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Design of a macroalgae amperometric biosensor; application to the rapid monitoring of organophosphate insecticides in an agroecosystem

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HIGHLIGHTS

- A macroalgae based biosensor based on tetracyanoquinodimethane (TCNQ) mediator was designed.
- The designed biosensor was used to analyze methyl parathion OP insecticide in water samples.
- The novel immobilization design led to enhanced stability and sensitivity of the biosensor.
- Macroalgae-biosensor could be used as a low-cost and sensitive screening method to detect target analyte.

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ABSTRACT

The immobilization of enzymes onto transducer support is a mature technology and has been successfully implemented to improve biocatalytic processes for diverse applications. However, there exists still need to design more sophisticated and specialized strategies to enhance the functional properties of the biosensors. In this work, a biosensor platform based on innovative fabrication strategy was designed, and employed for the detection of organophosphate (OP) in natural waters. The biosensor was prepared by incorporating acetylcholinesterase enzyme (AChE) to the graphite paste modified with tetracyanoquinodimethane (TCNQ) mediator, along with the use of a macroalgae (*Cladaphropsis* membranous) as a functional immobilization support. The novel immobilization design resulted in a synergic effect, and led to enhanced stability and sensitivity of the biosensor. The designed biosensor was used to analyze methyl parathion OP insecticide in water samples collected from a demonstrably contaminated lake of São Luis Island, Maranhão, Northeast of Brazil. Water analysis revealed that the aquatic ecosystem was polluted by sub-ppm concentrations of the OP insecticide, and a good correlation was found between values obtained through biosensor and GC–MS techniques. Our results demonstrated that macroalgae-biosensor could be used as a low-cost and sensitive screening method to detect target analyte.

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1. Introduction

Biosorption process has gained much attention in recent years for the removal of pollutants or recovery of metal ions by binding to biomass from aqueous solution. Various biomasses including bacteria, yeast, fungi, algae and mosses have reported for the cost effective removal of heavy metals (Low and Lee, 1991; Arica et al., 2004). For example, Algae biomasses present a suitable biosorption platform due to presence of biopolymers in their cell walls (Chojnacka et al., 2005). These functional groups results in metal ion binding by adsorption, ion exchange or covalent binding (Kalyani et al., 2004). The integration of biosorption process in biosensors could be an attractive alternative to the existing methods (Alpat et al., 2008). However, there is no study implying the algae biomass as immobilization support in biosensors.

Alginate is one of the basic component of the algae biomass, and is composed of chains of alternating α-L-guluronic acid and β-D-mannuronic acid residues(Taqieddin and Amiji, 2004). Based on above observation, macroalgae species *Cladophoropsis membranacea* due to its alginate content was used as enzyme immobilization support in the present work. Traditionally, different strategies to

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improve enzymatic assays performance have been reported as almost independent platforms. However, the use of different immobilization methods have shown synergic effect to improve the analytical performance of the biosensors (Cowan and Fernandez-Lafuente, 2011; Rodrigues et al., 2011). Despite of the potential advantages, there are only few studies on the coupled use of different tools to improve assays performance. The present study was aimed to explore the novel properties of macroalgae as immobilization support, along with use of coupled tools to enhance the assay performance. To best of our knowledge, such a study based on macroalgae as independent or in combination with other immobilization support has not been reported in the literature. The design platform was demonstrated by immobilizing acetylcholinesterase enzyme (AChE) to detect organophosphate in ecosystem.

Since the early twentieth century, the synthetic organic pesticides are widely used in pest control with the aim of increasing agricultural productivity. They are source of water contamination with serious risks to human and animal health. Additionally, they cause changes in ecosystems with detrimental consequences for the environment and agriculture domains (Menezes et al., 2010). The emergence of organophosphorus (OP) pesticides gradually replaced the chlorinated ones. But although less persistent than the chlorinated hydrocarbons, carbamate and organophosphate insecticides have a peculiar harmful characteristic to living organisms. They act directly on the nervous system by inhibiting the action of acetylcholinesterase enzymes, causing nerve dysfunction which can lead to death (Van Dyk and Pletschke, 2011). Over the past 20 years, the use of agrochemicals in Brazil has grown substantially, increasing the poisoning cases and contamination in soil, water and air. Because of this, some governmental programs have been developed in order to monitor and to control residues of such substances in the environment (Nunes et al., 2004).

Enzymatic biosensors are promising tools for the monitoring of pesticides due to their high sensitivity and selectivity, very short response time and minimal sample treatment (Andreescu and Marty, 2006; Dondoi et al., 2006; Sinha et al., 2010).

