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Validation of a conductometric bienzyme biosensor for the detection of proteins as marker of organic matter in river samples

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Abstract

This article describes a conductometric bi-layer based bienzyme biosensor for the detection of proteins as a marker of organic matter in rivers. Proteins were chosen to be used as indicators of urban pollution. The working mechanism of the bienzyme biosensor is based on the enzymatic hydrolysis of proteins into several fractions (peptides and amino acids), which results in a local conductivity change depending of the concentration of proteins. In this work, we began with the optimization of biosensor response using bovine serum albumin (BSA) as standard protein. For this objective seven enzymatic biosensors were prepared: four enzymatic sensors with only one layer of enzyme (proteinase K, trypsin, pronase or protease X) and three other enzymatic sensors with two layers (first layer: membrane containing proteinase K; second layer: one of the three other enzymes: trypsin, pronase or protease X). The biosensors were obtained through the deposition of enzymatic layers and the cross-linking process between enzymes and BSA in saturated glutaraldehyde vapour. The response of the various biosensors, described previously, were compared with the values of total organic carbon (TOC), and those of organic nitrogen (N_{org}), as determined by the laboratory accredits (CEMAGREF of Lyon) using the traditional method of analysis (NF EN 1484, infrared spectroscopy) and (NF EN 25663, mineralization/colorimetry assay) respectively for each water sample obtained from different sites in Lyon (France). The linear correlations obtained with the response of the seven biosensors showed the most important indices of correlations for the biosensor with two enzymatic layers: proteinase K + pronase (pkp). The optimum conditions for the preparation of the pkp biosensor increased the sensitivity and gave a limit of quantification of 0.583 μg/L for TOC and 0.218 μg/L for N_{org} in water samples. This sensor shows good reproducibility (2.28%), a capacity to be used at temperatures range 10–30°C (depending on the season) and moreover a long lifetime (5 weeks).

Key words: conductometric biosensor; proteins; enzyme; natural water analyses; total organic carbon; organic nitrogen
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Introduction

The analysis of various biological and chemical pollutants in environmental matrices has entered a new phase during the last decade. Improvements in instrumentation, sampling, and sample preparation techniques have become essential to keep up the requirements of detection at low levels such as the μg/L or ng/L range, as well as to achieve a fast analysis. Furthermore, analytical data have to fit to more and more stringent regulations especially in the fields of medicine, drinking water and food control. Biosensor can be considered as competitive tools for environmental monitoring because of their specificity, fast response and low cost.

Traditional systems of analysis are not in adequacy with the new needs such as *in situ* control of pollution and water quality control. For this reason, new microsystems should be designed to perform *in situ* continuous measurements. These microsystems should provide real time values of the organic matter contamination of the hyporheic zone which can be assimilated by microorganisms. The three types of organic matter which can be used to monitor the assimilation: lipids (10%–40% of oxidizable organic matter), glucides (10%–15%) and proteins (20%–30%). Among them, the protein content seems to constitute an excellent indicator of the hyporheic activity (Namour *et al*., 1998).

However, the most widely used tests are based on colorimetric procedures in which protein reacts with chromophores to produce colored complexes. These methods include the Biuret method (Sapan *et al*., 1999; Jdanova *et al*., 1996) and the Coomassie Blue (CB) G-250 dye binding (Bradford, 1976). These methods depend on a number of factors other than absolute protein quantity. For example, the CB test relies on the binding of dye to lysine and arginine residues. The Biuret method, whilst being independent of amino acid composition, is relatively insensitive and can vary according to sample purity. With the characteristics presented below, biosensors are the ideal tool for the detection of proteins.

In addition, unlike the optical methods of protein

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determination, the electrochemical sensors are able to measure turbid and colored samples. A number of biosensors for the detection of some protein have been developed (Morris and Sadana, 2005; Nordin *et al*., 2005).

Much work has been done with these conductometric biosensors for the detection of a variety of molecules (Dzyadevych *et al*., 2001; Anh *et al*., 2004). This type of biosensor presents many advantages: thin-film electrodes are suitable for miniaturization and large-scale production using low cost technology; they do not need any reference electrode and the transducers are not light sensitive. Finally, the supply voltage can be sufficiently low to significantly decrease the power consumption. In addition, the conductometric detection mode presents the major advantage that a large number of enzymatic reactions involve either consumption or production of charged species and therefore, lead to a detectable change in the ionic composition of the medium. This original concept allows its integration in a river automatic monitoring system for the assessment of the ecosystem chemical contamination (Chouteau *et al*., 2005).

