BIOLOGIJA. 2004. Nr. 4. P. 36–39

Effects of mixtures of oleic acid with chlorinated herbicides on *Vibrio fischeri* bacteria

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Abbreviations: D, diuron; EC_{50}, effective concentration,causing the inhibition of function fifty per cent; EDTA, ethylenediamine tetraacetic acid; FAs, fatty acids; M, monuron; OA, oleic acid; OP, organochlorine pollutants; POM, particular organic matter

The toxicity of chlorinated phenylurea herbicides and their mixtures with monounsaturated oleic (fatty) acid, OA, to bioluminescence of *Vibrio fischeri* was investigated. The EC_{50} of the inhibition of bioluminescence by mono- and di-chlorinated phenylureas such as monuron (M) and diuron (D) was found to be 13.5 and 1.05 ppm at 30 min. of exposure, respectively. The monuron at a concentration of 8 ppm inhibited bioluminescence by 40–43%, while diuron (0.6 ppm) decreased bioluminescence by 18% after 15 min of exposure. The toxicity of M and D at these concentrations was enhanced to 90% and 80%, respectively, by OA (up to 190 ppb) during the same exposure time. Different effects of phenylureas and OA were observed during a prolonged time of exposure up to 2 hours: (*a*) an increase in inhibition of bioluminescence during exposure to D and M at concentrations exceeding 0.8 and 10 ppm, respectively, and (*b*) slow recovery of bioluminescence after highest inhibition at 5–15 min of exposure to OA. Combined effects of OA and phenylurea herbicides depended on the time of exposure: an additive effect was observed after 5–15 min and synergistic after 1 h. The data show that monounsaturated fatty acid, which is usually used in formulations of technical pesticide preparations or is formed during (bio)degradation of the adjuvants to active ingredients (herbicides) can enhance the toxicity of herbicides to non-target species.

Key words: *Vibrio fischeri*, bioluminescence, toxicity, oleic acid, mixtures, monuron, diuron

INTRODUCTION

The accumulation, fate and toxicity of organochlorine pollutants, OP, in surface waters are mostly associated with a particular organic matter, POM, and sediment fractions of biogenic origin [1]. Fatty acids, FAs, and lipids in a water column of natural water bodies are mostly associated with POM rather than with very high and high molecular weight dissolved organic matter [2, 3]. The POM, for example, contains up to 50–140 μg/l (or μg/mg of organic carbon) of total FAs, and monounsaturated FAs can form from 0.1 to 0.3 of this amount [2]. Whereas, the amount of free FAs in sediments can reach 5%, and was found to be <500 μg/g (mg/kg or ppm) of dry sediments [4]. Lipids, FAs, their salts (like sodium oleate), esters (like triolein), phospholipids, glycolipids and other hydrophobic constituents or pollutants in POM and sediment fractions are bioavailable to ingestion by higher aquatic organisms [1, 5, 6]. These compounds are susceptible to different hydrolysis, oxidative transformation reactions catalyzed by microorganisms in sediments [5] and even in the intestine of biota [6]. The other important aspect of the toxicological risk to non-target terrestrial and freshwater species of such mixtures of OPs with FAs or their easily degradable derivatives is the application in agriculture of large quantities of technical herbicide preparations containing FAs or their esters as pesticides adjuvants [7]. The adjuvants are used for dissolution of hydrophobic chemicals in water environment; the characteristic examples used were Hilo Dry Clean Foam, Deo San and other surfactant-type technical mixtures containing from 4.48 up to 8% of oleic acid. Spray Fuse 90 (Cornbelt Chemical Co.) is still in use, and it is the 90% non-ionic spreader, activator used with insecticides, herbicides, acaricides consisting of alkylaryl polyethoxylene glycols + free fatty acids + isopropanol [7, 8]. Finally, data are available that marine phytoplankton and animal species undergo toxic exposure during oil spills and increase in toxicity during the use of contemporary, type II and III, and third generation dispersants, such as

