6 Biosensors for Analysis of Water, Sludge and Sediments with Emphasis on Microscale Biosensors

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1 INTRODUCTION

1.1 PRESENT AVAILABILITY OF SENSORS FOR LONG-TERM ENVIRONMENTAL MONITORING

Large resources have been allocated to the development of sensors for environmental monitoring. For some areas the work has been very successful, such as the development of sensors for automobile exhaust. For other areas the success with invention of sensors that have resulted in extensive use has been more limited. Currently the only sensors for analysis of chemical parameters in natural aquatic environments that are commercially available and have resulted in widespread use are electrochemical and optical $O_2$ and pH sensors as described elsewhere (Chapters 2 and 3) in this volume. It is apparent from the content of this volume that sensors may be used for many other analyses in the aquatic environment, but none of these sensors have until now gained widespread use, mostly because of unsatisfactory long-term stability, but also often because of the presence of interfering species in the natural environment. For scientific use many of these sensors are, however, highly interesting, as they can be used to collect essential information about the natural environment that could not be obtained by other means. In addition to the 'real' sensors, miniaturized analytical systems for continuous and long-term in situ use are under development (Chapter 12 this volume) [1,2,3], and UV absorption [4] or UV-caused fluorescence [5] are also used extensively.

1.2 ADVANTAGES OF MICROSCALE SENSOR DESIGN

The development of microscale sensors has been a key research field for the authors, and our description of environmentally relevant biosensors in this chapter is therefore heavily biased towards microscale biosensors. We thus do not attempt to give a full description of all types of environmentally relevant biosensors, but a brief overview of the various biosensor types is presented. We started to develop microscale sensors as we—being microbial ecologists—needed such tools to elucidate the chemistry and the transformation rates at a scale relevant to the world of bacteria. It turned out, however, that by making sensors small we gained more than just increased spatial resolution of our measurements. First of all the signals from microscale sensors are only marginally affected by changes in flow rate or diffusional characteristics of the medium. The small-scale spherical diffusion field around microsensor tips described in detail in Chapters 8 and 9 can explain this. Microscale sensors may also be characterized by very rapid responses to changes in analyte concentration. According to the Einstein–Smoluchowski equation [6] diffusion can be described by $I = (2Dt)^{1/2}$, where $I$ is diffusional path length covered during time $t$ and $D$ is the diffusion coefficient. According to this equation, a small molecule such as oxygen ($D = 2 \times 10^{-9}$ m$^2$s$^{-1}$ in water) will, on average, migrate about 0.06 mm in 1 s and 0.6 mm in 100 s. Sensors with external or internal concentration gradients extending $< 0.1$ mm will thus exhibit 90% response times of a few seconds or less, while sensors with longer diffusion distances may be slow. Examples of actual response times of small sensors are given for the nitrate biosensor treated below. Finally the small size of microsensors makes it possible to use completely new principles of detection as an effective supply of reactants can be mediated by diffusion from internal reservoirs. Both the NO$_3^-$ and the CH$_4$ biosensors described below are thus dependent on such efficient supply of reactants from internal reservoirs. The external dimensions of a sensor do, however, not need to be microscale even when the sensing elements inside the sensor are. Clark-type $O_2$ sensors may thus be made with a 1 mm tip consisting of almost solid glass, but with a membrane-filled pore of a few micrometers in diameter in the center and containing a cathode also being a few micrometers in diameter [7]. Such a sensor will still have the desired characteristics of low stirring sensitivity and rapid response, although the external diameter has been increased to improve physical sturdiness.

1.3 DEFINITION OF A BIOSENSOR

Much of the effort in sensor technology has been devoted to the development of biosensors, and a substantial part of this effort has been on the development of sensors for environmental use. The interest for biologically mediated reactions is, of course, caused by the extreme specificity of enzymatic and immunological reactions. The term 'biosensor' has been used for any biological component used to sense any parameter. Usually this biological component is attached to some piece of hardware such as an electrochemical detection system or an optical fiber, but the term biosensor has, as an example, also been used about genetically modified bacteria that emit light or show some other measurable gene expression when exposed to some environmental variable. The principles
of a 'classical' biosensor can be exemplified by the glucose biosensor [8], where a platinum anode is coated with a layer of the enzyme glucose oxidase. The glucose oxidase catalyzes the reaction between glucose and O₂, whereby hydrogen peroxide is formed. This hydrogen peroxide is oxidized at the anode, and the current generated by this oxidation is then a measure for the glucose concentration in the medium. The enzyme may be shielded from the sample by some kind of semi-permeable membrane.

1.4 (MACROSCLAE) BIOSENSORS OF RELEVANCE FOR ENVIRONMENTAL MONITORING

Although large resources have been allocated to biosensor development, the only biosensor (not counting immunological disposable strips for xenobiotics) regularly used for monitoring of aquatic environments is the so-called BOD (biological oxygen demand) sensor [9], which gives semi-quantitative estimates of the content of dissolved, easily degradable organic matter in water. It is basically just a layer of immobilized heterotrophic microorganisms placed in front of a conventional O₂ sensor. Because of the respiratory activity of the microorganisms, governed by the concentration of dissolved organics in the analyzed medium, they regulate the amount of O₂ reaching the O₂ sensor by diffusion from the medium. For interpretation of the signal from this sensor it is thus critical to know the O₂ concentration in the medium. It is apparent from the BOD sensor review of Praet et al. [10] that the signal from a BOD sensor may be quite difficult to calibrate to some well-defined expression of dissolved organic concentration, as the responses to different dissolved organic species differ, and the response to each individual species is furthermore dependent on the recent history of the sensor. Particulate organic matter is not included in the reading. BOD sensors are usually exposed to a pulse of the water to be analyzed which results in a peak in respiratory activity (i.e. lower reading by the O₂ sensor), and after this peak a considerable period of time is needed to approach some kind of baseline respiration. The lifetime of a macroscopic BOD sensor may be up to months [9] without replacement of the immobilized microbial cells. The same design as that of the BOD sensor has been used extensively with other microorganisms for detection of a wide range of chemical species [10], but apparently the characteristics of these sensors have not allowed for extensive practical use.

