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# Enzymatic Conductometric Biosensor Based on PVC Membrane Containing Methyl Viologen/Nafion®/Nitrate Reductase for Determination of Nitrate in Natural Water Samples

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A highly sensitive, fast, and stable conductometric enzyme biosensor for the determination of nitrate in water is described and validated in natural water samples. The nitrate biosensor is based on a methyl viologen mediator mixed with nitrate reductase (NR) from *Aspergillus niger* and Nafion® cation-exchange polymer dissolved in a plasticized PVC membrane deposited on the sensitive surface of interdigitated electrodes. The process parameters for the fabrication of the enzyme biosensor and various measuring conditions such as pH buffer concentration and temperature were investigated with regard to their effect on sensitivity, limit of detection, dynamic range and operational and storage stabilities. The sensitivity of the nitrate sensor was approximately 1.48 μS·L/mg, the detection limit was 1.2 μg/L, and linear calibration was in the range from $4\times10^{-3}$ to 8 mg/L with an application domain from $4\times10^{-3}$ to 50 mg/L. When stored in 20 mM phosphate buffer (pH 7.5) at 4°C, the sensor showed good stability over 2 months.

## 1. Introduction

The environmental water concentrations of the nitrogen oxy-anions, nitrate and nitrite, have progressively increased because of anthropogenic inputs. The presence of this anion is predominantly due to leaching from arable farmland owing to the extensive use of nitrogen-based fertilizers.[1,2]

The net results of the leaching of nitrates into environmental water are a depletion of dissolved oxygen and possible eutrophication because of algal blooms.[3] Nitrate enters the human body via the consumption of vegetables and drinking water. Nitrates are not

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considered toxic to humans, although they are reduced in the body to nitrites, which can then be further converted into *N*-nitrosoamine compounds. The latter compounds are possibly carcinogenic.[4,5]

For human health protection, the European Union (EU) has imposed limits on nitrate and nitrite in potable water of 50 mg/L (0.8 mM) and 0.1 mg/L (2.2 μM), respectively.[6] Nitrate and nitrite determination is mainly carried out by spectrophotometry (Griess reaction), ionic chromatography, polarography, capillary electrophoresis, colorimetry, ion-selective electrode techniques, and fluorescence spectrophotometry.[7]

However, these centralized and sophisticated analytical systems cause delays in solving problems in emergency situations where rapid food quality controls or environmental pollution monitoring are issues of critical concern. As a consequence, for the last three decades, there has been growing interest in the design of biosensors that intimately combine the recognition properties of biological macromolecules with the sensitivity of transducers, among them biosensors based on electrochemical transduction constituting the main category.

One of the most popular biosensor configurations consists of enzyme immobilized within a polymer, which is generated over an electrode. The electrosynthesis of organic conducting polymers indeed allows the reproducible deposition of biological macromolecules with controlled spatial resolution.[8] In addition, the electropolymerization of polymers functionalized by redox groups is an attractive approach for the immobilization of biomolecules where the electron transport to enzymes is ensured by electron hopping between immobilized redox centers.[9,10] In that sense, the majority of biosensors reported for the measurement of nitrate ions are molecular-based systems using a nitrate reductase enzyme purified from plant, fungal, or bacterial sources. Both electrochemical and optical biosensors have been reported.[11,12]

Nitrate reductase (NR) is the first enzyme involved in nitrate assimilation in higher plants.[13] The enzyme catalyzes the rate-limiting and regulated step, the two-electron reduction of $NO_3^-$ to $NO_2^-$, in the pathway of inorganic nitrogen assimilation.[14] The enzyme has been isolated from a variety of sources including algae, fungi, yeast, and higher plants[15,16] and, in all cases, has been shown to consist of multimeric proteins composed of identical subunits, each of which contains three cofactors, flavin adenine dinucleotide (FAD), a b-type cytochrome (cytochrome b557), and molybdenum cofactor prosthetic groups in a 1:1:1 stoichiometry per subunit. The assimilatory NR isolated from *Aspergillus niger* comprises a dimer of subunits each with a molecular mass of approximately 95 to 100 kDa.

