Manuscript Details

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Abstract

The study addresses biological effects of low-dose gamma-radiation. Radioactive 137Cs-containing particles were used as model sources of gamma-radiation. Luminous marine bacterium Photobacterium phosphoreum was used as a bioassay with the bioluminescent intensity as the physiological parameter tested. To investigate the sensitivity of the bacteria to the low-dose gamma-radiation exposure (≤ 250 mGy), the irradiation conditions were varied as follows: bioluminescence intensity was measured at 5, 10, and 20°C for 175, 100, and 47 h, respectively, at different dose rates (up to 4100 μGy/h). There was no noticeable effect of gamma-radiation at 5 and 10°C, while the 20°C exposure revealed authentic bioluminescence inhibition. The 20°C results of gamma-radiation exposure were compared to those for low-dose alpha- and beta-radiation exposures studied previously under comparable experimental conditions. In contrast to ionizing radiation of alpha and beta types, gamma-emission did not initiate bacterial bioluminescence activation (adaptive response). As with alpha- and beta-radiation, gamma-emission did not demonstrate monotonic dose-effect dependencies; the bioluminescence inhibition efficiency was found to be related to the exposure time, while no dose rate dependence was found. The sequence analysis of 16S ribosomal RNA gene did not reveal a mutagenic effect of low-dose gamma radiation. The exposure time that caused 50% bioluminescence inhibition was suggested as a test parameter for radiotoxicity evaluation under conditions of chronic low-dose gamma irradiation.

Keywords	low dose gamma-radiation; luminous marine bacteria; bioassay; radiotoxicity; mutagenic effect; temperature dependence
Taxonomy	Environmental Radioactivity, Environmental Monitoring, Environmental Science

Corresponding Author	Nadezhda Kudryasheva
Corresponding Author's Institution	Inst of Biophysics SB RAS
Order of Authors	Nadezhda Kudryasheva, Alena Petrova, Alexander Bondar, Dmitriy Dementyev
Suggested reviewers	Ludmila Shishkina, Alexander Kamnev, Anatoliy Popov, Gennadii Badun

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Reply to REVIEWER'S COMMENTS:

-Reviewer 1

(1) The Introduction is too general with some unnecessary references to various responses of biological objects to ionizing radiation, which were not addressed in the manuscript.

Reply:

INTRODUCTION was partly reconstructed. We removed some references on bacterial bioluminescent assay properties, several references on our previous studies of ionizing radiation effects, and reduced description of stochastic and deterministic effects. Withdrawn parts of the text are marked with red in attached file TextCorrections.docx. Adjunctions which were included according to Reviewer-2 suggestions are marked here with yellow.

(2) The main problem appear in the sections 2.3 "Evaluation of radiotoxicity of the test samples" and 3 "results and discussion". The results seems contradictory and inconsistent. *Authors formulated the main results as follows: bioluminescent response of bacteria does not depend on dose rate or dose of exposure; however, the response depends on time of exposure. This inconsistency of results can be explained by a methodological non-correctness in estimation of relative bioluminescent intensity Irel.* In the manuscript, Irel=Irad/Icontrol, where Irad is maximal bioluminescent intensity of exposed bacteria, and Icontrol- maximal bioluminescent intensity in the control. However, both Irad and Icontrol are functions of time Irad(t), Icontrol(t): in the control (at room temperature), bioluminescent intensity decreased from 11 down to 3 in the course of 50 hours (see fig.1); under radiation exposure, the response also is not immediate and is developing with time. Therefore, correct values of relative bioluminescent intensity Irel(t) shall be calculated in dynamics : Irel(t)= Irad(t)/Icontrol(t).

· The results need a revision with the correct calculation of the relative bioluminescent intensity of bacteria. *The "radiation effects" declared by the authors may disappear, when correct values of Irel (depending on time) are used in the result analysis.*

Reply:

We did not make this mistake. Analysis of time-course of control samples is a basic part of all chronoscopic measurements. Control samples are always supposed to be under conditions identical to those for exposed samples. This is why all publications of similar experiments should include time-courses of control samples (such as Fig.1).

We found the place in our manuscript that could mislead Reviewer, as well as probably other readers. This is section 2.3. We introduced the explanation: "Values of I^{rel} were calculated for all samples at different times of exposure. Times of control sample measurements corresponded to those of the exposed samples." Additionally, we removed word 'maximal', taking it as dispensable in this context. (This word implied only acceptable variations of bioluminescent intensity during measurement time, but not initial bioluminescent intensity). We hope that these changes make the procedure of experimental data treatment more understandable.

So, we suppose that the basis for our speculations and conclusions is correct.

Our group works with time-dependent responses of microorganisms to low-dose exposures for more than ten years, and our laboratory is included into toxicity measurements for almost five decades, so, we are highly experienced in treatment of experimental data of this type.

-Reviewer 2

(1) Although the impact of gamma irradiation is being studied intensively now, and a lot of works is concerned with this topic, the following aspect makes the current work specific and peculiar: the effects of gamma-radiation are compared here to those of alpha-and beta radiation that were studied at comparable conditions. The results of the comparison approached to revealing the physico-chemical basis of the biological effects of different radiation types: beta

and alpha-radiations ionize aquatic media forming active radicals, ion-radicals, including ROS and ACS (see Mishra et al. in the References), while gamma radiation is a high-energy electromagnetic waves with lower ionization ability of water media. It is known that the direct interaction of the waves with biological components of the cellular microorganisms under conditions of low-intensive gamma exposure is very low (Lampe, N. et al. (2016). Simulating the Impact of the Natural Radiation Background on Bacterial Systems: Implications for Very Low Radiation Biological Experiments. *PloS one*, *11*(11), e0166364 - to incorporate). Perhaps, the low interaction probability can explain time-response decay in fig.3. I think these positions and references can be added to the Conclusions.

Reply:

The following paragraphs were introduced as a discussion for

- fig.3:

"It is known that direct interaction of electromagnetic waves with biological components of cellular microorganisms under conditions of low-intensive gamma exposure is very low (Lampe, N. et al., 2016). Perhaps, a low interaction probability can be concerned with the low time-response decay in Fig.3."

-fig.4:

"The results of the comparison approached to revealing the physico-chemical basis of the biological effects of different radiation types: beta and alpha-radiations ionize aquatic media forming active radicals and ion-radicals, while gamma radiation is high-energy electromagnetic waves with lower ionization ability of water media."

(2) Additionally, the position is worthy for consideration that the hormesis model can be accepted as a basis one, while the threshold model can be considered as a derivative from the former coming into being under definite conditions (see Shi et al. in the References).

Reply:

The following phrases were included to:

- INTRODUCTION: "In (Shi et al., 2016), the hormesis model is suggested to be accepted as a basic one, while the other models (threshold and linear) can be considered as simplified derivatives from the former, coming into being under definite conditions"

- RESULTS AND DISCUSSION, while discussing fig 3**: "**All the curves are of 'threshold' character.".

- CONCLUSION: "Hence, time dependence of the bacterial response to low-level alpha and beta ionizing radiation can be discussed in term of 'radiation hormesis', while low-level gamma radiation reveals the threshold time dependence."

(3) It is important to note that DNA damage was excluded, as proved with sequence DNA analysis. However, the authors should pay attention that more delicate processes of genetic regulation cannot be excluded, and add corresponding references.

Reply:

The following sentence was added at the end of RESULTS AND DISCUSSION: "It should be paid attention that delicate processes of genetic regulation in bacterial cells cannot be excluded. As reported in (Bolsunovsky et al., 2016), two bacterial tests based on *E.coli* and *S.typhimurium* cells demonstrated a complex response to chronic low-dose gamma-exposure; this response included the induction of SOS-chromotest response and mutation frequencies, followed by the attenuation of the effects at longer exposure times."

(4) An additional comment: In the References some journal names are not abbreviated.

Reply: Abbreviations of some journals were introduced or corrected. They are marked in the list of references.

Withdrawn parts of the text are marked with red. Additions to the text are marked with yellow.

