https://doi.org/10.15407/microbiolj86.04.003

O.M. GROMOZOVA [1 *], V.S. MARTYNYUK [2], O.Yu. ARTEMENKO [2], I.O. HRETSKYI [1,3], JANEZ MULEC[4], Yu.V. TSEYSLYER [2]

[1] D.K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine, 154 Academika Zabolotnoho Str., Kyiv, 03143, Ukraine
[2] Institute of Biology and Medicine of Taras Shevchenko National University of Kyiv, 2 Akademika Hlushkova Avenue, Kyiv, 02000, Ukraine
[3] Kyiv National University of Technologies and Design, 2 Mala Shyianovska Str., Kyiv, 01011, Ukraine
[4] ZRC SAZU, Titov trg 2, SI-6230 Postojna, Slovenia
[*]Author for correspondence; e- mail: gren.elen@gmail.com

FEATURES OF BIOLUMINESCENCE DYNAMICS OF *PHOTOBACTERIUM PHOSPHOREUM* IMV B-7071

The problem of the prolonged and stable intensity of bioluminescent signals is relevant in the development of any test systems that use biological objects. The ***aim*** *of this work was to study the features of bioluminescence dynamics of Photobacterium phosphoreum IMV B-7071 in a liquid and on different stationary media.* ***Methods.*** *Bioluminescence studies were performed in liquid, agarose, and cellulose-cotton media. Bacterial suspensions were cultivated at 21°C in the mediums with standard composition. We studied both the background glow and its dynamics under conditions of mixing a liquid medium. Bioluminescence was recorded using digital photography with subsequent image processing of the samples. The measurements of luminescence were made by digital photo or video recording using Olympus digital camera SP560UZ, CANON 700D, and mobile device camera Samsung Galaxy 9 Note with specialized applications for mobile devices «Colorimeter (Lab Tools Apps)» and Camera Color Counter (Keuwsoft) at maximum light sensitivity in the automatic white balance mode at a fixed distance from the sample. Image processing was carried out using ImageJ and Origin Pro. Spectra of bacterial luminescence and its dynamics over time were measured using an LOMO MDR-23 spectrometer in the range of 200—750 nm.* ***Results.*** *The results of the study prove that in aqueous or solid agar and also on cellulose cotton medium, the intensity of bioluminescence of P. phosphoreum gradually increases, reaching a maximum within approximately 2 days, after which it slowly fades. It was established that the bioluminescence of photobacterium P. phosphoreum is a non-stationary process and has characteristic features of temporal dynamics associated with both the dynamics of the oxygen concentration in the environment of bacterial suspensions and the dynamics of the bacterial population density. Analysis*

Citation: Gromozova O.M., Martynyuk V.S., Artemenko O.Yu., Hretskyi I.O., Mulec Janez, Tseyslyer Yu.V. Features of Bioluminescence Dynamics of *Photobacterium phosphoreum* IMV B-7071. *Microbiological journal.* 2024 (4). P. 3—18. https://doi.org/10.15407/microbiolj86.04.003

of the luminescence spectra of bacteria shows that luminescence occurs mainly in the blue and green regions of the spectrum with luminescence maxima in the range of 460—520 nm, but the ultra-weak glow is also registered in the UV and red spectral ranges. The variability of photobacterial luminescence spectra over time in the spectral ranges of the main luminophores causes color fluctuations between the blue and green ranges. ***Conclusions.*** *The key parameters of multi-day background and short-term induced bioluminescence dynamics of photobacteria in different environments were clarified, and the certain variability of the spectral characteristics of luminescent radiation over time was shown. The revealed features of the dynamics of the bioluminescence of P. phosphoreum must be taken into account in practical application to assess the toxicity of substances of various nature, as well as in environmental monitoring.*

Keywords: *bacterial bioluminescence, Photobacterium phosphoreum, luminescence spectra, aqueous medium, solid agar, cellulose cotton medium*.

The interdisciplinary study of the phenomenon of luminescence in bacteria is one of the promising directions in modern science. The physical nature of the luminescence phenomenon is based on the transition of electrons in atoms or molecules from the excited state to the ground state. The cause of their initial excitation can be various factors: external radiation, temperature, chemical reactions, *etc*. The first definition of luminescence was given by S.I. Vavilov (1948): «Luminescence is an excess over the temperature radiation of the body in the case when this excess radiation has a finite duration of about 10^{-10} seconds or more».

It is known that the process of bacterial bioluminescence is caused by the reaction catalyzed by luciferase (flavin-dependent monooxygenase), requiring a reduced flavinmononucleotide ($FMNH_2$), O_2, and long-chain fatty aldehyde (RCHO). The reaction products are oxidized flavinmononucleotide (FMN), the corresponding fatty acid (RCOOH), H_2O, and visible light (Zhang et al., 2023).

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{bacterial luciferase}} FMN + RCOOH + H_2O + h\nu$$

This process is controlled by the *lux A, B, C, D, E* operons. It was found that the structural genes *lux A, B* encode luciferase, and *lux C, D, E* encode aldehyde synthesis. This part of the complex is the same for all studied bacteria (Brodl E. et al, 2018). The role of luminescence in the life of microorganisms is also poorly studied. Its participation in the defense of aquatic organisms, in the processes of intercellular communication, stress response, *etc*. has been considered (Lee, 2017; Li, 2022).

Today it is reliably shown that the luminescence reaction is a system indicator of the metabolic activity and functional state of bacterial cells. Due to high sensitivity, rapidity of reaction, and simplicity of registration, bioluminescent analysis is now widely used for express assessment of biologically active and toxic substances (Zhang, 2023; Hou, 2014).

The use of purified luciferase preparations and the use of genetically modified microorganisms possessing this property, as well as the use of a bioluminescent system for activation of light-sensitive proteins in optogenetics is considered a promising direction (Syed, 2021). Due to the high sensitivity of photobacteria to the influence of different factors, they were proposed for use as indicators of the biological effectiveness of electromagnetic fields of anthropogenic and natural origin. The high sensitivity of *Photobacterium phosphoreum* IMV B-7071 to a wide radio frequency range allowed the development of a biosensor device for detection of this physical factor (Hretskyi, 2019).

Usually, the assessment of the state of a luminescent biosystem under various influences is carried out by the optical signal value measured as a relative index compared to the control (not affected by the studied factors) sample. Approaches are being developed to quantify the signal by standardizing measurements to allow for photon counting at the cellular level (Niwa et al., 2023). According to the requirements of the International Committee

for Standardization (ISO), measurement systems must be reliable and reproducible.

Thus, the problem of prolonged and stable intensity of bioluminescent signals is relevant in the development of sensor test systems.

The **aim** of this work was to study the features of bioluminescence dynamics and assess its stability on photobacteria *Photobacterium phosphoreum* IMV B-7071 in a liquid and on different stationary media.

Materials and Methods. The culture of *Photobacterium phosphoreum* IMV B-7071 was the object of this study. It is included in the depositary of the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences NAS of Ukraine. The species identification of the bacteria was confirmed by the sequencing of 16S rRNA gene region. The nucleotide sequence was submitted to the GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov/genbank) under accession number KF656787.

The photobacteria were cultivated at 21°C in a liquid nutrient medium of the following composition (g/L): peptone — 5.0; yeast extract — 1.0; NaCl — 30.0; Na_2HPO_4 — 5.3; $KH_2PO_4 \times \times 2H_2O$ — 2.1; $(NH_4)_2HPO_4$ — 0.5; $MgSO_4 \times \times 7H_2O$ — 0.1; glycerol — 3.0 mL/L, distilled water — up to 1 L, pH 7.0. The solid medium was of the same content supplied with 2% of agar-agar. This nutrient medium was also used in experiments with cellulose-cotton plates.

In our previous research, the most intense bacterial glow was observed at the end of the exponential growth phase (Zelena, 2014), so we cultivated *P. phosphoreum* for 18 hours in 750 mL flasks with 100 mL of the nutrient medium at 145 rpm and 22 ˚C. The bacterial suspension with a concentration of 10^7 cells/mL was used for the research. The concentration of 10^7 cells/mL corresponds to the optical density of the suspension D = 0.1 measured on a KFK-3 photocolorimeter with a light filter of 670 nm and an optical path of 3 mm. In the previous studies, this concentration was determined as optimal for the study of *P. phosphreum* (Gorgo, 2018).

