throughout the experiment (Table 1).

The concentration of pollutant in the soil gradually decreased during microbial remediation (Fig. 1).

Integral toxicity of the contaminated soil before and after treatment with microorganisms-destructors was tested using Daphia bioassay. Before treatment, soil contaminated with aviation kerosene and formalin caused death in 100% of Daphnia. After 14 days of microbial remediation soil, toxicity decreased to reach a safe level (Table 2).

We defined the enzyme activity in the contaminated soil (dehydrogenase, hydrolase, and cellulase activity) before and after treatment with microorganisms-destructors. Soil contamination with aviation kerosene and formalin reduced the

Table 3. Phytotoxicity of the soil contaminated with formalin and aviation kerosene to oat seeds after the soil microbial remediation in laboratory experiments

Experiment variant	Measurement	Units	Mean value (M ± 6)	Number of seeds per iteration, *n*	Number of iterations
Soil + formalin + aviation kerosene (negative control)	Roots	mm	34.0 ± 23.9	25	3
	Sprouts	mm	46.5 ± 30.0		
	Weight (roots and sprouts)	g	3.5 ± 0.1		
	Number of ungerminated seeds	*n*	16		
Soil (control)	Roots	mm	62.0 ± 30.0	25	3
	Sprouts	mm	45.0 ± 24.6		
	Weight (roots and sprouts)	g	4.0 ± 0.4		
	Number of ungerminated seeds	*n*	7		
Soil + formalin + aviation kerosene + association 5G + 62M/3	Roots	mm	30.5 ± 22.8	25	3
	Sprouts	mm	58.0 ± 39.1		
	Weight (roots and sprouts)	g	3.3 ± 0.7		
	Number of ungerminated seeds	*n*	11		

Note: M — mean, 6 — standard deviation.

Table 4. The changes in microbial cell counts of microorganisms introduced into soil and saprophytic microbiota during the field experiment, CFU/g

Variant	Test duration, days					
	0	7	14	30	45	60
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	$(2.4 \pm 0.24) \times 10^5$ $(2.6 \pm 0.28) \times 10^5$ $(5.0 \pm 0.31) \times 10^4$	$(5.9 \pm 0.48) \times 10^6$ $(6.5 \pm 0.55) \times 10^6$ $(2.7 \pm 0.28) \times 10^5$	$(1.9 \pm 0.26) \times 10^6$ $(1.2 \pm 0.27) \times 10^6$ $(1.2 \pm 0.14) \times 10^6$	$(1.2 \pm 0.17) \times 10^6$ $(8.3 \pm 1.03) \times 10^5$ $(1.5 \pm 0.37) \times 10^6$	$(6.0 \pm 0.68) \times 10^5$ $(3.7 \pm 0.34) \times 10^5$ $(9.0 \pm 0.17) \times 10^6$	$(3.0 \pm 0.75) \times 10^5$ $(1.9 \pm 0.20) \times 10^5$ $(1.1 \pm 0.45) \times 10^6$
Soil + formalin + aviation kerosene (control)	– – $(2.3 \pm 0.41) \times 10^4$	– – $(3.2 \pm 0.24) \times 10^5$	– – $(2.1 \pm 0.21) \times 10^5$	– – $(2.0 \pm 0.31) \times 10^5$	– – $(7.6 \pm 0.27) \times 10^5$	– – $(6.54 \pm 0.48) \times 10^5$
Clean soil (control)	– – $(1.2 \pm 0.45) \times 10^6$	– – $(2.4 \pm 0.48) \times 10^6$	– – $(2.8 \pm 0.34) \times 10^6$	– – $(3.2 \pm 0.21) \times 10^7$	- - $(2.3 \pm 0.48) \times 10^6$	– – $(6.4 \pm 0.25) \times 10^6$

Note: the columns show indicators for strain 5G, strain 62M/3, soil saprophytes.

enzyme activity. After 30 days of microbial remediation enzyme activity gradually increased (Fig. 2–4).

