Journal of Environmental Science International
24(11); 1541~1546; November 2015

pISSN: 1225-4517 eISSN: 2287-3503
http://dx.doi.org/10.5322/JESI.2015.24.11.1541

ORIGINAL ARTICLE

Binary Mixture Toxicity of AROCLOR 1248, Oleic Acid, and Elemental Sulfur to Vibrio fischeri Luminescence

Virginija Kalciene*, Daiva Dabkeviciene[1)], Anolda Cetkauskaite[1)]

Centre for Ecology and Environmental Research, Faculty of Natural Sciences, Vilnius University, Vilnius LT-03101, Lithuania

[1)]Department of Biochemistry and Molecular Biology, Faculty of Natural Sciences, Vilnius University, Vilnius LT-03101, Lithuania

Abstract

The objective of this research was to evaluate the toxicity of the industial xenobiotic Aroclor 1248 (A) and natural origin substances~elemental sulfur (S80) and oleic acid (OA) and their binary mixtures to V. fischeri bioluminescence during the prolonged exposure time (up to 60 min). The bioluminescence quenching test was used to determine the toxic effects. Full factorial experiment design and multiple regression analysis and the comparison of binary mixture effect with the sum of effects of individual chemicals were used for the evaluation of combined effects of toxicants.

The analysis of general trend of mixture toxicity to bioluminescence showed that mixture toxic effects were reversible up to 60 min. Data analysis revealed different joint effects, which were depended on mixture composition. S80 enhanced toxic effect of A and acted additively with synergistic interaction. Hydrophobic OA in mixture with A acted antagonistically and in mixture with sulfur caused an additive effect with antagonistic component of interaction.

It was concluded that low concentrations of natural toxic substances present in environmental samples as mixtures of chemicals can define the toxicodynamic character of industrial xenobiotics.

Key words : Bioluminescence, Binary mixture toxicity, AROCLOR 1248, Oleic acid, Sulfur

1. Introduction

The standard *Vibrio fischeri* bioluminescence quen -ching test, Microtox® (EN ISO 11348-3:1998) is used in ecotoxicological analyses of single chemicals and their model or environmental 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 as indicated in 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 compiled by Kortenkamp et al.(2009).

The environmental abundance of these three chemicals was the reason for the choice for mixture toxicity analysis. Aroclor 1248 is a mixture of PCBs (polychlorinated biphenyls), which are very bioa -ccumulative and very persistent in the aquatic

Received 27 July, 2015; **Revised** 15 October, 2015;
Accepted 16 October, 2015
***Corresponding author** : Virginija Kalciene, Centre for Ecology and Environmental Research, Faculty of Natural Sciences, Vilnius University, Vilnius LT-03101, Lithuania
Phone: +370-5239-8750
E-mail: virginija.kalciene@gf.vu.lt

environment (Frame et al., 1996; Henry and DeVito, 2003). Oleic acid is one of most abundant free fatty acids in the aquatic environment particularly in sediments and water surface microlayer (Sodergren, 1987; Četkauskaitė, 2004). Free oleic acid and other free fatty acids can originate from many industrial processes including paper manufacture as was described by Morales et al.(1992). The cyclic form of elemental sulfur, S_8^0, is found in the aquatic environment (sediment, pore water) and its toxicity to *V. fischeri* in organic sediment extract was discussed earlier by Svenson et al.(1998).

The goal of this work was 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 during the prolonged expo -sure time (up to 60 min).

2. Materials and Methods

2.1. Materials

All the chemicals used in the assays (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 in ethanol (the final concentration of these solvents in the assays was 1% v/v). All growth and reaction media were prepared in Milli-Q water.

