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Effects of elemental sulfur and metal sulfides on *Vibrio fischeri* bacteria

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Elemental sulfur (S^0, S_8) and metal sulfides are abundant in anoxic sediments as metabolic products of microorganisms in an acidic (≥pH 5.0) environment, and toxic in the *Vibrio fischeri* bioluminescence (standart) test. The aim of this research is to compare the toxicity of S^0 and sulfides and to analyse the toxicity of iron and sodium sulfides, in particular to bioluminescence, and the respiration of *V. fischeri* at appropriate pH. The results indicated that S^0 was more toxic to *V. fischeri* bioluminescence during the first 15 min of exposure at concentrations by 2–3 orders lower than most abundant iron and sodium sulfides. Pyrite (FeS_2; ≤8 ppm) was non-toxic to bioluminescence at pH 7.0, but inhibited it up to 10–20% at pH 5.0. Bioluminescence was only slightly affected by FeS (0.4–8 ppm), but it was lower (up to 10–22%) at 1–15 min by exposure to Na_2S (5 ppm) at pH 7.0. The concentrations of Na_2S (0.05 ppm), FeS_2 (4, 8 ppm) and FeS (0.4 ppm) diminished bioluminescence up to 14–17%, 15–21%, and 4.5 %, respectively, at pH 5.0. The concentrations of FeS 4–8 ppm were only slightly toxic or non-toxic and had clear effects of bioluminescence restoration and even enhancement at pH 5.0 after 15 min of exposure. The respiration of *V. fisheri* cells increased 166–200% after 5 min of exposure to FeS (8 ppm), FeS_2 (8 ppm) or Na_2S (5 ppm), respectively, at pH 7.0. However, lower enhancement (149–111%) was observed at pH 5.0. In general, the bioluminescence decreased to 50% at pH 5.0 in comparison with that observed at pH 7.0, but pH itself had no effect on respiration.

Key words: sulfur, iron disulfide, sodium sulfide, toxicity, bioluminescence, respiration, *V. fischeri cells*

INTRODUCTION

Elemental sulfur (S^0, S_8) and sulfides are abundant in anaerobic sediments, soil, wastewaters, mining polluted freshwaters and as a product of metal corrosion in anaerobic environments [1, 2]. Atomic absorption (AA) analysis data of sediment samples from polluted sites of the Nemunas River Delta and Curonian Lagoon (CL) indicated, that: 1) the content of iron (2371–6529 mg/kg dry sediment weight) is by 6–7 or 5 to 4–3.5 and 3 orders of magnitude higher than the level of heavy metals (HM), such as Hg or Cd, to Cu, Pb, and Cr, Zn, respectively; 2) the level of sulfur (AA data) forms 1/4 to 1/20 of the content of iron [3] (unpublished data). Thus, because of the natural geochemical background, the high amount of ferrous/ferric ions usually forms large part of metal sulfides. Additionally, due to a higher solubility of iron salts (including FeS) than that of HM, they are responsible for a higher than HM bioavailability and the toxic effects of sediments or their porewater in biotests. Depending on the site of sampling, the content of $H_2S + HS^-$ in CL sediments varies from 0 to 224 mg/dm^3 of sediments [3]. Water content of total sulfide in polluted water bodies varies from 0.001 to 0.327 mg/l and H_2S from 0.001 to 0.097 mg/l as described for the US harbour sediment contaminants [4], whereas acid volatile sulfide (AVS) from river sediments varies from 0.3 to 46.9 mmol/kg (i. e. from 32 to 1504 ppm recalculated, respectively) [5].

Usually it is accepted that inorganic, weakly acid (1 N HCl) extractable sulfur reflects the amount of insoluble, relatively non-toxic heavy metals sulfides [5, 6]. So, the questions arise: 1) how toxic is this acid extractable fraction to a standard biotest, used for toxicity screening procedures, and 2) if the toxicity of these sulfides depends upon pH values observed in sediments under anoxic and anaerobic *in situ* conditions. The lowest pH value observed in such sediments is pH 5.2–5.0.