Among the enzymes employed for the construction of biosensors devoted to pesticide analysis, the acetylcholinesterase enzyme (AChE) is the most commonly used one. These devices are designed to supplement or replace the existing reference methods of analysis (i.e. chromatographic methods), simplifying or eliminating sample preparation and reducing the analysis time and the cost(Andreescu et al., 2002). Our results demonstrated that the present macroalgae-biosensor is sensitive and cost effective screening method to detect organophosphate in ecosystem.

2. Experimental

2.1. Materials

Chromatographic grade methanol and ultrapure sodium sulfate were obtained from Merck. Acetylcholinesterase (AChE 3.1.1.7 type-VI-S/1.5 mg, electric eel source, 500 U/1.5 mg) and acetylthiocholine chloride (ATChCl) were purchased from Sigma–Aldrich Chemicals Co., USA. AChE stock solutions were prepared directly in 0.9% NaCl (w/v) and stored at −18 °C. Methyl parathion standard (99% purity) was obtained from Riedel-de-Haen, Fluka, USA. Dithio-bis 5,5-(2-nitrobenzoic acid) (DTNB), hydroxyethylcellulose (HEC) and bovine serumalbumine (BSA) were purchased from Sigma–Aldrich Chemicals Co., USA. The graphite fine powder was procured from Merck, USA. All bio-reagents were prepared in phosphate buffer solution (PBS, pH = 7.2).

2.2. Macroalgae collection and hydrolysis

Macroalgae species *C. membranacea* has been collected by scrapping gently the mangrove root from the swamp, during the rainy season at the Anil River estuary, near the neighborhood Jaracati (São Luís, MA, Brazil), and stored in a plastic container at 4 °C. Afterwards, the macroalgae was air dried for 48 h. Then it was chopped and converted to the powder form. Papain solution, prepared in sodium acetate buffer (pH = 5.0), EDTA and cysteine was used to get a solution mixture of it. This mixture was exposed to a nitrogen gas flux for 10 min, and then the hydrolysis of the macroalgae was completed into a water bath at 50 °C for 3 h. After this, the material was filtered and the final residue was air-dried for a period of 5 h, and finely pulverized. The obtained powder was stored at 10 °C.

2.3. Instrumentation

Electrochemical measurements were performed using the biosensor connected to a potentiostat/galvanostat (Microautolab Type III), while spectrophotometric measurements were carried out with a UV/Vis spectrophotometer (Biospectro SSP-220). Neon lamp was used in the cold stage for enzyme polymerization onto the electrode surface.

Chromatographic analyses were performed with a gas chromatography model 3900 equipment containing a divider type split/splitless and a mass spectrometric detector model Saturn 2100T (both from Varian, Palo Alto, California). Chromatographic data were treated by comparing the mass spectra obtained with the NIST Library to those presented by the MS Workstation, version 6.9, from Varian. For the identification of the methyl parathion pesticide, the MS has selected the following ions: [M], [M-138] and [M-154] in the full scan mode in a 200–360 *m*/*z* range. The main chromatographic conditions were: capillary column VF-5-MS Factor Four (30 m × 0.25 mm id), helium as carrier gas (1 mL min^{-1} flow rate), injector temperature of 250 °C; temperature in the column programmed to 100 °C held for 1 min and then increasing to 250 °C, 30 °C min^{-1}; total run time of 8.5 min; retention time of 6.9 min. The SPME procedure is summarized as follow: The PDMS fiber was fixed to the extraction GC module. In a headspace vial of 20 mL capacity of 10 mL standard solution (prepared in methanol), was added or 10 mL of water sample plus Na_2SO_4 10% (*w*/*v*) solution. The extraction process occurred in a time of 40 min at 80 °C. After desorption, the compounds were analyzed by GC/MS according above.

2.4. Enzyme selection and biosensor preparation

Several tests were performed with three types of acetylcholinesterases, extracted from human erythrocyte, bovine erythrocyte and electric eel. The enzyme activity was measured before each immobilization by spectrometry. A two-steps immobilization method was employed for the biosensor preparation. Firstly, macroalgae powder was incorporated in a proportion of 7.5% onto the enzymatic paste prior to apply it at the working electrode surface. Thus, the modifying macroalgae paste was prepared by mixing macroalgae powder to 30% PVA–SBQ, 2% BSA and 1% HEC. Secondly, the AChE solution (pH 7.2) was added to this mixture, the final sensitive paste homogenized by vortexing at 5000 rpm during 10 s and following 2 μL of this paste was manually deposed on TCNQ-modified graphite working electrode prepared as previously described (Andreescu et al., 2002; Andreescu and Marty, 2006). At the end of this immobilization method, the enzymatic load was of 2 mU per sensor. The obtained AChE-biosensors were kept in the refrigerator at −4 °C for at least 2 days prior to use.