Phytotoxicity of the soil significantly decreased during the microbial remediation. Based on such parameters, as the "sprouts length" and the "number of ungerminated seeds" it approached the level of the conditionally clean soil (Table 3).

Field testing of the microbial destruction of formalin and aviation kerosene in soil by the association of microorganisms-destructors of RFC

Microbial destruction of formalin and aviation kerosene in the soil was studied under field conditions on the experimental plots. The working concentrations of pollutants in the soil were achieved by adding 0.1% formalin and 0.1% aviation kerosene. Microbial association of the destructor strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 with a concentration of 1×10^7 CFU/mL was added at a rate of 1 L/m^2 of soil. Soil samples for further assessment were collected within 60 days. The extract obtained from soil samples was cultured in Petri dishes with the fish meal hydrolysate and formalin (100 mg/L) for strain 5G, and in Petri dishes with the minimal salt medium and 1% of diesel fuel for strain 62M/3. The concentration of saprophytic (indigenous) bacteria was assessed using the GRM medium.

The field experiment included a whole range of studies, such as determination of the aviation kerosene concentration

Table 5. Integral toxicity of soil to Daphnia in the field experiment

Experiment variant	Number of surviving Daphnia, *n*		Daphnia mortality rate, %	Acute toxic effects/no acute toxic effects
	control (water)	experiment		
Baseline				
Soil + formalin + aviation kerosene	30	10	67	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 7 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	20	33	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	14	54	Acute toxic effects
After 14 days				
Soil + formalin + aviation kerosene + microbial association (5G+ 62M/3)	30	25	17	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	16	53	Acute toxic effects
After 30 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	27	10	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	15	50	Acute toxic effects
After 45 days				
Soil + formalin + aviation kerosene + microbial association (5G+ 62M/3)	30	27	10	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	14	46	No acute toxic effects
After 60 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	30	0	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	20	33	No acute toxic effects
Soil (control)	30	30	0	No acute toxic effects

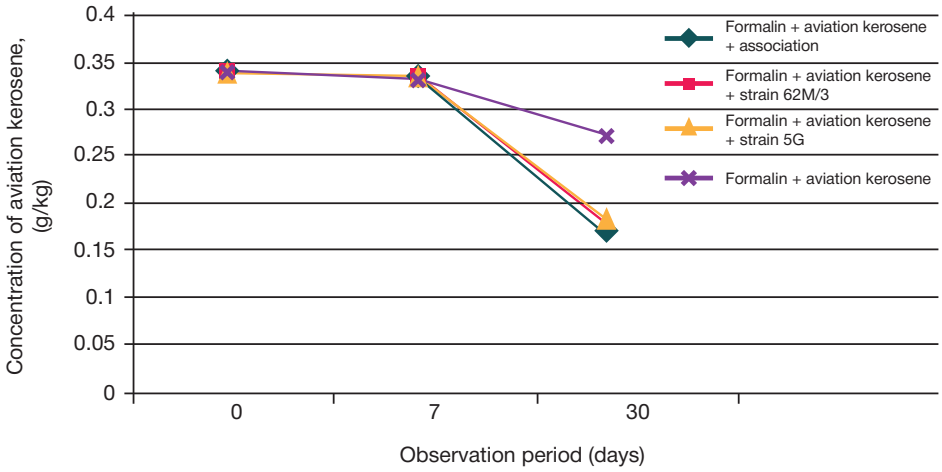

Fig. 5. Dynamic changes in the soil concentration of aviation kerosene during the field experiment, g/kg of soil

in the soil, integral toxicity of soil to Daphnia, bacteria counts for microorganisms-destructors and soil microbiota (Table 4), dehydrogenase, hydrolase, and cellulose-destroying activity, phytotoxicity.

