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Application of the luminous bacterium *Photobacterium phosphoreum* for toxicity monitoring of selenite and its reduction to selenium(0) nanoparticles

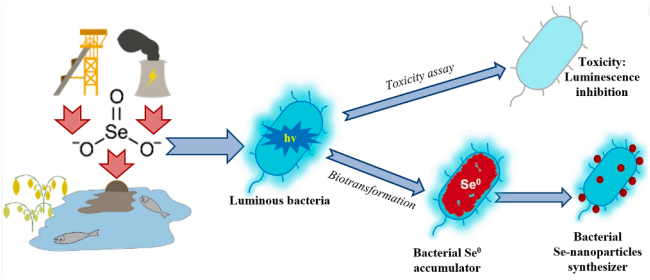
Andrei V. Zenkov [a], Ekaterina S. Sushko [a,b,c,*], Olga A. Mogilnaya [b], Mikhail N. Volochaev [c], Alexandr V. Shabanov [c], Alexander A. Kamnev [d], Anna V. Tugarova [d], Nadezhda S. Kudryasheva [a,b,*]

[a] School of Fundamental Biology and Biotechnology, Siberian Federal University, 660041 Krasnoyarsk, Russia
[b] Institute of Biophysics SB RAS, Federal Research Center "Krasnoyarsk Science Center SB RAS", 660036 Krasnoyarsk, Russia
[c] Institute of Physics SB RAS, Federal Research Center "Krasnoyarsk Science Center SB RAS", 660036 Krasnoyarsk, Russia
[d] Institute of Biochemistry and Physiology of Plants and Microorganisms, Saratov Federal Scientific Center of the Russian Academy of Sciences, 410049 Saratov, Russia

HIGHLIGHTS

- Luminescence of marine bacteria can be used to monitor toxicity of selenite ions.
- Bioluminescence activation and inhibition by SeO_3^{2-} is defined by ROS content.
- Bacteria change the ROS content in aqueous solutions of selenite.
- Marine bacteria reduce selenite ions to form elemental selenium.
- Luminous marine bacteria produce selenium nanoparticles.

GRAPHICAL ABSTRACT



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ABSTRACT

Luminous marine bacteria are traditionally used as a bioassay due to the convenience and high rate of registering the intensity of their physiological function – luminescence. This study aimed to develop the application of *Photobacterium phosphoreum* in traditional and novel fields – toxicity monitoring and biotechnology. We demonstrated (1) effects of selenite ions on bioluminescence, and (2) biotransformation of selenite to selenium(0) in the form of nanoparticles. The effects of selenite (SeO_3^{2-}) on the intensity of bacterial bioluminescence were studied, and its dependencies on exposure time and concentration of Na_2SeO_3 were analyzed. Bioluminescence activation and inhibition were revealed; dose–effect dependencies corresponded to the hormesis model. The toxicity of SeO_3^{2-} was characterized by an effective concentration of 10^{-3} M. Effects of SeO_3^{2-} on reactive oxygen species (ROS) in bacterial suspensions were studied. High positive correlations were found between the bioluminescence intensity and ROS content, which indicates the decisive role of ROS and associated redox processes in the bioeffects of selenite ions. Scanning and transmission electron microscopy revealed the presence of nano-structures in the bacteria exposed to selenite. The energy dispersion spectrum detected a high content of selenium in the nanoparticles. The particle size distribution depended on Na_2SeO_3 concentration; maxima of the distribution varied within 45–55 nm.

Abbreviations: NPs, Nanoparticles; ROS, Reactive oxygen species; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy.

* Corresponding authors at: 50/50 Akademgorodok, 660036 Krasnoyarsk, Russia.
E-mail addresses: kkovel@yandex.ru (E.S. Sushko), n-qdr@yandex.ru (N.S. Kudryasheva).

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1. Introduction

Selenium is a vital trace element. It is included in the active centers of various enzymes and protects organisms from oxidative stress. However, in high concentrations, selenium compounds can be acutely toxic [1–5]. The most toxic forms of selenium are its oxoanions – selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}). The toxicity of the oxoanions is related to their interaction with glutathione (an intracellular tripeptide, an active antioxidant) and the subsequent formation of reactive oxygen species (ROS) [5].

Natural selenium pollution occurs as a result of weathering of selenium-rich rocks and soils, as well as from volcanic eruptions. In addition, further industrial development can lead to high background selenium content. Selenium is released during the burning of fossil fuels, mining, agricultural activities, the use of nuclear fuel, oil production and metal smelting [1]. Getting into the air in the form of oxides, selenium is solubilized and passes into the soil and water in the form of oxoanions. The health-related consequences of selenium excess for humans are dermatitis, heart disease, joint pain, brittle nails [2]. Even a minor contamination with several micrograms of selenium can lead to impaired reproduction in fish [1]. A relation has been established between selenium levels and the development of type 2 diabetes mellitus [6].

There is a complete biogeochemical cycle of selenium compounds. Bacteria play a key role in this cycle, reducing selenium oxoanions. When the selenium content in the environment exceeds a critical level, the bacterial metabolism switches to neutralizing the excessive concentration of selenium oxoanions [1,2,7]. The oxoanions, reduced by bacteria to elementary selenium (Se^0), can form nanoparticles (NPs). Biogenic Se^0 NPs are known to be capped with biomacromolecules that provide stability to colloidal suspensions of such NPs [4], owing to a high adsorption capacity of Se^0 NPs and their ability to interact with functional groups of biomacromolecules: NH, C═O, COO^- and C—N [8]. The mechanisms of the formation of selenium NPs have not been fully elucidated. It is assumed that this occurs as a result of physicochemical processes – Ostwald maturation [9].