All known sequences of NR have been found to contain one conserved cysteine (Cys) residue that is located in the cytochrome *b* fragment of the enzyme.[17] Site-directed mutagenesis of the cytochrome *b* domain of corn leaf NADH:NR showed that this Cys residue is not essential for NADH binding or NADH:NR activity, but is essential for the highly efficient catalytic transfer of electrons from the NAD(P)H to FAD.[18] Reducing equivalents, donate to the enzyme from the physiological electron donor, NAD(P)H, ingress at the flavin prosthetic group and egress to nitrate via the molybdenum center.

In addition to the physiological NADH:NR activity, the enzyme catalyzes a variety of partial activities using artificial electron donors and acceptors in combination with one

or more of the enzyme prosthetic groups that have proven to be of value in dissecting the electron transfer sequence within this complex metalloflavoprotein[17,18] (cf. Fig. 1).

Viologens, derivatives of 4, 4-bipyridyl, continue to play an important role as electron relays in systems in which electron transfer is initiated by electrochemical processes.[19] They have been widely used as mediators for different amperometric and conductimetric nitrate reductase enzyme biosensors such as nitrate biosensors[20,21] and glucose oxidase biosensors.[19,22] Viologens are highly water soluble and very toxic. Therefore, any practical device containing these electron mediators should be based on immobilized viologens.[22,23] They exhibit fast reversible electrochemical responses at negative redox potentials, which make them useful as redox mediators for numerous enzymatic reactions .[19]

Of special significance are the reversibility of their redox processes and the chemical properties of their one-electron reduction products. The structure of methyl viologen consists of a hydrophobic part that is capable of hydrophobic-hydrophobic interaction with Nafion® solution and two cationic pyridinium groups that undergo ion exchange with the sulphonate sites of Nafion® polymer chains. This interaction results in the accumulation of methyl viologen in Nafion® films, as follows.

$$MV_{aq}^{2+}+2(SO_3^-Na^+)_{film} \rightarrow [(SO_3^-)_2MV^{2+}]_{film}+2Na_{aq}^{+} \quad (1)$$

Methyl viologen's electrochemical behavior involves the reduction of $MV_2^+$ by a reversible one-electron reaction to a blue radical cation, which can be further reduced to the neutral form that tends to adsorb on the electrode surface. A $MV_2^+/MV^+$ redox couple that has a high electron-exchange rate can facilitate the electron transfer between the biosensor and $O_2$. Most importantly, $MV^+$ as the electron reaction intermediate can also be entrapped in Nafion® film instead of diffusing rapidly away from the biosensor thereby maximizing signal strength.[19] The catalytic mechanisms can be expressed as follows.

$$[(SO_3^-)_2\,MV^{2+}]+1e^- \rightarrow [(SO_3^-)_2\,MV^+] \quad (2)$$

$$[(SO_3^-)_2\,MV^+]+1/2O_2+H^+ \rightarrow [(SO_3^-)_2\,MV^{2+}]+1/2H_2O_2 \quad (3)$$

In this work, we present a conductometric nitrate biosensor obtained using the co-immobilization of nitrate reductase from *Aspergillus niger* and methyl viologen with

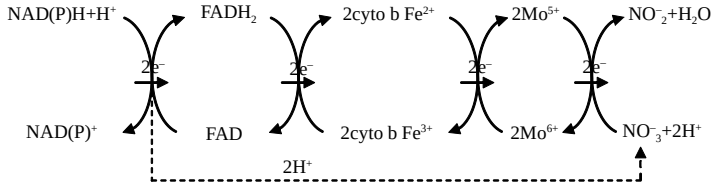



Fig. 1. Mechanism of nitrate reductase reaction.

Nafion® in a poly(vinylchloride) (PVC) matrix at an interdigitated thin-film electrode surface. Phthalates are used as plasticizers in PVC.

