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Biosensor-controlled degradation of chlorpyrifos and chlorfenvinfos using a phosphotriesterase-based detoxification column

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ABSTRACT

This works presents the development of a detoxification system based on bacterial phosphotriesterase (PTE) for the degradation of organophosphate (OP) insecticides in water. PTE was immobilised on an activated agarose gel via covalent coupling. Two different OPs were studied, chlorpyrifos and chlorfenvinfos, due to their importance in the field of water policy. The efficiency of insecticide degradation was controlled using a highly sensitive biosensor allowing the detection of OP concentration as low as 0.004 μg L^{-1}. Under optimum conditions, it was shown that a column incorporating 500 IU of PTE was suitable for the detoxification of solutions containing either isolated pesticides or pesticides mixtures, even at concentrations higher than authorized limits. Finally, the method was shown to be adapted to the decontamination of real samples of pesticides with concentrations up to 20 μg L^{-1}.

1. Introduction

Due to their high acute toxicity and risk towards the population, some directives have been established to limit the presence of pesticides in water and food resources. Concerning the quality of water for human consumption, the European Council Directive 98/83/EC (Drinking Water Directive) has set a maximum admissible concentration of 0.1 μg L^{-1} per pesticide and 0.5 μg L^{-1} for the total amount of pesticides. However, this directive does not mention the admissible concentrations in waters intended for human consumption before treatment, obliging each European country to establish internal rules. In France, the decree No. 2001-1220 of 20 December 2001 sets a maximum admissible concentration of 2 μg L^{-1} per pesticide and 5 μg L^{-1} for total pesticides.

Organophosphates (OPs) are a class of synthetic compounds developed from the Second World War, which are used as pesticides and nerve agents (Raushel, 2002; Bajgar, 2004). Since the removal of organochlorine insecticides from use, OPs have become the most widely used insecticides. They are normally used for agricultural, industrial, household and medical purposes. OPs poison insects and mammals by phosphorylation of the acetylcholinesterase enzyme (AChE) at nerve endings (Dubois, 1971; Ecobichon, 2001). Inactivation of this enzyme results in an accumulation of acetylcholine leading to an overstimulation of the effector organ (Aldridge, 1950; US EPA, 1999). The hazardous nature of OPs and their wide usage have led to concerted efforts for developing highly sensitive detection techniques as well as efficient destruction methods for these compounds (Gill and Ballesteros, 2000).

Detection techniques are fundamental in order to accurately determine the level of contamination of waters. They are classically based on extraction, cleanup and analysis using gas chromatography or liquid chromatography coupled to sensitive and specific detectors (Lacorte et al., 1993; Geerdink et al., 2002; Ballesteros and Parrado, 2004; Kuster et al., 2006). These methods are highly sensitive and allow the determination of a large number of compounds but they are costly, long and/or complex. Alternative methods are mainly based on the biosensor technology. Numerous sensors have been described for OP determination based on the inhibition of cholinesterases (Andreescu and Marty, 2006); some of them involve recombinant acetylcholinesterases specially tailored to enhance their sensitivity to specific inhibitors (Bachmann et al., 2000). Recently, the association of such a mutant enzyme with an original oriented immobilisation technique has led us to design a disposable sensor for the highly sensitive detection of OPs (Istamboulie et al., 2007).

Degradation of OPs is a mandatory step for the detoxification of waters devoted to human consumption. Conventional methods generally involve incineration and chemical hydrolysis of these products (Holm, 1996). However, these techniques are hindered by their high environmental impact as well as logistics and operational safety issues (Gill and Ballesteros, 2000). Efforts have thus been made to find safer technologies, mainly based on biotechnological degradation techniques. With this aim, enzymes able to degrade OPs have been the focus of several research studies in

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the environmental decontamination/detoxification field (Sogorb and Vilanova, 2002). OP-hydrolysing enzymes have been found in Bacteria, Archaea and Eukarya. The best-known and characterized enzyme is phosphotriesterase (PTE), firstly isolated from the soil bacterium *Pseudomonas diminuta*. Despite its natural substrate and main physiological function are still unknown (Merone et al., 2008; Elias et al., 2008), PTE has the ability of hydrolysing most OPs, including chemical warfare agents like sarin or sonan (Raushel, 2002; Ghanem and Raushel, 2005). Due to these properties, PTE has been also described as a potential catalytic scavenger for the treatment of OP poisoning (Masson et al., 1998).