2.5. Electrochemical characterization of the biosensor

The electrochemical characterization of the AChE-biosensor system was carried out using cyclic voltammetry and differential pulse voltammetry. Signal repeatability tests were performed, and the substrate concentration was optimized to get optimal enzyme loading concentration. The sensitivity and the reproducibility of biosensor were evaluated based on the optimized substrate concentration and working potential.

2.6. Pesticide detection in water samples

Natural water samples were collected from a lake located in an agricultural region of São Luis Island called "Cassaco", just close to the Quebra Pote district, in Maranhão State, Northeast of Brazil. Six sampling points were defined among the Cassaco Lagoon, considering its extension (total area of 1275 ha; perimeter of 583 m; minimum depth (dry season) of 2.5 m and maximum (rainy season) of 4 m. The collected samples were stored in amber vials of 20 mL, four samples for each point (two samples of surface water and 2 samples collected at 0.5 m deep), totalizing 24 bottles per day of collection. After collection and codification, the samples were kept at 4 °C before chromatographic analysis and also analyzed with designed biosensor in the laboratory. The study was carried out for a period of about three months with weekly tests.

3. Results and discussion

3.1. Characterization and demonstration of the designed sensor platform

AChE catalyzed reaction on the sensor surface are well reported in the literature (Campas et al., 2009; Rotariu et al., 2012). Similarly, the modification of the screen-printed graphite working electrode with TCNQ mediator provides the improved biosensor sensitivity and allowed to operate system at relatively lower working potential, even at relatively low substrate concentration. As can be seen from the Fig. 1, integration of TCNQ as mediator on the electrode surface resulted in low operating potential of the biosensor. An electrochemical oxidation peak was observed around 100 mV as function of sensor response. The low operating potential associated with the use of mediator effect can be considered advantageous for the anodic process because it prevents the oxidation of possible interfering species existing in the samples. Further to demonstrate the beneficial characteristic of macroalgae powder on the sensor surface, a comparative electrochemical study was performed in the presence and absence of macroalgae powder on the TCNQ modified sensor surface. As can be seen from the Fig. 2, the presence of macroalgae powder on the electrode surface shifted the working potential to lower values. The oxidation current intensities were recorded on the order of 5–15 μA in the presence and absence of macroalgae on the electrode surface, by keeping the same working condition for both sensors. Fig. 2 presents the differential pulse voltammogram obtained in the presence and absence of macroalgae on the transducer surface. In the presence of the macroalgae, the current intensity was increased about 3 times for the same substrate concentration as can be observed in Fig. 2. This increase of the current response allows to decrease the concentration of acetylthiocholine, making the process more economical in comparison to the already reported biosensors (Dondoi et al., 2006; Sinha et al., 2010). The shift of working oxidation potent towards lower value, and enhancement in the electrochemical signal for macroalgae biosensor clearly demonstrated the importance of incorporating macroalgae in sensor surface. These results provided an insight onto performance improved characteristics of macroalgae, and use of the two steps-immobilization method. Specifically, the immobilization technique based on TCNQ plus macroalgae addition is of fundamental importance, because this procedure provided a good stability of the enzyme, reduced cost and decreased time to the inhibition procedure. The final analytical process may become more economic, so that the sensor developed in this work can be used to detect AChE inhibitors such as organophosphates and carbamates, with the additional advantage of the enzyme could be replaced by others from different sources, such as genetically modified enzymes.

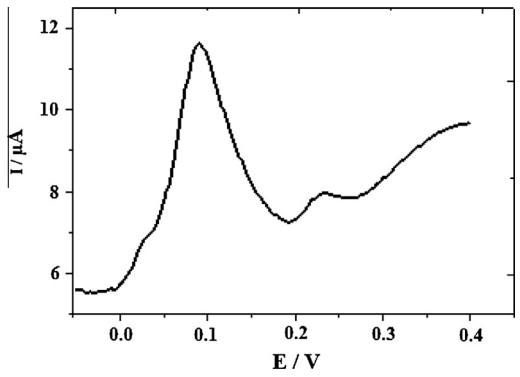

Fig. 1. Differential pulse voltammogram obtained with TCNQ-AChE-biosensor.