The field of biosensors for environmental use was recently reviewed in a special issue of Trends in Analytical Chemistry [11], and Table 1 is largely a summary of this issue. A lot of work has been devoted to the development of enzyme-based sensors for pesticides [16] or other pollutants such as phenolic compounds. [19] The pesticide sensors may be based on enzyme inhibition, where some enzyme-catalyzed reaction is inhibited owing to the presence of a pollutant, and they therefore cannot be used for real in situ monitoring, as the

<table>
<thead>
<tr>
<th>Chemical parameter</th>
<th>Bio-component</th>
<th>Detection principle</th>
<th>Analytical range (mol·L⁻¹)</th>
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<td>+</td>
<td>2 months</td>
<td>1, 2, 13,</td>
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<td>BOD</td>
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<td>H₂O</td>
<td>Bacteria</td>
<td>Oxygen cons.</td>
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<td>+</td>
<td>2 months</td>
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<td>Bacteria</td>
<td>Oxygen cons.</td>
<td>0.1-1</td>
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<td>+</td>
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<td>Nitrate + nitrite</td>
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<td>Phenols</td>
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<tr>
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<td>+</td>
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<tr>
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<td>Oxygen cons.</td>
<td>0.1-1</td>
<td>+</td>
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<td>2 months</td>
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primary substrates for the reaction must be added to the test medium. Immuno-
ological reactions have also been used for analysis of such xenobiotic com-
ounds [22]. It has, however, turned out to be extremely difficult to make
reversible enzyme or immunosensor membranes that can be used for direct long-
term monitoring of natural aquatic environments, either because the sensing
reaction itself results in inactivation or because humic substances etc. interfere
or gradually inactivate the sensor. The use of enzyme or antibody preparations
for use in sensors for environmental analysis thus seems to be highly problem-
atic when long-term stable biosensors are essential, but more sophisticated
approaches where highly selective membranes protect the biological compo-
ents [23] may in the future result in more stable enzyme sensors. The glucose
biosensor has at present, to our knowledge, the best long-term stability among
the enzyme-based sensors [24], as glucose oxidase is extremely stable. A micro-
scale version of this sensor has also been described [8]. Glucose is, however, not
an important freely dissolved chemical species of most natural environments. It
should be stressed that enzyme sensors may be used for detection of many
environmentally relevant chemical species if long-term stability is of minor
importance (Table 1), especially if pretreatment of the sample to remove inter-
fearing agents, to adjust pH, and to add essential chemicals for the reaction (and
also often to extract the analyte) is possible.

By using actively growing microorganisms instead of enzyme preparations
the essential enzyme(s) can be efficiently shielded against inactivation, and the
pool of enzymes is also continuously replenished by growth of the microorgan-
isms. It should be stressed that the idea of using bacteria or eucaryotic cells
instead of enzymes in biosensors was invented long ago. Examples of such
whole cell biosensors are the BOD sensor and similar designs sensing, among
other things, SO$_4^{2-}$, NO$_2^{-}$, CO$_2$, and NH$_4^{+}$ [12]. Most whole-cell biosensors
used until now are almost identical to the BOD sensor in terms of design, but
any microbiological reaction giving rise to a measurable product or consuming
a measurable reactant within the sensor may form the basis of a biosensor.
Compared with sensors based on purified enzyme preparations, whole cell
biosensors may seem less specific, as whole cells contain a large number of
different enzymes, but many bacteria are lithoautotrophic (cannot assimilate or
oxidize organic matter) and may rely on oxidation of only one or a few
inorganic species while assimilating CO$_2$. The O$_2$ consumption inside sensors
based on these organisms can thus be a measure of the concentration of such
inorganic species. Others use oxidized inorganic compounds as electron accep-
tors and reduce these to chemical species, which can be detected. An example
of this is the NO$_2^{-}$/NO$_3^{-}$ biosensor described below where NO$_2^{-}$ and NO$_3^{-}$ are
reduced to N$_2$O. The final option with whole cells is not to use the enzymes
themselves but rather gene expression (see section about bioluminescence
below) as a measure of the inducer concentration. Such gene expression is
often extremely specific for the chemical species in question. While metabolic
activity as such will only work in sensors specific to a few chemical species, gene
expression may be used for virtually all types of both inorganic and organic
species. As outlined below gene expression is, however, difficult to handle
owing to long response times and irreversibility.