The bacteria were sown on a solid agarose medium using 50 µl of bacterial suspension with a concentration of 10^7 cells/mL. The 2 mm thick sowing of the bacteria on dense cellulose cotton plates was carried out in plastic Petri dishes with a diameter of 2.5 cm. 1 mL of the nutrient medium of the above-specified composition was added to the cellulose cotton plates, and 0.2 mL of the bacterial suspension with a cell concentration of $2 \cdot 10^7$ cells/mL was added to their centers.

Registration of bacterial luminescence. Bioluminescence intensity was assessed using digital cameras, currently widely used to obtain images in the digital format, which allows measuring in relative units the intensity of the light flux entering the light-sensitive matrix. Although digital cameras of different manufacturers can differ in a certain way in terms of their spectral properties and sensitivity to light, they adequately convey the ratio of light intensities on a digital image. In this regard, such digital technology allows reliable comparison of digital images in terms of color and brightness.

The images of Petri dishes with bacterial colonies in solid agarose were captured with an Olympus digital camera SP560UZ under daylight and in the dark. Colony sizes were measured on the photographs either manually or using the program ImageJ 1.41 (http://rsb.info.nih.gov/ij)

The intensity of bioluminescence on the cellulose cotton plates was measured by digital photorecording using a CANON 700D digital camera in a manual mode with a maximum zoom at a distance of 50 cm from the samples, an exposure of 2 seconds and an aperture of 5.6, with the white balance mode automatically set. Similar methods of registering and evaluating the intensity of bioluminescence using a charge-coupled device (CCD) camera are used in experimental practice (Sakaguchi, 2003; Coates, 2004).

Digital images were processed to estimate the light flux intensity using the Origin Pro algorithm: Image to Matrix → Plot → Profile → Contour Profile (Fig. 2). As an indicator of bioluminescence intensity, the maximum values on

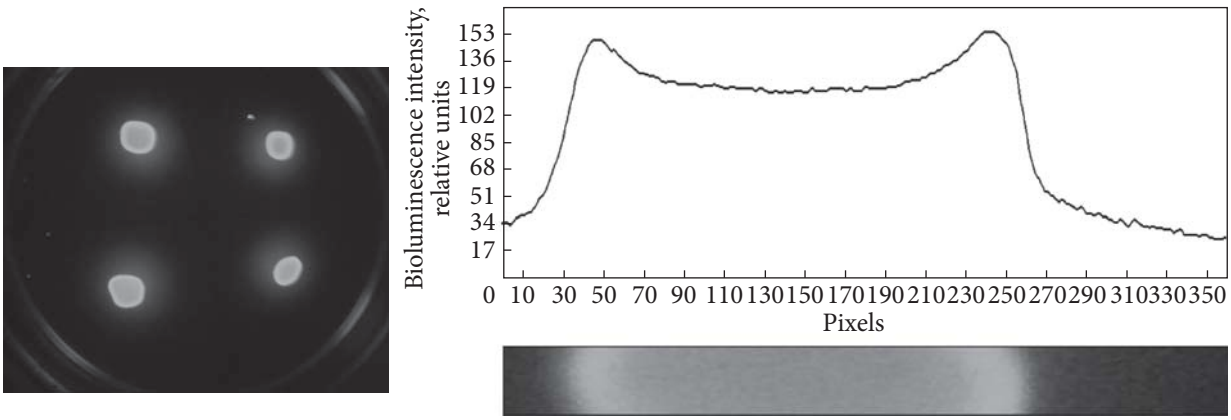

Fig. 1. An example of digital photorecording of *P. phosphreum* bioluminescence on a solid agarose medium and the results of image processing

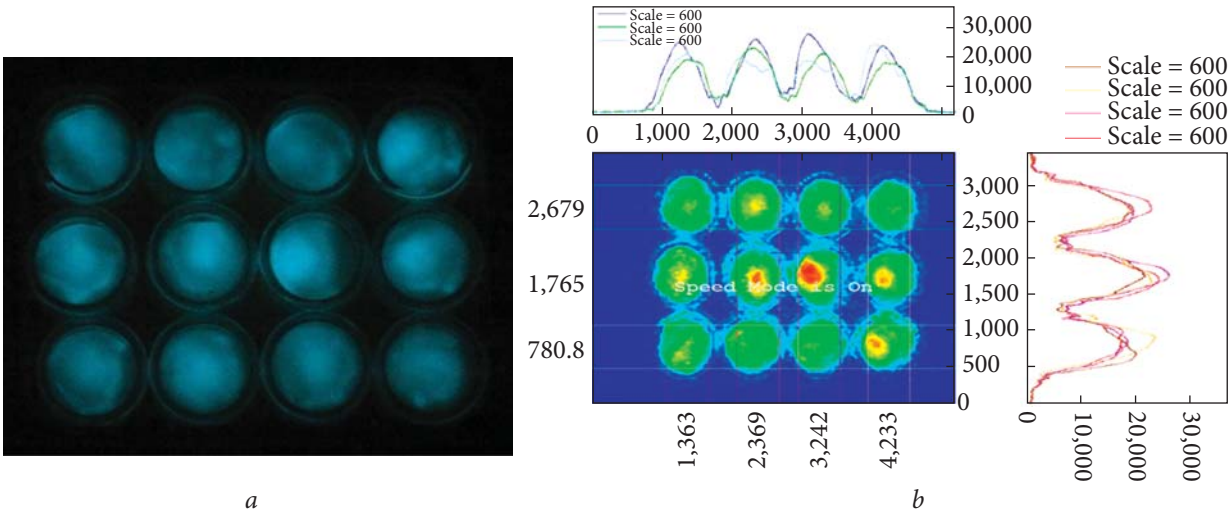

Fig. 2. An example of digital processing of bioluminescent light flux from individual samples of bacterial colonies on cellulose cotton plates: *a* — initial data of digital photorecording; *b* — results of digital processing

the graphs of luminescence distribution for each sample in two projections were used.

In the liquid medium, the luminescence dynamics in bacterial suspensions was recorded in a continuous manner. The bacterial suspension in a glass flask was shaken for 3 seconds, and photometric measurements of the luminescence of the upper layer of the suspension started simultaneously and lasted until the luminescence completely faded or the suspension reached the background mode of stationary low-intensity luminescence in the upper layer.

Photometric measurements were made by digital photo and video recording of luminescence using specialized applications for mobile devices or digital cameras at maximum light sensitivity in the automatic white balance mode at a fixed distance from the sample. The dynamics of luminescence intensity was observed using the following applications:

▪ Colorimeter (Lab Tools Apps), manual mode «Picker Mode», recording the signal intensity in the RGB format with an interval of 10—15 seconds. When processing the data, it is important

to take into account the fact that the luminescent signal corresponding to the emission of the main luminophores of the *P. phosphoreum* luminescent system corresponds to the blue and green spectral ranges, and the glow in the red range is extremely low and invisible against the background of noise fluctuations of the CCD. The CCD noise is correlated across RGB channels, so to increase the accuracy of the luminescence registration, the values for the red channel were subtracted from the values for the blue-green channel, thus reducing the contribution of the CCD noise to the signals recorded in other channels;

- Camera Color Counter (Keuwsoft), video recording mode with a graphical presentation of the signal dynamics in three color RGB channels, the area of the recording window is 10%. After recording the glow dynamics of the samples, a copy of the screen is made in jpg format (Fig. 3) for further processing using the appropriate algorithm in Origin Pro, which allows digitizing graphs displayed on the mobile device screen.

Blue and green lines correspond to the blue and green channels in RGB. A sharp flash of luminescence followed by a rapid decrease in the intensity corresponds to the phase of active mixing of the bacterial suspension (see in more detail Fig. 7). The dips in the graph that periodically occurred during the registration are related to the operation of the autofocus system; the horizontal axis is the registration time, and the vertical axis is the signal intensity in % relative to the maximum possible value in the digital RGB light intensity format, which corresponds to 255.