ABSTRACT

The study addresses biological effects of low-dose gamma-radiation. Radioactive ^{137}Cs-containing particles were used as model sources of gamma-radiation. Luminous marine bacterium *Photobacterium phosphoreum* was used as a bioassay with the bioluminescent intensity as the physiological parameter tested. To investigate the sensitivity of the bacteria to the low-dose gamma-radiation exposure (≤ 250 mGy), the irradiation conditions were varied as follows: bioluminescence intensity was measured at 5, 10, and 20°C for 175, 100, and 47 h, respectively, at different dose rates (up to 4100 μGy/h). There was no noticeable effect of gamma-radiation at 5 and 10°C, while the 20°C exposure revealed authentic bioluminescence inhibition. The 20°C results of gamma-radiation exposure were compared to those for low-dose alpha- and beta-radiation exposures studied previously under comparable experimental conditions. In contrast to ionizing radiation of alpha and beta types, gamma-emission did not initiate bacterial bioluminescence activation (adaptive response). As with alpha- and beta-radiation, gamma-emission did not demonstrate monotonic dose-effect dependencies; the bioluminescence inhibition efficiency was found to be related to the exposure time, while no dose rate dependence was found. The sequence analysis of 16S ribosomal RNA gene did not reveal a mutagenic effect of low-dose gamma radiation. The exposure time that caused 50% bioluminescence inhibition was suggested as a test parameter for radiotoxicity evaluation under conditions of chronic low-dose gamma irradiation.

Keywords: low-dose gamma-radiation; luminous marine bacteria; bioassay; radiotoxicity; mutagenic effect; temperature dependence

1. INTRODUCTION

Rapid development of nuclear energy and nuclear medicine has increased the background levels of radiation exposure of people and other living organisms. Recent years have seen a change in the approach in radiobiological studies: biota *in toto* is considered as a target of radiation impact, with the human included as part of biota integrated into the biosphere by a multiplicity of functional interrelations. Microorganisms play the fundamental role in the biosphere, and their physiological parameters are traditionally used to monitor environmental toxicity, including radiation toxicity. Marine luminous bacteria are an appropriate tool for such investigation, as they are highly sensitive to the presence of toxic compounds. Bioluminescence intensity, the main physiological parameter tested, can be easily measured instrumentally. It is also important that luminous bacteria-based assays are simple and not time consuming due to high rates of bioluminescence response. Hence, bioluminescent assays provide a large number of experimental results under comparable conditions, which is essential for their statistical treatment. These are the reasons why luminous bacteria, as well as their enzymes, have been used as toxicity bioassays for several decades (Roda et al., 2004; Girotti et al., 2008; Tarasova et al., 2012; Kudryasheva and Tarasova, 2015, Kratasyuk and Esimbekova, 2015; Kudryasheva et al., 2017). Bacterial

bioluminescent assays can be based on biological systems of different complexity – bacteria or their enzymes, providing comparison of toxic effects on microorganisms and their biochemical reactions (Kudryasheva et al., 1996; Kudryasheva et al., 1998; Rozhko et al., 2007; Tarasova et al., 2012; Selivanova et al., 2013; Kratasyuk and Esimbekova, 2015; Kudryasheva et al., 2016). These assays are used to study mechanisms of toxic effects on cellular and molecular levels. Physicochemical basis for toxic effects in bioluminescent systems processes taking place in the bioluminescent assay systems in the presence of exogenous compounds were addressed in the was elaborated in studies by Kudryasheva (2006) and Nemtseva and Kudryasheva (2007). Bacterial bioluminescent assay based on recombinant *Escherichia coli* has been previously used to test biological effects of high-dose gamma-radiation exposures; doses accumulated by the bacteria were 2.6 Gy (Ptitsyn et al., 1997) and 1-200 Gy (Min et al., 2003). The last decade has seen the application of luminous bacteria to monitor biological effects of low-dose ionizing radiation (Rozhko et al., 2007; Rozhko et al., 2011; Selivanova et al., 2013). (Kudryasheva and Rozhko, 2015).

Radiosensitivity of living organisms is usually expressed as a dose/effect relationship, and considerable uncertainty exists concerning low exposure doses. In addition to the linear dose/effect relationship, low-dose studies might be based on a threshold dose/effect relationship or the hormesis phenomenon (Burlakova et al., 2004; Calabrese, 2014; Baldwin and Grantham, 2015; Kudryasheva and Rozhko, 2015; Rozhko et al., 2016; Shi et al., 2016). The hormesis hypothesis suggests that low dose radiation can be favorable for living organisms. In (Shi et al., 2016), the hormesis model is suggested to be accepted as a basic one, while the other models (threshold and linear) can be considered as simplified derivatives from the former, coming into being under definite conditions.

In contrast to 'deterministic' effects of high doses, low-level radiation produces "stochastic" effects. , , which occur by chance. They are described in terms of 'randomnicity' and 'probability', and assume that the low dose exposures, below about 100-200 mSv, do not produce heritable effects in direct proportion to an equivalent dose. High doses produce 'deterministic' effects, "which is the severity of acute damage that is certain to happen; these effects are compared to the physical quantity absorbed dose" (ICRP, 2007).

A bioassay system based on luminous marine bacteria is a good candidate for monitoring the stochastic effects of low-dose radiation, due to high rates and simplicity of the assay procedure, as well as availability of reagents and devices. Modern microplate biochemiluminometers provide a technical support for such investigations. A review by Kudryasheva and Rozhko (2015) summarized the study of the effects of model solutions of alpha- and beta-emitting radionuclides (americium-241, uranium, and tritium) on marine bacteria under conditions of chronic low-dose irradiation. Non-linear dose-effect dependences were demonstrated. Three successive stages in the bioluminescent response to americium-241 and tritium were found: (1) absence of effects (stress recognition), (2) activation (adaptive response), and (3) inhibition (suppression of physiological function, i.e. radiation toxicity). The effects were attributed to the radiation hormesis phenomenon. The bacterial responses to alpha- and beta-emitting radionuclides were compared in a study by Selivanova et al. (2014); the difference in the effects was related to the content of reactive oxygen species and efficiency of redox processes (Alexandrova et al., 2011; Selivanova et al., 2014).

So far, the luminescent bacteria-based assay has not been used to evaluate low-dose effects of gamma-radiation. However, the gamma component of low-content radioactive contaminations might be extremely important, producing a harmful impact on living organisms due to its high penetrability and high energy. This

type of radiation has energy of a few hundred keV and can reach up to 10 MeV. Additionally, gamma-rays are less ionizing and more penetrative than alpha- or beta- particles: the maximal energy of tritium beta-particles is 5.7 keV, the maximal range of their path is about 1 cm (in the air, at 20°C), and specific ionization ability is 2.2×10^6 ions per cm (Selivanova et al., 2013).

Natural sources of low-intensive gamma irradiation include naturally occurring radioisotopes such as potassium-40 and atmospheric interactions with cosmic rays or particles. Natural exposure to gamma rays is about 1 to 2 mSv per year, and the average total amount of radiation received in one year per inhabitant in the U.S. is 3.6 mSv (UNSCEAR 1993). Artificial sources of gamma rays include radioactive decay in nuclear reactors, and high energy physics experiments, such as neutral pion decay or nuclear fusion.

Biological effects of high-dose gamma-radiation exposures have been tested previously using bacterial bioluminescent assay based on recombinant *Escherichia coli*; doses accumulated by the bacteria were 2.6 Gy (Ptitsyn et al., 1997) and 1-200 Gy (Min et al., 2003). Low-dose gamma-radiation was found to induce SOS-chromotest response of *Escherichia coli* and to increase mutation frequency in *S. typhimurium* cells (Bolsunovsky et al., 2016).

Comparison of low-dose biological effects of alpha-, beta-, and gamma-radiation in model experiments using the bacteria-based luminescent assay is a question of interest from both fundamental and applied points of view. The purpose of the present study was to determine the effects of low-dose gamma-radiation on *Photobacterium phosphoreum* and compare them with the effects of alpha- and beta-emitting radionuclides. The applied aspect of the work is the usage of the bacteria as a cellular bioassay to monitor toxicity of the gamma-radiation low-dose exposure. Irradiation conditions (temperature, dose rate, and exposure time) were varied. Radioactive ^{137}Cs-containing particles from the Yenisei River, which is affected by the operation of the Mining-and-Chemical Combine of Rosatom, were used as model sources of gamma-radiation. The radioactive isotope ^{137}Cs has the following characteristics: $E\gamma = 661.7$ keV, $T_{1/2} = 30.07$ y.

2. MATERIALS AND METHODS

2.1. Objects

Microbiotest 677F, preparation of lyophilized *Photobacterium phosphoreum* 1883 IBSO (Kuznetsov et al., 1996), was used as a bioassay to monitor toxicity of aquatic media exposed to gamma radiation. The preparation was obtained from the Institute of Biophysics SB RAS, Krasnoyarsk, Russia.

^{137}Cs-containing radioactive hot particles were used as the point sources of external gamma radiation. The particles were extracted from the floodplain soils and sediments of the Yenisei River in the area affected by the operation of the Mining-and-Chemical Combine of Rosatom (Bolsunovsky and Tcherkezian, 2001; Chuguyevskiy et al., 2010). Two hot particles were used in the experiments with luminous bacteria. Radioactivity of the particles, their distances from the bacterial samples, and the corresponding dose rates at these distances are given in Table 1.

