SCREENING OF VARIOUS HARMFUL COMPOUNDS IN A NEW BACTERIAL BIOLOGICAL MODEL

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Abstract. The rapidly increasing population corroborated with a growing industrialisation has resulted in the bioaccumulation in the environment of a large variety of chemical compounds. More than fifty thousand chemicals, most of which are xenobiotics, are in common use. Furthermore, new chemicals are continually and regularly introduced to the environment raising the concern about their bioaccumulation as well as the harmful effect of xenobiotics as such or as metabolite products. There are a few pollution treatment methods such as land-filling, recycling, pyrolysis and incineration, but unfortunately they can lead to the formation of toxic intermediates. In addition, these methods are expensive and sometimes more difficult to execute, especially in extensive agricultural sites where the use of pesticides is wide-spread. The harmful effect of chemical compounds on organisms living in a polluted area is assessed by acute and chronic toxicity tests performed on various biological models including fish, crustaceans, algae, etc. More recently, based on microorganism long-term ability to adapt to environments, they became an useful tool to assess the toxicity of harmful compounds released in the environment. In this study, we used a bacterial model as biosensors for detecting the harmful effect of pesticides as well as decontaminants of pesticide-infested environment. The gram-positive and gram-negative bacteria proved to be a simple, rapid and inexpensive tool to determine the ecotoxicity of Reldan EC40 pesticide.

Keywords: pollutants, toxic effect, bacteria, biological model.

AIMS AND BACKGROUND

The continuous growth of human population, as well as the increased industrialisation triggered a major ecological impact[1] by pollution, erosion, deforestation and climatic changes[2]. In general, the agricultural development is based on the technological progress and/or use of pesticides to eliminate or keep under control the pests such as insects, weeds, rodents, fungi or mold harmful to humans and to economy[3].

The pesticide amount used in the agriculture increased tremendously from 0.49 kg pesticide/ha in 1960 to 2 kg pesticide/ha in 2004 (environmental report, http://ec.europa.eu).

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The organophosphorus pesticides are the major pesticide group, accounting for more than 36% of the total world market[4]. The organophosphates possess an efficient insecticide activity, due to its characteristic of irreversibly inhibiting the enzyme acetylcholinesterase in the nervous system, which acts in both insects and in mammal. Unfortunately, the increased resistance of the pests to pesticides induced an 'arm race' between surviving the toxic effect of pesticides and the production of more structural diverse and aggressive chemical compounds. Furthermore, pesticides bioconcentrate because they are very persistent in the environment, thus representing a long-term danger as they biomagnify up the food chain and subsequently increasing the symptoms of acute and/or chronic toxicity to humans.

At the present, the evaluation of the toxic potential of pesticides to the environment is carried out on biological models belonging to all food chain levels from bacteria to fish. A particular emphasis has been put on bacterial microbiotests as an alternative method to vertebrate and invertebrate models used to detect the toxic effect of pesticides on the environment and its living organisms. The use of bacteria in the process of biodegradation of the pesticides is becoming an alternative treatment strategy that is effective, economical and environment friendly[5]. The biochemical and genetic basis of microbial degradation was linked to several genes/enzymes providing microorganism with the ability to degradate organopesticides[6,7].

In this study, we tested the toxicity of an organophosphorus pesticide, Reldan 40EC, using a bacterial microbiotest based on 4 types of gram-negative and gram-positive bacteria. In addition, we analysed the mechanisms by which bacteria adapts to the new environmental changes such as pesticide treatments.

EXPERIMENTAL

Reagents. Reldan 40EC (400 g/l chlorpyrifos-methyl) was a generous gift from ISTIS (Bucharest, Romania). HPLC grade dichloromethane was acquired from Sigma-Aldrich (Steinheim, Germany). Water for chromatography (18.3 Ω cm minimum resistivity and maximum 5 ppb TOC) was obtained within the laboratory by means of a MilliQ instrument. High purity gases (He, N_2, synthetic air, H_2) were obtained from Linde Gas Romania.