2 MICROSCALE WHOLE CELL BIOSENSORS—GENERAL
PRINCIPLES

It is possible to use bacteria or yeasts in macroscale biosensors as mentioned in
section 1. However, most macroscale designs impose some fundamental restric-
tions on the nutrient supply to the microorganisms, as all necessary chemical
species must be supplied through the membrane tip; otherwise, the sensor no
longer acts as a real sensor but rather as a microbiological assay in a stirred
liquid medium [25]. These limitations may be overcome by making the sensor
tip very small and conical. Then, all necessary growth components, except for
the one to be quantified by the sensor, can be readily supplied to the
microorganisms in the tip by diffusion from an internal reservoir (Figure 1).
The microsensor then works very much like a continuous culture vessel
where the growth-limiting factor is the supply of an electron donor or acceptor
through the tip membrane. The tip membrane may be more or less restrictive
in terms of permeability, one example being a silicone membrane only
allowing relatively small, uncharged molecules to pass, another example
being ion-permeable membranes made from cellulose acetate or other
materials.

The design illustrated in Figure 1 makes it theoretically possible to make
biosensors with an extremely long lifetime and stability, as the flux through the
tip membrane and thus also the need for nutrients from the internal reservoir
is extremely small. Often biosensors made this way have a "reaction chamber" in
the tip which is only 10$^{-7}$ times the volume of the 1 mL large medium reservoir,

![Figure 1](image-url)
and the reservoir may last for years. Another advantage is that the volume of bacteria mediating the reaction is extremely well defined, as the relative positions of the tip membrane and the internal sensing element are fixed. The metabolic status of the bacteria may, of course, vary as a result of their recent life history, but compared with macroscale analog the metabolic status is also more defined as only actively growing microorganisms are found in the tip, and excess microbial biomass is squeezed behind the tip where the microorganisms eventually perish. For the $\text{NO}_2^- + \text{NO}_3^-$ and $\text{CH}_4$ biosensors described in this chapter, the metabolic status of the microorganisms is actually irrelevance as long as the total population between the tip membrane and the sensing element is sufficient to mediate a full conversion. The length of the reaction chamber essentially governs the response time of the sensor, and it should thus be kept as short as possible. Not all microbial reactions are equally rapid, however, and the length needed for a full conversion of chemical species entering through the tip may thus vary. For the $\text{CH}_4$ and $\text{NO}_2^- + \text{NO}_3^-$ biosensors described below, the reaction chambers are 100–300 $\mu$m long.

One fundamental advantage of the sensor design shown in Figure 1 is that it is possible to change the supply of ions through sensors equipped with ion-permeable membranes by applying a voltage between environment and medium reservoir. For the $\text{NO}_2^- + \text{NO}_3^-$ biosensors it is thereby possible to increase the sensitivity by a factor of more than 10, and it is then possible to analyze $\text{NO}_2^- + \text{NO}_3^-$ down to 0.1 $\mu$mol L$^{-1}$ [26]. The use of such electrophoretic migration of ions has, to our knowledge, not been used successfully in macroscopic sensors, as it is essential to have a well-defined region where the electrical resistance of the sensor is located (i.e. our microsensor tip), and both internal electrolyte reservoir and electrode capacities must be large to minimize the effects of polarization.

The design shown in Figure 1 is not limited to analyses of growth limiting substrates entering the microsensor through the tip membrane. A fundamental condition for its performance is that growth is restricted to the tip, and at least one growth limiting substance must therefore enter through the tip. However, this growth limiting substance, typically $\text{O}_2$, may serve only to keep a microbial population dense and active in the tip, and a signal to be detected may then originate from a minor constituent also entering through the sensor tip. Such a signal could typically be bioluminescence, and bacteria emitting light by exposure to a large variety of environmental parameters have been engineered (e.g. ref. [46]). The approximately 1 h response time of bioluminescence, which is usually coupled to gene expression, has, however, limited the applicability of such techniques, but even with slow response there is no doubt that bioluminescence-based sensors will find widespread application in the future. Microorganisms may be engineered to emit light when exposed to various xenobiotics or to heavy metals, and sensors containing such bacteria may serve as warning systems for industrial effluents etc.

3 MICROSCALE BOD BIOSENSOR

A simple microscale, whole cell BOD sensor (Figure 2) not utilizing the continuous culture principle illustrated in Figure 1 has been described by Neudörfer and Meyer-Reil [27]. It is based on an $\text{O}_2$ microsensor situated behind poly(vinyl alcohol) immobilized yeast cells ($\text{Rhodotorula mucilaginosa}$). The glass surfaces were treated with a silane to facilitate adhesion of the polyvinyl alcohol to the glass. The sensor was shown to respond to glucose concentrations in a reproducible manner (Figure 3), and was subsequently used to quantify depth profiles of dissolved organic matter in a sediment. As mentioned in section 1, BOD sensors are difficult to calibrate to some well-defined expression of organic matter concentration as the response to different organic compounds differ.

A microscale design of a BOD sensor as shown in Figure 2 may result in faster response than those of macroscale analogs, but it is also clear that a design without a tip membrane will result in a limited lifetime (days) owing to growth of contaminating microorganisms in the tip. The greatest advantage of a microscale BOD sensor is the ability to measure the microdistribution of dissolved organic matter in sediments and similar stratified communities, but unfortunately this ability is restricted to the oxic zone where such concentrations are

Figure 2. Microscale BOD (biological oxygen demand) sensor based on immobilized yeast cells. The respiration of the yeast cells limits the amount of $\text{O}_2$ reaching the internal $\text{O}_2$ microsensor, and the respiration is governed by the concentration of dissolved organic matter in the surrounding medium. Redrawn from Neudörfer and Meyer-Reil [27] by permission of Inter-Research.
Figure 3. Response to various glucose concentrations of the microscale BOD sensor shown in Figure 2. The sensor was exposed to glucose at 'A' and reintroduced into water without dissolved organics at 'B'. Exposure to: ■, 100 μmol L⁻¹; ●, 500 μmol L⁻¹; *, 750 μmol L⁻¹. Redrawn from Neudörfer and Meyer-Reil [27].

low. The reading from the sensor is dependent on the concentration of O₂, so parallel readings of O₂ must always be performed.