This work aims to study the PTE-catalysed degradation of chlorpyrifos (CPO) and chlorfenvinfos (CFV), two insecticides included since 2001 in the list of priority substances in the field of water policy established by the European Parliament (Decision 2455/2001/EC). The ability of a detoxification column for degrading CPO and CFV has been investigated using simple solutions or mixtures of these pesticides. Concentrations much higher than the maximum concentration authorized before treatment have also been tested in order to evaluate the efficiency of the process. The efficiency of the detoxification process has been monitored using a highly sensitive biosensor based on recombinant acetylcholinesterase.

2. Materials and methods

2.1. Chemicals and stock solutions

PTE recombinant enzyme was kindly provided by Prof. D. Fournier (Toulouse, France). This enzyme was first isolated from *Flavobacterium* sp. and cloned in *Escherichia coli* (Denis-Quanquin et al., 2007). Paraoxon (phosphoric acid, O,O-diethyl O-(4-nitrophenyl) ester) (purity 97.6%) was used as reference substrate and was purchased from Sigma (St. Quentin Fallavier, France). A 0.1 M stock solution was prepared in acetonitrile and stored at 4 °C.

Genetically modified AChE from *Drosophila melanogaster* type B394 was produced by Protein Bio Sensor (PBS, Toulouse, France). The substrate acetylthiocholine chloride (ATCh) was purchased from Sigma. A 0.1 M ATCh stock solution was daily prepared in water and stored at 4 °C.

The two OP insecticides CPO (phosphoric acid, O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester) (purity 94%), and CFV (phosphoric acid, 2-chloro-1-(2,4-dichlorophenyl) ethenyl diethyl ester) (purity 95%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock solution of pesticides (10^{-3} M) were prepared in acetonitrile and stored at 4 °C.

Iminodiacetic acid (IDA)-pre-activated magnetic beads (diameter 300 nm) were from "Histidine adem-Kit for His-Tagged protein purification" provided by Ademtech SA (Pessac, France). Sepharose 4B activated by cyanogen bromide was purchased from Amersham (Buckinghamshire, UK).

The pastes used for screen-printing, i.e. Electrodag PE-410, 423SS and 6037SS were obtained from Acheson (Plymouth, UK). Cobalt-phtalocyanine-modified carbon paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK). A glycerophthalic paint (Astral, France) was used as insulating layer. Transparent PVC sheets (200 mm × 100 mm × 80.5 mm) were used as printing supports.

2.2. Apparatus

Spectrophotometric measurements were performed at 25 °C using a Hewlett Packard diode array 8451A spectrophotometer. Amperometric measurements were carried out at 30 °C with a 641VA potentiostat (Metrohm, Switzerland), connected to a BD40 (Kipp & Zonen, The Netherlands) flatbed recorder.

Screen-printed electrodes were produced using a semi-automatic DEK 248 printing machine according to a procedure previously described (Istamboulie et al., 2007). The working electrode was a 4-mm diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight line.

2.3. Chromatographic determination of CPO and CFV

Chromatographic determination of pesticides was carried out using a Merck L-6220 HPLC-system fitted with a Supelcosil LC18 reversed phase column (25 cm × 4 mm, 5 μm) (Supelco, France). The elution solvent used was acetonitrile/water (70:30), at a flow rate of 1 mL min^{-1}. The detection of pesticides was performed at 220 nm using a Merck L-4000 UV. The retention times observed for CPO and CFV were respectively 5.4 min and 6.3 min. The method did not involve any pre-concentration step, the quantification limits obtained for CPO and CFV were respectively 3×10^{-7} and 2.5×10^{-7} M.

2.4. Determination of enzymatic activities

Before immobilisation, enzymatic activity of B394 was measured spectrophotometrically at 412 nm using 5,5′-dithiobis(2-nitrobenzoic acid) according to Ellman's method (Ellman et al., 1961). PTE activity was measured in 10 mM phosphate buffer pH 8 by monitoring the formation of paranitrophenol at 405 nm using 1 mM of the reference substrate Paraoxon (Istamboulie et al., 2009).

2.5. Principle of OP insecticides detoxification by PTE

The activity of PTE for different OP substrates was already investigated and described in a previous work (Istamboulie et al., 2009). PTE was shown to exhibit a high affinity for both CPO and CFV, but with big differences in activities: the biodegradation rate of CPO was 10 000-fold faster than for CFV. Moreover it was demonstrated that CFV acts as a competitive inhibition of PTE for CPO degradation (Istamboulie et al., 2009).