Growth medium. Lauryl sulphate broth and azide broth were purchased from National Research and Development Cantacuzino (Bucharest, Romania). Acetamide nutrient broth from Sanimed International Company.

Bacteria. Gram-positive and gram-negative[8] bacteria were purchased from ATCC: *Salmonella* sp. (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 29212).

Growth inhibition assay. Every bacterial strain was initially grown on an agar plate O/N at 37˚C, then a single colony was transferred and incubated at 37˚C in 5 ml

medium (specific for each bacterial strain) treated or not with various concentrations of chlorpyrifos-methyl. Bacterial growing rate was monitored by spectrometry at an absorbance of 600 nm (A_{600}).

The effect of various concentrations of pesticide (from 0.04 to 4 g/l) was quantified in function of bacterial growing rate compared to control samples, no pesticide treatment. Each microbiological step had positive and negative control to ensure quality outcomes and efficiency of working methods.

Physicochemical analysis of the pesticide. Detection of chlorpyrifos-methyl was performed using an Agilent 7890A series GC system (Agilent, Waldbronn, Germany) containing a split-splitless injector and Flame Photometric Detector (FPD) with phosphorus filter. Data acquisition and analysis were performed using EZChrom software version 3.3.2 SP2 (Build 3.3.2 1031).

All chromatographic runs were carried out on a Zebron™ ZB5-MSi (60 m × 0.25 mm d.i., 0.25 µm film thickness) column from Phenomenex. GC inlet was maintained at 260°C and operated in Pulsed-Splitless mode with a pressure surge of 40 psi for 0.8 min. It (5.0) was used as carrier gas at a constant flow-rate of 1 ml/min. Detector flame gases flow-rates were 75 ml/min (H_2) and 100 ml/min (synthetic air), whereas make-up gas was N_2 at 60 ml/min. Detector acquisition data rate was set to 5 Hz.

After incubation, samples were extracted using dichloromethane (DCM). 100 µl sample accurately measured with an Eppendorf micropipette (20–200 µl) were transferred to an Eppendorf tube (1.5 ml). 1 ml DCM was added and the samples were thoroughly vortexed for 5 min at 2000 RPM. After phase separation (5 min), a 50-µl aliquot of the organic extract was transferred to a GC injection vial and then diluted with 950 µl DCM. The extracted sample results in a theoretical concentration of 2 µg/ml chlorpyrifos-methyl. Blank samples were subjected to the same liquid-liquid extraction procedure to ensure lack of interferences. Chlopyrifos-methyl quantitation was done by external standard calibration using the commercial product Reldan 40EC (400 g/l Chlorpyrifos-methyl).

Determination of biochemical oxygen demand after 5 days (BOD_5). Briefly, 0.4 g/l pesticide (recommended concentration for agricultural/farming field applications) were incubated at 20 ± 1°C, in the dark for 5 days in the presence of distilled water (control) and seeded dilution water enriched with phosphate buffer (pH 7.2), magnesium sulphate heptahydrate solution, calcium chloride solution and ferric chloride hexahydrate solution. The seed used in this assay was a settled effluent collected from a waste water treatment plant. A dilution water blank was determined in a parallel assay containing no test pesticide. The BOD is determined by the difference in dissolved oxygen (DO) content at the beginning (starting point of the assay) and at the end of the test (after 5 days of incubation). The DO was quantified by an oxygen-electrochemical meter.

RESULTS AND DISCUSSION

Pesticide biodegradability. Reldan 40EC is an organophosphorus insecticide with a large spectrum of action, used to eliminate pests from areas designated for agriculture and farms culturing, where chlorpyrifos-methyl is the active compound from Reldan EC40 composition.