4 CONTINUOUS CULTURE, WHOLE CELL MICROSCALE BIOSENSOR FOR NITRATE AND A SEMI-MICRO SENSOR FOR CONTROL OF WASTE WATER TREATMENT

4.1 GENERAL DESCRIPTION OF NITRATE BIOSENSOR

Nitrate can be reduced by heterotrophic bacteria to either NO₂⁻, N₂O, N₂, or NH₄⁺. Electrochemical microsensors for NO₂⁻, N₂O, and NH₄⁺ have been described [28–30], and a biosensor for NO₃⁻ can thus be made by a bacterial reduction of NO₃⁻ to one of these species. Nitrite will be measured as well if biosensors are based on electrochemical N₂O or NH₄⁺ microsensors. We have constructed [18] such a NO₃⁻ + NO₂⁻ biosensor (Figure 4) by using a N₂O microsensor as a transducer, but the NO₂⁻ microsensor may actually be sufficiently good for use as a transducer in an alternative biosensor design. We usually refer to the NO₃⁻ + NO₂⁻ biosensor as a NO₃⁻ biosensor, as most environments contain little NO₂⁻ compared with NO₃⁻. The liquid medium inside the sensor contains a high concentration (0.5 wt %) of tryptic soy broth, which supports rapid metabolism of the applied strain of the denitrifying bacterium Agrobacterium radiobacter. This strain has no N₂O reductase, and therefore the reduction of NO₃⁻ and NO₂⁻ stops at the N₂O stage. The medium is kept at relatively high salinity (about 1%) to facilitate electrophoretic attraction or repulsion (see section 2) of NO₃⁻ + NO₂⁻. The voltages used to mediate electrophoretic attraction are usually from +0.1 to +1.0 V versus an external standard calomel electrode, while a potential of −0.8 V practically excludes any entry of NO₃⁻ and NO₂⁻ through the ion-permeable tip membrane. Glass tips with inserted ion-permeable membranes are available commercially.

It should be stressed that the sensor actually senses NO₃⁻ + NO₂⁻, and any N₂O present will interfere. The interference of N₂O can, however, be compensated for by measuring the N₂O concentration while entry of NO₃⁻ and NO₂⁻ into the sensor is prevented by a high negative potential (−0.8 V) applied versus an external calomel electrode [26]. The concentration of N₂O in the environment is, however, rarely so high that it causes pronounced interference.

The NO₃⁻ biosensor is shown in Figure 4 with a tip diameter of about 25 μm, which is the smallest possible tip diameter if concentrations below 1 μmol L⁻¹ should be measured. It is still not known how large the diameter of the sensor tips can be made before the supply of electron donors from the internal reservoir starts to be limiting. The distance between membrane and transducer should be kept below 150 μm to obtain 90% response times of about 15–30 s, and at a maximum of 300 μm if a response time of 2 min is acceptable.

The response of the NO₃⁻ biosensor is linear as long as the bacteria can mediate a full conversion of NO₃⁻ in the reaction space between internal transducer tip and the tip membrane. Figure 5 shows calibration curves for a sensor measuring in air-saturated water and in O₂ saturated water. A significant proportion of the bacteria respires aerobically when the analyzed water is O₂ saturated, so the capacity to reduce NO₃⁻ is lowered, resulting in a lower range for linear response. The sensitivity in the linear range is, however, independent of O₂ concentration. This independence of O₂ concentration may not appear logical, but it can be shown both experimentally and by mathematical modeling.
medium is possible so that the sensor should operate over a wide range of pH values. Other types of bacteria are, however, very sensitive to toxic compounds, and biosensors containing nitrifying bacteria [13] have been constructed to serve as warning devices for toxic emissions.

Nitrate biosensors should be stored in NO$_3^-$-containing (e.g. 1 mmol L$^{-1}$) water when not in use, and a couple of days with continuous polarization is needed before a stable baseline for zero NO$_3^-$ is obtained. Continuous polarization is actually recommended for maximum lifetime and performance. The lifetimes of real microscale NO$_3^-$ biosensors have until now only been a maximum of a few weeks, but work is in progress to improve this. The problems causing a limited lifetime have apparently not been microbiological, but rather membrane and N$_2$O transducer stability.

4.2 MONITORING IN WASTE WATER TREATMENT PLANTS

There is a large demand for a stable and fast-responding NO$_3^-$ sensor for regulation and emission control of wastewater treatment plants, and a robust version of the NO$_3^-$ biosensor is therefore being developed for this purpose. A result of a test run in an alternating oxic/anoxic waste water treatment plant is shown in Figure 6 together with simultaneous readings of O$_2$ made by a robust version of an O$_2$ microsensor. It can be observed that nitrification, i.e. formation of NO$_3^-$ + NO$_2^-$, starts immediately after onset of aeration and that...
denitrification starts immediately after onset of anoxic conditions. It is also remarkable that nitrification was very intense during the initial 10–15 min of the oxic period owing to the presence of NH$_4^+$ formed during the preceding anoxic period, and that nitrification thereafter had to be based on the continuously liberated NH$_4^+$ resulting in a lower rate. From a manager’s point of view it is evident that the anoxic periods could have been reduced considerably with resulting increase in efficiency, as denitrification was complete within the first 30 min of the 60 min period without aeration. Use of a fast-responding NO$_3^-$ sensor in wastewater treatment plants could thus be of great value. At present it is possible to make fast-responding semi-micro NO$_3^-$ biosensors which exhibit < 20% drift in signal over the initial 2 months of continuous operation in a waste water treatment plant. The present dynamic range of these semi-micro sensors is from 5 to 3,500 µmol L$^{-1}$ at 20°C, but work is being conducted to insert the sensors in constant-temperature units so that the measuring range is independent of ambient temperature.