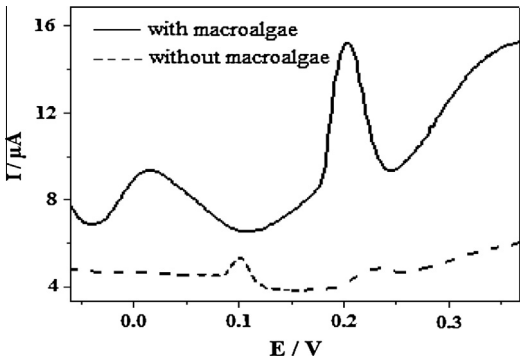

Fig. 2. TCNQ-modified biosensor with/without macroalgae incorporated to the sensitive AChE paste. Control performed with the biosensor in presence of buffer solution has indicated negligible current signal.

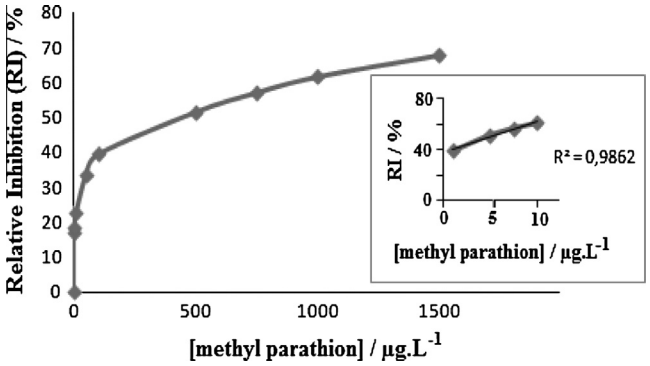

Fig. 3. Inhibition curve obtained for the methyl parathion pesticide. Inset is the inhibition curve into a lower concentration range, i.e., the practical linear range of the analytical method.

3.2. Optimization of working parameters and analytical characteristics of the sensor

The substrate concentration of 0.4 mmol L^{-1} was found optimal to perform further experiments. Similarly, a time 60 s was

Table 1
Results obtained with the use of the optimized AChE-biosensors in Cassaco Lake, São Luis Island, Maranhão, Brazil.

