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Bioluminescent Bioassays Based on Luminous Bacteria

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Bioluminescent analysis is one of the most promising express methods for biologically monitoring the environment because the luminescent system is highly sensitive to even micro quantities of pollutants. Bioassays based on luminous bacteria give an integral estimation of toxicity and frequently surpass other known bioassays in speed, accuracy, sensitivity and simplicity. The enzymes of bacterial luminescent system are also used in developing highly sensitive analytical methods for practical purposes. This paper considers the main features of bacterial bioluminescence and isolated luminescent system, and also the applications of bioluminescence. One part reviews the investigations on the influence of different chemical substances on bacterial luminescence, development and using of bioluminescent bioassays, in particular some of them, in environmental monitoring of several regions in Russia.
Luminous bacteria strains from the Culture Collection of Institute of Biophysics SB RAS (CCIBSO 836) are successfully used to produce new or improved bioassays based on lyophilized bacteria bearing the lux gene. Such bioassays are suitable for biotesting of water, air, soil, and the chemical substances used in everyday life. The «know-how» of kits for bioluminescent analysis and bioassays based on lyophilized natural luminous bacteria P.phosphoreum and recombinant E.coli strain with cloned lux genes is developed. The bioluminescent assay has certificate and is recommended as an additional method of ecological monitoring in parallel with the other bioassays. The sensitivity of the bioassays developed in the IBP SB RAS is comparable to that of the foreign analogues Microtox$^{©TM}$, ToxAlert$^{©TM}$, etc.

Keywords: bioluminescent assay, bioluminescence, biotesting, pollution.

Introduction

Bioluminescence in the visible spectrum area is a mysterious natural phenomenon. The ability to emit light in the visible spectrum is characteristic of living organisms which can be found on every branch of the evolution tree. At the same time, the majority of luminescent organisms inhabit aquatic ecosystems. The biological meaning of luminescence for many of these organisms is still unclear; and bioluminescence keeps on attraction for investigations. The particular interest is the possibility of using this trait for solving various basic and applied problems. Threat of increase of rates of environmental contamination puts a task to solve one of the important applied problems – development of express methods for an estimation of pollution.

High sensitivity and rapid response to action of different agents (in comparison with other biological tests) make bioluminescence of bacteria an efficient tool to determine of micro quantities of various inhibitors of biological activity (Stewart,

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Williams, 1992, Kudryasheva et al., 2002, Ren, Frymier, 2003, Roda et al., 2003). Environment toxicity monitoring is especial area of application where microbial bioassays are widely used simultaneously for both the integrated estimation of pollution in any given environment as well as the detection and biotic effect of specific substances. Luminescent bacteria – producers of luciferase and oxidoreductase – are applied to make different preparations for biotesting of various substances for almost a half of a century. Bioassays based on luminous bacteria quantify toxicity and are often quicker, simpler, more precise and sensitive than other bioassays based on ciliates, daphnia, algae, fish and ect. (Gupta, Lin, 1995, Kaiser, 1998, Hemming et al., 2002, Kim et al., 2003, Deheyn et al., 2004, Jos et al., 2005). They are applied to monitor a large number of toxicants. Bacteria of two natural marine genera, *Photobacterium* and *Vibrio* (*P.phosphoreum, P.leiognathi, V.fischeri and V.harveyi*) are widely used for this purpose. At present a lot of various bioluminescent tests have been developed. They are based on bacteria bearing the genes of bioluminescent systems of natural luminous bacteria and also enzyme-substrate systems isolated from them (Kratasyuk, Esimbekova, 2003; Rodicheva et al., 2004; Roda et al., 2004; Girotti et al., 2008a).

The Institute of Biophysics of Siberian Branch of Russian Academy Sciences (IBP SB RAS) carries out the studies of different aspects of bioluminescence for a long time. The luminous bacteria are studied intensively in different directions: distribution in the World ocean, symbiotic models, metabolic peculiarities and regulation of luminescent system, application in biotechnology, medicine, ecological monitoring. The Institute of Biophysics hosts and maintains the specialized Collection of luminous bacteria (CCIBSO 836) containing over 400 strains isolated in various regions of the World Ocean (Rodicheva et al., 1997, 1998). The Collection has also recombinant *Escherichia coli* strains bearing *lux* genes from *P.leiognathi* plasmids pUC18 and pHL1, which were designed by Dr. B. Illarionov (IBP SB RAS). CCIBSO is the main base for designation and development of bioassays which are used whole bacterial cells or selected from them luminescent system. The technology for production of highly cleared enzymes of luminescent systems and kits of reagents for the bioluminescent analysis (KRABs) have been developed. These bioassays and kits of reagents are used for development of various bioluminescent analysis techniques for medicine, biotechnology, ecology, education.

This review deals with the bacterial bioluminescence and the applications of bioluminescent bacteria and their luminescent system developed in different areas, especially in environmental items. The collected information has been divided in several parts. The first part concerns general features of bacterial luminescence. In the second part there is the main characteristic and peculiarities of bioluminescent analysis and bioluminescent bioassays based on both natural and genetically modified bacteria and luminescent systems selected from them. The third part informs about development of bioluminescent analysis in IBP RAS including studies on different effects of some chemical substances on bacterial luminescence and ultrastructure of luminous bacteria, and especial attention devotes to use of bioluminescent assays in environment monitoring of some regions of Russia.

1. Bacterial Bioluminescence

Bioluminescence is one of the most fascinating and unique phenomena of the living nature. The ability to emit visible light has emerged in the process of the evolution. The luminescent organisms even the most primitive (bacteria and protozoa) evolved special mechanisms regulating

this function. The major living emitters of light, both in species composition and in number, are the marine inhabitants. The smallest ones here are luminous bacteria. They are mainly represented by marine species, but the non-marine bacteria also occur. The process of emitting light by living organisms is based on chemiluminescence resulting from the formation of an electron-excited intermediate in the course of enzyme reaction. Reversion of this intermediate into the basic state is accompanied by the emission of visible light with certain wavelength, intensity and duration. The quantum yield of bioluminescence reactions is very high and amounts to 0.1 – 1.0. The bacterial bioluminescence is caused by the reaction catalyzed by luciferase (flavin dependent monooxygenase), requiring a reduced flavin mononucleotide ($FMNH_2$), O_2, and a long chain fatty aldehyde (RCHO). The reaction products are the oxidized FMN, the corresponding fatty acid (RCOOH), H_2O, and visible light (Hastings, 1968; Hastings, Johnson, 2003):

$$FMN\,H_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + light$$

The luminescence of bacteria in favorable growth conditions is rather bright; in a slightly blacked-out room it is well visible to an eye. Colonies of photobacteria grown on a solid nutrient medium emit cold greenish-blue light, reminding the light of snow-covered fields in a frosty moonlight night or the flickering star sky (Fig. 1). Usually, bacteria emit greenish-blue light (max ~ 490nm), but some strains of *V. fischeri* have been found to emit yellow light (max ~ 545nm).

The bacterial luciferase is a heterodimer consisting of two different polypeptides called α- and β-subunits (with a molecular mass being 40 kDa and 37 kDa, respectively), and encoded by genes *lux*A and *lux*B. The α-subunit has the active site. When the β-subunit is absent the α-subunit

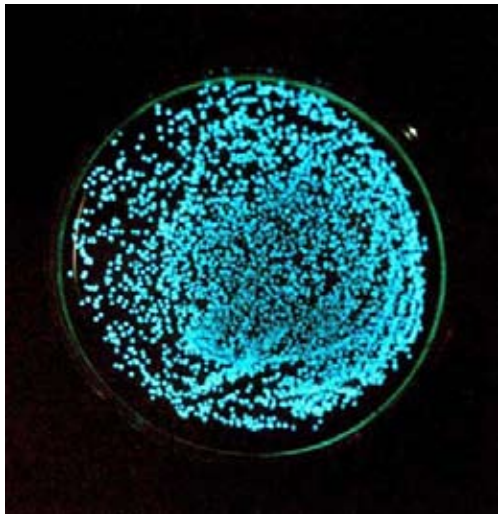

Fig. 1. Light emission in darkness from luminous bacteria colonies grown on solid nutrient medium

gives low level of light intensity. Basing on the available crystal structure of *V. harveyi* luciferase, it has been assumed that β-subunit plays a role of the support in conformational change of α-subunit in the catalysis (Meighen, 1994; Fisher et al., 1996).

Permanent light emission of luminescent bacteria is provided by different enzymes that constantly produce components necessary for bioluminescent reaction. To provide bioluminescence expression the DNA fragment of size 9000 p.n. is enough, which includes the following genes: *lux*R (repressor), *lux*I (inducer), *lux*A, *lux*B (luciferase), *lux*C, *lux*D *lux*E (aldehyde) (Engebrecht, Silverman, 1984, Meighen, 1994). S.C. Tu (2003) has published a review on the biochemistry of bacterial bioluminescence, focusing on the mechanisms and structure of luciferase. The sequence of structure genes (*lux*C, *lux*D, *lux*E), which code the synthesis of aldehyde factor, and luciferase genes (*lux*A and *lux*B) were the same in all bacteria species, indicating the similarity of luminescent systems. At the same time the regulator site of *lux* systems diverged appreciably; therefore, several regulator *lux* genes (*lux*I, *lux*R) found in *V.fischeri* didn't

reveal any analogs in *V.harveyi*. Other genes including *lux*F, *lux*G, and *lux*H, whose functions are not clearly identified yet and whose role for bioluminescence is not clear are also located in *lux* operon (Urbanczyk et al., 2008).