Reduced and oxidized iron (Fe^{2+} and Fe^{3+}) is among the most electroactive redox reactants in natural water systems, and their vertical distribution in lakes and streams is

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reflected in the distribution of redox potencials [7]. Additionally, changes in redox potencial are altered by pH. Hense, at neutral pH, ferrous iron (Fe^{2+}) is not stable in the presence of oxygen and is rapidly oxidized to ferric (insoluble) state. However, at acid pH ferrous iron is stable to chemical oxidation [8]. Ferrous ions (Fe^{2+}) diffuse readily from the sediments when redox potencials decline to about 200 mV. When the redox potencial declines below 100 mV, sulfate is reduced to hydrogen sulfide and some ferrous sulfide is precepitated [7].

Vibrio fischeri, a bioluminescence quenching standard biotest (earlier Microtox™) is used mostly for a rapid toxicity screening of field studies' samples, including those of sediments and wastewaters [9]. Sulfur is also known to be highly toxic to *V. fischeri* in extracts of sediments (prepared using water miscible solvents) which interferes with the toxicity of other environmental pollutants [10–12]. The toxicity of metal sulfides is evaluated less by this test due to their theoretically accepted relative insolubility and poor bioavailability. However, because of the large amount of sulfides in *in situ* anoxic fine and relatively acidic sediments and their porewater, it is actual to analyze the effects of most abundant sulfides in order to understand how much these effects can interfere with the toxicity of other pollutants under conditions close to environmental ones. Thus, in this work we aimed at analysing the effects of abundant ferrum and sodium sulfides (FeS, FeS_2, Na_2S) in *V. fischeri* bioluminescence and respiration under neutral (7.0) and acidic (5.0) pH.

MATERIALS AND METHODS

Reagents. Most of the chemicals used were of the highest obtainable purity (Sigma-Aldrich, Serva (US), Merck (Germany)); elemental sulfur, iron sulfide, iron disulfide (pyrite) and sodium sulfide, hydrochloric acid were from Reachim (Russia), all of chemical analytical grade. Salts (bacterial cultivation media) were obtained from Serva and Roth (Germany). Milli-Q water was used for growth and reaction media.

Bioluminescence measurements in bacterial cells. A bacterial culture of *V. fischeri* was prepared, stored and thawed as described earlier [13] and the dilution by factor 100 of stock solution ($OD_{590\ nm}$= 0.125) was used for bioluminescence measurements. The bioluminescence analyses were performed using a Model 1250 luminometer (LKB-Wallac, Sweden). One millilitre of pH 7.0 reaction medium consisted of 0.05 M KH_2PO_4 buffer (950 µl) containing 2% NaCl (pH 7.0), 50 µl of bacterial suspension and 10 µl of chemicals solution or 0.001 N HCl blank. The reaction medium pH 5.0 consisted of 2% NaCl. The kinetic measurements were performed after 5, 15, 30, 60 minutes of exposure to sulfur, iron sulfides or sodium sulfide, respectively at 20 ˚C.

Oxygen consumption. The respiration measurements *in vivo* were conducted using a polarograph (Clark-type oxygen electrode placed inside a cuvette-thermostate, Rank Brothers Ltd), (Cambridge, UK) in a 1 ml volume containing the same buffer (pH 7.0) or reaction medium (pH 5.0) as in bioluminescence measurements, and 20 µl of cells (final $OD_{590\ nm}$= 0.3). The evaluation of respiratory control was performed using an uncoupler (2,4-dinitrophenol), (36.8 ppm or 200 µM final concentration). The uncoupler, sulfur, iron sulfide and iron disulfide, sodium sulfide stock solutions and 0.1 or 0.001 N HCl blank were added in 10 µl volumes, respectively.

Calculation and statistical evaluation. Bioluminescence inhibition data *in vivo* were normalized to bioluminescence quenching in control samples by a conventional procedure and calculated as described earlier [13, 14]. The means presented in the figures and tables and standard deviations were calculated from three–six independent measurements.

RESULTS AND DISCUSSION

The analysis and prediction of the relative toxicity of metal ions in a widely used bioluminescence bioassay was performed mostly using soluble metal ions in the form of nitrate salts [15] which are hardly available and unnatural in big quantities in anoxic sediments. Thus, more actual is the analysis of possible adverse effects of most abundant constituents of anoxic sediment, such as elemental sulfur and sulfides.