<Table 1>

2.2. Experimental procedure

A radioactive particle was placed in the center of the experimental chamber. Eppendorf tubes with bacterial suspension in 1.5% NaCl were placed around the radioactive particle, at different distances from it. Dose rates of gamma-irradiation ranged from 0.2 to 137 μGy/h and from 122 to 4100 μGy/h for Particle 1 and Particle 2, respectively (Table 1). The average background exposure dose for the control bacterial samples was 0.1 μGy/h. The dose rate calculations were based on the activity of the ^{137}Cs source; they were additionally verified by direct measurements with a DKG-02U dosimeter (SPC "Doza", Ltd, Russia).

Bioluminescence kinetics of the bacterial samples (control and irradiated ones) was measured in 3% NaCl solutions using a CL3606 Biochemiluminometer (SDTB “Nauka” KSC SB RAS, Russia). Bacterial suspensions were exposed to the radiation in three experiments: at 5, 10, and 20°C. Fig. 1 shows bioluminescence kinetics of the control bacterial suspensions at the different temperatures.

<Fig.1>

Bioluminescent measurements of the control and irradiated samples were carried out and compared as described in the section below.

A mutagenic effect of low-dose gamma radiation was examined using sequence analysis of 16S ribosomal RNA gene of *P.Phosphoreum*. The analysis was performed on the samples of bacterial suspensions exposed to gamma radiation (4100 μGy/h, 20ºC); it was compared to that of the control bacterial suspensions.

2.3. Evaluation of radiotoxicity of the test samples

Radiotoxicity of a bacterial sample was assessed by relative bioluminescent intensity, I^{rel}, calculated as

$$I^{rel} = I_{rad} / I_{contr}$$

Here, I_{rad} is the average value of maximal bioluminescence intensity in the bacterial sample exposed to gamma radiation, and I_{contr} is the average value of maximal bioluminescence intensity in the control sample. The average values were obtained in four parallel experiments with five measurements for all irradiated and control bacterial suspensions. Experimental error did not exceed 10%.

Values of I^{rel} were calculated for all samples at different times of exposure. Times for control sample measurements corresponded to those of the exposed samples.

3. RESULTS AND DISCUSSION

We measured bioluminescence of bacterial suspensions exposed to gamma radiation at 5, 10, and 20ºC; values of I^{rel} were calculated and plotted vs. absorbed gamma-radiation dose. The 5ºC and 10ºC exposures did not show noticeable effects of gamma radiation. Fig. 2 shows results of the 10ºC exposure as an example. It can be seen from the graph that the bioluminescence intensities of the exposed bacterial test suspensions (I^{rel}) were close to 1 (black squares in Fig. 2), i.e. they demonstrate absence of authentic deviations from the control suspensions.

<Fig.2>

Results of bacterial exposure to gamma-radiation at 20ºC are also shown in Fig. 2. Bioluminescence inhibition (I^{rel} <1) occurred in this experiment for majority of bacterial test samples. Hence, the temperature rise

from 10ºC to 20ºC, with the latter being closer to the native bacterial temperature, made the microorganism more sensitive to low-dose gamma radiation. A possible reason for this may be higher rates of bacterial metabolic processes.

Fig. 2 demonstrates that monotonic dose dependency of I^{rel} was not observed at 20ºC. In contrast to 10ºC, the higher temperature conditions revealed 'stochasticity' of the bacterial response. The absence of linear dose vs. response dependence under low-dose exposure is usually associated with hormesis phenomenon (Calabrese, 2014; Baldwin and Grantham, 2015; Shi et al., 2016). Nevertheless, it is known that hormesis is characterized not only by inhibition, but also by low-dose stimulation (adaptive response), resulting in "either a J-shaped or an inverted U-shaped dose response". This gamma low-dose exposure does not reveal any distinct activation throughout the experimental dose range (Fig. 2). However, studies of low-dose alpha- and beta-radioactive exposures (Rozhko et al., 2007; Selivanova et al., 2013; Selivanova et al., 2014) showed a distinct activation stage in the bioluminescence kinetics followed by the inhibition (toxic) stage. Higher energy, penetrative ability, and low ionization ability of gamma irradiation are likely reasons for the lower adaptive response in bacterial cells. Moreover, active chlorine species, that can be produced in physiological solutions under gamma-irradiation exposure (Mishra et al., 2016), might contribute to the toxic effect.

Nonlinear biological effects of low dose gamma-radiation were demonstrated previously in (Zhikrevetskaya et al., 2015). It was shown that the identified changes in lifespan and gene expression in *Drosophila melanogaster* are not dose-dependent.

The 20ºC experimental data shown in Fig. 2 were analyzed, and the I^{rel} values were plotted in accordance with the time of exposure and dose rate. The results are shown in Figs 3 and 4, respectively.

Fig. 3 shows that all bacterial samples exposed to different dose rate radiation had similar time-courses of the bioluminescence intensity. All the curves are of 'threshold' character. In time interval 19-47 h, the time-course dependencies were approximated by linear dependencies:

$$I^{rel} = a - b \cdot t.$$

Here, t is the time of exposure.

<Fig.3>

Angular coefficients of these dependencies (b) were calculated as 0.028, 0.029, 0.026, and 0.025 for dose rates of 150, 460, 1040, and 4100 μGy/h, respectively. Close values of these coefficients confirm that bacterial response was not dependent on the dose rates under the conditions of low-dose gamma-radiation exposure.

It is known that direct interaction of electromagnetic waves with biological components of cellular microorganisms under conditions of low-intensive gamma exposure is very low (Lampe, N. et al., 2016). Perhaps, a low interaction probability can be concerned with the low time-response decay in Fig.3.

In (Selivanova et al., 2014), the time of conversion of bioluminescence activation to inhibition was suggested as a test parameter to evaluate the toxicity of solutions of alpha- and beta-emitting radionuclides for marine bacteria. The present study demonstrates that in the case of gamma irradiation, the exposure time appeared to be critical for the marine bacteria, too. Hence, the exposure time can be used as a test parameter for radiotoxicity evaluation under conditions of chronic low-dose gamma irradiation. For example, this can be the time of 50% bioluminescence inhibition (at $I^{rel} = 0.5$), $t_{1/2}$. As can be seen from Fig. 3, the $t_{1/2}$ values for the bacterial preparation were within 35-38 h at all dose rates applied.

Results of the experiment are presented in Fig. 4 as dose rate dependence of I^{rel}. In this graph, the horizontal lines connect dots of equal exposure times. The graph confirms that the bioluminescence intensity depended on the time of exposure and did not depend on the dose rates.

A similar conclusion was made in a study by Selivanova et al. (2013), in which beta-emitting radionuclide tritium was used as a source of irradiation for luminous bacteria. The authors of that study found that the bioluminescent intensity depended on the time of exposure, but did not depend on radioactivity concentration of tritiated water in a wide range of its radioactivity concentrations: between 0.0002 and 200 MBq/L.

<Fig.4>

In contrast to our current results for gamma irradiation, tritiated water produced distinct (up to 100-150%) bioluminescence activation followed by the inhibition stage (Selivanova et al., 2013). Activation bacterial response was found in high diluted solutions of alpha-emitting radionuclide Am-241, too (Rozhko et al., 2007; Alexandrova et al., 2011). As discussed before, the differences observed in the case of low-dose gamma-ray exposure might be attributed to higher energy of gamma radiation, its lower ionization ability in aqueous media, and possibility to form active chlorine species in physiological solutions.

The results of the comparison approached to revealing the physico-chemical basis of the biological effects of different radiation types: beta and alpha-radiations ionize aquatic media forming active radicals and ion-radicals, while gamma radiation is high-energy electromagnetic waves with lower ionization ability of water media.

Sequence analysis of bacterial DNA was performed for 16S ribosomal RNA gene of *P.Phosphoreum.* This gene was chosen for the genetic analysis as a model for evaluation of nonspecific DNA damage. The samples of the bacterial suspension exposed to low-dose gamma radiation (~0.2 Gy) were analyzed and compared with control samples. Under the conditions of the experiment no changes in the analyzed gene sequence were found.

Results of this study might be interpreted by using the novel “exposome” concept, which complements the genome and encompasses the totality of environmental (i.e. non-genetic) exposures (Rappaport and Smith, 2010; Wild, 2012). Although this term was initially introduced for human exposures, the study of simple model organisms might provide fundamental molecular, physicochemical, biochemical, and cellular bases for human exposure science.