The bacterial ability to form Se^0 NPs is prospective in different fields of biotechnology [10,11] and medicine [10,12,13]. They can be used as carriers for antitumor drugs and radioprotectors in radiation therapy [14]. Selenium NPs obtained using *Bacillus licheniformis JS2* have been shown to inhibit proliferation and cause necrosis of prostate adenocarcinoma cells; however, they were not toxic for blood cells [12]. Selenium NPs formed by bacteria can be used in food additives, providing human with bioavailable selenium. Lactobacilli with Se^0 NPs revealed an immunostimulating effect, prolonging the life of mice with cancer [13], and were also used to produce selenium-enriched yogurt [15]. There is an evidence of antimicrobial properties of selenium nanoparticles for gram-positive and gram-negative bacteria [11,16]. Additionally, Se^0 NPs are prospective for battery materials and photovoltaic devices [17].

The composition, properties and structure of selenium NPs obtained with bio-reduction of selenite by *Azospirillum brasilense* Sp7 and *Azospirillum thiophilum* BV-S strains were studied earlier [18–20]. It was shown that NPs included the amorphous modification of selenium and contained biomacromolecules (proteins, polysaccharides, and lipids) at the surface; the zeta potential of NP suspensions ranged from −18.5 to −23.7 mV, the size varied from 30 to 200 nm as a function of selenite concentration in the medium.

A large variety of other biotransformation methods and organisms capable of reducing selenium oxoanions have recently been reviewed [10], with discussions of possible reduction mechanisms, diversity and distribution of selenium-transforming microorganisms, mixed cultures, bioreactors, and biofilms including heterogeneous structures (e.g., containing diatoms or filamentous algae), multidisciplinary techniques used to characterize selenium speciation and Se(0)-containing NPs, bioremediation technologies applied to excessively Se-polluted environmental sites, as well as diverse biotechnological applications of Se-containing nano-sized biogenic products. In addition, a very recent comprehensive review [21] outlined the processes of selenium biovolatilization by microorganisms, microalgae, and plants as effective means of selenium removal with a promising future. It is shown that selenium-reducing bacteria can grow and function at higher concentrations than selenium-reducing plants. Therefore, bacterial bioremediation of selenium-polluted environments is of high interest.

Luminous marine bacteria can serve as an effective biological object for multifunctional applications involving toxicological monitoring of selenium oxoanions in waste waters, biotransformation of oxoanions, and production of selenium NPs. Luminous marine bacteria have been used as a toxicity bioassay for several decades [22–25]. The bioassay based on luminous bacteria was described first in 1969 by Kossler as reported in [26]. In the 1980s the bioassay was standardized in Germany as a method for pollutant detection. The tested parameter here is the bioluminescence intensity; it can be easily measured instrumentally with simple photophysical devices. High rates of registration and simplicity provide for simultaneous analyses of a series of tested samples resulting in a proper statistical treatment. Advanced research on the mechanisms of toxic effects of exogenous compounds with different structures is one more advantage of this bioassay [24,25,27,28]. The review [27] classified the mechanisms of influence of toxic compounds on the bacterial bioluminescence reactions at physical, chemical and biochemical levels; the effects were related to energy, electron, and hydrogen transfer efficiency, as well as interactions with substrates and enzymes. Additionally, the changes in membrane functions and coupled metabolic processes were considered to be important mechanisms of the toxicant's influence. The effects of groups of compounds were considered – organic oxidizers, dyes, salts of stable and radioactive metals [27,29,30]. The use of bioluminescent systems to evaluate the detoxifying (anti/pro-oxidant) ability of physiologically active compounds is being developed, taking examples of humic substances [31–35], fullerenols [34,36–41], iron-based nanostructures [42,43], surfactants [44], and oil refining waste [45].

The present work aimed at studying the effects of sodium selenite (Na_2SeO_3) on bacterial bioluminescence, as well as the bacterial ability to reduce selenite and accumulate selenium(0) in the form of NPs. We studied bacterial bioluminescence dependencies on time of exposure and concentration of selenite. Toxic and activation effects were determined and characterized. The content of ROS was measured in the experimental bacterial suspensions; correlations between concentrational dependencies of bioluminescence intensity and ROS content were analyzed, and the role of ROS in the bioeffects of selenite on the bacteria was revealed. Scanning and transmission electron microscopies were used to evaluate and characterize selenium NPs formed by the bacteria.

2. Materials and methods

2.1. Reagents

The reagents for bacterial cultivation were: peptone and yeast extract from Dia-M, Moscow, Russia; NaCl from Khimreactiv, Nizhny Novgorod, Russia; $MgCl_2{\cdot}6H_2O$, $CaCl_2$, and KCl from Pancreac AppliChem GmbH, Darmstadt, Germany; agar from Difco Laboratories, Detroit, MI, USA. To prepare solid semisynthetic medium, «GRM-Agar» from PMB, Obolensk, Russia, was used. The reagents for chemiluminescence assay were: luminol ($C_8H_7N_3O_2$); $K_3[Fe(CN)_6]$ from Sigma-Aldrich, St. Louis, MO, USA; KOH from Khimreactiv, Nizhny Novgorod, Russia; 3 % aqueous solution of H_2O_2 (v/v) from Tula Pharmaceutical Factory (Tula, Russia). Sodium selenite (anhydrous Na_2SeO_3) was obtained from Lenreactive, Saint-Petersburg, Russia.