Sampling point	Sampling week	Location	Inhibition (%)	Pesticide concentration ($\mu g\ L^{-1}$)	
				Biosensor (total)	SPME–GC/MS
Point 1	1	Surface water	45.3	85.4 ± 12.7	MP – 23.5 ± 3.1 MPO – 75.0 ± 7.5
		Deep	37.5	62.0 ± 8.2	MP – 15.0 ± 3.8 MPO – 48.7 ± 5.1
	2	Surface water	11.4	10.2 ± 1.5	n.c.
		Deep	28.3	34.4 ± 5.5	n.c.
	3	Surface water	51.3	103.2 ± 15.4	MP – 12.0 ± 3.4 MPO – 78.5 ± 8.5
		Deep	34.2	52.0 ± 6.6	MP – 10.6 ± 2.4 MPO – 41.0 ± 4.8
	4	Surface water	73.5	169.9 ± 12.3	MP – 15.0 ± 3.3 MPO – 88.3 ± 9.0
		Deep	20.0	9.6 ± 3.2	n.c.
	5	Surface water	58.1	123.7 ± 11.4	MP – 18.7 ± 3.3 MPO – 88.6 ± 9.5
		Deep	40.5	71.0 ± 6.0	MP – 16.4 ± 2.0 MPO – 47.0 ± 4.8
	6	Surface water	29.3	37.4 ± 5.8	n.c.
		Deep	28.9	36.2 ± 3.6	n.c.
	7	Surface water	28.5	34.9 ± 2.3	n.c.
		Deep	23.2	19.1 ± 1.6	n.c.
	8	Surface water	30.0	37.8 ± 5.7	MP – 12.8 ± 3.8 MPO – 30.0 ± 5.0
		Deep	25.6	22.9 ± 4.3	n.c.
	9	Surface water	42.0	74.4 ± 8.2	MP – 25.8 ± 5.5 MPO – 52.0 ± 6.6
		Deep	45.2	80.1 ± 6.9	MP – 30.3 ± 4.2 MPO – 56.0 ± 7.6
	10	Surface water	18.9	20.3 ± 3.1	n.c.
		Deep	53.7	110.5 ± 14.1	MP – 48.5 ± 3.5 MPO – 66.0 ± 7.9
Point 2	1	Surface water	26.4	28.7 ± 3.8	n.c.
		Deep	26.4	28.6 ± 2.6	n.c.
	2	Surface water	37.5	62.0 ± 5.3	MP – 16.5 ± 5.5 MPO – 58.5 ± 6.9
		Deep	41.3	73.3 ± 4.5	MP – 20.0 ± 3.5 MPO – 60.0 ± 7.7
	3	Surface water	54.2	111.9 ± 15.4	MP – 11.5 ± 1.8 MPO – 78.5 ± 7.3
		Deep	35.8	57.0 ± 6.8	MP – 16.0 ± 2.7 MPO – 85.0 ± 6.4
	4	Surface water	42.0	75.1 ± 7.5	MP – 22.0 ± 3.4 MPO – 67.5 ± 8.0
		Deep	35.6	56.3 ± 5.0	MP – 40.8 ± 5.5 MPO – 80.0 ± 10.6
	5	Surface water	28.7	35.6 ± 3.9	n.c.
		Deep	29.7	38.6 ± 2.3	n.c.
	6	Surface water	22.8	18.0 ± 1.4	n.c.
		Deep	28.4	34.7 ± 6.3	n.c.
	7	Surface water	21.2	13.2 ± 2.5	n.c.
		Deep	13.1	12.4 ± 0.9	n.c.
	8	Surface water	29.7	38.5 ± 3.5	n.c.
		Deep	24.5	22.6 ± 2.6	n.c.
	9	Surface water	10.2	9.6 ± 0.5	n.c.
		Deep	41.3	65.6 ± 5.3	MP – 21.2 ± 3.5 MPO – 42.5 ± 4.9
	10	Surface water	28.7	25.1 ± 1.7	n.c.
		Deep	56.9	125.2 ± 15.8	MP – 45.7 ± 5.5 MPO – 88.5 ± 9.2
Point 3	1	Surface water	36.2	58.2 ± 3.8	MP – 8.4 ± 0.96 MPO – 24.8 ± 3.7
		Deep	54.2	111.9 ± 8.6	MP – 12.0 ± 1.9 MPO – 78.5 ± 8.0
	2	Surface water	33.8	50.8 ± 6.2	MP – 21.2 ± 2.6 MPO – 62.5 ± 5.6
		Deep	26.7	29.7 ± 1.7	n.c.
	3	Surface water	29.7	38.7 ± 7.0	n.c.
		Deep	40.2	70.0 ± 5.5	MP – 34.4 ± 4.3 MPO – 85.6 ± 7.0
	4	Surface water	47.3	91.2 ± 8.2	MP – 32.0 ± 2.7 MPO – 55.6 ± 5.6
		Deep	40.0	69.5 ± 4.8	MP – 32.0 ± 3.2

