

A Microscale NO_3^- Biosensor for Environmental Applications

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A biosensor for NO_3^- containing immobilized denitrifying bacteria and a reservoir of liquid growth medium for the bacteria was constructed. The bacteria did not have a N_2O reductase and therefore reduced NO_3^- to N_2O , which was then subsequently quantified by a built-in electrochemical transducer for N_2O . The only agents interfering with the determination of NO_3^- were NO_2^- and N_2O . The sensitivity for NO_2^- was identical to the one for NO_3^- whereas the sensitivity for N_2O was 2.4 times higher than for NO_3^- . Diffusive supply of electron donors to the bacteria from the built-in reservoir of growth medium ensured that the biosensor could work for 2–4 days. The tip diameter was down to 20 μm , and the sensors exhibited perfectly linear responses to nitrate in both freshwater and seawater. The detection limit was $\sim 1 \mu\text{M}$. The 90% response time to changes in NO_3^- concentration was from 15 to 60 s at room temperature and about twice that at 6 $^\circ\text{C}$, which was the lowest temperature for successful operation. The new NO_3^- biosensor is a very useful tool for the study of nitrogen metabolism in nature.

Great economic and environmental importance has made nitrogen metabolism subject to intensive research. Nitrate (NO_3^-) is a key element in the nitrogen cycle as it is the link between the nitrification and denitrification processes. These processes often take place in biological systems characterized by steep chemical gradients, e.g., biofilms and sediments. Determination of NO_3^- concentration profiles at high spatial resolution is therefore essential for the study of nitrogen metabolism in nature. A NO_3^- microsensor¹ as well as microsensors for ammonia (NH_4^+)² and nitrous oxide (N_2O)³ were therefore developed previously and studies using these sensors have yielded important information about the parameters controlling transformations of nitrogen compounds in nature.^{4,5} However, the Liquid Ion eXchanger (LIX)-type NO_3^- microsensors used in these studies suffer from interferences from common ions. Interference from HCO_3^- especially makes the LIX-type NO_3^- sensor difficult to use in biologically active environments characterized by steep HCO_3^- gradients, and the use of the LIX sensor in marine environments is impossible due to Cl^- interference.

The development of nitrate reductase-based biosensors has solved the problem with interfering ions,^{6,7} and these sensors have the ability of selective and precise detection of NO_3^- . However, these enzyme-based NO_3^- sensors have been restricted to measurements in buffered and defined solutions, and there are no reports of the development of enzyme-based microelectrodes for NO_3^- . Kobos et al.⁸ coupled the bacterium *Azotobacter vinlandii* with an ammonia gas-sensing electrode and obtained a NO_3^- biosensor. The bacteria reduced NO_3^- to ammonia, which was sensed by the electrode. This biosensor measured NO_3^- without the conventional interference from common negative ions, but it lacked the ability to carry out measurements in natural environments with nitrogen metabolism due to interference from ammonia.

Recently it was reported⁹ how a NO_3^- microsensor could be made by applying a microcapillary containing an immobilized culture of denitrifying bacteria in front of a N_2O electrode. This NO_3^- biosensor was based on bacterial reduction of NO_3^- to N_2O and a subsequent electrochemical detection of N_2O . This simple principle resulted in a linear response of the N_2O sensor as a function of the concentration of NO_3^- , with no interference from substances other than NO_2^- and N_2O . However, this NO_3^- biosensor was limited in its use because of two major disadvantages:

(1) The production of N_2O was dependent on acetylene blockage of the enzyme N_2O reductase. Otherwise the bacteria produced N_2 , which was not sensed by the microsensor. Addition of acetylene to environments with denitrifying activity would cause formation of large amounts of N_2O , and this N_2O would interfere with the NO_3^- measurements. Acetylene also inhibits the production of NO_3^- by nitrification,¹⁰ and this inhibition could also constitute a major problem, as the NO_3^- pool may be highly dynamic.

(2) The activity of the denitrifying bacteria was dependent on a supply of electron donor and nutrients from the solutions in which the biosensor was immersed. Exposed to tap water, it ceased functioning after 2 h.

