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 $l = (2Dt)^{1/2}$, where l is diffusional path length covered during time t and D is the diffusion coefficient. According to this equation, a small molecule such as oxygen ($D = 2 \times 10^{-9}\,m^2s^{-1}$ 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 O_2, 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 (MACROSCALE) 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 waste water. It is basically just a layer of immobilized heterotrophic microorganisms placed in front of a conventional O_2 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 O_2 reaching the O_2 sensor by diffusion from the medium. For interpretation of the signal from this sensor it is thus critical to know the O_2 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 O_2 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 1. Examples of biosensors that could possibly be used for environmental monitoring (cons. = consumption)

Chemical parameter	Bio-component	Detection principle	Lifetime	Analytical range (mol L^{-1})	Interferences / comments	Continuous operation	References
Ammonium	Bacteria	Oxygen cons.	?	?	?	+	12, 13
BOD	Bacteria/yeasts	Oxygen cons.	months	Ill-defined	Ill-defined	–	9, 10
CO_2	Bacteria	Oxygen cons.	1 month	3–12% sat.	None	+	14, 15
Herbicides	Algae	Photosyn. inh.	?	Very variable	?	–	16
Methane	Bacteria	Oxygen cons.	months	10^{-6}–10^{-3}	Sulfide	+	17
Nitrite	Bacteria	Oxygen cons.	?	?	?	+	12
Nitrate + nitrite	Bacteria	N_2O evol.	> 2 months	10^{-7}–10^{-2}	N_2O	+	18
Pesticides	Enzymes	Enzyme inh.	?	$\gg 10^{-8}$	?	–	16
Phenols	Enzyme(s)	Oxygen cons.	?	$\gg 10^{-8}$	Pre-treatment is necessary	–	19
Surfactants	Bacteria	Oxygen cons.	?	?	?	–	20
Toxicity	Bacteria	Respiration inh.	?	?	?	+	13, 21
Xenobiotics	Antibody	Many detection principles	?	Very variable	Highly specific	–	22

primary substrates for the reaction must be added to the test medium. Immunological reactions have also been used for analysis of such xenobiotic compounds [22]. It has, however, turned out to be extremely difficult to make reversible enzyme or immunological sensors 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 problematic when long-term stable biosensors are essential, but more sophisticated approaches where highly selective membranes protect the biological components [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 microscale 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 interfering 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 microorganisms. 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_3^{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 acceptors 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_2O. 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 restrictions 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,

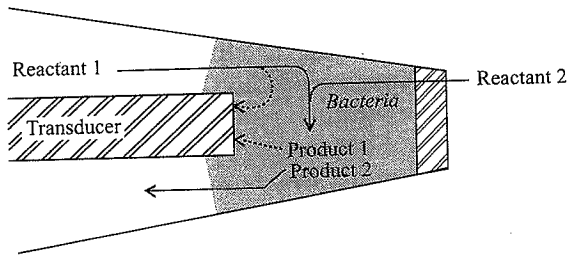

Figure 1. Microscale continuous-culture principle of microscale biosensors based on living microorganisms. The growth of microorganisms is limited to the tip region as one or more growth-limiting chemical species enter through the tip membrane

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 $NO_2^- + NO_3^-$ and CH_4 biosensors described in this chapter, the metabolic status of the microorganisms is actually irrelevant 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 CH_4 and $NO_2^- + NO_3^-$ biosensors described below, the reaction chambers are 100–300 μm 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 $NO_2^- + NO_3^-$ biosensors it is thereby possible to increase the sensitivity by a factor of more than 10, and it is then possible to analyze $NO_2^- + NO_3^-$ down to 0.1 μmol 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 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 O_2 microsensor situated behind poly(vinyl alcohol) immobilized yeast cells (*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