It should be paid attention that delicate processes of genetic regulation in bacterial cells cannot be excluded. As reported in (Bolsunovsky et al., 2016), two bacterial tests based on *E.coli* and *S.typhimurium* cells demonstrated a complex response to chronic low-dose gamma-exposure; this response included the induction of SOS-chromotest response and mutation frequencies, followed by the attenuation of the effects at longer exposure times.

CONCLUSIONS

In this work, we investigated the low-intensive gamma radiation effects on the luminescence bacteria-based assay. Low dose effects of gamma radiation on the luminous bacteria were studied and compared with those of beta- and alpha- radiation that had been studied previously under comparable conditions.

We determined suitable conditions for evaluation of gamma-radiation effects. Three series of experiments were carried out: at 5°C, 10°C, and 20°C. The results showed the absence of authentic deviations from the control bacterial suspension for bacterial samples at 5°C and 10°C. The 20°C exposure revealed authentic bioluminescence inhibition. Higher rates of metabolic processes at higher temperature may make the bacteria more sensitive to gamma radiation.

Results of the 20°C exposure were analyzed in detail. The absence of the monotonic dose/effect dependencies was demonstrated under conditions of the experiment. Bioluminescence inhibition efficiency was found to be related to the exposure time, while no relationship to the dose rate was found. Similar effects were found earlier in experiments with luminous marine bacteria exposed to low-level alpha- and beta-radiation. However, in contrast to the alpha- and beta- exposures, gamma radiation exposure did not reveal distinct bioluminescence activation (adaptive response). Hence, time dependence of the bacterial response to low-level alpha and beta ionizing radiation can be discussed in term of 'radiation hormesis', while the low-level gamma radiation reveals the threshold time dependence. Such dissimilarity might be caused by higher energy of gamma radiation, its lower ionization ability in aqueous media, and active chlorine species production.

Further experiments should elucidate the molecular and physicochemical basis for the difference in biological low-dose effects of alpha, beta, and gamma radiation. Apart from the quantitative evaluation, bacterial cells are a very convenient tool for a number of methods applicable to studying intracellular processes: from membrane penetrability to gene regulation, enzyme activity, ATP consumption and crystallinity of intracellular macrocomponents.

From the applied point of view, the temperature of 20°C was found to be optimal for using luminous marine bacteria in low-dose gamma-radiation toxicity monitoring, providing a convenient combination of radiosensitivity and duration of the bioassay procedure. As the exposure time appeared to be critical for the bacteria, it can be suggested as a test parameter for radiotoxicity evaluation under conditions of chronic low-dose gamma irradiation. For example, this can be the time for 50% bioluminescence inhibition.

The sequence analysis of 16S ribosomal RNA gene did not reveal a mutagenic effect of low-dose gamma radiation. The results of this study can be interpreted by using the novel approach based on the "exposome" concept of complementing the genome.

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Figure Captions

Fig. 1. Bioluminescent kinetics of *P.phosphoreum* at 5, 10, and 20ºC.

Fig. 2. Relative bioluminescence intensity of *P.phosphoreum*, I^{rel}, vs. dose of gamma irradiation. Temperature of the experiments: 10 ºC (■) and 20ºC (o). An error for I^{rel} measurements was 10%.

Fig. 3. Bioluminescence kinetics of *P.phosohoreum* exposed to gamma radiation of different dose rates, Particle 2, 20ºC. An error for I^{rel} was 10%.

Fig. 4. Relative bioluminescence intensity I^{rel} vs. gamma-radiation dose rate. Particle 2, 20ºC. Horizontal lines connect dots of equal exposure times. An error for I^{rel} was 10%

ABSTRACT

The study addresses biological effects of low-dose gamma-radiation. Radioactive ^{137}Cs-containing particles were used as model sources of gamma-radiation. Luminous marine bacterium *Photobacterium phosphoreum* was used as a bioassay with the bioluminescent intensity as the physiological parameter tested. To investigate the sensitivity of the bacteria to the low-dose gamma-radiation exposure (≤ 250 mGy), the irradiation conditions were varied as follows: bioluminescence intensity was measured at 5, 10, and 20°C for 175, 100, and 47 h, respectively, at different dose rates (up to 4100 μGy/h). There was no noticeable effect of gamma-radiation at 5 and 10°C, while the 20°C exposure revealed authentic bioluminescence inhibition. The 20°C results of gamma-radiation exposure were compared to those for low-dose alpha- and beta-radiation exposures studied previously under comparable experimental conditions. In contrast to ionizing radiation of alpha and beta types, gamma-emission did not initiate bacterial bioluminescence activation (adaptive response). As with alpha- and beta-radiation, gamma-emission did not demonstrate monotonic dose-effect dependencies; the bioluminescence inhibition efficiency was found to be related to the exposure time, while no dose rate dependence was found. The sequence analysis of 16S ribosomal RNA gene did not reveal a mutagenic effect of low-dose gamma radiation. The exposure time that caused 50% bioluminescence inhibition was suggested as a test parameter for radiotoxicity evaluation under conditions of chronic low-dose gamma irradiation.

Keywords: low-dose gamma-radiation; luminous marine bacteria; bioassay; radiotoxicity; mutagenic effect; temperature dependence

1. INTRODUCTION

Rapid development of nuclear energy and nuclear medicine has increased the background levels of radiation exposure of people and other living organisms. Microorganisms play the fundamental role in the biosphere, and their physiological parameters are traditionally used to monitor environmental toxicity, including radiation toxicity. Marine luminous bacteria are an appropriate tool for such investigation, as they are highly sensitive to the presence of toxic compounds. Bioluminescence intensity, the main physiological parameter tested, can be easily measured instrumentally. It is also important that luminous bacteria-based assays are simple and not time consuming due to high rates of bioluminescence response. Hence, bioluminescent assays provide a large number of experimental results under comparable conditions, which is essential for their statistical treatment. These are the reasons why luminous bacteria, as well as their enzymes, have been used as toxicity bioassays for several decades (Roda et al., 2004; Girotti et al., 2008; Tarasova et al., 2012; Kudryasheva and Tarasova, 2015, Kratasyuk and Esimbekova, 2015; Kudryasheva et al., 2017). Physicochemical basis for toxic effects in bioluminescent systems was elaborated in studies by Kudryasheva (2006) and Nemtseva and Kudryasheva (2007).

Bacterial bioluminescent assay based on recombinant *Escherichia coli* has been previously used to test biological effects of high-dose gamma-radiation exposures; doses accumulated by the bacteria were 2.6 Gy (Ptitsyn et al., 1997) and 1-200 Gy (Min et al., 2003). The last decade has seen the application of luminous bacteria to monitor biological effects of low-dose ionizing radiation (Kudryasheva and Rozhko, 2015).

Radiosensitivity of living organisms is usually expressed as a dose/effect relationship, and considerable uncertainty exists concerning low exposure doses. In addition to the linear dose/effect relationship, low-dose studies might be based on a threshold dose/effect relationship or the hormesis phenomenon (Burlakova et al., 2004; Calabrese, 2014; Baldwin and Grantham, 2015; Kudryasheva and Rozhko, 2015; Rozhko et al., 2016). The hormesis hypothesis suggests that low dose radiation can be favorable for living organisms. In (Shi et al., 2016), the hormesis model is suggested to be accepted as a basic one, while the other models (threshold and linear) can be considered as simplified derivatives from the former, coming into being under definite conditions.

In contrast to 'deterministic' effects of high doses, low-level radiation produces "stochastic" effects. They are described in terms of 'randomnicity' and 'probability', and assume that the low dose exposures, below 100-200 mSv, do not produce heritable effects in direct proportion to an equivalent dose.

A bioassay system based on luminous marine bacteria is a good candidate for monitoring the stochastic effects of low-dose radiation, due to high rates and simplicity of the assay procedure, as well as availability of reagents and devices. Modern microplate biochemiluminometers provide a technical support for such investigations. A review by Kudryasheva and Rozhko (2015) summarized the study of the effects of model solutions of alpha- and beta-emitting radionuclides (americium-241, uranium, and tritium) on marine bacteria under conditions of chronic low-dose irradiation. Non-linear dose-effect dependences were demonstrated. Three successive stages in the bioluminescent response to americium-241 and tritium were found: (1) absence of effects (stress recognition), (2) activation (adaptive response), and (3) inhibition (suppression of physiological function, i.e. radiation toxicity). The effects were attributed to the radiation hormesis phenomenon.

So far, the luminescent bacteria-based assay has not been used to evaluate low-dose effects of gamma-radiation. However, the gamma component of low-content radioactive contaminations might be extremely important, producing a harmful impact on living organisms due to its high penetrability and high energy. This type of radiation has energy of a few hundred keV and can reach up to 10 MeV. Additionally, gamma-rays are less ionizing and more penetrative than alpha- or beta- particles: the maximal energy of tritium beta-particles is 5.7 keV, the maximal range of their path is about 1 cm (in the air, at 20°C), and specific ionization ability is 2.2×10^6 ions per cm (Selivanova et al., 2013).