Here we present an improved NO_3^- biosensor, based on the same principles, which does not suffer from these disadvantages. An N_2O reductase-deficient denitrifying strain is used in the

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biosensor, and the NO_3^- reducing activity of the bacteria is kept high by continuous supply of electron donors and other nutrients from a "built-in" reservoir. This improved biosensor is therefore able to measure NO_3^- concentration profiles in gradient systems with high spatial resolution without the conventional interferences, thus making it a very useful tool in the study of NO_3^- metabolism in natural environments.

EXPERIMENTAL SECTION

Isolation of Denitrifying Bacteria without N_2O Reductase.

We isolated NO_3^- -reducing bacteria from soil and sediments as described by Larsen et al.⁹ and among these we looked for strains that denitrified but lacked the enzyme N_2O reductase. The isolates were grown in 20-mL preautoclaved test tubes containing 10 mL of an ethanol medium with an inverted test tube (1 mL) to test for gas production. The medium contained the following (in g L^{-1}): ethanol, 4; tryptic soy broth, 0.1; K_2HPO_4 , 1; KH_2PO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; NH_4Cl , 0.2; KNO_3 , 3; and 1 mL of trace metals solution.¹¹ The pH was adjusted to 7.1. The test tubes were inoculated and subsequently placed in an anaerobic jar and incubated for 4 days at 20 °C, and as growth of the isolates only could be supported by NO_3^- reduction, NO_2^- , N_2O , or N_2 would be produced. Any production of N_2 by denitrifying cultures caused formation of a gas phase in the small test tube due to the low solubility of N_2 in water. Test tubes without N_2 production were checked for NO_2^- content by use of Griess reagent (Prolabo, Fontenay-Sous-Bois, France). Any coloration of the culture by the reagent due to NO_2^- formation was unacceptable, as the stoichiometry of NO_3^- reduction to N_2O was then unknown. Cultures that would not produce either NO_2^- or N_2 were supposed to have the more soluble N_2O as the end product of denitrification. However, only one of the isolates produced N_2O as the only product of NO_3^- reduction. By use of an API 20 NE test (Bio Merieux SA/69280 Marcy-l'Etoile/France), this isolate was identified to be a strain of *Agrobacterium radiobacter*.

Construction of a NO_3^- Biosensor. We inserted a N_2O microelectrode into a tapered glass casing (the media chamber) so that a small reaction chamber in front of the tip of the N_2O electrode was created (Figure 1).

The N_2O microelectrode, which is the sensing element of the NO_3^- biosensor, was of the microelectrode-type originally described by Revsbech et al.³ It was constructed partly as recommended in their article, but it was not necessary to apply an O_2 -removing cathode in front of the N_2O -reducing silver cathode as the bacteria consumed all the O_2 before it reached the N_2O electrode. The N_2O electrode consisted of a silver cathode, a silver guard cathode, and an internal Ag/AgCl reference anode contained within a tapered outer glass casing equipped with a silicone membrane in the tip opening (Figure 1). The tip diameter was from 20 to 40 μm and the silicone membrane had a thickness of $\sim 10 \mu\text{m}$. The electrolyte contained 0.5 M KCl and 0.1 M NaOH. Before use, the N_2O electrodes were connected to a custom-made picoammeter with a built-in electronically stabilized polarization source. A potential of -1.05 V versus the Ag/AgCl anode was applied on the two cathodes, and the electrodes were tested for N_2O sensitivity. Electrodes that had linear responses to N_2O and a detection limit below 1 μM (data not shown) were chosen for further use.