Fig. 1 presents the reactions catalysed by PTE for the oxidation of CPO and CFV, the main products of hydrolysis are respectively O,O-diethyl phosphoric ester, 3,5,6-trichloro-2-pyridinol and 2-chloro-1-(2′,4′-dichlorophenyl)vinyl alcohol. It is important to mention that these detoxification products are not inhibitors of AChE and are not pointed out as harmful chemicals by European regulation and WHO recommendations on water quality (Sogorb and Vilanova, 2002).

2.6. Immobilisation of PTE on Sepharose Gel 4B

The gel was prepared by mixing 1 g of Sepharose 4B in 10 mL of HCl 1 mM, this suspension was then gently casted in an Omnifit column (internal volume 10 mL) (Cambridge, England). The column was cleaned with 200 mL of 1 mM HCl and then equilibrated using 50 mL of 0.1 M phosphate buffer pH 8. After the determination of enzyme activity (Istamboulie et al., 2009), PTE solutions of different concentrations were gently mixed with the Sepharose gel for 2 h at room temperature (25 °C). The saturation step was then performed by passing 10 mL of 1 M ethanolamine through the gel for another 2 h. Finally, the gel was cleaned using 20 mL of 0.1 M acetate buffer pH 4 and equilibrated with 20 mL of 0.1 M phosphate buffer pH 8. The final bed volume was 4 mL, before use the column was stored at 4 °C.

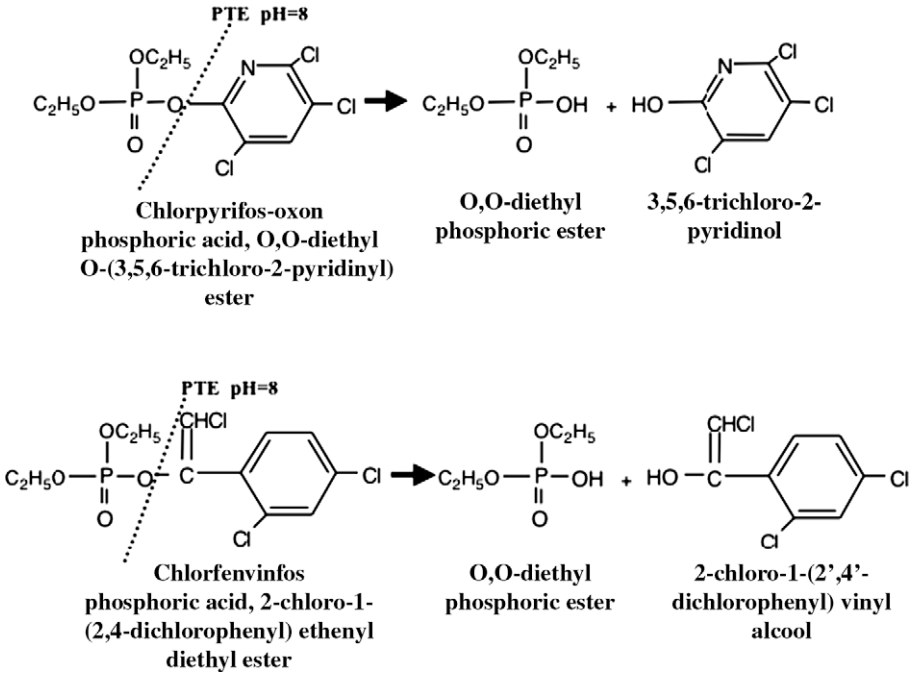

Fig. 1. Molecular structures of CPO (MW = 334 g mol^{-1}) and CFV (MW = 359.6 g mol^{-1}) and their hydrolysis reactions catalysed by phosphotriesterase (PTE).

2.7. Immobilisation of AChE on magnetic nanoparticles via Ni-His affinity

This method is based on the ability of some metal ions (Ni^{2+}, Cu^{2+}, Zn^{2+}) to bind strongly but reversibly to peptides and proteins containing histidine or cysteine residues. In affinity chromatography, chelating agents like nitrilotriacetic acid or IDA are generally used to bind these metal ions on solid supports to perform His-tagged-enzyme purification. In this work, pre-activated magnetic beads carrying Ni-IDA complexes have been used to immobilise a genetically modified AChE (B394) having a hexa (histidine) tail. The general principle of this procedure, the immobilisation protocol and biosensor fabrication were described in previous works (Istamboulie et al., 2007). The biosensor was fabricated by placing 1 μL of enzyme functionalised beads suspension on the surface of the working electrode, beforehand fitted with a 4-mm diameter magnet on the back of the electrode.