There are several classes of proteases depending on their active site (Cormish-Bowden *et al*., 2004). For example, trypsin is a serine based protease of only one polypeptide chain of 223 amino acids. It catalyses the hydrolysis of the peptide connection at the end of the residues of amino acids, lysine, and arginine (basic amino acid) (Walsh, 1970). Proteinase K is a serine based protease of 28.9 kDa that hydrolyzes any protein of any origin in a few hours, preferentially the peptide connections located after the hydrophobic amino acids (leucine, for example). Protease X is a thermostable (thermophilic) extracellular metalloendopeptidase containing four calcium ions, its cofactors are zinc and calcium. It hydrolyzes the protein bonds on the N-terminal side of hydrophobic amino acid residues and is often used to do limited proteolysis for peptide mapping and studies of protein structure and conformational changes (Lazic *et al*., 2007). Finally, pronase is a mixture of endo- and exo-proteinases; it cuts almost any peptide bond. For its stability, the pronase requires the presence of calcium ions (Branden and Tooze, 1996).

The working mechanism of the bienzyme biosensor is based on the enzymatic hydrolysis of proteins into several fractions (peptides and amino acids), which results in a local conductivity change depending of the concentration of proteins. The objective of this work is the conception and validation of a conductometric protease biosensor allowing the *in situ* monitoring of organic matter assimilation *in situ* through continuous measurements of protein concentration in water samples.

The performance of each biosensor was tested and optimized. The validation of the optimum biosensor was performed using natural water samples in parallel with classical analysis methods in terms of total organic carbon (TOC), according to the European Standards NF EN 1484 (infrared spectroscopy technique), and organic nitrogen (N_{org}), according to the European Standards NF EN 25663 (mineralization/colorimetry assay).

1 Materials and methods

1.1 Reagents and chemicals

Trypsin (EC 3.4.21.4, freeze-dried from bovine pancreas, DIFCO), and Proteinase K (EC 3.4.21.64, freeze-dried 2989 U/mg from *Tritirachium album*) were provided by Fluka (France). Pronase (*Streptomyces griseus*), freeze-dried, 7 U/mg was purchased from Roche. It is an enzymatic preparation exhibiting a nonspecific protease activity. Indeed, this preparation contains endo- and exo-peptidases and, in particular, chymotrypsin, trypsin, carboxypeptidase and aminopeptidase. Its optimum pH is in the range of 7–8. Protease X 38 U/mg, bovine serum albumin (BSA) and glutaraldehyde (GA) (grade II, 25% aqueous solution) were purchased from Sigma-Aldrich (France). All other chemicals were of analytical grade. Millipore Milli-Q ultrapure water (resistivity 18.2 MΩ·cm) was used throughout for the preparation of solutions.

1.2 Design of conductometric transducers

Conductometric transducers (Fig. 1), consisting of two identical pairs of gold interdigitated thin film electrodes, were obtained from Research Institute of Microdevices (Kiev, Ukraine) (Chouteau *et al*., 2005; Marrakchi *et al*., 2005). Two pairs of Au interdigitated electrodes (150 nm thick) were fabricated by lift-off on a ceramic substrate (5 × 30 mm). A 50-nm thick intermediate Ti layer was used to improve the adhesion of Au to the substrate (Hoogvliet