The spectra of bacterial luminescence and its dynamics in time were measured using a LOMO MDR-23 spectrometer in the range of 200—750 nm. The culture medium in which the bacteria were cultivated was used as a control for the dark glow. The background signal obtained in this way, which was actually the dark current of the photoelectronic multiplier, was subtracted from the primary data when recording the luminescence spectra of bacterial suspensions.

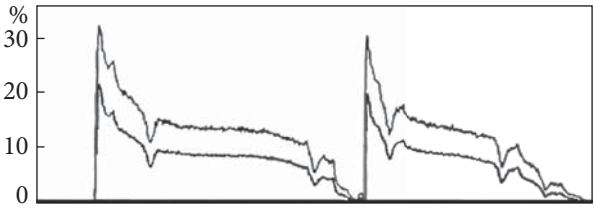

Fig. 3. Graphical representation of the time dynamics of luminescence in the upper layer of *P. phosphreum* suspensions on the screen of a mobile device.

Oxygen concentration measurement. The oxygen concentration in liquid bacterial suspensions was studied using an EZODO-7031 oximeter (GonDO Electronic, Ltd., Taiwan) based on a Clarke membrane electrode with a temperature correction function.

Statistical processing of the experimental data was based on the generally accepted algorithms of variation statistics. In particular, the mathematical expectation and its error were calculated. The reliability of the difference between the statistical samples was assessed through analysis of variance using the ANOVA algorithm.

Results. ***Bacterial luminescence on solid agarose medium.*** The analysis of the dynamics of bacterial luminescence intensity showed that the luminescence of colonies on solid agarose medium was most intense after 24 hours of cultivation (Fig. 4). Over time, the intensity of bioluminescence gradually decreased, and after 92 hours we observed it only at the periphery of the colony. In this part of the colony, there are young and more active cells, the concentration of which is lower than in the center of the colony. It is worth noting that under these cultivation conditions, the colony's growth stops when it reaches approximately 7 mm in diameter, despite the sufficient amount of nutrients in the medium.

Bacterial luminescence on cellulose cotton plates. The dynamics of the intensity of bacterial luminescence on cotton media is similar to that on a solid medium. After sowing photobacteria on a cellulose base saturated with a nutrient medium, the luminescence gradually increases, reaching a

Fig. 4. Bioluminescence intensity of bacterial colonies on solid agarose medium for 168 hours (7 days)

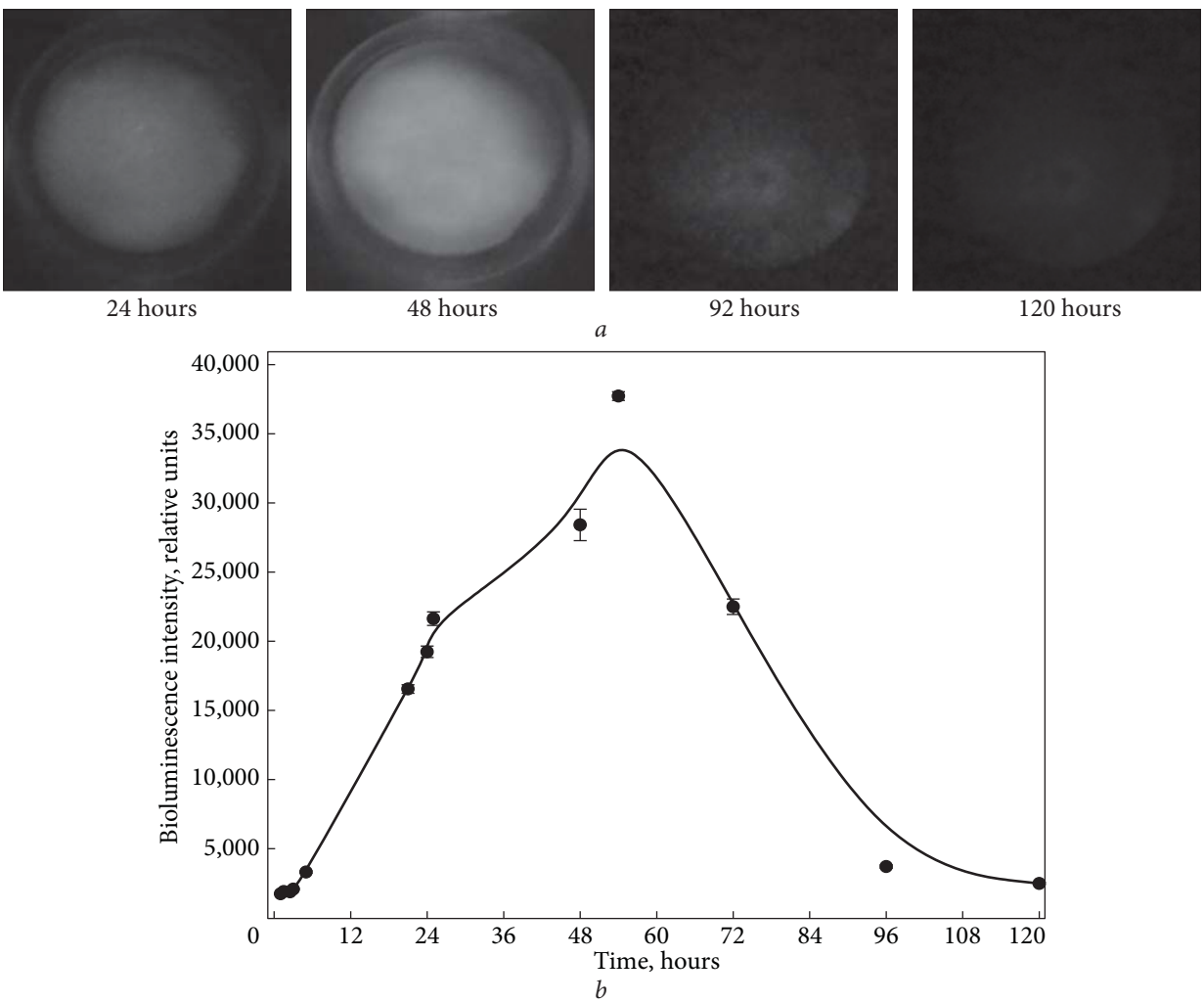

Fig. 5. The typical dynamics of the background bioluminescence of *P. phosphreum* on cellulose cotton plates for 5 days: A — an example of digital photorecording of bioluminescence of samples; B — the result of statistical processing of digital images of individual samples (n=12)

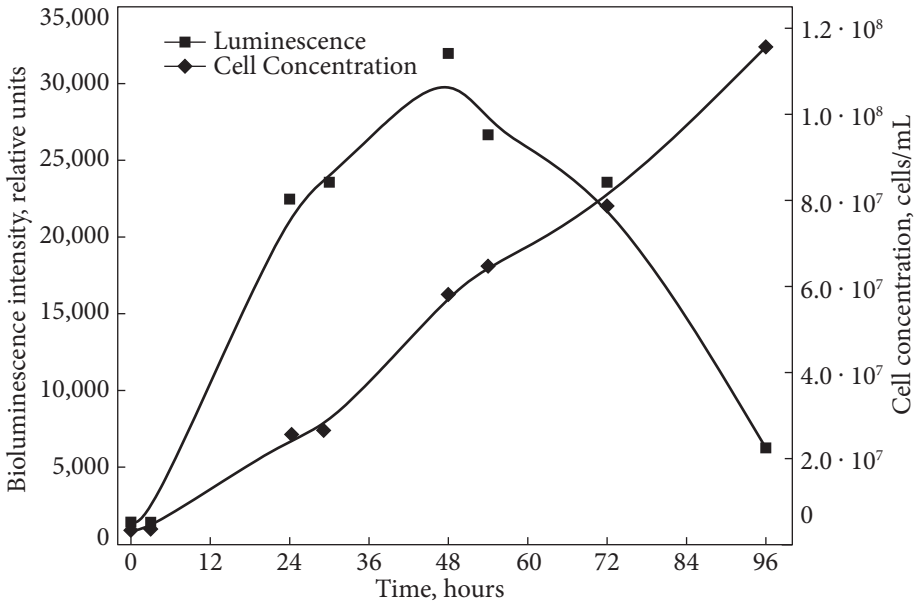

Fig. 6. Dynamics of background bacterial luminescence (black line) and cell concentration (red line) in the liquid medium for 96 hours (4 days).

maximum intensity on the second or third day, then it fades after 92—120 hours (4—5 days) and enters the mode of ultra-weak luminescence that can be barely seen by the eye only with the prolonged adaptation of the human visual analyzer in the dark (Fig. 5). Similar dynamics of *P. phosphreum* bioluminescence was observed by other researchers (Tanet, 2019), but they observed the maximum intensity of bacterial luminescence during the first or second day. Importantly, such a bioluminescence dynamics weakly correlates with the activity of the bacterial quorum sensing system (Tanet, 2019), which raises new questions about the functional and general biological significance of the light generation process, which requires high energy expenditure.

