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Bioluminescence as a tool for studying detoxification processes in metal salt solutions involving humic substances

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ABSTRACT

The paper considers effects of humic substances (HS), as natural attenuators of toxicity, on solutions of model inorganic pollutants, metal salts – $Pb(NO_3)_2$, $CoCl_2$, $CuSO_4$, $Eu(NO_3)_3$, $CrCl_3$, and $K_3[Fe(CN)_6]$. Luminous bacteria *Photobacterium phosphoreum* and bioluminescent system of coupled enzymatic reactions were used as bioassays to monitor toxicity of salt solutions. The ability of HS to decrease or increase toxicity was demonstrated. Detoxifying concentrations of HS were determined; detoxification coefficients were calculated at different times of exposure of salt solutions to HS. To study the combined effects of HS and salts on bioluminescent assay systems, the rates of biochemical reactions and bacterial ultrastructure were analyzed. The detoxifying effects were explained by: (1) decrease of free metal content in water solutions under metal–HS binding; (2) increase of biochemical reaction rates in a bioluminescent assay system under HS effect; (3) enhancement of mucous layers on cell surface as a response to unfavorable impact of toxicants. Detoxifying mechanisms (2) and (3) reveal the active role of bioassay systems in detoxification processes.

1. Introduction

Salts of metals are leaders among environmental pollutants. As exogenously introduced contaminants, metals penetrate into humus horizons of soils, as well as water reservoirs. Humic substances (HS) are known as natural attenuators of metal toxicity [1–5]. Elucidation of detoxification mechanisms of metal salt solutions by HS is of great challenge for researchers now; it provides a basis for elaboration of effective and ecological methods for remediation of contaminated waters.

HS are the products of natural transformation of organic matter in soil and sediments. They are supposed to be irregular polymers of a complex structure [1,2]. Another concept for HS molecular organization, supramolecular one, assumes HS to consist of relatively small molecules linked by hydrogen, hydrophobic, or π–π bonds, as well as polyvalent cations [6,7].

The ability of HS to detoxify toxic solutions is a subject of interest for researchers [2,8–11]. Carboxyl, quinoid, phenolic, SH–, and other electron-donating groups of HS are known to be responsible for binding, and hence, decrease of content of free metal ions in water ecosystems [3,12–15]. Phenolic, SH–, and other groups of HS macromolecules reduce toxic effects of oxidizers. Redox activity of HS was studied in [16,17]. So, it is currently supposed that detoxifying ability of HS deals with their complexing and redox processes resulting in the decrease of free toxic compound concentration in water solutions. However, the term "toxicity" is not only chemical, but biological, too. It implies an active role of organisms in response to unfavorable impact of toxicants. Therefore, the way organisms react to toxic compounds in the presence of HS is the point of attention of biophysicists and toxicologists. The responses could be studied on the levels of different complexity – biochemical, cellular, tissue, as well as individual organ, whole-organism, and population.

Biological assays are conventional tools to monitor toxicity in aquatic media. The classic bioassays are based on fish, algae, crustaceans, plants, etc. [9,18,19]. It is assumed that the results of such monitoring can be extrapolated, to a certain extent, to higher organisms.

The assay systems involving luminous marine bacteria are good candidates for toxicity monitoring. Bacterial bioluminescent assays have been widely used to assess environmental toxicity for more

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than 40 years [20–25], and now they are the traditional and important biotechnological applications of bioluminescence phenomenon. The tested parameter here is luminescence intensity that can be easily measured instrumentally. The advantages of bioluminescent assays are sensitivity, simplicity, rapidity, and availability of devices for toxicity registration [20]. This is a reason why structure and functions of luminous bacteria, as well as effects of exogenous compounds on the bacteria, are the objects of intensive research [23,26].

The bacterial bioluminescent assays can base on biological systems of different complexity – bacteria or enzymes, providing study of the effects of toxic compounds on enzymes and bacterial cells [17,23,27].

Bacterial bioluminescent enzyme system was suggested as a bioassay for the first time in 1990 [28]. The advantages for this approach were demonstrated later [23,24,29–31]. Mechanisms of interactions of exogenous compounds with bioluminescent enzyme systems were reviewed in [32].

The bacterial bioluminescent enzyme system involves two coupled enzyme reactions. The first one, catalyzed by NADH:FMN-oxidoreductase, is a reduction of FMN by NADH:

$$\mathrm{NADH} + \mathrm{FMN} \xrightarrow{\mathrm{NAD(p)H:FMN-oxidoreductase}} \mathrm{FMN}\cdot\mathrm{H}^- + \mathrm{NAD}^+ \qquad (1)$$

In the second reaction, catalyzed by bacterial luciferase, the reduced flavin (ionized form) and the long-chain aldehyde are oxidized by molecular oxygen to yield the corresponding acid, H_2O, FMN, and light (λ_{max} = 490 nm):

$$\mathrm{FMN}\cdot\mathrm{H}^- + \mathrm{RCHO} + \mathrm{O}_2 \xrightarrow{\mathrm{luciferase}} \mathrm{FMN} + \mathrm{RCOO}^- + \mathrm{H}_2\mathrm{O} + h\nu \qquad (2)$$

Peculiarity of the enzymatic assay system is its specificity to oxidizers. Oxidizers are capable of competing with FMN in reduction by NADH in the first reaction and, hence, inhibiting the second (luminescent) reaction. In this case, specific changes take place in light emission kinetics: delay period appears; its duration depends on concentration and redox potentials of the oxidizers [33,34]. Fig. 1a, curve 2, illustrates bioluminescent kinetics in solution of a model inorganic oxidizer.