2.2. Methods

2.2.1. Bioluminescence measurements in bacterial cells

A bacterial culture of *V. fischeri* (NRRL B-11177) was prepared, stored and thawed as described earlier by Četkauskaitė et al.(2004). Aluminometer Model 1250 (LKB-Wallac, Sweden) with 1 ml vials was used for luminescence measurements. One ml of the sample consisted of 50 mM KH2PO4 buffer (940 $\mu\ell$), containing 2% NaCl (pH 7.0), 50 $\mu\ell$ of bacterial suspension (OD590 = 0.125) and 10 $\mu\ell$ of chemicals solution or solvent blank. The measurements of bioluminescence were performed after 5, 30, 60 min of exposure to inhibitors or their mixtures, respec -tively, at room temperature. Experiments with binary mixtures of A, OA and S80 were undertaken following a two level full factorial experimental design. Con -centrations 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) S80: 0.027 and 0.055 mg/L. The reference toxicant was 3,5-dichlorophenol.

2.2.2. Data analysis

Statistical analyses of data were performed using t 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.

2.2.3. Sequence of statistical analysis

The significance of chemicals and their interac -tions to overall mixture toxicity was evaluated using two-way ANOVA (for 30 min exposure measurements). Then a reduced multiple regression polynomial models were used to confirm the interaction and to define the character of the combined effect (antagonistic, additive, synergis -tic). Low and high concentrations of chemicals in mixture were coded to the values of -1 and +1, res -pectively, according the calculations used by Ren et al.(2004) in order to avoid multicolinearity using two-way ANOVA and multiple regression procedures.

The experimental data were fitted to the reduced polynomial model:

$$BL(\%) = b_0+b_xC_x+b_yC_y+b_{xy}C_x\cdot\times C_y\pm \varepsilon \quad (1)$$

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 com -ponents 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 $C_x\cdot\times C_y$, 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 ade -quacy of the models was evaluated considering: determination coefficient (R^2),Fischercriteria(*F*) with *P* value.

3. Results and Disccusion

3.1. Trend of binary mixture toxicity

Data on toxicity of binary mixtures of A, OA and S_8^0 to *V. fischeri* bioluminescence at different expo -sure durations are presented in Fig. 1-3. The analysis of general trend of toxicity to bioluminescence 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 up to 60 min.

Hence, it was deduced that inhibition of biolumine -scence caused by the most part of tested concen -trations of A, OA and S80 in mixtures was rever -sible. Similar time-dependent effects of hydrophobic organic chemicals (non-polarnarcotics) on biolumine -scence of *V. fischeri* (*in vivo*) were obtained earlier by Dawson et al.(2006).

3.2. Joint effects of Aroclor 1248, oleic acid and sulfur in binary mixtures

Preliminary two- way ANOVA analysis at fixed different exposure time revealed, that different combi -nations 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 expo -sure time was selected for further analysis of effects of binary mixture on bioluminescence.

Data presented in Fig. 1 demonstrates that toxic effects of binary mixtures of A and S_8^0 increase with the enhancement of the concentration of any mixture component after 30 min exposure. Significant contri

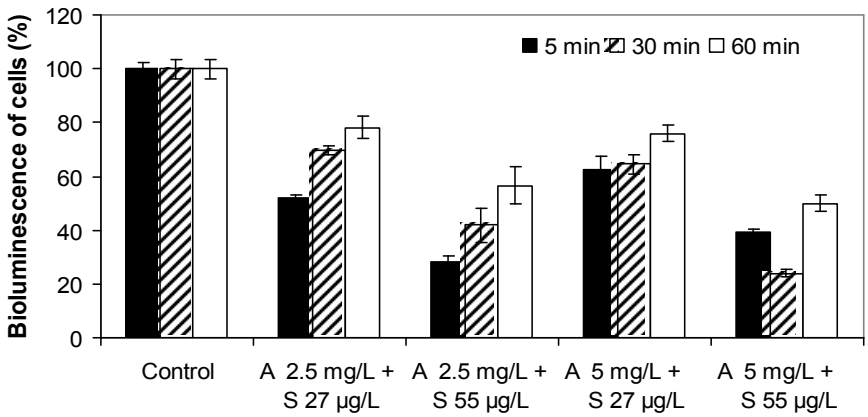

Fig. 1. Effects of Aroclor 1248 and sulfur mixtures to *V. fischeri* bioluminescence after 5, 30 and 60 min exposure. Results are expressed as a mean of three separate experiments ± standard deviation.

