Biodegradation (2011) 22:1203–1213
DOI 10.1007/s10532-011-9475-z

ORIGINAL PAPER

Removal of methyl parathion and tetrachlorvinphos by a bacterial consortium immobilized on tezontle-packed up-flow reactor

Gustavo Yáñez-Ocampo · Enrique Sánchez-Salinas · M. Laura Ortiz-Hernández

Received: 1 December 2010 / Accepted: 15 April 2011 / Published online: 1 May 2011

Abstract A tezontle-packed up-flow reactor (TPUFR) with an immobilized bacterial consortium for biological treatment of methyl-parathion and tetrachlorvinphos was evaluated. These organophosphate pesticides are widely used in Mexico for insect and mite control, respectively. With the aim of developing a tool for pesticide biodegradation, four flow rates (0.936, 1.41, 2.19, and 3.51 l/h) and four hydraulic residence times (0.313, 0.206, 0.133, and 0.083 h) were evaluated in a TPUFR. In the bioreactor, with an operating time of 8 h and a flow of 0.936 l/h, we obtained 75% efficiency in the removal of methyl-parathion and tetrachlorvinphos. Their adsorptions in the volcanic rock were 9% and 6%, respectively. It was demonstrated that the removal of pesticides was due to the biological activity of the immobilized bacterial consortium. We confirmed the decrease in toxicity in the treated effluent from the bioreactor through the application of acute toxicity tests on *Eisenia foetida*. Immobilization of a bacterial consortium using tezontle as a support is innovative and an economical tool for the treatment of mixtures of organophosphorus pesticide residues.

G. Yáñez-Ocampo · E. Sánchez-Salinas · M. L. Ortiz-Hernández (✉)
Laboratorio de Investigaciones Ambientales, Centro de Investigación en Biotecnología, Universidad Autónoma del Estado Morelos, Cuernavaca, Mexico
e-mail: ortizhl@uaem.mx

Keywords Methyl-parathion · Tetrachlorvinphos · Bacterial consortium · Tezontle · Removal

Introduction

Agricultural activities around the world demand large amounts of pesticides, among which the organophosphates (OPs) are the most commonly used to increase crop yields and to control livestock ectoparasites. Their sales are estimated at 34% of the total pesticides in the world (Singh and Walker 2006). Over the last 60 years, approximately 150 OP chemicals have been used to protect crops, livestock, and human health. From the millions of tons of pesticides applied annually at a worldwide level, liquid and solid wastes are generated. In addition, the containers for these chemicals are transferred directly, without control, into the environment, particularly into the soil and water, and, therefore, are a risk to the environment and public health due to their toxicity (Schwarzenbach et al. 2010; Aktar et al. 2009; Pimentel 2005).

In addition, in a number of developing and transitional countries, there are more than half a million tons of obsolete, unused, banned, or outdated pesticides, which endanger the environment and the health of millions of people. In the absence of a clear strategy for obsolete pesticide management, significant amounts of obsolete pesticides have been stockpiled over the years in developing countries (Dasgupta et al. 2010).

In 29 of the 33 states of Mexico, there are about 26,725.02 l and 147,274 kg of obsolete stored pesticides, both liquid and solid. In addition, there are 28 reports of pesticide-contaminated sites in 15 states of the Mexican Republic and 500 m^3 of highly polluted soils. Furthermore, approximately 7000 tons of empty pesticide containers could be produced annually (Ortiz-Hernández et al. 2011). Owing to the damage caused to the environment and human health, and the existence of obsolete pesticides, it is necessary to develop technologies that guarantee pesticide elimination in a safe, efficient, and economical way.

Among the technologies that currently exist, there are those that apply physical treatments, such as adsorption and percolator filters, and chemical treatments, such as advanced oxidation, inverse osmosis, and incineration. These treatment options are not usually available in developing countries (Karstensen et al. 2006). However, a treatment method that promises to be efficient, economical, and safe is biological treatment through reactions catalyzed by enzymes of specific microorganisms. This kind of treatment has been developed to provide a methodology that is safer and more economical than conventional treatments, and to avoid additional damage to the environment. Biological processes have been used to treat wastes and sites polluted with pesticides (Moens et al. 2004; Khan et al. 2004; Yair et al. 2008). Biodegradation of these pesticides provides a cheap and efficient solution for their final disposal or for the treatment of agricultural soils, contaminated water, or polluted ecosystems (Yair et al. 2008). In 1973, the first bacteria with the capability of degrading organophosphorus compounds were described (Sethunathan and Yoshida 1973; Singh 2009). Since then, a number of different genera have been identified, and enzymes involved in pesticide degradation have been widely studied.

A great number of bacterial species that degrade OP pesticides has been reported. Such bacteria are capable of degrading the latter because of the presence of some enzymes, mainly related to the phosphotriesterase, which is capable of hydrolyzing OP pesticides through a nucleophilic attack on phosphorous central atom in the molecule. The hydrolysis is essential for complete degradation of OP pesticides and phosphotriesterase activity is the first and the most important step in the detoxification of OP waste or OP-contaminated sites (Yáñez-Ocampo et al. 2009; Sorgob and Vilanova 2002; de la Peña et al. 2006).

Cell immobilization has been employed for biological removal of pesticides because it confers the possibility of maintaining catalytic activity over long periods of time (Richins et al. 2000; Chen and Georgiou 2002; Martin et al. 2000). Whole-cell immobilization has been shown to have remarkable advantages over conventional biological systems using free cells, such as the possibility of employing a high cell density, the avoidance of cell washout, even at high dilution rates, easy separation of cells from the reaction system, repeated use of cells, and better protection of cells from harsh environments. Previous reports have suggested that this higher productivity results from cellular or genetic modifications induced by immobilization. There is evidence indicating that immobilized cells are much more tolerant to perturbations in the reaction environment and less susceptible to toxic substances, which makes immobilized cell systems particularly attractive for the treatment of toxic substances like pesticides (Ha et al. 2008). In addition, the enhanced degradation capacity of immobilized cells is due primarily to the protection of the cells from inhibitory substances present in the environment. The degradation rates for repeated operations were observed to increase for successive batches, indicating that cells became better adapted to the reaction conditions over time (Ha et al. 2009).