Conductometric transducers present many advantages: thin-film electrodes are suitable for miniaturization and large-scale production using low-cost technology; they do not require 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 has a major advantage because a large number of enzymatic reactions involve either the 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 an automatic river-monitoring system for the assessment of the ecosystem chemical contamination in an ecosystem.[24]

In this study, the electrochemical biosensor operates with the mechanism depicted in Fig. 2. The subsequent local changes of conductance inside the membrane are dependent on the enzymatic reaction with nitrate. The performance of the conductometric nitrate biosensor was validated by the detection of nitrate in natural water samples.

## 2. Materials and Methods

### 2.1 *Materials*

Purified *Aspergillus niger* nitrate reductase (NR) (EC 1.1.6.6.2), bovine serum albumin (BSA), and aqueous solutions (25%, w/v) of glutaraldehyde (GA) were purchased from Sigma-Aldrich (France). Methyl viologen and Nafion® (perfluorosulfonated ion-exchange resin, 5% (w/v) in an 80% aliphatic alcohol and 20% water mixture) were purchased from Sigma-Aldrich Chemie GmbH. Diisononyl phtalate (DNP approx. 80%) and tetrahydrofuran (THF > 99%) were purchased from Sigma. Polyvinylchloride (PVC) (EC 2.0.8.7.5.0.2) and $Ca(NO_3)_2.4H_2O$ (> 99.0%) were purchased from Fluka. All other chemicals were of analytical grade. Millipore Milli-Q ultrapure water (resistivity 18.2 MΩcm) was used throughout for the preparation of solutions.

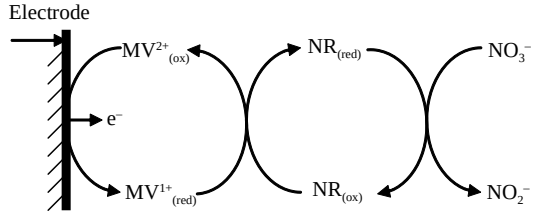



Fig. 2. Operating mechanism for nitrate determination.

### 2.2 *Sensor chip*

The conductometric transducer consisted of two identical pairs of gold interdigitated (IDT) microelectrodes (150 nm thick). It was made at the Research Institute of Microdevices (Kiev, Ukraine)[25] by lithography on a ceramic substrate (5×30 mm). The digit width and interdigital distance were both 20 μm, and their length was about 1.0 mm. As a result, the "sensitive" area of each electrode was about 1 $mm^2$.

The deposition was by the thermal vacuum sputtering of chromium (5 nm) and gold (150 nm) on a glass ceramic substrate to improve the adhesion of gold.[26]

The lithographic process used[25] is as follows.

1. The photoresist is positive with a thickness of 3 μm and a resolution of 400 lines/mm. After deposition by centrifugation, the photoresist is dried under air, heated at 60°C, then finally heated at 120°C.
2. The photolithography is contact optical positive. After exposition under UV, development in 0.5% KOH and the washing-off at the photoresist from the exposed area are carried out.
3. The wet etching of gold in nitrohydrochloric acid and etching of chromium in a special solution are carried out.
4. The washing-off of photoresist using dimethylformamide, washing using distilled water, and drying are carried out.

### 2.3 *Measurements*

Many enzyme reactions may be monitored using conductometric devices with interdigitated microelectrodes. Because the sensitivity of the measurement is hindered by the series conductance of the sample solution, a differential measurement is usually performed between an electrode with the enzyme and an identical one without the enzyme but loaded with bovine serum albumin. Thus, enzymatic reactions produce ions or electrons that induce changes in the overall conductivity or resistivity of the enzymatic membranes.

These changes are measured and calibrated to a suitable scale. An electric field is generated using a 10 mV sinusoidal voltage (AC) at high frequency (typically 100 kHz), which helps minimize undesirable effects such as Faradaic processes, double layer charging, and concentration polarization.[27]

The differential output signal (in-phase) between the working IDT electrodes (working sensor) and reference IDT electrodes (reference sensor) was logged using an SR 510 lock-in amplifier (Stanford Research System), and $\Delta S$ ($\Delta S = (S_n - S_0)$, where $S_n$ is the steady-state signal in the presence of the enzyme and $S_0$ is the steady-state signal obtained in the absence of the enzyme.

