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A bi-enzymatic whole cell conductometric biosensor for heavy metal ions and pesticides detection in water samples

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Abstract

A conductometric biosensor using immobilised *Chlorella vulgaris* microalgae as bioreceptors was used as a bi-enzymatic biosensor. Algae were immobilised inside bovine serum albumin membranes reticulated with glutaraldehyde vapours deposited on interdigitated conductometric electrodes. Local conductivity variations caused by algae alkaline phosphatase and acetylcholinesterase activities could be detected. These two enzymes are known to be inhibited by distinct families of toxic compounds: heavy metals for alkaline phosphatase, carbamates and organophosphorous (OP) pesticides for acetylcholinesterase. The bi-enzymatic biosensors were tested to study the influence of heavy metal ions and pesticides on the corresponding enzyme. It has finally appeared that these biosensors are quite sensitive to Cd^{2+} and Zn^{2+} (limits of detection (LOD) = 10 ppb for a 30 min long exposure) while Pb^{2+} gives no significant inhibition as this ion seems to adsorb on albumin preferably. For pesticides, first experiments showed that paraoxon-methyl inhibits *C. vulgaris* AChE contrary to parathion-methyl and carbofuran. Biosensors were then exposed to different mixtures (Cd^{2+}/Zn^{2+}, Cd^{2+}/paraoxon-methyl) but no synergetic or antagonist effect could be observed. A good repeatability could be achieve with biosensors since the relative standard deviation did not exceed 8% while response time was 5–7 min.

A comparison between inhibition levels obtained with biosensors (after a 30 min long exposure) and bioassays (after a 240 min long exposure) has finally shown a similar LOD for both Cd and Zn (LOD = 10 ppb).

Keywords: Bi-enzymatic biosensor; *Chlorella vulgaris* microalgae; Heavy metal ions; Pesticides; Alkaline phosphatase activity (APA); Acetylcholinesterase activity (AChE)

1. Introduction

Aquatic ecosystem management requires early warning systems (EWS) for on line and in situ monitoring. Biosensors can be considered as competitive tools for environmental monitoring because of their specificity, their fast response and their low cost (Dennison and Turner, 1995).

Recently, many works have led to the development of biosensors using immobilised enzymes. These sensors have been used for the detection of toxic compounds such as heavy metals, pesticides, phenols, etc. However, the family of pollutants that can be detected depends on the enzyme used: for instance, organophosphorous and carbamates with acetylcholinesterase (Marty et al., 1993; Andres and Narayanaswamy, 1997; Dzyadevych et al., 2002; Ciucu et al., 2003; Wan et al., 1999), heavy metals with urease (Zhylyak et al., 1995), phenols with tyrosinase (Mai Anh et al., 2002, 2004). Because of their different optimal operational conditions, these enzymes cannot be immobilised on the same sensor easily. Arkhipova et al. (2001) have proposed a multibiosensor based on enzyme inhibition

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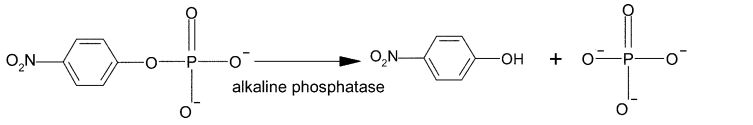
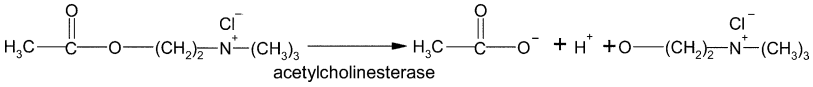
for pollutant detection (Arkhipova et al., 2001). However, they have faced stability problems when immobilising several enzymes on a multi-detection array. Furthermore, as generally commercially available enzymes are expensive, costs for multi-detection biosensors can be relatively high.

The use of micro-organisms for multi-detection can be a good alternative, each living cell containing a large number of enzymes. For electrochemical detection, membrane-bound enzymes are of particular interest since enzymatic reactions occur on cell surface. It has been shown that for *Chlorella vulgaris* microalgae, some alkaline phosphatases (Durrieu and Tran-Minh, 2003) and esterases (Durrieu et al., 2004) belong to the cell wall, their activities can then be monitored rapidly. They also enable electrochemical detection using conductometric biosensors as these two enzymatic reactions involve either consumption or production of charged species and, therefore, lead to a global change in the ionic composition of the sample as shown on the following equations:





A previous study has shown that conductometric biosensors using immobilised *C. vulgaris* can be used to follow alkaline phosphatase activity (Chouteau et al., 2004). This work aims at monitoring *C. vulgaris* alkaline phosphatases and cholinesterases activities with the same conductometric biosensor. Indeed, these two enzymes are known to be inhibited by distinct families of pollutants: heavy metals for alkaline phosphatase (Durrieu and Tran-Minh, 2003), organophosphorous and carbamates for esterases as previously mentioned. On the same biosensor, it could then be possible to detect heavy metals and some pesticides. Enzyme stability would not be a problem since algal enzymes would remain in their natural cellular environment. Besides, cost would be low since algae can be grown easily.

