VILNIUS UNIVERSITY

Virginija Kalcienė

TOXICITY OF ENVIRONMENTAL POLLUTANTS AROCLOR 1248, OLEIC ACID AND SULFUR TO *VIBRIO FISCHERI* AND BIOCHEMICAL MECHANISMS

Summary of doctoral dissertation
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VILNIAUS UNIVERSITETAS

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APLINKOS TARŠALŲ AROCLOR 1248, OLEINO RŪGŠTIES IR SIEROS TOKSIŠKUMAS *VIBRIO FISCHERI* BAKTERIJOMS IR JO BIOCHEMINIAI MECHANIZMAI

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INTRODUCTION

The standard *Vibrio fischeri* bioluminescence quenching test, Microtox® (EN ISO 11348-3:1998) is used in ecotoxicological analyses of single chemicals and their binary mixtures. For more than two decades, the *V. fischeri* bioluminescence inhibition test has been individually or in conjunction with other tests utilized as one of the Techniques for Early Warning Systems, i.e. for toxicity evaluation of individual chemicals and for monitoring of environmental samples of chemical mixtures in contaminated drinking water, effluents, sediments, etc. (US EPA, 2005). The importance of risk assessment of chemical mixtures in environment is accepted by the European Commission (EC). It is emphasized that there is a demand for analysis of mixtures of chemicals that are produced and discharged from industrial processes or occur in the same environmental compartment c.f. EC report (Kortenkamp et al., 2009). Over the past ten years *V. fischeri* bioluminescence quenching test has been utilized in mixture toxicity analysis of heavy metals (Mowat ir Bundy, 2002) or organic compounds (Altenburger et al., 2000; Backhaus et al., 2000; Fernández-Alba et al., 2002; Christensen et al., 2006; Cedergreen et al., 2008). However, testing of mixtures containing widely divergent components (e.g., organic with inorganic compounds) is uncommon (McCarty, Borgert, 2006). Moreover, little attention has been given to mixtures of industrial and naturally occurring chemicals. According to our knowledge over past 10 years only one study on heavy metals and humic acids mixtures toxicity to *V. fischeri* cells was reported (Tsiridis et al., 2006). In our research, three chemicals were selected for mixture toxicity analysis using bacterial bioluminescence test; these compounds originated either from industrial or natural sources: 1) Aroclor 1248 (A); 2) oleic acid (OA); and, 3) elemental octahedral (orthorhombic) sulfur (S_8^0).

The environmental abundance and the bioaccumulative potential individually of these three chemicals was the reason for the choice for mixture toxicity analysis. Aroclor 1248 is an example of a dielectric chemical (now phased out), which is a mixture of PCBs (polychlorinated biphenyls) and is highly bioaccumulative and persistent in the aquatic environment (Frame et al., 1996; Henry, DeVito, 2003). The sensitivity of bacterial bioluminescence to individual commercial PCB mixtures (Aroclor 1242, 1260

and total PCBs) differ at least ten times (Chu et al., 1997; Salizzato et al., 1998a; Salizzato et al., 1998b). However, there are no available data on toxicity of Aroclor 1248. Oleic acid is one of most abundant toxic free fatty acids in the aquatic environment particularly in sediments and water surface microlayer (Patty, 1963, Odham et al., 1978; Sodergren A. 1987; Сиренко, Козицкая, 1988). Additionally, oleic acid and oleates are toxic to aquatic organisms(Četkauskaitė, 2004; Četkauskaitė, Bražėnaitė, 2004; REACH, 2006). Free oleic acid was found in toxic amounts sufficient for bioluminescence test in a pine forest ecosystem river sediments and shore soil (Četkauskaitė, 2004). Free oleic acid and other free fatty acids can originate from many industrial processes including paper manufacture (Morales et al., 1992). S_8^0 – the cyclic form of S is found in the aquatic environment (sediment, pore water) and its toxicity to *V. fischeri* in organic sediment extract was discussed earlier (Svenson et al., 1998). Geological analyses show, that in the marine environment, it is found mostly as S_8^0 as an essential elemental sulfur speciation and it co-exists with other sedimentary sulfur forms (Beffa et al., 1993; Gagnon et al., 1996). Hence, in this research, all three selected chemicals (A, OA and S_8^0) individually and in mixtures are relevant candidates for toxicity analysis to marine bacteria *V. fischeri*.

Analysis of the effects of hydrophobic substances, acting on biological membranes, i.e. chemicals with a dominant narcotic mode of action revealed that they are toxic to *V. fischeri* after a short incubation time (up to 5-15 min), (Hermens et al., 1985; Kaiser, Palabrica, 1991), and that reversibility of bioluminescence can occur in response to non-polar narcotics (e.g. ketones), (Dawson et al., 2006). In this work, the prolonged exposure time (up to 60 min) was used in *in vivo* system in order to clarify the trend of action of three hydrophobic toxicants.

There are a number of studies evaluating the effects of individual chemicals and their mixture to the bioluminescence of *V. fischeri*. However, the additional experimental studies are rarely used for the explanation of the mechanisms of toxic action. Over past 10 years the mechanisms of action of quinones and phenols only were analyzed (Ismailov et al., 2000; Kudryasheva et al., 2002; Vetrova et al., 2007; Wang et al., 2009b). Various biomarkers of toxicity have been used including: 1) respiration; 2) ROS production; 3) induction of antioxidant enzymes (superoxide dismutase and

catalase); 4) growth (aerobic and anaerobic); 5) activity of bioluminescence enzyme complex and luciferase; and, 6) NADH oxidation (Ismailov et al., 2000; Kudryasheva et al., 2002; Vetrova et al., 2007; Wang et al., 2009b). It is valuable to note that only some of these biomarkers were chosen for toxicity assesment in these scarce works. Thus, in our research the effects of A, OA and S_8^0 on *V. fischeri* systems *in vivo* and *in vitro* were analyzed for the explanation of mechanisms of their action.

The objectives of this work were to evaluate the toxicity of the industial xenobiotic Aroclor 1248 (A) and natural origin substances – elemental sulfur (S_8^0) and oleic acid (OA) and their binary mixtures to *V. fischeri* bioluminescence, and their biochemical mechanisms of action.

Aims:

1) determine the toxicodynamic effects of Aroclor 1248, oleic acid, sulfur and their binary mixtures to *V. fischeri* bioluminescence *in vivo* and *in vitro.*

2) evaluate the effects of Aroclor 1248, oleic acid and sulfur on the reducing capacity of *V. fischeri* and ROS generation.

3) evaluate the effects of Aroclor 1248, oleic acid and sulfur on respiration and membrane potential in *V. fischeri* cells.

4) determine the effects of Aroclor 1248, oleic acid and sulfur to *V. fischeri* NADH:FMN oxidoreductase-luciferase complex functions: a) NADH oxidation *in vitro;* b) O_2 consumption *in vivo* (after inhibition of respiratory chain with KCN); and, c) the restoration of bioluminescence by *n*-decanal *in vivo*.

Scientific novelty. For the first time, the toxicodynamic effects of binary mixtures of an industrial pollutant (Aroclor 1248) and environmental toxic substances (sulfur, oleic acid) to bioluminescence of *V. fischeri in vivo* and *in vitro* using multiple regression analysis were determined. Additionally, it was shown that Aroclor 1248, oleic acid and sulfur had different *in vivo* and *in vitro* trends of bioluminescence inhibition. A thorough search of *Vibrio* spp. (and *V. fischeri*) protein and gene data bases suggested that cells have a self-protection systems (xenobiotic biotransformation, efflux) capable to restore inhibited functions. It was showed experimentally that *V. fischeri* have ferric reducing/antioxidant capacity *in vivo*, which can be induced by sulfur. Moreover, sulfur diminished the generation of ROS in *V. fischeri* cells. It was indicated that Aroclor 1248

had multiple effects in *V. fischeri*: it inhibited bioluminescence slightly, caused enhancement of oxygen consumption, generated ROS, and diminished membrane potential *in vivo*. It was observed that oleic acid had the capacity to induce ROS production in *V. fischeri* cells equally as other multiple effects. Moreover, the same concentrations of Aroclor 1248 inhibited oxidation of NADH and bioluminescence *in vitro*; however the effects of oleic acid and sulfur to NADH oxidation were weak.

Thesis content. The doctoral thesis (in Lithuanian) contains the following parts: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusions, List of references (247 citations), List of publications (2 articles), 14 tables and 21 figures. The material of the doctoral thesis is presented in 131 pages.

Abbreviations

$\Delta\psi$ – membrane potential; $\Delta\mu_{H+}$ – electrochemical proton gradient; $\Delta\mu_{Na+}$ – electrochemical Na^+ gradient; ΔpH – proton concentration gradient; 2,4-DNP – 2,4-dinitrophenol; A – Aroclor 1248, commercial mixture of polychlorinated biphenyls; ANOVA – analysis of variance; CCCP – carbonyl cyanide m-chlorophenylhydrazone; DCDHF-DA – 2',7'-dichlorodihydrofluorescein diacetate; DCF – 2',7'-dichlorofluorescein; Dec – *n*-decanal; DTT – 2,4-dithiothreitol; *F* – Fisher criteria for variance homogenicity test; FMN – flavine mononucleotide oxidized; $FMNH_2$ – flavine mononucleotide reduced; FRAP – ferric reducing ability of plasma as "antioxidant power"; HC – high concentration(s); KC-400 – Kanechlor-400, polichlorintų bifenilų mišinys; KCN – potassium cyanide; LC – low concentration(s); $NAD(P)^+$ – nicotinamide adenine dinucleotide (phosphate), oxidized; NAD(P)H – nicotinamide adenine dinucleotide (phosphate), reduced; NEM – N-ethylmaleimide; OA – oleic acid; OD_{590nm} – optical density at wavelength of 590 nm; *P* – significance level; PCBs – polychlorinated biphenyls; P_i – inorganic phosphate; R^2 – coeficient of determination; ROS – reactive oxygen species; S_8^0 – orthorhombic sulfur containing eight elemental sulfur atoms; TCB – tetrachlorobiphenyl; TPP^+ – tetraphenylphosphonium ions; TPTZ – 2,4,6-tri(2-pyridyl)-1,3,5-triazine; v/v – volume percent.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the assays (substrates, inhibitors, reducing agents, reagents, solvents) were of the highest obtainable purity (Sigma-Aldrich, USA; Serva, USA; Merck, Germany), and elemental sulfur of analytical grade was obtained from Reachim (Russia). Aroclor 1248 was from Supelco (USA). Salts (bacterial cultivation media) were obtained from Serva (USA) and Roth (Germany). The stock solutions of chemicals were prepared either in ethanol or isopropanol (the final concentration of these solvents in the assays was 1% v/v). All growth and reaction media were prepared in Milli-Q water.