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Tergo R 40, some Corexits (likely numbers 9550, 9527, 7664) [9, 10]. Oil dispersants usually contain 15–75% of nonionic surfactants, such as relatively biodegradable (up to fatty acids and respective alcohols) sorbitan monooleate, ethoxylated sorbitan monooleate, polyethylene glycol esters of unsaturated fatty acids [9]. Consequently, the accumulation and co-existence of organic pollutants together with lipids and free FAs in the POM or sediments are obvious and frequent, however, changes in the toxicity of OP in the presence of natural concentrations (up to hundreds ppb) of free FAs is not well documented. **Thus, this work presents analysis of toxicity of chlorinated (phenylurea) herbicides to bioluminescent bacteria *Vibrio fischeri*, as well as toxicity of mixtures of low concentrations of oleic (fatty) acid with phenylurea herbicides containing different levels of chlorine substituents.**

MATERIALS AND METHODS

Reagents. Acetone and acetonitrile from Merck; ethanol (redistilled); NaCl, KCl, $MgCl_2$, KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2HPO_4$, and glycerol from Reachim; EDTA and peptone from Serva; 3,5-dichlorophenol from Aldrich Chem.; oleic acid from Merck; monuron, diuron – purity of gass chromatography (analytical reference standard grade), Research Triangle Park, US EPA.

Liquid medium for growth of bacteria: Beneckea harvey broth (BHB) was used as described [11]. **Reaction medium** (RM): 50 mM of potassium phosphate and 2.5% of sodium chloride, pH 7.3. **The evaluation of toxicity to the bioluminescence of *Vibrio fischeri*** was performed as described earlier [12–14]. The EC_{50}, Effective Concentration causing 50 per cent bioluminescence inhibition, was calculated using the linear regression method from the data of three independent inhibition experiments.

RESULTS AND DISCUSSION

Toxicity of Phenylurea Herbicides to* Vibrio fischeri *bioluminescence. The effects of different concentrations of the phenylurea herbicides monuron and diuron on bioluminescence of photobacteria were measured at a standard time of exposure (15 and 30 min) proposed for Microtox [7] and during a time period up to 2 h in order to obtain a clear pattern of inhibition kinetics (Figs. 1 and 2). The effective concentrations of monuron causing 40–60% inhibition of bioluminescence were found in concentration range over 10 ppm: EC_{50} = 13.02 ppm and 13.5 ppm for 15 min and 30 min of exposure, respectively (data calculation not shown). The effective concentrations of diuron causing a 40–60% inhibition of bioluminescence were found in the concentration range over 1.0 ppm: EC_{50} = 1.15 ppm and 1.05 ppm for 15 min and 30 min of exposure, respectively. These data on a higher toxicity of diuron *versus* monuron are similar to the data on bioluminescence quenching toxicity of diuron and monuron presented in old *Vibrio fischeri* (former *Photobacterium phosphoreum*) Toxicity Database (EC_{50} = 16.5 ppm for D and at EC_{50} = 228 ppm for M at 5 min of exposure) [15, 16]. It should be noted that data presented in this database are not fully reliable, because they were collected from earlier works when standard biotesting procedures and protocols on growth and bioluminescence inhibition measurements had not been prepared [15]. However, it should be useful to compare data obtained on chlorinated herbicides with oleic acid toxicity data. Oleic acid caused a 40–60% inhibition of bioluminescence at a concentration range of a tenth or hundredth of ppb's interval (15 min EC_{50} = 37 ppb; 30 min EC_{50} = 110 ppb) [14]. This comparison of our data (EC_{50} at 30 min of exposure) shows that OA is at least 10 and 100 times more toxic than diuron and monuron, respectively.