Finally, contrary to biosensors using pure enzymes, whole cells can give information on the ecotoxicological effects of pollutants as parameters such as bioavailibility, external parameters influence (temperature, pH, etc.), cell sensitivity will be integrated to the biosensor response.

This paper presents the last developments concerning multi-detection of *C. vulgaris* alkaline phosphatase and acetylcholinesterase activities with conductometric biosensors. In a recent paper, it has been shown that heavy metal ions (Cd^{2+}, Zn^{2+} and Pb^{2+}) could be detected using algal conductometric biosensors (Chouteau et al., 2004). Further experiments with heavy metal ions are presented herein as well as toxicity tests using organophosphorous and carbamates.

2. Experimental

2.1. Materials

The *C. vulgaris* strain (CCAP 211/12) was purchased from the culture collection of Algae and Protozoa at Cumbria, UK. The axenic algal strain was grown in the culture medium and under conditions described by the international organisation for standardisation (ISO 8692, 1989). APA measurements require a 21 day long starvation period in culture medium without phosphate (Fitzgerald and Nelson, 1966). Algal concentrations were 2 to 5×10^7 cells/ml (Algae are counted under a microscope using a Thoma numeration cell).

Bovine serum albumin (BSA) and 25% aqueous solution of glutaraldehyde (GA) were purchased from Sigma–Aldrich.

Paranitrophenyl phosphate (pNPP) and methyl-umbelliferyl-phosphate (MUP) from Sigma–Aldrich were used as substrates to determine alkaline phosphatase activity. Three substrates were tested for acetylcholinesterase activity: fluorescein diacetate (FDA), acetylcholine chloride (AChCl) and butyrycholine chloride (BChCl) from Sigma–Aldrich.

$Cd(NO_3)_2$, $ZnSO_4$ and $Pb(NO_3)_2$ salts (of analytical grade) were used as potential inhibitors for alkaline phosphatase activity. For esterase inhibition, carbuforan (CF), parathion-methyl (MP) and paraoxon-methyl (MPx) were tested. All other reagents were of analytical grade.

2.2. Sensor design

The conductometric transducers were fabricated at the Institute of Chemo- and Biosensorics (Munster, Germany) (Trebbe et al., 2001). Two pairs of Pt (150 nm thick) interdigitated electrodes were made by the lift-off process on the Pyrex glass substrate. The Ti intermediate layer of a 50 nm thick was used to improve adhesion of Pt to substrate. Central part of the sensor chip was passivated by Si_3N_4 layer to define the electrodes working area. Both the digits width and interdigital distance were 10 μm, and their length was about 1 mm. Thus, the sensitive part of each electrode was about 1 mm^2.

Measurements are based on the detection of solution conductivity variations inside BSA membranes containing microalgae. Alkaline phosphatases and cholinesterases, as many other enzymes, induce catalytic reactions

consuming/producing different ionic species resulting in measurable conductivity changes.

2.3. Algae immobilisation

In a previous work, algae have been immobilised successfully using bovine serum albumin and glutaraldehyde (GA) as a crosslinker (Chouteau et al., 2004).

The active membrane was formed by cross-linking algae with BSA in saturated GA vapours. This protocol was adapted from pure enzyme membranes used with some conductometric and ISFET biosensors (Dzyadevych et al., 1994; Shul'Ga et al., 1994). A mixture containing 100 μl algae solution and 10% (w/v) BSA was deposited on the sensitive area of the electrode using a drop method. Another mixture of 100 μl culture medium without phosphate and 10% (w/v) BSA was deposited on the other electrode used as a reference for differential measurements.

The sensor chips were placed in saturated GA vapours for 20 min. After exposure, membranes were dried at room temperature from 15 to 30 min. Enzymatic activity was stable for 20 days of storage in culture medium without phosphate at 4 °C.

2.4. Measurements

2.4.1. Enzymatic reaction measurements

For *biosensors*, measurements were carried out in daylight at room temperature in a 5 ml glass cell filled with

- for APA, Tris–HCl buffer (10 mM, pH 8.5) and $MgCl_2$ (1 mM);
- for AChE, KH_2PO_4 buffer (2.5 mM or 5 mM, pH 8).

Biosensors were immersed in this vigorously stirred solution. After stabilisation of the output signal, different aliquots of the substrate stock solution were added into the vessel. The differential output signal (d*S*) was registered using a "home made" conductometric laboratory apparatus and the steady state response of the biosensor was plotted against the substrate concentration.

For *bioassays*, free algae are used and measurements in microplates are based on fluorescence detected by a spectrofluorimeter (Fluostar, BMG). The alkaline phosphatase and esterase enzymatic reactions using MUP (2 mM) and FDA (2 mM) respectively as substrates give fluorescent products, MUF (methyl-umbelliferone) and fluorescein, that can be easily detected using an optic fibre (APA: $\lambda_{excitation} = 365$ nm and $\lambda_{emission} = 460$ nm, AE: $\lambda_{excitation} = 480$ nm and $\lambda_{emission} = 538$ nm) (Durrieu et al., 2003). Assays were carried out in 96 wells microplates (volume: 300 μl). For each substrate concentration, eight replicates were carried out. The composition in each well is:

- for APA: Tris–HCl buffer (0.1 M, pH 8.5), $MgCl_2$ (1 mM);
- for AE: citrate buffer (10 mM, pH 5.4), $MgCl_2$ (1 mM).