The baseline microbial cell counts of both strains (after being added to the soil) were at the level of 10^5 CFU/g of soil, and the bacteria counts of soil saprophytes were at the level of 10^4 CFU/g of soil. The highest microbial cell counts were observed on day 7: $(5.9 \pm 0.48) \times 10^6$ CFU/g of soil for strain 5G, $(6.5 \pm 0.55) \times 10^6$ CFU/g of soil for strain 62M/3. The microbial cell counts of biodestructors slightly decreased starting from day 45 due to degradation of soil pollutants

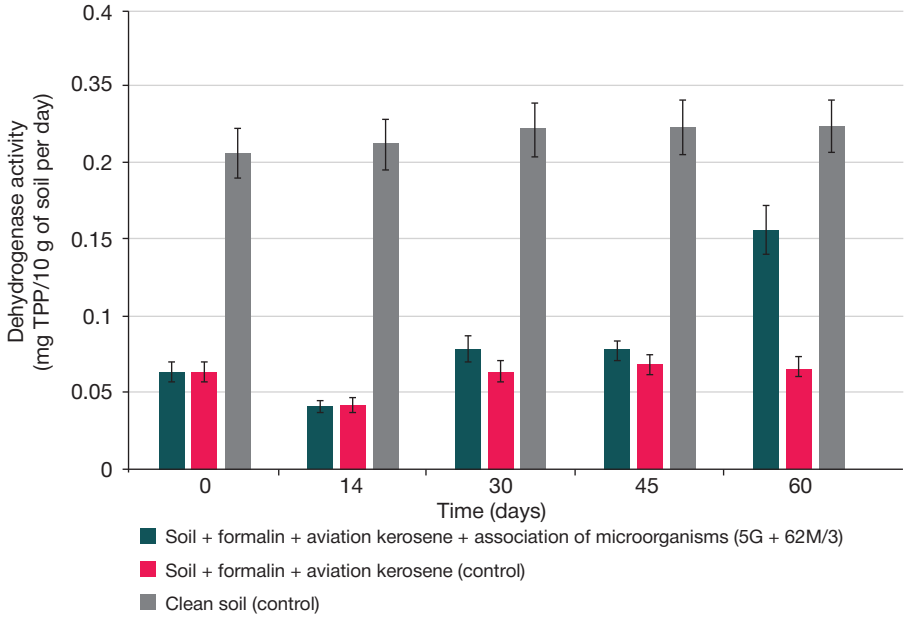

Fig. 6. Dehydrogenase activity of the soil in the field experiment

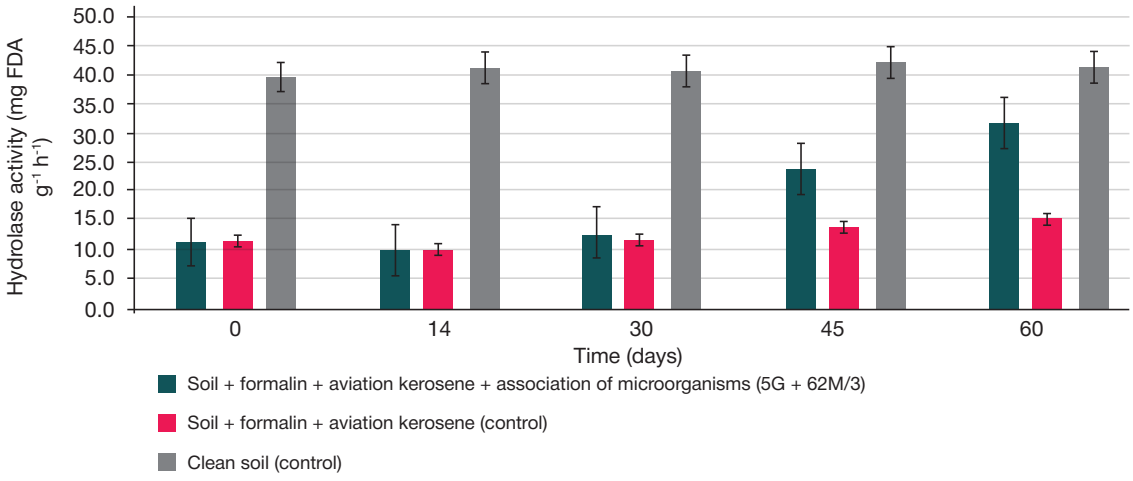

Fig. 7. Hydrolase activity of the soil in the field experiment

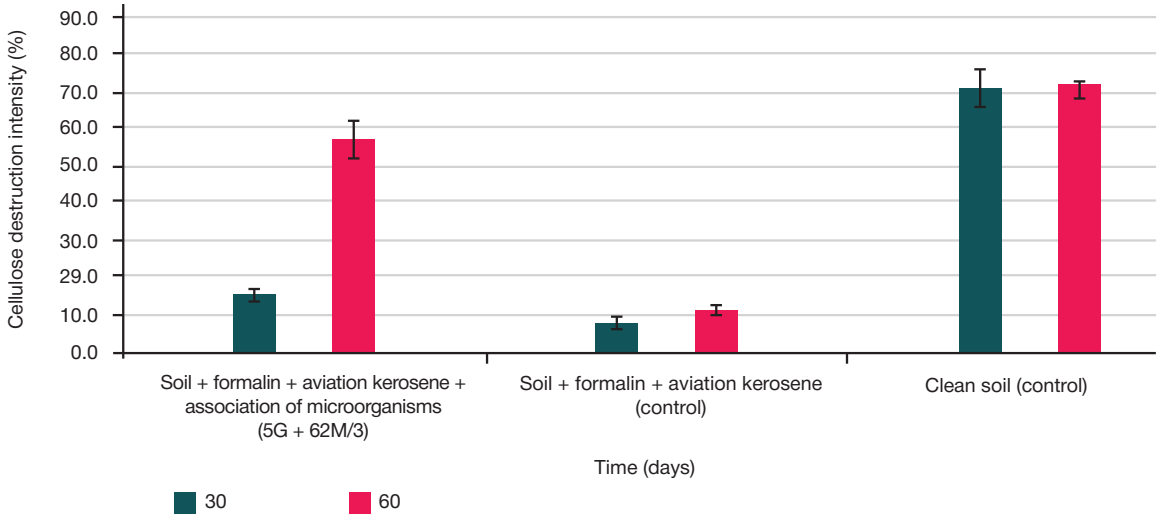

Fig. 8. Cellulase activity of the soil (%) in the field experiment

and a corresponding decrease of the substrates essential for the biodestructor existence. Bacteria counts of saprophytic bacteria reached their maximum on day 60 of the experiment. Microbial cell counts of saprophytic bacteria in the clean soil remained almost the same throughout the experiment.

Soil treated with formalin and aviation kerosene at the start of the experiment was highly toxic to Daphnia (Table 5). Contaminated soil, that was treated with microorganisms, became non-toxic by day 7 of the experiment.

The concentration of aviation kerosene in the soil gradually decreased during bioremediation (Fig. 5).

Soil enzyme activity changed considerably during bioremediation (Fig. 6–8).

The findings showed that biological activity of the soil significantly decreased after adding formalin and aviation kerosene. Thus, on day 7 of the field experiment, dehydrogenase and hydrolase activity of soil in the studied variants was 19% and 24%, respectively, compared to the clean soil (control). The increase in biological activity was observed in the contaminated soil, treated with microorganisms-destructors, starting from day 30 of the experiment. After 60 days the values of soil enzyme activity significantly exceeded that of the contaminated soil not subjected to microbial remediation: dehydrogenase activity increased by 2.4 times, hydrolase activity by 2.1 times, and cellulase activity by 5.1 times.