Effects of S^0, FeS_2, FeS and Na_2S on cell bioluminescence *in vivo*. The data presented in Figure, indicate that S^0 was more toxic to *V. fischeri* bioluminescence (a decrease to 20% in ppb concentration range) during the first 5–15 min of exposure to concentrations by 2–3 orders lower than most abundant iron and sodium sulfides (a decrease to 90% in ppm concentration range). Pyrite (FeS_2; ≤8 ppm) was nontoxic to bioluminescence at pH 7.0, but inhibited it up to 10–20% at pH 5.0 (Table 1). Bioluminescence was

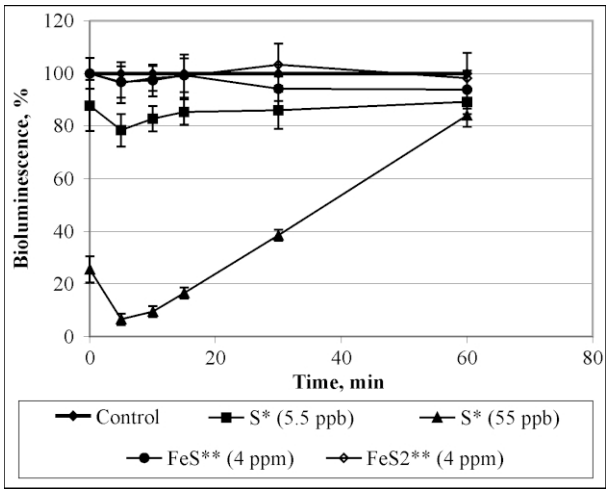

Figure. Effects of elemental sulfur and ferrum sulfides on bioluminescence of *V. fischeri* cells
Reaction medium: 50 mM K_2HPO_4, 2% NaCl , pH 7.0;
* Stock solution prepared in ethanol;
** Stock solution prepared in 0.001 N HCl.

Table 1. **Effects of metal sulfides on *Vibrio fischeri* bioluminescence***

Time, min	Bioluminescence, %							
	Control	Na_2S (0.05 ppm)	Na_2S (2.5 ppm)	Na_2S (5 ppm)	FeS_2 (4 ppm)	FeS_2 (8 ppm)	FeS (0.4 ppm)	FeS (4 ppm)	FeS (8 ppm)
pH 7.0**									
1	100	99.2 ± 10.6	89.8 ± 6.3	95.1 ± 10.6	100.0 ± 2.1	100.9 ± 1.1	97.1 ± 4.9	100.0 ± 5.8	96.8 ± 2.6
5	100	107.4 ± 7.9	86.7 ± 6.9	82.3 ± 2.4	96.5 ± 7.8	98.1 ± 1.9	97.8 ± 5.4	102.8 ± 5.9	99.3 ± 5.1
15	100	104.4 ± 6.9	90.0 ± 9.5	78.1 ± 0.9	99.0 ± 8.2	100.5 ± 1.4	99.4 ± 8.3	99.1 ± 6.4	94.2 ± 6.1
30	100	103.1 ± 4.5	94.8 ± 11.7	81.5 ± 1.7	103 4 ± 8.0	104.0 ± 2.7	105.2 ± 4.5	97.1 ± 4.7	93.9 ± 5.9
60	100	110.0 ± 6.0	107.5 ± 11.3	92.9 ± 5.7	98.2 ± 9.7	103.0 ± 3.6	105.4 ± 4.7	94.5 ± 7.2	95.6 ± 5.2
pH 5.0***									
1	100	72.9 ± 4.7	101.8 ± 3.1	118.7 ± 4.4	86.9 ± 3.5	78.8 ± 0.8	99.5 ± 0.7	95.3 ± 8.1	102.0 ± 5.3
5	100	72.5 ± 5.9	107.0 ± 3.7	112.5 ± 7.0	88.9 ± 0.9	85.0 ± 1.7	95.5 ± 10.6	93.9 ± 10.1	102.3 ±12.7
15	100	76.1 ± 8.7	108.0 ± 5.1	113.4 ± 5.6	85.3 ± 6.6	88.8 ± 7.2	95.5 ± 11.2	106.5 ± 7.2	104.8 ± 0.2
30	100	84.8 ± 7.0	115.0 ± 5.8	124.3 ± 7.9	90.1 ± 2.6	89.3 ± 2.8	99.0 ± 12.5	107.5 ± 8.7	108.6 ± 0.6
60	100	97.8 ± 10.4	114.7 ± 4.2	133.5 ± 6.4	90.6 ± 6.6	83.5 ± 7.1	98.3 ±13.1	113.3 ± 9.2	107.7 ± 9.6