2.5. Toxicity measurements

In this work, enzymatic activities are "early toxicity signals" and their inhibitions can be considered as efficient signals of the presence of pollutants in samples.

For *biosensors*, d*S* was measured for a definite substrate concentration. The biosensor was then preincubated in a test solution for 30–60 min. After washing, d*S* before (dS_{before}) and after exposure (dS_{after}) to the test solution were compared and the residual activity rate was calculated.

For *bioassays*, 48 wells microplates were filled with algal solution. After sedimentation, culture medium could be removed and replaced by the test solution. Exposures last 120 and 240 min. After removing the test solution and resuspending algae in distilled water, fluorescence measurements were carried out in 96 wells microplates. Residual alkaline phosphatase and esterase activity rates could be estimated using the decrease of fluorescence of the product (MUF/fluorescein) after exposure to the test solution.

3. Results and discussion

3.1. Enzymatic activity detection using conductometric biosensors

3.1.1. Alkaline phosphatase activity detection

In a previous work, it has been proved that APA could be monitored for immobilised *C. vulgaris* using conductometric biosensors (Chouteau et al., 2004). As shown in Fig. 1, the enzymatic activity follows a classical Michaelis–Menten behaviour. The relative standard deviation of the sensor did not exceed 8%.

Signal amplitudes are also dependent on the algal concentration in the active membrane (i.e. the enzyme concentration): the optimal algal concentration is 4 to 5×10^7 cells/ml (Chouteau et al., 2004).

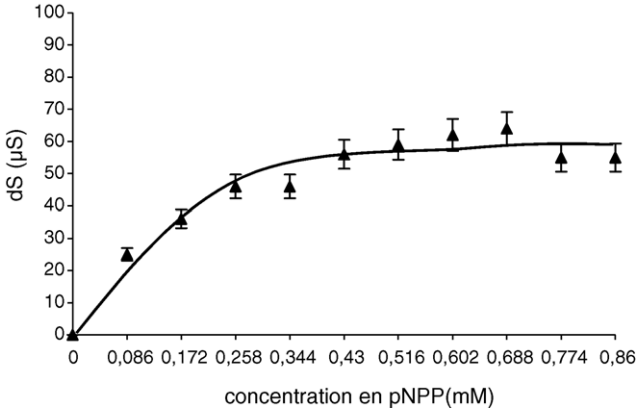


Fig. 1. Alkaline phosphatase activity measured with a conductometric biosensor (10 mM Tris–HCl, pH 8.5; 1 mM $MgCl_2$).

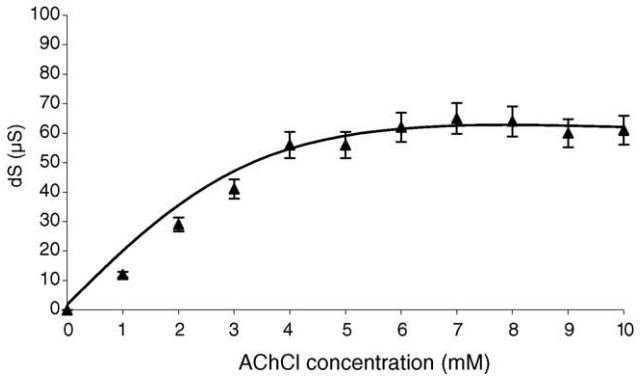


Fig. 2. Acetylcholinesterase activity measured with a conductometric biosensor (2.5 mM KH_2PO_4, pH 8).

3.1.2. Cholinesterase activity detection and protocol optimisation

In animals, acetylcholinerase is an enzyme from the nervous system. Few studies have been interested in non-neuronal cholinesterases in plants. However, Gupta et al. (1998) have shown that cholinesterase activity could be detected in algae (Gupta et al., 1998).

In this work, conductometric biosensors have been used to detect the AChE activity of immobilised *C. vulgaris*. A kinetic with a Michaelis–Menten behaviour could be plotted as shown in Fig. 2. The relative standard deviation of the biosensor is similar to APA measurements and did not exceed 8%.

The influence of the phosphate buffer concentration was considered for AChE measurements. Using the same biosensor, the AChE enzymatic kinetic was plotted using two KH_2PO_4 concentrations (2.5 and 5 mM, pH 8) (see Fig. 3). It appeared that the signal amplitude was strongly dependent on the buffer concentration. This can be explained by the increase of the global conductivity of the solution for increasing buffer concentrations leading to a higher background noise that finally reduces the signal amplitude (Hanss and Rey, 1971; Lawrence and Moores, 1972). Moreover, increasing ionic strength by adding inorganic salts is known to inhibit

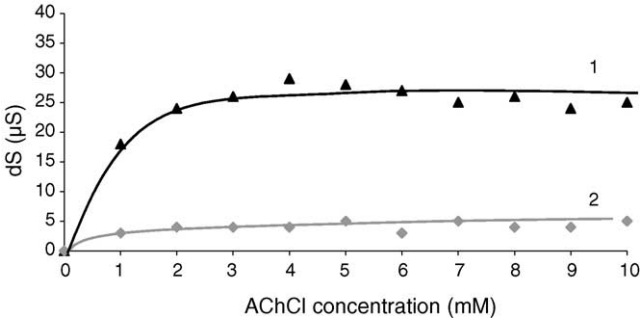


Fig. 3. Acetylcholinesterase activity measured with a conductometric biosensor for two buffer concentrations: (1) KH_2PO_4 = 2.5 mM; (2) KH_2PO_4 = 5 mM.