Methods

Bioluminescence measurements in bacterial cells (in vivo). A bacterial culture of *V. fischeri* (NRRL B-11177) was prepared, stored and thawed as described earlier (Četkauskaitė et al., 2004). Toxicity of whole cells was analyzed using a luminometer Model 1250 (LKB-Wallac, Sweden) with 1 ml vials. One ml of the sample consisted of 50 mM KH_2PO_4 buffer (940 µl), containing 2% NaCl (pH 7.0), 50 µl of bacterial suspension (OD_{590} = 0.125) and 10 µl of chemicals solution or solvent blank. The measurements of bioluminescence were performed after 5, 15, 30, 60 min of exposure to inhibitors or their mixtures, respectively, at room temperature. Experiments with binary mixtures of A, OA and S_8^0 were undertaken following a two level full factorial experimental design. Concentrations of these chemicals tested in all possible binary combinations were as follows: 1) A: 2.5 and 5 mg/L; 2) OA: 0.012 and 0.195 mg/L; 3) S_8^0: 0.027 and 0.055 mg/L. It is emphasized that in the presence of K^+ and Na^+ in the test media (pH 7.0), OA will be in the form of the appropriate salt. The reference toxicant was 3,5-dichlorophenol.

Preparation of NADH:FMN oxidoreductase-luciferase cell-free complex (in vitro). The cell-free bacterial bioluminescence complex extract was prepared as described earlier by Četkauskaitė et al. (2004). Protein concentration was determined using the method of Bradford (1976).

Bioluminescence assay in vitro. The medium for bioluminescence measurement was prepared according to Данилов, Егоров (1990), and modified for 300 µl volume vials, which were used for luminometric measurements with a Fluoroscan (BioLab Systems, Finland) in micro plates as described earlier by Četkauskaitė et al. (2004). The experiments were performed in following order: 1) enzyme extract (50 µl) was added to 150 µl sample (buffer and solvent blanks, or inhibitors, or their mixtures, as appropriate at their respective dilutions) and incubated 5, 15, 30 min at room temperature, respectively; and, 2) the measurement of luciferase activity commenced immediately after the addition of the substrates and residual volume of buffer as described earlier (Četkauskaitė et al., 2004). The crude enzyme activity remained at 80% in control samples up to the end of the assay; this confirmed that bacterial luciferase enzyme is stable below +35°C as indicated in http://www.brenda-enzymes.org. The calibration experiments indicated that the lowest bioluminescence level measured by Fluoroscan was comparable fully with luminescence units obtained by Luminometer Model 1250 (LKB-Wallac, Sweden). Control blanks contained 1% (v/v) ethanol, i.e. the same volume as the inhibitor-containing samples. A full factorial experiment design was performed with following concentrations of: 1) A: 2.5 and 5 mg/L; 2) OA: 6.25 and 12.5 mg/L; and, 3) S_8^0: 0.55 and 1.1 mg/L. The final concentration of the protein was 0.1 mg/ml in the bioluminescence measurement media with substrates. Alternatively, -SH group inhibition/protection assay *in vitro* was performed after 30 min exposure to N-ethylmaleimide (NEM) or sulfur (S_8^0) either alone or in combination with 1,4-dithiothreitol (DTT). The final concentration of the protein was 0.05 mg/ml in this case.

NADH oxidation in NADH:FMN oxidoreductase-luciferase pathway in vitro. The conditions of preparation of NADH:FMN oxidoreductase-luciferase cell-free bacterial enzyme extract were as described above. The media, substrates at their respective concentrations and experimental design were identical to those described for bioluminescence inhibition assay *in vitro*. Briefly, the enzyme extract was exposed to inhibitors for 5, 15, 30 min of incubation at room temperature, respectively. The measurement of NADH oxidation was started immediately after addition of substrates. NADH:FMN oxidoreductase activity was measured according to the decrease of NADH absorbance at 340 nm in one min interval, using FMN as electron acceptor. Reaction was

monitored using absorbance plate reader Tecan Infinite M200 (Tecan Group LTd., Männedorf, Switzerland). NADH concentrations reduction was calculated using the extinction coefficient 6,220 $M^{-1}.cm^{-1}$ of NADH.

Determination of ferric reducing antioxidant power (FRAP) of V. fischeri cells. The FRAP assay is a simple direct test of antioxidant capacity, that was described for evaluation of blood plasma reducing ability (Benzie, Strain, 1996). It measures the change in absorbance at 593 nm due to the formation of the blue colored Fe^{2+}-2,4,6-tri(2-pyridyl)-1,3,5-triazine compound from the colorless oxidized Fe^{3+} form by the action of electron donating antioxidants. The FRAP reagent was prepared by mixing 10 parts of 300 mM $CH_3COONa.3H_2O$ buffer (pH 3.6) with one part of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) in 40 mM HCl and with one volume of 20 mM $FeCl_3$. All solutions were used on the day of preparation. *V. fischeri* cells diluted with 2% NaCl media (OD_{590} = 0.3) were incubated with different concentrations of S_8^0 or with ethanol blank for up to one h. Reaction commenced after 50 µl of the sample (exposed *V. fischeri*) were added to 200 µl freshly prepared FRAP reagent. Absorbance readings were taken at 593 nm after 15 min incubation with FRAP reagent by using an absorbance plate reader ASYS UVM 340 (Asys Hitech GmbH, Eugendorf, Austria) in micro Brandplates™. FRAP data, which were obtained after *V. fischeri* incubation with S_8^0 or blank ethanol, were calculated as follows: total values of OD_{593} (FRAP in the presence of cells) minus OD_{590} of *V. fisheri* cells. The technical control of FRAP assay, S_8^0 (0.11 and 2.2 mg/L) without cells in 2% NaCl, did not enhanced the blue color (i.e. Fe^{2+} complex) formation. Technical controls (without cells) showed that A (2.5 mg/L) and OA (3.12 mg/L) were affecting the FRAP reagent color formation slightly, thus these control OD_{593} values were also subtracted from the values of OD_{593} (FRAP in the presence of cells). Final value of OD was used for further calculations. The results were expressed as concentration of Fe^{2+} in µM according $FeSO_4.7H_2O$ standard curve.

Determination of intracellular ROS. The evaluation of possible ROS stress, induced by A, OA and S_8^0 in *V. fischeri* cells, was performed using the fluorescent dyestuff 2',7'-dichlorodihydrofluorescein diacetate (DCDHF-DA). This dyestuff diffuses across cell membrane and undergoes deacetylation by intracellular esterases to 2',7'-dichlorodihydrofluorescein. This product is oxidized by intracellular ROS, forming a

highly fluorescent product, 2',7'-dichlorofluorescein (DCF). Fluorescence of DCF was detected with a fluorescence plate reader Tecan Infinite M200, using the excitation and emission wavelengths of 485 and 538 nm, respectively. Experiments were performed as follows: a) the suspension of *V. fischeri* cells (OD_{590} = 0.3) was exposed to the fluorescent dye, DHDCF-DA (10 μM) 30 min; b) the exposed suspension was centrifuged (4400g, 15 min), and the supernatant discarded; c) the cells in the pellet were suspended to obtain an initial density (OD_{590} = 0.3) with 50 mM KH_2PO_4 buffer, containing 2% NaCl (pH 7.0); d) the separate samples of *V. fischeri* suspension (OD_{590} = 0.3) were exposed to A, OA, S_8^0 or ethanol blank; and, e) fluorescence was recorded every 15 min for two h at the respective excitation and emission wavelengths.

Respiration measurements in V. fischeri cells were performed with a polarograph (Rank Brothers Ltd., Bottisham, Cambridge, England), using a Clark-type polarographic electrode in the same media as used for bioluminescence quenching measurements (pH 7.0), and additionally, at lower pH (pH 6.0). Total volume of the cell suspension in the cuvette/thermostat of the polarograph was one ml (final OD_{590nm} = 0.3), mixing speed 85.0 rpm, at room temperature. The classical uncoupler 2,4-dinitrophenol (2,4-DNP) were used to check sensitivity of method. Oxygen consumption due to luciferase in *V. fischeri* cells was evaluated in a medium containing 1 mM KCN – inhibitor of cytochrome oxidase. *n*-Decanol (0.06 mM; inhibitor of luciferase), OA or S_8^0,or control (ethanol) were added, respectively, after five min exposure to KCN (1 mM) and measurements were performed after a further 5 min.

Uptake of the lipophilic cation, tetraphenylphosphonium (TPP^+) in V. fischeri cell was measured as described earlier (Beržinskienė, Četkauskaitė, 1996) with a TPP^+-selective electrode, kindly presented by Doc. Dr. A. Zimkus from our laboratory. The volume of reaction medium (50 mM KH_2PO_4, 2 % NaCl, pH 7.0) was one ml. Final concentration of TPP^+ (0.4 μM) was used in order to avoid possible toxic effects of TPP^+. *V. fischeri* were grown as described earlier (Četkauskaitė et al., 2004), cells were washed, and resuspended in the reaction medium, and stored on ice until required. The uptake of the lipophilic cation, TPP^+, by freshly cultivated cells (OD_{590nm} = 0.9), was observed after exposure to A, OA and S_8^0 within 10 min. Further calculations were based on assumption that similarly to *E. coli V. fischeri*, where OD_{590} = 0.9 corresponds to 0.5

mg of dry mass, and the intracellular water volume is 2.7 µl/mg of dry mass (Hirota et al., 1981). The calculations of the changes in membrane voltage were conducted using a modified Nernst equation, as described previously (Četkauskaitė et al., 2006).