* See Materials and Methods for conditions and design of experiment;
** 50 mM K_2HPO_4, 2% NaCl, pH 7.0;
*** 2% NaCl , pH 5.0 (background conditions as in ISO Standard Method).

diminished at 1–15 min by exposure to Na_2S (5 ppm) by 10–22% and by FeS (8 ppm) to 6% at pH 7.0. Other concentrations at pH 7.0, including these of FeS (0.4–8 ppm), were only slightly toxic. Concentrations of Na_2S (0.05 ppm), FeS_2 (4, 8 ppm) and FeS 0.4 ppm) diminished the bioluminescence by 14–17%, 15–21%, and 4.5%, respectively, at pH 5.0. Other concentrations, including these of FeS (4-8 ppm), were only slightly toxic or non-toxic and had a clear effect of bioluminescence restoration and even enhancement at pH 5.0 after 15 min of exposure. The general conclusion would be that the concentrations of sulfides (0.05 ppm Na_2S, 0.4 ppm FeS) that were closer to the highest naturally observed $H_2S + HS^-$ (Curonian Lagoon sediments, [3]) caused a slight bioluminescence enhancement (up to 110 or 105%) after one hour of exposure at pH 7.0. The possibility of Na_2S to restore the bioluminescence up to 15% (from a 27% inhibition) at pH 5.0 during one hour could be attributed to alkalinization of standard 2% NaCl medium. This was confirmed by analysis of pH dependence, where a higher bioluminescence level was observed towards neutral and slightly alkaline media (Table 2 and unpublished results). In general, despite the bioluminescence decrease to 50% at pH 5.0 (Table 2) as compared with that observed at pH 7.0, pH itself had no effect on respiration (data not presented).

Table 2. **Effects of pH on *Vibrio fischeri* bioluminescence in control samples**

Time, min	Bioluminescence, %	
	pH 7.0*	pH 5.0*
1	100 ± 11.4	50 ± 3.1
5	100 ± 6.8	51 ± 3.3
15	100 ± 5.7	48 ± 9.6
30	100 ± 7.2	43 ± 10.7

* The conditions and media of experiment are the same as in Table 1.

Table 3. **Enhancement of *Vibrio fischeri* respiration by metal sulfides**

Time, min	Respiration rate, %			
	Control	Na_2S (5 ppm)	FeS_2 (8 ppm)	FeS (8 ppm)
pH 7.0*				
1	100**	170 ± 7.1	170 ± 11.2	200 ± 2.3
3	100	220 ± 4.7	208 ± 6.8	164 ± 4.9
5	100	212 ± 6.1	212 ± 2.5	166 ± 7.8
pH 5.0*				
1	100**	100 ± 2.1	100 ± 7.1	100 ± 4.2
3	100	104 ± 6.4	118 ± 4.9	163 ± 7.3
5	100	111 ± 8.3	122 ± 2.2	149 ± 6.9

* The conditions of experiment are described in Materials and Methods section;
** No differences in *V. fischeri* respiration rate at pH 7.0 and pH 5.0 in control samples.

Effects of FeS_2, FeS and Na_2S on cell respiration *in vivo*. Our earlier results indicated that S^0 enhanced the respiration in *V. fischeri* cells (unpublished data). The respiration of *V. fisheri* cells increased by 170–200 % after one minute of exposure to FeS (8 ppm), FeS_2 (8 ppm) or Na_2S (5 ppm) at pH 7.0. However, a lower enhancement (149–111%) was observed at pH 5.0. In general, it shows effects similar to those of S^0 of sodium disulfide, iron disulfide on oxygen consumption by *V. fischeri* cells. The bioavailability of metal sulfides as of water insoluble chemicals could not be ignored as their hydrophobic properties are confirmed and used in practice. For example, the capability of metal sulfides to adhere to hydrophobic phase is well known and used in industry for extraction of sulfide minerals using flotation oils, such as PHILFLO, ORFOM and others [16] and for cleanup of contaminated land [17]. Additionally, the possible enhanced bioavailability of sulfides or their complexation/penetration into biological membranes could be indicated by these geochemical or technical data, which show the capability of inorganic sulfur compounds (H_2S, polysulfides, or other reduced sulfur species) to react with double bond and to form sulfurized lipids and sugars [18, 19], to enhance hydrocarbon accumulation, foam formation (FeS properties) [20]. All these facts, together with our experimental data on enhancement of the respiration of *V. fischeri* cells, allow us to suggest that in contrast to the general opinion [4–6], the bioavailability of iron sulfides and their effects in biomembrane and enzyme activities take place, at least in a sulfide-rich environment, such as anoxic sediments and/or pore water, and affect sediment dwelling organisms at a different natural acidity of environment (pH 5.0 and pH 7.0). If sulfur and sulfides can enter the hydrophobic membrane of *V. fischeri*, it is possible to suggest that sulfides, exactly as sulfur, could be slightly or reversibly toxic to benthos feeding organisms, and sulfides bioavailability through the intestinal tract could be enhanced by hydrophobic components, such as fats or fatty acid fractions. The enhancement of respiration/electron transfer could be explained by the influence of Fe_xS_y centres in respiratory enzymes of pro- and eukaryotic membranes [21] by the elemental sulfur and abundant hydrophobic iron sulfides.