Luminescence in a liquid medium. The background level of *P. phosphreum* luminescence in the liquid culture medium, as well as in the model of colonies on agar medium and cotton carriers, increases for two days, after which it drops, reaching a very-low-intensity mode of luminescence with spontaneous low-amplitude fluctuations of the light flux. At the same time, photobacteria continue to actively multiply after 2 days, but this active growth of the bacterial population is accompanied by a decrease in bioluminescence up to an extremely low level after 4—5 days (Fig. 6). This fact is an additional argument for doubts about a strong connection between bacterial quorum sensing and bioluminescence intensity in bacterium *P. phosphoreum* IMV B-7071 compared to *Vibrio fishery* (Tanet, 2019).

***Dynamics of bioluminescence in a liquid medium when a bacterial suspension* is shaken.** When a bacterial suspension is shaken (actively mixed) for 1—3 seconds, a flash of luminescence is observed (phase 1), the intensity of which decreases rapidly and the suspension enters the mode of active but less intense luminescence (phase II), which for some time remains at a relatively constant level for tens of seconds to tens of minutes and then slowly decreases (Fig. 7). Phase III begins with a rapid decay of bioluminescence in the suspension volume within ten seconds to one minute to the background level (Fig. 7). The background luminescence is phase IV in the luminescence dynamics in this study protocol, characterized by a long-lasting but extremely weak luminescence in the volume of the bacterial suspension, which is almost invisible to the eye and at the same time a weak luminescence in the surface layer of the sus-

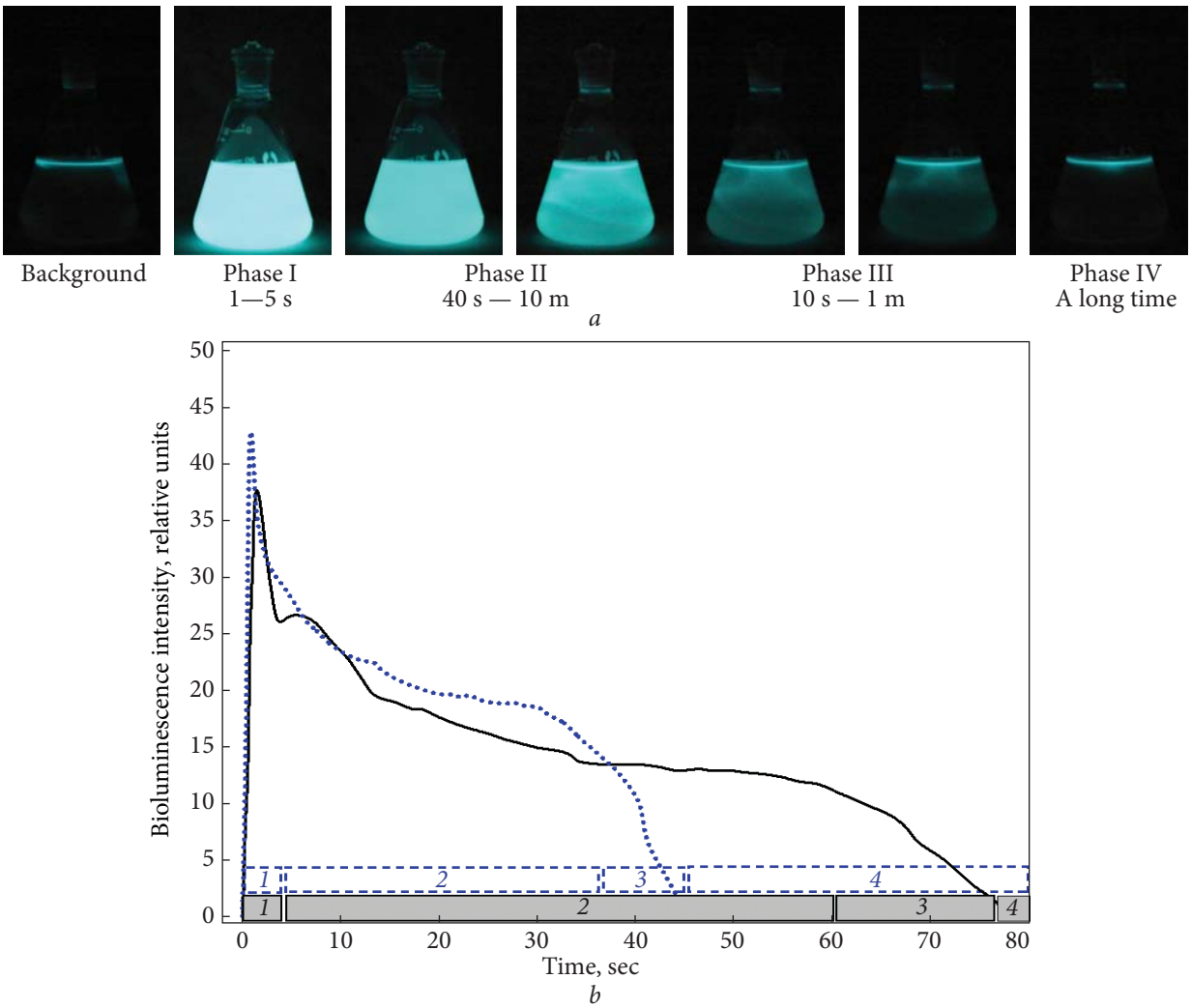

Fig. 7. Dynamics of luminescence intensity inside the aqueous suspension of *P. phosphreum* after its shaking: A — photorecording of the luminescence dynamics (the duration of the luminescence phases is indicated); B — an example of recording the dynamics of bioluminescence intensity in a continuous mode (for the blue RGB channel) for two samples of bacterial suspensions, which indicates the variability of the time dynamics of luminescence of individual samples; 1, 2, 3, 4 corresponded to phases I — IV of the bioluminescence dynamics when two different bacterial suspensions were shaken at the same experimental conditions and bacterial cell concentration, respectively.

pension 3—5 mm deep from the surface, which is nevertheless sufficient for reliable registration. Thus, phase II is characterized by a relatively high intensity of luminescence of bacterial suspensions and a certain stability within a short time, and phase IV is the state of background luminescence of bacterial suspensions in the upper layer, which is more oxygen-enriched compared to the lower layers in the suspension volume (Fig. 8).