Yáñez-Ocampo et al. (2009) reported removal efficiencies of methyl-parathion (MP) and tetrachlorvinphos (TCV) of 40% and 50%, respectively, by a bacterial consortium grown in suspension in a liquid medium of mineral salts with a mixture of these pesticides. In the same study, it was found that bacterial immobilization on volcanic rock significantly increases the removal efficiency by up to 66% for both pesticides. Compared with microbial cultures in suspension, cell immobilization can increase the length of time of bacterial catalytic activity; in addition, bacterial immobilization on porous supports allows cells to tolerate high concentrations of toxic compounds (Martin et al. 2000; Santacruz et al. 2005; Galíndez-Nájera et al. 2009).

The TPUFRs have been successfully used in industrial effluent treatment, as well as for hazardous waste mixtures, such as pesticides, hydrocarbons, and polychlorinated biphenyls. This system, based on the formation of biofilm on porous supports, can work

under aerobic and anaerobic conditions; sludges are not formed as a byproduct of this method, and biomass and material are recovered and reused. Bacteria of the genus *Flavobacterium*, *Pseudomonas*, *Rhodococcus*, *Acinetobacter*, and other members of the Enterobacteriaceae family have the ability to produce compounds with adhesive and biosurfactant properties, allowing them to be fixed to surfaces. This also facilitates the bioavailability of some compounds with a low solubility. In addition, an up-flow system operates with the recirculation of wastes through several cycles, increasing the removal efficiency (Jajuee et al. 2007; Singh and Walker 2006; Jin-Woo et al. 2002).

The purpose of this study was to evaluate the removal efficiency of MP and TCV by a bacterial consortium immobilized on tezontle in an up-flow packed bed bioreactor.

Materials and methods

Tezontle description

Tezontle (in Nahuatl, *tezt* means rock and *zontli* means hair) is a native volcanic rock of Morelos state (central Mexico). This rock is highly porous, provides a large contact surface, and can also be sterilized and reused. The presence of micropores allows the establishment of bacterial microcolonies (Godon et al. 1997). Other characteristics of tezontle include density, 2.24–2.93 g/ml; water retention capacity, 12.91–43.3%; total porosity, 37.91–63.8%; pH, 6.88–7.35; organic material, 0%; Ca: 22.0 mg/l; Mg: 10.09 mg/l; K, 2.74 mg/l; and P: 0.31 mg/l (Yáñez-Ocampo et al. 2009; Galíndez-Nájera et al. 2009). The tezontle rocks were broken using a metal hammer to obtain fragments that were sifted using a 3-mm mesh sieve. These particles of tezontle were sterilized three times in an autoclave at 121°C for 20 min and then left to rest for 24 h.

Organophosphate pesticides

Methyl-parathion—MP (*O*,*O*-dimethyl *O*-4 nitrophenyl phosphorotioate) and tetrachlorvinphos—TCV (phosphoric acid, 2-chlore-1-(2,4,5-trichlorephenyl) dimethyl ester, vinyl) are authorized OP pesticides used in Mexico's livestock and agricultural industries. These substances were purchased from Chemservice (99% purity) (http://web1.chemservice.com). Reactive grade ethyl acetate was used as a solvent for pesticide extraction; HPLC grade methanol from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA) was used to inject samples into the gas chromatograph. All other chemicals were of reagent grade and were obtained from J.T. Baker, Mexico City.

Culture medium composition

A mineral salt medium (MSM) described by O'Reilly and Crawford (1989) was modified and used for the cultivation of the consortium, which had the following composition (in g/l): 0.82 K_2HPO_4; 0.19 KH_2PO_4; 0.20 $MgSO_4{\cdot}7H_2O$; 2.0 KNO_3; 0.99 $(NH_4)_2SO_4$; and 2 ml/l of a trace element solution with a composition of (in g/l): 2.8 H_3BO_3; 2.55$MnSO_4{\cdot}H_2O$; 0.17 $CuSO_4{\cdot}5H_2O$; $CoCl_2{\cdot}6H_2O$, 2.43; and 0.25 $ZnSO_4{\cdot}7H_2O$. The pH of the medium was 7 $\pm$ 0.05. The carbon sources tested were pesticides, and the nitrogen source was ammonium and nitrate salts.

Bacterial consortium

The bacterial consortium used in this study was previously isolated from agricultural soils from Morelos State (central Mexico), where OP pesticides had been constantly applied. The isolated consortium had an adaptation process during several weeks, using mineral medium without an additional carbon source, plus untreated OP pesticide wastes generated from livestock dipping operations containing an OP pesticide mixture. This consortium was subcultured and continuously improved by the selection pressure. The consortium was formed for 14 bacterial strains (Yáñez-Ocampo et al. 2009).

Tezontle-packed up-flow reactor (TPUFR)

The pesticide removal experiments were conducted in a bench-scale up-flow tubular column reactor with a 6-cm inner diameter, a height of 35.4 cm, and a 1000 cm^3 nominal volume (Nv). The column reactor consisted of a feed tank, a feed pump, and the column itself with an inlet and an outlet. It was constructed with high-quality sterilization-resistant glass. The column reactor was loaded to 70% Nv with volcanic rocks of an average diameter of 2.5 mm. This was considered as the packing volume (Pv)

Preliminary experiments were conducted to standardize the peristaltic pump operation by testing different conditions for flow rate (l/h), hydraulic residence time (h), tezontle packing density and times, and favorable growth media for bacterial colonization on tezontle (data not shown).