In [30,33], the bioluminescence delay period was used to monitor oxidative toxicity of solutions of organic oxidizers, quinones. The one was observed for quinones of high redox activity and was absent for those of low redox activity. Similar effects took place in solutions of metal salts: metals of higher standard redox potential, e.g. iron(III), developed the delay period, thus demonstrating oxidative toxicity [34]; while metals of lower redox potentials – did not [20].

In [34], the detoxification of solutions of model inorganic oxidizer, potassium ferricyanide, was attributed to both binding and reduction properties of HS. It was noted in this paper that two types of chemical processes can be responsible for detoxification: (1) 'external' mechanism, resulting from binary interactions outside the bioassay system; (2) 'internal' mechanism, with biochemical processes of the assay system involved into detoxification. The 'internal' mechanism pointed to an active role of bioassay system in evaluation of toxicity, and it is currently most interesting. The active role of the assay system in the process of detoxification should be substantiated in detail using a number of toxic compounds.

Our work focuses on chemical, biochemical, and cellular processes taking place in bioluminescent assay systems in the process of detoxification of metal salt solutions by HS. Such metal salts as $Pb(NO_3)_2$, $CoCl_2$, $CuSO_4$, $Eu(NO_3)_3$, and $CrCl_3$ were used as model inorganic toxic compounds.

The applied aspect of our work focuses on the use of bioluminescent assays for evaluation of detoxification efficiency. Detoxification coefficients were determined with two biological assays

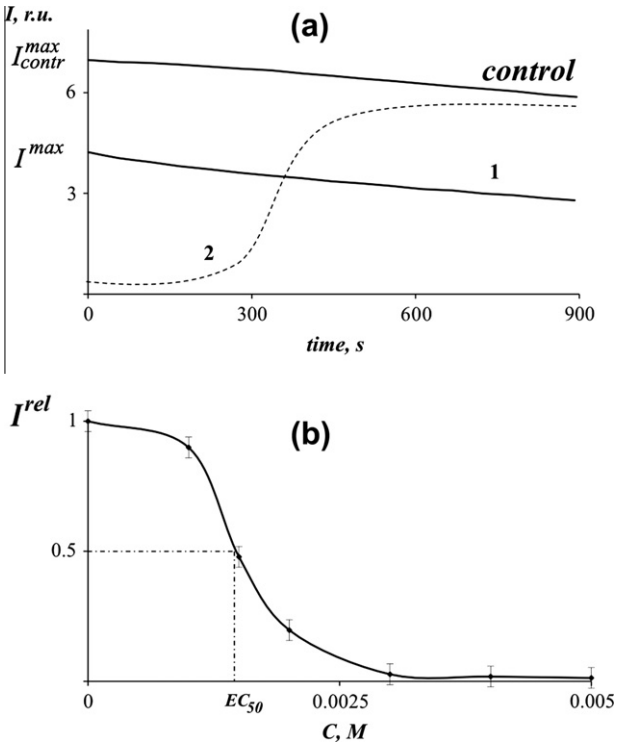

Fig. 1. Bioluminescence intensity in enzymatic assay: (a) bioluminescence intensity (I) in the absence of metal salts (control) and in the presence of $Eu(NO_3)_3$, 1.5×10^{-3} M (1), and $K_3[Fe(CN)_6]$, 8×10^{-5} M (2); (b) relative bioluminescent intensity (I^{rel}) at different concentrations of $Eu(NO_3)_3$.

(bacterial and enzymatic ones) at various time of exposure of metal salt solutions to HS and at various HS concentrations. Conditions for toxicity decrease and increase by HS were characterized.

Section 3 sequentially describes the results of our research

- evaluation of the effects of (i) metal salt solutions, (ii) HS, and (iii) complex salt + HS solutions on bioassay systems;
- the analysis of chemical and biochemical rates in salt + HS solutions;
- the study of ultrastructure of bacterial cells in salt + HS solutions.

2. Materials and methods

2.1. Reagents and equipment

Toxicity of metal salt solutions was assessed using two bioluminescent assays: (1) bacterial – Microbiosensor 677F, based on the lyophilized luminous bacteria *Photobacterium phosphoreum*, and (2) enzymatic – the preparation based on a coupled enzyme system NADH:FMN-oxidoreductase from *Vibrio fischeri* (0.15 a.u.) and luciferase from *Photobacterium leiognathi*, 0.5 mg/ml [35]. All the biological preparations were produced at the Institute of Biophysics SB RAS (Krasnoyarsk, Russia). Additionally, the intact *P. phosphoreum* (strain 1883 IBSO from the Collection of the Institute of Biophysics SB RAS, Krasnoyarsk, Russia) was applied in electronic microscopy studies.

The chemicals used were: NADH from ICN, USA; FMN and tetradecanal from SERVA, Germany; $Pb(NO_3)_2$, $CoCl_2$, $CuSO_4$, $Eu(NO_3)_3$, $CrCl_3$, and $K_3[Fe(CN)_6]$ of analytical grade, Khimreactiv, Russia.

The influence of salts and HS on bioluminescent intensity of bacteria was studied in 0.3 ml of 3% NaCl solution at room temperature. The measurement conditions excluded growth of bacteria.