4.3 IN SITU MONITORING OF NATURAL AQUATIC ENVIRONMENTS

The only field experiment conducted until now with a microscale NO$_3^-$ biosensor was performed in a Danish lake while the water temperature was 8°C (L.H. Larsen et al., unpublished results). The biosensor was mounted on a benthic lander [32] and profiles of NO$_3^-$ in the sediment were measured at intervals during a 10 d period. The experiment was very successful, and detailed NO$_3^-$ profiles were obtained although the water phase NO$_3^-$ concentration was only 8 µmol L$^{-1}$. The signal drift of the NO$_3^-$ sensor during the period was less than 1% d$^{-1}$.

4.4 EFFECTS OF CHANGES IN DIFFUSIVITY AND FLOW ON MEASUREMENTS

The obvious advantage of microsensors is that they may be used to analyze the spatial distribution of chemical or physical parameters. Much work of this type has, however, been done without taking into account that the sensor should be characterized by very low stirring effect (< 2%) if reliable results should be obtained [33]. A sensor with a 2% difference between stirred and stagnant water with identical concentration of the species being sensed will typically exhibit a 3–6% difference between a reading in a sediment matrix and in stirred water because of the low transport coefficients in a stagnant sediment matrix, and even a 2% stirring effect may thus be critical for calculations based on concentration gradients near the sediment–water interface. When used without electrophoretic transport of ions into the NO$_3^-$ biosensor, there is only a small sensitivity to stirring. The same is not always the case if the sensitivity is improved by applying a positive potential to the sensor (see section 2), but for 25 µm thick sensors experiments showed relatively small effects (< 5% change from stirred to stagnant water). There is, however, one additional problem which should not be neglected: when relatively thick and conical microsensors are approaching a sediment they affect the water flow in the immediate vicinity of the sensor, and the readings are thus made under another flow regime than found in the absence of the sensor [34]. The diffusive boundary layer above the sediment may be eroded down from, for example, 200 to 100 µm, and the concentration of NO$_3^-$ at the sediment surface is therefore increased. Such an effect is negligible if the NO$_3^-$ penetration is several millimeters, but the effect can be pronounced in very active systems where large concentration changes occur over less than 1 mm [35], as a significant proportion of the decrease in NO$_3^-$ concentration then occurs in the diffusive boundary layer. The effect on the local flow conditions is smaller when very thin sensors are used, but introduction of even very thin O₂ sensors with tip diameters < 10 µm did result in significant effects on the thickness of the diffusive boundary layer [34].

4.5 MEASUREMENT IN MARINE ENVIRONMENTS WITH LOW NITRATE CONCENTRATIONS

An example of a NO$_3^-$ profile in a marine sediment as obtained by a 25 µm thick biosensor is shown in Figure 7. The readings were performed while sensitivity was improved by applying a potential of +0.6 V to the biosensor.

Figure 7. Profile of NO$_3^-$ in a marine sediment at 16°C. Nitrification results in a peak of NO$_3^-$ up to 10 µmol L$^{-1}$ in the (oxic) 0–3.5 mm surface layer while denitrification causes NO$_3^-$ depletion in the (anoxic) 3.5–4.5 mm layer.
versus an external calomel reference electrode. It should be noticed that the NO$_3^-$ concentration in the overlying water was only 4 µmol L$^{-1}$, and that the resolution of the readings was about 0.1 µmol L$^{-1}$. There was a peak in NO$_3^-$ (+NO$_2^-$) caused by NO$_3^-$ (+NO$_2^-$) production (nitrification) in the upper 3-3.5 mm of the sediment followed by NO$_3^-$ (+NO$_2^-$) consumption (denitrification) below ca. 3.5 mm depth.

4.6 DETAILED MAPPING OF MICROSCALE DISTRIBUTION OF NITRIFICATION AND DENITRIFICATION IN A SEDIMENT

It is obvious from the data presented in Figure 7 that NO$_3^-$ distribution in sediments can be analyzed at great accuracy by the use of NO$_3^-$ biosensors, and that these sensors can thus be used to study nitrification and denitrification. The data presented in Figure 8 illustrate this in more detail (see Lorenzen et al. [36] for a thorough discussion of similar data). The data were recorded in a diatom-covered sediment core from a freshwater lake that was exposed to 12 h light and 12 h dark diurnal cycles. The NO$_3^-$ and O$_2$ profiles in Figure 8A represent steady-state conditions during the night, whereas the data of Figure 8B represent steady-state light conditions. During the night, O$_2$ penetrated to only 1 mm depth. The NO$_3^-$ profile through the oxic layer was almost linear, indicating no net transformation of NO$_3^-$, whereas denitrification in the anoxic layers below 1 mm depth caused depletion of NO$_3^-$ at a depth of about 2 mm. In the light, the diatoms in the top 1.5 mm produced O$_2$, and the maximum O$_2$ concentration was about three times air saturation. The O$_2$ penetration was increased from 1 mm in the dark to 5 mm in the light. The NO$_3^-$ profile was also heavily affected by the light and associated microphytobenthic photosynthesis. There was thus a minimum in NO$_3^-$ in the diatom layer caused by assimilation. In the oxic zone below the diatom layer there was a peak in NO$_3^-$ caused by nitrifying bacteria oxidizing NH$_4^+$ to NO$_3^-$, followed by NO$_3^-$ depletion in the anoxic layers below 4.3 mm depth due to denitrification.