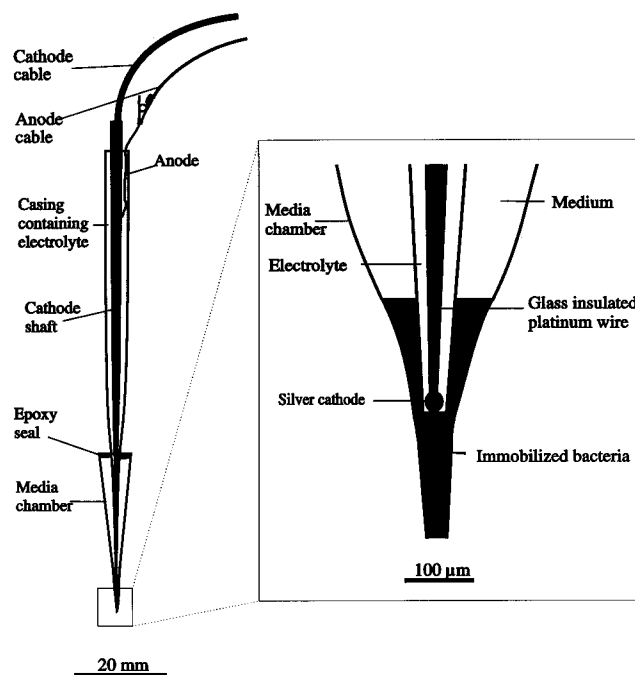


Figure 1. NO_3^- biosensor based on bacterial reduction of NO_3^- to N_2O with subsequent detection of the N_2O .

The media chamber was made from a soda-lime glass Pasteur pipet using general procedures.¹² By use of a gas flame and a heated platinum loop (and by the use of a micromanipulator while watching at 50 times magnification in a dissection microscope) the pipet was shaped to a $\sim 3\text{-cm}$ -long glass tube with the requested dimensions. The tip of the casings had openings from 20 to 50 μm , and the outer 175–275 μm was made parallel-sided or only slightly conical, depending on the desired sensor characteristics. Between 175–275 and 1000 μm from the tip, the casings widened to a diameter of 200–300 μm , and between 1 and 3 cm from the tip the casings widened to reach the internal diameter (6.2 mm) of the Pasteur pipet. The N_2O electrode was then inserted into the casing and carefully manipulated to a position so its tip was placed 150–250 μm from the tip of the casing. In this position the casing was fixed to the shaft of the N_2O sensor with epoxy resin (Super Epoxy, Hisingplast AB, Göteborg, Sweden).

A reaction chamber for the reduction of NO_3^- (and NO_2^-) to N_2O was formed in the 150–250 μm long and 20–50 μm wide space between the tip of the N_2O sensor and the opening of the casing. Because the casing was much more conical than the N_2O sensor, a large (0.2-mL) medium reservoir with a 10^6 times larger volume than the reaction chamber was formed behind the tip of the N_2O sensor. The tip of the N_2O sensor was fitted into the casing so that a narrow passage between the reaction chamber and the medium chamber was formed. After assembling the N_2O sensor and the casing, the reaction chamber and the medium chamber were filled with liquid nutrient medium through an opening in the epoxy seal. The medium contained the following (in g/L): tryptic soy broth, 5; NaCl, 10; and tris(hydroxymethyl)-aminomethane (Tris), 1. The pH was adjusted to 7.8.

The bacteria to be inoculated into the reaction chamber were grown aerobically on agar plates based on the same medium as described above. After inoculation and an incubation period of

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24 h at 30 °C, cell mass from these plates was scraped off and mixed with an equal volume of 1% sodium alginate solution. This bacteria/alginate mixture was drawn a few millimeters into a Pasteur pipet and, by use of two micromanipulators and while watching in a microscope at 100× magnification, the tip of the NO_3^- biosensor was positioned so it touched the bacteria/alginate mixture in the Pasteur pipet. By applying a gentle vacuum, the mixture was aspirated into the biosensor. When the reaction chamber and ~1 mm of the medium chamber was filled, atmospheric pressure was re-established inside the sensor, and the tip of the biosensor was immersed in tap water containing 500 μM NO_3^- . The alginate solidified after being exposed to the Ca^{2+} content (2 mM) in the tap water.

After incubation of the biosensor for 6–10 h in the presence of 500 μM NO_3^- , the activity of the bacteria in the reaction chamber was sufficiently high for calibration and use of the sensor, and the N_2O transducer of the NO_3^- biosensor was connected to a combined picoammeter and polarization source. Before any use of the NO_3^- biosensors, they were placed in vials containing NO_3^- -free water, and various amounts of N_2O saturated water were added in order to confirm sufficient sensitivity of the N_2O electrode.