All the reagents were of analytical or chemical grade and used as received.

2.2. Bacterial luminescence assay

The intact marine luminous bacterium, strain *Photobacterium phosphoreum* 1883 IBSO [46], was used as a bioassay to evaluate the effects of selenite on the bacterial cells. The strain was obtained from the Collection of Luminous Bacteria CCIBSO-863, Institute of Biophysics SB RAS, Krasnoyarsk, Russia.

2.2.1. Bacterial growth conditions

For cultivation of *P. phosphoreum*, a liquid semisynthetic medium containing 10 g/L Peptone, 28.5 g/L NaCl, 4.5 g/L $MgCl_2{\cdot}6H_2O$, 0.5 g/L $CaCl_2$, 0.5 g/L KCl, 3 g/L yeast extract was used. The solid semisynthetic medium contained 38 g/L «GRM-agar»; also, 10 g/L agar and 26 g/L NaCl were added.

P. phosphoreum was plated on 25 mL of semisynthetic agar and incubated at 25 °C for 24 h (stationary growth phase, corresponding to maximum bioluminescence) in an incubator (WIS-20R, WiseCube Laboratory Instruments, Wertheim, Germany).

2.2.2. Luminescence measurements

Bioluminescence kinetics was studied in the presence of bacteria; chemiluminescence kinetics was studied either in the presence of bacteria or in bacteria-free media. Chemiluminescence was registered immediately following the bioluminescence measurements in the same bacterial samples.

(1) Bacterial media: bacteria (control samples); bacteria + Na_2SeO_3 (experimental samples). Bacterial suspensions were prepared as follows. Control samples: 45 μL of bacterial suspensions were added to 155 μL of 3 % NaCl solution (w/w). Experimental samples: 45 μL of bacterial suspensions and 20 μL of Na_2SeO_3 (10^{-8}–10^{-1} M) were added to 135 μL of 3 % NaCl solution.
(2) bacteria-free media: 3 % NaCl solution (control samples); 3 % NaCl solution + Na_2SeO_3 (experimental samples). The bacteria-free media were prepared as follows. Control samples: 200 μL of 3 % NaCl solution. Experimental samples: 20 μL of Na_2SeO_3 were added to 180 μL of 3 % NaCl solution.

Na_2SeO_3 was dissolved in 3 % solution of NaCl.

Optical densities of all Na_2SeO_3 solutions did not exceed 0.1 in the maxima of the bioluminescence and chemiluminescence (490 and 425 nm, respectively); hence, the effect of «optical filter» was excluded [47].

(A) Bioluminescence assay system and experimental data processing

To investigate the effects of selenite on the bacterial cells, standard procedures for bioluminescence measurements were used [46,48]. Prior to experimentation, the bacteria were collected at the stationary growth phase (Section 2.2.1) by pipetting 3 % NaCl solution directly onto the agar to release the bacteria. The bacterial suspension was diluted to Abs_{660} = 0.1 and stored at 4 °C for 30 min to allow bioluminescence stabilization prior to experimentation. The 3 % NaCl solutions were used to imitate a marine environment for the bacterial cells and to maintain osmotic processes.

The biological effects of selenite on bioluminescence of the bacterium were characterized by the relative bioluminescence intensity, I^{rel}:

$$I^{rel} = I_{Se}/I_{contr} \quad (1)$$

where I_{Se} and I_{contr} are the maximum bioluminescence intensities in the presence and absence of Na_2SeO_3, respectively.

The time courses of I^{rel} were studied at different concentrations of Na_2SeO_3 for 18 h. The effective concentration of Na_2SeO_3 inhibiting the bioluminescence intensity by 50 % (I^{rel} = 0.5), EC_{50}, was determined to evaluate its toxic effect.

(B) Chemiluminescence assay for evaluating the content of reactive oxygen species

We used the luminol chemiluminescence assay [49,50] to evaluate the content of ROS in the experimental bacterial media and bacteria-free media (3 % NaCl solutions). This technique determines an integral content of ROS, assuming that there occurs a dynamic equilibrium among different types of ROS. The calibration dependency was preliminarily determined as chemiluminescence intensity vs·H_2O_2 concentration in 3 % NaCl solution; the dependency was used to determine the content of ROS in experimental solutions; H_2O_2 was considered as a representative of the ROS family.

The stock luminol solution (10^{-2} M) was prepared as follows: luminol powder was dissolved in 5 mL 1 M solution of KOH, and then, 5 mL of distilled water was added. To provide chemiluminescence reaction, 70 μL of luminol solution (10^{-4} M) were added to the bacterial media or bacteria-free media (pH 11.24), Section 2.2.2 (1) and (2). Then, the chemiluminescence luminol reaction was initiated by adding 40 μL of $K_3[Fe(CN)_6]$ solution (10^{-3} M) through the injection system, and the maximum value of the chemiluminescence intensity was determined in bacterial media and in bacteria-free media. The concentrations of luminol and $K_3[Fe(CN)_6]$ in the experimental samples were 2.26×10^{-5} M and 1.3×10^{-4} M, respectively.