Measurements were carried out in daylight at room temperature in a strongly agitated 5 ml glass cell filled with 1 mM phosphate buffer of pH 7.5. After the stabilization of the signal, the substrate was added to the phosphate buffer.

### 2.4 *Enzyme immobilization*

The following protocols were used for the membrane preparation (reference and working sensors).

- Enzymatic membrane for the working sensor: 50 µl of 10% (w/w) NR enzyme and BSA, 3% (v/v) Nafion® (5% in alcohol), 10% (w/w) methyl viologen, and 10% glycerol in 20 mM phosphate buffer (pH 7.5) were mixed with 50 µl of the PVC membrane components (7 mg PVC and 3 mg of DNP at a ratio of 7:3 (w/w)) diluted in 1 ml of THF. It was deposited on the sensitive area of the sensor (working sensor).
- Membrane for the reference sensor: 50 µl of 10% (w/w) BSA, 3% (v/v) Nafion® (5% in alcohol), 10% (w/w) methyl viologen, and 10% (w/w) glycerol in 20 mM buffer (pH 7.5) were mixed with 50 µl of the PVC membrane components (7 mg PVC and 3 mg DNP at a ratio of 7:3 (w/w)) diluted in 1 ml of THF. It was then deposited on the reference sensor.

The sensor chips were placed in saturated glutaraldehyde vapor for 10 min followed by drying at room temperature for 30 min. Moreover, the hydrophobic methyl groups at the surface ensure the good adhesion of the PVC membrane on the ceramic support.

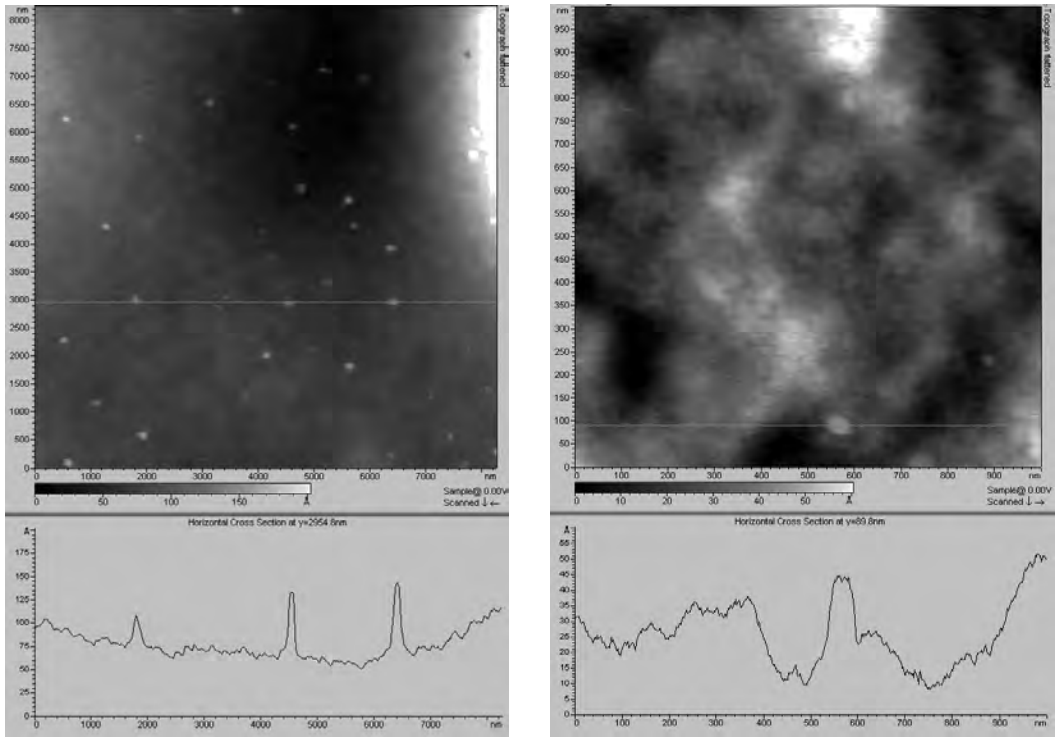
The surface topography of two types of enzymatic membrane, one obtained by enzyme cross-linking with glutaraldehyde vapor and the other by entrapment in the plasticized PVC membrane, was observed by AFM. The surface of the PVC/NR membrane is relatively smooth compared with that of the cross-linked enzymatic membrane (cf. Figs. 3(a) and 3(b), respectively), showing a good wettability of the ceramic support by the PVC membrane.