Data Analysis. Statistical analyses of data were performed using three different procedures: three-way and two-way ANOVA, and multiple regression. For all statistical methods significance level was 0.05. Toxicity data were presented as a mean of three or four separate experiments ± standard deviation. Sigma Plot10 software was used for statistical analysis.

Sequence of statistical analysis. Three-way ANOVA was used to evaluate changes of mixture toxicity within exposure time. Thereafter mixture effects at 30 min exposure time were evaluated in depth. Initially, the significance of chemicals and their interactions to overall mixture toxicity was evaluated using two-way ANOVA (for 30 min exposure measurements). ANOVA data evaluated on the basis of Fischer criteria and an analysis of *P* values. Then a reduced multiple regression polynomial models were used to confirm the interaction and to define the character of the combined effect (antagonistic, additive, synergistic). Low and high concentrations of chemicals in mixture were coded to the values of -1 and +1, respectively, according the calculations used by Ren et al. (2004) in order to avoid multicolinearity using two-way ANOVA and multiple regression procedures.

Mathematical expression of the full second order multiple regression polynomial model is as follows:

$$BL(\%) = b_0 + b_x C_x + b_y C_y + b_{xy} C_x \times C_y + b_x C_x^2 + b_y C_y^2 \pm \varepsilon \quad (1)$$

The experimental data were fitted to the reduced model:

$$BL(\%) = b_0 + b_x C_x + b_y C_y + b_{xy} C_x \times C_y \pm \varepsilon \quad (2)$$

where *BL*(%) represents the response, i.e. bacterial luminescence level; b_0 is an intercept; *b* – regression coefficients; *C* – the coded (calculated) concentration of chemical; subscripts *x*, *y* represents different components in a mixture (the C_A, C_{OA}, C_S, i.e. coded concentration of A, OA, S_8^0, respectively); *xy* is a subscript to regression coefficient of $C_x \times C_y$, which denotes interaction between chemicals *X* and *Y,* ε – standard error of estimate (*BL*).

In order to produce the best quality models, only terms with significant regression coefficients were retained, terms with insignificant coefficients were eliminated from the respective equation. The adequacy of the models was evaluated considering: determination coefficient (R^2), Fischer criteria (F) with P value.

The interpretation of the character of the combined effects was based on the signs of the regression coefficients of the equation terms. The effects of individual mixture components on binary mixture toxicity are indicated as:

1) additive, in case of negative regression coefficients (b_x, b_y), and when $C_x \times C_y$ interaction is insignificant;

2) additive with synergistic interaction, in case 1 and when $C_x \times C_y$ interaction is significant with negative b_{xy};

3) additive with a component of antagonistic interaction, in case 1 and when $C_x \times C_y$ interaction is significant with positive b_{xy};

4) antagonistic, in case of different signs (+ and/or -) of regression coefficients (b_x, b_y), and when $C_x \times C_y$ interaction is insignificant; and,

5) antagonistic with significant interaction, in case 4 when $C_x \times C_y$ interaction is significant with negative or positive b_{xy}.

RESULTS AND DISCUSSION

A significant amount of works analyze the data of xenobiotic mixture toxicity to *V. fischeri* using Concentration Addition (CA) and Independent Action (IA) concepts (Altenburger et al., 2000; Backhaus et al., 2000). However, the use of other methods is uncommon. It was suggested that the multiple regression method is suitable for the characterization of toxicodynamic effects of chemicals on bioluminescence bacteria (Bois et al., 1986; Ren et al., 2004). Thus, in our work the effects of binary mixtures of A, OA and S_8^0 to bioluminescence *in vivo* and *in vitro* were analyzed using this method. The interpretation of toxicodynamic effects is difficult because of the lack of knowledge on the target sites of chemicals in *V. fischeri* bacteria. There are only few studies, where the toxicity of xenobiotics are analyzed according to the data from related biochemical (enzymological) field on known and possible target sites in bioluminescence systems (*in vivo* and *in vitro*), (Ismailov et al., 2000; Kudryasheva et al., 2002; Wang et al., 2009b). Hence, in order to understand the mechanisms of chemical action the effects of A, OA and S_8^0 on the systems of redox enzymes (*in vivo*) and individual enzymes (*in vitro*) of *V. fischeri* bacteria were analyzed in this research.

Effects of single substances (Aroclor 1248, oleic acid and sulfur) to bioluminescence *in vivo* and *in vitro*

In vivo data indicated that the highest toxicity of A to *V. fischeri* cells bioluminescence (Fig. 1) reached 19% and 23% at concentrations 0.625 mg/L and 5 mg/L, respectively after 15 min exposure. OA at a lower concentration range (0.390–3.12 mg/L) caused the highest inhibition (from 64 up to 71%) after 15 min (Fig. 2). The high toxicity (up to 85%) of OA in similar concentration (1.56–3.12 mg/L) range was observed previously (Četkauskaitė, Beržinskienė, 2000). S_8^0 (0.11–0.27 mg/L) inhibited the bioluminescence (Fig. 3) from 90% up to 99% after 15 min exposure. The aforesaid results confirmed earlier obtained results by Četkauskaitė et al. (2004) that S_8^0 (0.11–0.22 mg/L) is very toxic to *V. fischeri* after short incubation time (5, 15 min). From the above, it was deduced that OA and S_8^0, individually were more toxic to *V. fischeri* cells in 11 μg/L–3 mg/L concentration range than A in the 0.625–10 mg/L concentration

range. Toxicity caused by A (0.625, 10 mg/L), OA (12–195 μg/L) and S_8^0 (11–220 μg/L) to bioluminescence *in vivo* was diminishing with increasing exposure time up to 60 min (Fig. 1–3). Hence, it was deduced that inhibition of bioluminescence *in vivo* caused by most of concentrations of A, OA and S_8^0 was reversible. Similar time-dependent effects of hydrophobic organic chemicals (non-polar narcotics) on bioluminescence of *V. fischeri* (*in vivo)* were obtained earlier (Dawson et al., 2006).

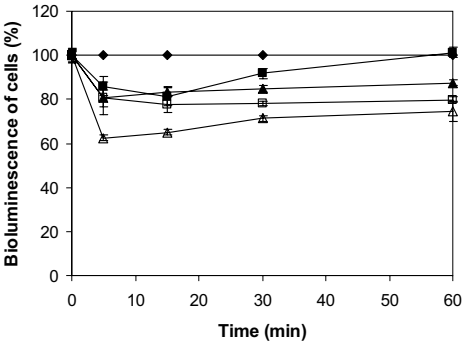

Fig. 1. Bioluminescence of *V. fischeri* (*in vivo*) cells at different time exposure with Aroclor 1248. Results are expressed as a mean of four separate experiments ± standard deviation.
Concentrations of Aroclor 1248: -◆- 0 mg/L; -■- 0.625 mg/L; -▲- 2.5 mg/L; -□- 5 mg/L; -△- 10 mg/L.

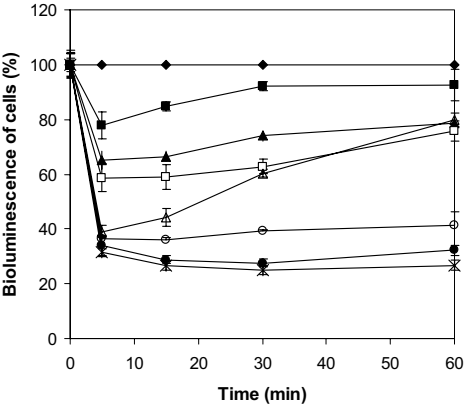

Fig. 2. Bioluminescence of *V. fischeri* (*in vivo*) cells at different time exposure with oleic acid. Results are expressed as a mean of four separate experiments ± standard deviation.
Concentrations of oleic acid: -◆- 0 μg/L; -■- 12 μg/L; -▲- 24 μg/L; -□- 48.5 μg/L; -△- 195 μg/L; -○- 390 μg/L; -●- 1560 μg/L; -✳- 3120 μg/L.

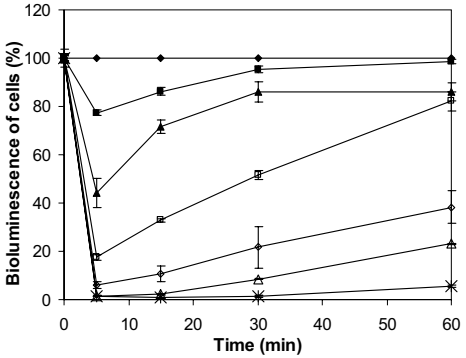

Fig. 3. Bioluminescence of *V. fischeri* (*in vivo*) cells at different time exposure with elemental sulfur. Results are expressed as a mean of four separate experiments ± standard deviation.
Concentrations of sulfur: -◆- 0 μg/L; -■- 11 μg/L; -▲- 27 μg/L; -□- 55 μg/L; -◇- 110 μg/L; -△- 220 μg/L; -✳- 270 μg/L.

Table 1. Effects of Aroclor 1248, oleic acid and sulfur to bioluminescence complex of *V. fischeri* (*in vitro)*

Concentration mg/L	Bioluminescence[a], % (related to control)		
	5 min	15 min	30 min
Aroclor 1248			
2.5	52.99 ± 7.27*	55.92 ± 1.63*	51.08 ± 6.63*
5	65.68 ± 4.26*	64.18 ± 1.01*	44.17 ± 7.17*
10	72.52 ± 2.38*	68.61 ± 5.88*	63.64 ± 4.13*
Oleic acid			
3.12	73.34 ± 1.48*	93.95 ± 4.86	87.62 ± 4.22
6.25	62.36 ± 1.56*	69.22 ± 2.60*	51.81 ± 0.46*
12.5	58.04 ± 3.50*	58.17 ± 6.90*	43.81 ± 1.85*
25	43.60 ± 0.76*	37.83 ± 1.69*	43.84 ± 2.62*
Sulfur			
0.27	76.92 ± 8.03*	78.95 ± 0.70*	56.23 ± 9.10*
0.55	68.66 ± 7.03*	50.72 ± 6.76*	32.15 ± 2.25*
1.1	78.90 ± 2.89*	53.82 ± 3.28*	32.67 ± 4.98*
2.2	70.20 ± 2.69*	55.45 ± 0.09*	30.10 ± 2.80*

[a] Results are expressed as a mean of four separate experiments ± standard deviation. The bioluminescence values of exposed samples were compared with the respective control values (*P < 0.05).