Calibration of the NO_3^- Biosensor and Tests for Stirring Sensitivity and Response Time. The NO_3^- biosensors were calibrated in a vial containing NO_3^- -free water with approximately the same ionic strength and temperature as the environment where the measurements were to be performed. A calibration curve was obtained by plotting the current from the N_2O electrode against various NO_3^- concentrations obtained by repeated addition of NO_3^- from a concentrated stock solution.

A test for stirring sensitivity was done by keeping the NO_3^- concentration constant and observing the change in response between vigorously stirred and unstirred water.

The response time was defined as the time it takes a sensor to exhibit 90% of the complete change in signal after being exposed to a change in NO_3^- concentration.

Interference Test. A NO_3^- biosensor made as described above was tested for possible interference from different substances relevant to microbiology and from changes in pH. Tests of interference from NO_2^- , NH_4^+ , N_2O , HCO_3^- , acetate, and O_2 were performed in both tap water and an aqueous 2.5% NaCl solution, corresponding to the salinity of Aarhus Bay water, whereas a possible interference from SO_4^- and pH was investigated only in the saline solution.

NO_3^- was added to the tap water and to a saline solution to a concentration of 500 μM , and the change in sensor signal was observed after the addition of the following (in mmol/L): NO_2^- , 0.1; N_2O , 0.1; NH_4^+ , 10; HCO_3^- , 10; and acetate, 10.

Calibration curves were made both in anaerobic and O_2 -saturated water to test whether changes in O_2 concentration affected the response to NO_3^- .

A possible interference by SO_4^- was checked by preparing a 200 μM NO_3^- solution in water with 2.5% NaCl and checking as to whether an addition of 28 mM of SO_4^- changed the sensor signal. After this it was checked as to whether changes in pH ranging from 6.5 to 9.5 affected the signal.

Temperature and Salinity Effects. The activity of the denitrifying bacteria in the reaction chamber was influenced by the physical and chemical conditions in the environment in which the sensor was immersed. The most important parameters

relevant to microbial ecology that influenced the activity were temperature and salinity. Therefore, calibration curves were made in tap water and artificial seawater (2.5% NaCl) at different temperatures in order to get an impression of the temperature and salinity range in which the sensor can operate.

Measurements in Sediment. NO_3^- concentration profiles were recorded in undisturbed sediment cores from the freshwater lake Søbygård Sø and from the marine Aarhus Bay. The cores were incubated in water from the sampling sites, but because the water from Aarhus Bay was almost NO_3^- depleted, NO_3^- was added in order to demonstrate the ability of the sensor to record a NO_3^- concentration profile at high salinity. After preincubation for a day in the laboratory, a NO_3^- microsensor was attached to a motor-driven micromanipulator so that the tip of the sensor was positioned just above the sediment surface. The sensor was then introduced vertically into the sediment at 100- μm steps while the signal from the sensor was collected by a personal computer equipped with a 12-bit A/D converter.

RESULTS AND DISCUSSION

Functioning of the NO_3^- Sensor. Bacterial reduction of NO_3^- to N_2O and a subsequent electrochemical detection of the produced N_2O are the basic principles of the NO_3^- biosensor. The immobilized bacteria in the reaction chamber of the tip region are constantly supplied with electron donors and other nutrients by diffusion through the space between the N_2O electrode and the glass casing used for medium chamber. The medium chamber is so big compared to the consumption rate in the reaction chamber that the reservoir is virtually inexhaustible. The bacteria are facultatively anaerobic and have the potential to oxidize organic material from the medium chamber in the presence of O_2 or NO_3^- as electron acceptors. The bacteria closest to the opening of the sensor reduce all incoming O_2 . This leaves most of the chamber anoxic and thereby makes denitrification, which cause N_2O production, the only possibility of respiratory metabolism in this zone. As silicone is very permeable for gases, some of the N_2O produced from NO_3^- reduction will diffuse into the N_2O electrode where it is reduced on the negatively polarized silver cathode. If no N_2O is found in the environment in which the NO_3^- biosensor operates, a signal from the biosensor could only originate from bacterial reduction of $\text{NO}_3^-/\text{NO}_2^-$, as the N_2O electrode is insensitive to substances other than N_2O and O_2 . The amount of N_2O molecules per unit time reduced on the silver cathode of the N_2O sensor and, thereby the diffusive transport of N_2O through the silicone membrane, determines the signal of the NO_3^- biosensor.