The performance of the biosensor, in terms of sensitivity and long-term stability, is strictly dependent on the enzyme loading and the amount of BSA. The increase in the amount of enzyme loading might lead to an increase in the substrate diffusion resistance then to a decrease in the biosensor response.

On the other hand, if the enzyme concentration is too low, there are not enough enzymes involved in the reaction, which leads only to a slight variation in conductance. In addition, a high concentration of BSA may effectively decrease the NR activity.[28] In a previous work,[21] higher sensitivity of a biosensor was observed at the concentration of 3% (v/v) Nafion®. However, a further increase in the negatively charged Nafion® polymer content in the composite membrane increases the hydrophobicity of the membrane and also diffusion barriers of the negatively charged substrate through the polymer membranes, resulting in, respectively, a slow response time and moderate sensitivity.

In addition, since Nafion® is dissolved in ethanol, the increase in the Nafion® ratio in the composite membrane could lead to a greater deactivation of NR by ethanol. An increase in the mediator concentration to over 10% (w/v) in the enzyme mixture solution did not lead to an increase in the sensor response. We concluded that the methyl viologen concentration of 10% (w/w) was sufficient to saturate the electrode surface with mediator molecules.

Without the enzyme or mediator, no signal was observed, implying that no direct reduction of $NO_3^-$ occured. This indicates that the conductometric biosensor detection process is nitrate-dependent and enzyme-catalyzed. Glycerol and phthalate (DNP) were




(a) (b)

Fig. 3. (a) Representative AFM image of nitrate reductase (NR) plasticized by PVC membrane. (b) Representative AFM image of NR immobilized by cross-linking with glutaraldehyde.

used as plasticizers to avoid cracks appearing in the biolayer. Electrodes were kept overnight at 4°C in a 20 mM phosphate buffer. This time lapse is necessary to allow the enzyme to reorganize in the layer so as to have optimal activity. Biosensors are prepared and then stored at 4°C in a 20 mM phosphate buffer solution of pH 7.5 between the experiments.

## 3. Results and Discussion

### 3.1 *Effect of pH, buffer concentration and temperature*

Since the enzyme activity is markedly affected by the solution pH, the effect of pH on the biosensor response was examined for 5 mg/L $NO_3^-$ concentration in 1 mM phosphate buffer. The result is shown in Fig. 4. The maximum response of the biosensors is observed at pH 7.5; below and above this value, decreases in response were observed. This result is strongly correlated to those obtained from the enzymatic membrane fabricated by BSA-glutaraldehyde immobilization, for the determination of nitrate.[21]

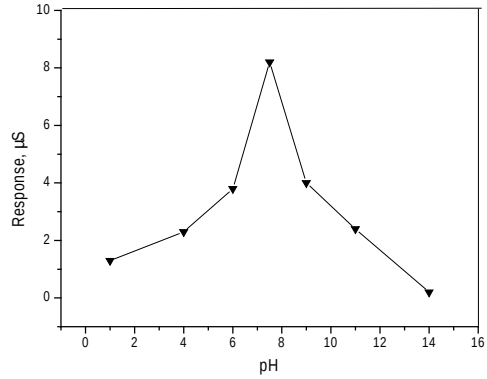



Fig. 4. Dependence of biosensor response on pH. Measurements were conducted using 5 mg/L of nitrate in 1 mM phosphate buffer.