Combined Iinhibitory effects of oleic acid and phenylurea herbicides. A combined action of oleic acid with mo-

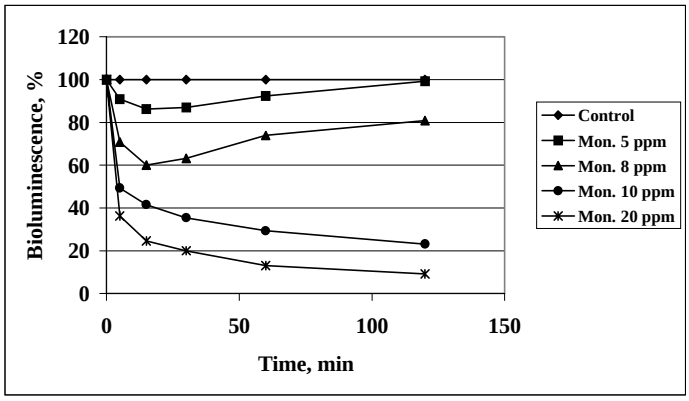

Fig. 1. Inhibition of *Vibrio fischeri* bioluminescence by different concentrations of monuron.

Reaction medium (RM) as described in "Materials and methods". RM volume 1 ml; Control – acetonitrile 1%

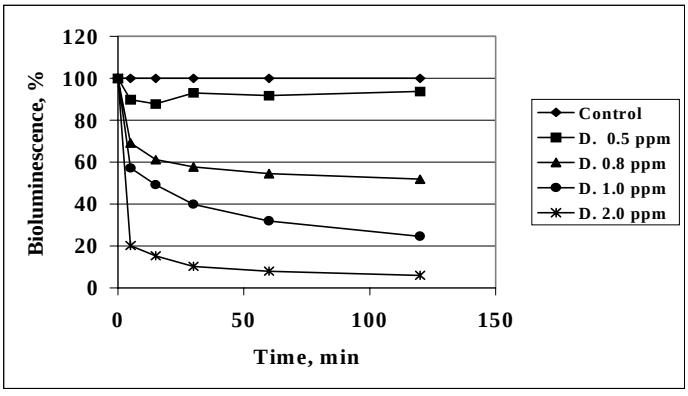

Fig. 2. Inhibition of *Vibrio fischeri* bioluminescence by different concentrations of diuron.

Reaction medium and control as in Fig. 1

nuron and diuron on bioluminescence of *V. fischeri* cells was tested in a low concentration range using an exposure time up to two hours (Figs. 3 and 4). Oleic acid alone (190 ppb, at 5 and 15 min of exposure) inhibited the bioluminescence up to 71% and 65%, respectively; this was followed by a subsequent restoration of bioluminescence up to 83% of control (17% of inhibition) after two hours. Monuron (8 ppm) and diuron (0.6 ppm) caused a similar bioluminescence inhibition (up to 46% and 18%, respectively, during 5 min of exposure) with its subsequent slow restoration during 2h up to 98% of control. These changes of toxic effects of oleic acid (primary inhibition and further restoration of bioluminescence) was described earlier as characteristic effects at lower oleic acid concentrations [14]. It could be explained partially by the known competition of oleic acid at the substrate decanal (hydrophobic aldehyde) binding side of the luciferase enzyme [14]. The recovery of bioluminescence at low concentrations of oleic acid could be caused by a higher affinity of decanal than that of oleic acid during formation of the substrate–enzyme complex. Equally this could be a consequence of a possible oxidative transformation [17–20] of oleic acid inside the luciferase enzyme with a subsequent dissociation of a less hydrophobic product from the affinity site. We have reviewed earlier [14] that the toxicity of oleic acid is observed in various bioobjects at the following concentrations: 1) 50% uncoupling of oxidative phosphorylation in eukaryotic mitochondria at 5.64 ppm (20 mM) [21]; 2) 60% ATP leakage in Ehrlich ascites tumour cells at 25–50 ppm [22]; 3) an 18% inhibition of a whole cell Na^+ current through the inhibition of the acetylcholine receptor in ovocytes at 20 mM, *i.e.* 5.64 ppm [23]; 4) a 50% inhibition of hormone-sensitive lipase at 500 nM and to other 15 enzymes at 500 nM – 750 mM concentrations [24].