-bution to mixture toxicity by A($P < 0.001$) and S ($P < 0.001$) and their interaction ($P < 0.015$) was deter -mined by two-way ANOVA. Multiple regression analysis of binary mixture toxicity indicated the synergistic interaction of A and S (all regression coefficients were negative - Table 1; Eqn. 1). Addi -tionally, this was confirmed by fact that toxic effect of mixture was stronger than the sum of toxic effects of individual mixture components (Fig. 1; Table 2).

Table 1. Best fitted multiple regression equations[a,b], describing Aroclor 1248, oleic acid and elemental sulfur binary mixtures toxicity for *V. fischeri* after 30 min exposure

Aroclor 1248 and sulfur
1) BL (%) = 50.0 − 6.0 C_A − 17.4 C_S − 3.2 $C_A \times C_S$ ± 3.6 R^2=0.98, F = 165.47, P < 0.001
Aroclor 1248 and oleic acid
2) BL (%) = 83.8 − 5.0 C_{OA} ± 3.1 R^2=0.76, F = 31.15, P < 0.001
Oleic acidand sulfur
3) BL (%) = 41.3 − 18.8 C_{OA} − 8.2 C_S + 7.3 $C_{OA} \times C_S$ ± 4.3 R^2=0.98, F = 98.40, P < 0.001

[a] - descriptions of equation variables q.v. Materials and Methods;
[b] - where R^2(determinationcoefficient), F and P represents ade -quacy and statistical significance of regression equations;

Table 2. Effects of AROCLOR 1248, oleic acid and elemental sulfur to the bioluminescence of *V. fischeri* cells after 30 min exposure

Concentration, mg/L	*Bioluminescence[a],% (relatedtocontrol)*
AROCLOR 1248	
2.5[b]	84.73±1.70*
5[b]	78.00±1.00*
Oleic acid	
0.024	73.99±1.76*
0.195[b]	60.33±1.53*
Sulfur	
0.027[b]	86.10±4.28
0.055[b]	51.53±1.81*

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

Mixtures of A and OA caused lower toxicity on bioluminescence (Fig. 2) than mixtures of A and S_8^0 (Fig. 1). 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 indicated that the toxicity of binary mixture was defined only by changing concentrations of OA (Table 1; Eqn. 2). Hence, the effect of A in mixture with OA on bioluminescence was reduced, however the same concentrations of A in mixture with S_8^0 were signi -ficant determinant of bioluminescence inhibition. Additionally, inhibition caused by OA (195$\mu\ell$/L) individually (by up to 40%; Table 2) was higher than inhibition caused by mixture (by up to 23%) of high concentrations of A (5 mg/L) and OA (195 $\mu\ell$/L) after 30 min (Fig. 2). The reduction of OA toxic effect (inhibition) in mixture to bioluminescence 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.

Results presented in Fig. 3 showed that the alter -native increase in OA or S concentration in mixture caused a significant enhancement of mixture toxicity after 30 min. However, the increase in both toxicants concentrations together did not enhanced mixture toxicity significantly. The results of two-way ANO -VA showed significant determination of mixture toxicity by OA, $S_8^0$and the irinteraction($OA \times S_8^0$)($P <$ 0.001 in all cases). Multiple regression equation describing toxicity of OA and S mixture (Table 1; Eqn. 3) indicated additive toxic effect with component of antagonistic interaction. The component of anta -gonistic interaction was confirmed by fact that combined effect of high concentrations of OA and S (Fig. 3) was less than the sum of their individual effects (Table 2).