CONCLUSIONS

The most abundant iron and sodium sulfides were less toxic to *V. fischeri* bioluminescence in concentrations by 2–3 orders higher than that of elemental sulfur. Iron sulfides (≤ 8 ppm) were non-toxic or slightly toxic to bioluminescence at neutral pH. The relatively acidic environment characteristic of anoxic sediments (pH 5.0) increased bioluminescence inhibition caused by exposure to FeS (0.4 ppm), Na_2S (0.05 ppm), FeS_2 (4, 8 ppm), from 4.5 up to 21%. These observations suggest that the toxicity of metal sulfides to bioluminescence depends on the acidity of the environment. Exposure of *V. fischeri* cells to different sulfides (FeS, FeS_2, Na_2S) caused the increase in cell respiration which was lower (104–163%) at pH 5.0 than at pH 7.0 (212–200%). Thus, in general, the ability of metal sulfides to enhance *V. fisheri* respiration rate depended on pH and decreased with increasing the acidity of the environment.

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ELEMENTINĖS SIEROS IR METALŲ SULFIDŲ POVEIKIS *Vibrio fischeri* BAKTERIJOMS

Santrauka

Elementinė siera (S^0, S_8) ir metalų sulfidai, kaip mikroorganizmų apykaitos produktai rūgštinėje (≥pH 5,0) aplinkoje, yra plačiai paplitę anoksiniuose sedimentuose ir yra toksiški *Vibrio fischeri* bioliuminescencijos (standartiniam) testui. Šio darbo tikslas – palyginti S^0 ir sulfidų toksiškumą, taip pat įvertinti geležies ir natrio sulfidų toksiškumą *V. fischeri* bioliuminescencijai ir kvėpavimui, esant atitinkamam pH. Tyrimų duomenys rodo, kad 2–3 eilėmis mažesnės S^0 koncentracijos buvo toksiškesnės *V. fischeri* bioliuminescencijai per pirmąsias 15 poveikio minučių nei labiausiai paplitę geležies ir natrio sulfidai. Piritas (FeS_2; ≤8 ppm) neveikė bioliuminescencijos, esant pH 7,0, tačiau jis inhibavo bioliuminescenciją iki 10–20%, esant pH 5,0. Bioliuminescencija buvo tik silpnai paveikiama FeS (0,4–8 ppm), veikiant 1–15 minučių, tačiau ji sumažėjo iki 10–22%, paveikta Na_2S (5 ppm) (pH 7,0). Tokių sulfidų, kaip Na_2S (0,05 ppm), FeS_2 (4, 8 ppm), FeS (0,4 ppm), koncentracijos sumažino bioliuminescencijos aktyvumą atitinkamai iki 14–17%, 15–21%, ir 4,5%, esant pH 5,0. Kitos FeS (4-8 ppm) koncentracijos buvo nedaug toksiškos arba netoksiškos, rodančios aiškų bioliuminescencijos atsistatymą ir net padidėjimą per pirmas 15 minučių pH 5,0 terpėje. *V. fischeri* ląstelių kvėpavimas padidėjo 166–200% po 5 minučių poveikio FeS (8 ppm), FeS_2 (8 ppm) ar Na_2S (5 ppm), esant pH 7,0. Mažesnis padidėjimas (149–111%) buvo stebimas pH 5,0 terpėje. Apskritai bioluminescencija sumažėjo iki 50% pH 5,0 terpėje, lyginant su stebėta pH 7,0, tačiau pH neturėjo įtakos ląstelių kvėpavimui.