Table 1
Comparison between esterase substrates for enzymatic detection using conductometric biosensors

	Concentration range (µM)	Sensitivity (µS/mM)	Response time (min)	R.S.D. (%)
FDA	0–2	ND	ND	ND
BChCl	0–10	ND	ND	ND
AChCl	0–10	8	<5	8

AChE. It has been hypothesized that salt cation binding on enzyme anionic groups and/or screening of favourable electrostatic interactions for substrate binding caused by ionic strength could explain the reduction in affinity of AChE for their substrates (Tougu and Kesvatera, 1996). A 2.5 mM concentration has finally been chosen for phosphate buffer.

Further measurements with biosensors were carried out to compare three esterase substrates: FDA, AChCl and BChCl (Table 1). AChCl is the only appropriate substrate for conductometric detection since FDA and BChCl gave no detectable signals. FDA is a substrate used for esterase measurements based on fluorescence but its transformation during the enzymatic reaction gives no modification of ionic charges. This explains why conductometric biosensors could not detect any signal variation. Concerning BChCl, this substrate is specific for butyrylcholinesterases which concentration is usually lower in organisms compared to acetylcholinesterases. This could explain why no enzymatic activity could then be detected for *C. vulgaris*. AChCl has finally been chosen for further experiments.

3.1.3. Signal variability

As previously mentioned, a good repeatability (measurements repeated three times for each substrate concentrations) for APA and AChE measurements can be observed (R.S.D. < 8%). However, signal amplitude variability is important when comparing different biosensors as illustrated in Figs. 2 and 3. This can be explained by differences in algal loading and distribution within each active membrane because of the manual drop method. A microscopic observation of active membranes has shown that algal distribution is inhomogeneous: zones with no algae can be seen as well as algae clusters. This inhomogeneity in algae distribution has already been observed with enzymes immobilised on electrodes. In their work, Danzer and Schwedt (1996) have shown a high variability in enzymatic responses measured with pH electrodes when comparing different active membranes (Danzer and Schwedt, 1996). They have justified this poor reproducibility by differences in enzyme loading between sensors and by an inhomogeneous distribution of bioreceptors. Lastly, using whole cells as bioreceptors can also contribute to explain variability between biosensors since organisms have their own response to stimuli.

One of the main consequences of d*S* variations between biosensors is the difference in inhibition levels as it will be explained later on.

Table 2
Substrate concentrations used for toxicity assays

For APA (mM)	0.344	0.86
For AChE (mM)	4	10

3.2. Optimisations of the protocol for toxicity assays

3.2.1. Activity residual rate calculation

Some optimisations of the protocol for toxicity arrays were then considered. dS was measured twice ($t = 0$ and t = exposure time) for two substrate concentrations (see Table 2) before preincubation in a test solution. This protocol allowed to control signal stability during immersion since incomplete reticulation can sometimes occur and gives membranes with poor mechanical resistance: these biosensors would not be used for further experiments. Moreover this double measurement of dS before exposure to a test solution was used to calculate the residual activity rate (APA_{res} and $AChE_{res}$) using the mean value $dS_{before} = \frac{dS_0 + dS_t}{2}$ and dS_{after} corresponding to the signal variation measured after incubation in the test solution.

$$A_{res}(\%) = 100 - 100 \times \frac{dS_{before} + dS_{after}}{dS_{before}}$$

3.2.2. Parameters influencing enzymatic inhibition

Enzymatic inhibition depends on several parameters that had to be considered when toxicity assays were carried out.

First, algae concentration influences inhibition levels. Indeed for a same toxic concentration, low algae concentration in the active membranes (corresponding to low dS) means less target organisms and thus higher inhibition rates. It has then been decided to use an algal concentration in active membranes of 2 to 3×10^7 cells/ml as a compromise between the signal amplitude and a significant inhibition level.

Algal distribution within active membranes is of particular interest since it governs accessibility to algae for toxic compounds. For instance, algal clusters can reduce binding to membrane-bound enzymes. One of the main consequences of this inhomogeneous distribution is that responses of different active membranes to a test solution can vary.

Finally, it has been previously mentioned that organisms can respond differently to the same stimulus, in particular to the presence of a toxic compound.

3.2.3. Protocol optimisations for toxicity assays

First experiments with Cd^{2+} were carried out with long exposure times (from 60 to 240 min) and allowed to compare the efficiency of conductometric biosensors using immobilised *C. vulgaris* with bioassays (Chouteau et al., 2004). However Rogers and Lin (1992) have underlined some of the major requirements of environmental biosensors and they have pointed out that exposure plus measurements must be less than 60 min (Rogers and Lin, 1992). Consequently, it has been decided in this study to preincubate biosensors 30 and 60 min in test solutions.