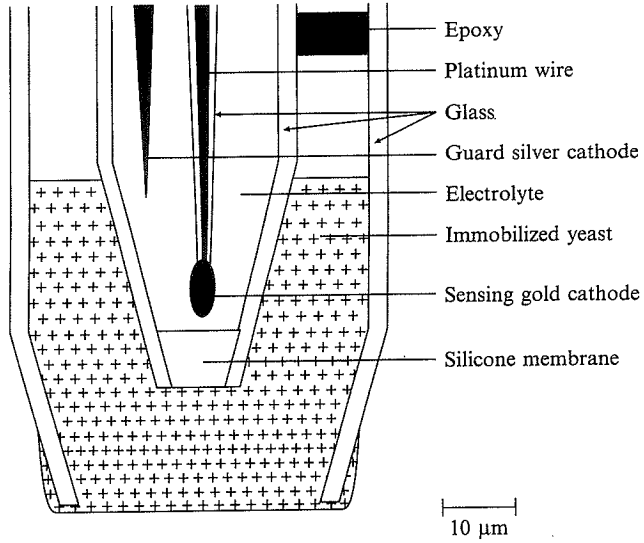

Figure 2. Microscale BOD (biological oxygen demand) sensor based on immobilized yeast cells. The respiration of the yeast cells limits the amount of O_2 reaching the internal O_2 microsensor, and the respiration is governed by the concentration of dissolved organic matter in the surrounding medium. Redrawn from Neudörfer and Meyer-Reil [27] by permission of Inter-Research

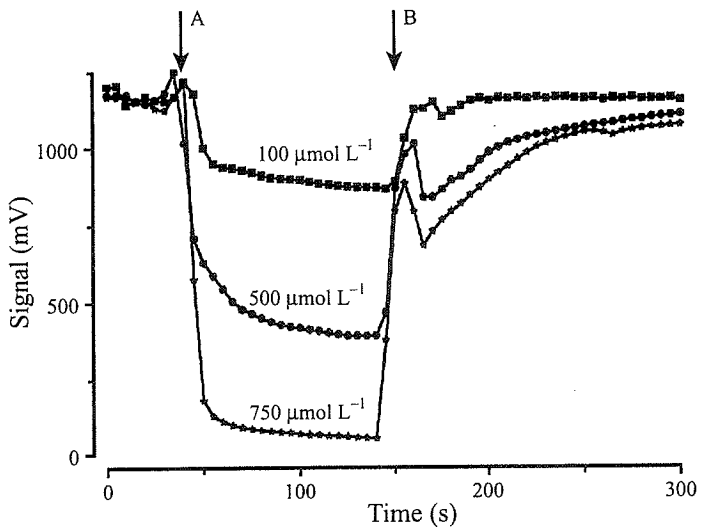

Figure 3. Response to various glucose concentrations of the microscale BOD sensor shown in Figure 2. The sensor was exposed to glucose at 'A' and reintroduced into water without dissolved organics at 'B'. Exposure to: ■, 100 µmol L^{-1}; ●, 500 µmol L^{-1}; *, 750 µmol L^{-1}. Redrawn from Neudörfer and Meyer-Reil [27].

low. The reading from the sensor is dependent on the concentration of O_2, so parallel readings of O_2 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 NO_2^-, N_2O, N_2, or NH_4^+. Electrochemical microsensors for NO_2^-, N_2O, and NH_4^+ have been described [28–30], and a biosensor for NO_3^- can thus be made by a bacterial reduction of NO_3^- to one of these species. Nitrite will be measured as well if biosensors are based on electrochemical N_2O or NH_4^+ microsensors. We have constructed [18] such a $NO_3^- + NO_2^-$ biosensor (Figure 4) by using a N_2O microsensor as a transducer, but the NO_2^- microsensor may actually be sufficiently good for use as a transducer in an alternative biosensor design. We usually refer to the $NO_3^- + NO_2^-$ biosensor as a NO_3^- biosensor, as most environments contain little NO_2^- compared with NO_3^-. The liquid medium inside the sensor contains a high concentration (0.5 wt %) of tryptic soy broth,

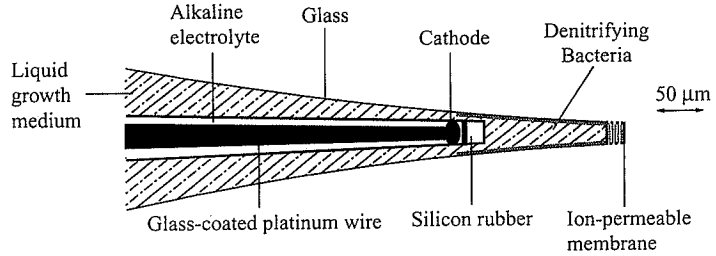

Figure 4. Tip of biosensor for NO_3^- based on bacterial conversion of NO_3^- to N_2O and subsequent electrochemical detection of the N_2O

which supports rapid metabolism of the applied strain of the denitrifying bacterium *Agrobacterium radiobacter*. This strain has no N_2O reductase, and therefore the reduction of NO_3^- and NO_2^- stops at the N_2O stage. The medium is kept at relatively high salinity (about 1 %) to facilitate electrophoretic attraction or repulsion (see section 2) of $NO_3^- + NO_2^-$. 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 NO_3^- and NO_2^- 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 $NO_3^- + NO_2^-$, and any N_2O present will interfere. The interference of N_2O can, however, be compensated for by measuring the N_2O concentration while entry of NO_3^- and NO_2^- into the sensor is prevented by a high negative potential (−0.8V) applied versus an external calomel electrode [26]. The concentration of N_2O in the environment is, however, rarely so high that it causes pronounced interference.