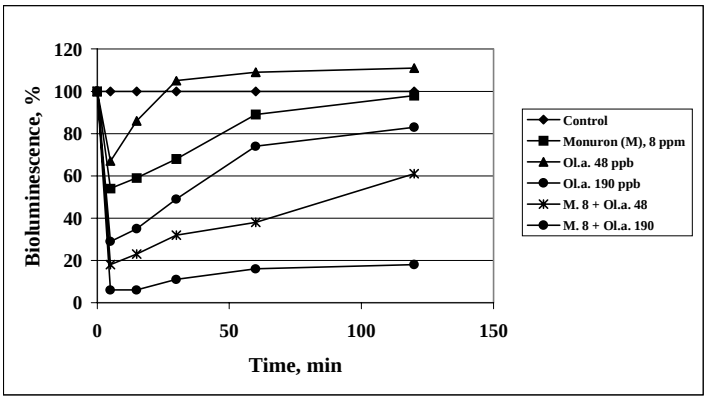

Fig. 3. Enhancement of bioluminescence inhibition in *Vibrio fischeri* caused by oleic acid and monuron.

Reaction medium as in Fig. 1. RM volume 980 ml. Control – acetonitrile 2%

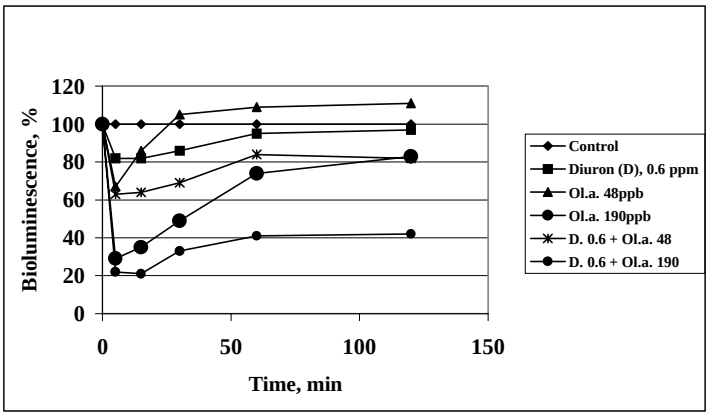

Fig. 4. Enhancement of bioluminescence inhibition in *Vibrio fischeri* caused by oleic acid and diuron.

Reaction medium, its volume and control as in Fig. 3

Mixtures of low concentrations of oleic acid with monuron and oleic acid with diuron had a higher inhibitory effect than the chlorinated phenylureas or oleic acid alone. The general character of bioluminescence inhibition kinetics of a mixture (a rapid increase at 5–15 min of exposure and a slow restoration up to 2 h) was the same as for oleic acid and chlorinated phenylureas. The excess amount of oleic acid only in hundreds ppb in the mixture helped to maintain the toxicity level of the mixture higher than EC_{50} for 2 h though the general toxic effect of different tested concentrations of phenylurea herbicides and oleic acid decreased with time. For example, for monuron (8 ppm) at 5 min of exposure only 8.8 ppb of oleic acid was needed to reach the EC_{50} of a mixture, but after 15, 30 or 120 min of exposure this OA concentration increased from 16.7 ppb to 31 ppb or 121 ppb, respectively. This example shows that during the exposure time far exceeding standard test requirements, oleic acid at a concentration of up to 200 ppb is capable of ensuring the toxic effect of mixture at least up to the value of EC_{50}. Thus, the increasing content of oleic acid in a mixture enhanced the inhibitory effect of monuron and diuron, and the character of bioluminescence inhibition caused by the mixture changed during the exposure time from additive after 15 min to synergistic after 1 h.