Chemiluminescence intensity was used to calculate the ROS content in the experimental solutions via the calibration dependency. Relative values of ROS content were calculated as ratios of the ROS content in experimental solutions, ROS_{Se}, to that in the control solutions (without selenite), ROS_{contr}:

$$ROS^{rel} = ROS_{Se}/ROS_{contr} \quad (2)$$

ROS^{rel} values were evaluated in the bacterial media and bacteria-free media at different concentrations of Na_2SeO_3 (10^{-8}–10^{-1} M) and various times of exposure. Kinetics of ROS^{rel} was plotted.

2.2.3. Formation of selenium NPs

Prior to experimentation, bacteria were collected at the stationary growth phase (Section 2.2.1) by pipetting of 3 % NaCl solution directly onto the agar to release bacterial cells. Biogenic selenium NPs were obtained by adding solutions of 5, 25, 100 mM selenite to the purified bacterial cells according to [18]. Cell suspensions were obtained by washing with sterile 3 % NaCl. Optical density of bacterial suspensions (Abs_{660}) was 1.0. After washing, the bacterial suspension was centrifuged (6000*g*, 5 min) and washed twice with a sterile 3 % NaCl solution.

Solutions of 5, 25, and 100 mM Na_2SeO_3 were prepared in sterile 3 % NaCl; the solutions were added to the cell pellets, which were resuspended. The samples were incubated for 24 h at 24 °C in a thermostat (TSO-1/80 SPU, SKTB-SPU, Russia).

2.2.4. Microscopy

Bacterial samples for electron microscopy (bacterial suspensions in the absence and presence of selenite) were prepared as described in Section 2.2.2A. All samples were allowed to react for 24 h prior to fixation.

Samples for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were purified twice with NaCl solution (3 %) and phosphate-saline buffer by centrifugation (6000*g*, 5 min). Bacterial samples for SEM were prepared according to the protocol described in [51]. The samples were initially fixed with 2.5 % glutaraldehyde solution (1:1 v/v); after 4–19 h they were purified with distilled water and dehydrated in ethanol solutions.

SEM supports were used according to a method developed for biological objects on inverse opals, as previously described in [52]. Briefly, silica inverse opals were prepared by a sol–gel method to study biological objects. Submicron spherical particles were obtained from polymethylmethacrylate to produce 100–500 nm particles that were

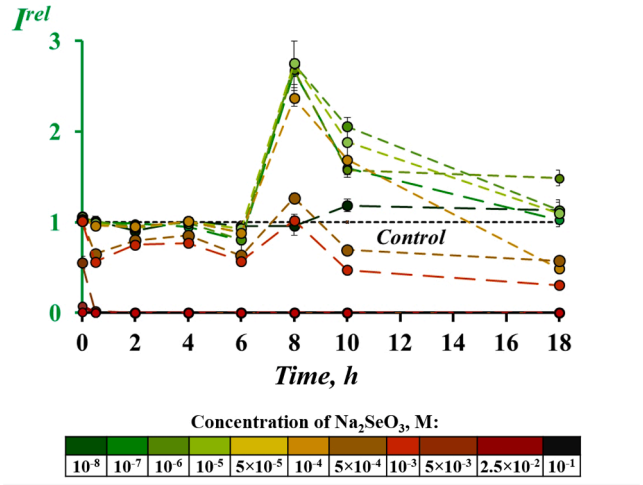

Fig. 1. Relative intensity of bacterial luminescence, I^{rel}, at different concentrations of selenite. «Control» corresponds to the absence of selenite.

ordered into an inverse opal crystal matrix. This created an open system of pores up to 400 nm in size that was used for sample adsorption.

TEM monitoring of the samples was carried out using the scanning-transmission (STEM) mode for better visualization of bacteria and NPs. Bacterial suspensions were deposited on supporting copper grids with a transparent carbon coating and dried at room temperature. Elemental analysis of the extracellular NPs was identified by energy dispersive spectroscopy (EDS). Logarithmic normal size distribution of selenium NPs was calculated using OriginPro 2022.

2.2.5. Bioluminescence spectra measurements

Bacterial samples for bioluminescence spectral measurements were prepared as described in Section 2.2.2A. Bioluminescence spectra were recorded in the range from 370 to 600 nm. The scanning rate was 600 nm/min and the spectral bandwidth was 10 nm, with a wavelength accuracy ±1.5 nm. A quartz cuvette with a rectangular cross-section (2 × 10 mm) was used to register the spectra. The spectra were measured at 20 °C for 12.5 s. OriginLab 2018 (FFT, 15 points) was used for smoothing the spectra. Final spectra were averaged over 10 spectral measurements.

2.3. Equipment

Luminoskan Ascent (Thermo Electron Corporation, Solon, OH, USA) was used to measure both the bioluminescence and chemiluminescence intensities. All the luminescence measurements were carried out at 25 °C. The optical density of the sodium selenite solutions and that of the bacterial suspensions were measured using a double-beam spectrophotometer UVIKON-943 (KONTRON Instruments, Milano, Italy). Bioluminescence spectra were measured with a Cary Eclipse-2000 spectrofluorometer (Agilent, Santa Clara, USA).

A scanning electron microscope SU3500 (Hitachi, Tokyo, Japan) was used to visualize bacterial cells and inorganic particles (morphological analysis). Visualization of the shape and size and EDS elemental analysis of extracellular NPs were performed using a transmission electron microscope HT7700 (Hitachi, Tokyo, Japan) equipped with a Bruker Xflash 6 T/60 energy dispersive spectrometer.

2.4. Statistical processing

All the bioluminescence and chemiluminescence measurements were conducted in 5 replicates for all the solutions. Standard errors for I^{rel}, ROS^{rel} were calculated and presented in Figures; they did not exceed ±15 %.