Luminescence is closely connected with their general metabolism and depends on the stage of culture growth, environment conditions. Bioluminescence is governed by common and specific regulation of bacterial operon expression. In the laboratory the luminous bacteria growing in liquid media at low cell density emit minimum of the light due to the weak expression of *lux*CDABE genes and deficiency of precursors of bacterial luciferase reaction. From the middle till the end of exponential growth phase the intensity of light emission increases abruptly due to the quick accumulation of synthesized substrates and enzymes for the activation of *lux*CDABE gene expression. Studies with luminous bacteria *V.fischeri* and *V.harveyi* (Nealson 1977; Nealson, Hastings, 1979; Meighen, 1991, 1994) led to the discovery of «autoinduction», now referred to as «quorum sensing» and shown to be functionally important in a wide number of bacterial groups, including pathogenic and symbiotic, gram-negative and gram-positive ones (Miller, Bassler, 2001; Whitehead et al., 2001; Waters, Bassler, 2005; Camilli, Bassler, 2006). The bioluminescence appears at threshold concentration of autoinducer N-(3-oxohexanoyl) homoserine lactone (3-oxo-C_6-HSL) at the density of culture that exceeds 10^7 cells/mL (Eberhard et al., 1981). Variable conditions of environment, damaging cells result in decreasing or increasing luminescence. At the same time the decrease or increase of the expression level of bioluminescence has been caused by changes in *lux* operon regulation (Medvedeva et al., 2006). Therefore, high sensitivity of the bioluminescence response to any changes of environment (physical, chemical, radiological and etc.) determines their wide use as bioassays to estimate the toxicity and pollution in the environment. The mechanisms underlying the toxic effects of chemicals in these assays are diverse and complex (Cronin, Schultz, 1998; Ismailov et al., 2000; Davidov et al., 2000; Kudryasheva, 2006). For example, toxicity may involve interactions with cell surface receptors, disruption of cell membrane functions, and chemical reactions with cellular components or inhibition/competition of enzyme systems (Sixt et al., 1995; Gustavson et al., 1998; Mariscal et al., 2003). Antagonistic and synergistic interactions with other compounds are complicating factors that can dramatically affect toxic responses both inside and outside the cell and within the cell membranes.

2. Bioluminescent analysis

The presence of toxic compounds and the potential associated ecological risks can be determined by using bioassays and toxicity tests. Tests using alive organisms as indicators are widely employed to identify the toxicity of chemicals or commercial products, and environmental pollution. The biological biotesting method is based on changing of important parameters of an organism and takes into account change and interactions of polluting substances. Death rate, survivability, intensity of breath and other metabolic processes of various test-organisms are the most often used parameters among known bioassays. Bioassays allow evaluating the changes in physiology or behavior of living organisms resulting from stresses induced by biological or chemical toxic compounds, which can cause disruption of the metabolism. The estimation of the observable effect on the whole population is considered as perspective and more informative. The biological method of biotesting allows estimating not only sample toxicity, but also integrated influence of toxicants on ecosystem as a whole. Change of

luminescence intensity of the test – organism testifies to presence of conditions unusual for ecosystems. Quenching of luminescence speaks about presence of substances dangerous to life, and stimulation of luminescence can be estimated as presence of the substances strengthening metabolic activity of test – object. Since bacterial bioluminescence is tied directly to cellular respiration, any inhibition of cellular metabolism due to toxicity results in a decrease in the light emission of the affected cells. But there are some limitations with the bioluminescent bacterial assay. Living cells are complex systems, and light output of the bioluminescent biosensors depends not only on the chemical complexity of the sample but also on variations of the physiological state of the cells, including changes in the rate of gene transcription, protein synthesis, membrane permeability, and metabolism. These facts should de taken into account during application. Luminescent bacteria are included now in the list of tests – objects used in the ecological control in different countries together with the classical objects: algae *Scenedesmus quadricauda* Turp., *Chlorella vulgaris* Beije, bacteria *Escherichia coli*, *Salmonella* sp., infusorian *Paramecium caudatum* Ehr., daphnia *Daphnia magna* Straus and fishes *Poecilia reticulata* Peters and *Brachydanio rerio* Hamilton-Buchanan (Zhmur, 1997; ISO 11348-2; Van Hamme et al., 2003; Reshetilov, 2007).

The main measured parameter in bioluminescent bioassay is luminescence. The test is standardized as a method for pollutants' detection, and used now in different countries (ISO 11348-2). The high sensitivity of the luminescent system, up to micro-quantities of toxicants, the rapid results, the exact quantification of changes in the luminescence level and convenience in work make the bioluminescent analysis suitable for quick biological monitoring the environment (Bulich, Isenberg, 1981; Gitelson et al., 1984; Isenberg, Bulich, 1994; Kaiser et al., 1998; Wolska, Polkowska, 2001; Ulitzur et al., 2002; Wang et al., 2002; Parvez et al., 2006; Grande et al., 2007; Girotti et al., 2008a; Zhu et al., 2009). Bioassay is a standard technique to measure total toxicity of studied samples; it excludes the necessity to cultivate and maintain bacterial culture with *lux* gene. A bioluminescent assay is often chosen as the first screening method in a test battery. The luminous bacteria bioassay takes not more than 30 min (compare to the other bioassays that may take up to 48-96 hours). Such characteristics provide to luminous bacteria doubtless advantage in comparison with other biological tests. It is known that the response of luminescent bacteria to toxic substances fully correlates with that at other biological organisms, and the value EC_{50} (50 % quenching luminescence) fully correlates with the LD_{450} for the higher animals (Ganshin et al., 2001). Moreover, it becomes unnecessary to perform costly chemical analysis of all samples. This will be done only if the bioluminescent sensor signals an alarm.

Methods of biological monitoring of environment condition are the major component of a complex of nature protection measures. The bioassay applying for inclusion in an expert system should meet strong requirements: 1) availability, simplicity tests – objects cultivation, 2) an opportunity of precise registration of effects, 3) simplicity of performance with bioassay, 4) high-speed reply quick response time, 5) accuracy, reproducibility and reliability of results, 6) high enough sensitivity, 7) profitability. Bioluminescent methods of the analysis based on the intact or lyophilized bacteria emitting visible light meet all of the listed requirements. The enzyme systems selected from luminous organisms including luciferases, which translate energy of chemical reactions catalyzed by them, in radiation of an optical range, are also used in the bioluminescent analysis. Bioluminescent

bioassays allow to receive a prompt reply about toxicity of a sample and to make the decision on carrying out further of the expensive chemical analysis. Measurement of intensity of luminescence with bioluminometer usually takes 5-30 min after addition of a studied sample to a test – object. Procedure of measurement is simple, especially at study of water samples. Bacterial bioluminescence is one of the most used bioluminescent systems in ecological monitoring. While chemical analysis shows biodegradation of compounds, the bioluminescent biosensor assays report on changes in bioavailability and potentially toxic integrative effect of contaminants. Thus, they are alarm about toxicity in environment.

The change in the luminescence intensity of bioluminescent bioassays after the action of an analyzed substance fulfills modern requirements for estimating substance toxicity. Bioluminescent analysis is used to determine such toxicologically accepted parameters as: efficient concentration (EC_{50}) – the substance concentration that inhibits the luminescence function by 50 %, and threshold concentration (TC-0), or biologically safe dilution level (BSDL) – the substance concentration (dilution) at which the luminescence level of analyzed solutions is equal to the luminescence intensity in the control sample. The most important toxicological parameter (EC_{50}) is the substance concentration that reduces bacterial luminescence by 50 % (Drzyzga et al., 1995).

In the bioluminescent analysis the whole cells of the natural or genetically modified bacteria bearing genes of luminescent system, and also the luminescent system selected from luminous organisms are used most frequently as a test – object. Some terms for a designation of developed preparations for biotesting are used in the literature: biosensor, bioassay (biotest), bioreporter. The lyophilization or immobilization of a test – object in the certain carrier are used to bioassay making, devices for maintenance of a prolonged quality monitoring of toxicity of an environment are developed.

Creating bioluminescent bioassays usually consists of three stages: 1) preparing the test object; 2) measuring assay luminosity in the presence or absence of the analyzed substances; and 3) establishing the relationship between luminosity parameters and the quantitative characteristics of the medium's toxicity.

2.1. Preparing the test object

2.1.1. Alive test bacterial culture

Bacterial test-objects are usually standardized in two ways: either by using a continuous culture of luminous bacteria or by producing reagents based on freeze-dried or immobilized bacteria. In continuous cultivation in a luminostat or a bioluminostat the bacterial culture can be maintained in a certain physiological state, with constant luminescence intensity, by controlling the nutrient solution flow (Gitelson et al., 1984, Pooley et al., 2004). A new portion of media is added when bacterial luminescence exceeds the predetermined level of luminosity. The supply of nutrient solution is stopped when bacterial luminescence falls to the target intensity. The luminescence then continues to fall due to reduction in a specific luminescence, but this is counterbalanced by the increase in the number of bacterial cells which increases the intensity of the luminescence.

Schwedt G. with co-authors (Schwedt et al., 1997) have offered the bioluminescent bacterial bioassay for continuous monitoring of water. The computer control system optimized the following parameters: mixing of analyzed solution and bacterial suspension, intensity and the period of vibration during mixing, a delay of time at temperature alignment, time of stabilization of bacteria and an estimation of the measured signals. All measurements have been carried out with use of organic and inorganic pollutants

(formaldehyde, phenol, 2,4-dichlorphenol, cyanides, ions of lead, etc.). Scheerer S. and co-workers (Scheerer et al., 2006) studied the conditions for continuous cultivation of the luminous bacterium, *Vibrio fischeri* NRRL-B-11177, in a fermenter. They reported that the designed system provided a reliable long-term (more than 1 month) continuous culture facility for the reproducible measurement of perturbation of *V. fischeri* metabolism by monitoring changes in its luminescence. The other systems for continuous tracking toxicity of the water, which fixed the response from one strain or at once from several recombinant strains with a *lux* gene, were developed by scientists from USA (Ripp et al., 2000), South Korea (Lee, Gu, 2005) and Germany (Stolper et al., 2008).