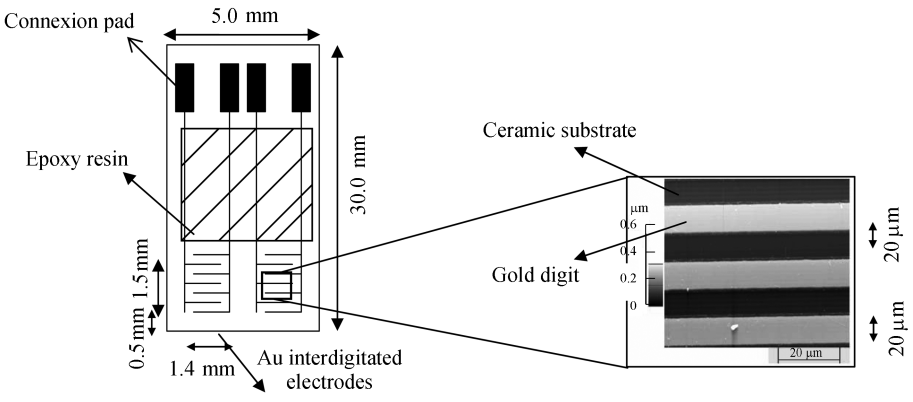

Fig. 1 General view of the transducer conductometric.

and Bennekom, 2001). The pads of the transducteur were covered manually with epoxy resin for their insulation. Both the digit width and the interdigital distance were 20 μm, and their length was about 1.0 mm. As a result, the "sensitive" area of each electrode was about 1 mm^2.

1.3 Conductometric measurements

With each pair of interdigitated electrodes, conductometric measurements were performed by applying a small-amplitude alternating voltage (10 mV peak-to-peak at about 0 V) of 100 kHz generated by a low-frequency wave-form generator (Schlumberger type 4431). The differential output signal between working and reference sensor was recorded using a Stanford Research Systems lock-in amplifier SR510 and the responses of the biosensor were recorded as a function of substrate concentration. The reason for choosing the frequency indeed, at frequencies higher than 10 kHz, the impedance of diffusion can be neglected and the total impedance of the system consists mainly of the resistance of the electrolyte solution in series with, the double layer capacitance and the polarization resistance in parallel to each other. The phase signals from both sensors were compared only in terms of the resistance contribution and its variation was recorded.

Measurements were carried out in daylight at room temperature in a 5-mL glass cell filled with the 5 mmol/L phosphate buffer pH 7.5 strongly agitated. After stabilization of output signal, different aliquots of substrate solution were added to the vessel.

1.4 Enzyme immobilization

1.4.1 Preparation of the enzymatic membranes

The following protocols were used for the membrane preparation (reference and working sensors). (1) The enzymatic membrane for the working sensor was: a mixture of 4% of enzyme and 6% of BSA prepared in 20 mmol/L phosphate buffer at pH 7.5, and then 10% of glycerol was added to the mixture. (2) The membrane for reference sensor was a mixture of 10% BSA and 10% glycerol prepared in 20 mmol/L phosphate buffer.

Bovine serum albumin (BSA) was used in addition to the enzyme before the co-reticulation, to allow a better distribution of the various proteins, leading to a better control of the enzymatic activity without deteriorating the mechanical properties of the membranes. Glycerol plays the role of a plasticizer, which makes it possible to avoid the formation of cracks in the membrane during storage, and to obtain a better homogeneity and a better adherence of the membrane onto the sensor surface.

Seven enzymatic biosensors were prepared: four enzymatic sensors with only one layer of enzyme (proteinase K, trypsin, pronase or protease X) and three other enzymatic sensors with two layers (first layer: membrane containing proteinase K; second layer: one of the three other enzymes: trypsin, pronase or protease X). The immobilization of the bioreceptor is one of the key points as it can also influence the operational stability, shelf-life, recovery time, sensitivity, linear range, accessibility to the transducer and response time (Hamlaoui *et al*., 2002).

1.4.2 Immobilization on the sensor surface

After the optimization of the enzymatic membrane formulation, the deposition procedure was performed as the following. (1) Preparation of the first layer: 0.1 μL of each membrane preparation (as defined in Section 1.4.1) was deposited on the sensitive area of the sensor using a microsyringe, BSA membrane for the reference sensor and enzymatic membrane for the working sensor. Sensors were then placed in a saturated GA vapour during a given optimal time according to the enzyme used and then dried and left at room temperature. As shown in Fig. 2, the optimal exposure time in GA vapour is 10–12 min for each layer. GA is a bifunctional agent, having two aldehyde groups at its ends that react with the amine functions of the enzyme or proteins to form imine. This results in a considerable increase of the molecular mass, giving place to an insoluble compound. (2) Preparation of the second layer: a second layer was deposited on the sensitive area of the sensor, BSA membrane for the reference sensor, and enzymatic membrane (protease, pronase or trypsin) for the working one. Then the sensor was again placed in an atmosphere saturated with glutaraldehyde. The biosensor was then dried at room temperature for 30 min and then put into 20 mmol/L phosphate buffer pH 7.5 during 15 to 30 min to eliminate non-immobilized enzyme molecules and to produce a homogenous membrane.