Natural sources of low-intensive gamma irradiation include naturally occurring radioisotopes such as potassium-40 and atmospheric interactions with cosmic rays or particles. Natural exposure to gamma rays is about 1 to 2 mSv per year, and the average total amount of radiation received in one year per inhabitant in the U.S. is 3.6 mSv (UNSCEAR 1993). Artificial sources of gamma rays include radioactive decay in nuclear reactors, and high energy physics experiments, such as neutral pion decay or nuclear fusion.

Comparison of low-dose biological effects of alpha-, beta-, and gamma-radiation in model experiments using the bacteria-based luminescent assay is a question of interest from both fundamental and applied points of view. The purpose of the present study was to determine the effects of low-dose gamma-radiation on *Photobacterium phosphoreum* and compare them with the effects of alpha- and beta-emitting radionuclides. The applied aspect of the work is the usage of the bacteria as a cellular bioassay to monitor toxicity of the gamma-radiation low-dose exposure. Irradiation conditions (temperature, dose rate, and exposure time) were varied. Radioactive ^{137}Cs-containing particles from the Yenisei River, which is affected by the operation of the Mining-and-Chemical Combine of Rosatom, were used as model sources of gamma-radiation. The radioactive isotope ^{137}Cs has the following characteristics: $E\gamma = 661.7$ keV, $T_{1/2} = 30.07$ y.

2. MATERIALS AND METHODS

2.1. Objects

Microbiotest 677F, preparation of lyophilized *Photobacterium phosphoreum* 1883 IBSO (Kuznetsov et al., 1996), was used as a bioassay to monitor toxicity of aquatic media exposed to gamma radiation. The preparation was obtained from the Institute of Biophysics SB RAS, Krasnoyarsk, Russia.

^{137}Cs-containing radioactive hot particles were used as the point sources of external gamma radiation. The particles were extracted from the floodplain soils and sediments of the Yenisei River in the area affected by the operation of the Mining-and-Chemical Combine of Rosatom (Bolsunovsky and Tcherkezian, 2001; Chuguyevskiy et al., 2010). Two hot particles were used in the experiments with luminous bacteria. Radioactivity of the particles, their distances from the bacterial samples, and the corresponding dose rates at these distances are given in Table 1.

<Table 1>

2.2. Experimental procedure

A radioactive particle was placed in the center of the experimental chamber. Eppendorf tubes with bacterial suspension in 1.5% NaCl were placed around the radioactive particle, at different distances from it. Dose rates of gamma-irradiation ranged from 0.2 to 137 μGy/h and from 122 to 4100 μGy/h for Particle 1 and Particle 2, respectively (Table 1). The average background exposure dose for the control bacterial samples was 0.1 μGy/h. The dose rate calculations were based on the activity of the ^{137}Cs source; they were additionally verified by direct measurements with a DKG-02U dosimeter (SPC "Doza", Ltd, Russia).

Bioluminescence kinetics of the bacterial samples (control and irradiated ones) was measured in 3% NaCl solutions using a CL3606 Biochemiluminometer (SDTB “Nauka” KSC SB RAS, Russia). Bacterial suspensions were exposed to the radiation in three experiments: at 5, 10, and 20°C. Fig. 1 shows bioluminescence kinetics of the control bacterial suspensions at the different temperatures.

<Fig.1>

Bioluminescent measurements of the control and irradiated samples were carried out and compared as described in the section below.

A mutagenic effect of low-dose gamma radiation was examined using sequence analysis of 16S ribosomal RNA gene of *P.Phosphoreum*. The analysis was performed on the samples of bacterial suspensions exposed to gamma radiation (4100 μGy/h, 20ºC); it was compared to that of the control bacterial suspensions.

2.3. Evaluation of radiotoxicity of the test samples

Radiotoxicity of a bacterial sample was assessed by relative bioluminescent intensity, I^{rel}, calculated as

$$I^{rel} = I_{rad} / I_{contr}$$

Here, I_{rad} is the average value of bioluminescence intensity in the bacterial sample exposed to gamma radiation, and I_{contr} is the average value of bioluminescence intensity in the control sample. The average values were obtained in four parallel experiments with five measurements for all irradiated and control bacterial suspensions. Experimental error did not exceed 10%.

Values of I^{rel} were calculated for all samples at different times of exposure. Times for control sample measurements corresponded to those of the exposed samples.

3. RESULTS AND DISCUSSION

We measured bioluminescence of bacterial suspensions exposed to gamma radiation at 5, 10, and 20ºC; values of I^{rel} were calculated and plotted vs. absorbed gamma-radiation dose. The 5ºC and 10ºC exposures did not show noticeable effects of gamma radiation. Fig. 2 shows results of the 10ºC exposure as an example. It can be seen from the graph that the bioluminescence intensities of the exposed bacterial test suspensions (I^{rel}) were close to 1 (black squares in Fig. 2), i.e. they demonstrate absence of authentic deviations from the control suspensions.

<Fig.2>

Results of bacterial exposure to gamma-radiation at 20ºC are also shown in Fig. 2. Bioluminescence inhibition (I^{rel} <1) occurred in this experiment for majority of bacterial test samples. Hence, the temperature rise from 10ºC to 20ºC, with the latter being closer to the native bacterial temperature, made the microorganism more sensitive to low-dose gamma radiation. A possible reason for this may be higher rates of bacterial metabolic processes.

Fig. 2 demonstrates that monotonic dose dependency of I^{rel} was not observed at 20ºC. In contrast to 10ºC, the higher temperature conditions revealed ‘stochasticity’ of the bacterial response. The absence of linear dose vs. response dependence under low-dose exposure is usually associated with hormesis phenomenon (Calabrese, 2014; Baldwin and Grantham, 2015; Shi et al., 2016). Nevertheless, it is known that hormesis is characterized not only by inhibition, but also by low-dose stimulation (adaptive response), resulting in “either a J-shaped or an inverted U-shaped dose response”. This gamma low-dose exposure does not reveal any distinct activation throughout the experimental dose range (Fig. 2). However, studies of low-dose alpha- and beta-radioactive exposures (Rozhko et al., 2007; Selivanova et al., 2013; Selivanova et al., 2014) showed a distinct activation stage in the bioluminescence kinetics followed by the inhibition (toxic) stage. Higher energy, penetrative ability, and low ionization ability of gamma irradiation are likely reasons for the lower adaptive response in bacterial cells. Moreover, active chlorine species, that can be produced in physiological solutions under gamma-irradiation exposure (Mishra et al., 2016), might contribute to the toxic effect.

Nonlinear biological effects of low dose gamma-radiation were demonstrated previously in (Zhikrevetskaya et al., 2015). It was shown that the identified changes in lifespan and gene expression in *Drosophila melanogaster* are not dose-dependent.

The 20ºC experimental data shown in Fig. 2 were analyzed, and the I^{rel} values were plotted in accordance with the time of exposure and dose rate. The results are presented in Figs 3 and 4, respectively.

Fig. 3 shows that all bacterial samples exposed to different dose rate radiation had similar time-courses of the bioluminescence intensity. All the curves are of 'threshold' character. In time interval 19-47 h, the time-course dependencies were approximated by linear dependencies:

$$I^{rel} = a - b \cdot t.$$

Here, t is the time of exposure.

<Fig.3>

Angular coefficients of these dependencies (b) were calculated as 0.028, 0.029, 0.026, and 0.025 for dose rates of 150, 460, 1040, and 4100 μGy/h, respectively. Close values of these coefficients confirm that bacterial response was not dependent on the dose rates under the conditions of low-dose gamma-radiation exposure.

It is known that direct interaction of electromagnetic waves with biological components of cellular microorganisms under conditions of low-intensive gamma exposure is very low (Lampe, N. et al., 2016). Perhaps, a low interaction probability can be concerned with the low time-response decay in Fig.3.

In (Selivanova et al., 2014), the time of conversion of bioluminescence activation to inhibition was suggested as a test parameter to evaluate the toxicity of solutions of alpha- and beta-emitting radionuclides for marine bacteria. The present study demonstrates that in the case of gamma irradiation, the exposure time appeared to be critical for the marine bacteria, too. Hence, the exposure time can be used as a test parameter for radiotoxicity evaluation under conditions of chronic low-dose gamma irradiation. For example, this can be the time of 50% bioluminescence inhibition (at $I^{rel} = 0.5$), $t_{1/2}$. As can be seen from Fig. 3, the $t_{1/2}$ values for the bacterial preparation were within 35-38 h at all dose rates applied.