Continuous cultuvation as the bioassay is not widely used up to now. A luminostat significantly increases the measurement precision, but the assay sensitivity is almost 10 times lower than in the case of a batch culture. Another drawback of continuous cultivation is its technical complexity and great expenditure of nutrient solution.

2.1.2. Reagents based on freeze-dried or immobilized bacteria

Lyophilization of luminous bacteria is most frequently used to produce standardized and stabile bioluminescent assay to analyze different luminosity-inhibiting substances. The batch culture of bacteria with high luminescence intensity level is usually used to prepare bioluminescent assay. There are some bacterial based systems in use at present, e.g. Microtox™ (AZUR Environmental, Carlsbad, CA, USA), ToxAlert™ (Merck, Germany), BioTox™ (Hidex Oy, Turku, Finland); they use the luminous bacterium *Vibrio fischeri.* In addition, ToxScreen assay (CheckLight Ltd., Qiyrat Tiv'on, Israel) uses *P.leiognathi,* and Microbiosensor (IBP SB RAS, Russia) uses *P.phosphoreum*, while others use genetically constructed luminous bacteria. Bioluminescent bioassays based on luminous bacteria, Microtox®, BioTox™, LUMIStox™, ToxAlert™, SOS-LUX-TOXICITY-Test, etc., are used in different countries (Bulich, Isenberg, 1981; Richardson, 1996; Jennings et al., 2001; Wolska, Polkowska, 2001; Wang et al., 2002; Elke et al., 2002; Loibner et al., 2004; Micevska et al., 2006; Rabbow et al., 2006; Grande et al., 2007). Flash Assay protocol is also successfully used for analysis of suspensions, turbid and colored samples, as well as for high throughput evaluation of harmful properties of chemicals, including organic and inorganic NPs, to bacteria (Lappalainen et al., 1999; Mortimer et al., 2008).

In Russia there are some bacterial bioluminescent bioassays. Bioassays Microbiosensor-B17 (based on luminous lyophilized *P. phosphoreum*) and Microbiosensor-ECK (based on *E.coli Z905* carrying pHL1 plasmid from *P. leiognathi*) were developed at the Institute of Biophysics of SB RAS using bacteria from the Culture Collection IBSO (Kuznetsov et al., 1990, 1996, 2000). A few bioassays «Ecolum» (on the basis of genetically modified *E.coli* strain with luminescent system genes from luminous bacteria *V.fischeri* and *V.harveyi*) were developed in the Moscow State University (Ganshin et al., 1998; Danilov et al., 2002) and bioassays based on genetically modified *E.coli* strain with *lux* operon from bacteria *V. fischeri* and *Photorhabdus luminescens* were developed in the State Research Institute for Genetics and Selection of Industrial Microorganisms (Manukhov et al., 1999; Rastorguev, Zavil'gelsky, 2001). The microbioluminescent indicator of toxicity (MIT) (on a basis of genetically modified *E.coli* strain) was also created in the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Science (Pshenichnov et al., 2005).

The most common is Microtox™, which is widely used in laboratory and field experiments to monitor the quality of industrial and natural waters and to determine the degree of toxicity of pharmaceutical preparations and newly devised chemical compounds (Backhaus et al., 2000; Jennings et al., 2001; Hyung, Gu, 2002; Pham et al., 2004). Microtox™ is used to test micro quantities of aromatic hydrocarbons, heavy metal salts, pesticides, respiration poisons, and other water contaminants (Ritchie et al., 2001; Wolska, Polkowska, 2001; Fernández-Alba et al., 2002; Wang et al., 2002; Kim et al., 2003; Phyu et al., 2005; Girotti et al, 2008b). It is also used in medical investigations (Simon et al., 2001; Pellinen et al., 2002).

Some researchers have proposed a bioassay using immobilized luminous bacteria (Schreiter et al., 2001; Premkumar et al., 2001; Christo et al., 2002; Lee et al., 2003; Park et al., 2005; Sakaguchi et al., 2007; Tamia, 2007; Chu et al., 2009). Cells of native or genetically modified luminous bacteria are immobilized by gelatin, Na alginate or polyvinyl (alkyl-styrylpyridinium) polymer, encapsulated in a sol-gel matrix, immobilized in a chip assembly or onto a glass surface. Immobilized preparations were stable and used to monitor the toxicity of different substances (Lee et al., 2003; Tani et al., 2004; Yoo et al., 2007; Yu et al., 2008).

2.1.3. Genetically-modified organisms

Luminescent genetically-modified microorganisms (*Pseudomonas* genus, *E. coli,* etc.) with *lux* genes from marine *Vibrio* and *Photobacterium* species or from *Photorhabdus* (*Xenorhabdus) luminescens* are widely used as test-objects among bioluminescent bioassays to determine the presence of different substances in water and soil samples (Gu, Choi, 2001; Rastorguev, Zavil'gelsky, 2001; Abd-El-Haleem et al., 2002, 2006; Bechor et al., 2002; Ren, Frymier, 2003, 2005; Berno et al., 2004; Ivask et al., 2004; Lovanh, Alvarez, 2004; Lang et al., 2005; Toba, Hay, 2005; Wiles et al., 2005; Lee et al., 2006, 2007a; Leedjarv et al., 2006; Grande et al., 2007; Chinalia et al., 2008). The possibility of using *lux* genes as markers of gene expression is important for studying pathogeny, virulence, adaptations and secondary metabolism (Van Dyk, 1998; Lovanh, Alvarez, 2004; Qazi et al., 2004). The bioluminescent reporter strains were created to investigate and control the survivability of bacterial species in different host cells (Francis et al., 2000; Van Dyk, 2001; Gillor et al., 2003). The strain *Salmonella hadar,* which had a complete set of luminescent system (*lux* CDABE) from *P.phosphoreum,* was used to study bacteria's ability to restore their original properties after stress action (Bautista et al., 1998). Bioassays based on recombinant bioluminescent strains have being developed to determine the presence and effectiveness of various antibiotics (Kurittu et al., 2000; Simon et al., 2001; Pellinen et al., 2002; Vesterlund et al., 2004) and narcotics (Valtonen et al., 2002). Correlation of a *lux*-label with the PCR-analysis (Hill et al., 1994; Bechor et al., 2002; Park et al., 2004; Urbanczyk et al., 2008) have shown, that put cloned marker *lux*-genes in the common line with other modern methods of identification.

Strains were constructed in which the luminescence increased after the action of studied substance. Some examples were used to determine the mercury presence (Stewart, Williams, 1992; Yamagata et al., 2002; Endo et al., 2003), some metals (Riether et al., 2001; Rastorguev, Zavil'gelsky, 2001; Fulladosa et al., 2005a). Other *lux*-fusions were constructed to monitor the expression of catabolite genes, including those for degradating isopropylbenzene (Selifonova, Eaton, 1996) and toluene (Applegate et al., 1998), mixture of naphthalenes (Ford et al., 1999), expression of heat shock genes (Van

Dyk et al., 1994, 1995; Rupani et al., 1996), oxidative stress (Belkin et al., 1997; Szpilewska et al., 2003; Lee et al., 2007b; Niazi et al., 2008). Some bioassays were developed on the basis of pseudomonads. Applegate et al (1998) constructed a strain using *Pseudomonas putida* F1 contained a complete *lux*-casette (*lux*CDABE) to investigate the induction of *tod*-operon after exposition with benzene, toluene, ethylbenzene and xylene compounds. The tod-*lux*-reporter was very sensitive and allowed bacterial luminescence in whole cells to be measured without added aldehyde substrate. In addition, the constructed strain was sensitive to benzene, ethylbenzene, m- and p-xylene, and may be used as a reporter to estimate hydrocarbon fuel pollution that contains these substances. The bioluminescent reporter based on *Pseudomonas fluorescens* HK44 is capable to emit light under action of naphthalene, salicylates and other analogues of substrats (Ripp et al., 2000). Using recombinant strains of *E. coli* with a cloned luciferase gene is successful to develop a bioluminescent bioassay that can be used to analyze fresh waters. Scientists have already shown how genetically engineered strains of *E. coli* with a complete set of luminescent system (*lux*CDABE) from the luminescent bacteria *V.fischeri* can be used to identify 23 out of 25 investigated toxicants in mixtures of various compositions and to assess the their degree of toxicity (Ben-Israel et al., 1998). Genetically engineered microorganisms, tailored to respond by a dose-dependent signal to the presence of toxic chemicals, are a potentially useful tool for environmental monitoring. Several reporters, which based on a panel of luminescent bacterial strains, harboring fusions of the *lux*CDABE operon to various stress-responsive gene promoters, were obtained using this approach. Different bioluminescent reporters were designed to register heavy metals and phenols in water and soil samples (Bechor et al., 2002; Stoyanov et al., 2003; Ivask et al., 2004; Alkorta et al., 2006; Dawson et al., 2006; Heinlaan et al., 2007; Trott et al., 2007; Elad et al., 2008), and mutagenic pollution of environment (Czyz et al., 2002; Guzzella et al., 2006; Podgorska, Wegrzyn, 2006; Heinlaan et al., 2007).

Methods based on biochemiluminescent reactions play an important role not only in environmental research, but also in biomedical studies and clinical medicine (Rathinam, Mohanan, 1998; Simon et al., 2001; Kuklin et al., 2003; Gad et al., 2004; Lang et al., 2005; Deriabin, Poliakov, 2005; Vlasova et al., 2007), as well as in immunology (Kurtasova et al., 2001; Edinger et al., 2003). The new generation of bioreporters for *in vivo* monitoring and diagnostics technology was developed to investigate the expression of the bacterial luciferase system in mammalian cells (Patterson et al., 2005). Development of the bioluminescent reporters containing simultaneously genes of luminescent systems from luminous bacteria or firefly *Photinus pyralis* and green fluorescent protein from *Aequorea victoria* considerably expand the spheres of their use in medicine and ecological monitoring (Pellinen et al., 2002; Lehtinen et al., 2003; Mitchel, Gu, 2004; Yagi et al., 2007, Li et al., 2008).