BOD determination is the principle test to give an idea of the aerobic biodegradability of any sample or chemical solution. Reldan EC40 biodegradability which was quantified by measuring BOD_5 increased more than 2 folds, pointing to a bacterial accelerated pesticide degradation compared to control sample (pesticide without bacterial incubation) (Fig. 1).
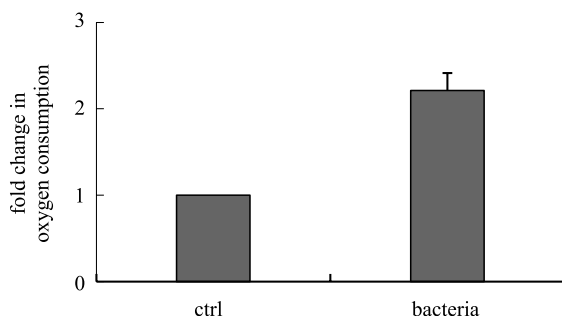

Fig. 1. Biochemical oxygen demand (BOD_5)

Chlorpyrifos-methyl was incubated for 5 days in the absence (ctrl) or presence of aerobic microorganism (bacteria) and DO content was measured. The chlorpyrifos-methyl biodegradability rate due to bacterial activity was quantified by BOD_5 fold change between bacterial treated and untreated assay. All studies represent one of at least two independent experiments.

Organophosphorus pesticide modulates the bacterial growth. In order to establish the effect of pesticide on the bacterial growth, we initially analysed the optimum growing conditions for each bacterial strain used in this study (Fig. 2). All four bacterial strains tested have a significant detectable growth rate after 4 h of monitoring; *E. coli* have the highest growth rate and *P. aeruginosa* the slowest one. *S. enterica* and *E. faecalis* have similar growing rates which are between *E. coli* and *P. aeruginosa*. Based on these results we decided to use 0.2 absorbance at 600 nm as bacterial cultures starting point for the next experimental assays.
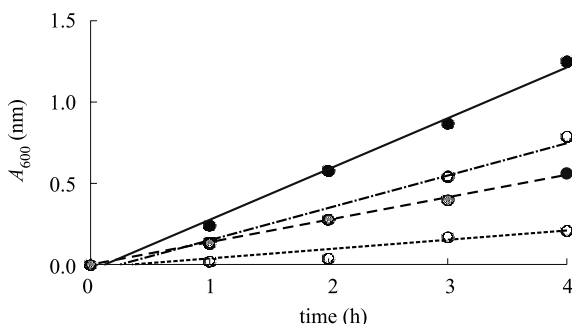
Fig. 2. Bacterial growth
S. enterica (-----), *E. coli* (-), *E. faecalis* (—·–), and *P. aeruginosa* (····)

Bacteria, *S. enterica*, *E. coli*, *E. faecalis* and *P. aeruginosa* were incubated and their specific growing was monitored at an absorbance of 600 nm (A_{600}). All studies represent one of at least two independent experiments.

The toxic effect of chlorpyrifos-methyl, active compound from Reldan 40EC with a large spectrum of action, was assessed by 1 h bacterial growth (Fig. 3). In spite of the fact that recommended concentration for the agricultural/farming fields is 0.4 g/l chlorpyrifos-methyl, we observed that a much lower concentration of pesticide inhibit by 50% the bacterial growth (Fig. 3). If 50% *S. enterica* growth inhibition (Fig. 3*A*) was achieved by a chlorpyrifos-methyl concentration close to the recommended dose of 0.4 g/l, in case of *P. aeruginosa* (Fig. 3*B*), 50% inhibition growth rate was achieved around 10 fold less chlorpyrifos-methyl.

The results showed that 0.4 g/l chlorpyrifos-methyl (recommended concentration for agricultural/farming field applications) inhibited more than 50% the *E. coli* growth (Fig. 3*C*), but it induced no significant growth inhibition for *E. faecalis* (Fig. 3*D*). The concentration of chlorpyrifos-methyl should be raised around 10 fold (0.4 g/l) in order to reach 50% bacterial growth inhibition during 1 h testing.

S. enterica, *P. aeruginosa*, *E. coli* and *E. faecalis* were incubated in the presence of chlorpyrifos-methyl (0.04–4 g/l) and their inhibition growth rate was determined in function of the bacteria growth without pesticide treatment. All studies represent one of at least two independent experiments.