The empty volume (Ev) was calculated from the difference between the column Nv and the Pv:

$$Ev = Nv - Pv.$$

The Ev was used to calculate the hydraulic residence time (HRT) of the pesticides in the packed column, using the following equation:

$$HRT = Ev\ (l)/Flow\ (l/h).$$

Biofilm formation on tezontle particles in TPUFR

Tripticasein soy (TS) broth (Bioxon, Becton–Dickinson, Mexico) was used for promoting the formation of biomass. The previously adapted consortium was cultured in Petri dishes with TS agar for 24 h at 28°C to obtain the bacterial inoculum. After that, the consortium was inoculated in flasks containing 50 ml of TS broth and a mixture of MP–TCV at a final concentration of 25 mg/l. These flasks were incubated for 72 h at 28°C with 100 rpm shaking. After that, the biomass was collected by centrifuging at $1000 \times g$ for 20 min and then discarding the supernatant. The biomass was used for the inoculation of the experimental bioreactor packed with particles of tezontle that had previously been sterilized as described above, containing TS broth, MP at final concentration of 25 mg/l, and TCV at final concentration of 10 mg/l. This bioreactor was incubated for 7 days at room temperature to promote the formation of a biofilm and the colonization of tezontle particles. After this period, the culture medium was removed to eliminate excess broth and the biomass in suspension. The particles of tezontle were washed with a sterile solution of 0.5% NaCl. To confirm bacterial immobilization, tezontle particles were collected, treated, and observed with a Carl Zeiss EVO®40 scanning electron microscope, according to methods adapted from a previous report (Yáñez-Ocampo et al. 2009) (Fig. 1).

Degradation experiments in TPUFR

After the biofilm was formed in the TPUFR, different flows were evaluated (3.51, 2.19, 1.41, and 0.936 l/h), and as a result, four hydraulic retention times

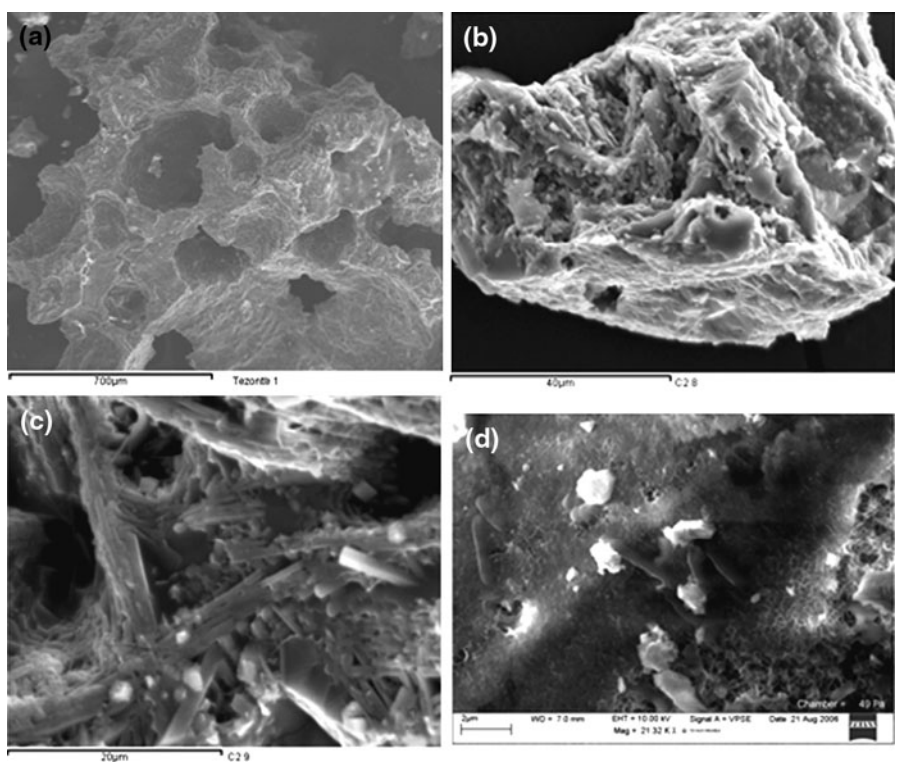

Fig. 1 Scanning electron micrographs of tezontle colonized by the consortium. **a** Control without biomass, **b–d** tezontle particle colonized with biofilm at 40, 20, and 2 μm, respectively

(HRT) were investigated (0.085, 0.136, 0.212, and 0.320 h). The initial pH of the bioreactor was 7.0. The reactor was operated at room temperature (approximately 25°C). A Kitasato flask was connected to the column via a peristaltic pump. All silicone tubing used was Masterflex type (Cole Parmer 96400-35). The liquid from the top of the column was recycled through the inlet positioned at the bottom of the column (Bianchi et al. 2008).

The influent of the system was placed at the base of the column to ensure the homogenization of the mineral salt medium supplemented with 10 mg/l of TCV and 25 mg/l of MP. The effluent was located 5 cm from the top of the column and was connected to a 1000-ml Kitazato flask that stored the culture medium for recirculation.

As a control, a sterile TPUFR without biomass was installed. For its sterilization, a column packed with volcanic rock was treated with 0.5% nitric acid for 24 h, then washed three times with distilled water, and finally, autoclaved at 15 kg/cm^2 at 121°C for 20 min; all of this was done to remove organic matter and microorganisms. Sterilization was intermittent and was repeated three times, with 24-h interval between each sterilization cycle.

After the reactor was started, at different time intervals during the experiment, a 2-ml aliquot was collected from the Kitasato flask and placed in glass tubes. To extract both OP pesticides, these samples were extracted three times with equal volumes of ethyl acetate; after 1 min of vortexing, the organic phase was removed and filtered using glass wool and anhydrous sodium sulfate-packed glass funnels. This operation was performed sequentially. The filtrates were mixed, evaporated, and then reconstituted in 1 ml of HPLC grade methanol for analysis by GC/MS.

Analytical methods

Both MP and TCV were quantified by gas Trace GC chromatograph coupled to a Polaris Q Thermo Finnigan mass spectrometer (GC–MS) using the EPA8141 method under the following conditions: equity column-5; 30 m × 0.25 mm ID; 0.25 µm, oven at 120°C (3 min) and at 270°C at 5°C/min, injector 250°C, MSD detector, scan range 45–450 amu, 325°C transfer line, helium flow 30 cm/s @ 120°C, injection 1.0 µl, splitless (0.3 min), splitless liner, and double taper.

Pesticide adsorption on tezontle

The pesticide adsorption on tezontle was measured according to the methods described in a previous report (Yáñez-Ocampo et al. 2009).