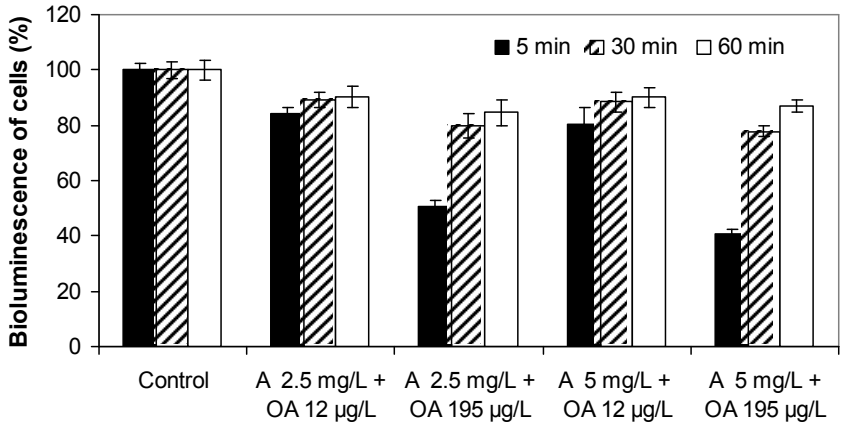

Fig. 2. Effects of Aroclor 1248 and oleic acid mixtures to *V. fischeri* bioluminescence after 5, 30 and 60 min exposure. Results are expressed as a mean of three separate experiments ± standard deviation.

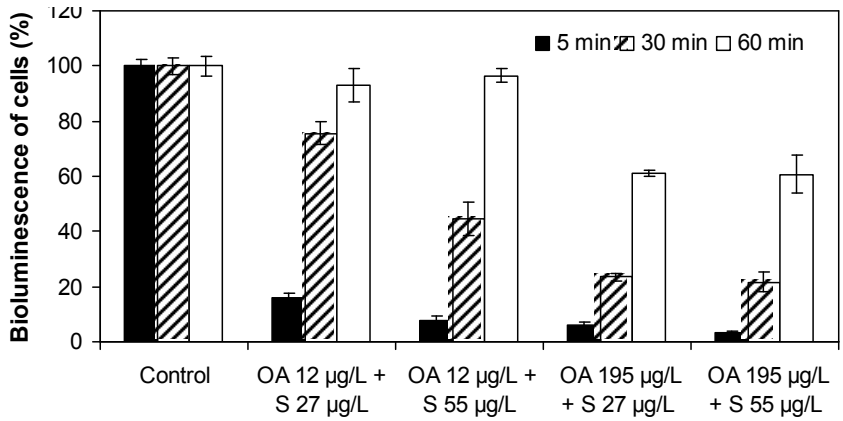

Fig. 3. Effects of oleic acid and sulfur mixtures to *V. fischeri* bioluminescence after 5, 30 and 60 min exposure. Results are expressed as a mean of three separate experiments ± standard deviation.

A, OA and S_8^0 are hydrophobic substances, whichh have the potential to bioaccumulate and cause the toxic effect on biological membranes (Svenson, 1998; Pardos, 1999; Cetkauskaite et al., 2004). Due to their hydrophobic properties all these substances are found in polluted sediments. The concentrations of natural toxicants (OA and S_8^0), which are found in sediments are at least ten times higher than concentrations of PCBs (µg/kg dry wt.) (Sun et al., 1997; Scrimshaw et al., 1994; Jonsson, 2000; Smith and Klug, 1981; Cetkauskaite, 2004). Hence, it is realistic, that natural toxicants can influence results of sediment toxicity tests, especially using *V. fischeri* assay.

4. Conclusions

The present study demonstrated that environmen -tally relevant, low (µg/L) concentrations of natural hydrophobic and toxic substances, i.e. S_8^0 and OA, which can be found in sediments, were more toxic than A individually, and they increased toxicity of Acontaining binary mixtures to *V. fischeri* biolumin -escence *in vivo*. It is concluded that low concen -trations of natural toxic substances present in environ-

mental samples as mixtures of chemicals can define the toxicodynamic character of industrial xenobiotics.

Acknowledgement

The authors express their thanks to Mervyn Richar -dson, the UN Consultant, Office Birch Assessment Servises for Information on Chemicals, Crawley, GBR, for the valuable remarks and recommendations.

Paper is a part of Virginija Kalciene thesis "Toxicity of environmental pollutants Aroclor 1248, oleic acid and sulfur to *Vibrio fischeri* and biochemical mecha -nisms".