We used a pH of 7.5 for the buffer solution throughout the experiments to obtain the maximum sensitivity.

The effect of buffer concentration on the biosensor response was tested for different phosphate concentrations; the effect was examined in the presence of 5 mg/L $NO_3^-$. At a higher buffer concentration, the response decreases. The maximum response of the biosensor occurs at 1 mM phosphate concentration (Fig. 5). The 1 mM phosphate buffer concentration of pH 7.5 was chosen to be optimal for biosensor working conditions.

Because of the nature of the biocomponent used in the biosensor construction and also the effect of temperature on the enzymatic activity, the effect of temperature on the biosensor responses was studied. The experiments were carried out between 2 and 50°C in 1 mM phosphate buffer of pH 7.5 using 5 mg/L $NO_3^-$.

Like most enzymes, the activity of NR is related to temperature; it can be seen that with increasing temperature, the biosensor sensitivity also increases. According to the results, the highest biosensor response was observed between 30 and 35°C. Below and above these temperatures, decreases in the biosensor response were observed (Fig. 6).

It is noted that the response is faster when the temperature increases because the kinetics of the enzymatic reaction increases owing to the decrease in the diffusion resistance, thus, the conductance increases.

All enzymes are proteins, which can easily be denatured at higher temperatures. For practical reasons, room temperature (20–25°C) is chosen as our working temperature to prolong the lifetime of the biosensor.

### 3.2 *Sensitivity of nitrate biosensor*

After the optimization of the bioactive membrane and the working conditions of the biosensor, a calibration curve was obtained for different concentrations of the calcium nitrate diluted in 1.0 mM phosphate buffer of pH 7.5 under optimized conditions of enzyme immobilization using the plasticized PVC membrane. From Fig. 7, it can be

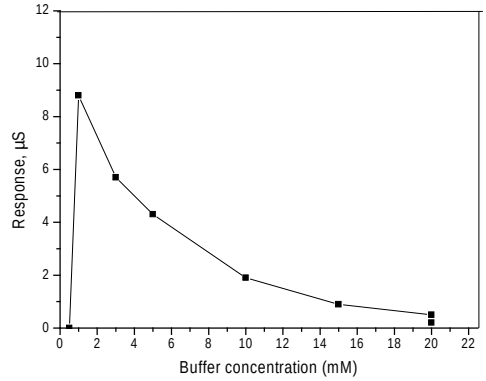



Fig. 5. Dependence of biosensor response on phosphate buffer concentration. Measurements were conducted using 5 mg/L nitrate in a phosphate buffer of pH 7.5.

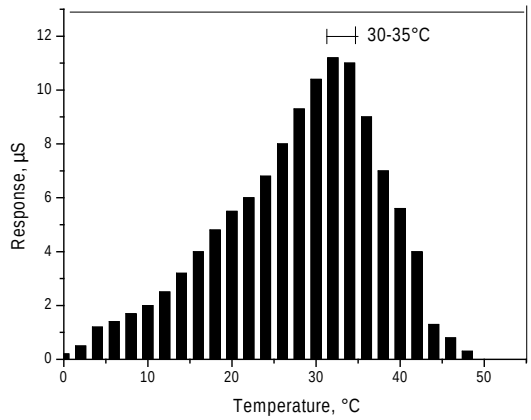



Fig. 6. Dependence of biosensor response on temperature. Measurements were conducted using 5 mg/L nitrate in 1 mM phosphate buffer of pH 7.5.

observed that the biosensor response depends linearly on nitrate concentration between $4\times10^{-3}$ and 8 mg/L, for an application domain of up to 50 mg/L with a slope of 1.486 µS·L/mg and $r^2 = 0.9953$, and the detection limit is 1.2 µg/L.