The comparison of ***in vitro*** A, OA and S_8^0 effects on bioluminescence complex activity after 5 min showed that A (2.5 mg/L) inhibited enzymes more effectively than OA (3.12 mg/L) and S_8^0 (2.2 mg/L). However, S_8^0 developed the strongest inhibition (by up to 70%) after 30 min at the same concentration (Table 1). Additionally, toxicity caused by A (2.5–10 mg/L), OA (the highest tested concentrations 6.25–25 mg/L) and

S_8^0 (0.27–2.2 mg/L) to bioluminescence *in vitro* was enhancing with increasing exposure time up to 30 min (Table 1). From the above, it was deduced that inhibition caused by A, OA and S_8^0 *in vitro* was irreversible.

Thus in general, the toxicity to bioluminescence of individual toxicants A, OA and S_8^0 had characteristic opposite trends *in vivo* and *in vitro*. These results could indicate that *in vivo V. fischeri* have self-protection systems, i.e. coping with hydrophobic xenobiotic biotransformation (Jegathesan, Paramasivam, 1976; Byers, 1989; Villa, Willetts, 1997; Kwak et al., 2003; Jiang et al., 2010), or efflux (Antunes et al., 2007; Gu et al., 2009; Kuroda, Tsuchiya, 2009) systems.

Effects of binary mixtures of Aroclor 1248, oleic acid and sulfur to bioluminescence *in vivo* and *in vitro*

Effects of binary mixtures* to bioluminescence *in vivo. *General trend of toxicity*. Data on toxicity of binary mixtures of A, OA and S_8^0 to *V. fischeri* bioluminescence *in vivo* at different exposure durations are presented in Fig. 4–6. The analysis of general trend of toxicity to bioluminescence *in vivo* led to conclusion that binary mixtures of low and high concentrations of OA and S_8^0, A and OA, and even many of A and S_8^0 concentrations caused the partial restoration of bioluminescence function (i.e. reversible toxicity) up to 60 min. Three-way ANOVA analysis data revealed that changes of all three binary mixtures toxicity (Fig. 4–6) to *V. fischeri* cells were significant within 60 min ($P < 0.001$). Hence, toxicity of binary mixtures of partially polar A, polar OA and oxidoreductive S_8^0 was reversible.

Preliminary two- way ANOVA analysis at fixed different exposure time revealed, that different combinations of chemical concentrations almost in all cases caused significant different effects and contribution of chemicals to mixture toxicity were significant after 30 min. From the foregoing, 30 min standard exposure time was selected for further analysis of effects of binary mixture on bioluminescence *in vivo*.

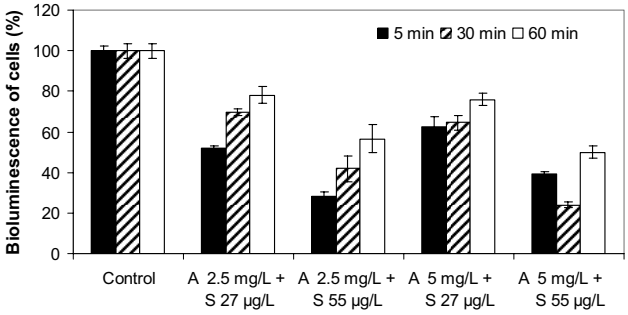

Fig. 4. Effects of Aroclor 1248 and sulfur mixtures to *V. fischeri* cells bioluminescence after 5, 30 and 60 min exposure. Results are expressed as a mean of three separate experiments ± standard deviation.

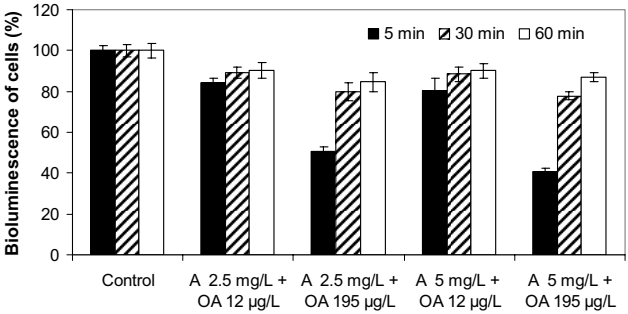

Fig. 5. Effects of Aroclor 1248 and oleic acid mixtures to *V. fischeri* cells bioluminescence after 5, 30 and 60 min exposure. Results are expressed as a mean of three separate experiments ± standard deviation.

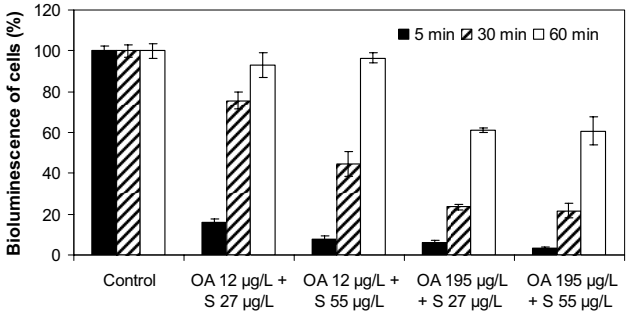

Fig. 6. Effects of oleic acid and sulfur mixtures to *V. fischeri* cells bioluminescence after 5, 30 and 60 min exposure. Results are expressed as a mean of three separate experiments ± standard deviation.

Toxicodynamic effects. Effects of binary mixture of A and S_8^0 on bioluminescence *in vivo* are presented in Fig. 4. After 30 min exposure, significance contribution to mixture toxicity by A ($P < 0.001$) and S_8^0 ($P < 0.001$) and their interaction ($P < 0.015$) was determined by two-way ANOVA. Multiple regression analysis indicated this interaction as synergistic (all regression coefficients were negative) i.e. toxic effects of mixture were stronger than simple additive effects of individual chemicals (Table 2; Eqn. 1).

A and OA combined effects on bioluminescence *in vivo* are presented in Fig. 5. Insignificant interaction between A and OA was indicated by two-way ANOVA ($P = 0.71$) and only OA was significant determinant of mixture toxicity ($P < 0.001$) after 30 min exposure. The best fitted multiple regression equation for 30 min exposure indicated that the toxicity of binary mixture was defined only by changing concentrations of OA (Table 2; Eqn. 2). Hence, the effect of A in mixture with OA on bioluminescence *in vivo* was reduced, however the same concentrations of A in mixture with S_8^0 were significant determinant of bioluminescence inhibition. Additionally, inhibition caused by OA (195 μg/L) individually (by up to 40%; Fig. 2) was higher than inhibition caused by mixture (by up to 23%) of high concentrations of A (5 mg/L) and OA (195 μg/L) after 30 min (Fig. 5). The reduction of OA toxic effect (inhibition) in mixture to bioluminescence *in vivo* by up to 17%, and the reduction of A contribution to overall mixture toxicity led to the deduction that A and OA acted with antagonistic character.

Data on binary mixture of OA and S_8^0 toxicity to bioluminescence *in vivo* are presented in Fig. 6. The results of two-way ANOVA showed significant determination of mixture toxicity by OA, S_8^0 and their interaction (OA × S_8^0) at 30 min exposure ($P < 0.001$ in all cases). Multiple regression equation describing toxicity of OA and S_8^0 mixture after 30 min (Table 2; Eqn. 3) indicated additive toxic effect with component of antagonistic interaction. The increase in OA or S_8^0 concentrations caused an enhancement of mixture toxicity after 30 min, but the combined effect was less than the sum of their individual effects.

Table 2. Character of Aroclor 1248, oleic acid and elemental sulfur binary mixtures toxicity for *V. fischeri in vivo*

Multiple regression equation[a,b]	Remarks	Character of toxicodynamic effects
Aroclor 1248 and sulfur		
1) BL (%) = 50.0 – 6.0 C_A – – 17.4 C_S – – 3.2 $C_A \times C_S \pm 3.6$ $R^2 = 0.98$, $F = 165.47$, $P < 0.001$	All reg. coef.(s)[c] were significant ($P < 0.015$)	Additive toxic effect of A and S_8^0; synergistic interaction
Aroclor 1248 and oleic acid		
2) BL (%) = 83.8 – 5.0 $C_{OA} \pm 3.1$ $R^2 = 0.76$, $F = 31.15$, $P < 0.001$	C_A and $C_A \times C_{OA}$ were eliminated, as their reg. coef.(s) were insignificant ($P = 0.462$; $P = 0.706$, respectively); reg. coef. for C_{OA} was significant ($P < 0.001$)	Toxic effect of mixture depends only on concentration of OA; no interaction
Oleic acid and sulfur		
3) BL (%) = 41.3 – 18.8 C_{OA} – 8.2 C_S + + 7.3 $C_{OA} \times C_S \pm 4.3$ $R^2 = 0.98$, $F = 98.40$, $P < 0.001$	All reg. coef.(s) were significant ($P < 0.001$)	Additive toxic effect of OA and S_8^0; with the component of antagonistic interaction.

[a] – descriptions of equation variables q.v. Materials and Methods;

[b] – where R^2 (determination coefficient), F and P represents adequacy and statistical significance of regression equations;

[c] – reg. coef.(s) – regression coefficient(s).

Effects of binary mixtures to bioluminescence in vitro. The analysis of *general trend of toxicity* to bioluminescence *in vitro* (Data not shown) led to conclusion that all tested binary mixtures of low and high concentrations of OA and S_8^0, A and S_8^0, and even many mixtures of A and OA inhibited bioluminescence up to 30 min (i.e. caused irreversible toxicity). Three-way ANOVA data confirmed that pre-treatment time of the bioluminescence complex *in vitro* with binary mixtures of A , OA and S_8^0 was a significant factor of toxicity ($P < 0.001$). Thus in general, the toxicity to bioluminescence of binary mixtures of A, OA and S_8^0 had opposite trends in bioluminescence *in vivo* and *in vitro* systems.