For comparison, bioassays are presented in this work. However as they require higher algal concentrations, longer exposures to test solutions had to be performed.

After exposure to the test solution, biosensors must be washed. Different washing solutions (Millipore water, buffer solution) were tested for a few seconds to 5 min. No influence of any of these two parameters was observed. Finally a simple rinsing with Millipore water for a few seconds has been chosen.

3.3. Toxicity measurement assays

Variability has appeared to be a major problem in this study since it prevents from comparing residual activity rates calculated for different biosensors. As a consequence, no correlation between heavy metal/pesticide concentration and residual activity rate will be done, this study will only be concerned with the possible detection of different toxic compounds. Consequently, inhibition rates will be considered as significant when they exceed 15% for biosensors (i.e. residual activity rate < 85%) and 10% for bioassays (i.e. residual activity rate < 90%).

Considering limits of detection achieved, they are determined for a specific exposure time for a toxic concentration corresponding to a 15% inhibition for biosensors and a 10% inhibition for bioassays.

3.3.1. Heavy metals

Three heavy metals were tested (Cd^{2+}, Zn^{2+} and Pb^{2+}) and APA inhibition after exposure was studied depending on the ion concentration.

APA_{res} for two pNPP concentrations after exposure to Cd^{2+} and Zn^{2+} (1 ppb to 1 ppm) are given in Figs. 4 and 5. For both metal ions, concentrations of 1 ppm and 100 ppb gave significant inhibitions for a 30 min long exposure. For Cd^{2+}, APA_{res} was less than 85% for 100 ppb and less than 50% for 1 ppm. For Zn^{2+}, APA_{res} was less than 75% for 100 ppb and less than 70% for 1 ppm. For Cd^{2+} as well as Zn^{2+}, 10 ppb

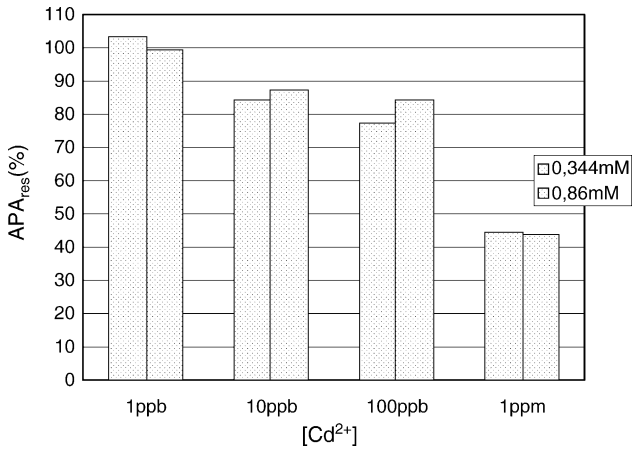


Fig. 4. APA_{res} for a 30 min long exposure to Cd^{2+} (measurements with biosensors for two pNPP concentrations: 0.344 and 0.86 mm).

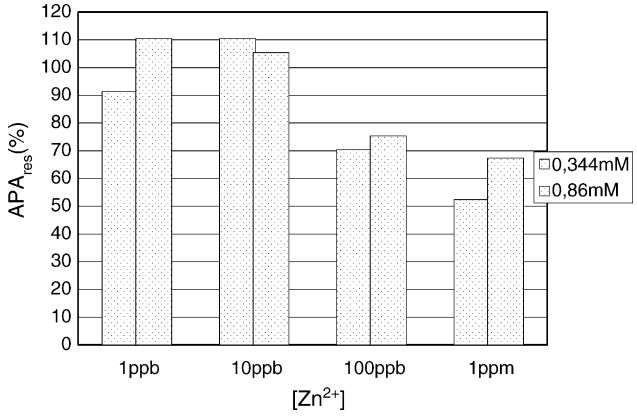


Fig. 5. APA$_{res}$ for a 30 min long exposure to Zn^{2+} (measurements with biosensors for two pNPP concentrations: 0.344 mM and 0.86 mM).

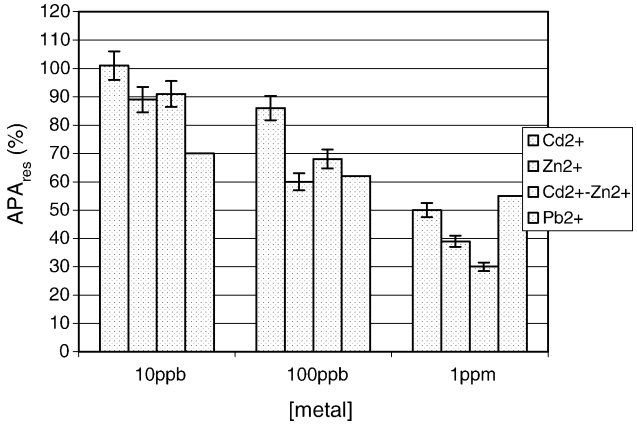


Fig. 7. APA$_{res}$ for a 240 min long exposure to heavy metal ions (measurements with bioassays).

could be considered as the limit of detection. A 60 min long exposure for 1 ppb was tested for Cd^{2+} and Zn^{2+} but no significant inhibition was obtained.