					MPO – 70.0 ± 6.1
	5	Surface water	25.8	26.9 ± 2.7	n.c.
		Deep	30.8	41.8 ± 3.9	MP – 24.5 ± 2.2
					MPO – 56.0 ± 6.5
	6	Surface water	33.4	46.7 ± 5.3	MP – 25.0 ± 2.3
					MPO – 38.8 ± 3.9
		Deep	15.5	12.6 ± 0.9	n.c.
	7	Surface water	31.1	42.9 ± 2.8	MP – 9.5 ± 0.4
					MPO – 39.0 ± 4.6
		Deep	15.6	12.6 ± 1.7	n.c.
	8	Surface water	24.2	25.4 ± 3.0	n.c.
		Deep	8.4	8.2 ± 1.5	n.c.
	9	Surface water	38.5	72.8 ± 4.9	MP – 23.2 ± 2.3
					MPO – 54.5 ± 4.5
		Deep	29.3	28.3 ± 2.6	n.c.
	10	Surface water	36.4	70.3 ± 8.0	MP – 32.4 ± 6.5
					MPO – 55.8 ± 6.7
		Deep	19.4	21.4 ± 3.4	n.c.
Point 4	1	surface water	38.8	65.8 ± 6.9	MP – 22.4 ± 2.3
					MPO – 56.0 ± 5.5
		Deep	29.2	37.3 ± 3.3	n.c.
	2	surface water	44.5	83.0 ± 9.5	MP – 20.5 ± 2.8
					MPO – 69.5 ± 6.0
		Deep	42.3	76.4 ± 7.5	MP – 14.8 ± 2.4
					MPO – 86.0 ± 6.6
	3	surface water	65.0	144.4 ± 12.1	MP – 32.0 ± 3.8
					MPO – 88.0 ± 9.5
		Deep	26.6	29.3 ± 3.8	n.c.
	4	surface water	35.6	56.2 ± 4.6	MP – 15.6 ± 1.8
					MPO – 65.2 ± 3.0
		Deep	37.5	62.0 ± 6.2	MP – 23.6 ± 2.4
					MPO – 56.4 ± 4.5
	5	surface water	18.0	3.7 ± 0.8	n.c.
		Deep	29.8	38.8 ± 5.0	n.c.
	6	surface water	40.80	71.9 ± 6.6	MP – 30.0 ± 3.5
					MPO – 69.0 ± 6.0
		Deep	25.8	27.0 ± 3.6	n.c.
	7	surface water	20.5	11.0 ± 2.2	n.c.
		Deep	24.9	24.2 ± 4.1	n.c.
	8	Surface water	18.6	22.8 ± 3.0	n.c.
		Deep	17.9	21.8 ± 2.8	n.c.
	9	Surface water	19.1	23.7 ± 3.5	n.c.
		Deep	45.2	84.5 ± 7.7	MP – 38.6 ± 2.9
					MPO – 53.0 ± 6.2
	10	Surface water	48.7	94.4 ± 9.6	MP – 38.0 ± 3.1
					MPO – 113.0 ± 9.5
		Deep	45.9	86.4 ± 8.9	MP – 38.0 ± 2.7
					MPO – 113.0 ± 9.0
Point 5	1	Surface water	60.0	129.0 ± 12.0	MP – 38.0 ± 4.5
					MPO – 113.0 ± 10.8
		Deep	42.9	78.2 ± 6.5	MP – 24.7 ± 3.5
					MPO – 67.8 ± 6.5
	2	Surface water	19,7	8.5 ± 0.92	n.c.
		Deep	28.3	34.3 ± 4.4	n.c.
	3	Surface water	39.4	67.6 ± 8.0	MP – 17.2 ± 2.2
					MPO – 82.9 ± 8.8
		Deep	27.5	31.9 ± 3.5	n.c.
	4	Surface water	37.2	61.1 ± 5.7	MP – 24.2 ± 4.5
					MPO – 65.8 ± 5.9
		Deep	34.5	52.9 ± 4.8	MP – 20.0 ± 3.7
					MPO – 58.0 ± 6.2
	5	Surface water	28.0	33.5 ± 3.7	n.c.
		Deep	24.3	22.4 ± 2.5	n.c.
	6	Surface water	33.9	51.2 ± 5.2	MP – 18.0 ± 2.7
					MPO – 47.0 ± 6.0
		Deep	39.1	66.1 ± 7.7	MP – 25.0 ± 3.3
					MPO – 59.0 ± 7.5
	7	Surface water	19.1	6.8 ± 0.45	n.c.
		Deep	24.4	22.6 ± 2.3	n.c.
	8	Surface water	24.4	22.8 ± 3.1	n.c.
		Deep	23.8	21.8 ± 3.5	n.c.
	9	Surface water	31.2	39.5 ± 4.7	MP – 27.8 ± 2.9
					MPO – 28.6 ± 2.2
		Deep	4.2	2.1 ± 0.7	n.c.
	10	Surface water	31.0	42.3 ± 3.8	MP – 22.7 ± 2.5
					MPO – 30.5 ± 2.8
		Deep	42.3	81.5 ± 8.4	MP – 25.6 ± 3.6

(*continued on next page*)

Table 1 (*continued*)

Sampling point	Sampling week	Location	Inhibition (%)	Pesticide concentration (μg L^{-1})	
				Biosensor (total)	SPME–GC/MS
					MPO – 68.4 ± 4.0
Point 6	1	Surface water	53.1	108.7 ± 13.7	MP – 35.6 ± 3.2
					MPO – 84.8 ± 8.3
		Deep	54.0	111.5 ± 12.3	MP – 62.0 ± 2.8
					MPO – 98.5 ± 6.5
	2	Surface water	33.5	50.1 ± 6.5	MP – 19.5 ± 2.8
					MPO – 46.3 ± 6.0
		Deep	25.3	25.5 ± 2.5	n.c.
	3	Surface water	64.1	141.9 ± 22.0	MP – 28.6 ± 4.5
					MPO – 87.4 ± 7.7
		Deep	29.2	37.0 ± 3.8	n.c.
	4	Surface water	34.2	52.0 ± 5.5	MP – 16.7 ± 2.4
					MPO – 49.0 ± 4.5
		Deep	37.0	60.6 ± 12.3	MP – 23.0 ± 6.4
					MPO – 47.0 ± 6.8
	5	Surface water	23.3	19.2 ± 2.5	n.c.
		Deep	33.9	51.2 ± 6.9	MP – 15.0 ± 4.5
					MPO – 67.0 ± 9.6
	6	Surface water	19.1	7.0 ± 2.5	n.c.
		Deep	27.7	32.5 ± 8.5	n.c.
	7	Surface water	18.8	6.0 ± 0.5	n.c.
		Deep	17.2	12.0 ± 1.3	n.c.
	8	Surface water	27.6	35.0 ± 6.5	n.c.
		Deep	20.5	9.2 ± 2.8	n.c.
	9	Surface water	52.2	107.4 ± 13.2	MP – 36.8 ± 4.5
					MPO – 88.5 ± 12.3
		Deep	28.0	33.2 ± 6.5	n.c.
	10	Surface water	41.0	72.0 ± 11.5	MP – 14.0 ± 2.5
					MPO – 57.2 ± 8.7
		Deep	43.8	83.6 ± 12.5	MP – 25.6 ± 6.5
					MPO – 67.0 ± 7.6