The NO_3^- 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 NO_3^- biosensor is linear as long as the bacteria can mediate a full conversion of NO_3^- 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 O_2 saturated water. A significant proportion of the bacteria respires aerobically when the analyzed water is O_2 saturated, so the capacity to reduce NO_3^- is lowered, resulting in a lower range for linear response. The sensitivity in the linear range is, however, independent of O_2 concentration. This independence of O_2 concentration may not appear logical, but it can be shown both experimentally and by mathematical modeling

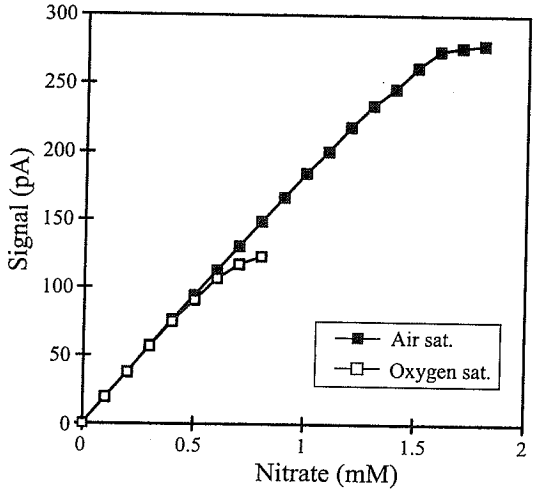

Figure 5. Calibration curves for NO_3^- biosensor in air- and O_2 saturated water. Within the linear range the calibration curves are identical, but the maximum NO_3^- concentration for linear response is lower at high O_2 as the bacteria prefer O_2 to NO_3^-. Reprinted with permission from Larsen *et al.* [31]. Copyright American Chemical Society

of the concentration gradients within the sensor (T. Kjær *et al.*, unpublished results). The linearity is even unaffected by the shape of the tip region (parallel sided or conical). There are no interfering substances except for the N_2O mentioned above. In the first publication on the NO_3^- biosensor [31] sulfide was mentioned as an irreversible inhibitor, but a change in the cathode material plated onto the platinum electrode from silver to palladium has removed the sulfide interference [26].

The bacteria (*Agrobacterium radiobacter*) used in the sensor are remarkably active under a wide range of environmental conditions. The NO_3^- biosensor has thus been used successfully in a temperature range from 3 to 42 °C, but the dynamic range was very low at 3 °C, and for routine operation the temperature should be above 5 °C. The bacteria are also active under a wide range of salinities, and the sensor has thus been used in freshwater as well as oceanic strength seawater. Toxic chemical species seem also to have relatively little effect on the bacteria, probably because they experience relatively low concentrations owing to an efficient diffusional exchange between the sensor tip region and the large internal medium reservoir. Phenol, which goes readily through the membrane, was thus in one instance used to stop bacterial activity in a model wastewater treatment plant while a NO_3^- biosensor was inserted. All biological activity in the plant ceased immediately after the phenol addition, but the biosensor still functioned. The tolerable extremes of external pH have still not been determined for the NO_3^- biosensor, but strong buffering of the internal

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_2O 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

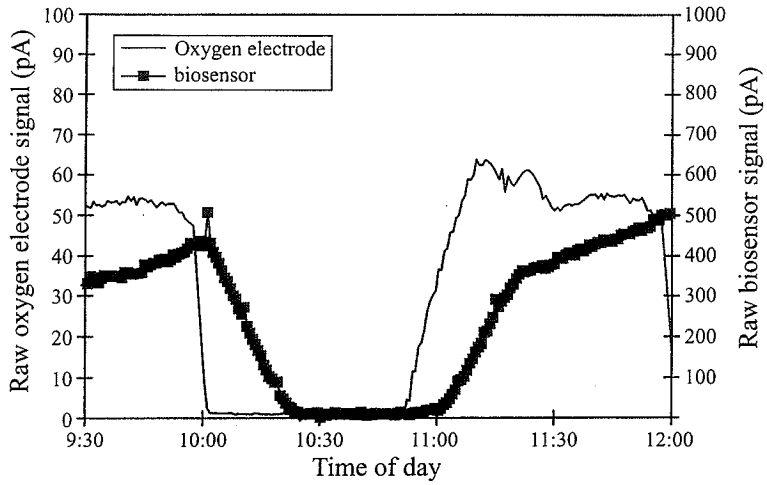

Figure 6. O_2 and NO_3^- concentrations during an aeration cycle of an activated sludge tank in a waste water treatment plant. Nitrification results in a rapid increase in NO_3^- after start of aeration, and denitrification consumes the NO_3^- after onset of anoxic conditions