It is important to note the high variability in the duration of the 2nd and 3rd phases of luminescence of bacterial suspensions under shaking. Possible reasons for the variability of bacterial bioluminescence were considered by Satoshi Sasaki (Sasaki, 2012), but the issue remained unresolved. Each experimental sample under turbulent perturbation of a bacterial suspension demonstrates own luminescence dynamics,

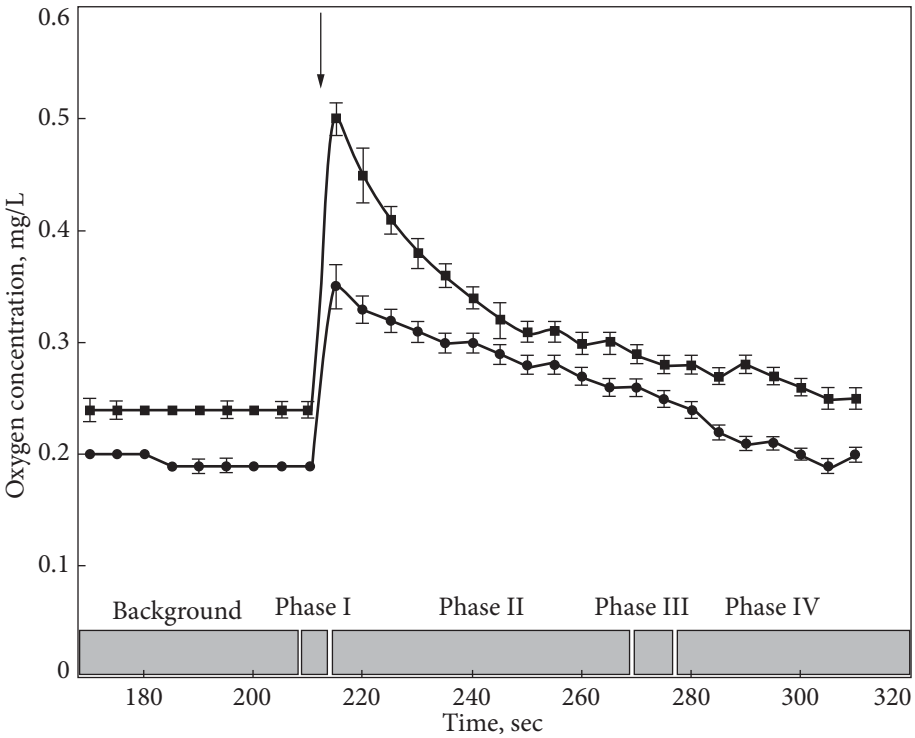

Fig. 8. An example of recording the dynamics of oxygen concentration in aqueous suspensions of *P. phosphreum* after shaking. The upper line corresponds to the top layer of the suspension at a depth of 0.5 cm from the surface, the lower line — in the middle of the suspension volume. The arrow indicates the moment of shaking of the bacterial suspension.

which indicates that each isolated population of photobacteria that is formed in the volume of the nutrient medium is a separate «ecosystem» with own peculiarities of behavior in time and space.

Bacterial bioluminescence is an oxygen-dependent process, so the oxygen concentration in the medium is one of the key factors affecting the luminescence intensity of photobacteria. The measurements of this parameter in bacterial suspensions indicate that the oxygen concentration is very low and in the state of background luminescence does not exceed 0.25—0.3 mg/L (Fig. 8), which is about 3% and less of the oxygen concentration in water saturated with air under normal atmospheric pressure conditions. However, as it turns out, this amount of oxygen in the culture medium is sufficient for the bacterial luciferase system to function, which indicates its high efficiency in conditions with low oxygenation. The similar high activity of the bacterial luciferase system under extremely low oxygen concentrations was also observed by other researchers (Prante, 2018). When the suspension is shaken, the dynamics of oxygen content in the medium generally corresponds to the dynamics of luminescence in the first phase, but subsequently, the amount of oxygen slowly decreases without significant fluctuations in phase III, which testifies to the corresponding threshold oxygen-dependent mechanism of «on-off» bioluminescence in bacterial cells.

The above variable behavior of bacterial suspensions is confirmed by studies of the dynamics of luminescence intensity (Fig. 9), which was recorded using an LOMO MDR-23 spectrometer at a wavelength of 489 nm. According to the literature (Lee, 2019; Cifra, 2014), the spectral range of 485—505 nm corresponds to the maximum luminescence of the flavin of bacterial luciferase, flavin-4a-hydroxide, produced by the enzymatic redox reaction. The luminescence recording began 10—20 seconds after pouring the bacterial suspension into a cuvette and mechanical mixing, which corresponds to phase II of the dynamic pro-

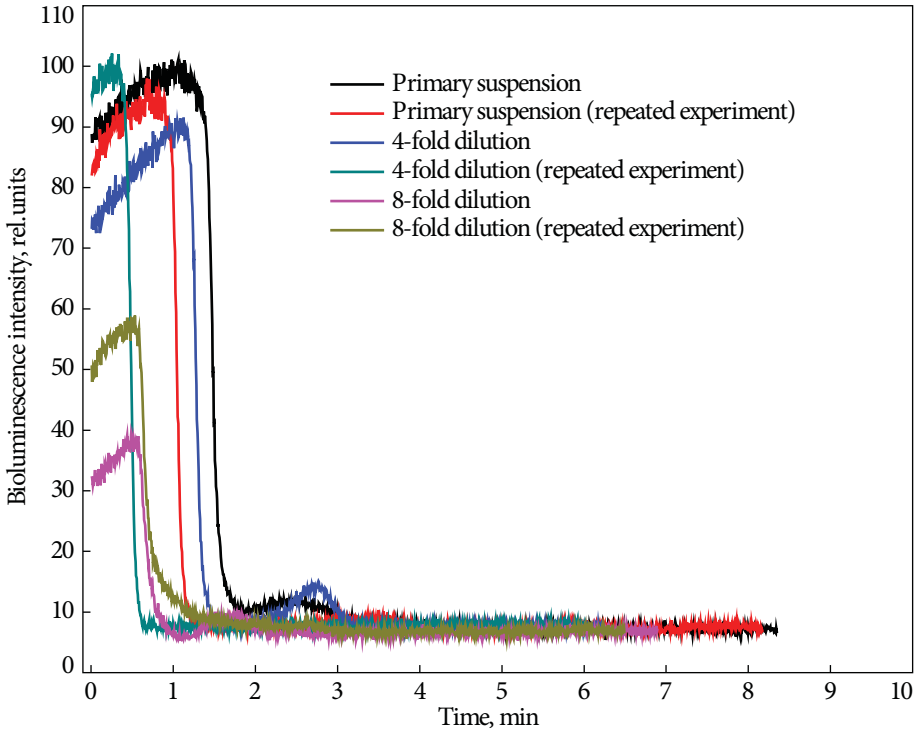

Fig. 9. Dynamics of the luminescence intensity of bacterial suspensions at the wavelength λ_{max} = = 489 nm after addition into the cuvette and mixing.

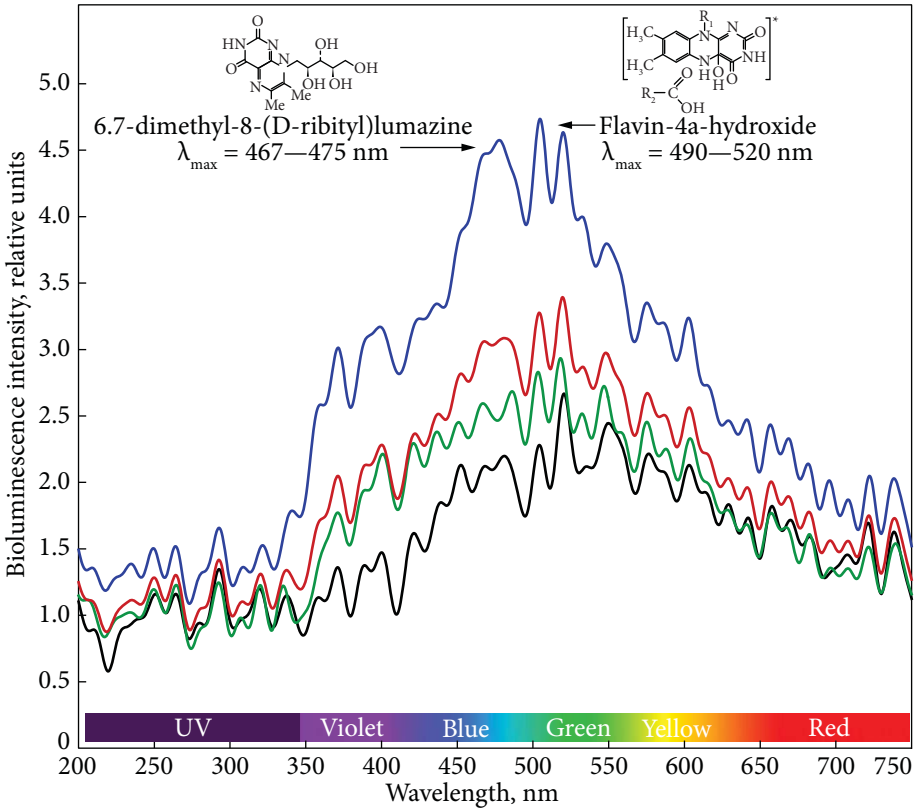

Fig. 10. Spectral variability of background luminescence of bacterial suspensions in phase IV for three samples under the same cultivation conditions. The bioluminescence spectra of individual samples are shown by thin lines, the thick line is the averaged spectrum. Arrows indicate the luminescence maxima of lumazine and flavin hydroxide.