2.1.4. Enzyme systems

The enzyme bioluminescent analysis is based on measuring of several important parameters (intensity, light-emission decay rate constant, general quantum output) of light emission resulted from two conjugated reaction luciferase- NAD(P)H- oxidoreductase (coupled enzyme system). Enzymes in the NAD(P)H-oxidoreductase family catalyze the reduction of flavin at the expense of NAD(P)H. Bacterial luciferase utilizes oxygen, reduced FMN ($FMNH_2$) and a long-chain aliphatic aldehyde as substrates for prolonged light emission:

$$NAD(P)H + FMN + H + \xrightarrow{\text{reductase}} NAD(P) + + FMNH_2$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + light$$

Thus, the light-emitting system in this case contains two enzymes and three substrates added in a reaction mixture. Coupled enzyme system is mainly used in world practice for development of specific methods of the analysis of various metabolites and enzyme activity (Brolin et al., 1971; Middleton, Smith, 1976a,b; Ismailov et al., 1981; Petushkov et al., 1984; Stanley, 1996). Information on enzyme luciferase systems use continues to appear till now (Mourad et al., 2000; Kudryasheva, 2006; Liu et al., 2008). It was shown that advantages of bioluminescent methods *in vitro* are promptness, high (femto- and picomol substances) sensitivity, simplicity, a small error of measurements, repeatability (Kratasyuk, Gitelson, 1987; Kratasyuk et al, 1991; 1996). This also enables the analysis of small-volume samples, which leads to the development of miniaturized and high-throughput assays.

Immobilization of the connected system in various supports is used to increase stability of enzymes and provide an opportunity to get light reply in one sample. A systematic review on the methods to immobilize the different luminescent enzymes and microorganisms (natural and genetically engineered) and to use them in biosensors and bioinvestigations was made by V. Kratasyuk and E. Esimbekova (2003). Stability, sensitivity, precision, and effects of interfering substances and microenvironment were outlined. Advantages and limitations of immobilized enzyme biosensors in bioluminescent analyses were also highlighted in this review. At present, a creation of the prototype of the bioluminescent biochip based on nanotechnology develops also this direction (Puzyr et al., 2004).

3. Development of bioluminescent analysis in IBP SB RAS

As it was mentioned above, tests – objects of different levels of the organization are used during development of the bacterial bioluminescent bioassays: alive organisms (luminous bacteria) and biochemical (enzyme) system. The important advantage of bioluminescent bioassay is the opportunity of quantitative estimation of the substances dissolved of water on magnitude of change of the luminescence level. The bioluminescence index $BI=I_e/I_c$ (where I_c – luminescence intensity in control cuvette, I_e – luminescence intensity in experimental cuvette) is used to estimate the effect of different factors on bacterial luminescence. For the analysis 10^9 microbe cells/ml are used to guarantee average statistically authentic estimation of toxicity.

Four species of luminous bacteria *P. phosphoreum, P.leiognathi, V. harveyi* and *V.fischeri* from the Culture Collection of Institute of Biophysics of the Siberian Branch of the Russian Academy of Science (CCIBSO 836) were used for a choice of test – object for design of the bioassay based on lyophilized cells and studying of influence of various chemical substances on luminescence (Rodicheva et al., 1997, 1998; Medvedeva et al., 2005). Comparison of luminescence intensity of different luminous bacteria species before and after lyophilization has shown that *P.phosphoreum* culture had the greatest intensity after re-conservation, that's why it became the basic object for the further researches.

3.1. Effect of different chemical substances on bacterial luminescence

To develop the bioassay numerous experiments have been carried out for revealing influence of various substances on a cell as a whole and luminescent system of native luminous bacteria *P.phosphoreum* and genetically modified

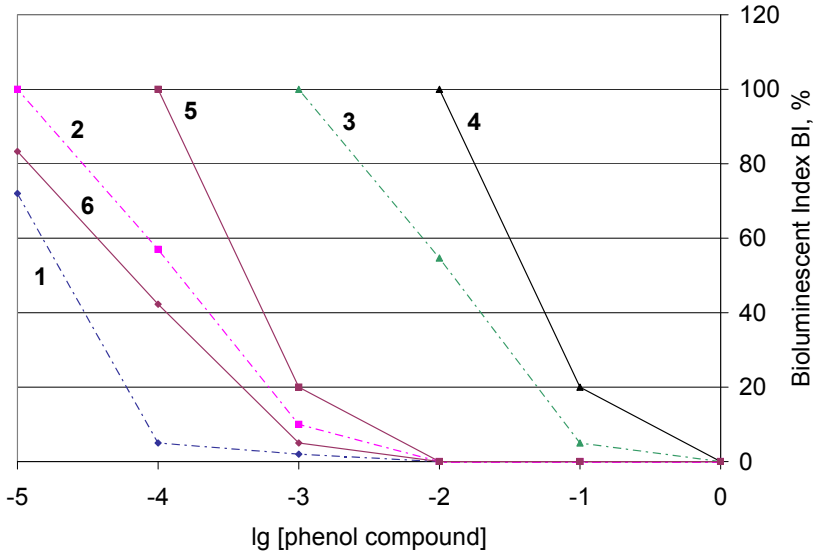

Fig. 4. Bioluminescent index vs. concentration of phenol compounds (concentration of phenol compound at lg (x) = 0 corresponded to 1 mg/ml). Notations: parabenzoquinone (1 – *E. coli*, 6 – *P.phosphoreum*); hydroquinone (2 – *E. coli*, 5 – *P. phosphoreum*); pyrocatechol (3 – *E. coli*, 4 – *P. phosphoreum*). BI norm = 80-120 %. (from Rodicheva et al., 2004)

E.coli strain bearing plasmid with *lux*AB genes from luminous bacteria *P.leiognathi*. It is known that phenolic compounds and heavy metals are one of the most widespread pollution coming in superficial waters with drains of the enterprises. Dumping of phenolic waters in reservoirs and water-currents sharply worsens their common sanitary condition, influencing on alive organisms not only due to toxicity, but also due to significant change in form of nutrients and the dissolved gases (oxygen and carbon dioxide). The solutions of easily oxidized diphenols (pyrocatechin, hydroquinone) and the products of their oxidation (p-benzoquinone), metaphenol (resorcinol), as well as heavy metal salts and other substances were used to study their action on luminous bacteria (Stom et al., 1992; Kuznetsov et al., 1998, 2000; Medvedeva, 1999). Comparing the effect of model substances on luminescence of both luminous strains revealed that the luminescence level dependence on the substance's concentration was similar in the concentration ranges studied. But the analysis of these results demonstrates that the luminescence sensitivity of recombinant *E. coli* to phenol compounds was higher than that for *P.phosphoreum* (Fig. 4). It was shown that parabenzoquinone was the most toxic, and the inhibition of luminescence was revealed at concentration 10^{-5} – 1 mg/mL. In reservoirs the maximum concentration limit for phenol is established 10^{-3} mg/L. At the same time the effective concentration (EC_{50}) of parabenzoquinone for *E.coli* was 2 x 10^{-5} mg/ml after 5 min of action. For hydroquinone the effective concentration (EC_{50}) was 2 x 10^{-4} mg/ml. EC_{50} for pyrocatechol was 10^{-2} mg/ml after 5 min of action. This phenols' toxicity row for recombinant *E. coli* corresponds to the phenols' toxicity row determined on the intact cells of luminescent bacteria *P. phosphoreum* and various hydrobionts. The luminescence sensitivity of recombinant *E. coli* to heavy metals was also higher than that for *P.phosphoreum* (Fig. 5).

Studies of the combined effects of heavy metal salts on luminescence show that in most cases their action is not additive (Kuznetsov et al., 1998), i.e. the effect of the sum of the substances isn't equal to the sum of the effects of each

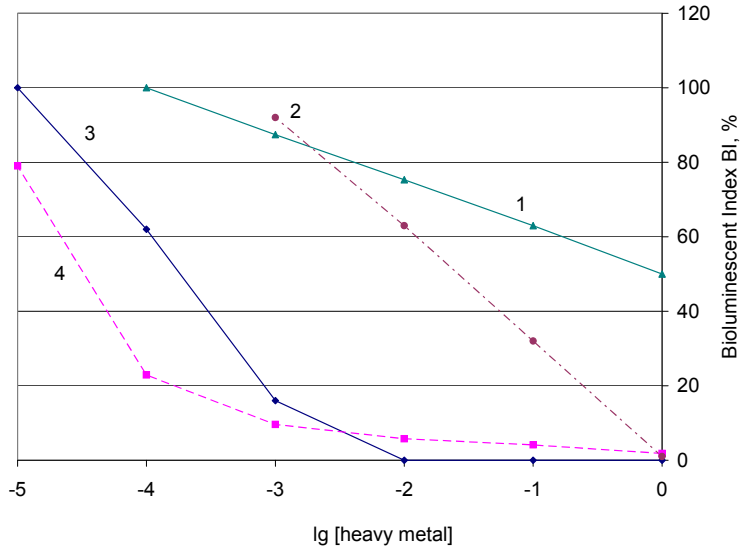

Fig. 5. Bioluminescent index vs. concentration of heavy metal salts. Notations: $CdCl_2$ (1 – *P.phosphoreum*, 3 – *E. coli*); $CuSO_4$ (2 – *P. phosphoreum*, 4 – *E. coli.* BI norm = 80-120 %

component in a mixture (Fig. 6). Any individual salt (curves 1, 2) inhibited the luminescence intensity. But the sum of the coupled effect of luminescence inhibition (curve 3) was less than the theoretical additive effect (curve 4). Thus, the nonadditivity of the effects of these salts was ascertained. The data obtained show the necessity to take into account the combined effect of different substances of effluents to give a more accurate forecast of the ecological situation. The results obtained by Fulladosa E. with co-authors (2005b) confirmed such data. The studied mechanism of the complex effect allowed us to predict more accurately effect of phenols and heavy metals on the natural biocenosis.