Results of the experiment are presented in Fig. 4 as dose rate dependence of I^{rel}. In this graph, the horizontal lines connect dots of equal exposure times. The graph confirms that the bioluminescence intensity depended on the time of exposure and did not depend on the dose rates.

<Fig.4>

A similar conclusion was made in a study by Selivanova et al. (2013), in which beta-emitting radionuclide tritium was used as a source of irradiation for luminous bacteria. The authors of that study found that the bioluminescent intensity depended on the time of exposure, but did not depend on radioactivity concentration of tritiated water in a wide range of its radioactivity concentrations: between 0.0002 and 200 MBq/L.

In contrast to our current results for gamma irradiation, tritiated water produced distinct (up to 100-150%) bioluminescence activation followed by the inhibition stage (Selivanova et al., 2013). Activation bacterial response was found in high diluted solutions of alpha-emitting radionuclide Am-241, too (Rozhko et al., 2007; Alexandrova et al., 2011). As discussed before, the differences observed in the case of low-dose gamma-ray exposure might be attributed to higher energy of gamma radiation, its lower ionization ability in aqueous media, and possibility to form active chlorine species in physiological solutions.

The results of the comparison approached to revealing the physico-chemical basis of the biological effects of different radiation types: beta- and alpha-radiations ionize aquatic media forming active radicals and ion-radicals, while gamma radiation is high-energy electromagnetic waves with lower ionization ability of water media.

Sequence analysis of bacterial DNA was performed for 16S ribosomal RNA gene of *P.Phosphoreum.* This gene was chosen for the genetic analysis as a model for evaluation of nonspecific DNA damage. The samples of the bacterial suspension exposed to low-dose gamma radiation (~0.2 Gy) were analyzed and compared with control samples. Under the conditions of the experiment no changes in the analyzed gene sequence were found.

Results of this study might be interpreted by using the novel "exposome" concept, which complements the genome and encompasses the totality of environmental (i.e. non-genetic) exposures (Rappaport and Smith, 2010; Wild, 2012). Although this term was initially introduced for human exposures, the study of simple model organisms might provide fundamental molecular, physicochemical, biochemical, and cellular bases for human exposure science.

It should be paid attention that delicate processes of genetic regulation in bacterial cells cannot be excluded. As reported in (Bolsunovsky et al., 2016), two bacterial tests based on *E.coli* and *S.typhimurium* cells demonstrated a complex response to chronic low-dose gamma-exposure; this response included the induction of SOS-chromotest response and mutation frequencies, followed by the attenuation of the effects at longer exposure times.

CONCLUSIONS

In this work, we investigated the low-intensive gamma radiation effects on the luminescence bacteria-based assay. Low dose effects of gamma radiation on the luminous bacteria were studied and compared with those of beta- and alpha- radiation that had been studied previously under comparable conditions.

We determined suitable conditions for evaluation of gamma-radiation effects. Three series of experiments were carried out: at 5°C, 10°C, and 20°C. The results showed the absence of authentic deviations from the control bacterial suspension for bacterial samples at 5°C and 10°C. The 20°C exposure revealed authentic bioluminescence inhibition. Higher rates of metabolic processes at higher temperature may make the bacteria more sensitive to gamma radiation.

Results of the 20°C exposure were analyzed in detail. The absence of the monotonic dose/effect dependencies was demonstrated under conditions of the experiment. Bioluminescence inhibition efficiency was found to be related to the exposure time, while no relationship to the dose rate was found. Similar effects were found earlier in experiments with luminous marine bacteria exposed to low-level alpha- and beta-radiation. However, in contrast to the alpha- and beta- exposures, gamma radiation exposure did not reveal distinct bioluminescence activation. Hence, time dependence of the bacterial response to low-level alpha and beta ionizing radiation can be discussed in term of 'radiation hormesis', while the low-level gamma radiation reveals the threshold time dependence. Such dissimilarity might be caused by higher energy of gamma radiation and its lower ionization ability in aqueous media.

Further experiments should elucidate the molecular and physicochemical basis for the difference in biological low-dose effects of alpha, beta, and gamma radiation. Apart from the quantitative evaluation, bacterial cells are a very convenient tool for a number of methods applicable to studying intracellular processes: from membrane penetrability to gene regulation, enzyme activity, ATP consumption and crystallinity of intracellular macrocomponents.

From the applied point of view, the temperature of 20°C was found to be optimal for using luminous marine bacteria in low-dose gamma-radiation toxicity monitoring, providing a convenient combination of radiosensitivity and duration of the bioassay procedure. As the exposure time appeared to be critical for the bacteria, it can be suggested as a test parameter for radiotoxicity evaluation under conditions of chronic low-dose gamma irradiation. For example, this can be the time for 50% bioluminescence inhibition.

The sequence analysis of 16S ribosomal RNA gene did not reveal a mutagenic effect of low-dose gamma radiation. The results of this study can be interpreted by using the novel approach based on the "exposome" concept of complementing the genome.

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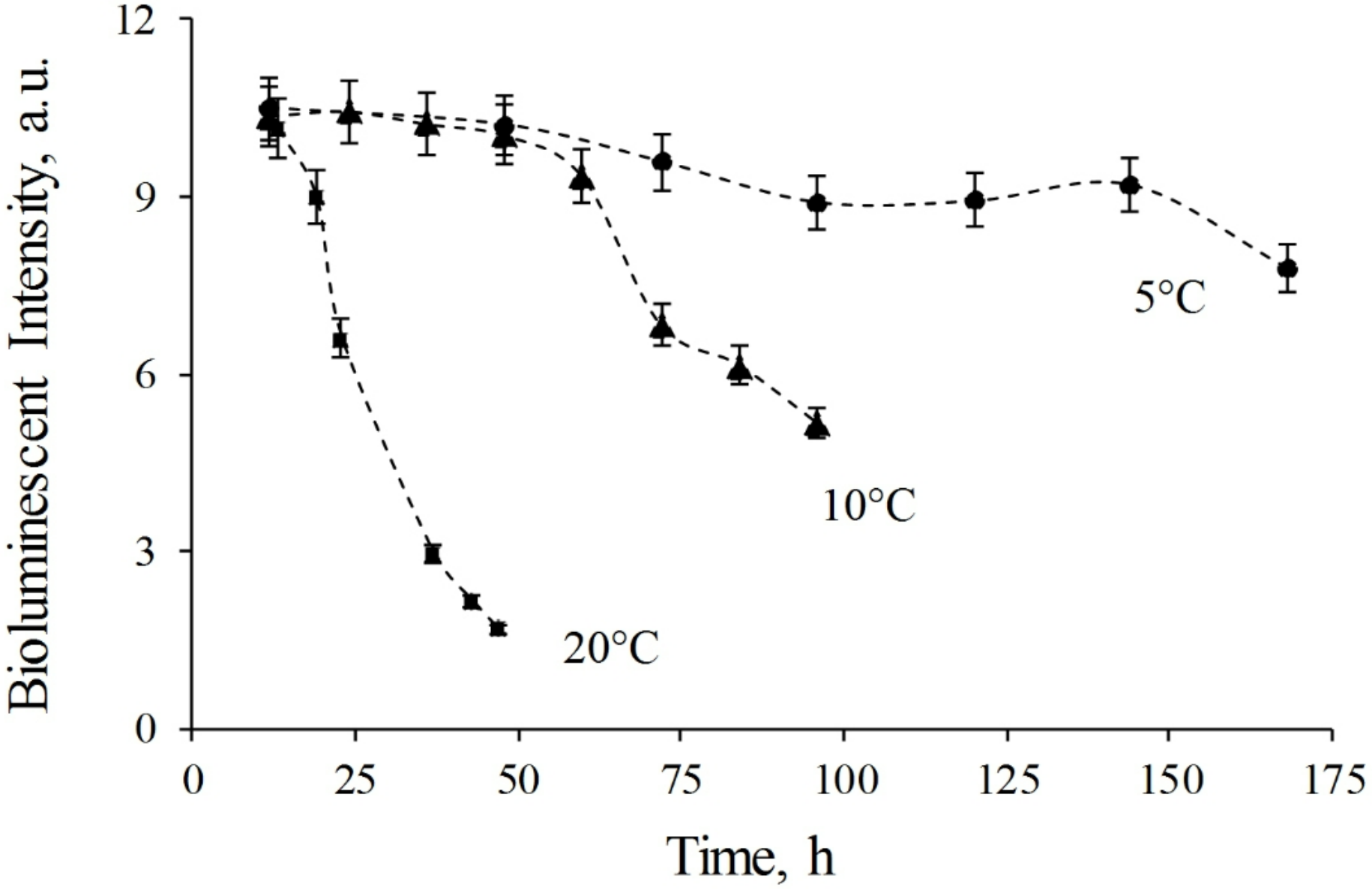
Figure Captions