Toxicodynamic effects. The multiple regression equations, describing A and S_8^0 mixture effects after 30 min, indicated that A and S_8^0 inhibited bioluminescence *in vitro* additively without significant interaction. The fitted multiple regression equation indicated that A and OA in mixture after 30 min caused antagonistic effects; and, OA and S_8^0 in mixture acted additively with synergistic interaction (Data not shown).

In summary, different characters of combined effect at 30 min exposure were determined which were dependent on binary mixture composition, and on investigated bioluminescence system. A and S_8^0 in admixture acted additively with synergistic interaction and without significant interaction in *in vivo* and *in vitro* system, respectively. Effects of A and OA in mixture had antagonistic character in both investigated systems. OA and S_8^0 acted additively with antagonistic component of interaction and synergistic interaction in bioluminescence *in vivo* and *in vitro* systems, respectively.

Hence, different toxicodynamic characters of the combined effects depends on chemical composition observed in *in vivo* and *in vitro* system together with literature data shows that different binding sites of hydrophobic toxic substances exist in *in vitro* system (Curry et al., 1990; Abu-Soud et al., 1993; Lei et al., 1994; Koike et al., 1998; Low ir Tu, 2002). Different toxicity trends observed in this study, suggested that *in vivo* system developed protective enzymatic activities capable of removing or transforming these toxic compounds. These results induced further investigation of action sites of these compounds in both (*in vivo* and *in vitro)* systems.

Effects of individual toxicants (A, OA and S_8^0) on the state of *V. fischeri* oxidoreductive enzyme systems and cytoplasmic membrane *in vivo*

The second stage of this research was to analyze the possible effects of pollutants on oxidoreductive state of *V. fischeri* cells: 1) ROS generation by cells; 2) reducing capacity of cells; 3) oxygen consumption; and, 4) membrane potential.

Changes in ROS generation. Detectable ROS were produced when *V. fischeri* cells were exposed to A, OA and S_8^0 (Fig. 7). A (5 mg/L) and OA (6.25 mg/L) caused an increase; however, S_8^0 (1.1 mg/L) caused a decrease of ROS production in comparison with control (Fig. 7). In general, the amount of ROS in exposed cells was increasing during 2 h.

Literature data indicate that in eukaryotic hepatocytes some PCB congeners at wide concentration range (28.9 µg/L–33 mg/L) can cause the enhancement of ROS formation (Schlezinger et al., 2006; Aly, Domenech, 2009) and the depletion of antioxidant enzymes (glutathione peroxidase and glutathione reductase), (Aly, Domenech, 2009). Data presented in our studies indicates that PCBs (Aroclor 1248) in 5 mg/L concentration can induce ROS in *V. fischeri* cells.

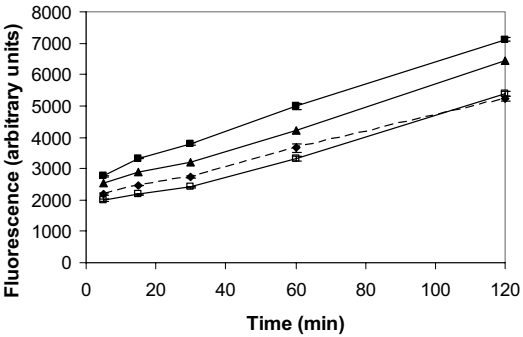

Fig. 7. The formation of ROS in *V. fischeri* cells, caused by Aroclor 1248, oleic acid and sulfur. The final optical density (OD_{590}) of *V. fischeri* cells was 0.3; for other details see Section Materials and Methods. Results are expressed as a mean of three independent experiments ± standard deviation. --◆-- Control, -■- A 5 mg/L, -▲- OA 6.25 mg/L, -□- S 1.1 mg/L.

Earlier it was indicated that free fatty acids increased mitochondrial generation of ROS at the forward electron transport but caused a decrease of ROS during reverse transport (Schonfeld, Wojtczak, 2007). Thus, data from our studies on ROS generation and respiration enhancement (i.e. forward electron transport) caused by OA in *V. fischeri* are comparable with Schonfeld and Wojtczak (2007) results obtained in experiments with mitochondria.

Effects on cell reducing capacity. One of the causes of the reversible inhibition of bioluminescence *in vivo* was deduced to be the activity of self-protection systems in *V. fischeri* cells. One of the possible cell self-protection systems is antioxidant activity of *V. fischeri*, and this was evaluated using FRAP method. *V. fischeri* cells had relatively small FRAP values (Fig. 8). However, S_8^0 (0.55–2.2 mg/L) increased their antioxidant capacity even at one (data not shown) and at five min, resulting two - four times higher FRAP values in comparison with control (technical controls showed that S_8^0, ethanol, had no effect on FRAP reaction in the absence of cells). These values were decreasing further with an incubation time ≤ 60 min. Contrary, a very slight enhancement of FRAP (close to control values) was observed at 5-60 min with other two compounds A (2.5 mg/L) and OA (3.12 mg/L). Hence, it was deduced, that S_8^0 was inducing the antioxidant

capacity of *V. fischeri* cells much more powerfully, than A and OA. The amount of reducing equivalents, formed by cells, was dissipating with time (≤ 60 min).

FRAP method is based on reduction of reagents equally as other methods of the reduction of tetrazolium salts, resazurin, which are used for the detection of the dehydrogenase activity (Lopez et al., 1986; Gabbita ir Hang, 1984; Liu, 1986). This means that the reducing ability of *V. fischeri* cells, which is induced by S_8^0, and is detected by FRAP method, reflects the activity of redox enzymes. This leads to the question "what kind of reducing equivalents were produced by *V. fischeri* oxidoreductases"?

It was reported that *Vibrio* spp. bacteria were able to perform the reduction of various compounds (chromate, algal mycosporine-like amino acids, ferric salts), (Fulladosa et al., 2006; Shick, Dunlap, 2002; Hasanuzzaman, Araki, 2001). Thus, there is possibility that *V. fischeri* is able to reduce S_8^0 to H_2S.

There is possibility that *V. fischeri* are able to perform other biotransformation reactions. Četkauskaitė with co-authors (2004) proposed that reduction of S_8^0 toxicity to bioluminescence is due to oxidation of S_8^0. It is useful to note that S_8^0 oxidizing bacteria (e.g. *Acidithiobacillus thiooxidans*) have sulfur dioxygenase, which requires reduced glutathione, for initial stage, the non-enzymatic reaction with S_8^0, and, paradoxically, oxidizes S_8^0 through the formation of H_2S and monoorganylpolysulfane (GS_nH, n>1), (Rohwerder, Sand, 2003).

Furthermore, it is known that H_2S production from proteins was a marker for the identification of many heterotrophic bacteria (including *Vibrio* spp.), (Jegathesan, Paramasivam, 1976). Thus, when considering all these facts mentioned above, *V. fischeri* cells might remove S_8^0 as H_2S, which can enhance the reductive power of cells involved in a FRAP reaction.

Additionally, the FRAP reaction *in vivo* could indicate, that H_2S is produced by *V. fischeri*, since various Fe^{3+} containing salts (sulfate, citrate, chloride) together with organic redox compounds are used as H_2S detection (with other redox organic compounds) or removal agents (http://lydakis.chania. teicrete.gr/; http://www.wipo.int/).

It can be noted, that the FRAP assay is an indirect test of total antioxidant power (it does not measure the –SH group containing antioxidants); however, in serum samples

FRAP data have weak, but statistically significant linear correlation with oxygen (or pro-oxidant - peroxyl, hydroxyl) radical absorbance capacity (ORAC), (Prior, Cao, 1999). Hence, the reduction of ROS content (Fig. 7) and the activity of antioxidant power (Fig. 8) of *V. fischeri* cells in the presence of S_8^0 (1,1 mg/L) during 5–60 min shows the possible induction of redox enzyme system.

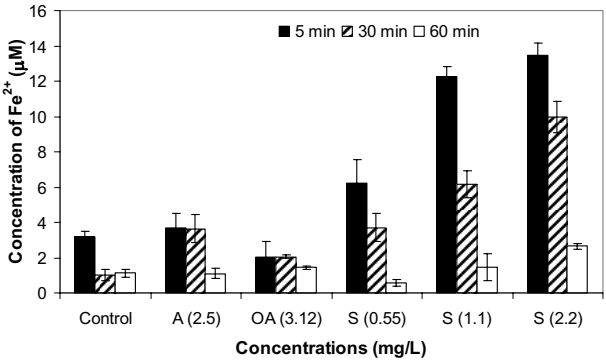

Fig. 8. Changes in reducing capacity of *V. fischeri* cells after exposure to Aroclor 1248, oleic acid and sulfur. The final optical density (OD_{590}) of *V. fischeri* cells was 0.3. Results are expressed as a mean of three independent experiments ± standard deviation.

Enhancement of oxygen consumption. Oxygen consumption or respiration in most prokaryotic cells and eukaryotic mitochondria reflects the state of oxidative phosphorylation. The classical weak acid respiratory uncoupler 2,4-dinitrophenol (200 μM or 36,8 mg/L) used in this work as reference chemical and A (5 mg/L) enhanced oxygen consumption in *V. fischeri* cells by up to 31% and 13%, respectively (data not shown) at pH 7.0. Similarly, OA was able to activate the respiration of *V. fischeri* cells by up to 14% at 2.5–3.12 mg/L (Fig. 9a). Only high S_8^0 concentration (2.2 mg/L) was able to enhance respiration by up to 20% at pH 7.0 (Fig. 9b).