Pb^{2+} is also known to inhibit *C. vulgaris* APA (Durrieu et al., 2003). However with conductometric biosensors, no inhibition could be detected for high Pb^{2+} concentrations (1 ppm and 100 ppb).

A last study was led for heavy metals using a mixture containing Cd^{2+} and Zn^{2+} to study the possible synergetic or antagonist effects of this test solution. Biosensors were incubated 30 min and APA$_{res}$ are given in Fig. 6. For a mixture containing 1 ppm Cd^{2+}/1 ppm Zn^{2+} and 100 ppb Cd^{2+}/100 ppb Zn^{2+}, significant inhibitions could be detected: for 100 ppb, APA$_{res}$ was less than 85% and less than 40% for 1 ppm. Ten parts per billion Cd^{2+}/10 ppb Zn^{2+} was considered as the limit of detection. As it is difficult to compare APA$_{res}$ calculated with different biosensors, bioassays using free *C. vulgaris* exposed to Cd^{2+}/Zn^{2+} for 240 min were carried out (see Fig. 7). However, from these different results, it has not been possible to determine any synergetic or antagonist effects.

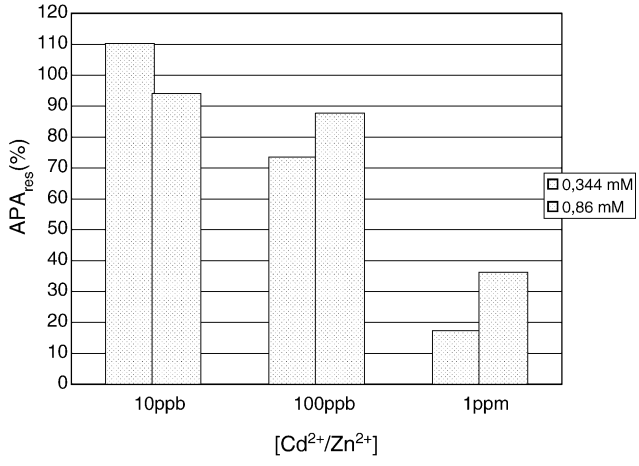


Fig. 6. APA$_{res}$ for a 30 min long exposure to Cd^{2+}/Zn^{2+} (measurements with biosensors for two pNPP concentrations: 0.344 mM and 0.86 mM).

Several conclusions can be drawn from these results.

Firstly, comparing APA$_{res}$ calculated for biosensors and bioassays has shown that biosensors tend to be more sensitive than bioassays. For shorter exposures, APA$_{res}$ were indeed higher for biosensors: for example, APA$_{res}$ was 50% after a 30 min long exposure to 1 ppm Cd^{2+} with biosensors but a 240 min long exposure was required with bioassays to achieve the same inhibition level. The same conclusion can be given for Zn^{2+} and Cd^{2+}/Zn^{2+}. The main explanation for this higher sensitivity is the lower quantity of target organisms exposed to toxic compounds with biosensors compared to bioassays.

Heavy metal bioavailibility has also appeared to be an important issue. Indeed, contrary to bioassays for which Pb^{2+} strongly inhibited APA (see Fig. 7), biosensors could not detect this ion.

This result can be explained by a limited bioavailibility of Pb^{2+} ions in BSA membranes caused by metal adsorption on albumin (Martins and Drakenberg, 1982; Sadler and Viles, 1996; Bal et al., 1998). This metal adsorption on BSA has already been considered by Zhylyak et al. (1995) who have shown that heavy metal inhibition was higher for free urease compared to immobilised urease. The authors have finally concluded to a reduced bioavailibility of metal ions in BSA membranes (Zhylyak et al., 1995). In this study, it has also appeared that Pb^{2+} is less bioavailable for algae than Cd^{2+} and Zn^{2+}. However, few works on metal adsorption on albumin are available (Bal et al., 1998; Martins and Drakenberg, 1982; Sadler and Viles, 1996) and no classification of metal ions towards their affinities to albumin can confirm our results.

Finally, performances (LOD) for heavy metal ions detection for this conductometric biosensor using immobilised *C. vulgaris* and for biosensors presented in different papers were compared in Table 3. It appears that limits of detection obtained with conductometric biosensors using immobilised *C. vulgaris* are comparable to those obtained with *C. vulgaris* optical biosensors. When compared to pure enzyme (urease) biosensors, *C. vulgaris* conductometric biosensors have lower limits of detection which can be explained by a lower quantity of bioreceptors in active membranes.