cess shown in Fig. 7. The results of the experiment indicate a certain nonlinearity of the dependence of the intensity of photobacterial luminescence during phase II on their concentration in the suspension. In particular, diluting the initial suspensions several times with the culture medium does not lead to the expected decrease in luminescence intensity. A noticeable decrease in luminescence intensity is observed only at higher dilutions (Fig. 9). This fact raises new questions, in particular: Why, at certain dilutions, does the intensity of the luminescence of bacterial suspensions not decrease during phase II of intense luminescence? Is this due to the fact that not all bacteria glow in the initial and more concentrated suspension and after certain dilution other cells begin to glow, which maintains the overall level of the luminescent signal, or is this due to the additional activation of the intracellular enzymatic oxidative process catalyzed by bacterial luciferase when the bacterial suspension is diluted? Finding answers to these questions requires separate studies.

A significant time variation in the luminescence phases is noteworthy. Their duration can vary greatly depending on the day the suspension was obtained, the time it was kept at room temperature at rest or under conditions of active aeration during growth, and the concentration of bacterial cells. In particular, the variability of the luminescence duration of bacterial suspensions in phase II can range from tens of seconds to tens of minutes (Fig. 9). Thus, the results of the analysis of spectrometric data confirm the significant variability of the dynamics of the luminescence of bacterial suspensions after their mixing. Similar variability in bacterial bioluminescence was observed by other researchers (Sasaki, 2012). As shown above, when bacterial suspensions are kept at room temperature and without mechanical stress for several days, a gradual decrease in the luminescence intensity is observed, which is associated with an increase in the density of bacterial suspensions (Fig. 6). However, the variability of the duration of the phases of the luminescence dynamics of the suspensions after their single shaking is still observed.

The variability of the luminescence of bacterial suspensions is also characteristic of the weak background luminescence during phase IV when not only a certain variation in the total luminescence flux can be observed but also certain fluctuations of the intensity of the luminescent light flux in different spectral ranges (Fig. 10).

The luminescence spectra show that the bacterial suspension glows in almost the entire range of 200—700 nm and the overall spectral profile corresponds to the background luminescence of *P. phosphoreum* suspensions without stirring (Sasaki, 2012). That is, the redox transformations of substrates occurring in the active center of bacterial luciferase are a very powerful energy process that leads to the appearance of powerful electron-excited states in the active center of the enzyme, the relaxation of which leads to the emission of light with subsequent scattering it in a wide range — from the ultraviolet to the red. M. Cifra's work (Cifra, 2014) theoretically and experimentally proves that the energy of electronic excitation in certain chemical transformations exceeds 380 nm, which, in our opinion, is sufficient for electronic excitation of basic luminophores of the luciferase system in bacterial cells. This fact of broad-spectrum radiation with the dominance of certain spectral bands explains the color features of bacterial luminescence, which is perceived by the human eye as a milky-blue-green glow.

As seen in Fig. 10, the luminescence spectra show the emission lines of the two main luminophores of the bacterial luminescent system, namely lumazine and flavin hydroxide. Excited flavin hydroxide (4a-hydroxy-1,5-dihydroflavin mononucleotide), formed as a result of the luciferase reaction, is an emitter of blue-green light with a maximum emission in the range of 490—520 nm. Lumazine (6,7-dimethyl-8-(D-ribityl)lumazine) is a luminophore of the fluorescent protein lumase (LumP), which forms a complex with bacterial luciferase and glows in the blue region with a maxi-

mum in the range of 467—475 nm. The question of the energy mechanism of such excitation of the electronic structure of lumazine is controversial. Many years ago, there was an attempt to substantiate the possibility of transferring the excitation energy from flavin hydroxide to lumazine (Lee, 1989), but the transfer of energy from a «colder» electronic state to a «hotter» one is unlikely. In this regard, approximate estimates of the energy capacity of redox enzymatic catalysis should be taken into account that allow the generation of energy at the level of ultraviolet radiation.

Firstly, the maximum in the luminescence spectrum of *P. phosphoreum* is observed in the range of 480—490 nm, which formally corresponds to about 60 Kcal/mol of energy. This is almost eight times higher than the enthalpy of hydrolysis of ATP to ADP (Medintz, 2020). Secondly, more powerful energy can be generated in the molecular redox transformations corresponding to the UV range (Cifra, 2014). Therefore, it is likely that the electronic excitation energy generated by the enzymatic process in the reaction center of bacterial luciferase is sufficient for the electronic excitation of both luminophores — lumazine and flavin hydroxide. The echo of the energetic power of this process is the weak glow of the bioluminescent reaction in the UV range (Fig. 10).

It is also important to note the fact that the luminescence spectra vary over time, primarily in the luminescence range of the main emitters of bacterial luminescence, namely flavin hydroxide and lumazine. The luminescence of lumazine has a typical light blue luminescence color, and flavinoxide glows in the blue-green range. Superimposing the luminescence spectra of these two luminophores gives the average blue glow that fluctuates between blue and blue-green shades. The analysis of digital images confirms this color variation in the luminescence of bacterial suspensions over time (Fig. 11). In particular, when analyzing digital images of bacterial bioluminescence in cellulose cotton media in the magnification mode, randomly located areas («domains») of green and blue are distinctly visible. This fact indicates that the luminescence of lumazine dominates in certain areas of the bacteria and flavinoxide luminescence — in others. In our opinion, this phenomenon is interesting and requires more detailed study. Some researchers explain this color variation in bioluminescence by different ratios of «dark» and «light» cells in different phases of the cell cycle (Sasaki, 2012). At the same time, the reason for a certain difference in the bioluminescence spectra of bacterial suspensions at rest and under active stirring remains unclear. In particular, the maximum bioluminescence shifts to the green region in the resting state, and when the suspension is stirred, the maximum shifts to the blue region (Sasaki, 2012).

Our study suggests that in limited volumes of bacterial suspensions, the «blue-green» transitions occur even at the rest state but rather quickly and for a short time (Fig. 11 A). Similar but spatial color spectral differences are observed in experiments on cellulose cotton carriers, where the green and blue zones are distinctly visible. At low bioluminescence intensity, the green zones become more noticeable (Fig. 11 B), and the blue zones dominate when the luminescence intensity is increased (Fig. 11 C).

Discussion. Bacterial luminescence is commonly used as a bioecological test system to assess biological activity or environmental toxicity. Any test system should be characterized by stability and accuracy. When considering the photobacterium *P. phosphoreum* as a model for developing such test systems, several questions arise that require further investigation and solution.