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 $\mu mol\ L^{-1}$ 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 $\mu mol\ L^{-1}$. 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 O_2 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

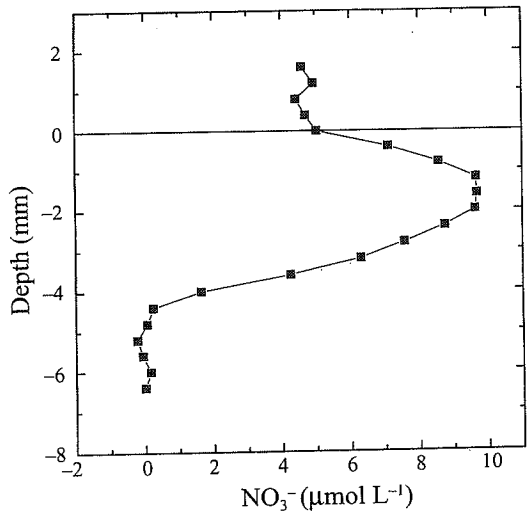

Figure 7. Profile of NO_3^- in a marine sediment at 16 °C. Nitrification results in a peak of NO_3^- up to 10 $\mu mol\ L^{-1}$ in the (oxic) 0–3.5 mm surface layer while denitrification causes NO_3^- depletion in the (anoxic) 3.5–4.5 mm layer.

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

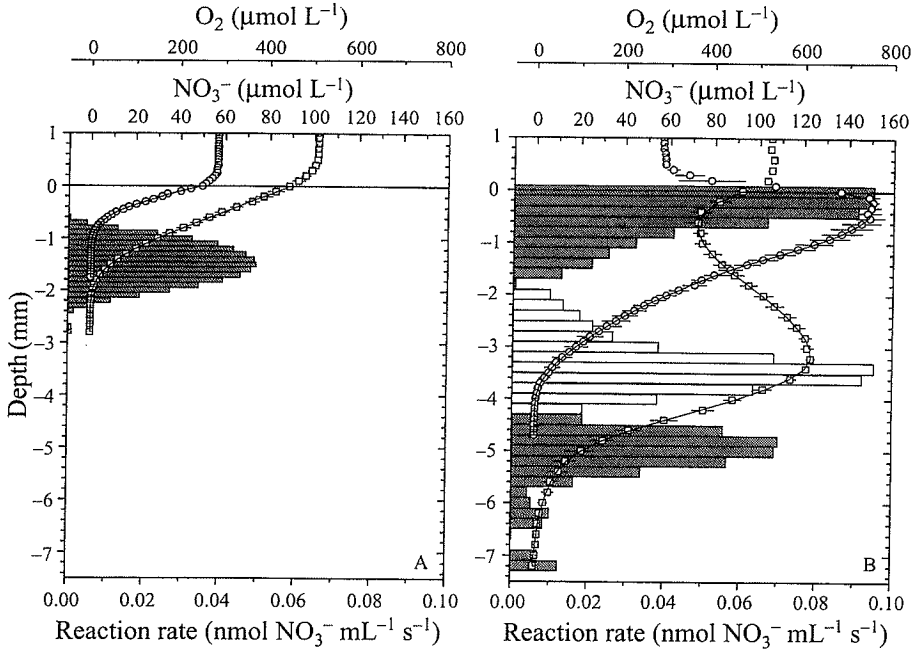

Figure 8. Profiles of O_2 (○, mean values with bars indicating SD, $n = 6$), NO_3^- (□, mean values with bars indicating SD, $n = 6$), rates of NO_3^- assimilation (grey bars in the 0–2 mm layer of panel B), rates of nitrification (light bars), and rates of denitrification (grey bars in panel A and the 4.4–7.2 mm layer of panel B) in a diatom-covered sediment during darkness (A) and during illumination (B). All profiles were measured at different sites in the sediment core, but as shown by the standard deviations, the different profiles were very similar