REFERENCE

Cetkauskaite, A., Pessala, P., Sodergren, A., 2004, Elemen -tal sulfur: toxicity *in vivo* and in vitro to bacterial luciferase, in vitro yeast alcohol dehydrogenase, and bovine liver catalase, Environ., Toxicol., 19, 372-386.

Cetkauskaitė, A., Pessala, P., Sodergren, A., 2004, Elemen -tal sulfur: toxicity *in vivo* and *in vitro* to bacterial luciferase, *in vitro* yeast alcohol dehydrogenase, and bovine liver catalase, Environ., Toxicol., 19, 372-386.

Cetkauskaitė, A., 2004, Oleic acid: detection in environ -mental samples, modes of toxic action, Biologija, 1, 13-16.

Dawson, D. A., Poch ,G., Schultz, T. W., 2006, Chemical mixture toxicity testing with *Vibrio fischeri*: Com -bined effects of binary mixtures for ten soft electro -philes, Ecotoxicol., Environm., Saf., 65, 171-180.

EN ISO 11348-3:1998, Water quality-Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)-Part 3: Method using freeze-dried bacteria.

Frame, G. M., Cochran, J. W., Bowadt, S. S., 1996, Complete PCB congener distribution for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, quantitative congener specific analysis, J., High, Resol., Chromatogr., 19, 657-668.

Henry, T. R., DeVito, M. J., 2003, Non-dioxin-like PCBs: effects and consideration in ecological risk assessment. ERASC-003, NCEA-C-1340, US: Environment Protec -tion Agency.

Jonsson, P., 2000, Sediment Burial of PCBs in the Off -shore Baltic Sea, Ambio, 29, 260-267.

Kortenkamp, A., Backhaus, T., Faust, M., 2009, State of the art report on mixture toxicity - Final Report, Executive Summary, Contract No. 070307/2007/485 103/ETU/D.1, http://ec.europa.eu/ environment/chemi cals/pdf/report_Mixture%20toxicity. pdf.

Morales, A., Birkholz, D., Hrudey, S. E., 1992, Analysis of pulp mill effluent contaminants in water, sediment, and fish bile - fatty and resin acids, Water, Environ., Res., 64, 660-668.

Pardos, M., Benninghoff, C., Thomas, R. L., Khim-Heang, S., 1999, Confirmation of elemental sulfur toxicity in the Microtox assay during organic extracts assessment of freshwater sediments, Environ., Toxicol., Chem., 18, 188-193.

Ren, S., Mee, R. W., Frymier, P. D., 2004, Using factorial experiments to study the toxicity of metal mixtures. Ecotoxicol., Environ., Saf., 59, 38-43.

Scrimshaw, M. D., Bubb, J. M., Lester, J. N., 1994, Magnitude and distribution of contaminants in salt marsh sediments of the Essex coast, IV, Organo -chlorine insecticides and polychlorinated biphenyls, Sci., Tot., Environ., 155, 73-81.

Smith, R. L., Klug, M. J., 1981, Reduction of sulfur compounds in the sediments of a eutrophic lake basin. Appl., Environ., Microbiol., 41, 1230-1237.

Sodergren, A., 1987, Origin and composition of surface slicks in lakes of differing trophic status, Limnol., Oceanogr., 32, 1307-1316.

Sun, M. Y., Wakeham, S. G., Lee, C., 1997, Rates and mechanisms of fatty acid degradation in oxic and anoxic coastal marine sediments of Long Island Sound, New York, USA. Geochim., Cosmochim., Acta, 61, 341-355.

Svenson, A., Viktor, T., Remberger, M., 1998, Toxicity of elemental sulphur in sediments, Environ., Toxicol., Water, Qual., 13, 217-224.

U.S. Environmental Protection Agency, 2005, Technologies and techniques for Early Warning Systems to monitor and evaluate drinking water quality, A State-of-the-art review, Research report, EPA/600/R-05/156., Office of Research and development, National Homeland Security Research Center.