Metabolic rates (bars in Figure 8) were calculated from the concentration profiles by a computer-implemented diffusion-reaction model [37]. To do this it is, however, necessary to know the depth profiles of diffusivity, but this is now a relatively simple task as a microsensor for the determination of microscale water flow or sediment diffusivity has been developed [38] (see also section 6). It should be stressed that the modeled rates in Figure 8 are net rates, so in principle a rate of zero at some depth could be due to identical production and consumption rates at that depth.

4.7 COMPARISON OF NITRATE BIOSENSOR WITH ION-EXCHANGER BASED SENSORS

As compared to the liquid ion-exchanger (LIX) type NO$_3^-$ [39] and NO$_2^-$ [28] micro- and macrosensors (see Chapter 5), the NO$_3^-$ biosensor has both advantages and limitations. The LIX electrodes are relatively easy to make, whereas the biosensors require great skill. The LIX electrodes can also be made with extremely small tips (at least for the NO$_3^-$ electrodes down to sub-micrometer diameter), whereas the biosensors lose sensitivity if made with tip diameters below about 25 µm. The biosensors are, however, able to measure accurately in water containing interfering ions, including seawater. When operated with an applied positive tip potential the biosensors may be much more sensitive than the ion-exchanger electrodes, where the practical detection limit in environmental waters is very dependent on the concentration of interfering ions. Finally it is possible to make biosensors which are extremely long-term stable. We still have not found procedures that reproducibly result in long-term stable microscale biosensors, but as described above the semi-microscale NO$_3^-$ biosensor may operate continuously, even in wastewater, for periods of months.
Ion-exchanger-type NO₃⁻ electrodes are marketed [4], but the stability in waste water is apparently too poor for widespread use in waste water treatment.

5 MICROSCALE BIOSENSOR FOR METHANE

5.1 PREVIOUS METHODS FOR RESOLVING METHANE GRADIENTS

Usable electrochemical sensors for CH₄ have not been described, as CH₄ is very inert, so the spatial resolution of CH₄ in sediments has been determined by gas sampling through membrane-equipped capillaries with subsequent GC analysis of the collected gas [40], or by membrane-inlet mass spectrometry [41]. These methods suffer, however, from the need for a relatively large and highly permeable membrane-covered window to ensure a sufficient gas flux for the analysis, and the probes are therefore characterized by a high stirring sensitivity of about 100–200%. As described for the NO₃⁻ biosensors above, high stirring sensitivities lead to inaccurate readings when the diffusive properties of analyzed stagnant media change, and changes in the reading may then be due both to real changes in concentration and to local changes in diffusivity. A so-called biosensor for CH₄ has also been described [25], but it was based on addition of large samples to a stirred culture of methane-oxidizing bacteria with subsequent monitoring of the decrease in O₂ concentration. Methane is, however, mostly present in anaerobic environments, so the ideal sensor would be one that could measure without the need for external oxygen.

5.2 GENERAL DESCRIPTION OF METHANE BIOSENSOR

A biosensor for the determination of CH₄ under anoxic conditions was developed by using the design illustrated in Figure 9, where CH₄-oxidizing bacteria are cultured in the thin microsensor tip [17]. The principle is basically the same microscale continuous culture vessel as illustrated in Figure 1, but the CH₄ sensor is made a little more complicated by the supply of O₂ to the tip via an internal gas-filled capillary containing an O₂ microsensor with its tip permanently positioned near the surface of the membrane covering the gas-filled capillary. The O₂ microsensor monitors the O₂ gradient within the biosensor as illustrated in Figure 10. The current in the measuring circuit is high for zero CH₄ and decreases with increasing methane concentration. The calibration curve may be linear over the full range from 0 to 100% CH₄ saturation (Figure 11), or it may be linear only at relatively low CH₄ concentrations. The O₂ partial pressure is always constant at the inner surface of the silicone rubber (Figure 10), as the diffusion coefficient of O₂ in air is about 10⁴ times higher than the diffusion coefficient in water or silicone rubber. The response time is determined by the relatively long distance from the air reservoir to the biosensor.

Figure 9. Microscale biosensor for CH₄ based on CH₄-oxidizing bacteria living in a gradient of CH₄ from the analyzed medium and O₂ from an internal reservoir. An internal O₂ microsensor monitors the O₂ gradient within the sensor. Left: entire sensor. Right: enlarged section through the tip region. Reprinted with permission from Damgaard and Revsbech [17]. Copyright (1997) American Chemical Society

Figure 10. Functioning of the CH₄ biosensor. The sensor tip is shown schematically to the left. The two diagrams (A) and (B) illustrate how changes in CH₄ concentration affect the O₂ gradient and thereby the signal (illustrated with an arrow) from the internal O₂ microsensor. Reprinted with permission from Damgaard and Revsbech [17]. Copyright (1997) American Chemical Society
The CH₄ biosensor may function for months, but calibration should be performed at regular intervals. The bacteria in the tip respond rapidly by increased respiration rate when they are exposed to CH₄, but there will always be some residual metabolism even in the absence of CH₄, and such a residual metabolism may change as a function of the life history of the biosensor. It is actually strange that such a residual metabolism does not result in pronounced baseline problems as are known for BOD sensors [10], where the usual practice is to incubate the sensor in nutrient-free medium for a considerable period, so that the metabolism can stabilize at a low level before each exposure to a new sample.