When the sensor is exposed to increasing NO_3^- concentrations, NO_3^- will diffuse further into the reaction chamber. The concentration of NO_3^- outside the tip that causes unreduced NO_3^- to reach the tip of the N_2O electrode is the maximum linearly detectable concentration of NO_3^- . The NO_3^- reduction capacity of the bacteria depends on the length of the anoxic zone in front of the N_2O electrode and of the bacterial activity in this zone, and therefore, the maximum detectable concentration of NO_3^- is higher in anoxic than in oxic environments. Any physical or chemical conditions in the environment being analyzed that negatively affect the activity of the bacteria will limit the maximum detectable concentration of NO_3^- , but it will not affect the response as long as the bacteria are sufficiently active to prevent any NO_3^- from reaching the tip of the N_2O sensor.

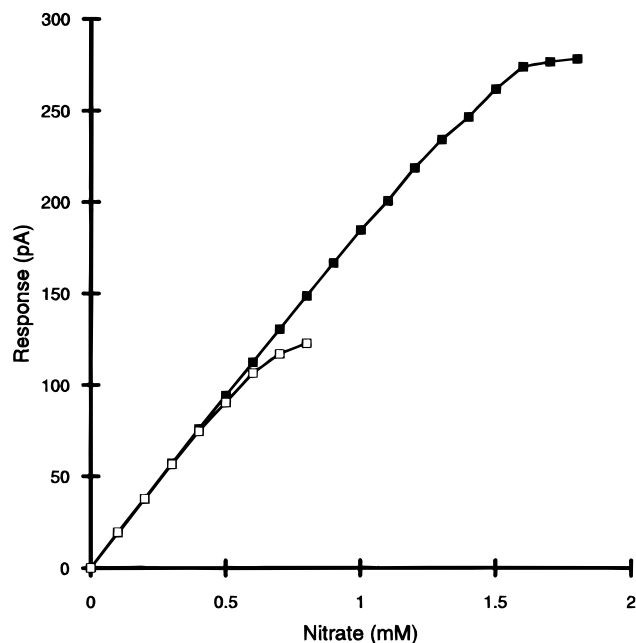


Figure 2. Calibration curves obtained with the NO_3^- biosensor. The closed symbols illustrate how the biosensor responded to NO_3^- in anoxic tap water whereas the open symbols illustrate the response in tap water saturated with O_2 (1 atm). Regression equation of the linear domain of the curve obtained in anaerobic water: $188.0x + 0.29$; $r^2 = 0.9999$. Regression equation of the linear domain of the curve obtained in O_2 -saturated water: $188.2x + 0.27$; $r^2 = 0.9998$.

A minor part of the N_2O produced will pass the tip of the N_2O electrode and diffuse into the medium chamber. A buildup of N_2O behind the N_2O sensor would disturb the NO_3^- measurements, and therefore, it is important that the medium chamber is conical so that the expansion of the volume will dilute any N_2O that may enter the medium chamber.

Calibration, Sensitivity, and Response to Changing O_2 Concentrations. By varying the relation between the size of the opening and the volume of reaction chamber, NO_3^- biosensors with different characteristics can be made. The calibration curve in Figure 2 shows how a NO_3^- biosensor responded linearly to changes in NO_3^- concentration in tap water in the range from 0 to $800 \mu\text{M}$ NO_3^- under anoxic conditions and from 0 to $300 \mu\text{M}$ in water saturated with O_2 gas ($P_{\text{O}_2} = 1 \text{ atm}$). Although the maximum detectable NO_3^- concentration was lower in solutions with high O_2 concentration than in anoxic solutions, the sensitivity in the linear range was exactly identical. The reaction chamber of this sensor had a length of $240 \mu\text{m}$. The diameter of the opening as well as the diameter of the reaction chamber was $50 \mu\text{m}$, meaning that the reaction chamber was parallel sided. The detection limit for this particular sensor was $5 \mu\text{M}$.