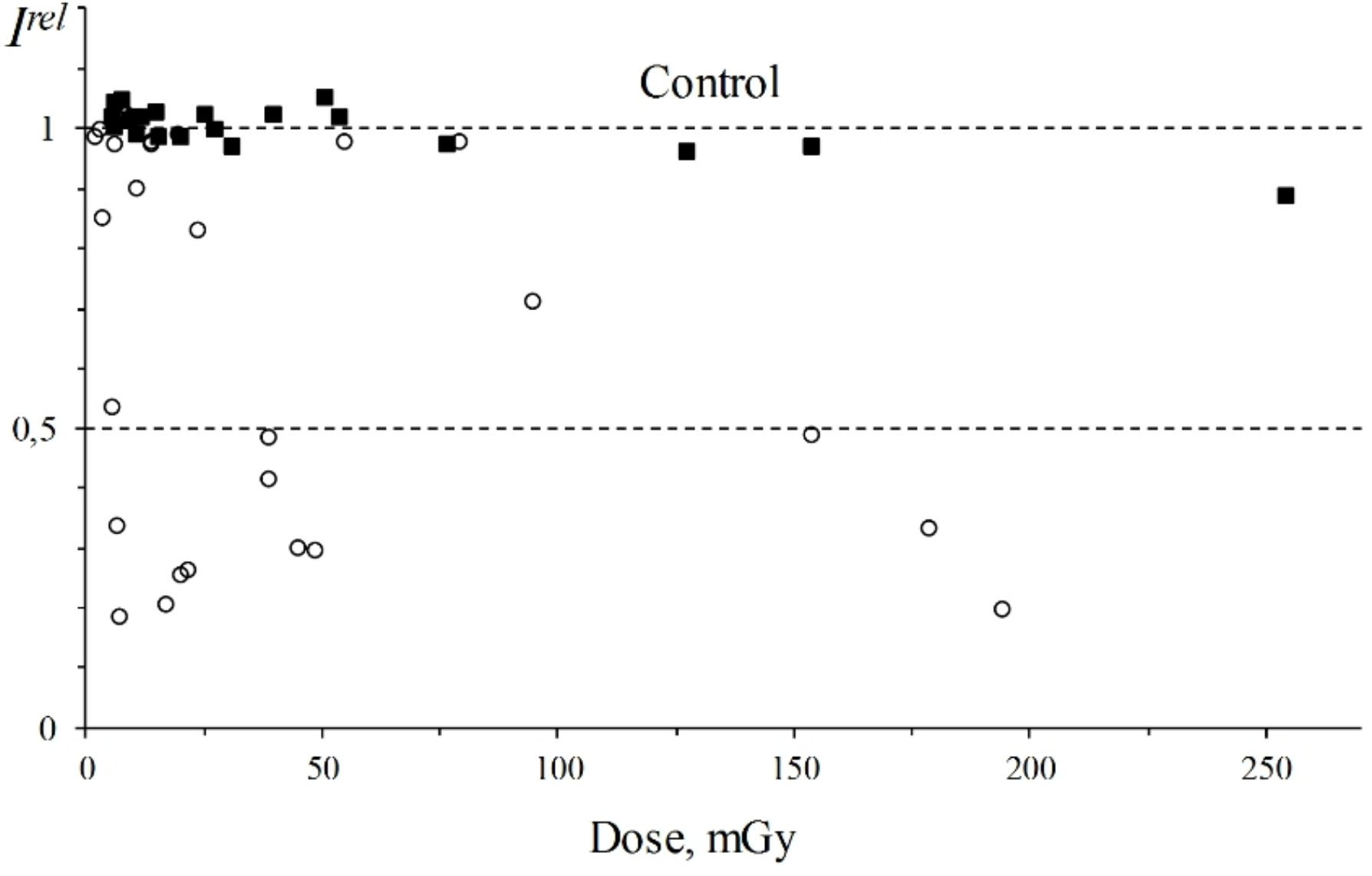
Fig. 1. Bioluminescent kinetics of *P.phosphoreum* at 5, 10, and 20ºC.

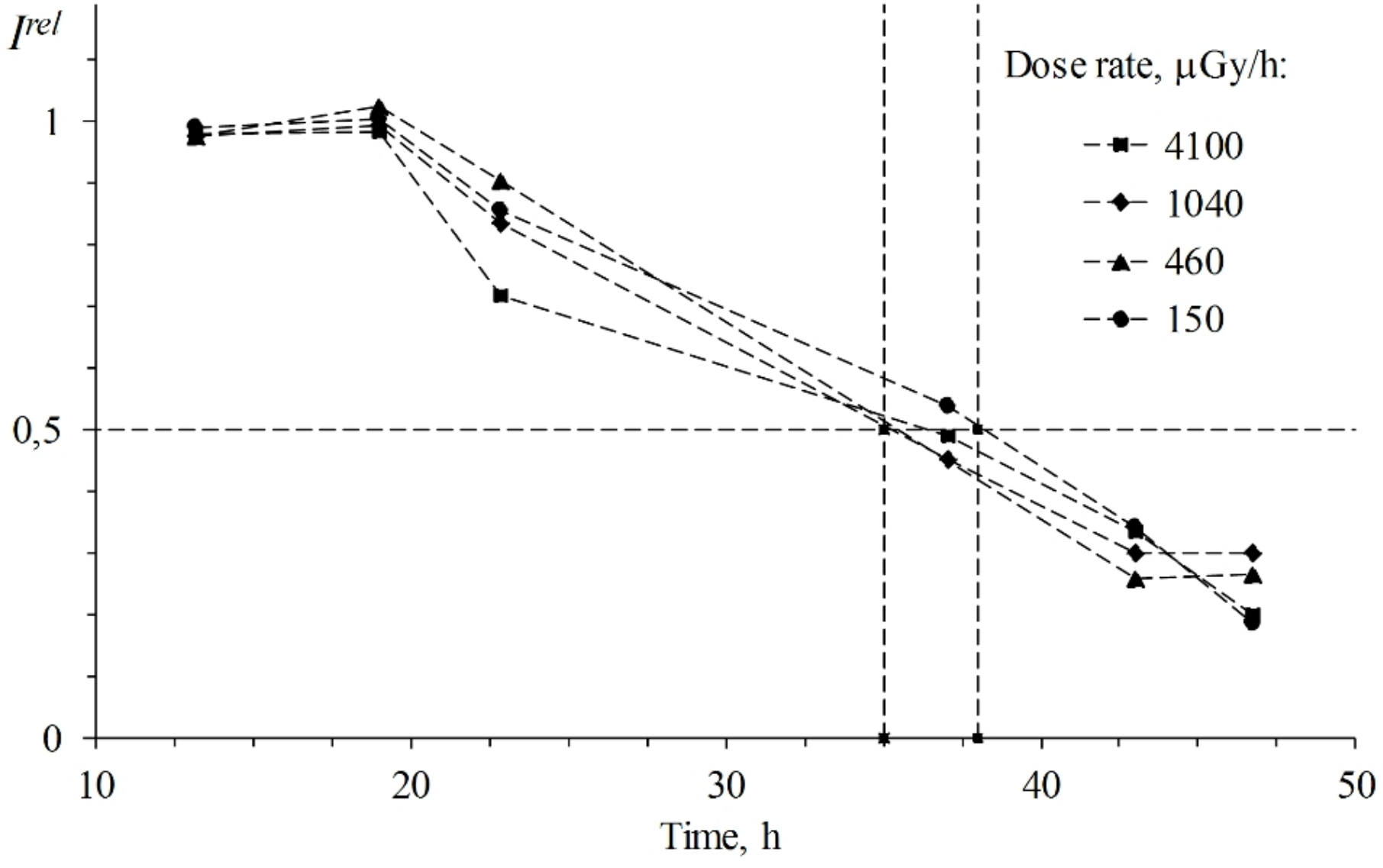
Fig. 2. Relative bioluminescence intensity of *P.phosphoreum*, I^{rel}, vs. dose of gamma irradiation. Temperature of the experiments: 10 ºC (■) and 20ºC (o). An error for I^{rel} measurements was 10%.