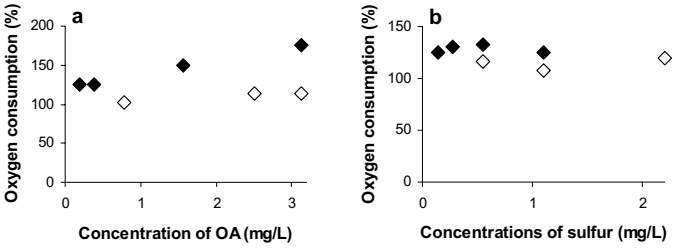

Fig. 9. Enhancement in oxygen consumption of *V. fischeri* after 5 min exposure to oleic acid (a) and sulfur (b) at different pH. The final optical density (OD_{590}) of *V. fischeri* cells was 0.3. -◆- pH 6.0, -◇- pH 7.0.

The data in this work indicated, that at pH 6.0 the respiration enhancement caused by OA and S_8^0 was significantly higher ($P < 0.05$), than at pH 7.0 (Fig. 9). It was indicated earlier, that uncouplers in general were less active at ≥ pH 7.0 (Tokuda, Unemoto, 1983; Wada et al., 1992). *V. harveyi* growth, luminescence and respiration resistant to the classical uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), (10 μM or 2 mg/L) was observed at at pH 7.5–8.5, where the electrochemical gradient was created mostly by Na^+ ions (Wada et al., 1992; Fujiwara-Nagata et al., 2003). Thus, our data showed, that OA and S_8^0 tested in the 0.14–3.12 mg/L concentration range were acting similarly as classical uncouplers, which dissipate proton gradient preferably at pH 6.0, where the value of electrochemical energy depends mainly on H^+, but not on the Na^+ gradient.

Respiration of *V. fisheri* during NADH oxidation reflects oxygen usage by: 1) respiration chain enzymes (including Na^+-pumping NADH:quinone oxidoreductase, which is capable of translocating Na^+ from inside to outside of cells); and, 2) bioluminescence complex enzymes. The respiration enhancement in electrochemical proton or ion gradients generating membrane (that is also a characteristic of the marine *V. fischeri*) means uncoupling effect (Skulachev, 1998).

Thus, the data in this work on the two on-going simultaneously effects, i.e. the decrease in bioluminescence and an increase in oxygen consumption under exposure to A, OA or S_8^0 indicated, at least, a few mechanisms of action: 1) no efficient electron entry into the bioluminescence complex pathway; 2) possible inhibition of bioluminescence complex enzymes; and, 3) possible cytoplasmic membrane

permeability enhancement (specific or not), because of the uncoupling, entropic decoupling or general narcotic action.

Effects on membrane potential. The bioluminescence inhibition, enhancement of respiration, changes in ROS generation caused by A, OA and S_8^0, which were observed in this work, shows that the sites of action of these toxicants are located in cytoplasmic membrane. This led to the question "if these toxicants change inner membrane permeability"?

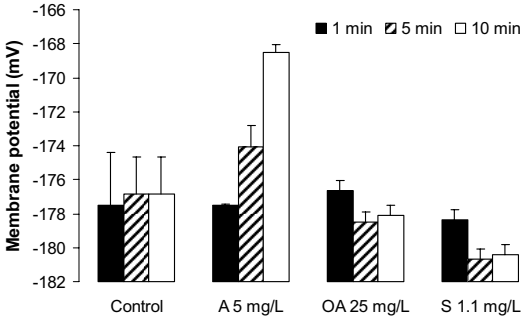

Fig. 10. Changes of membrane potential of *V. fischeri* after exposure to Aroclor 1248, oleic acid and sulfur. The final optical density (OD_{590}) of *V. fischeri* cells was 0.9. Inhibitors or control (ethanol) were added at time zero. Results are expressed as a mean of three separate experiments ± standard deviation.

According literature data, the additional Na^+ ions gradient to proton gradient is produced at pH 7.0–8.5 in marine *Vibrio* spp. due to Na^+ pumping NADH-quinone oxidoreductase and Na^+/H^+ antiporter (Oh et al., 1991; Hayashi et al., 2001). Electrochemical gradient of protons ($\Delta\mu_{H+}$) and its buffering form – gradient of Na^+ ($\Delta\mu_{Na+}$) can be used for ATP synthesis, for transport of P_i, metabolites and toxins, for the motility of flagellum, and could be dissipated by a proton leak though the membrane (Скулачев, 1989). Since, $\Delta\mu_{H+}$ and $\Delta\mu_{Na+}$ are used for the maintenance of membrane potential, the sensitivity of membrane potential is lower at pH 7,0, than at acidic pH, where $\Delta\mu_{H+}$ is dominating.

In this work, A (5 mg/L or 17.12 μM) caused reduction of membrane potential of *V. fischeri* by 8 mV in 10 min (Fig. 10). It is useful to reiterate that A in the same concentration range slightly enhanced oxygen consumption and generation of ROS.

Similar effects were observed earlier when rat liver mitochondria were exposed to structural analog of Aroclor 1248 – PCB mixture Kanechlor-400 (KC-400) (30 mg/L). KC-400 enhanced state 4 respiration of rat liver mitochondria with α-ketoglutarate/malate as substrate, dissipated membrane potential, and caused intracellular K^+ leakage (Nishihara, 1985). It was demonstrated that non-planar tetrachlorobiphenyls, TCB (containing at least 2 Cl atoms at the 2,2‘ and any other, e.g. 6,6‘ positions), caused uncoupling of the oxidative phosphorylation, membrane release of Ca^{2+}, K^+ ions, and dissipation of membrane potential at 40–80 μM concentration range in state 4 respiration of mitochondria (Nishihara et al., 1987). Contrary, non-planar 2,2’,5,5’-TCB (5.8 mg/L or 20 μM) increased inner membrane permeability to ions, slightly enhanced membrane potential, and inhibited the enzyme system of respiration and phosphorylation in state 3 respiration of rat liver mitochondria (Mildažienė et al., 2002a, 2002b). Aroclor 1248 contains sufficient amount of non-planar PCBs (~42%) and cause similar effects in *V. fischeri* like other non-planar PCBs in mitochondria. Since A as PCB mixture has no dissociable organic acid group, it most likely that it does not act as classical protonophoric uncoupler. Inhibitory effects in the complex I of the respiratory chain of rat liver mitochondria PCBs was earlier observed due to exposure to Aroclor 1257 (10–60 μM or 6.5–33 mg/L concentration range), (Aly, Domenech, 2009), and 2,2’,5,5’-TCB (5.8 mg/L) (Mildažienė et al., 2002b). It is possible that A (5 mg/L) action on *V. fischeri* membrane permeability was specific, and followed by ROS formation and the reduction of membrane potential. Hence, in our work A (5 mg/L) acted in few ways: a) enhanced oxygen consumption; b) reduced membrane potential; c) enhanced ROS formation, acting specifically on the components of respiration chain (e.g. NADH dehydrogenases), and had non-specific activity on membrane permeability of *V. fischeri* bacteria similarly as other non-planar PCBs in mitochondria, as was mentioned above.

In our work OA (25 mg/L) and S_8^0 (1.1 mg/L) enhanced the membrane potential by ~2 mV during 10 min, respectively (Fig. 10). Since both compounds caused slight respiration enhancement (Fig. 9), it was suggested that facilitated TPP^+ uptake was depending on changes of electrochemical gradient of H^+, and of other ions (Na^+, K^+). Such small effects of OA and S_8^0 on membrane potential could be explained by strong

buffering Na^+ gradient (in addition to $\Delta\mu_{H+}$) or observed earlier K^+ efflux from cells caused by hydrophobic herbicides in similar OA concentrations range (Beržinskienė, Četkauskaitė, 1996). The resistance of membrane potential to OA and S_8^0 could be created by the existence of multiple drug resistance pumps, which are known in *Vibrio* spp. (Ruby et al., 2005; Antunes et al., 2007; Gu et al., 2009; Kuroda, Tsuchiya, 2009).

Hence, the different physiological-biochemical effects of A, OA and S_8^0, which were related to inhibition of bioluminescence in *V. fischeri* cells, were determined. It was found that A and OA, but not S_8^0, enhanced ROS generation in *V. fischeri* cells. Contrary, S_8^0 (greater than A and OA) induced the reducing ability (FRAP) of *V. fischeri* cells. These results indicate the activity of the protective system of redox enzyme in *V. ficheri*. The fact that A, OA and S_8^0 (0.14–5 mg/L) enhanced oxygen consumption of *V. fischeri* indicate that these compounds have uncoupling activity. However, different effects on membrane potential (A diminished membrane potential, but effects of OA and S_8^0 were negligible) led to the suggestion that mechanisms of chemicals action are more complicated.

Effects of individual toxicants on bioluminescent enzyme systems

In order to predict the possible sites of pollutants action in enzyme systems, their effects on bioluminescence complex dependent oxygen consumption, changes of toxicity after addition of *n*-decanal (the substrate of luciferase) on bioluminescence *in vivo* were analyzed, and comparison of their effects on NADH oxidation and bioluminescence *in vitro* were undertaken. Additionally, effects of S_8^0 and N-ethylmaleimide (NEM) on bioluminescence were compared in order to clarify the sites of S_8^0 action.

Effects of individual toxicants on bioluminescence complex dependent oxygen consumption in vivo. It was reported, that the bioluminescence pathway in *V. fischeri* consumes 17–20.7% of oxygen in total respiration (Makemson, 1986; Bourgois et al., 2001). Our results confirmed that 18% of the total respiration belonged to oxygen consumption caused by luciferase (Table 3). The addition of OA and S_8^0 after inhibition of the respiratory chain of *V. fischeri* cells with 1 mM KCN, inhibited residual (luciferase pathway) oxygen consumption similarly to the known luciferase inhibitor – *n*-decanol. Hence, the inhibition of *V. fischeri* respiration after KCN addition caused by

OA and S_8^0 may indicate that the target sites of OA and S_8^0, similarly to *n*-decanol are located at the luciferase enzyme of *V. fischeri* cells. It was deduced that OA and S_8^0 can uncouple the transfer of $FMNH_2$ from NADH:FMN oxidoreductase to luciferase in the complex of these enzymes.