Electron microscopy employed to study the action of various phenols, quinones, and heavy metals on bacterial cells demonstrated the different cell damages after chemical treatment (Stom et al., 1992). The solutions of phenols, the products of their oxidation (p-benzoquinone) and heavy metal salts were used in the concentration, which caused 50 % quenching of luminescence. The tested compounds produced the different effect on the luminous bacteria cells. Cadmium chloride and resorcinol had the greatest effect, resulting in considerable damage in the cell ultrastructure (Fig. 7a, b). During a prolonged treatment the cleavages in the cell wall were seen more clearly. Hydroquinone and p-benzoquinone had a more specific effect on the luminescent system and did not cause severe structural changes (Fig. 7c). Simultaneously, the number of living cells in the population and their viability were evaluated. Cadmium chloride had the greatest effect, killing of approximately half of the population. It was followed by resorcinol, p-benzoquinone, hydroquinone, mercury chloride, and pyrocatechin.

An assay based on luminous bacteria often involves a procedure to increase the sensitivity of bacterial cells to low concentrations of toxic compounds. This can be achieved by varying the cultivation conditions and the treatment procedure with a toxicant to increase cell membrane permeability (Medvedeva, 1999; Mariscal et al., 2003), as well as by using specific sensitive mutant strains. The bacterial luminescence decreased under action of high concentration of EDTA and did not decrease under short-term treatment

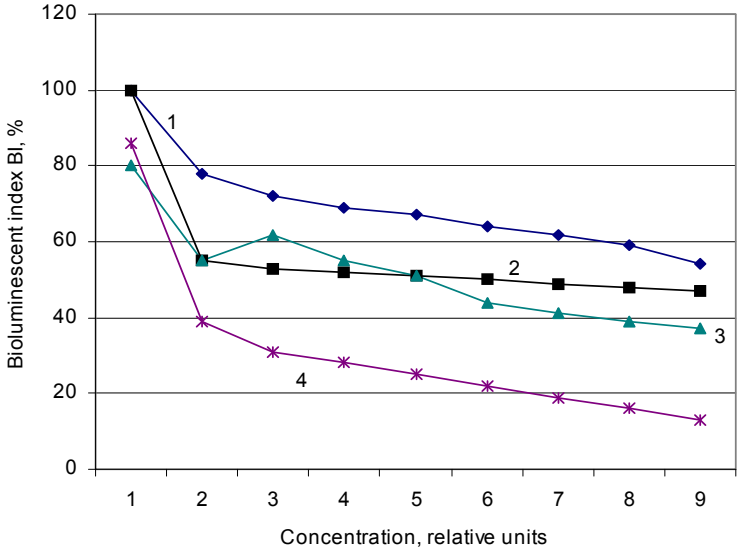

Fig. 6. Combined effects of Co-, and Cu-salts on bacterial bioluminescence. 1 – Cu-salt, 2 – Co-salt, 3 – mixture of Co- and Cu-salts, 4 – theoretical curve of additive action of Cu- and Co-salts mixture. (from Kuznetsov et al., 1998)

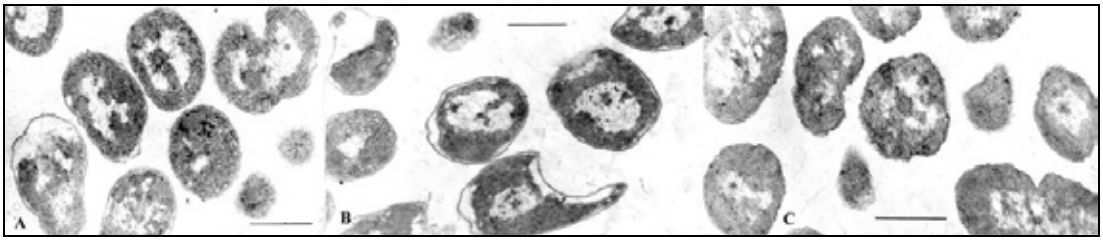

Fig. 7. Effects of cadmium chloride (A), resorcinol (B) and p-benzoquinone (C) on luminous bacteria. (from Kuznetsov et al., 1998)

with low concentration of EDTA and toluene (Fig. 8). It was shown that the condensation of DNA-fibrils occurred after short-term action of EDTA, toluene and 2,4-dinitrophenole (DNP). The most cell damage was revealed after action of 10^{-4} mol/L DNP on bacterial cells (Fig. 9), while the noticeable change of cell membranes did take place during short-term treatment with 10^{-2} mol/L toluene (Fig. 10).

However, the long-term action of these substances changed the membrane permeability resulting in increased sensitivity of bacterial luminescence to different concentrations of 2,4-DNP. *P.leiognathi* cells with maximal luminescence were more sensitive to this substance under long-term cultivation in mediums containing 10^{-4} mol/L EDTA or toluene (Fig. 11). Thus, the luminescence intensity can be used as a parameter indicative for cell vital functions.

To conclude, we used several approaches to devise an assay system that determined the action of various phenols and their derivatives, heavy metal salts and the insecticide (hexachlorancyclohexane) on luminous bacteria (Stom et al., 1992; Popova et al., 1994) and developed bioluminescent assays (Rodicheva et al, 2004). As a result, microbiosensors B17 and ECK were developed in IBP SB RAS (Kuznetsov et al., 1996, 2000), which are widely used in medicine and ecological monitoring in Russia and countries of former Soviet Union (Kuxnetsov et al., 1999; Kurtasova et al., 2001;

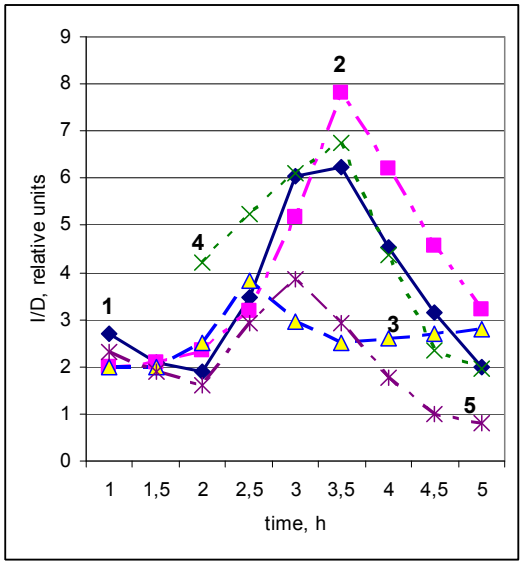

Fig. 8. Prolonged action of EDTA and toluene on *P.leiognathi* 213 luminescence: 1 – control, 2 – 10^{-5} mol/L EDTA, 3 – 10^{-4} mol/L EDTA, 4 – 10^{-3} mol/L toluene, 5 – 10^{-2} mol/L toluene

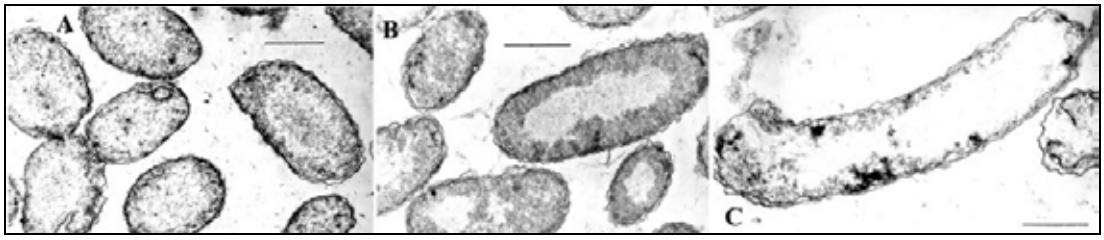

Fig. 9. Short-term action (5 min) of 10^{-5} mol/L EDTA (A), 10^{-3} mol/L DNP (B), 10^{-4} mol/L DNP (C) on luminous bacteria *P.leiognathi*

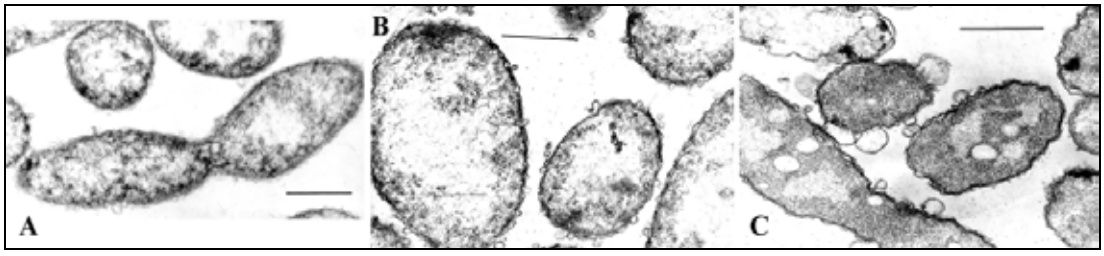

Fig. 10. Prolonged action of 10^{-3} M toluene added in nutrient medium: A – phase of logarithmic grown, B – phase of state grown, C – short-term action (5 min) of 10^{-2} M toluene on luminous bacteria *P.leiognathi*