Next, we tested for longer periods of time the effect of recommended chlorpyrifos-methyl concentration (0.4 g/l) on the bacterial growth. *S. enterica*, a pathogenic gram-negative bacteria[9] and *P. aeruginosa*, gram-positive bacteria[10,11] showed a similar growth inhibition curve in presence of 0.4 g/l chlorpyrifo-methyl (Fig. 4). After only 1 h incubation, bacterial growth was 75% inhibited compared to the control sample, bacteria without pesticide treatment. Then for the next 3 h, both bacterial strains, *S. enterica* (Fig. 4*A*) and *P. aeruginosa* (Fig. 4*B*) kept almost the same growth rate inhibition.
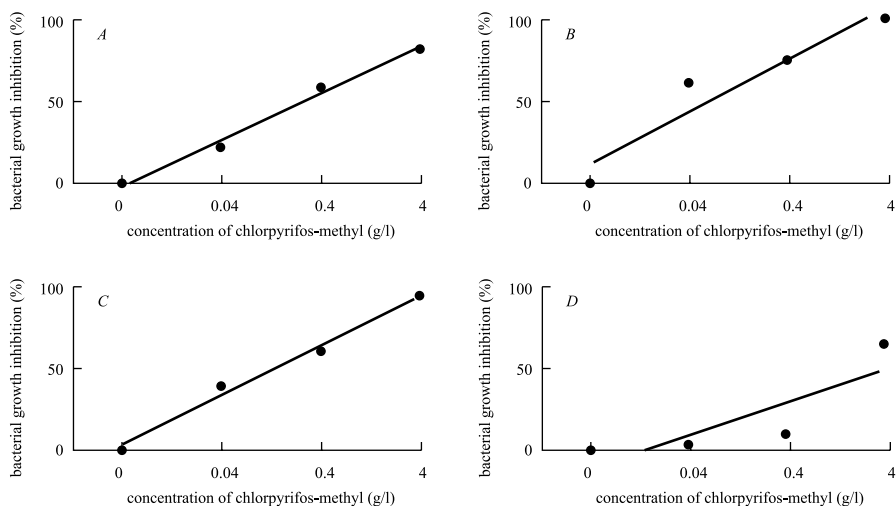
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Fig. 3. Modulation of bacterial growth rate by various concentrations of chlorpyrifos-methyl *S. enterica* (*A*), *P. aeruginosa* (*B*), *E. coli* (*C*) and *E. faecalis* (*D*)

E. coli, gram-negative bacteria, is present at the human intestinal level[12], but its ability to survive outside the humans implied a particular resistance to the environment[13]. Bacteria incubated with Reldan EC40 reached very fast a growth inhibition up to 60% (Fig. 4*C*, 1 h) compared to the control samples (bacteria without Reldan EC40 treatment). Then, *E. coli* started to grow again, so the inhibition rate decreased by 4 h of treatment to around 30%, compared to the control (Fig. 4*C*). *E. faecalis*, a facultative anaerobic commensally gram-positive bacteria localised in the gastro-intestinal track in mammals[14], in the presence of 0.4 g/l chlorpyrifos-methyl decreased gradually and steadily the growth rate (Fig. 4*D*).