Na = not calculated; MP = methyl parathion; MPO = methyl paraoxon $N = 3$.

obtained for the completion of electrochemical measurements. Between measurements, the biosensor was washed with PBS buffer (pH = 7.2). In order to verify the stability/reuse of the electrode, ten chronoamperometric measurements were carried out with the same sensor. No significant difference in the electrochemical signal was observed between first and last measurement. The robustness of the developed biosensor was evaluated by studying the concepts of repeatability (new electrode, same standard and substrate solutions, same day, same analyst) and reproducibility (new electrode, new standard and substrate solutions, different day, different analysts). The results indicated that the biosensor possess good repeatability (CV = 12.5%) and reproducibility (CV = 16.0% intra measurements) as if it was an ideal single sensor. The long term stability of the AChE biosensor was investigated under the storage conditions (at 4 °C), it was noticed that the activity of immobilized AChE was stable up to three months. Raghu et al. (2012) have recently constructed an AChE-biosensors based on the enzyme entrapment into silica sol–gel onto the carbon paste electrode which was stable for 1 month. All the analytical parameters for the designed biosensor are summarized in the Supplementary Table 1. These results further supported the importance of macroalgae integration in onto sensor surface. The improved stability of the sensor could be contributed to the alginate component of the macroalgae, which is known to immobilize biomolecules.

3.3. Detection of AChE-inhibitors in the aquatic ecosystem

After optimization and demonstration, the newly designed sensor platform was used to detect AChE inhibitors in the aquatic system. Environmental monitoring generally requires the analysis of a large number of samples and there is a need for low, rapid and sensitive methods of analysis. Here, the insecticide content was determined by AChE-biosensors in natural water samples without any previous extraction, clean-up and preconcentration steps, whereas for GC/MS a previous SPME procedure had to be carried out.

An inhibition curve was constructed for the methyl parathion pesticide (Fig. 3). The limit of detection was defined as 10% inhibition of the total enzymatic activity. With this novel AChE-macroalgae-biosensor, a higher sensitivity was observed in comparison to other reported methods (Dondoi et al., 2006; Rodrigues et al., 2011; Van Dyk and Pletschke, 2011). The detection limit obtained by using the linear regression equation for the methyl parathion was found to be in the range of 1.5–1.8 μg L^{-1}. These LD values are much lower than that found by Dondoi et al. (2006), for example, which have found 7.5 ppb for the oxidized form of parathion, paraoxon. It is convenient to mention that in this study the authors performed a solid phase preconcentration by using a XAD2 column. In the present study, no pretreatment of water samples was carried out previously inhibition assay, which leads us to conclude that a previous pre-concentration step could make the LOD even lower, perhaps at subppt levels. The sensitivity achieved with this biosensor makes it suitable for environmental control, since its LD can be highly improved through a pre-concentration step, making it below to the concentration limits recommended by the Environment Protection Agency (EPA) and by the Brazilian Ministry of Environment which are 0.1 ppb (for a unique pesticide) (Fer, 1979) and 40 ppb (Brazil, 2005), respectively (1979). It is convenient to mention that numerous events involving human poisoning and environmental contamination with methyl parathion led the US-EPA to cancel the registrations of emulsifiable concentrate formulations in 1997(Agrow). When large concentrations of methyl parathion reach the soil, degradation will occur only after many years (Lewis, 1989). While the Ministry of Environment of Brazil is considering changing its legislation and finally ban this pesticide from

the market, rapid monitoring campaigns should be made aiming to control its use and consequently its effects. Thus, even without a pre-concentration step, the biosensor described in this work is sufficiently sensitive for this purpose, because of its LD below the limit content for this pesticide in natural waters recommended by current Brazilian law which is 40 ppb (2005).