Deriabin, Poliakov, 2005; Kolenchukova et al., 2008). Bioassays are used successfully in many ways: for continuous on-the-spot monitoring of the environment in industrial areas and human-managing ecosystems, for monitoring harmful industrial discharges, in estimating the efficiency of the methods used in environmental detoxification and the operation of purification facilities, and in environmental certification of industrial facilities and regions (Stom et al., 1992; Kuznetsov et al., 1998, 1999, 2000; Rodicheva et al., 2004). One flask of Microbiosensor can be used to measure nearly 100 experimental water samples. Microbiosensor activity is stable for 6

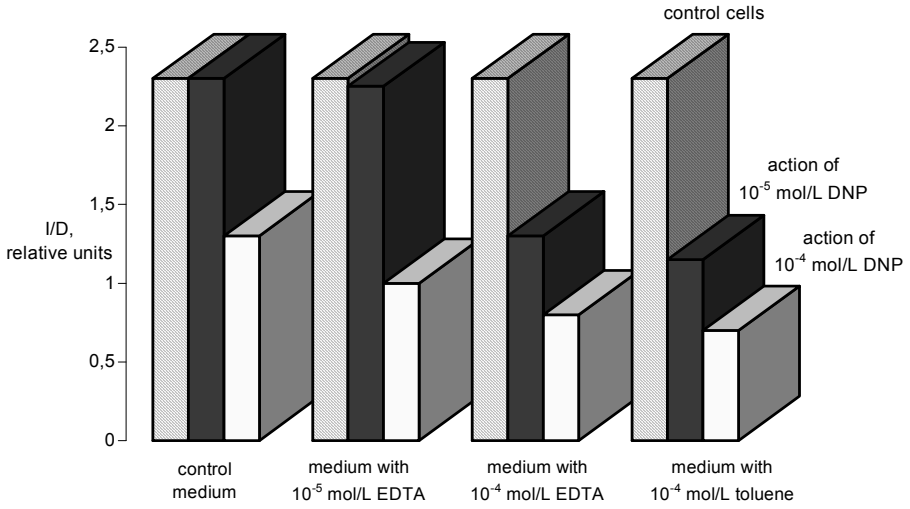

Fig. 11. *P.leiognathi* cells with maximal luminescence after cultivation in control medium, medium with 10^{-5} mol/L EDTA, medium with 10^{-4} mol/L EDTA and medium with 10^{-4}-mol/L toluene. The long-term treatment by these substances increased cell sensitivity to 10^{-5} mol/L 2,4-DNP (dark column) and 10^{-4} mol/L 2,4-DNP (white column) in comparison with cells grown in control medium (the shaded column). The first (shaded) column of control cells' luminescence is presented with other data to make more evident the action of substances on each medium (from Medvedeva, 1999)

months in freezer storage at +5-10° C and more than 1 year at –18°C.

3.2. Enzyme bioluminescent analysis

Necessary condition of wide use of the bioluminescent analysis is creation of highly effective and stable preparations. The luciferase from any luminous bacteria species can be used for the bioluminescent analysis. At the Institute of Biophysics reagent sets for bioluminescent analysis have been developed. The luciferase is isolated from the recombinant strain of *Escherichia coli* SL60 carrying *lux*A and *lux*B genes of *P. leiognathi* luminous bacteria from the Culture Collection of the Institute of Biophysics SB RAS (Tyulkova, 1990; Illarionov, Tyulkova, 1997). Two types of NAD(P)H:FMN-oxidoreductase are extracted and purified from *V. fischeri* and *V. harveyi* with specific activity to NADH and NAD(P)H. To produce a set of reagents the flavin reductase from *V. fischeri* species is used which employs NADH and NAD(P)H with similar efficiencies.

Parameters of light-emission of the coupled enzyme system considerably depend on a ratio luciferase and NAD (P) H-FMN – oxidoreductase in a reagent. The maximal activity of the coupled enzyme system depends on physical and chemical conditions of a reaction, such as pH, temperature and molarity of buffers (Fig. 2). Studies of influence of environment factors on the bioluminescent connected reaction have shown that the maximal intensity is achieved in a range of temperatures from 20 up to 30°C, at neutral pH and low molarity of buffer solution (Tyulkova, Antonova, 1991; Kuznetsov et al., 1997).

The coupled enzyme system provides the bioluminescent analytical methods to be widely used while maintaining stable sensitivity to NADH up to 0.01 pycomol (Fig. 3). The kits for bioluminescent analysis are used in applied microbiology and environmental monitoring, in biochemistry, clinical laboratory tests for determination of the activity of NAD-dependent dehydrogenases, their substrates, proteases and antiproteases in biological objects.

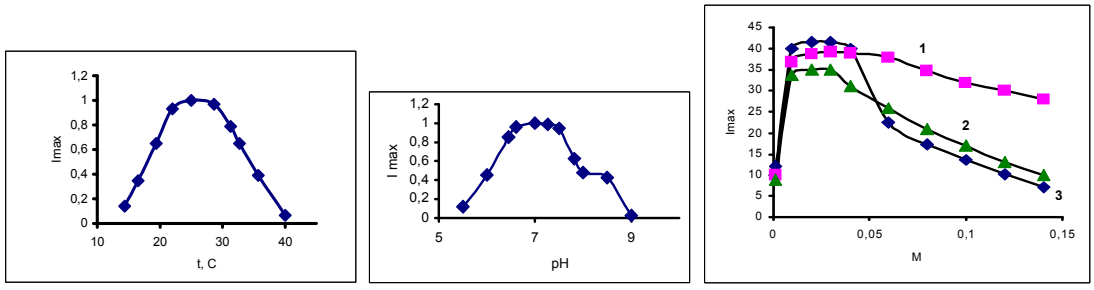

Fig. 2. Luminescence intensity depending on temperature (A), pH of medium (B), composition and molarity of used buffer (C). 1- NaCl with 0.01 M phosphate buffer, 2 – phosphate buffer, 3 – tris-HCl buffer (from Tyulkova, Antonova, 1991)

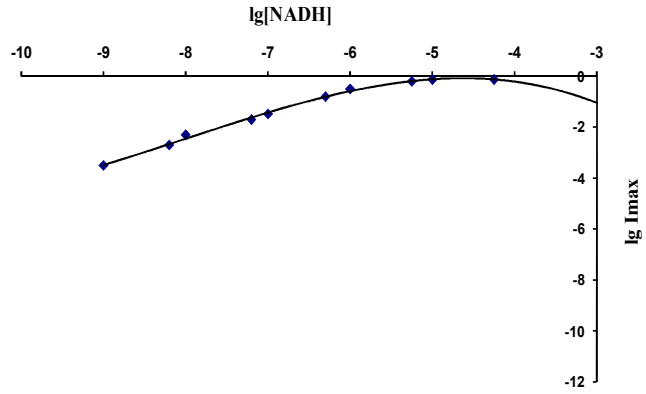

Fig. 3. Relationship between the light intensity and concentration of NADH in coupled enzyme system (from Tyulkova, Antonova, 1991)

It is obvious that change in the reaction conditions or addition of studied samples to the coupled enzyme system affect both enzymes, and result is interfering effect on coupled reaction kinetics. Studying of influence of factors of the reaction environment on stability and kinetic parameters of separate enzymes included in a reagent (Sukovataya, Tyulkova, 2000, 2001, 2003) allows to interpret bioassay results adequately and to develop new methods (Krasnova et al., 2008).

Bioluminescent methods, developed for enzymes isolated from luminous bacteria can conveniently be divided into the following groups: 1) analysis of substrates of luminescence reaction; 2) analysis of the activity of enzymes and their substrates in multicomponent chains of coupling; 3) analysis of bioluminescence inhibitors.

In Russia V.A. Kratasyuk and her team have developed since 1980-th bioluminescent enzymatic toxicity assays through a series of studies of bioluminescence sensitivity to exogenous toxic substances. Use of bioluminescent enzyme bioassay *in vitro* was offered for the analysis of integrated toxicity of various objects. Over the years, this team have developed bioluminescent assays for environmental control of native ecosystems and industry wastewaters, medical diagnostics of endotoxicosis, sports medicine (athletic coaching control), control of food quality (corn and bread infection by fungi), toxicology and biotechnology (control of biologically active substances) (Petushkov et al., 1984; Kratasyuk, Gitelson, 1987; Kratasyuk et al, 1991, 1996, 1998, 1999, 2001; Esimbekova et al., 1999; Vetrova et al., 2007).

Coupled enzyme system includes more components in comparison with mono-enzyme reaction and, thus, more «targets» of the toxicants action. It provides specificity of this kind of bioluminescent analysis. In other side, the enzyme method is more complex in comparison with the bacterial assay because it needs using 5 components in reaction mixture in one measurement that also increases time of carrying out of testing. A multicomponent reagent is developed to provide measurement of a luminescence after simple addition of sample in reagent (Kratasyuk , Esimbekova, 2005; Esimbekova et al, 2009).

Comparing the influence of a great number of various pollutants on bioluminescence of bacterial cells and enzyme systems isolated from them has shown that there is a correlation between the toxicity level of pollutants and the change of luminescence parameters of both systems. Correlations between changes of kinetic parameters of reactions and physical-chemical peculiarities of pollutants have been found (Kudryasheva et al., 1994; Kratasyuk et al., 1996). Regularities of influences of model range of pollutants (fluorescent organic molecules of different spectral-luminescent peculiarities, molecules containing atoms of different atomic mass, metal salts whose cations differ by the affinity for an electron, groups of redox-active compounds with different redox-potentials) on enzyme systems have been studied (Kudryasheva et al., 2002; Kudryasheva, 2006). Physico-chemical patterns of influence of exogenous compounds on the bioluminescent system of the coupled enzyme reactions serve as theoretical basis for bioluminescent bioassay. It can be applied to interpret and predict the results of bioassay and to construct complex systems of coupled enzymatic reactions with optimal sensitivity to various pollutants (Kudryasheva, 2006). Using various variants of interface of enzyme reactions with bacterial bioluminescence is possible to create complex enzyme tests that will be sensitive to various groups of pollutants (Vetrova et al., 2007).