2.8. Amperometric measurements of acetylcholinesterase activity

Amperometric measurements were performed in stirred solutions using a 10 mL cell, the temperature being regulated at 30 °C by a water circulation system. The electrodes were tested in 10 mL of 0.1 M phosphate buffer at a working potential of +100 mV vs. Ag/AgCl, which corresponds to the oxidation of thiocholine, the product of the enzymatic hydrolysis of the acetylthiocholine, in the presence of cobalt phtalocyanine as electron mediator. The current intensity was recorded and, after current stabilisation, 1 mM ATCh (final concentration in the cell) was injected. The time necessary to reach the plateau was 2–3 min. The measured signal corresponded to the difference of current intensity between the baseline and the plateau. The cell was washed with distilled water between measurements.

The pesticide detection was made in a three-step procedure as follows: first, the initial response of the electrode at the injection of the ATCh (1 mM) was recorded three times, then the electrode was incubated in a solution containing a known concentration of insecticide, and finally the residual response of the electrode was recorded again in the same conditions. The percentage of inhibition was correlated with the insecticide concentration. The limit of detection was calculated as the insecticide concentration inducing a 10% decrease of the biosensor response.

3. Results and discussion

3.1. Biosensor characterisation and kinetic study of the immobilised enzyme

The stability of the sensors needs to be evaluated in order to ensure that the decrease in the signal during inhibition measurements is due to enzyme inactivation and not to enzyme leaking. The operational stability of immobilised B394 acetylcholinesterase was estimated by successively measuring the response of the same enzyme electrode to 1 mM ATCh, at 100 mV vs. Ag/AgCl. The sensors were washed between tests with distilled water. All three types of sensors were shown to be stable for at least 10 consecutive measurements. The biosensors presented a good reproducibility, with a signal maximum variation of 5%. The average response to 1 mM ATCh for $n = 5$ electrodes prepared in the same experimental conditions was 895 ± 56 nA. These reproducibility data refers to the mean value of ten assays for each electrode.

The incubation time of each sensor with various concentrations of pesticides was optimized in order to achieve the highest sensitivity of detection. It was observed that an incubation time of 10 min was sufficient to achieve the maximum inhibition whatever the pesticide concentration used. This incubation time was thus applied in further experiments.

The inhibition effect of CPO and CFV on sensors incorporating 1 mU of AChE immobilised on the electrode has been already described in previous works (Istamboulie et al., 2007). The limit of detection I_{10}, the I_0, I_{50}, and I_{100} were calculated corresponding to the pesticide concentration inducing respectively 10%, 0%, 50% and 100% inhibition. These results are summarized in Table 1. It is important to note that 0% inhibition corresponds to a pesticide concentration of 0.002 μg L^{-1}, a concentration 50-fold lower than

Table 1
Characteristics of the biosensors based on B394-AChE (1 mU/sensor) for the detection of CPO and CFV (*I*% = percentage of AChE inhibition, C = pesticide concentration in μg L^{-1}).

	Equation	I_0 (μg L^{-1})	I_{10} (μg L^{-1})	I_{50} (μg L^{-1})	I_{100} (μg L^{-1})
CPO	$I\% = 15.8 \text{ Ln (C)} + 96.49$ $r^2 = 0.9946$	0.002	0.004	0.053	1.248
CFV	$I\% = 10.7 \text{ Ln (C)} + 67.35$ $r^2 = 0.9965$	0.002	0.005	0.197	21.26

the maximum concentration tolerated for drinking water (0.1 μg L^{-1}).

3.2. Optimisation of the detoxification column

The hydrolysis of high concentrations of CPO and CFV by immobilised PTE was firstly studied to evaluate the efficiency of the detoxification process for these two pesticides. This study was carried out using a 2.5 mL syringe packed with 0.8 mL of Sepharose Gel 4B containing 8 IU of PTE. All experiments were performed in 0.1 M phosphate buffer pH 8, at 25 °C. Each experiment was repeated at least three times.

In first attempts, the control of the pesticide solution flow rate (1 mL min^{-1}) was ensured using a peristaltic pump fitted with Tygon tubes. However, a very strong adsorption of both CPO and CFV was observed in these conditions, leading to aberrant and non-reproducible results. To overcome this problem, it was opted for the use of a classical HPLC pump (Merck L-6220) fitted with polytetrafluorethylene (Teflon) tubes.