S. enterica (*A*), *P. aeruginosa* (*B*), *E. coli* (*C*) and *E. faecalis* (*D*) were incubated 4 h at 37ºC in the presence of chlorpyrifos-methyl (0.4 g/l) and their inhibition growth rate was determined as a function of the bacteria growth without pesticide treatment. All studies represent one of at least two independent experiments.
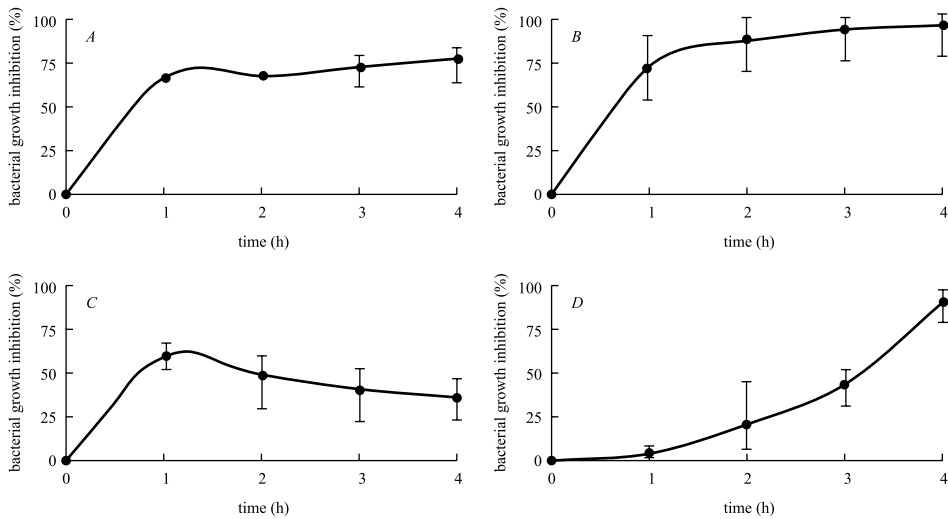
Fig. 4. Inhibition of bacterial growth by pesticide
S. enterica (*A*), *P. aeruginosa* (*B*), *E. coli* (*C*) and *E. faecalis* (*D*)

Monitoring of chlorpyrifos-methyl concentration during bacterial growth. Bacteria are very resistant and adaptive microorganisms to almost all changes occurred in the environment. One of the main defense bacterial mechanism is based on the efflux pumps which eliminate the toxic compounds from inside cells back to the medium. In the first step, we analysed the chlorpyrifos-methyl stability for each growing medium used for the bacterial growth. 0.4 g/l chlorpyrifos-methyl incubated during 4 h at 37°C in both growing bacteria mediums showed no significate variations in concentrations through the test (data not shown). On the other hand, we detect various changes in pesticide final concentrations by adding bacteria to the growing medium.

In the presence of *S. enterica*, during 2 h of bacterial incubation, the concentration of chlorpyrifos-methyl decreased gradually to 70% of the initial amount, then remained stabilised up to 4h of further incubation (Fig. 5*A*). The loss of chlorpyrifos-methyl is more drastic even after 1 h of incubation in presence of *P. aeruginosa*, when its concentration level reached 25% of the initial concentration (Fig. 5*B*). It seems that major changes happened only in the 1 h of incubation, then the pesticide concentration remains without any significant changes at around 25% compared to the starting point (100% chlorpyrifos-methyl). Addition of bacterial strains induced changes in pesticide final concentrations, especially in the presence of *E. faecalis* where after 4 h of incubation the final concentration of the pesticide decreased to 50% of the starting point (Fig. 5*D*). Moreover, concentration of chlorpyrifos-methyl constantly decreased, during 4 h incubation in *E. coli* growing medium, up to 70–75% of the initial concentration (Fig. 5*C*).
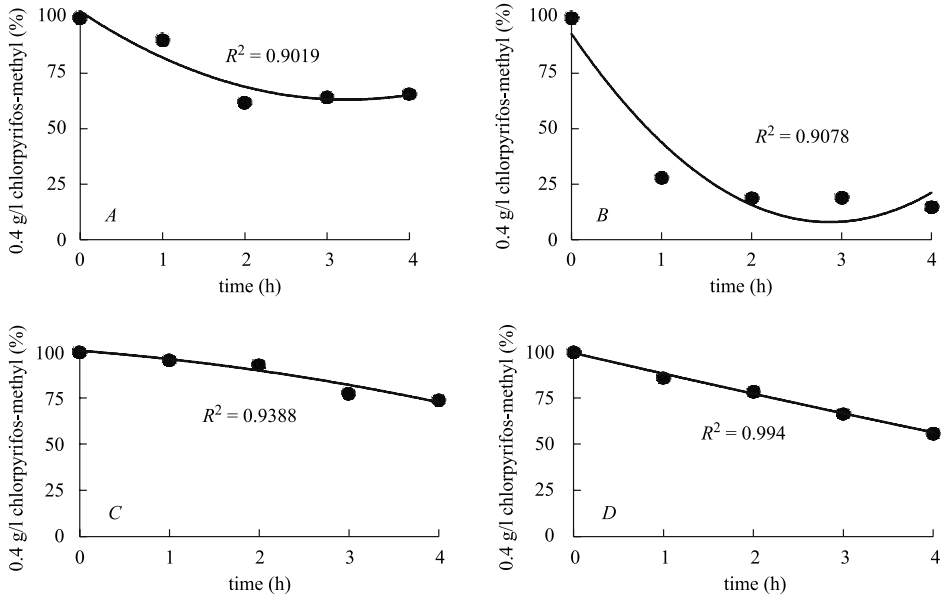
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Fig. 5. Influence of bacteria on pesticide concentration
S. enterica (*A*), *P. aeruginosa* (*B*), *E. coli* (*C*), *E. faecalis* (*D*)