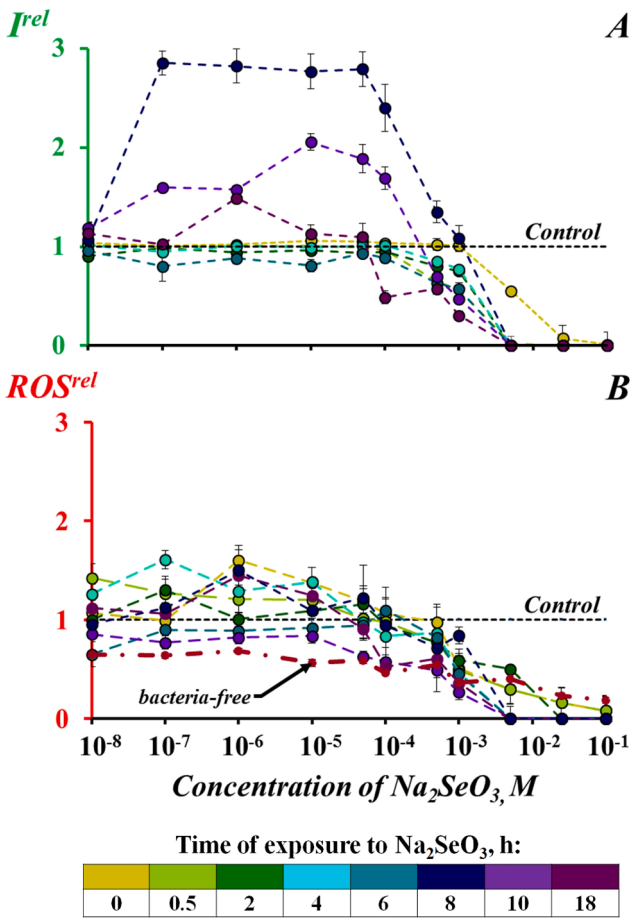

Fig. 2. Dependence of the relative bioluminescence intensity, I^{rel} (A), and relative ROS content, ROS^{rel} (B), in the bacterial suspension on Na_2SeO_3 concentration. «Control» corresponds to the absence of Na_2SeO_3. The ROS content in the control bacterial suspension was 3.7×10^{-7} M; in bacteria-free media (3 % NaCl solution) – 7.6×10^{-7} M.

To evaluate correlations between time courses of relative bioluminescence intensity and the ROS content (I^{rel} and ROS^{rel}, respectively), a statistical dependency between the rankings of the two variables was analyzed [53], and Spearman's rank correlation coefficients (r) were calculated. The application of this method was justified with a moderate kit of data sets, as well as a lack of normal distribution of I^{rel} and ROS^{rel}.

3. Results and discussion

3.1. Effects of sodium selenite on bacterial luminescence

We monitored the bacterial bioluminescence intensity in solutions of sodium selenite, Na_2SeO_3. Fig. 1 presents time courses of the bacterial luminescence intensities in the presence of selenite at different concentrations. The dependencies demonstrate delays of the bioluminescence response (till 6 h of exposure) with the following bioluminescence activation up to 270 % (10^{-7}–5×10^{-4} M). Selenite concentrations exceeding 5×10^{-4} M did not activate bioluminescence; bioluminescence inhibition only was observed, thus revealing toxicity of the selenite solutions.

The results of the experiment are presented in Fig. 2A in other coordinates: bioluminescence intensity, I^{rel}, vs. selenite concentration. This Figure also illustrates intervals of bioluminescence activation ($I^{rel} > 1$) and inhibition ($I^{rel} < 1$, toxic effect). According to Fig. 2A, the EC_{50} value appeared to be ca. 10^{-3} M.

The response of the bacteria to selenite corresponds to the generally

accepted model of «hormesis» [54–59]. This model includes three stages of a biological dose-dependent response: stress recognition (I), activation (II), and suppression of physiological functions, i.e., toxic effect (III). The activation stage is a necessary attribute of the hormesis model. Other models (linear and threshold) can be considered as simplified derivatives of hormesis. It should be noted that low-concentration activation usually does not exceed 50–60 %, however, our experiment demonstrated very effective activation of bioluminescence (up to 200 %, Fig. 2A). Previously, we observed similar values only in the case of low-dose exposure to radionuclide tritium [60].

As Fig. 1 shows, the bioluminescence kinetics includes two stages (I and II) in the Na_2SeO_3 concentration interval 10^{-7}–10^{-4} M. Higher concentrations of Na_2SeO_3 correspond to threshold or linear models and involve stage I and III, or stage III only, respectively. The hormesis effect is evident from the dependence of I^{rel} on Na_2SeO_3 concentration, Fig. 2A: lower concentrations of Na_2SeO_3 stimulate a compensatory effect in bacteria that is visualized as bioluminescence activation ($I^{rel} > 1$).

Hormesis effects of selenium compounds were demonstrated previously for red-winged blackbirds [61]. In [62], the hormetic effect was attributed to the chelating ability of selenium.

The role of ROS in redox-active processes in biological systems might be essential. This is a reason why we analyzed the ROS content in bacterial suspensions at different concentrations of Na_2SeO_3. The ROS content, ROS^{rel}, should be compared to the characteristics of bacterial physiological activity – bioluminescence intensity, I^{rel} (Fig. 2B and A, respectively).