5.3 METHANE BIOSENSOR WITH OXYGEN GUARD AND ITS USE IN RICE PADDY SOIL

Methanogenic environments may be investigated in great detail by use of the CH₄ biosensor shown in Figure 9, but it only works under anoxic conditions as all O₂ must be supplied from the internal reservoir. It is, however, possible to add an O₂ scavenging system to the sensor tip as shown in Figure 12. The sensor...

![Figure 11. Calibration of a CH₄ biosensor performed twice with an intervening 18 h interval: ○, calibration at start of experiment; □, calibration after 18 h. Reprinted with permission from Damgaard and Revsbech [17]. Copyright American Chemical Society](image)

![Figure 12. Tip of CH₄ biosensor equipped with an O₂ guard capillary containing the heterotrophic bacterium Agrobacterium radiobacter in a 1% tryptic soy broth medium. Reproduced from Damgaard et al. [42] by permission of American Society for Microbiology](image)
shown in Figure 12 used heterotrophic bacteria immobilized in front of the capillary with the CH₄-oxidizing bacteria to remove the O₂ [42], but a higher efficiency may theoretically be obtained by using a 0.5 mol L⁻¹ solution of ascorbate at pH 13, and it should thereby be possible to reduce the distance between the two membranes to 30 μm. By adding this O₂ guard the CH₄ sensor is made insensitive to external O₂, and the (small) stirring effect seen by the ‘simple’ CH₄ biosensor is practically removed, so the modification could seem to be ideal. There are, however, also negative aspects of the O₂ guard. First of all, the level of complexity is increased, and the construction of a complete sensor is quite difficult and tedious. The addition of a guard does, however, also lead to lower signal and to slower response. The distance between the exterior and the internal O₂ microsensor is increased, and as the response time increases with the square of the distance (twice the distance gives four times longer response time) this is in itself a problem. What is worse, however, is that CH₄ may accumulate in the O₂ guard behind the tip of the CH₄ biosensor, and this gives a very slow response to large changes in CH₄ concentration. It can thus only be recommended to use an O₂ guard when absolutely necessary. When aerobic CH₄ oxidation is studied there is, however, no choice. Overlapping CH₄ and O₂ profiles from a rice paddy as measured with an O₂ microsensor and a CH₄ biosensor with O₂ guard are shown in Figure 13. During darkness

![Figure 13](image)

**Figure 13.** Oxygen (□) and CH₄ (■) profiles in a rice paddy soil as measured by microsensors. (A) Profiles during the day with deep O₂ penetration caused by cyanobacterial photosynthesis. (B) Profiles at night. The O₂ and CH₄ profiles were measured with different sensors, so they may not be perfectly aligned. Reproduced from Damgaard et al. [42] by permission of American Society for Microbiology

(Figure 13B) the O₂ penetration was less than 1 mm, but extensive CH₄ oxidation in this 1 mm led to almost full CH₄ depletion below the sediment surface. During the day (Figure 13A) illumination caused O₂ production by cyanobacteria living in the top soil layers so that the CH₄ oxidation horizon was now found at 4–6 mm depth.

6 CALCULATION OF METABOLIC RATES BASED ON DIFFUSIVITY SENSORS AND DEPTH PROFILES OF CHEMICAL SPECIES MEASURED WITH MICROSCALE BIOSENSORS

The results shown in Figure 8 illustrate how detailed data obtained by microscale (bio)sensors can be, but they also illustrate how calculations of depth profiles of metabolic rates can be performed based on the chemical profiles. The diffusivity profiles necessary for performing such calculations based on Fick’s first and second laws of diffusion can now be measured with a diffusivity sensor [38]. This diffusivity sensor contains a reservoir of tracer gas which diffuses out into the surrounding medium through a membrane in the sensor tip while a built-in microsensor for the gas in question monitors the gas concentration at the membrane surface. A low diffusivity in the surrounding medium will result in impeded diffusion of the tracer gas away from the sensor tip and thus in a high reading from the built-in sensor, whereas the opposite is the case for a high diffusivity. The same sensor can also be used to quantify flow rates down to very low values (<10 μm s⁻¹). Flow/diffusivity sensors based on O₂ as a tracer are commercially available, but for environmental applications more inert tracers such as acetylene (L.R. Damgaard et al., unpublished results) should be used. An alternative optical determination of microscale diffusivity distribution has also been described [43].

7 FUTURE DEVELOPMENTS IN MICROSCALE BIOSENSORS FOR ENVIRONMENTAL MONITORING

As mentioned in the section about NO₃⁻ biosensors a long-term stable NO₃⁻ sensor for control of waste water treatment has been developed. At present this sensor and the O₂ and pH sensors are, to our knowledge, the only real chemical sensors (i.e. not counting miniaturized flow injection and spectroscopic devices) that will function continuously on-line for periods of months while immersed in complex media such as waste water. There are, however, several other possibilities for new types of microscale biosensors for long-term environmental monitoring, and we expect that such biosensors will be based on whole cells, as enzyme-based sensors most probably cannot be made sufficiently long-term stable. Biosensors with a short lifetime (for measuring, e.g.
xenobiotic compounds) may, however, be used extensively in the future, and there may be advantages of applying microscale designs here also. In the beginning of the chapter the possibility of making bioluminescence-based biosensors has already been mentioned, and this is probably the most extensive open area for new developments as such sensors may detect very low concentrations.