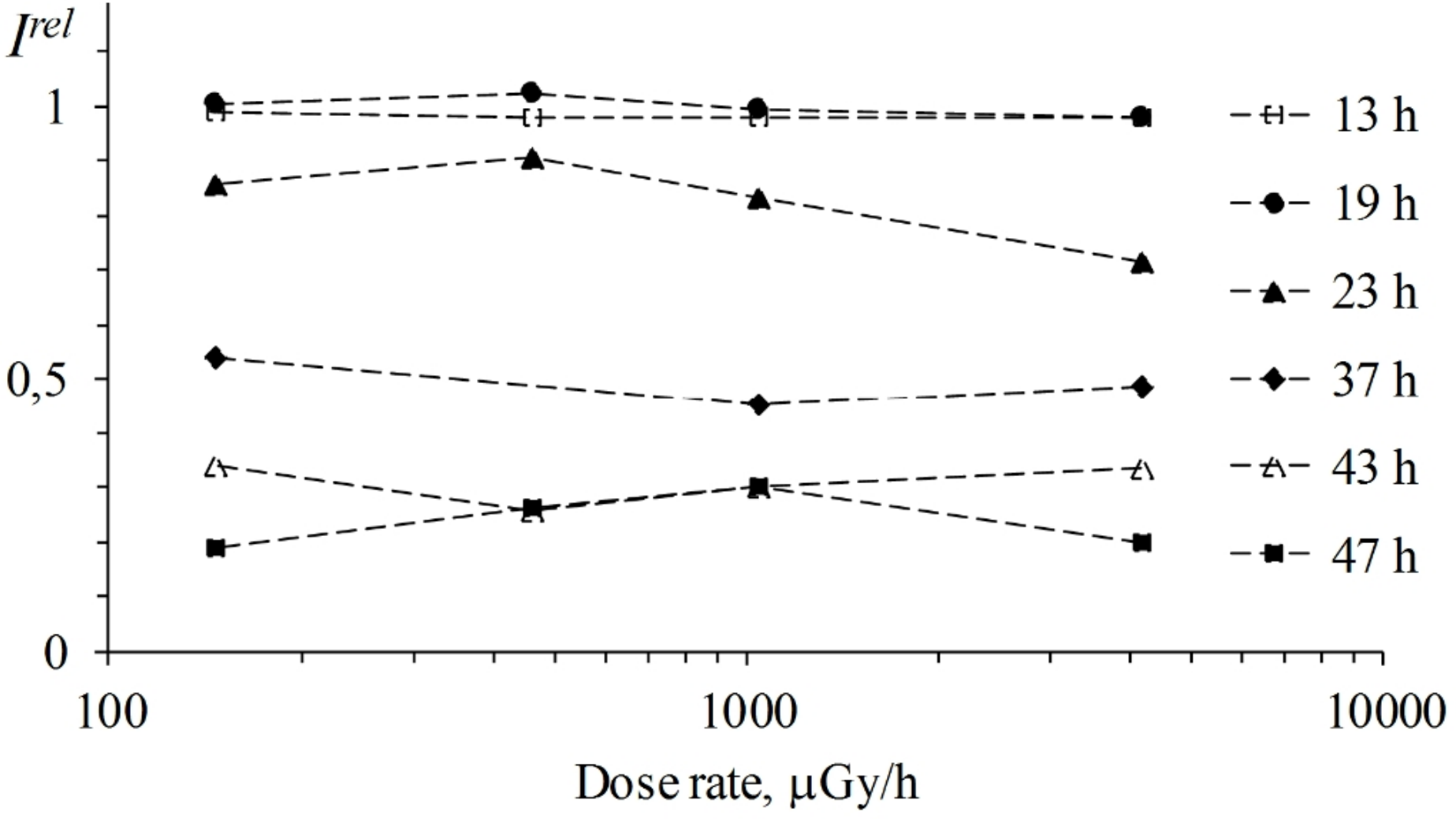
Fig. 3. Bioluminescence kinetics of *P.phosohoreum* exposed to gamma radiation of different dose rates, Particle 2, 20ºC. An error for I^{rel} was 10%.

Fig. 4. Relative bioluminescence intensity I^{rel} vs. gamma-radiation dose rate. Particle 2, 20ºC. Horizontal lines connect dots of equal exposure times. An error for I^{rel} was 10%

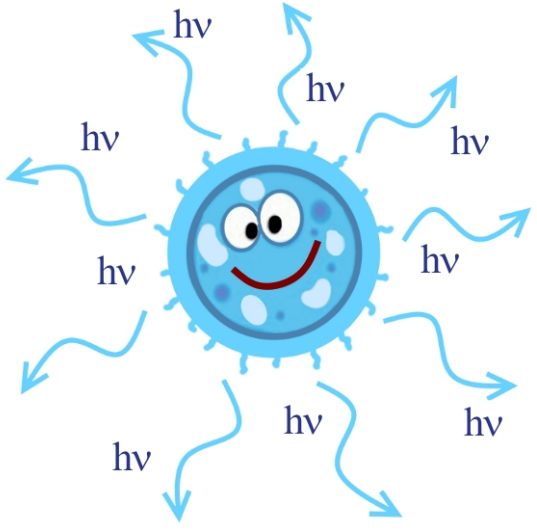

Bioluminescent Intensity, a.u.
12
9
6
3
0
0
25
50
75
100
125
150
175
Time, h
5°C
10°C
20°C


I^{rel}
Control
1
0,5
0
0
50
100
150
200
250
Dose, mGy


I^{rel}
1
0,5
0
10
20
30
40
50
Time, h
Dose rate, µGy/h:
4100
1040
460
150


I^{rel}
1
0,5
0
100
1000
10000
Dose rate, μGy/h
13 h
19 h
23 h
37 h
43 h
47 h

Photobacterium Phosphoreum



Low-dose gamma-radiation

≤ 250 mGy

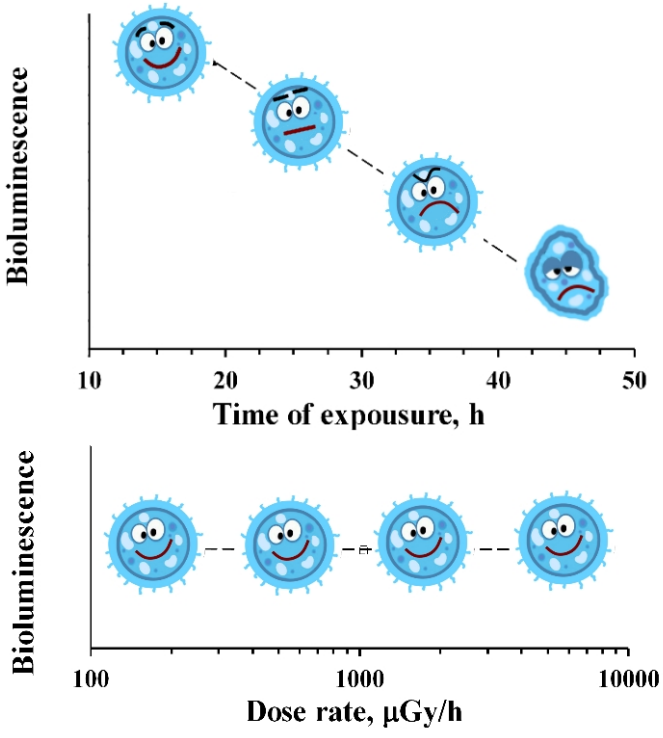


Table 1. Characterization of gamma-emitting ^{137}Cs-containing particles

	Particle 1	Particle 2
Radioactivity, MBq	0.41	12.4
Distances of bacterial samples from the particle, cm, (corresponding dose rates, μGy/h)	1.5(137); 3(34); 4.5(15); 6(8.6); 9(3.8);13.5(1.7); 19.5(0.8); 39(0.2)	1.5(4100); 2(2070); 3.5(830) 4.5(415); 6(244); 8.5(122)

Bacterial exposure to gamma radiation did not reveal monotonic dose-effect dependency

Luminous bacteria response to low dose gamma radiation depended on exposure time

Luminous bacteria response to low dose gamma radiation did not depend on dose rate

Luminescent activation was not observed under low dose gamma radiation exposure

Rise of temperature to 20°C makes bacteria more sensitive to low dose gamma radiation

Exposure of luminous marine bacteria to low-dose gamma-radiation

N.S. Kudryasheva[1,2*], A.S. Petrova[1,3], D.V. Dementyev[1], A.A. Bondar[4]

[1]Institute of Biophysics SB RAS, Federal Research Center 'Krasnoyarsk Science Center SB RAS', 50/50 Akademgorodok, Krasnoyarsk, 660036, Russia

[2]Siberian Federal University, 79 Svobodny Prospect, Krasnoyarsk, 660041, Russia

[3]Krasnoyarsk State Agrarian University, 90 Mira Prospect, Krasnoyarsk, 660049, Russia

[4]SB RAS Genomics Core Facility, Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, 630090, Russia

e-mail: n_qdr@yahoo.com