Growth of consortium immobilized on tezontle

The growth of the consortium immobilized on tezontle was measured by counting viable cells. One tezontle particle was placed in a glass tube with 1 ml of 0.4 M potassium phosphate solution. Vigorous homogenization was applied for approximately 1 or 2 min. Serial dilutions of this solution were carried out, and 0.1 ml was inoculated in TS agar for 24 h at 28 °C. Finally, the number of colony-forming units was recorded as CFU/tezontle.

MP and TCV removal in TPUFR

To evaluate pesticide removal from the culture medium, the removal percentage, removal rate, and removal efficiency of both pesticides were calculated. In addition, the residual concentration data versus TPUFR operating time data were plotted.

The value of delta OP (ΔOP) was calculated to evaluate the performance of pesticide removal by the TPUFR. This value allows knowing the percent of each OP removed for each hydraulic residence time (HRT) in the packed reactor. The ΔOP was calculated as the difference between the initial concentration (Iop) and the residual concentration (Rop) for each HRT. ΔOP was calculated for each HRT and was expressed as a percentage.

$$\Delta OP = Iop - Rop.$$

The percentages of removed MP and TCV in each flow were transformed using an angular transformation ($\arcsen\sqrt{yi}$) and analyzed using the general linear models procedure; through an analysis of variance with a factorial arrangement of treatments. Post-hoc analysis of differences in means was conducted with the Tukey test ($\alpha = 0.05$) (SAS Institute 2003).

Toxicity evaluation of the treated effluent in TPUFR

The toxicity of the effluent from the TPUFR subjected to the mixture of pesticides was evaluated by an acute toxicity test using the earthworm *Eisenia foetida* as a biological model. This procedure was conducted according to Olvera-Velona et al. (2008). After 24 and 48 h of exposures, muscular effects in the worms were observed, such as decreased coordination and stiffness. The mortality of the organisms after 72 h of exposure was evaluated simultaneously. The data were analyzed using chi-squared test ($\alpha = 0.05$). The conditions of the experiment are shown in Table 1.

Results and discussion

Biological removal of MP and TCV in the TPUFR

The OP pesticide removal was evaluated in the TPUFR, and the results were compared between the TPUFR control (without cells) and experimental TPUFR (with immobilized consortium).

Figure 2a and b show the concentration of MP and TCV during the TPUFR operation at different tested flows. The amount of pesticides removed from the culture medium by the immobilized consortium in the experimental TPUFR was significantly greater than in the TPUFR control. The biological activity of the immobilized bacterial consortium was the main factor responsible for the removal of MP and TCV. During the first 8 h of TPUFR operation, the pesticide concentration decreased, apparently as a function of increasing flow rate. Under the 3.51-l/h flow, changes in the OP pesticide concentrations were not as evident as in the 0.936-l/h flow, suggesting that the catalytic activity of the consortium requires more contact time with pesticides to carry out transformation reactions (Sheeja and Murugesan 2002; Rama Krishna and Ligy 2009). High flow rates could limit the spread of the pesticides and, thereby, the degradation ability of a biofilm system (Mansee et al. 2000; Schmid et al. 2001).

In parallel, the adsorption of the pesticides by tezontle in the control TPUFR was quantified. The percentage of adsorption of MP was 9%, while it was 6% for TCV. Santacruz et al. (2005) and Mondragón-Parada et al. (2008) reported adsorption percentages of phenol and simazine on this type of volcanic rock as 8.12% and 4.0%, respectively. The amount of adsorption observed in our experiment was similar to and consistent with these previous reports,because of the presence of an aromatic ring in the molecular structure of the OP pesticides tested. When these pesticides are hydrolyzed, phenolic groups could form and then be absorbed by tezontle.

Owing to the catalytic activity of the consortium used in this study, the removal of both MP and TCV may depend on the chemical composition and structure of each of these compounds (Rama Krishna and Ligy 2009). Ortiz-Hernández et al. (2003) found that the molecular weight of pesticides was inversely proportional to enzymatic activity (PM in comparison with TCV). They found that there was steric hindrance of TCV degradation by *Flavobacterium* ATCC 27 551, which only degraded 8% of the TCV added to culture medium, in contrast to its ability to degrade other OP pesticides. The molecular structure of TCV probably confers a higher degree of recalcitrance because of the presence of a chlorinated ring. Singh and Walker (2006) reported that the biodegradation of several OPs depends on the chemical structure related to where the phosphotriester bond is present, which is the case also of MP, TCV, ethyl parathion, and chlorpyrifos.

Table 1 Experimental design of acute toxicity tests

Treatment	Characteristics
Control (distilled water)	Aliquots of 2 ml of distilled water and 0.4% (v/v) methanol were added to Petri dishes.
TPUFR effluent control	A 2-ml aliquot from the effluent of the TPUFR control
TPUFR effluent with biofilm	Aliquots of 2 ml from effluents were added at 0, 4 and 8 h after treatment of the TPUFR