It may be possible to make new microscale biosensors based on whole cells for chemical species such as NH₄⁺ [12] and SO₄²⁻. Ammonium-oxidizing bacteria (Nitrosomonas sp.) that might be used in a possible micro-biosensor are, however, very sensitive to variations in environmental parameters, and as quite good electrochemical NH₄⁺ sensors exist, the niche for use of an NH₄⁺ biosensor will be relatively narrow. Analysis of NH₄⁺ in marine sediments could be done with such sensors, but a more interesting possibility is long-term monitoring of NH₄⁺ in waste water, where a biosensor might outperform purely electrochemical sensors in terms of lifetime and long-term stability if based on a microscale design. A microscale SO₄²⁻ biosensor would be of great scientific interest, as no reliable electrochemical sensor for SO₄²⁻ exists, and the principle of bacterial SO₄²⁻ reduction followed by electrochemical detection of the sulfide evolved should be tested in microscale biosensors.

By applying the proper microorganisms and membranes, the CH₄ biosensor design shown in Figure 9 can be used for analysis of many different organic or inorganic compounds. The main problem is, however, that except for CH₄, HS⁻, and NH₄⁺, most oxidizable low-molecular weight chemical species do not build up as large dissolved pools in natural sediments and biofilms. An exception to this is acetate (and other short-chain carboxylic acids), as many methanogenic environments contain freely dissolved acetate in appreciable concentrations (10⁻⁵ – 10⁻² mol L⁻¹). A modified CH₄ biosensor containing acetate-oxidizing bacteria such as a Pseudomonas sp. may thus be used for analysis of acetate. Other easily degradable organic species are usually found in concentrations below the few micromolar level necessary for detection by the O₂ consumption within the sensor. Iron and manganese may build up to appreciable concentrations, but various aspects of solubility, diffusivity, and possible interferences do not make the construction of biosensors for these species feasible. The tip of a biosensor based on oxidation of Fe³⁺ or M²⁺ would rapidly be filled with insoluble oxides and hydroxides, and the very slow diffusion into the sensor (the diffusivity of these ions is much lower than for NO₃⁻, CH₄, O₂, etc.) [44] would also give a poor sensitivity. At present voltammetry (Buffer, Chapter 9) and dialysis methods (Davison, Chapter 11) seem to be the best in situ detection principles for Fe²⁺ and Mn²⁺.

One sensor that would be extremely valuable and where a satisfactory detection scheme still has to be devised is a phosphate sensor. Many biological reactions and transport systems are highly specific for phosphate, but although attempts have been made it has until now not been possible to couple this specificity with a satisfactory detection principle.

8 CONCLUSION

Taken as a whole, the combination of available microscale electrochemical sensors, optodes, and biosensors (a short review was presented by Kühl and Revsbech [45]) now makes it possible to analyze the microscale chemistry of aquatic environments in great detail, although especially a phosphate sensor is still missing. A considerable amount of information about our environment has already been gained by use of these sensors, but the potential for considerable expansion of our knowledge is still there. The development of microscale sensors does, however, also have a broader scope. The analytical schemes utilized in the microscale NO₃⁻ and CH₄ biosensors also work in sensors with diameters up to about 0.5 mm, and such semi-macro biosensors may in the future contribute significantly to environmental monitoring and to efficient control of waste water treatment.

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GLOSSARY

Axenic  Culture of organisms with only one type being present
Benthic lander  Instrument made for in situ investigation of the sediment-water interface.
Bioluminescence  Biological emission of light based on enzymatic oxidation of an aldehyde.
Biosensor  Often used for sensors based on any biological component that can be used to obtain a signal for a chemical parameter. In this chapter, biosensor is used in a more restricted sense, i.e. it is a physical device based on the combination of microorganisms and a detection system that enables the measurement of chemical species.
Cyanobacteria  Photosynthetic microorganisms also often referred to as blue-green algae.
Denitrification  Bacterial respiration with NO₃⁻ and NO₂⁻ whereby NO₃⁻ and NO₂⁻ are reduced to N₂ or N₂O.
Diatoms  Eucaryotic photosynthetic microorganisms with a silica shell.
Diffusive boundary layer  The thin layer just above a surface where diffusional transport of dissolved species perpendicular to the surface dominates over transport by flow.
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Gene expression  Translation of the genetic code in DNA to RNA and often further to protein.
Inducer  In molecular biology, this term is used for some chemical species that causes gene expression (see above).
Eucaryotic  Organisms having a nuclear membrane as opposed to bacteria. Eucaryotic cells are usually larger than bacterial cells.
Heterotrophic  Organisms assimilating organic species as opposed to the autotrophic ones assimilating CO₂.
LIX  Liquid ion exchanger, i.e. some ion exchanger for a specific ion dissolved into a hydrophobic liquid.
Methanogenic  Methane-producing.
Microphytobenthos  Photosynthetic microorganisms living on the sediment surface.
Nitrification  Oxidation of NH₄⁺ to NO₃⁻ by one type of bacteria followed by further oxidation of NO₃⁻ to NO₂⁻ by another type of bacteria.
Strain  Bacterial species are difficult to define, and it is therefore common to refer to specific isolates (or mutants), also called strains.
 Xenobiotic  Non-biological, man-made chemical species.

REFERENCES