Moreover, since the response of the biosensor, in terms of intensity of current, increased in the presence of macroalgae, then the enzyme loading may be decreased. For this novel biosensor, the AChE charge was reduced to 1/3 of that required for the construction of biosensor without macroalgae. This means an economy of total analytical process.

The water samples which provided AChE inhibitions higher than 30% were analyzed by SPME–GC/MS for the final confirmation of the presence of methyl parathion and methyl paraoxon insecticides. Table 1 presents the results obtained with natural water samples analysis. The inhibition values were obtained directly in water samples, and they were equivalent to the inhibitory effect induced by the compounds methyl paraoxon and methyl parathion, in higher and lower level, respectively.

GC/MS method has presented detection and quantification limits of 14.5 $\mu g\,L^{-1}$ and 21.9 $\mu g\,L^{-1}$, respectively. Results by GC/MS presented in Table 1 were calculated on basis of the sum of two compounds identified through the use of the MS library. Confidence limits calculated based on 95% probability are presented in each value of concentration obtained by two methods in each sample data. Results obtained for the samples that presented higher inhibitions allowed us to evidence a relatively good correlation between the two analytical techniques (Table 1) which shows the total concentration data obtained in sampling point 1 over 10 weeks by using both analytical techniques (Fig. 4). In fact, for the majority of points the correlation between the total OP pesticide amount estimated by AChE-based biosensor and determined by SPME–GC/MS is quite good, with a statistical deviation lower than 20%, indicating that the relative inhibition obtained by the biosensor was actually due to the presence of methyl parathion and its major degradation product, methyl paraoxon.

It was observed a large variation of results over time and according to the collection site, so that the pollution is not constant in all seasons (Table 1, Fig. 4). This phenomenon was also observed by Raghu et al. (2012) and Pedrosa et al. (2008) by analyzing natural water samples with AChE-biosensors. Moreover, the pesticide concentration in the water surface seems to be always superior to the concentration at the deep. In general, the results showed very strong inhibitions in most samples, indicating a significant pollution of the investigated aquatic ecosystem. Cassaco lake is situated in a lower zone, above which are installed several plantations of vegetables that have been using for years the pesticide methyl parathion to protect against insects, resulting in high rate of water pollution.

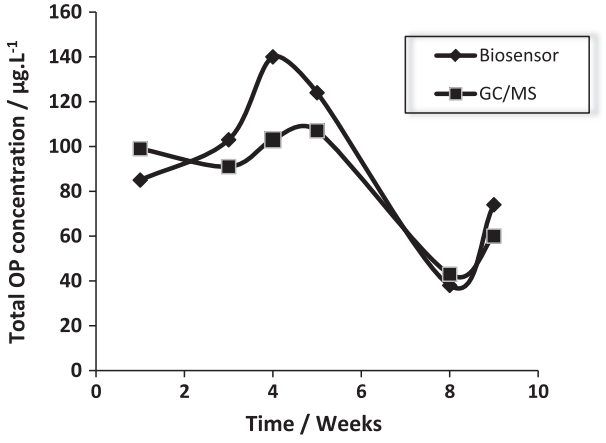

Fig. 4. Total AChE-inhibitor concentration obtained by using TCNQ-macroalgae-modified biosensor and SPME–GC/MS techniques over 10 weeks in collecting point 1, Cassaco Lake, São Luis Island, Maranhão State, Brazil.

4. Conclusion

Biosensors are a promising tool to supplement existing techniques, due to their unique characteristics, such as selectivity, relatively low cost of construction and storage. However, these tools still need to be improved to encourage the replacement of classical analytical procedures. In this work, we have designed a macroalgae based biosensor and evaluated its performance towards polluted aquatic environment. The biosensor system was showed to be successful in screening of OP insecticides in water samples. The use of hydrolyzed macroalgae as immobilization matrix reduced both enzyme amount and substrate concentration, which allowed the biosensor to operate at a relatively low cost. A good quantitative correlation between inhibition of AChE obtained by using the biosensors develop in this work and the level of methyl parathion and its degradation product found by GC/MS was observed. The screening was enough to establish the level of pollution to which the aquatic environment is currently subjected.

These results can be photography of Brazilian reality. In fact, chemical contamination is extremely severe in Brazil; the only other countries that can be compared are China and some of the Eastern Europe countries. The present study emphasizes thus the need for local public politic to reduce the environmental and health impacts caused by the indiscriminate use of pesticides.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.05.028.

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