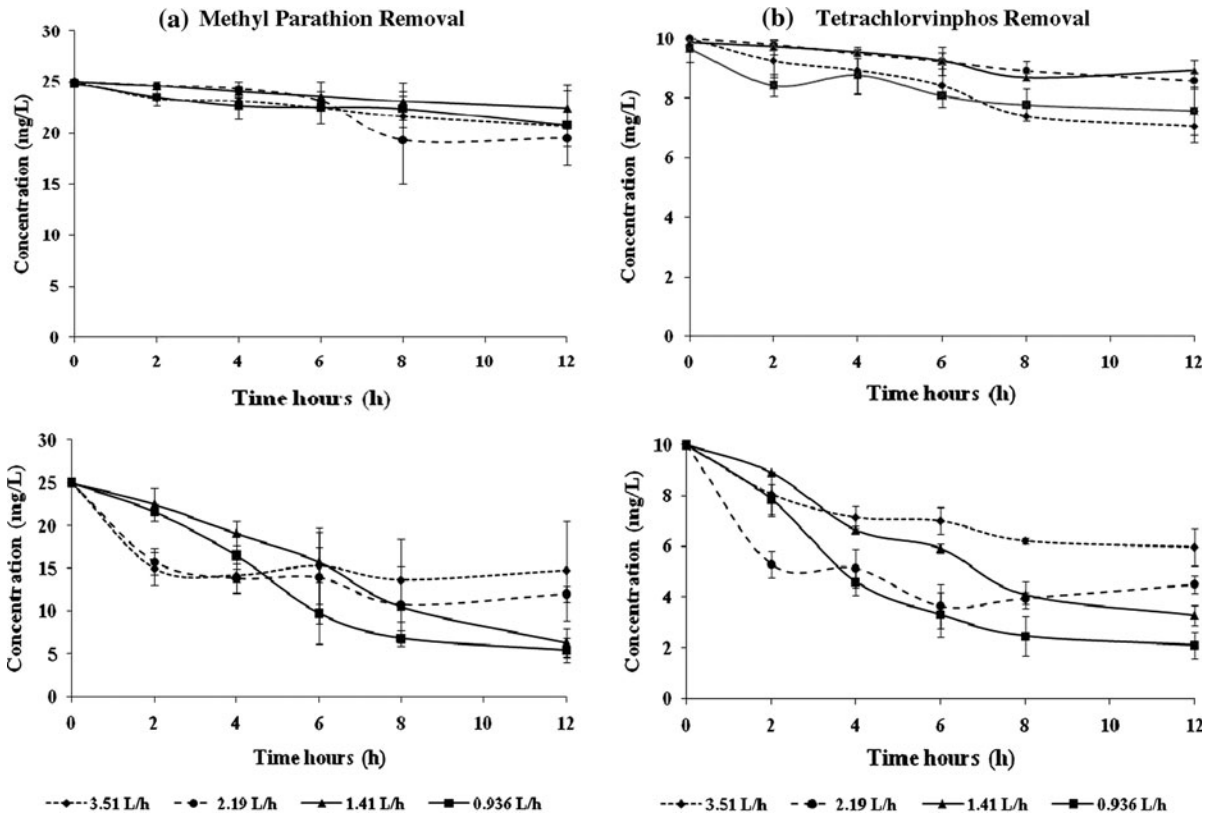

Fig. 2 Removal of **a** MP and **b** TCV in the TPUFR control without biofilm (superior) and in the TPUFR with immobilized bacterial consortium (below)

Evaluation of the biological removal efficiency of MP and TCV in the TPUFR

Table 2 shows the results of the evaluation of the TPUFR with the biofilm obtained at 8 h. The population of the bacterial consortium remained viable during the four HRTs evaluated. However, a decrease in the bacterial population of 10^{11}–10^{9} CFU/tezontle particle was observed. This phenomenon could have been the result of biomass dragging due to the increased shear stress (friction) caused by both the stream flow of 3.51 l/h (0.083 h HRT) and the biofilm on the porous support. Increased flow can create unstable conditions in the TPUFR, such as biofilm detachment and low diffusion through the substrate (Morgan-Sagastume and Noyola 2008; Mondragón-Parada et al. 2008).

The removal percentage of both pesticides was higher with a longer contact time between the

Table 2 Evaluation of TPUFR with immobilized cells after 8 h of operation

Flow (l/h)	HRT (h)	Removal (%)		ΔOP (%)		Removal rate (mg/l*h)		CFU/tezontle
		MP	TCV	MP	TCV	MP	TCV	
3.51	0.083	$35.05^{a} \pm 3.39$	$37.83^{a} \pm 2.10$	$0.36^{a} \pm 0.11$	$0.779^{a} \pm 0.06$	1.09 ± 0.106	0.81 ± 0.11	2.4×10^{9}
2.19	0.133	$57.05^{b} \pm 1.78$	$60.50^{b} \pm 2.40$	$0.95^{b} \pm 0.21$	$1.40^{b} \pm 0.01$	1.78 ± 0.55	0.75 ± 0.03	1.2×10^{10}
1.41	0.206	$57.76^{b} \pm 1.60$	$59.16^{b} \pm 5.21$	$1.48^{c} \pm 0.03$	$2.15^{c} \pm 0.11$	1.80 ± 0.05	0.74 ± 0.19	2.6×10^{10}
0.936	0.313	$72.71^{d} \pm 3.58$	$75.45^{d} \pm 5.54$	$2.84^{d} \pm 0.091$	$3.52^{d} \pm 0.12$	2.27 ± 0.11	0.94 ± 0.14	3.1×10^{11}

CFU/tezontle colony-forming units on tezontle particle. Similar letters means not significantly different; different letters express significant differences ($\alpha = 0.05$). *HRT* Hydraulic residence time, *ΔOP* removal pesticide percentage (MP and/or TCV) in each HRT

solution of pesticides and the immobilized consortium. Thus, with an HRT of 0.83 h (the lowest), the removal percentage was approximately 35% for MP and 37% for TCV, whereas with a longer HRT, removals up to 72% and 75% were achieved for MP and TCV, respectively. This situation may have been caused by an increase of the organic load. When flow increases (l/h) and the initial concentration of pesticides (mg/l) remains constant, the mass by time unit (mg/h) that is transferred into the packed column increases, saturating the consortium's catalytic activity, resulting in a low efficiency in the removal of both pesticides (Hallas et al. 1992; Burton 2001; Li et al. 2005).

The removal efficiencies of MP and TCV with an HRT of 0.313 h was significantly different ($\alpha = 0.05$) in comparison with the other three HRTs studied, and it was the highest removal percentage observed. With an HRT of 0.083 h, we found that the removal percentage of pesticides was significantly lower ($\alpha = 0.05$). In the present study, 75.45% TCV removal was achieved; the initial concentration of 10 mg/l allowed a better removal efficiency over that reported by Yáñez-Ocampo et al. (2009), where the initial concentration of TCV was 25 mg/l (water solubility = 11 mg/l). Thus, in this study, we obtained higher removal efficiency, suggesting an effect of bioavailability, particularly for TCV. From a practical point of view, it is important to know the concentration of pesticide wastes before applying a biological treatment to establish appropriate treatment strategies (Struthers et al. 1998; Mulchandani et al. 1999).

Apparently, the proportion of the biological removal of both pesticides in each HRT was the same, which represents an advantage from a practical point of view because mixtures of pesticide residues are generated daily, and pesticides are used at different concentrations (Konstantinou et al. 2006). In this study, it was observed that the investigated consortium grew and maintained its viability; in addition, it removed the mixture of two OP pesticides efficiently.