By diminishing the opening of the NO_3^- biosensor compared to the volume of the reaction chamber, biosensors with higher maximum detectable NO_3^- concentration can be made, but this also causes a drop in sensitivity. Biosensors capable of measuring NO_3^- up to 6 mM have been made (data not shown). Biosensors with detection limits to $1 \mu\text{M}$ can be made by shortening the distance between the N_2O sensor and the opening to $\sim 150 \mu\text{m}$ and by increasing the tip diameter to $\sim 40 \mu\text{m}$. These sensors have a lowered maximum NO_3^- concentration for linear response.

As it is impossible to reproduce exact dimensions of a biosensor and because these dimensions have great influence on the sensor

characteristics (e.g., slope of the calibration curve and linear domain range), all biosensors must be individually calibrated.

The stirring sensitivity has been less than 1% for all sensors made so far.

Influence of Temperature and Salinity on Sensor Response. The maximum detectable NO_3^- concentration of the biosensor must be higher than the maximum expected NO_3^- concentration in the investigated environment. Therefore physical or chemical parameters in the investigated environment that negatively affect the activity of the bacteria in the reaction chamber must be included in the calibration conditions. The sensor shown in Figure 1 measured up to $500 \mu\text{M}$ NO_3^- in air-saturated water at 20°C and had a maximum detectable NO_3^- concentration of $300 \mu\text{M}$ at 15°C , $200 \mu\text{M}$ at 10°C , and $\sim 100 \mu\text{M}$ at 6°C . Below 5°C it ceased working. In artificial seawater containing 25 g/L NaCl , the biosensor ceased working below 12°C .

Due to the temperature influence on diffusion coefficients, the response of the biosensor to NO_3^- changes by $\sim 2\%/^\circ\text{C}$ (data not shown).

Response Time. Response time relies on the sensor design (the most important parameter being the length of the reaction chamber) and is in the range of 15–60 s for a 90% response. The sensor, by which the calibration curve shown in Figure 2 was obtained, had a response time of 30 s. Due to a lowering of the diffusion coefficients, decreases in temperature led to a slower response and a decrease from 21 to 6°C thus increased the response time from 30 to 50 s.

Interferences. As the detection of N_2O is highly specific, nothing except for NO_2^- and N_2O itself should interfere with the measurement of NO_3^- . Because NO_3^- reduction to N_2O involves both NO_3^- and NO_2^- reductases, the bacteria in the reaction chamber produce N_2O from NO_2^- as well as from NO_3^- . As the diffusion coefficients of NO_3^- and NO_2^- are approximately the same, the responses for NO_2^- as for NO_3^- were identical. N_2O should theoretically result in a response ~ 2.5 times the response for NO_3^- in equal concentrations, taking into account that it takes two NO_3^- molecules to make one N_2O molecule and that the diffusion coefficient for NO_3^- is ~ 0.8 times the one for N_2O . For the biosensor tested, the response for N_2O was 2.4 times the response for NO_3^- . Environments producing considerable amounts of N_2O are unsuitable for NO_3^- analysis by the NO_3^- biosensor, but in environments producing smaller amounts of N_2O , it is possible to correct for the N_2O interference by use of a combined N_2O and O_2 microsensor.³

A minor interference from the other substances tested in freshwater (HCO_3^- , NH_4^+ , acetate) was observed, but it was less than 1% of the NO_3^- response for equal concentrations. This minor "interference" relied in an increased salinity in the water after addition of the species that affected the diffusion of NO_3^- into the sensor. None of the substances tested affected the NO_3^- response in seawater as the relative increases in salinity after the addition of the substances was negligible. The NO_3^- biosensor must, because of the salinity effect, be calibrated in water containing approximately the same ionic strength as the environment investigated. A 25% increase in sensor response per micromolar NO_3^- occurred in water added 25 g/L NaCl relative to the response obtained in tap water (5 mM bicarbonate with Ca^{2+} as the dominant cation), but the zero reading remained unchanged.