    S. enterica, *P. aeruginosa*, *E. coli*, and *E. faecalis* (D) were incubated for 4 h at 37ºC in the presence of chlorpyrifos-methyl (0.4 g/l) and the pesticide concentration was monitored by GC system. All studies represent one of at least two independent experiments.
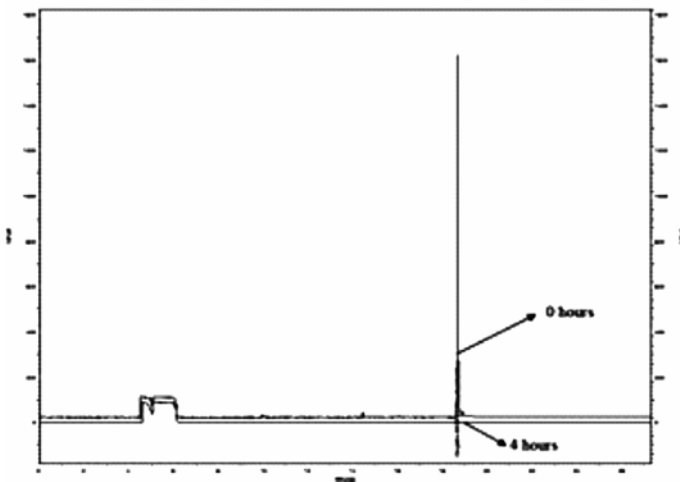

Fig. 6. Example of GC chromatogram with chlorpyrifos-methyl detection

The loss of chlorpyrifos-methyl concentration detected by GC showed no addition of news peaks on the chromatogram (Fig. 6).

CONCLUSIONS

In spite of the fact that organophosphates are targeted to control a variety of pests that attack crops, the excessive use of organophosphorus in agriculture generate serious problems to the environment[15] as well as they accumulate in humans[16,17]. The toxic effect of pesticides or their metabolites on the environment could be assessed through acute and/or chronic toxicity tests using fish and invertebrates models[18], however, the microbial biotests are very efficient to assess the short-term (acute) toxicity[19–21]. Based on bacterial cells behaviour in presence of Reldan EC40 pesticide, we observed that *S. enterica* and *P. aeruginosa* exhibited an overall similar profile of bacterial growth and pesticide degradation. This suggests a rapid bacterial defense response by metabolising the pesticide, but due to pesticide high concentration and its toxic effect these bacterial cells dramatically decreased their growth. The remaining bacterial cells, most likely acquired resistance by genetic mutations[22] or used the efflux pumps, pumping harmful intracellular substance directly to the external medium[23–26].

Based on *E. coli* growth behaviour in the presence of Reldan EC40 pesticide, we suggested that a part of bacterial population survived by metabolising the pesticide to 3,5,6-trichloro-2-pyridinol, which is a metabolite undetectable on GC chromatogram. A similar continuous degradation profile of the pesticide was observed in presence of *E. faecalis*. In spite of pesticide degradation, the bacterial growth inhibition seemed to steadily increase during the inhibition time. It suggested a possible metabolisation of the pesticide, but not at a rate to ensure viability of bacterial strain especially due to the high concentration of pesticide.

As a conclusion, we showed a bacterial model composed by *E. coli* and *E. faecalis* which could be used as biodetector of toxicity as well as biodegradation promoter of chlorpyrifos-methyl.

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