Fig. 2B presents the ROS content in bacteria-free solutions of Na_2SeO_3, as shown with dark-red curve. It is seen that selenite suppresses the ROS content ($ROS^{rel} < 1$) within the whole concentration range. This effect might be explained by the chemical oxidation of SeO_3^{2-} to SeO_4^{2-} with concomitant consumption of ROS (as oxidizing agents) from aqueous media.

It is seen that addition of the bacteria to these solutions increases the ROS content at selenite concentrations $<10^{-3}$ M and suppresses it at higher concentrations (Fig. 2B). These effects might be explained by the ability of bacteria to compensate for the lack of ROS in the medium through metabolic activity at moderate stress levels (i.e., at lower concentrations of Na_2SeO_3), and by the loss of this ability at higher stress levels (i.e., at higher concentrations of selenite).

It is noticeable that a concentration of ca. 10^{-3} M demonstrates an evolution of bioluminescence activation (or absence of the effect) to bioluminescence inhibition, Fig. 2A. Values of correlation coefficients r between concentrational dependencies of ROS^{rel} and I^{rel} (Fig. 2A and B) are positive and mostly of high value: 0.83, 0.97, 0.80, 0.66, 0.77, 0.89, 078, and 0.98 for exposure times 0, 0.5, 2, 4, 6, 8, 10, and 18 h, respectively. They show that changes in the intensity of bioluminescence (inhibition and activation) are concerned with changes in the content of ROS in bacterial suspensions. Similar results were obtained earlier for other compounds [40,41], and similar conclusions were drawn.

Hence, as an outline, luminescence of marine bacteria is sensitive to selenite; their dose response corresponds to the hormesis model and can include activation or inhibition phases, as well as a delay in the response. Toxicity of SeO_3^{2-} is characterized by the effective concentration $EC_{50} = 10^{-3}$ M. Selenite reduces the ROS content in aquatic environments; bacteria can change this effect by increasing or decreasing the ROS content. Correlations between concentration dependencies of ROS^{rel} and I^{rel} confirmed that the changes in the intensity of bacterial bioluminescence (inhibition and activation under exposure to selenite) is determined by the ROS content in the bacterial suspensions.

The bioluminescence spectra of the bacterial samples, that were used to monitor effects of Na_2SeO_3 on the formation of electron-excited states in intracellular enzymatic reactions, did not change with time (not shown). All spectra contained a structureless band with a maximum at 481 ± 3 nm and a width at half maximum ca. 83 ± 3 nm. A typical bioluminescence spectrum is presented in Fig. 3. Similarity of the spectra both for the control sample and in the presence of SeO_3^{2-} shows that it influences the bacterial bioluminescence at stages prior to the formation of electron-excited states [63].

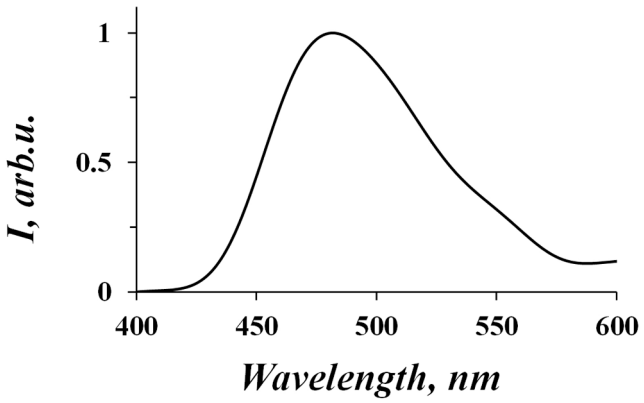

Fig. 3. A typical bioluminescence spectrum of *P. phosphoreum*.

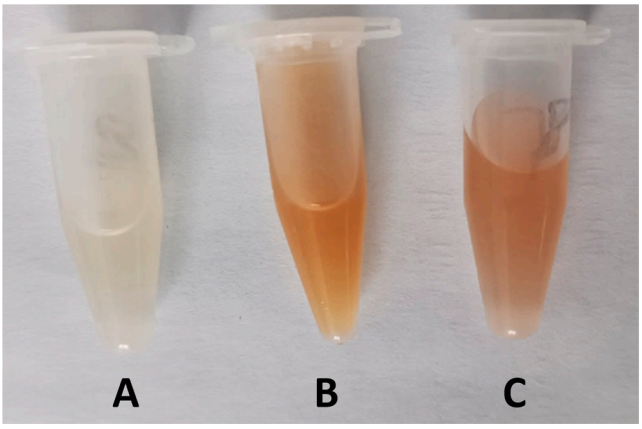

Fig. 4. Suspensions of *P. phosphoreum* (in 3 % NaCl) after 7 days of exposure to Na_2SeO_3. Concentrations of Na_2SeO_3: A – 0 (control), B – 10^{-3} M, C – 2.5 × 10^{-3} M.

3.2. Microscopy of bacterial cells

In preliminary studies, we tested the ability of *P. phosphoreum* to transform Na_2SeO_3 and accumulate elementary selenium(0). As is known [10,18], the formation of selenium NPs by bacterial cells should be accompanied by color change (commonly a reddish-orange tint). Our experiments revealed similar color changes, as illustrated by Fig. 4.

Microscopic images of bacterial cell were obtained to reveal (1) changes in the morphology of cells exposed to SeO_3^{2-}, (2) biotransformation of water-soluble Na_2SeO_3 to insoluble NPs; (3) size distribution of NPs; (4) the elemental composition of NPs using EDS analysis. Toxic concentrations of Na_2SeO_3, which resulted in bioluminescence inhibition, were chosen: 5 × 10^{-3}, 2.5 × 10^{-2}, and 10^{-1} M. Unexposed bacterial cells were used as control samples.