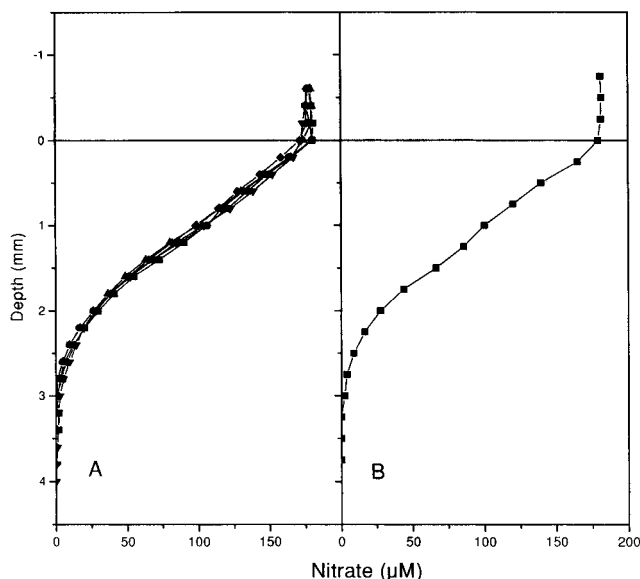


Figure 3. (A) NO_3^- concentration profiles in a marine sediment measured with the NO_3^- biosensor and (B) a similar profile from a freshwater lake.

Measurements in Sediments and Biofilm. The vertical distribution of NO_3^- in marine sediment from Aarhus Bay is shown in Figure 3A. The five profiles are almost identical due to very homogeneous sediment. In the upper 1.5 mm of the sediment, only O_2 was used as electron acceptor for heterotrophic processes (oxygen profile not shown) and therefore the NO_3^- profiles are linear in this zone. From 1.5–3-mm depth, curvature of the profiles indicates decreased diffusion flux with depth due to NO_3^- respiration (denitrification). The NO_3^- concentration profile in sediment from Lake Søbygård (Figure 3B) shows likewise how the sediment consumed NO_3^- from the water phase by heterotrophic processes. The sensor has also been used for analysis of biofilms.¹³

Stability. A major disadvantage of the sensor is a limited lifetime, primarily due to a decreasing catalytic activity of the silver cathode in the N_2O electrode. Most N_2O electrodes reach unacceptable low sensitivities within 2 days. A decreasing sensitivity of the N_2O electrode causes drift of the response to NO_3^- , and frequent recalibration of the NO_3^- biosensor is

therefore necessary while working with the biosensor. The N_2O electrode can be reactivated by reversal of the polarity of the voltage applied on the silver cathode for a few minutes, but the signal must subsequently be allowed to stabilize for at least 1 h before measurements can be performed again. The quality of the N_2O electrodes differs from electrode to electrode but normally the lifetime is less than 1 week. The stability of the N_2O electrode does not depend on whether the biosensor perform on-line measurements of NO_3^- or it is stored without polarized cathodes.

Improvements To Be Done. The development of a more long-term stable N_2O electrode as transducer would greatly improve the performance of the NO_3^- biosensor, but only little is known about the reduction of N_2O of metal surfaces.¹⁴

We will try to isolate strains of psychrophilic and halotolerant denitrifying bacteria without N_2O reductase. The application of such an organism would allow the use of the biosensor at lower temperatures and higher salinities.

An ion-permeable membrane applied in the opening of the reaction chamber is necessary in order to make the biosensor long-term stable. The application of such a membrane is necessary to prevent undesired organisms such as N_2 -producing denitrifying bacteria from contaminating the reaction chamber. The maximum lifetime of a NO_3^- biosensor constructed as shown in Figure 1 is only a few days, mostly because organic material precipitates out in the opening of the sensor and renders it impermeable to NO_3^- . An ion-permeable membrane might also alleviate this problem.

Although the sensor still can be improved, many important studies of nitrogen metabolism in nature can now be done. The integration of NO_3^- , NO_2^- , and N_2O by the sensor will not be a major problem in most contexts as the integral is the parameter of most importance in wastewater treatment, and in most other environments, the concentrations of NO_2^- and N_2O are very low.

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