First, the dynamics of a bioluminescent system over time and its variability in intensity during the hydrodynamic movement of the medium need to be addressed. This movement is inherent when working with liquid samples of bacterial suspensions. In the studies of other researchers (Sasaki, 2012), there was also observed such dynamism in bioluminescence. However, our research indicates that media movement can be effectively

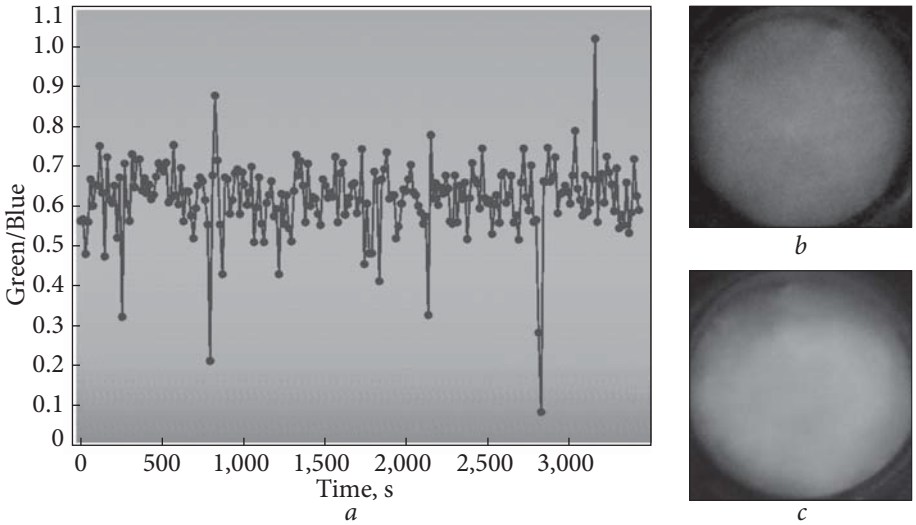

Fig. 11. Color variability of bioluminescence of *P. phosphoreum* suspensions. A — dynamics of the ratio of signals in the green and blue spectral ranges when the recording of background luminescence was in the suspension volume in the digital RGB mode. The background color gradient visualizes fluctuations in the color properties of the luminescence of bacterial suspensions over time; B — blue-green spatial zonation of weak background bacterial luminescence on cellulose cotton plates; C — dominance of blue color at high luminescence intensity

controlled using conventional inexpensive, relatively inert, and highly porous cellulosic media. This methodological approach yields more stable indicators of baseline bioluminescence intensity and is also technologically convenient.

Second, the variability of bacterial luminescence spectra is of significant theoretical and practical interest. Our findings demonstrate the presence of a light flux in the UV range, which opens new possibilities for using bacterial luminescence to study the biophysical mechanisms of energy transformation in electron-excited states within biomolecular structures (Cifra, 2014). Moreover, the temporal variability in the spectral composition of bacterial luminescence may reflect the spatial-time organization of bacterial metabolism and intercellular communication. These parameters can be employed for more detailed studies of the molecular mechanisms underlying their sensitivity to various chemical and physical factors. Currently, a review of the literature reveals a lack of significant progress in addressing these questions.

Conclusion. Thus, the results of our study prove that the bioluminescence of the bacteria *P. phosphoreum* is not a stationary process and has characteristic features of time dynamics associated with both the dynamics of oxygen concentration in the medium of bacterial suspensions and the dynamics of the bacterial population density. In aqueous, solid agarose, and cellulose cotton media, the luminescence intensity gradually increases reaching a maximum within 1—2 days, after which it slowly fades. The drop in bioluminescence is probably due to an increase in the density of the bacterial culture under conditions of sufficient nutrients.

In a liquid medium, intense shaking of bacterial suspensions leads to a sharp powerful flash of bioluminescence followed by intense luminescence for tens of seconds to tens of minutes, after which the luminescence quickly fades to a low-intensity background level. During this process, the dynamics of oxygen concentration only partially corresponds to the dynamics of luminescence in the first phases. The phase of the rapid drop in bioluminescence in the volume of the bacterial suspension characterizes the intracellular regulatory mechanisms, which most likely

control the luminescence process according to the threshold «on-off» principle.

Bacterial luminescence is observed at very low oxygen concentrations, which indicates the high energy efficiency of the photobacterial bioluminescent system. The analysis of bacterial luminescence spectra shows that luminescence occurs in the range of 200—700 nm. This fact proves that the redox transformations of substrates that occur in the active center of bacterial luciferase are a very high-energy enzymatic process that leads to the emergence of powerful electron-excited states in the active center of the enzyme, the relaxation of which leads to the emission of light by the main luminophores with subsequent light scattering in a wide range.

The variability of the bacterial luminescence spectra over time, primarily in the luminescence spectral ranges of the main emitters of bacterial luminescence, flavinoxide and lumazine, causes color fluctuations between the blue and green ranges observed during the monitoring of the spectral features of the bioluminescent light flux.

So, the key parameters of multi-day background and short-term induced bioluminescence dynamics of photobacteria on different media have been clarified, and the certain variability of the spectral characteristics of luminescent radiation over time has been shown in this research. The revealed features of the time dynamics of the bioluminescence of *P. phosphoreum* must be taken into account in practical use to assess the toxicity of substances of various nature, as well as in environmental monitoring.

Funding. This work is supported by project «Magnetism for Ukraine 2022» Program under Grant Agreement #9918 with the support of the IEEE Magnetic Society

REFERENCES

Brodl, E., Winkler, A., & Macheroux, P. (2018). Molecular mechanisms of bacterial bioluminescence. Computational and structural biotechnology journal, 16, 551-564. https://doi.org/10.1016/j.csbj.2018.11.003

Cifra, M., & Pospíšil, P. (2014). Ultra-weak photon emission from biological samples: definition, mechanisms, properties, detection and applications. Journal of Photochemistry and Photobiology, B: Biology, 139, 2-10. https://doi.org/10.1016/j.jphotobiol.2014.02.009

Coates, C. G., Denvir, D. J., McHale, N. G., Thornbury, K. D., & Hollywood, M. A. (2004). Optimizing low-light microscopy with back-illuminated electron multiplying charge-coupled device: enhanced sensitivity, speed, and resolution. Journal of biomedical optics, 9(6), 1244-1252. https://doi.org/10.1117/1.1805559

Hou, C., Liu, Y. J., Ferré, N., & Fang, W. H. (2014). Understanding bacterial bioluminescence: a theoretical study of the entire process, from reduced flavin to light emission. Chemistry-A European Journal, 20(26), 7979-7986. https://doi.org/10.1002/chem.201400253

Hretskyi, I., Zelena, L., & Gromozova, E. (2019). Effect of radiofrequency electromagnetic radiation on Photobacterium phosphoreum luminescence. Mikrobiolohichnyi zhurnal, 81(6), 58-68. https://doi.org/10.15407/microbiolj81.06.058

Gorgo, Y., Greckiy, I. O., & Demydova, O. I. (2018). The use of luminos bacteria Photobacterium phosphoreum as a bioindicator of geomagnetic activity. Innov Biosyst Bioeng, 2(4), 271-277. https://doi.org/10.20535/ibb.2018.2.4.151459

Lee, J., O'Kane, D. J., & Gibson, B. G. (1989). Bioluminescence spectral and fluorescence dynamics study of the interaction of lumazine protein with the intermediates of bacterial luciferase bioluminescence. Biochemistry, 28(10), 4263-4271. https://doi.org/10.1021/bi00436a022

Lee, J. (2017). Perspectives on bioluminescence mechanisms. Photochemistry and Photobiology, 93(2), 389-404. https://doi.org/10.1111/php.12650

Lee, J., Müller, F., & Visser, A. J. (2019). The sensitized bioluminescence mechanism of bacterial luciferase. Photochemistry and Photobiology, 95(3), 679-704. https://doi.org/10.1111/php.13063

Li, Y., He, X., Zhu, W., Li, H., & Wang, W. (2022). Bacterial bioluminescence assay for bioanalysis and bioimaging. Analytical and Bioanalytical Chemistry, 1-9. https://doi.org/10.1007/s00216-021-03695-9

Niwa, K., Kubota, H., Enomoto, T., Ichino, Y., & Ohmiya, Y. (2023). Quantitative Analysis of Bioluminescence Optical Signal. Biosensors, 13(2), 223. https://doi.org/10.3390/bios13020223

Prante, M., Ude, C., Große, M., Raddatz, L., Krings, U., John, G., & Scheper, T. (2018). A portable biosensor for 2, 4-dinitrotoluene vapors. Sensors, 18(12), 4247. https://doi.org/10.3390/s18124247

Sakaguchi, T., Kitagawa, K., Ando, T., Murakami, Y., Morita, Y., Yamamura, A., & Tamiya, E. (2003). A rapid BOD sensing system using luminescent recombinants of Escherichia coli. Biosensors and Bioelectronics, 19(2), 115-121. https://doi.org/10.1016/S0956-5663(03)00170-2

Samanta, A., & Medintz, I. L. (2020). Bioluminescence-based energy transfer using semiconductor quantum dots as acceptors. Sensors, 20(10), 2909. https://doi.org/10.3390/s20102909