The efficiency of the developed detoxification column was studied using CPO and CFV. The effect of contact time on the hydrolysis of a 0.33 mg L^{-1} (10^{-6} M) CPO solution was studied by varying the flow rate from 1.5 to 0.1 mL min^{-1}. The hydrolysis efficiency was controlled using a biosensor based on B394-AChE, which allows detecting as low as 4 ng L^{-1} of insecticide (Istamboulie et al., 2007). As expected, decreasing the flow rate allows increasing the contact time thus the percentage of hydrolysis. An optimum flow rate of 0.33 mL min^{-1} was selected, corresponding to a contact time of 2.5 min. In these conditions, no inhibition of the biosensor was observed, allowing us to assume that the CPO was completely hydrolysed. The same conditions applied to a 0.36 mg L^{-1} (10^{-6} M) solution of CFV lead to the hydrolysis of only 50% of the pesticide. Lowering the flow rate did not allow reaching a total hydrolysis of CFV, even when using low concentrations. For instance, the hydrolysis percentage of a 3 μg L^{-1} (10^{-8} M) solution was respectively 70%, 80% and 90% using flow rates of 0.33, 0.2 and 0.1 mL min^{-1}. Consequently, it was decided to increase dramatically the amount of immobilised PTE to achieve a satisfactory degradation of CFV, whatever its concentration. A total amount of immobilised PTE of 500 IU was thus chosen for further experiments.

3.3. Bio-detoxification of CPO and CFV in a flow system

A detoxification system was prepared using a 10 mL Omnifit column filled with 4 mL of Sepharose Gel 4B previously loaded with 500 IU of PTE. The column was connected via Teflon tubes to a HPLC pump, and the flow rate was set to either 0.5 or 1 mL min^{-1}, equivalent to contact times of respectively 8 and 4 min. Due to the high activity of PTE for CPO, the limiting factor lies in the ability of the column to efficiently degrade CFV as well as mixtures of CPO and CFV. Concentrations of CFV ranging from

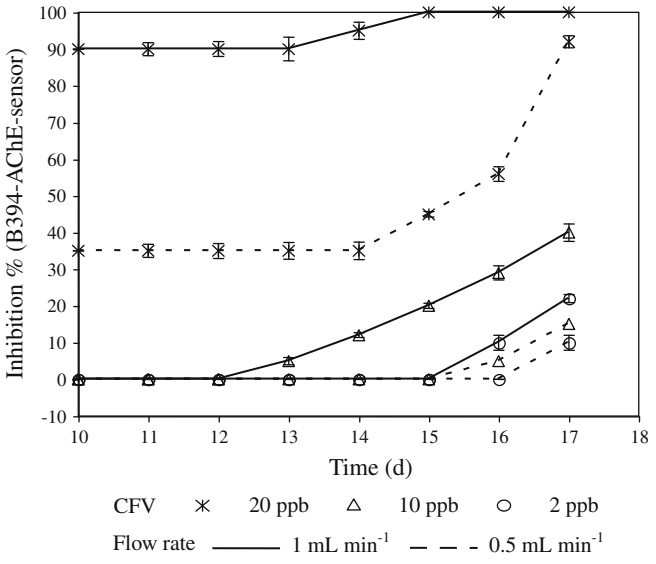

Fig. 2. Evaluation of the stability in time of the detoxification column by monitoring its ability to degrade CFV. Determination of the inhibition rate of a B394-AChE-based sensor as a function of storage time at 25 °C, using various concentrations of CFV and two different flow rates: 0.5 mL min^{-1} and 1 mL min^{-1}.

Table 2
Concentrations of CPO and CFV measured using a B394-AChE-based biosensor before and after treatment on a detoxification column loaded with 500 IU of PTE.