Phytotoxicity of the soil contaminated with formalin and aviation kerosene was determined during the field experiment. The decrease in phytotoxicity of the recultivated soil to oat seeds to the level of the clean soil was revealed by day 60 based on such parameters, as the “sprouts length” and the “number of ungerminated seeds” (Table 6).

The field experiment revealed a decrease in the acute toxicity and phytotoxicity of the cleaned soil, as well as an increase in the soil biological activity (levels of dehydrogenases, hydrolases, cellulases).

Microorganisms-destructors of RFC *Ps. putida* (strain 5G) and *Rh. erythropolis* (strain 62M/3) were deposited in the All-Russian Collection of Microorganisms and assigned the numbers BKM Ac-2933D and BKM B-3636 D.

DISCUSSION

We isolated microorganisms capable of degrading dimethylhydrazine and aviation kerosene from soils that had been contaminated with these substances for a long time. When performing laboratory testing, we selected two active

Table 6. Soil phytotoxicity to oat seeds on day 60 of the field experiment

Experiment variants	Measurement	Units	Mean value (M ± 6)	Number of seeds per iteration, *n*	Number of iterations
Control (clean soil)	Roots	mm	76.9 ± 45.3	25	3
	Sprouts	mm	49.8 ±18.7		
	Weight (roots and sprouts)	g	4.2 ± 0.1		
	Number of ungerminated seeds	*n*	6		
Soil + formalin + aviation kerosene	Roots	mm	72.2 ± 37.3	25	3
	Sprouts	mm	46.2 ± 16.9		
	Weight (roots and sprouts)	g	3.9 ± 0.1		
	Number of ungerminated seeds	*n*	14		
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	Roots	mm	78.4 ± 29.0	25	3
	Sprouts	mm	53.4 ± 20.4		
	Weight (roots and sprouts)	g	4.4 ± 0.1		
	Number of ungerminated seeds	*n*	7		

Note: M — mean, 6 — standard deviation.

strains of microorganisms: biodestructor of formaldehyde *Ps. putida*, strain 5G, and biodestructor of aviation kerosene *Rh. erythropolis*, strain 62M/3. The conditions for the separate cultures and co-culture were worked out.

The results of toxicity testing performed in white mice and rats showed that *Ps. putida*, strain 5G, and *Rh. erythropolis*, strain 62M/3, were not pathogenic (harmless) to warm-blooded animals based on their virulence, toxicity, toxigenicity, and dissemination patterns. Microorganisms can be used for bioremediation of contaminated soils without limitation.

Laboratory experiments showed that treatment of the soil, contaminated with formalin and aviation kerosene, with the association of microorganisms-destructors of RFC for 30 days resulted in the reduced soil contamination, as well as in the decreased soil integral toxicity and phytotoxicity. Soil enzyme activity gradually increased to reach the level of the clean soil during the microbial remediation.

The field experiment showed that the association of microorganisms consisting of the formalin (heptyl) biodestructor *Ps. putida*, strain 5G, and the aviation kerosene biodestructor *Rh. erythropolis*, strain 62M/3, was suitable for cleaning of soils contaminated with RFC. Microorganisms-destructors of RFC added to the contaminated soils are not suppressed by indigenous microflora and grow actively.

The isolated strains of microorganisms-destructors of RFC can be used at the Russian cosmodromes and Baikonur Cosmodrome (Kazakhstan) to clean up the polluted areas.

The package of measures aimed at detoxication of soil contaminated with RFC will reduce the risk of occupational disorders in the employees engaged in bioremediation of the soil and disposal of space rockets.

CONCLUSIONS

Laboratory and field testing of the association of microorganisms-destructors of propellant components (RFC) *Pseudomonas putida*, strain 5G, and *Rhodococcus erythropolis*, strain 62M/3, showed its high efficiency for decontamination of RFC, as well as its environmental and toxicological safety. The association of microorganisms *Ps. putida*, strain 5G, and *Rh. erythropolis*, strain 62M/3, can be recommended for practical use, i.e. for bioremediation of soil contaminated with RFC.

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