Under the same conditions, we tested the performances (sensitivity, specificity, and detection limit) of the nitrate reductase biosensor for nitrate determination in water samples obtained from different sites in France (around Lyon). The results presented in Fig. 7 show the linear correlation between sensor response and nitrate concentration ($a = 1.486x\pm0.957$ µS·L/mg, $r^2 > 0.995$), demonstrating the remarkable capacity of this biosensor for the determination of nitrate in natural water samples.

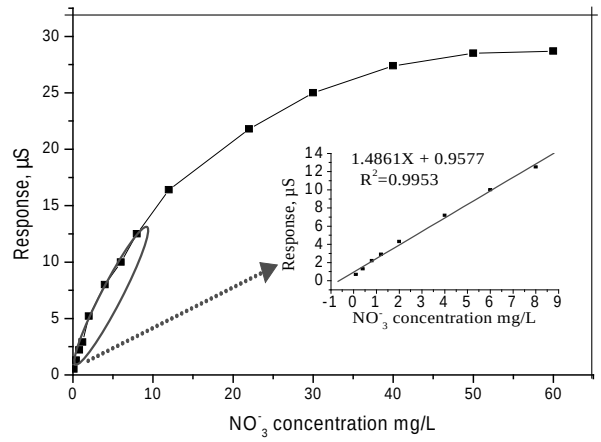



Fig. 7. Dependence of biosensor response (µS) on $NO_3^-$ concentration. Measurements were conducted in 1 mM phosphate buffer of pH 7.5.

It is noteworthy that the sensitivity to nitrate in natural water is ten times higher than that in synthetic samples. This effect is not easy to explain. It will be necessary to calibrate the sensor by adding natural samples with known concentrations of nitrate.

The biosensors were tested in terms of their reproducibility by plotting the calibration curves for five different concentrations of the nitrate diluted in 1.0 mM phosphate buffer of pH 7.5, and the measurements were repeated five times within 1 day, using the same biosensor (cf. Fig. 8).

The coefficient of variation of the biosensor (CV %) was found to be 7.6% for one sensor; a CV % of 15% was obtained for five different sensors.

### 3.3 *Effect of interfering compounds*

Environmental samples contain not only the target analyte but also other interfering compounds that could interfere with the biosensor response. It is important to consider the possible interference from extraneous compounds, particularly when applying a biosensor system to river water monitoring. Therefore, a variety of probable interferents were selected and their effect on the biosensor system was examined.

The interference was tested using a 0.1 mM standard solution of nitrate with varying concentrations of $Cu^{2+}$, $Ca^{2+}$, $K^+$, $Na^+$, $Cl^-$, and $SO_4^{2-}$ ions, the results are presented in Table 1. The biosensor response did not change appreciably at concentrations of up to 1 mM for each compound; thus, this system can be applied to river water monitoring.

### 3.4 *Lifetime of the nitrate biosensor*

The lifetime of enzymatic membranes constitutes a limiting factor in biosensor applications. The variation of the sensitivity of the sensor as a function of storage time is presented in Fig. 9. The enzymatic PVC membrane sensor was stored in 20 mM phosphate buffer (pH 7.5) at 4°C between measurements, and the effect was examined in the presence of 5 mg/L $NO_3$.

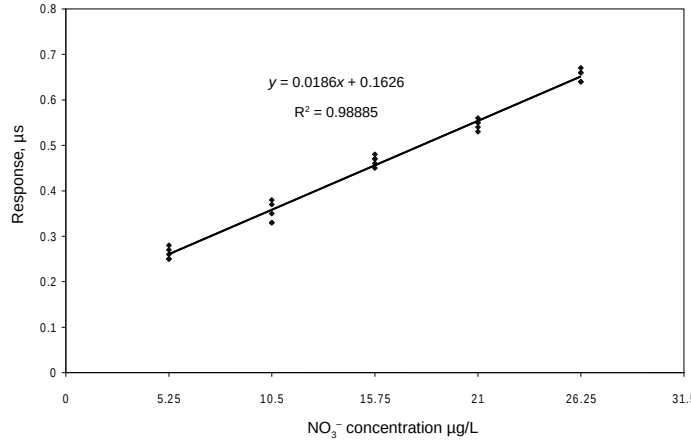



Fig. 8. Calibration curve of biosensor for five different $NO_3^-$ concentrations (µg/L). Measurements were conducted in 1 mM phosphate buffer of pH 7.5.