Table 3
Comparisons between limits of detection for different biosensors

	Exposure	M^{n+} concentration			Reference
		Cd^{2+}	Zn^{2+}	Pb^{2+}	
C. vulgaris optical biosensor	Direct	10 ppb	Not tested	10 ppb	Durrieu and Tran-Minh (2002)
Immobilised urease conductometric biosensor	10 min	700 ppb	Not tested	6 ppm	Zhylyak et al. (1995)
Urease optical biosensor	20 min	1 ppm	2.5 ppm	21 ppm	Tsai et al. (2003)
BOD biosensor (cells: *Bacillus subtilis*)	Direct	No detectable effect for $[Cd^{2+}] = 560$ ppm	130 ppm	Not tested	Zhenrong and Tan (1999)
Antibody biosensors	10 min	30 ppt	Not tested	Pb-complex 1 ppb	Blake et al. (2001)
DNA amperometric biosensor	15 min	0.1 ppb	Not tested	0.2 ppb	Babkina and Ulakhovich (2004)
C. vulgaris conductometric biosensor	30 min	10 ppb	10 ppb	Not determined	This work

Furthermore, enzyme sensitivity to a pollutant also depends on its nature (alkaline phosphatase seems to be more sensitive to heavy metals than urease) as well as its origin (alkaline phosphatases from different sources, animal or plant cells for instance, can have different sensitivities to a same pollutant, see following paragraph). Finally, *C. vulgaris* conductometric biosensors are much more sensitive than biosensors based on global metabolic perturbations (BOD) but their performances are clearly lower in comparison to DNA and antibody biosensors. However, for these two biosensors, an expensive cost prevents from extensive use as on line monitoring devices.

3.3.2. Organophosphorous and carbamate pesticides

Carbamate and organophosphorous pesticides are known to inhibit AChE and have been used widely as toxic references to test pure acetylcholinesterase biosensors (Marty et al., 1993; Andres and Narayanaswamy, 1997; Dzyadevych et al., 2002; Ciucu et al., 2003; Wan et al., 1999).

Algal conductometric biosensors were incubated in carbofuran and parathion-methyl for 30 min. However, they showed no signal decrease. This absence of inhibition of *C. vulgaris* AChE in presence of CF and MP was confirmed with bioassays. In literature, these two pesticides have been widely used to study their impacts on pure acetylcholinesterases immobilised on biosensors and significant inhibitions have generally been observed for CF (Marty et al., 1993; Andres and Narayanaswamy, 1997; Ciucu et al., 2003; Dzyadevych et al., 2002). Concerning MP, fewer studies have been led. The main reason is probably its low toxicity for pure acetylcholinesterase (Dzyadevych et al., 2002). However this organophosphorous is known to degradate easily into different products, one of them (paraoxon-methyl) has a highly inhibiting potential on acetylcholinesterase.

Consequently, this organophosphorous was tested on *C. vulgaris* acetylcholinesterase. For biosensors incubated 30 min in 1 ppm and 100 ppb, an inhibition of AChE could be detected for both AChCl concentrations (see Fig. 8): for 100 ppb, $AChE_{res}$ was 80% for 4 mM AChCl and for 1 ppm, $AChE_{res}$ was less than 85% for both AChCl concentrations. Bioassays confirmed this inhibition for *C. vulgaris* esterases as shown in Fig. 9 ($AChE_{res}$ was 70% for 100 ppb and 50% for 1 ppm).

These results have provided information on *C. vulgaris* sensitivity to carbamates and organophosphorous. For carbofuran and parathion-methyl this sensitivity is low compared to electric eel acetylcholinesterase used classically for pure enzyme biosensors (Dzyadevych et al., 2002). Villatte et al. (1998) have studied the sensitivity of different acetylcholinesterases to CF and OP and have finally shown different responses to inhibitors depending on the enzyme origin.

For instance, they have pointed out that insect acetylcholinesterase is far more sensitive to CF and OP pesticide (Villatte et al., 1998). Sensitivity to a toxic compound is then partly dependent on the enzyme origin but the use of membrane-bound enzymes must also be taken into consideration. Indeed, these enzymes remain in their natural environment and their activities could be influenced after exposure to a toxic compound by cellular protection mechanisms.

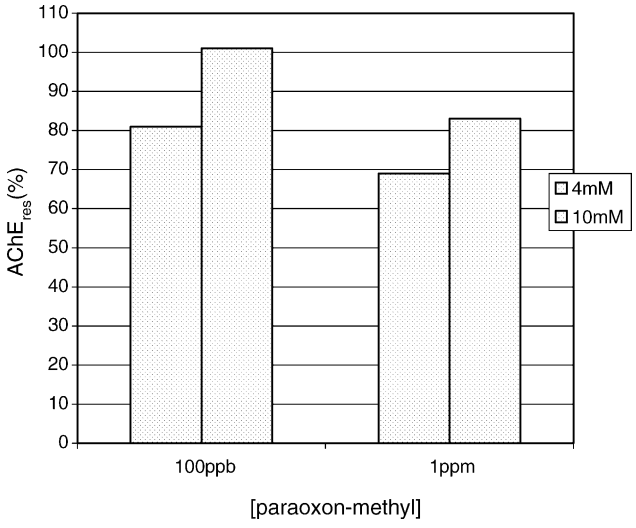


Fig. 8. $AChE_{res}$ for a 30 min long exposure to paraoxon-methyl (measurements with biosensors for two AChCl concentrations: 4 mM and 10 mM).

Table 4
Drinking water standards in USA and UE

	US standards	UE standards
Cadmium	5 ppb	5 ppb
Zinc	5 ppm	5 ppm
Lead	15 ppb	Reduction from 25 ppb (2003) to 10 ppb (2013)
Pesticides	Standards depending on the pesticide	0.1 ppb for one pesticide or metabolite and 0.5 ppb for all pesticides and metabolites found in the sample

As a consequence, *C. vulgaris* can be considered as an efficient bioreceptor only for MPx (i.e. highly inhibiting organophosphorous).