Control samples of the bacteria had an average diameter 0.5–1.0 μm, Fig. 5A. Fig. 5B presents a bacterial SEM image after 24 h exposure to selenite. No changes in bacterial morphology were found; however, white inorganic inclusions were identified on the surface of the cells, which could be presumably attributed to NPs of elementary selenium(0).

TEM was applied to confirm biotransformation of water-soluble Na_2SeO_3 to selenium NPs, to study the size distribution of the NPs, and to determine the elemental composition of the NPs. Bacterial TEM images are presented in Fig. 6. Electron-dense inclusions of spherical shape on the bacterial images are evident in Fig. 6B and C.

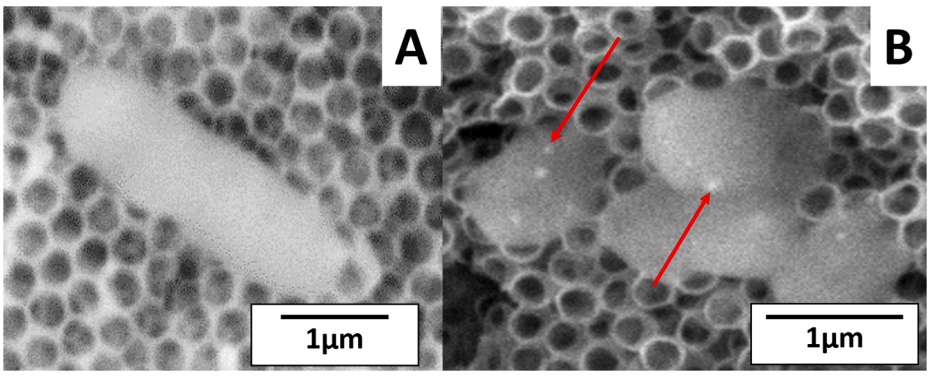

Fig. 5. SEM images of *P. phosphoreum;* A – control sample, B – after 24 h exposure to Na_2SeO_3 (2.5×10^{-2} M).

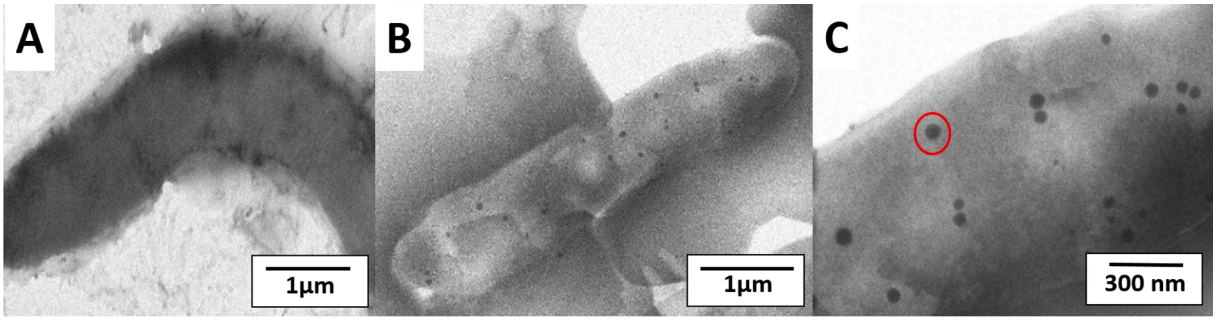

Fig. 6. TEM images of *P. phosphoreum*. A – control sample; B – after 24 h exposure to Na_2SeO_3 (2.5×10^{-2} M); C – magnified fragment of image B; the circle marks the area analyzed by EDS.

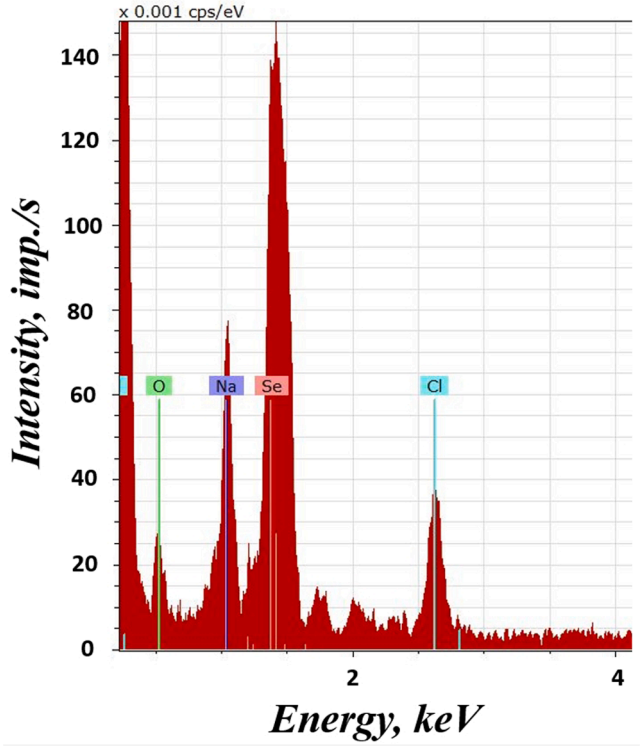

Fig. 7. Energy dispersive spectrum of the element composition of the NP environments.