	Before treatment		After treatment – flow rate 1 mL min^{-1}		After treatment – flow rate 0.5 mL min^{-1}	
	Actual concentration (μg L^{-1})	Inhibition (%)	Inhibition (%)	Calculated concentration (μg L^{-1})	Inhibition (%)	Calculated concentration (μg L^{-1})
CFV	0.1	43	0	<0.005	0	<0.005
	0.3	55	0	<0.005	0	<0.005
	0.5	60	0	<0.005	0	<0.005
	0.7	64	0	<0.005	0	<0.005
	1	67	0	<0.005	0	<0.005
	2	75	0	<0.005	0	<0.005
	5	84	5	<0.005	0	<0.005
	10	95	8	<0.005	0	<0.005
	12.5	100	43	0.1	0	<0.005
	15	100	78	2	8	<0.005
	20	100	90	10	35	<0.1
CPO	334	100	0	<0.004	0	<0.004
CPO + CFV	334 (CPO) + 20 (CFV)	100	90	10	35	<0.1 (CPO + CFV)

Table 3
Analyse of mixtures of CPO and CFV before and after treatment with the detoxification column. Concentrations of CPO and CFV measured using a B394-AChE-based biosensor in surface water samples spiked with CPO and CFV.

Sample	Before treatment			After treatment – flow rate 1 mL min^{-1}		After treatment – flow rate 0.5 mL min^{-1}	
	CPO ($\mu g\ L^{-1}$)	CFV ($\mu g\ L^{-1}$)	Inhibition (%)	Measured inhibition (%)	Calculated concentration ($\mu g\ L^{-1}$)	Measured inhibition (%)	Calculated concentration ($\mu g\ L^{-1}$)
A	10	0	100	0	<0.004	0	<0.004
B	0	10	100	15	0.007	0	<0.005
C	2	2	100	0	<0.005	0	<0.005
D	5	5	100	0	<0.005	0	<0.005
E	10	10	100	21	0.01	0	<0.005

0.1 to 20 $\mu g\ L^{-1}$ were thus passed through the column, and the pesticide concentration was checked before and after treatment using a biosensor based on B394-AChE. The maximum CFV concentration, *i.e.* 20 $\mu g\ L^{-1}$, was chosen in order to amply fulfil the French regulation, which sets at 2 $\mu g\ L^{-1}$ the maximum pesticide concentration allowed for drinking water intended for human consumption before treatment (IFEN, 2007). Table 2 summarizes the results obtained after detoxification for each pesticide solution. Although CFV was shown to be a very poor substrate of PTE (Istamboulie et al., 2009), a high enzyme loading (500 IU) allowed achieving a satisfactory degradation of CFV when the flow rate was set to 0.5 mL min^{-1}. In that case, the output pesticide concentration was always lower than 0.1 $\mu g\ L^{-1}$, which corresponds to the maximum pesticide concentration allowed for drinking water. At higher flow rate (1 mL min^{-1}), CFV solutions with concentrations higher than 12.5 $\mu g\ L^{-1}$ were only partially degraded, so that the output water was not suitable for human consumption. The mixture of a high concentration of CPO (334 $\mu g\ L^{-1}$) and 20 $\mu g\ L^{-1}$ of CFV was also satisfactory degraded on this column using a flow rate of 0.5 mL min^{-1}.

The stability with time of the detoxification column was studied during 18 d using different concentrations of CFV (2, 10, 20 $\mu g\ L^{-1}$). A very good stability was achieved for 2 week using a flow rate of 0.5 mL min^{-1} (Fig. 2).

3.4. Application to real samples

Five water samples taken from a lake (Villeneuve-de-la-Raho, France) were spiked with known concentrations of CPO and CFV. Spiked and unspiked water were analyzed using the sensor before and after treatment by the detoxification column, using flow rates of either 1 or 0.5 mL min^{-1}. Unspiked samples revealed no inhibitory effect, thus proving the absence of matrix effects. The inhibition ratios and the corresponding pesticide concentrations obtained with spiked samples are presented in Table 3. It was shown that whatever the flow rate used, the pesticide concentration after treatment was compatible with EC regulation for drinking water.

4. Conclusions

This work shows the potentiality of using phosphotriesterase as a degradation enzyme for waters polluted by OP pesticides. Two different insecticides were studied, chlorpyrifos and chlorfenvinfos, due to their importance in the field of water policy and different behaviours towards PTE. Under optimum conditions, a column containing 500 IU of phosphotriesterase was shown to immediately detoxify CPO solutions, whatever their concentration. In the case of CFV, the detoxification was much lower, due to the very slow hydrolysis of this pesticide by PTE. However, CFV solutions with concentrations up to 15 $\mu g\ L^{-1}$ could be efficiently detoxified, thus fulfilling the European regulations for human consumption water. Pesticides mixtures were also satisfactory degraded, even at concentrations higher than authorized limits. Finally, the method was shown to be adapted to the decontamination of real samples previously spiked with pesticides concentrations up to 20 $\mu g\ L^{-1}$.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2009.10.037.

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