The time of exposure to GA vapour strongly influences the biosensor quality. In fact, for a longer immobilization time (> 25 min), a dramatic decrease of response was obtained. This phenomenon can be related to the formation of a large number of covalent bonds between glutaraldehyde and the enzyme molecules, which lead to the blocking of the enzymes active centers. Moreover, such a dense membrane can reduce the diffusion of substrates and products of the biochemical reaction that can result in broadening the dynamic range of the sensor response. On the other hand, for short exposure times (< 10 min) the enzyme leakage through the membrane can take place due to insufficient covalent bonding thus, the stability of the biosensor becomes poor, and the response of the sensors decreases markedly with the number of measurements.

1.5 Storage

Biosensors were stored at 4°C in the 20 mmol/L phosphate buffer solution, pH 7.5, between the experiments.

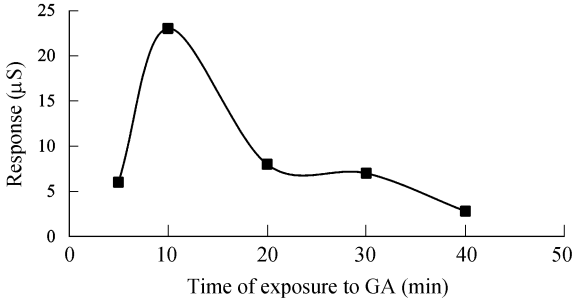

Fig. 2 Effect of immobilization time with glutaraldehyde (GA) on the biosensor response for monolayer of proteinase K. Measurements were performed in 5 mmol/L phosphate buffer, pH 7.5 for 2 mg/L bovine serum albumin (BSA).

1.6 Preparation of the natural water samples

Different samples were taken from different sites around Lyon in France: Ribes (hyporheic, surface water), Chaudanne (surface water), Yzeron (hyporheic, surface water) were filtered using a 0.45-μm filter to eliminate the insoluble microorganisms and compounds. The removal of the microorganisms is important to improve the stability of the physicochemical characteristics of the samples throughout the experiments.

Traditional methods of analysis for the total organic carbon (NF EN 1484) and for the organic nitrogen (NF EN 25663) were applied to the filtered water samples (Table 1). We used these eight different samples for comparing the responses obtained with seven biosensors. But, for the characterization of the pkp biosensor (proteinase K + pronase), a sample was prepared by mixing this eight different samples (working natural water sample).

2 Results and discussion

The response of the various biosensors, described previously (as defined in Section 1.4.1 and Section 1.4.2), were compared to the concentration of TOC and N_{org}, as determined by the analysis laboratory accredits (CEMAGREF of Lyon) for each water sample (Table 1). The linear correlations obtained for the response of the seven biosensors versus TOC concentration and N_{org} concentration respectively are displayed in Table 2. The conductometric biosensor with one layer of proteinase K allowed us to show the feasibility of this type of biosensor for protein detection (Marrakchi *et al*., 2005, 2007). Nevertheless, the higher correlations indices are obtained for the biosensor with two enzymatic layers: proteinase K + pronase (pkp). Indeed, the membrane compositions can be classified according to the increasing indices of correlation as follows: protease X < trypsin < pronase < proteinase K < proteinase K + protease X < proteinase K + trypsin < proteinase K + pronase (pkp).

The two histograms present hereafter (Fig. 3) the responses obtained for the seven biosensors for the different natural water samples. In Fig. 3a, the responses are compared to TOC reference concentrations whereas in Fig. 3b, they are compared to N_{org} reference concentrations. The biosensor with proteinase K + pronase (pkp) gives the higher signal and a response in close agreement with the variations in TOC and N_{org} concentrations, this biosensor was then selected, compared to the first conductometric biosensor for protein detection (Marrakchi *et al*., 2005, 2007). In fact, the pronase composition (endo- and exo-peptidases and in particular: chymotrypsine, trypsin, carboxypeptidase and aminopeptidase) and the association with proteinase K confer to the biosensor the capability to catalyze the hydrolysis of all the different proteins contained in the water samples.