The coupled enzyme system has been used as an analytical tool for quantification of the activity of different enzymes, including NAD(P)H –dependent ones. It should be noted that the bioluminescent method of NADH analysis possesses higher sensitivity in comparison with spectrophotometric method. High sensitivity of the method allows carrying out researches in microsamples of biological material with a minimum quantity of reactants. Wide spectrum of NAD-and NADP-dependent dehydrogenase activities in blood lymphocytes of the patients with various autoimmune diseases have been studied in medical and biological researches. For example, the developed methods are involved for an estimation of functional activity of immune-competent cells at autoimmune pathologies (Prokhorenkov et al., 2000; Kurtasova et al., 2001). Methods of an estimation of functional parameters of lymphocytes, which have diagnostic and forecasting importance, are developed (Inzhevatkin et al., 2007; Kolenchukova et al., 2008).

3.3 Applications of bioluminescent bioassays developed in IBP in environmental monitoring

The use of bioluminescent bacterial bioassays that were developed in IBP SB RAS to test the pollution of sewage and various water resources in different regions of Siberia are represented below. The bioluminescence index (BI) was used to estimate the action of water pollution on bacterial luminescence. It was found that treatment of luminous bacteria with water from different sources increased or decreased bacteria luminescence, or didn't influence its intensity. The inhibition of luminescence testifies toxic effect of a studied sample on the test-

object. Stimulation of luminescence should be considered as the deviation from the norm and necessity to accept the additional researches for correct interpretation of the received data.

It is known that one of the sources of phenol compounds and heavy metals in water objects is industrial wastes. Bioluminescent bioassay was used to determine toxic effect of phenol compounds and products of their destruction in effluents from several enterprises (Geel et al., 1993, Kusnetsov et al., 1999, 2002; Rodicheva et al., 2004). Different parameters, luminescence quenching of luminous bacteria, suppression of dehydrogenase activity and reproductive ability of *Dunaliella* cells, were employed in attempts to determine the sewage toxicity. Luminescence quenching proved to be the most sensitive and the least inert. It was found that dilution of sewage of the pulp-and-paper industry (PPP) in 400 times caused *Dunaliella* cells to stop moving and reduced the reproductive and dehydrogenase activities. Bioluminescent bioassay Microbiosensor B-17 was more sensitive in this case: luminescence quenching of lyophilized bacteria on 50 % (EC_{50}) was observed after sewage dilution in 100 times and the waste waters of the PPP became non-toxic when diluted a 1000 times. It was also shown that these substances caused the damages in cell membrane structures (Stom et al., 1992).

The lyophilized luminous bacteria bioassays were also applied to test the sites of water pollution in the Yenisei River. Several zones were studied downstream of Krasnoyarsk city. The analysis of the bioluminescent bioassay data and the redox test data leaded to the same conclusions. It was shown that the heaviest pollution of water was recorded in the zones 0-116 km (Krasnoyarsk and satellite towns), and 318-370 km (large settlements). We should mark that 0-116 km zone had the heaviest pollution both in the middle stream and near the both banks (Kuznetsov et al., 1998). A great number of enterprises are located in this zone: in and around Krasnoyarsk and along the the Yenisei banks. The stimulative effect was the most evident in the water samples taken at the place where the Yenisei receives its largest tributary, the Angara River. That's why the lyophilized luminous bacteria bioassay Microbiosensor B17 was used in the analysis of sewage of some factories to estimate their contribution to pollution of the Yenisei. The sewage of most factories was found to be toxic. The index of biologically safe dilution ranged from 10 to 1000 for different factories. Results showed (Fig. 12) that the genetically modified *E.coli* strain was more sensitive to the pollutants presented in the medium than was the *P.phosphoreum*'s bioassay (Rodicheva et al., 2004). This can be explained by: 1) the higher permeability of *E.coli* cell wall to inhibiting substance, 2) the salting-out of toxic agents (decrease in the concentration of inhibiting substances after sodium chloride was added to the solution) in bioassay using marine luminous *P.phosphoreum* bacteria.

The underground and surface waters of the Altai Territory were studied with the bioluminescent assay Microbiosensor B17 (Rodicheva et al., 2004). It was shown that the waters of the Katun and Biya Rivers were uncontaminated, and those of the Alei and Choumych Rivers were slightly toxic. The presence of organic substances in water samples could cause marked stimulation of bioassay luminescence (Fig. 13, curves 1, 2). There were deviations from the norm in most water samples taken from village wells; the assay luminescence was inhibited. The water samples taken from the lakes of Novenkoye, Bezymyannoye, Lapunikha, Ivanovskoye, Gorkoye, Bolshoye Ostrovnoye had different levels toxicity. The surface water pollution of Bolshoye Ostrovnoye Lake was demonstrated by the inhibition of bioluminescence in all water samples (Fig. 13, curve 3). This data correlated with optical and hydrobiological estimates of

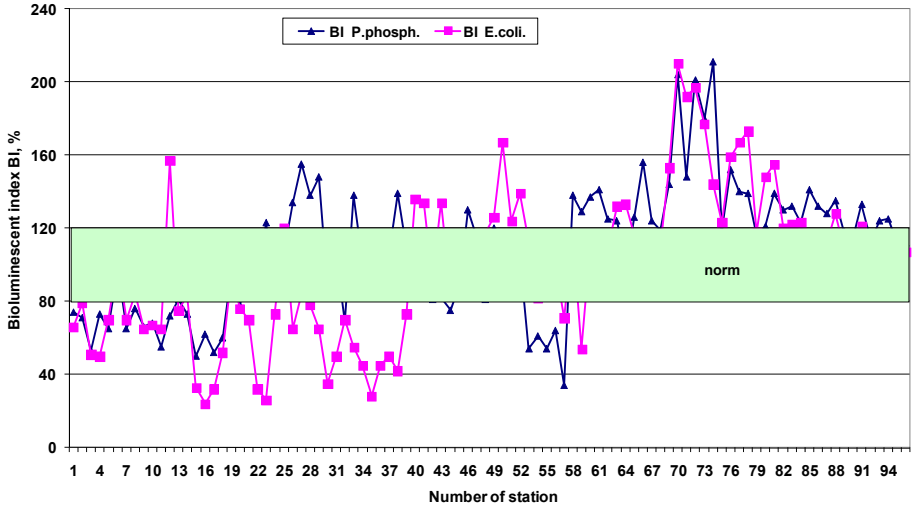

Fig. 12. Zones of different water pollution in the Yenisei River detected by bioluminescent bioassays Microbiosensor B17 and Microbiosensor ECK. BI norm = 80-120 %. (from Rodicheva et al., 2004)

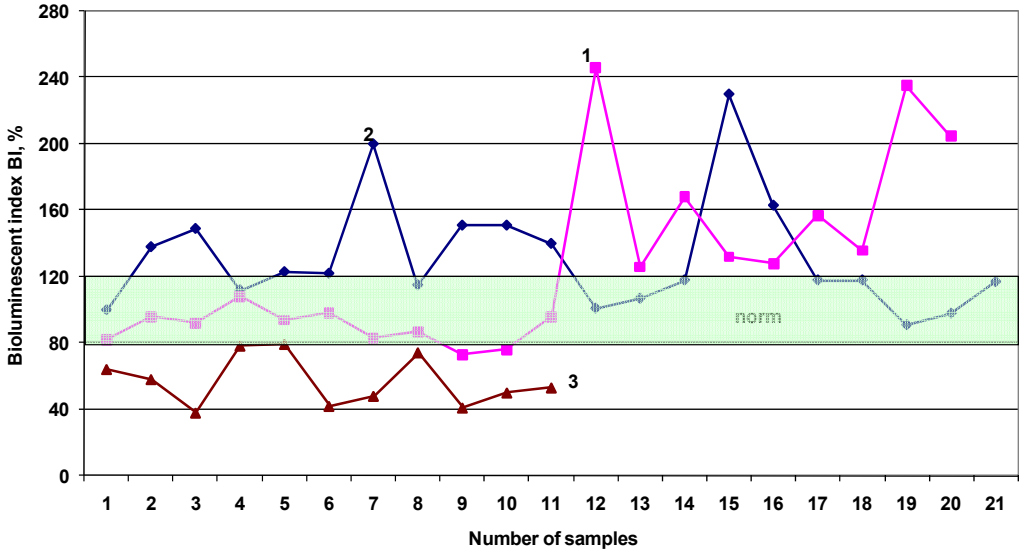

Fig. 13**.** Zones of different water pollution in the Alei (1) and Choumysh (2) Rivers and Bolshoye Ostrovnoye Lake (3) detected by bioluminescent bioassay Microbiosensor B17. BI norm = 80-120 %

water quality, as well as with the effect of sample water on the ultrastructure of intact luminous bacteria cells. The electron microscopy studies confirmed such data. It was obtained that a high poison concentration damaged the cell structure, while a low poison concentration changed the membrane penetrability. A lot of cells treated with water from the Bolshoye Ostrovnoye Lake had increased periplasmic zone or local cytoplasm damages likely due to penetration changes of cell envelope. It was shown that the water from the Bezymyannoye Lake was more toxic. Majority of luminous bacteria cells had different damages of cell envelope, only some cells were safe.