Table 2 shows the values of ΔOP calculated for MP and TCV expressed as the percentage of pesticides removed in each HRT in the TPUFR with the immobilized consortium. The relationship between ΔOP and HRT is an approximation of the pesticide proportion that is removed each time it is recycled and travels through the TPUFR to be transformed. While the HRT decreased from 0.313 to 0.083 h, the number of passages of the mixture of pesticides increased; however, this does not mean that degradation was more efficient. Therefore, when the mixture of pesticides remains at a time of 0.313 h, the removal of MP and TCV was significantly higher ($\alpha = 0.05$). Based on our experimental results, under the experimental conditions of this study, we suggest that the biological treatment of mixtures of OP pesticides in a TPUFR could be more efficient using an HRT longer than 0.313 h, which can be achieved by lowering the flow. TCV and MP pesticides were removed at different rates with an HRT of 0.313 h, such that MP was removed more rapidly. This suggests that the enzymes responsible for pesticide removal have a greater affinity for MP than TCV (Buchbinder et al. 1998).

The removal rates were calculated, assuming their equivalence with the reaction rate of the enzymes involved in pesticide degradation. The results presented in Table 2 show the rates of the removal of pesticides in the TPUFR. These results indicate that reaction rates do not change with the HRT evaluated, although they are greater in the case of MP. The highest removal rates were 2.7 mg/l*h for MP and 0.94 mg/l*h for TCV, which were found at an HRT of 0.313 h and a flow of 0.936 l/h. This confirms that with a slow flow, we can obtain better removal. According to Morgan-Sagastume and Noyola (2008) and Mondragón-Parada et al. (2008), an increase in flow can create unstable conditions in a TPUFR system, such as biofilm detachment and lower diffusion of pesticides.

In previous reports, in cultures carried out in a batch reactor, we found removal percentages of 41, 72, and 66% for MP and 53, 65, and 47% for TCV using suspended, alginate-immobilized and tezontle-immobilized consortia, respectively (Yáñez-Ocampo et al. 2009). In this study, we showed that using a TPUFR with an immobilized consortium, it was possible to achieve more than 70% removal of a pesticide mixture, in an economical and efficient way.

Acute toxicity test with *Eisenia foetida*

Following biological treatment of the mixture of OP pesticides in the TPUFR, the detoxification of the effluent was evaluated through toxicity tests using *Eisenia foetida* as an indicator organism. The results are shown in Fig. 3a–c at 0, 24, and 48 h of exposures, respectively. The percentage of the

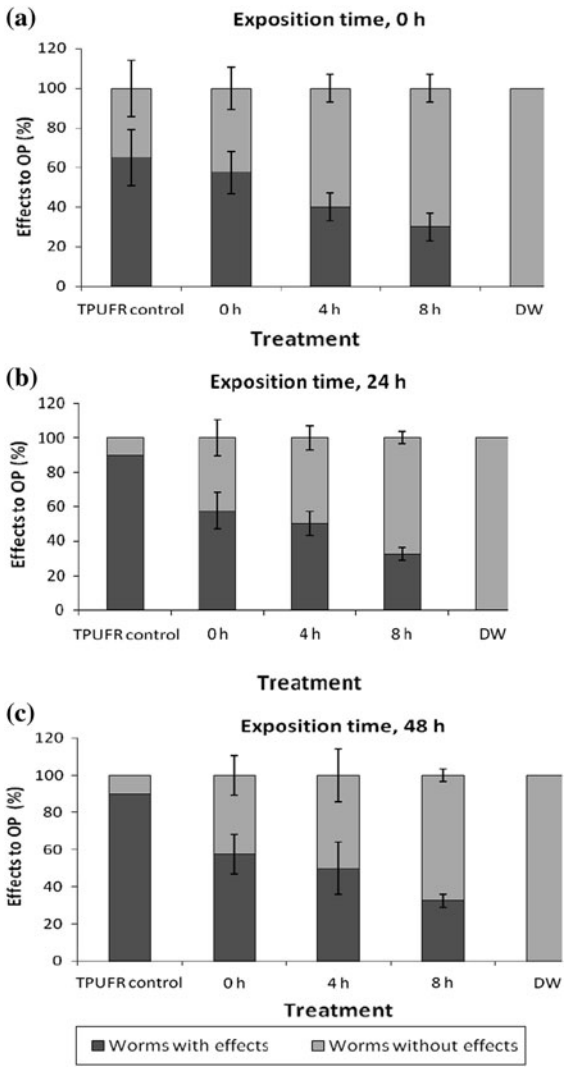

Fig. 3 Acute toxicity of organophosphates (stiffness and muscular incoordination) on *Eisenia foetida,* after being exposed to 0, 24, and 48 h to the effluent of the treatments. *TPUFR control* Effluent from the bioreactor control (tezontle sterile, mineral salts medium, and the mixture of MP and TCV); 0 h, 4 h, and 8 h, treatment times of pesticide mixture in TPUFR with immobilized bacteria, *DW* Distilled water–methanol (0.4% v/v)

affected individuals diminished gradually in the TPUFR effluent after biological treatment times of 0, 4, and 8 h. This behavior suggests that as a result of the biological removal process, the effluent is detoxified. The TPUFR effluent toxicity control was also assessed, and the percentage of worms exhibiting an effect of the treatment was still 90% after 48 h of exposure. *Eisenia foetida* proved to be an appropriate

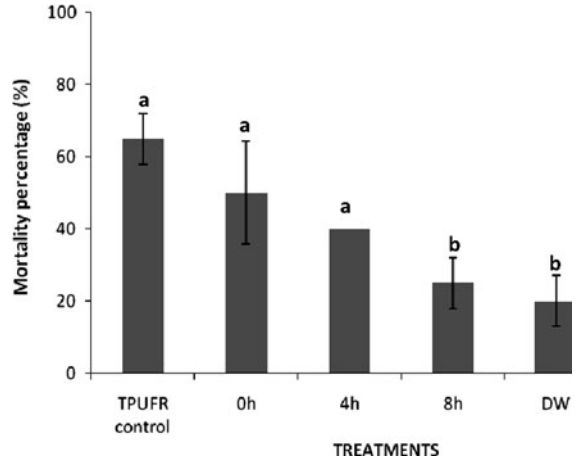

Fig. 4 Mortality (%) of *Eisenia foetida* after 72 h of exposure to the mixture of MP and TCV of bioreactor effluent. *TPUFR control* Effluent from the bioreactor control (tezontle sterile, mineral salts medium, and the mixture of MP and TCV); 0 h, 4 h, and 8 h, treatment time of pesticide mixture in TPUFR with immobilized bacteria, *DW* distilled water–methanol (0.4% v/v). *Different letters* express significant statistical differences ($\alpha = 0.05$)

biological model to evaluate the toxicity of the effluent treated with TPUFR because the symptoms of exposure were expressed conspicuously in this species.