Sasaki, S. (2012). Oscillation in bacterial bioluminescence. Bioluminescence-Recent advances in oceanic measurements and laboratory applications. London: InTech, 167. https://doi.org/10.5772/37065

Syed, A. J., & Anderson, J. C. (2021). Applications of bioluminescence in biotechnology and beyond. Chemical Society Reviews, 50(9), 5668-5705. https://doi.org/10.1039/D0CS01492C

Tanet, L., Tamburini, C., Baumas, C., Garel, M., Simon, G., & Casalot, L. (2019). Bacterial bioluminescence: light emission in Photobacterium phosphoreum is not under quorum-sensing control. Frontiers in microbiology, 10, 365. https://doi.org/10.3389/fmicb.2019.00365

Zelena, L., Gretsky, I., & Gromozova, E. (2014). Influence of ultrahigh frequency irradiation on Photobacterium phosphoreum luxb gene expression. Open Life Sciences, 9(10), 1004-1010. https://doi.org/10.2478/s11535-014-0347-5

Zhang, K., Liu, M., Song, X., & Wang, D. (2023). Application of Luminescent Bacteria Bioassay in the Detection of Pollutants in Soil. Sustainability, 15(9), 7351. https://doi.org/10.3390/su15097351

Received 08.08.2023

О.М. Громозова [1*], *В.С. Мартинюк* [2], *О.Ю. Артеменко* [2],
І.О. Грецький [1,3], *Жанез Мулек* [4], *Ю.В. Цейслер* [2]

[1] Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,
вул. Академіка Заболотного, 154, Київ, 03143, Україна
[2] Інститут біології та медицини Київського національного університету імені Тараса Шевченка,
проспект Академіка Глушкова, 2, Київ, 02000, Україна
[3] Київський національний університет технологій та дизайну,
вул. Мала Шияновська, 2, Київ, 01011, Україна
[4] ZRC SAZU,
Titov trg 2, SI-6230, Постойна, Словенія

ОСОБЛИВОСТІ ДИНАМІКИ БІОЛЮМІНЕСЦЕНЦІЇ
PHOTOBACTERIUM PHOSPHOREUM IMV B-7071

На цей час надійно доведено, що біолюмінесцентна реакція є системним показником загальної метаболічної активності клітин та їх функціонального стану. Біолюмінесценція бактерій забезпечується спеціальною бактеріальною монооксигеназою — люциферазою, яка локалізується в цитоплазмі і здійснює спряжене окислення NADPH і довголанцюгового альдегіду. Продуктами реакції є окислений флавінмононуклеотид (FMN), відповідна жирна кислота (RCOOH), H_2O і видиме світло. У зв'язку з високою чутливістю, швидкістю реакції та простотою реєстрації біолюмінесцентний аналіз широко використовується для експрес-оцінки біологічно активних та токсичних речовин. Проблема тривалої та стабільної інтенсивності біолюмінесцентного сигналу є актуальною при розробці будь-яких тест-систем, що використовують біологічні об'єкти. **Метою** даної роботи було вивчити особливості динаміки біолюмінесценції *Photobacterium phosphoreum* IMV B-7071 у різних середовищах. **Методи.** Об'єктом дослідження була культура *Photobacterium phosphoreum* IMV B-7071 з депозитарію Інституту мікробіології і вірусології ім. Д.К. Заболотного НАН України. Дослідження біолюмінесценції проводили у рідинному, агаризованому і целюлозно-ватному середовищах. Бактеріальні суспензії культивували за температури 21 °С у рідкому поживному середовищі наступного складу (г/л): пептон — 5.0; дріжджовий екстракт — 1.0; NaCl — 30.0; Na_2HPO_4 — 5.3; KH_2PO 4 × 2H_2 O — 2.1; $(NH_4)_2HPO_4$ — 0.5; $MgSO_4$ ×

× $7H_2O$ — 0.1; гліцерин — 3.0 мл/л, вода дистильована — до 1 л, pH 7.0. Тверде середовище було такого ж вмісту з 2% агар-агару. В експериментах на целюлозно-ватному середовищі на ватні диски поміщали 1 мл поживного середовища зазначеного складу, після чого в центральну частину додавали 0.2 мл бактеріальної суспензії з оптичною густиною D = 0.100, що відповідало концентрації клітин $2*10^7$ клітин/мл. Вимірювання люмінесценції проводили методом цифрової фото- або відеофіксації за допомогою цифрових фотоапаратів Olympus SP560UZ, CANON 700D та камери мобільного пристрою Samsung 9 Note зі спеціалізованими додатками для мобільних пристроїв «Colorimeter (Lab Tools Apps)» та Camera Color Counter (Keuwsoft) за максимальної світлочутливості в режимі автоматичного балансу білого на фіксованій відстані від зразка. Обробку зображень проводили за допомогою програм ImageJ та Origin Pro. Спектри люмінесценції бактерій та їх динаміку вимірювали за допомогою спектрометра LOMO MDR-23 в діапазоні 200—750 нм. Як контроль темнового світіння використовували поживне середовище, в якому культивувались бактерії. Статистичну обробку експериментальних даних здійснювали згідно із загальноприйнятими алгоритмами варіаційної статистики. **Результати.** Результати дослідження доводять, що біолюмінесценція фотобактерій *P. phosphoreum* не є стаціонарним процесом і має характерні особливості часової динаміки, пов'язаної як з динамікою концентрації кисню в середовищі бактеріальних суспензій, так і з динамікою щільності бактеріальної популяції. У водних, на твердих середовищах і на целюлозно-ватних носіях інтенсивність світіння поступово зростає, досягаючи максимуму на другу добу, після чого повільно затухає. Затухання біолюмінесценції, ймовірно, пов'язано зі зростанням щільності бактеріальної культури в умовах достатньої кількості поживних речовин. У рідинному середовищі активне збовтування бактеріальних суспензій приводить до різкого потужного спалаху біолюмінесценції з подальшим інтенсивним світінням впродовж від десятка секунд до десятка хвилин, після чого світіння швидко затухає за декілька секунд до низькоінтенсивного фонового рівня. Під час цього процесу динаміка концентрації кисню лише частково відповідає динаміці світіння на перших фазах. Фаза швидкого затухання біолюмінесценції в об'ємі бактеріальної суспензії характеризує особливості роботи внутрішньоклітинних регуляторних механізмів, які, ймовірніше за все, контролюють процес світіння за пороговим «on-off» принципом. Бактеріальна люмінесценція спостерігається за дуже низьких концентраціях кисню, що свідчить про високу енергоефективність функціонування біолюмінесцентної системи фотобактерій. Аналіз спектрів бактеріальної люмінесценції свідчить про те, що світіння відбувається у всьому дослідженому діапазоні 200—700 нм. Цей факт свідчить, що окисно-відновні перетворення субстратів, які відбуваються в активному центрі люциферази *P. phosphoreum* IMV B-7071, є ферментативним високоенергетичним процесом, що супроводжується виникненням потужних електрон-збуджених станів в активному центрі ферменту, релаксація яких приводить до випромінювання світла головними люмінофорами з подальшим розсіюванням його в широкому діапазоні. Часова варіативність спектрів світіння фотобактерій, насамперед у спектральних діапазонах люмінесценції головних емітерів бактеріальної люмінесценції — флавіноксиду і люмазину, є причиною кольорових коливань світіння між синім і зеленим діапазонами, які спостерігаються при моніторингу спектральних особливостей біолюмінесцентного світлопотоку. **Висновки.** З'ясовано ключові параметри динаміки багатоденної фонової та короткочасної індукованої біолюмінесценції фотобактерій на різних середовищах, а також показано певну мінливість спектральних характеристик люмінесцентного випромінювання у часі. Виявлені особливості динаміки люмінесценції фотобактерій *P. phosphoreum* мають обов'язково враховуватись у практичному використанні з метою оцінки токсичності речовин різної природи, а також в екологічному моніторингу.

Ключові слова: *бактеріальна біолюмінесценція, Photobacterium phosphoreum, спектри люмінесценції, водне, тверде агаризоване та целюлозно-ватне середовища.*