Table 3. Effects of *n*-decanol, oleic acid and sulfur on bioluminescence complex dependent oxygen consumption *in vivo*[a]

Parameter	O_2 consumption (%)
KCN	18 %
KCN + *n*-decanol	0 %
KCN + OA	9 %
KCN + S_8^0	9 %

[a] – Reaction medium: 50 mM K_2HPO_4, 2% NaCl, pH 6.0. The final optical density (OD_{590}) of *V. fischeri* cells was 0.3. Concentrations: KCN – 1 mM; *n*-decanol – 0.06 mM; OA – 0.78 mg/L; S_8^0 – 0.55 mg/L.

Protecting effects of n-decanal (Dec) to V. fischeri bioluminescence in mixtures with inhibitors A, OA and S_8^0. The inhibition caused by binary mixture of A (5 mg/L) and the luciferase substrate Dec (3.12 mg/L) after 30 min was significantly (P <0.05) higher, than effects of Dec (3.12 mg/L) and A (5 mg/L) individually (Fig. 11). These observations led to the proposition that A possibly do not compete with Dec for luciferase active site binding *in vivo*.

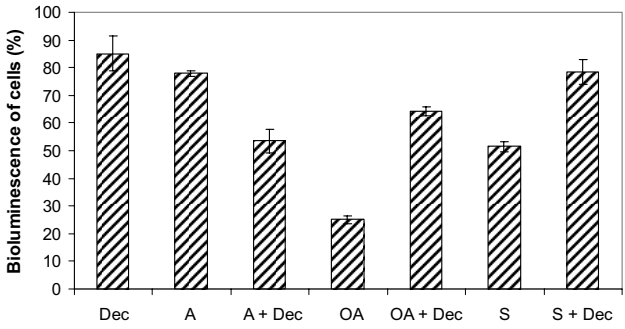

Fig. 11. The effects of luciferase substrate – *n*-decanal (Dec) in the presence of individual toxicants A, OA and S_0^8 on *V. fischeri* bioluminescence *in vivo* after 30 min exposure. Results are expressed as a mean of three independent experiments ± standard deviation. Concentrations in mg/L: A – 5.0; OA – 3.12; Dec – 3.12; S_8^0 – 0.055.

OA (3.12 mg/L) was added to cells suspension prior to Dec (3.12 mg/L). It should be noted that the bioluminescence of cells exposed to their mixture was enhanced by ~40% compared to samples with OA individually after 30 min (Fig. 11). Hence, Dec in mixture with OA showed protective, possibly, an antagonistic effect against OA toxicity (Fig. 11). This indicated that OA binding site was at least close to site of Dec binding as of luciferase substrate.

The luciferase substrate, Dec (3.12 mg/L) added after S_8^0 (0.055 mg/L) was able to reduce S_8^0 toxicity by 30% after 30 min (Fig. 11). Moreover, the difference between bioluminescence inhibition caused by binary mixture and Dec individually was insignificant ($P > 0.05$). Thus, the analysis of the character of inhibition showed an antagonistic action of Dec against S_8^0 toxicity in bioluminescence. This indicated that S_8^0 action site is related also to hydrophobic Dec binding sites.

Effects of A, OA and S_0^8 on NADH oxidation in vitro. The general reason for inhibition of bioluminescence *in vitro*, and the reduction of bioluminescence complex dependent oxygen consumption *in vivo* (i.e. in the presence of KCN) observed in this study could be the inhibition of NADH oxidation in NADH:FMN oxidoreductase. In this study, A (5 mg/L) inhibited NADH oxidation *in vitro* by up to 53% after 5 min (Fig. 12), and 66% after 30 min. Hence, it could be deduced that A at 5 mg/L concentration acted as an irreversible inhibitor in the NADH:FMN oxidoreductase. However, the same concentration of A (5 mg/L) inhibited bioluminescence *in vitro* by up to 35 and 56% after 5 and 30 min, respectively (Table 1). Higher inhibition of NADH oxidation than of resulting of bioluminescence *in vitro* showed that the main binding site of A is located in the NADH:FMN oxidoreductase, and it is possibly NADH binding site. The literature data on inhibition of respiration complex I (NADH-quinone oxidoreductase) of eukaryotic mitochondria caused by 2,2',5,5'-TCB (20–140 μM), (Mildažienė et al., 2002b) and Aroclor 1254 (20–60 μM), (Aly, Domenech, 2009) support data of this work that A (having ~42% of 2,2' non-planar PCBs) binding location is close to NADH binding site(s) in the NADH:FMN oxidoreductase.

NADH oxidation by bioluminescence complex *in vitro* was reduced by OA (6.25 mg/L) by up to 13% and 28% after 5, 30 min, respectively (Fig. 12). Additionally, the same concentration of OA inhibited bioluminescence *in vitro* by up to 38%, 48% after 5,

30 min, respectively (Table 1). Such low inhibition of NADH oxidation, rather than of bioluminescence (both *in vitro*) caused by OA indicated that OA has a greater affinity to luciferase rather than NADH:FMN oxidoreductase. It is useful to recall that *in vitro* experiments were performed with 22 μM (6.25 mg/L) OA, which was about two times higher than the Dec concentration (13 μM or 2 mg/L). OA is a hydrophobic compound with a linear structure as is Dec, hence it is possible that one target site of OA in bioluminescence complex is the luciferase substrate Dec binding site. Possibly $FMNH_2^{\cdot}$produced by oxidoreductase could not restore bioluminescence *in vitro*, because of formation of dead-end complex between inhibitor and luciferase enzyme.

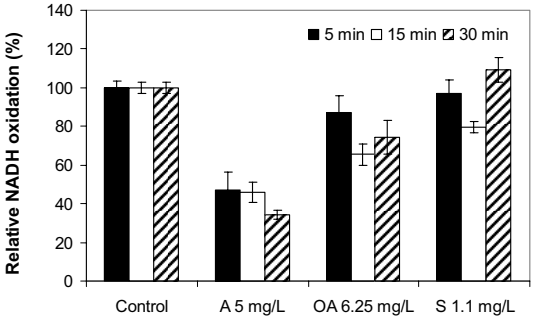

Fig. 12. NADH oxidation by bioluminescence complex *in vitro* after exposure to Aroclor 1248, oleic acid and sulfur. Results are expressed as mean of three separate experiments ± standard deviation.

S_8^0 (1.1 mg/L) did not inhibit NADH oxidation after 5 min exposure and after 30 min. enhanced oxidation by up to 9% in comparison with control (Fig. 12). However, the same S_8^0 (1.1 mg/L) concentration inhibited bioluminescence *in vitro* by up to 21 and 67% after 5 and 30 min, respectively (Table 1). These observations support the deduction that S_8^0 acted more effectively on luciferase as its inhibitor, rather than in NADH:FMN oxidoreductase.

Hence, the data obtained from inhibition of different *in vivo* and *in vitro* functions of bioluminescence complex indicated that: 1) bioluminescence complex dependent oxygen consumption is sensitive to OA and S_8^0 inhibition, similarly to *n*-decanol (luciferase inhibitor) inhibition; 2) Dec (luciferase substrate) can protect from OA and

S_8^0, but not from A bioluminescence inhibition *in vivo*; and, 3) inhibition of NADH oxidation caused by A is the greatest. All these data collectively give evidence that A contrary to OA and S_8^0 has different site(s) (toxic action) in bioluminescence (i.e. NADH:FMN oxidoreductase-luciferase) complex and such site(s) belong to the NADH:FMN oxidoreductase and not to luciferase.

Effects of agents reacting with the sulfhydryl groups of enzymes on bioluminescence in vivo and in vitro. S_8^0 has been known for some time as an inhibitor of enzymes –SH groups. For example in mitochondria S_8^0 is acting on –SH groups of adenine nucleotide translocase and adenylate kinase (Džėja et al., 1993; Russell et al., 1995). Alternatively, most bacterial luciferases are αβ dimers having in total > 10 cysteinyl residues (*V. harveyi* -14 Cys residues). The most reactive one is on α subunit, e.g. Cys-α106 of *V. harveyi*, which was identified as located at (or near) the aldehyde inhibitor site (Lei et al., 1994). In order to confirm that S_8^0 acts as –SH group inhibitor, the effects of S_8^0 and the –SH group inhibitor N-ethylmaleimide (NEM) on *V. fischeri* bioluminescence *in vivo* and *in vitro* were compared. Additionally, the possible protective action against inhibition of this function provided by the –SH group maintaining agent, 1,4-dithiothreitol (DTT) was analyzed (Fig 13).

S_8^0 (5.5 μg/L) and NEM (31 μg/L) decreased the bioluminescence *in vivo* by up to 15% and 62% after 30 min, respectively (Fig. 13a). DTT (5 mg/L) did not affect the bioluminescence, and, when added in combination with NEM or with S_8^0, afforded *in vivo* protection from the effect of the each –SH groups inhibitor, respectively.

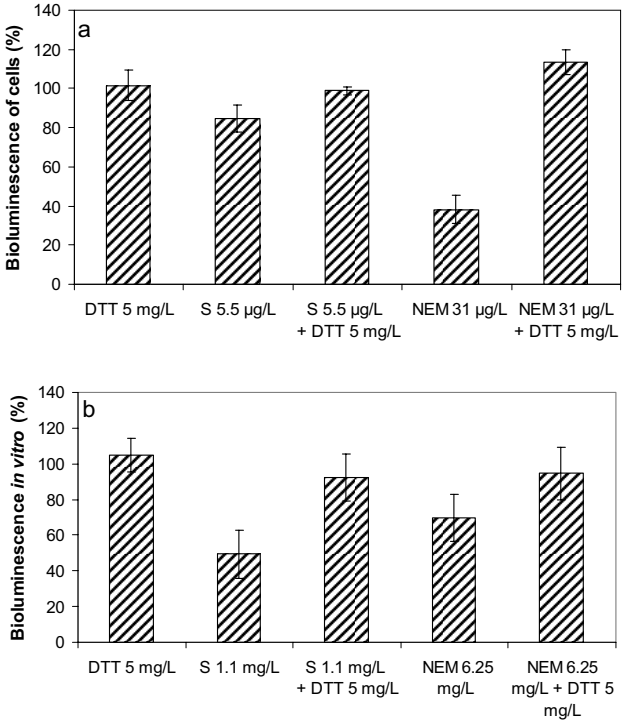

Fig. 13. Bioluminescence of *V. fischeri* cells (a) and bioluminescence complex *in vitro* (b) after 30 min exposure to DTT, S_8^0, NEM, and their mixtures. Results are expressed as a mean of four separate experiments ± standard deviation.