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 also 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 NO_3^- 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 CH_4 have not been described, as CH_4 is very inert, so the spatial resolution of CH_4 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 NO_3^- 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 CH_4 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 O_2 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 CH_4 under anoxic conditions was developed by using the design illustrated in Figure 9, where CH_4-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 CH_4 sensor is made a little more complicated by the supply of O_2 to the tip via an internal gas-filled capillary containing an O_2 microsensor with its tip permanently positioned near the surface of the membrane covering the gas-filled capillary. The O_2 microsensor monitors the O_2 gradient within the biosensor as illustrated in Figure 10. The current in the measuring circuit is high for zero CH_4 and decreases with increasing methane concentration. The calibration curve may be linear over the full range from 0 to 100% CH_4 saturation (Figure 11), or it may be linear only at relatively low CH_4 concentrations. The O_2 partial pressure is always constant at the inner surface of the silicone rubber (Figure 10), as the diffusion coefficient of O_2 in air is about 10^4 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

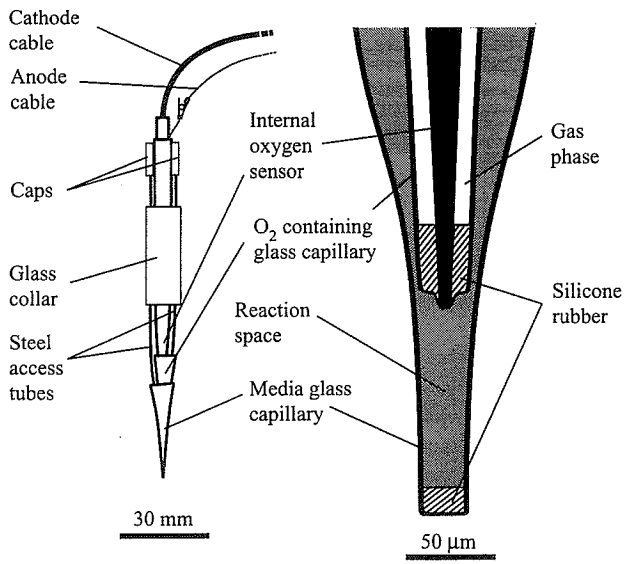

Figure 9. Microscale biosensor for CH_4 based on CH_4 – oxidizing bacteria living in a gradient of CH_4 from the analyzed medium and O_2 from an internal reservoir. An internal O_2 microsensor monitors the O_2 gradient within the sensor. Left: entire sensor. Right: enlarged section through the tip region. Reprinted with permission from Damgaard and Revsbech [17]. Copyright (1997) American Chemical Society

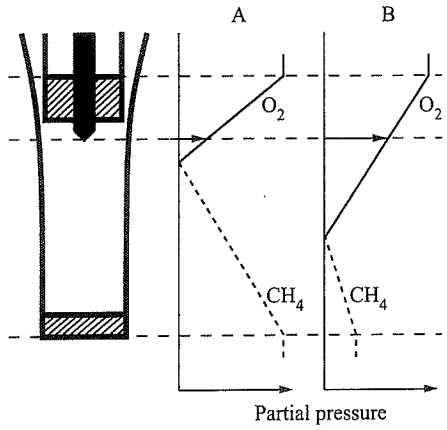

Figure 10. Functioning of the CH_4 biosensor. The sensor tip is shown schematically to the left. The two diagrams (A) and (B) illustrate how changes in CH_4 concentration affect the O_2 gradient and thereby the signal (illustrated with an arrow) from the internal O_2 microsensor. Reprinted with permission from Damgaard and Revsbech [17]. Copyright (1997) American Chemical Society

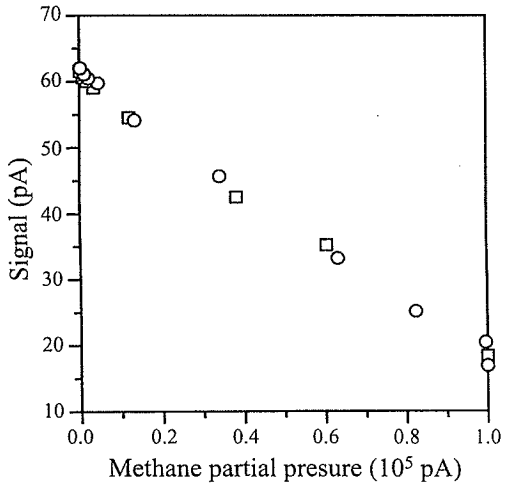

Figure 11. Calibration of a CH_4 biosensor performed twice with an intervening 18 h interval: ○, calibration at start of experiment; □, calibration after 18 h. Reprinted with permission from Damgaard and Revsbech [17]. Copyright American Chemical Society

tip, and the 90 % response time for a sensor with dimensions as shown in Figure 9 is about 30 s.