It is worth noting that during exposure the character of inhibition caused by this mixture containing constant monuron (8 ppm) or diuron (0.6 ppm) concentrations changed significantly: 1) at 15 min the character of inhibition was less than additive (for diuron) or additive (for monuron) at 48–190 ppb concentrations of oleic acid, and 2) after 60 and 120 min of exposure the character of inhibition was synergistic. The published experimental data on a combined action of oleic acid with hydrophobic organic pollutants are scarce: it has been shown that oleic acid at a concentration of 14.2 ppm (50 mM) enhances DNA damage in the Sister Chromatide Exchange test in the presence of genotoxins [25] and increases the toxicity of the standard reference toxicant 3,5-dichlorophenol [14]. So, data pre-

sented here on gram-negative bioluminescent *Vibrio fischeri* cells confirm the enhancement by the natural hydrophobic polar compound, oleic acid, of its inhibitory action in a mixture with chlorinated herbicides.

CONCLUSIONS

1. Oleic acid was much more toxic to bioluminescence of *Vibrio fischeri* than chlorinated phenylurea herbicides. Oleic acid caused a 14–65% inhibition of bioluminescence at a 48 and 190 ppb concentration range during 15 min of exposure, while efective concentrations of monuron and diuron were in the ppm range (15 min EC_{50} = 13 ppm for monuron and 1.15 ppm for diuron).

2. A two-phase inhibition kinetics for bioluminescence was observed in *Vibrio fischeri* cells during an acute exposure time up to 2 h to a mixture of oleic acid and monuron (or oleic acid and diuron) at the concentration ranges tested. It was an increase in the inhibition of bioluminescence during exposure up to 5 (15) min and a slow recovery of bioluminescence in the case of oleic acid.

3. Oleic acid slowly enhanced the inhibitory effects of chlorinated phenylureas, as the combined effects of oleic acid and monuron or diuron depended on the time of exposure: the additive effect was observed after 15 min and the synergistic after 1 and 2 hours.

Received 3 February 2004
Accepted 25 November 2004

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OLEINO RÛGÐTIES MIÐINIØ SU CHLOROORGANINIAIS HERBICIDAIS POVEIKIS BAKTERIJOMS *VIBRIO FISCHERI*

Santrauka

Darbe tirta chlorintø fenilkarbamidiniø herbicidø ir jø miðiniø su monosoèiàja oleino (riebalø) rûgðtimi (OR) toksiðkumas *Vibrio fischeri* làsteliø bioliuminescencijai. Mono- ir dichlorintø fenilkarbamidø efektyvioji bioliuminescencijos inhibicijos koncentracija (EC_{50}) per 30 min. buvo: 13,5 ppm monurono (M) ir 1,05 ppm diurono (D). Išlaikius 15 min. monurono 8 ppm koncentracija inhibavo bioliuminescencijà 40–43%, tuo tarpu diuronas (0,6 ppm) jà sumaþino iki 18%. Oleino rûgðtis (iki 190 ppb) padidino ðiø monurono ir diurono koncentracijø toksiðkumà 90% ir 80% atitinkamai per tà patá laikotarpá. Skirtingi oleino rûgðties ir fenilkarbamidø efektai buvo stebëti veikiant *V. fischeri* làsteles 2 val.: a) bioliuminescencijos inhibicijos didëjimas, veikiant 0,8 ir 10 ppm diurono ir monurono koncentracijomis, ir b) lëtas bioliuminescencijos atsistatymas po didþiausios inhibicijos oleino rûgðtimi 5–15 minuèiø. Oleino rûgðties ir fenilkarbamidiniø herbicidø miðinio bendri efektai bioliuminescencijai priklausë nuo bandymo trukmës: adityvûs efektai stebëti po 5–15 min. ir sinerginiai – po 1 val. Gauti duomenys rodo, kad toks miðinio komponentas, kaip laisvoji, nesoèioji riebalø rûgðtis, kuri paprastai naudojama techniniuose pesticidø preparatuose arba susidaro juose dël veikliosios medþiagos (herbicido) priedø biodegradacijos, gali padidinti herbicidø toksiðkumà organizmø rûðims, nesanèioms tiesioginiais jø taikiniais.