Table 1
Effect of interfering compounds on the biosensor response at 0.1 mM nitrate concentration for different concentrations of ions. Measurements were conducted in 1.0 mM phosphate buffer of pH 7.5.

| Ion | Concentration mM | Relative signal change % |
|---|---|---|
| $Cu^{2+}$ | 0.05 | +2 |
| | 0.1 | +3 |
| | 1 | +8 |
| $Ca^{2+}$ | 0.05 | +2 |
| | 0.1 | +4 |
| | 1 | +12 |
| $K^+$ | 0.05 | +1 |
| | 0.1 | +3 |
| | 1 | +5 |
| $Na^+$ | 0.05 | +3 |
| | 0.1 | +5 |
| | 1 | +10 |
| $Cl^-$ | 0.05 | +4 |
| | 0.1 | +5 |
| | 1 | +14 |
| $SO_4^{2-}$ | 0.05 | +1 |
| | 0.1 | +3 |
| | 1 | +7 |

During the first period, a decrease of 10% from the initial response owing to membrane conditioning was observed. Then, the response of the sensor remained stable, and after more than two months, the membranes were still operational, and only a slight decrease in the linear response range was observed. After more than two months some cracks appeared in the membrane.

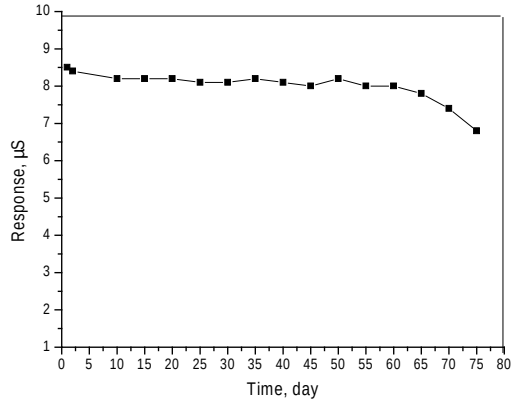



Fig. 9. Biosensor stability. Measurements were conducted using 5 mg/L nitrate in 1 mM phosphate buffer of pH 7.5.

The lifetime of the biosensor with the enzymatic membrane prepared by cross-linking with BSA-glutaraldehyde was determined.[21] When the biosensor was stored in 5 mM phosphate buffer (pH 7.5) at 4°C, there was a decrease of 30% from the initial response after 3 days, and the enzymatic activity decreased markedly after 2 weeks of storage. These results show that better storage stability is obtained using a PVC membrane than by glutaraldehyde cross-linking.

## 4. Conclusion

In this study, we compared two different procedures used for the immobilization of enzymes on a conductometric transducer for detecting nitrate, to be used for monitoring river pollution. In this work, nitrate reductase (NR) from *Aspergillus niger* mixed with a methyl viologen mediator and Nafion® cation-exchange polymer were entrapped in a plasticized PVC membrane. This method of immobilization was compared with that of enzyme cross-linking with glutaraldehyde.

The PVC enzymatic membrane shows a high sensitivity of around 1.486 µS.L/mg, the detection limit is 1.2 µg/L, and linear calibration is in the range from $4\times10^{-3}$ to 8 mg/L, for an application domain of up to 50 mg/L. It shows a good reproducibility (7.6%), a capacity to be used at temperatures varying between 10 and 40°C, and above all, a longer lifetime of around 2 months. It has been shown that a linear relationship with a strong correlation is obtained between sensor response and nitrate concentration in natural water samples.

As a further development, we propose to optimize these biosensors. The optimal temperatures of the current biosensor should be decreased to between 5 and 15°C, which is compatible with the natural temperature of river water. To enable the use of our biosensor at low temperatures, it is necessary to use enzymes that are active at low temperatures.

## Acknowledgements

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