In vitro experimental data indicated that DTT (5 mg/L) protected the bioluminescence function during 30 min from S_8^0 (1.1 mg/L) and from NEM (6.25 mg/L) by up to 40% and 25%, respectively (Fig. 13b). Bearing in mind the molecular mechanism of DTT action (–SH group restoration i.e. disulfide bridge reduction), all *in vivo* and *in vitro* results indicate that DTT has a protective effect on –SH groups, maintaining the redox environment. Additionally, bioluminescence *in vivo* was inhibited by two orders magnitude lower concentrations of S_8^0 and NEM than *in vitro* (bioluminescence complex). These observations showed that S_8^0 similarly to NEM acts more effectively on –SH groups *in vivo*, and this *in vivo* system is more sensitive to S_8^0 inhibition. As bioluminescence intensity *in vivo* is regulated by both luciferase and

respiratory chain enzymes (both containing –SH group in active centers), this explains high sensitivity *in vivo* system to S_8^0.

Thus, it was concluded, that exposure of *V. fischeri* cells to A, OA and S_8^0 in the concentration range of 5.5 μg/L–10.0 mg/L can cause pleiotropic effects depending on the concentration range inside coupling membranes and separate oxidoreductive enzyme chains. The mechanisms (sites) of action are multiple, and the strength of these effects differs, when electron transfer processes are coupled (i.e. regulatory sensitive *in vivo*) or otherwise (*in vitro*). The main toxic effects of A, OA and S_8^0 in *V. fischeri* involve quenching of bioluminescence, enhancement of respiration, generation of ROS, inactivation of bioluminescence complex enzymes. The effects of A, OA and S_8^0 shows that chemical have different sites of action and this confirms the data on multiple toxicodynamic effects on bioluminescence *in vivo* and *in vitro.*

CONCLUSIONS

1. Binary mixtures of Aroclor 1248 (A), oleic acid (OA) and elemental sulfur (S_8^0) caused different toxicodynamic effects on bioluminescence of *Vibrio fischeri* and NADH:FMN oxidoreductase – luciferase *in vitro*. A and S_8^0 acted additively with synergistic interaction on system *in vivo* and additively without significant interaction on system *in vitro*; A and OA acted antagonistically in both systems. OA and S_8^0 caused an additive effect with antagonistic component of interaction on system *in vivo* and had additive effect with synergistic interaction *in vitro*.
2. Toxicity of the individual pollutants *(*A, OA and S_8^0) and their binary mixtures to bioluminescence diminished with increasing exposure time (30, 60 min) in the *in vivo*, but not in the *in vitro* system.
3. Aroclor 1248 and oleic acid, but not sulfur, enhanced ROS generation in *V. fischeri* cells. Contrary, S_8^0 (greater than A and OA) enhanced the reducing ability of *V. fischeri* cells.
4. The principle target site of oleic acid and sulfur in the bioluminescence complex is the active center of the luciferase enzyme.
5. The principle target site of Aroclor 1248 is a NADH:FMN oxidoreductase of the bioluminescence complex.

LIST OF PUBLICATIONS

ARTICLES

Kalcienė V, Četkauskaitė A. 2006. Effects of elemental sulfur and metal sulfides on *Vibrio fischeri* bacteria. *Biologija* 2:42-46. ISSN 1392-0146.

Kalcienė V, Četkauskaitė A. 2007. Environmental and synthetic sulfhydryl group inhibitors: effects on bioluminescence and respiration of *Vibrio fischeri*. *ATLA: Alternatives to Laboratory Animals* 35: 93-100. ISSN: 0261-1929

ABSTRACTS

Kalcienė V, Četkauskaitė A. 2005. Environmental and synthetic –SH groups inhibitors: effects on bioluminescence and respiration of *Vibrio fischeri*. Joint Conference of Estonian Society of Toxicology & Scandinavian Society for Cell Toxicology. Progamme and abstracts. "Chemicals, Human and Environment", October 20-23, 2005. Toila, Estonia.

Kalcienė V, Četkauskaitė A. 2006. Elementinės sieros ir metalų sulfidų poveikis *Vibrio fischeri* bakterijoms. IX-sis suvažiavimas-konferencija "Biochemija:mokslas ir žinių visuomenė", 2006 m. birželio mėn. 16-18 d. Tolieja (Molėtų raj.).

Kalcienė V, Četkauskaitė A. 2006. *Vibrio fischeri* bioliuminescencijos kvėpavimo pokyčiai –SH grupių inhibitorių aplinkoje. Ketvirtoji mokslinė konferencija "Mokslas Gamtos mokslų fakultete", 2006 m. lapkričio mėn. 23-24 d., GMF, VU.

Kalcienė V, Četkauskaitė A. 2008. The toxicity of time-depending exposure to mixtures of AROCLOR 1248, oleic acid and elemental sulphur on *Vibrio fischeri*. SETAC Europe 18th Annual Meeting. Abstract book. Word under stress: scientific and applied issues in environmental toxicology and chemistry", 25-29 May 2008, Warsaw, Poland.

REZIUMĖ

Standartinis *Vibrio fischeri* bioliuminescencijos gesimo bandymas, Microtox® (EN ISO 11348-3:1998) yra naudojamas vertinant cheminių medžiagų ir jų binarinių mišinių ekotoksiškumą. Nors iki šiol atlikta daug mišinių tyrimų, visai mažai nagrinėtas pramoninės ir gamtinės kilmės cheminių medžiagų mišinių toksiškumas, panaudojant šį testą. Antra vertus, cheminių medžiagų mišinių toksiškumo prognozavimui yra svarbios žinios apie jų veikimo būdą *V. fischeri* bakterijose. Tačiau pripažįstamas šių žinių trūkumas. Siekiant apibūdinti ksenobiotikų veikimo mechanizmus, yra vertinga nustatyti jų poveikio vietas *V. fischeri* bakterijų fermentų sistemų (oksidoredukcinių procesų) *in vivo* bei atskirų fermentų (*in vitro*) lygmenyse. Šio darbo tikslas buvo įvertinti pramoninio ksenobiotiko Aroclor 1248 (A) ir gamtinės kilmės medžiagų – elementinės sieros (S_8^0) bei oleino rūgšties (OR) ir jų binarinių mišinių poveikį *Vibrio fischeri* bioliuminescencijai bei šių medžiagų biocheminius veikimo mechanizmus.

Šio darbo pirmame etape daugialypės regresijos metodu buvo įvertintas A, OR ir S_8^0 binarinių mišinių poveikio bioliuminescencijai pobūdis. Šios medžiagos pasižymėjo skirtingais toksikodinaminiais efektais *Vibrio fischeri* ląstelėms ir NADH:FMN oksidoreduktazės-liuciferazės kompleksui *in vitro*: A ir S_8^0 veikė bioliuminescencijos *in vivo* sistemą adityviai su sinergistine sąveika, o sistema *in vitro* buvo veikiama adityviai be reikšmingos sąveikos; A ir OR veikė antagonistiškai abiejose sistemose; OR ir S_8^0 poveikis sistemai *in vivo* buvo adityvus su antagonistinės sąveikos komponente, o sistemai *in vitro* – adityvus su sinergistine sąveika. Individualių taršalų (A, OR ir S_8^0) ir jų binarinių mišinių slopinamasis poveikis mažėjo, ilgėjant poveikio laikui (30, 60 min.) bioliuminescencijos *in vivo*, bet ne *in vitro* sistemoje. Tai leido teigti, kad bioliuminescencijos *in vivo* sistemoje buvo indukuojami apsauginiai fermentai, kurie šalina arba transformuoja šias toksines medžiagas.

Antrame tyrimų etape, buvo įvertintas A, OR ir S_8^0 poveikis oksidoredukcinių fermentų ir citoplazminės membranos būklei. Nustatyta, kad A, OR, bet ne S_8^0 skatino aktyviųjų deguonies formų susidarymą *V. fischeri* ląstelėse. Priešingai, S_8^0 (daugiau nei A ir OR) didino *V. fischeri* ląstelių redukcinę gebą. Tai leido teigti, kad *V. fischeri* ląstelėse yra aktyvios apsauginės oksidoredukcinių fermentų sistemos. Buvo parodyta,

kad A, OR ir S_8^0 skatino *V. fischeri* bakterijų kvėpavimą, tačiau šios medžiagos skirtingai veikė membranos potencialą: A mažino, o OR ir S_8^0 neturėjo reikšmingos įtakos. Todėl manoma, kad individualių junginių veikimo mechanizmai yra daug sudėtingesni.

Trečiajame šio darbo etape buvo ištirtas A, OR ir S_8^0 poveikis *V. fischeri* NADH:FMN oksidoreduktazės-liuciferazės komplekso atskiroms funkcijoms. Nustatyta, kad OR ir S_8^0 slopino deguonies sunaudojimą bioliuminescencijos kompleksu *in vivo* (esant KCN) ir pačią bioliuminescenciją, kurią nuo OR poveikio apgynė *n*-dekanalis, o nuo S_8^0 – 2,4-ditiotreitolis. Tai liudija, kad OR ir S_8^0 poveikio vietos yra aktyviajame liuciferazės centre. Parodyta, kad A poveikis bioliuminescencijai *in vitro* buvo silpnesnis nei NADH oksidacijai *in vitro*. Tai leidžia teigti, kad bioliuminescencijos komplekse A pagrindinė poveikio vieta yra NADH:FMN oksidoreduktazė.

A, OR ir S_8^0 poveikis bioliuminescencijai *in vivo*, kvėpavimui, aktyviųjų deguonies formų susidarymui, redukcinei gebai ir fermentų sistemai *in vitro* patvirtina, kad šios toksinės medžiagos turi daugybines poveikio vietas *in vivo* ir *in vitro* sistemose, ir tai paaiškina jų binarinių mišinių skirtingus toksikodinaminius efektus.

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