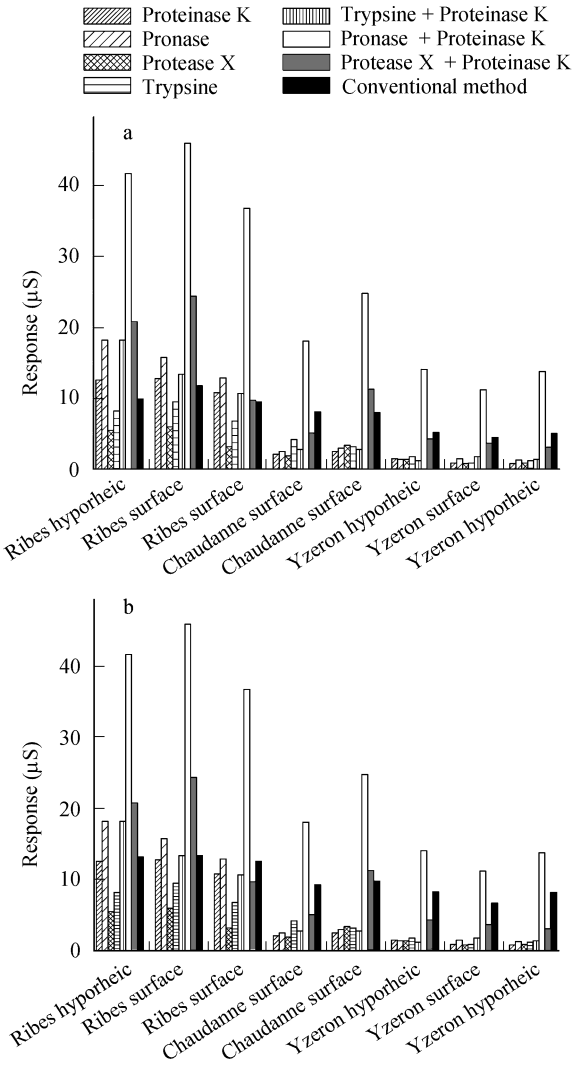

Fig. 3 Biosensors response for different natural water samples and comparaison to their TOC (a) and N_{org} (b). Measurements were performed in 5 mmol/L phosphate buffer at pH 7.5.

Table 1 Chemical content of the natural water samples (mg/L)

Site	Nkj	NH_4^+-N	N_{org}	TOC	NO_3^--N	NO_2^--N
Ribes (hyporheic water)	2	0.88	1.32	9.9	2	0.16
Ribes (surface water)	2.8	2	1.24	11.8	1.2	0.14
Ribes (surface water)	1.3	0.05	1.26	9.5	1.6	0.38
Chaudanne (surface water)	1	0.09	0.93	8.1	2.2	0.07
Chaudanne (surface water)	1	0.02	0.98	8	2.3	0.11
Yzeron (hyporheic water)	0.9	0.09	0.83	5.2	1.3	0.1
Yzeron (surface water)	0.7	0.04	0.67	4.5	1.3	0.1
Yzeron (hyporheic water)	0.9	0.1	0.82	5.05	0.9	0.08

N_{Kj}: kjeldal nitrogen; N_{org}: organic nitrogen; TOC: total organic carbon.

Table 2 Correlation indices obtained for the response of the seven biosensors versus TOC and N_{org}

Sensor	TOC		N_{org}	
	R^2	Sensitivity ((μS·L)/μmol)	R^2	Sensitivity ((μS·L)/μmol)
Protease X only	0.779	0.284	0.693	0.0244
Trypsin only	0.683	0.329	0.838	0.0332
Pronase only	0.740	0.3125	0.875	0.0309
Proteinase K only	0.787	0.425	0.896	0.0413
Proteinase K + protease X	0.863	1.216	0.802	0.1066
Proteinase K + trypsin	0.898	0.0685	0.890	0.771
Proteinase K + pronase	0.901	0.1811	0.928	0.0167

2.1 Analytical characteristics of the pkp biosensors

2.1.1 Specifity of the pkp biosensor response

The differential mode measurement used is able to avoid any interference that should be caused by charged species. In order to check or verify this effect, the differential mode measurement was performed with two reference sensors (BSA membrane on both sensors). The small peak observed after injection of 2 mg/L of BSA in the measuring cell is due to a heterogeneous distribution of BSA between both electrodes at the time of the injection. However, with the magnetic stirring, the medium of measurement quickly became uniform and a nil response was detected.