It was also found that long-term anthropogenic and technogenic effects on the organisms living in investigated reservoirs were accompanied by adaptations of some species to

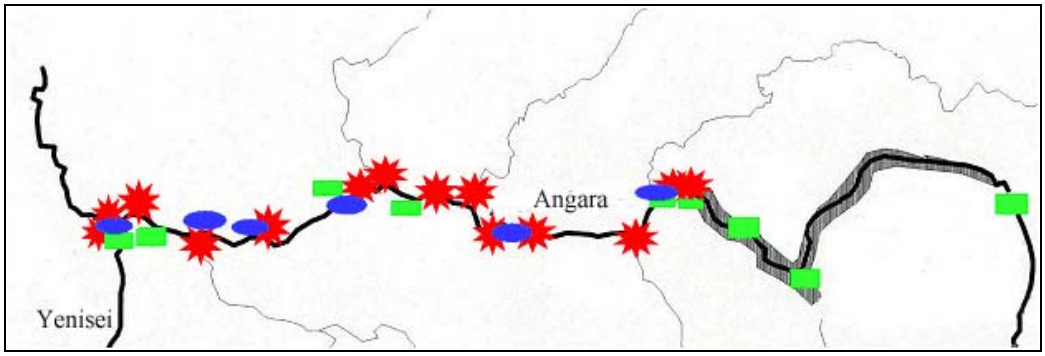

Fig. 14. Pollution testing water in the Angara River detected by bioassay Microbiosensor B17: Rectangular – BI is norm; Oval – BI is near norm (deviation not more than 20 % from norm); Star – BI is increased by 1,5-2,5 times in comparison with norm. (from Rodicheva et al., 2004)

changed conditions. So, ultrastructure of cells of green algae and cyanobacteria from Lakes Bezymiyannoye, B.Ostrovnoye, Noven'koye, Gor'koye and Alei River was appreciably varied. The greatest ultrastructural changes were shown in thilakoid's swelling, periplasm expansion, amplified accumulation of any kind of reserve inclusions, or significant destruction of cytoplasm. The other bacterial species that lived in studied reservoirs had no appreciable structural changes (Medvedeva et al., 2000).

Measurement of pollution of Baikal water by bioluminescent bioassay has yielded inconsistent results. Depending on depth and the location of station of tests fence, values of luminescence varied. The inhibition of luminescence below norm is seldom, however, stimulation of luminescence of both bioassays was characteristic for the majority of tests from various stations. We can conclude that in Baikal the cleanest water there is on depth of 500-750 meters (on Microbiosensor B17 data), the Microbiosensor ECK gave more favorable forecast for depths up to 20 m, 200-1000 m and near the Cape Talyi – on 150 m.

Monitoring waters from the Angara River by bioluminescent bioassay showed that its water was nontoxic at the studied sites because inhibition of bioluminescence was not revealed (Fig. 14) (Rodicheva et al., 2004). However, the water cannot be considered completely clean, since many samples stimulated bioassay luminescence, in some cases considerably. This testifies to the presence of many organic substances of both natural and anthropogenic origin in the samples. Bioluminescent bioassays (*in vivo* and *in vitro*) were simultaneously used in ecological monitoring of effluents and natural reservoirs in the Altai and Krasnoyarsk Territories and also of artificial water ecosystems (Kratasyuk et al., 1996). Bioluminescent three-enzyme systems – NADH:FMN-oxidoreductase – luciferase with alcoholdehydrogenase, lactatedehydrogenase and trypsin have been used for ecological monitoring in water reservoirs (Kratasyuk et al., 2001; Kudryasheva et al., 1998, 2003). The researches of action of phenols and quinones on bioluminescence were carried in parallel. It was shown that influence of these compounds on luminescence of three bacterial bioluminescent systems of various complexity was determined by a structure and characteristics of molecules-inhibitors (Kudryasheva et al., 2002). Quinones were more effective inhibitors of bioluminescence than phenols. The luminous bacteria had the great sensitivity to quinones, while the bioluminescent system of coupled enzyme reactions was more sensitive to phenols. The authors supported that efficiency

of inhibition of bacterial bioluminescence by quinones was determined by size of quinone redox-potential. The induction period and time of an output for a maximum of bioluminescence in enzyme systems appeared to grow at presence of quinones. The magnitude of these parameters was proportional to quinone concentration and depended on its standard redox-potential.

Some experiments were recently carried out to estimate the possibility of use of luminous bacteria in control of low-level radiation. As a result, the technique of definition of low-level α-radiation with use of bioluminescent assay systems of various complexity was developed (Rozhko et al., 2007). It was also shown that the bioluminescent assay system *in vivo* was highly sensitive to $^{241}Am^{3+}$ (up to 10^{-17}M) in comparison with lyophilized bacteria and bioluminescent system of coupled enzyme reactions (Rozhko et al., 2008).

The other of the important tasks of modern ecology and biotechnology is the search of methods to detoxify the environmental toxicants. Encouraging data about photoinduced UV detoxification of the phenol solutions were obtained as well as the data about reduction of toxic action of phenols and quinones after treatment of samples by humic substances (Fedorova et al., 2007; Tchaikovskaya et al., 2007; Tarasova et al., 2008).

Thus, the light-emitting system is particularly attractive for use in bioassays as readily available and sensitive to a wide variety of different biological compounds. The combined application of designed bioassays allows the range of the analyzed substances to be widened. The comparison of the results on the *in vivo* and *in vitro* bioassays provides additional information how the toxicants act and guarantees a more complete inspection of water bodies, irrespective of the structure, physical and chemical properties of their toxicants.

Conclusion

The methods based on biochemiluminescent reactions play now the important role among ecological, biomedical researches, clinical medicine and immunology. The most important toxicological parameter, $EC_{50,}$ is the concentration of substance reducing a bacterial luminescence in 50 %. Bioluminescent assays can be unhesitatingly used for detecting the toxicity of certain chemical compounds because toxicants directly affect the luminescent system. Bioluminescent bioassays can be recommended for: continuous express-monitoring of the environmental conditions of industrial regions and natural-economic complexes, controlling a releases volley of pollutants by factories, estimating how well fresh water cleans an industrial enterprise and the methods used for environmental detoxification, and ecologically certifying enterprises and regions. There is bioluminescent assay for detecting soil toxicity, used water extracts from soil and quantitatively assessed toxicants in them. Such biosensors are used to estimate the luminescence inhibition by all toxicants contained in soil. The information about detection of radiation effects (Min et al., 2000, 2003), especially effect of low-level α-radiation on luminous bacteria (Rozhko et al., 2008) allows to develop new approach for environment monitoring.

Bioassays are standard test-objects used to detect the integral toxicity of water samples; there is no need for cultivating and maintaining bacterial cultures with the marker *lux*-gene. Microbiosensor ECK and Microbiosensor B17 developed in IBP SB RAS are successfully used for defining effluent toxicity and purifying installations in different cities of Russia. The bioluminescent methods using bacterial luminous cells and isolated from them luminescent system were approved by experts in ecology, recommended as an additional method for ecological monitoring,

and Microbiosensor had also certificate from Federal Agency of Russia for technical regulation and metrology (№ 224.01.13.151/2007). The sensitivity of the bioassays developed in the IBP SB RAS is comparable to that of the foreign analogues Microtox, ToxAlert, Lumistox, etc. Bioluminescent assays using bacteria and enzyme system are mutually complementary in ecological and biomedical researches. The simultaneous use of bioassays allows expanding a range of analyzed toxicants. Application of bioluminescent methods along with increase in sensitivity allows in many cases to reduce cost of analyses and time spent in comparison with traditional methods of the analysis.

The Culture Collection at the Institute of Biophysics of Siberian Branch of Russian Academy of Sciences, operating under the acronym CCIBSO 836, contains more than 700 strains of luminous bacteria *P.phosphoreum, P.leiognathi, V.harveyi,* and *V.fischeri*, and genetically modified strains of *E. coli* bearing *lux*-genes from the luminescent cells of *P.leiognathi*. The collection provides many possibilities for perfecting bioassays and finding strains that are sensitive to certain toxicants, and have know-how for producing kit reagent for bioluminescent analysis based on the isolated bacterial luminescent system. Information about numerous topics of bioluminescence and bioluminescent analysis is available at the Web-portal «Bioluminescence and luminous organisms» (http://bl.ibp.ru), which is developed in Culture Collection IBSO.

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Биолюминесцентные биотесты на основе светящихся бактерий

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Биолюминесцентный анализ является одним из наиболее перспективных экспрессных методов биологического мониторинга окружающей среды, поскольку люминесцентная система отличается высокой чувствительностью к микроколичествам загрязняющих веществ. Биотесты на основе светящихся бактерий дают интегральную оценку токсичности и часто

превосходят другие известные биотесты в скорости, точности, чувствительности и простоте использования. Ферменты бактериальной люминесцентной системы также используются в разработке высокочувствительных аналитических методов для практических целей. В статье рассматриваются основные особенности бактериальной биолюминесценции как на уровне клетки, так и выделенных ферментов люминесцентной системы, а также применение биолюминесценции в различных сферах. Одна из частей статьи представляет собой обзор исследований о влиянии различных химических веществ на бактериальную люминесценцию, развитие и использование биолюминесцентных биотестов, в частности, даны некоторые примеры их использования в области экологического мониторинга в ряде регионов России.

Светящиеся штаммы бактерий из Коллекции Культур Института биофизики СО РАН (CCIBSO 836) успешно применяются для создания новых или усовершенствованных биотестов на основе лиофилизированных бактерий, несущих lux гены. Такие биотесты находят применение для анализа загрязнения воды, воздуха, почвы. В изготовлении наборов реагентов для биолюминесцентного анализа из ферментов люминесцентной реакции и биолюминесцентных биотестов на основе природных лиофилизированных светящихся бактерий P.phosphoreum и рекомбинантного штамма E.coli с клонированными lux генами используются авторские «ноу-хау». На биолюминесцентный биотест получен сертификат, и этот тест рекомендован в качестве дополнительного метода экологического мониторинга параллельно с другими биотестами. Чувствительность биотестов, разработанных в ИБФ СО РАН сопоставима с зарубежными аналогами Microtox ©TM, ToxAlert ©TM и т.д.

Ключевые слова: биолюминесцентный биотест, биолюминесценция, биотестирование, загрязнение