3.3.3. Mixture Cd^{2+}/paraoxon-methyl

It has previously been shown that APA as well as AChE could be detected using the same biosensor and that these two enzymes were inhibited by distinct toxic compounds, heavy metals for alkaline phosphatases and some organophosphorous for acetylcholinesterases. First experiments were carried out to study the inhibition of both enzymatic activities after a 30 min long exposure to a mixture containing 25 ppb Cd^{2+}/50 ppb MPx (see Fig. 10). APA_{res} and $AChE_{res}$ obtained with two different biosensors were compared to those obtained after exposure to Cd^{2+} and MPx solutions (see Figs. 4 and 9). For APA, 25 ppb Cd^{2+} gave no significant inhibition for both biosensors. This concentration was probably to close to the limit of detection (10 ppb) to give a significant decrease of APA. For AChE measured on the same biosensors, an inhibition was reported ($AChE_{res}$ is 80% and 65% depending on the biosensor) for 50 ppb MPx confirming results obtained for MPx alone (see previous paragraph). It can also be noted that MPx did not inhibit APA. Furthermore no synergetic or antagonist effects of the mixture could be observed on both enzymatic activities (Fig. 10).

These first experiments using a mixture as a test solution has confirmed that this bi-enzymatic biosensor can be of great interest to discriminate toxic compounds in an unknown sample depending on the enzymatic activity inhibited.

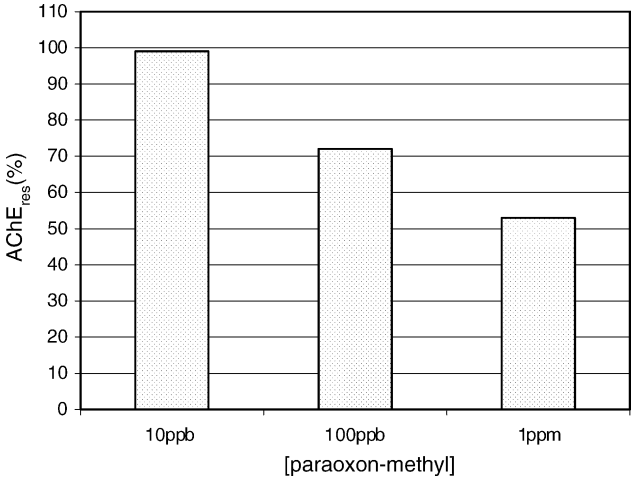


Fig. 9. $AChE_{res}$ for a 240 min long exposure to paraoxon-methyl (measurements with bioassays).

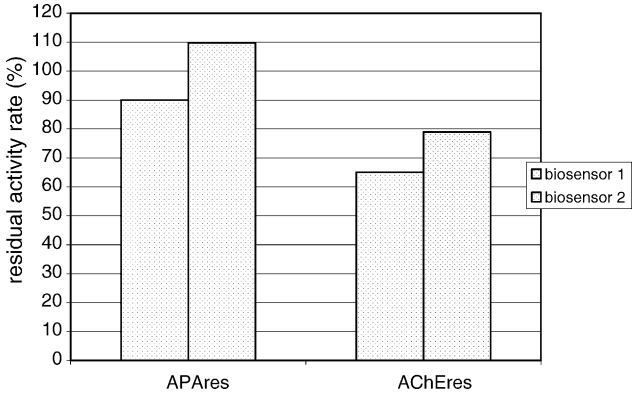


Fig. 10. $AChE_{res}$ for a 30 min long exposure to 50 ppb MPx/25 ppb Cd^{2+} (measurements with biosensors).

4. Conclusion

This paper presents a bi-enzymatic conductometric biosensor using immobilised *C. vulgaris* for the detection of heavy metal ions and organophosphorous compounds in water samples. The performances of these biosensors for heavy metals and organophosphorous compounds are encouraging regarding US and UE drinking water standards (see Table 4). However, further work is necessary before considering these biosensors as competitive tools for on line and in situ monitoring that can be used as early warning systems for qualitative analysis.

Moreover as two enzymatic activities inhibited by different families of pollutants can be detected, these biosensors can be helpful for further lab analysis with conventional analytical techniques. Using whole cells is also particularly interesting since ecotoxicological parameters can be integrated, especially the true toxicity of a compound for an organism.

However, algae immobilisation in BSA membranes has appeared to reduce the efficiency of these whole cell biosensors for compounds as Pb^{2+} for instance. Further optimisations of biofunctionalisation will have to be investigated as well as the use of other organisms as bioreceptors. Recent works have shown that yeast (*Saccharomyces cerevisiae*) could be interesting, firstly as a source of enzymes (acid and alkaline phosphatases, esterases, deshydrogenases, etc.),

secondly because they have their own sensitivity to pollutants and can be more adapted than algae for the detection of some compounds.

Finally, as irreversible inhibition prevents from reusing these biosensors, further experiments will be led on enzymatic reactivation. First assays have already been carried out with EDTA, however no reactivation of alkaline phosphatase could be observed.

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