2.1.2 Effect of pH concentration

The influence of pH on the response of the pkp biosensor has been studied. Since the enzyme activity is strongly affected by the solution pH. The effect of pH on the biosensor response was determined by injecting the working natural water sample (as defined in Section 1.6), in 5.0 mmol/L phosphate buffer at pH range 3–12. The final TOC concentration being 10.5 μg/L and the final N_{org} concentration being 3.95 μg/L. The highest signal of the biosensor occurs at pH 7.5. We then used the buffer solution at pH 7.5 throughout the experiments to obtain the highest sensitivity.

2.1.3 Effect of temperature

The effect of temperature on the biosensor response was monitored by injecting the working natural water sample (as defined in Section 1.6.) in 5.0 mmol/L phosphate buffer pH 7.5 for different temperatures varying from 4 to 45°C. The final TOC concentration was 10.5 μg/L and the final N_{org} concentration was 3.95 μg/L. As the majority of enzymes, the activity is temperature dependent. It can be observed that with increasing temperature the biosensor sensitivity increased. The optimum performance was reached between 20 and 30°C. This study shows the capacity of the biosensor to work at low temperatures (⩽ 10°C), which is very important for it's on site use (expected change of in water temperature according to the season). These results (effects of pH and of temperature) are similar to those observed for a sensor with only one enzyme layer, proteinase K for BSA (Marrakchi, 2005).

2.1.4 Sensitivity of the pkp biosensor for BSA detection

With the pkp sensor, it is possible to obtain a detection range of BSA between 0.4 and 8 mg/L (Fig. 4).

2.1.5 Lifetime of the biosensor (pkp)

Long-term storage stability is one of the key factors of a biosensor performance. After each measurement, the pkp biosensor was rinsed with 20 mmol/L phosphate buffer solution at pH 7.5 and stored at 4°C. The pkp biosensor presents a storage stability of more than 4 weeks when BSA samples are measured and 5 weeks when working water samples were measured (Fig. 5). During the first two days, the response decreased due to the salting out of certain molecules of the enzymes not well immobilized. However, after this first period a quite stable response was observed for more than 1 month for both types of samples.

2.2 Validation of the pkp biosensor with the working natural water samples

For validation of the pkp biosensor, several European standards such as T 90-552, XP T 90-210, ISO 15839 and ISO 5725-1-6 can be used. The validation procedure used in this work is the AFNOR XP90-210 standard. This standard procedure allows us to evaluate an alternative method of quantitative physicochemical analysis compared to a reference method.

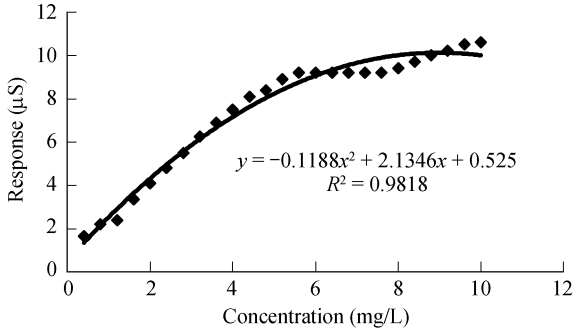

Fig. 4 Dependence of the biosensor (pkp) response on BSA concentration. Measurements were performed in 5 mmol/L phosphate buffer, pH 7.5. The fitting curve was obtained according to second degree equation.