To prove biotransformation of SeO_3^{2-} to selenium(0) NPs, an energy dispersive spectrum of the element composition was measured; the results are shown in Fig. 7. The cell surface region analyzed is marked with a circle in Fig. 6C.

The spectrum obtained from the nanoparticle area indicates a high content of selenium (Fig. 7), whereas the spectra obtained from areas without particles (not shown) contain several times less selenium, which can be attributed to the residual content of Na_2SeO_3 in the bacterial culture.

Size distributions of the NPs were of lognormal character; they are presented in Fig. 8**.** It is seen that the rise in Na_2SeO_3 concentration shifts the distribution to lower-size NPs: maxima of the distributions were 55, 50 and 45 nm for 5×10^{-3} M (Fig. 8A), 2.5×10^{-2} M (Fig. 8B), and 10^{-1} M (Fig. 8C), respectively. Note, that a similar, although more strongly expressed effect had been reported earlier for Se^0 NPs obtained by selenite reduction using bacteria of the genus *Azospirillum* [18]. Narrowing of the distribution was observed in the same series: the half-abundance widths of the distributions were 40, 28, and 23 nm, respectively.

As an outline, two bacterial imaging techniques were used – SEM and TEM. After incubation with selenite, the cells retained their morphological characteristics. However, exposure to SeO_3^{2-} resulted in the formation of clearly visible NPs. The use of the two microscopy techniques, including EDS analysis, has shown and confirmed the formation of selenium(0) NPs; SEM images confirm that NPs are localized on the surface of bacteria. In SEM images they look like whitish granules, in TEM they are electron-dense and are visualized as dark round spots. Maxima of size distribution varied from 45 to 55 nm as a function of the initial selenite concentration. The energy dispersive spectrum confirmed a high content of selenium in NPs.

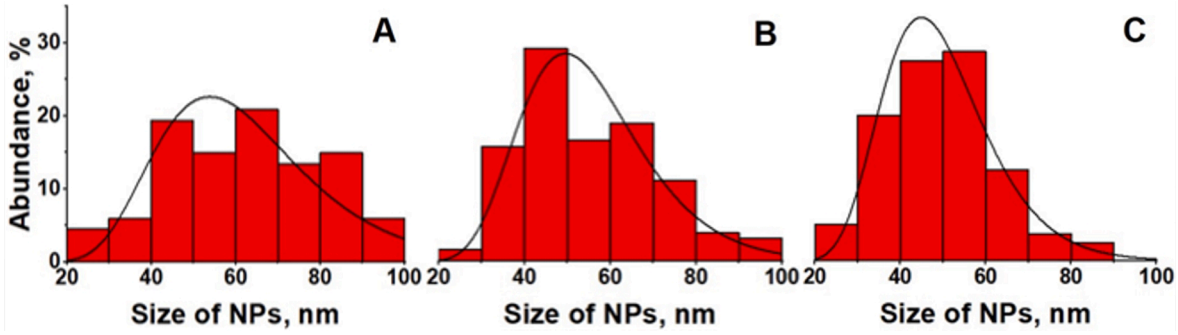

Fig. 8. Size distribution of selenium NPs: A – 5×10^{-3} M, B – 2.5×10^{-2} M, C – 10^{-1} M.

4. Conclusions

The present study revealed (1) the effects of selenite ions on bioluminescence that was used as an inherent physiological function of luminous marine bacteria, (2) biotransformation of selenite to selenium(0) NPs. The results of the study are both of fundamental and practical interest.

The luminous marine bacterium *P. phoshporeum* is widely used as a bioassay due to the convenience and high rate of registering the intensity of its physiological function – luminescence. The effects of selenite (SeO_3^{2-}) on the intensity of bacterial bioluminescence were explored at different concentrations and times of exposure to Na_2SeO_3. Bioluminescence activation and inhibition were revealed and analyzed; the dose–effect dependencies were attributed to the hormesis model. The toxicity of Na_2SeO_3 was characterized by an effective concentration of $EC_{50} = 10^{-3}$ M. The effects of Na_2SeO_3 on the ROS content in bacterial suspensions were studied. High positive correlations were found between the intensity of bioluminescence and the ROS content, which indicates the decisive role of ROS and associated redox processes in the bioeffects of selenite ions.

Scanning and transmission electron microscopy revealed the presence of nano-structures in the bacteria exposed to Na_2SeO_3. The energy dispersive spectrum revealed a high content of selenium in the NPs. The particle size distribution depended on the concentration of Na_2SeO_3; maxima of the size distribution were within 45–55 nm.

The applied aspect of the study is related to the use of luminescence bacteria as a polyfunctional biological product for (1) monitoring the toxicity of selenium-containing water environments, (2) detoxification of selenium-containing solutions, (3) biotransformation of selenite ions to selenium NPs. The applied aspect of the work is extremely important for toxicity monitoring and bioremediation of aquifers polluted with selenium compounds, as well as for a number of areas in biotechnology, such as nanobiotechnology, production of food additives and products enriched with selenium. Thus, nanobiotechnological applications of luminescence bacteria are well worth to be further developed.

The observed high sensitivity of *P. phosphoreum* to selenite as compared to that for other microorganisms [4,9,10] is an additional factor facilitating its use as a test object for bioluminescent monitoring of selenite pollution.

In addition, the biotransformation of soluble forms of selenium is of fundamental interest from the viewpoint of understanding the role of microorganisms in the migration of selenium compounds and in microbial ecology.

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Declaration of competing interest

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Data availability

Data will be made available on request.

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