As a part of the toxicity test, the mortality of the worms was quantified after 72 h of exposure to each of the treatments (Fig. 4). The mortality of the worms exposed to the control TPUFR effluent was 65%. This value was due to the non-biodegraded pesticides present; in the case of distilled water (DW), the mortality percentage was 20%, which was due to starvation because worms were kept unfed during the experiment.

In addition, worms exposed to the treated effluent for 0, 4, and 8 h of treatment in the TPUFR with the immobilized consortium of a biofilm on tezontle were observed to exhibit mortality rates that decreased significantly to levels that can be compared to treatment with distilled water.

Conclusions

The bacterial consortium immobilized in a biofilm on tezontle exhibited a considerable capacity for the removal of MP and TCV, which are the pesticides widely used in agriculture and stockbreeding in Mexico. Through the operation of a TPUFR

colonized by a bacterial consortium, a mixture of two OPs was successfully removed and detoxified. Acute toxicity tests with *Eisenia foetida* confirmed the detoxification of the solution containing the mixture of pesticides.

References

Aktar W, Sengupta D, Chowdhury A (2009) Impact of pesticides use in agriculture: their benefits and hazards. Interdisc Toxicol 2(1):1–12. doi:10.2478/v10102-009-0001-7

Bianchi E, Censabella I, Fascetti E (2008) Aerobic biodegradation of MtBE in an upflow fixed bed reactor. J Chem Technol Biotechnol 84(6):871–876. doi:10.1002/jctb.2133

Buchbinder J, Stephenson R, Dresser M, Pitera J, Scanlan T, Fletterick R (1998) Biochemical characterization and chrystallographic structure of an *Escherichia coli* protein from the phosphotriesterase gene family. Biochemistry 37(15):5096–5106. doi:10.1021/bi971707+

Burton S (2001) Development of bioreactors for application of biocatalysts in biotransformation and bioremediation. Pure Appl Chem 73(1):77–83

Chen W, Georgiou G (2002) Cell-surface display of heterologous proteins: from high throughput screening to environmental applications. Biotechnol Bioeng 5:496–503. doi:10.1002/bit.10407

Dasgupta S, Meisner C, Wheeler D (2010) Stockpiles of obsolete pesticides and cleanup priorities: a methodology and application for Tunisia. J Environ Manage 91(4): 824–830. doi:10.1016/j.jenvman.2009.10.012

de la Peña MM, Tehara SK, Hong T, Keasling JD (2006) Mineralization of paraoxon and its use as a sole C and P source by a rationally designed catabolic pathway in *Pseudomonas putida*. Appl Environ Microbiol 72(10): 6699–6706. doi:10.1128/AEM.00907-06

Galíndez-Nájera SP, Llamas-Martínez MA, Ruiz-Ordaz N, Juárez-Ramírez C, Mondragón-Parada ME, Ahuatzi-Chacón D, Galíndez-Mayer J (2009) Cyanuric acid biodegradation by a mixed bacterial culture of *Agrobacterium tumefaciens* and *Acinetobacter* sp. in a packed bed biofilm reactor. J Ind Microbiol Biotechnol 36:275–284. doi: 10.1007/s10295-008-0496-5

Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl Environ Microbiol 63(7):2802–2813

Ha J, Engler CR, Lee SJ (2008) Determination of diffusion coefficients and diffusion characteristics for chlorferon and diethylthiophosphate in Ca-alginate gel beads. Biotechnol Bioeng 100(4):698–706. doi:10.1002/bit.21761

Ha J, Engler CR, Wild JR (2009) Biodegradation of coumaphos, chlorferon, and diethylthiophosphate using bacteria immobilized in Ca-alginate gel beads. Bioresour Technol 100(3):1138–1142. doi:10.1016/j.biortech.2008.08.022

Hallas L, Adams W, Heitkamp M (1992) Glyphosate degradadtion by bacteria immobilized: field studies with industrial wastewater effluent. Appl Environ Microbiol 58(4):1215–1219

Jajuee B, Margaritis A, Karamanev D, Bergougnou M (2007) Kinetics of biodegradation of *p*-xylene and naphthalene and oxygen transfer in a novel airlift immobilized bioreactor. Biotechnol Bioeng 96(2):232–243. doi:10.1002/bit.21106

Jin-Woo K, Rainina I, Mulbry W, Engler R, Wild R (2002) Enhanced-rate biodegradation of organophosphate neurotoxins by immobilized nongrowing bacteria. Biotechnol Prog 18(3):429–436. doi:10.1021/bp0200346

Karstensen KH, Nguyen KK, Le BT, Pham HV, Nguyen DT, Doan TT, Nguyen HH, Tao MQ, Luong DH, Doan HT (2006) Environmentally sound destruction of obsolete pesticides in developing countries using cement kilns. Environ Sci Policy 9(6):577–586. doi:10.1016/j.envsci.2006.05.005

Khan FI, Husain T, Hejazi R (2004) An overview and analysis of site remediation technologies. J Environ Manage 71:95–122. doi:10.1016/j.jenvman.2004.02.003

Konstantinou IK, Hela DG, Albanis TA (2006) The status of pesticide pollution in surface waters (rivers and lakes) of Greece. Part I. Review on occurrence and levels. Environ Pollut 141:555-570. doi:10.1016/j.envpol.2005.07.024

Li F, Xu P, Feng J, Meng L, Zheng Y, Luo L, Ma1 C (2005) Microbial desulfurization of gasoline in a *Mycobacterium goodii* X7B immobilized-cell system. Appl Environ Microbiol 71(1):276–281. doi:10.1128/AEM.71.1.276-281.2005