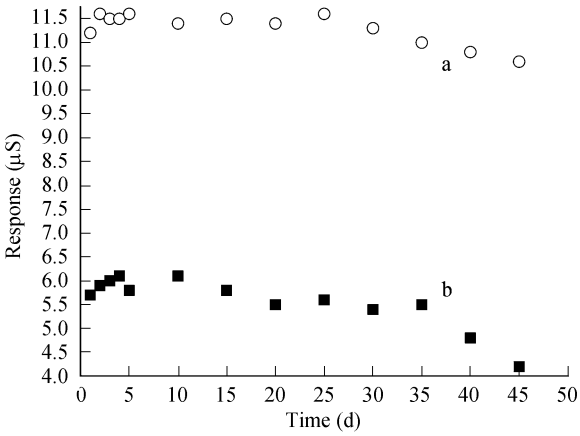

Fig. 5 Study of the biosensor (pkp) stability for 3.95 μg/L N_{org} concentration (a) and 10.54 μg/L TOC concentration (b) in natural water samples and for 1 mg/L BSA concentration. Measurements were performed in 5 mmol/L phosphate buffer, pH 7.5.

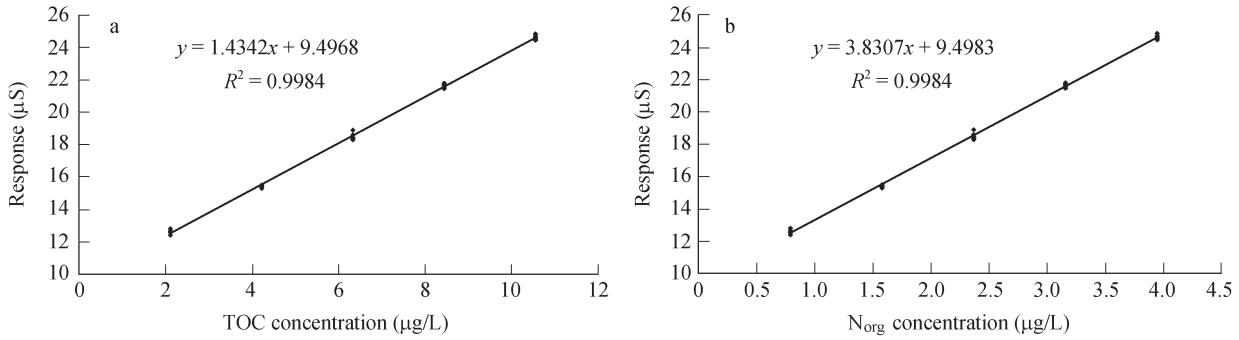

Fig. 6 Calibration curve of biosensor (pkp) for TOC (a) and N_{org} (b) concentrations. Measurements were performed in 5 mmol/L phosphate buffer, pH 7.5.

For this purpose, five different aliquots of the working water sample were injected in 5.0 mmol/L phosphate buffer, pH 7.5. The measurements were repeated 5 times for each concentration. The results are displayed in Fig. 6, as corresponding to conductivity measurements versus concentration of TOC and of N_{org}, respectively. An acceptable linear regression was obtained in both cases. The repeatability is constant, the standard deviation being equal to 2.28% for five determinations using one sensor. The sensor-to-sensor reproducibility was found to be 11%.

The quantification limits were 0.583 μg/L of TOC and 0.218 μg/L of N_{org}, respectively. This increase in sensitivity compared to the detection of BSA can be explained by the possible presence in natural river water of a heterogeneous composition of proteins. In addition, proteins found in natural water are different in size and nature which favors their hydrolysis and detection by the biosensor, in comparison to BSA that is a large and complex protein.

3 Conclusions

In this work, a conductometric bienzyme biosensor optimized for organic mater determination in water samples was developed using two sets interdigitated thin-film planar electrodes and immobilization of an enzyme bilayer (pronase and proteinase K) with BSA in glutaraldehyde vapour. The optimum conditions for the preparation of the biosensor increased the sensitivity and gave a limit of quantification of 0.583 μg/L for total organic carbon and of 0.218 μg/L for organic nitrogen in water samples. This sensor shows good reproducibility (2.28%), a capacity to be used at temperatures varying between 10 and 30°C (temperature depending on the season), and a long lifetime (5 weeks).

In future developments, these biosensors will be specifically designed for an *in situ* use by adding a physical protection around the sensitive part to protect the enzymatic membranes from water current during their use. The main interest of these *in situ* sensors will be the real monitoring of organic matter (during storm for instance) which is not realistic when samples are analysed.

Acknowledgments

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