Mansee AH, Chen W, Mulchandani A (2000) Biodetoxification of coumaphos insecticide using immobilized *Escherichia coli* expressing organophosphorus hydrolase enzyme on cell surface. Biotechnol Bioprocess Eng 5:436–440

Martin M, Mengs G, Plaza E, Garbi C, Sánchez A, Gibello A, Gutierrez F, Ferrer E (2000) Propachlor removal by *Pseudomonas* strain GCH1 in a immobilized-cell system. Appl Environ Microbiol 66(3):1190–1194

Moens T, Araya M, Swennen R, Waele DD (2004) Enhanced biodegradation of nematicides after repetitive applications and its effect on root and yield parameters in commercial banana plantations. Biol Fertil Soils 39:407–414. doi: 10.1007/s00374-004-0726-6

Mondragón-Parada ME, Ruiz-Ordaz N, Tafoya-Garnica A, Juárez-Ramírez C, Curiel-Quesada E, Galíndez-Mayer J (2008) Chemostat selection of a bacterial community able to degrade s-triazinic compounds: continuous simazine biodegradation in a multi-stage packed bed biofilm reactor. J Ind Microbiol Biotechnol 35:767–776. doi:10.1007/s10295-008-0347-4

Morgan-Sagastume JM, Noyola A (2008) Evaluation of an aerobic submerged filter packed with volcanic scoria. Bioresour Technol 99:2528–2536. doi:10.1016/j.biortech.2007.04.068

Mulchandani A, Kaneva I, Chen W (1999) Detoxification of organophosphate nerve agents by immobilized *Escherichia coli* with surface-expressed organophosphorus hydrolase. Biotechnol Bioeng 63(2):216–223. doi:10.1002/(SICI)1097-0290(19990420)63:2<216:AID-BIT10>3.0.CO;2-0

O′Reilly K, Crawford R (1989) Kinetics of p-Cresol degradation by an immobilized *Pseudomonas* sp. Appl Environ Microbiol 55:866–870

Olvera-Velona A, Capowiez I, Mascle O, Ortiz-Hernández L, Benoit P (2008) Assessment of the toxicity of ethyl-parathion to earthworms (*Aporrectodea caliginosa*) using behavioural, physiological and biochemical markers. Appl Soil Ecol 40(3):476–483. doi:10.1016/j.apsoil.2008.07.002

Ortiz-Hernández ML, Quintero-Ramírez R, Nava-Ocampo A, Bello-Ramírez AM (2003) Study of the mechanism of *Flavobacterium* sp. for hydrolyzing organophosphate pesticides. Fundametal Clin Pharmacol 17:717–723. doi:10.1046/j.1472-8206.2003.00202.x

Ortiz-Hernández ML, Sánchez-Salinas E, Olvera-Velona A, Folch-Mallol JL (2011) Pesticides in the environment: impacts and their biodegradation as a strategy for residues treatment. In Stoytcheva M (ed) Pesticides-formulations, effects, fate. ISBN: 978-953-307-532-7. InTech. Rijeka, pp 551–574

Pimentel D (2005) Environmental and economic costs of the application of pesticides primarily in the United States? Environ Dev Sustain 7:229–252. doi:10.1007/s10668-005-7314-2

Rama Krishna K, Ligy P (2009) Biodegradation of mixed pesticides by mixed pesticide enriched cultures. J Environ Sci Health 44:18–30. doi:10.1080/03601230802519520

Richins R, Mulchandani A, Chen W (2000) Expression, immobilization, and enzymatic characterization of cellulose-binding domain-organophosphorus hydrolase fusion enzymes. Biotechnol Bioeng 69:591–596. doi:10.1002/1097-0290(20000920)69:6<591:AID-BIT2>3.0.CO;2-X

Santacruz G, Bandala E, Torres LG (2005) Chlorinated pesticides (2, 4–D and DDT) biodegradation at high concentrations using immobilized Pseudomonas fluorescens. J Environ Sci Health 40:571–583. doi:10.1081/PFC-200061545

SAS Institute (2003) SAS 9.1. Institute Inc., Cary, NC, USA

Schmid A, Dordick J, Hauer B, Kiener A, Wubbolts M, Witholt B (2001) Industrial biocatalysis today and tomorrow. Nature 11:258–268. doi:10.1038/35051736

Schwarzenbach R, Egli T, Hofstetter T (2010) Global water pollution and human health. Annu Rev Environ Resour 35:109–136. doi:10.1146/annurev-environ-100809-125342

Sethunathan N, Yoshida T (1973) A *Flavobacterium* sp. that degrades diazinon and parathion. Can J Microbiol 19:873–875. doi:10.1139/m73-138

Sheeja RY, Murugesan T (2002) Mass transfer studies on the biodegradation of phenols in up-flow packed bed reactors. J Hazard Mater 9:287–301. doi:10.1016/S0304-3894(01)00319-3

Singh B (2009) Organophosphorus-degrading bacteria: ecology and industrial applications. Nat Rev Microbiol 7:156–164. doi:10.1038/nrmicro2050

Singh B, Walker A (2006) Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev 30(3):428–471. doi:10.1111/j.1574-6976.2006.00018.x

Sorgob MA, Vilanova E (2002) Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol Lett 128(1–3):215–228. doi:10.1016/S0378-4274(01)00543-4

Struthers JK, Jayachandran K, Moorman TB (1998) Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. Appl Environ Microbiol 64(9):3368–3375

Yair S, Ofer B, Arik E, Shai S, Yossi R, Tzvika D, Amir K (2008) Organophosphate degrading microorganisms and enzymes as biocatalysts in environmental and personal decontamination applications. Crit Rev Biotechnol 28(4):265–275. doi:10.1080/07388550802455742

Yáñez-Ocampo G, Sanchez-Salinas E, Jimenez-Tobon GA, Penninckx M, Ortiz-Hernández ML (2009) Removal of two organophosphate pesticides by a bacterial consortium immobilized in alginate or tezontle. J Hazard Mater 168:1554–1561. doi:10.1016/j.jhazmat.2009.03.047