The signal from the CH_4 biosensor is not as ideal as the signal from the NO_3^- biosensor. First of all, there is a high current from the internal O_2 microsensor at low CH_4 concentration and a low current at high CH_4. This results in lower accuracy at low CH_4 concentrations, as a temperature change of only 1°C affects the current in the circuit by about 3 %. The 'simple' CH_4 biosensor shown in Figure 9 is not as insensitive to stirring as is the case with the NO_3^- biosensor, and the stirring effect is an offset of the whole calibration curve corresponding to 2–4 $\mu mol\ L^{-1}$ CH_4, so this creates problems in the quantification of very low methane concentrations. The problems with stirring effects described here are, however, negligible as compared with the > 100 % stirring effect of the alternative membrane probe sampling procedures. Sulfide interferes. The sensitivity to sulfide is about 25 % of that to CH_4 at pH = 7. Long-term exposure to sulfide may, lead to depositions of elemental sulfur inside the sensor, but such long-term exposure has not yet been tested. Hydrogen may also interfere, but this interference is apparently due to a non-axenic (i.e. contaminated) methanotrophic culture in the microsensor tip and might be alleviated by sterilization of the sensor before the bacteria are added. The signal for hydrogen has, however, been much lower than that for CH_4 in the sensors investigated, and as hydrogen in methanogenic environments is usually present at concentrations < 1 $\mu mol\ L^{-1}$ this interference is in most cases irrelevant.

The CH_4 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 CH_4, but there will always be some residual metabolism even in the absence of CH_4, 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 CH_4 biosensor shown in Figure 9, but it only works under anoxic conditions as all O_2 must be supplied from the internal reservoir. It is, however, possible to add an O_2 scavenging system to the sensor tip as shown in Figure 12. The sensor

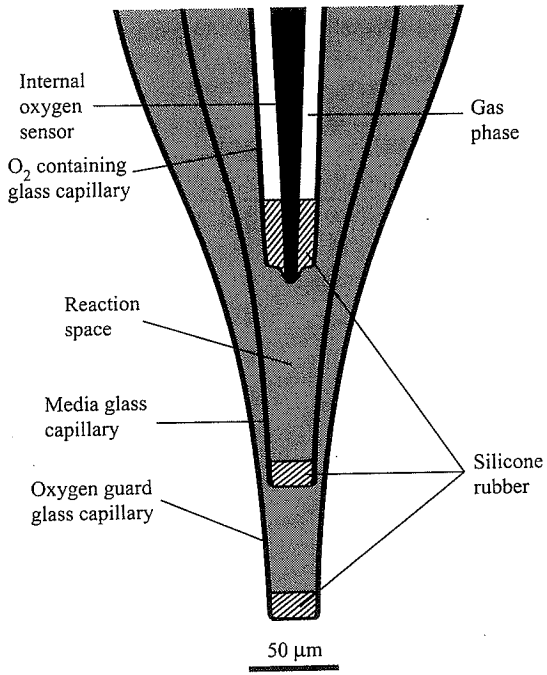

Figure 12. Tip of CH_4 biosensor equipped with an O_2 guard capillary containing the heterotrophic bacterium *Agrobacterium radiobacter* in a 1 % tryptic soy broth medium. Reproduced from Damgaard *et al.* [42] by permission of American Society for Microbiology

shown in Figure 12 used heterotrophic bacteria immobilized in front of the capillary with the CH_4-oxidizing bacteria to remove the O_2 [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 O_2 guard the CH_4 sensor is made insensitive to external O_2, and the (small) stirring effect seen by the 'simple' CH_4 biosensor is practically removed, so the modification could seem to be ideal. There are, however, also negative aspects of the O_2 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 O_2 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 CH_4 may accumulate in the O_2 guard behind the tip of the CH_4 biosensor, and this gives a very slow response to large changes in CH_4 concentration. It can thus only be recommended to use an O_2 guard when absolutely necessary. When aerobic CH_4 oxidation is studied there is, however, no choice. Overlapping CH_4 and O_2 profiles from a rice paddy as measured with an O_2 microsensor and a CH_4 biosensor with O_2 guard are shown in Figure 13. During darkness

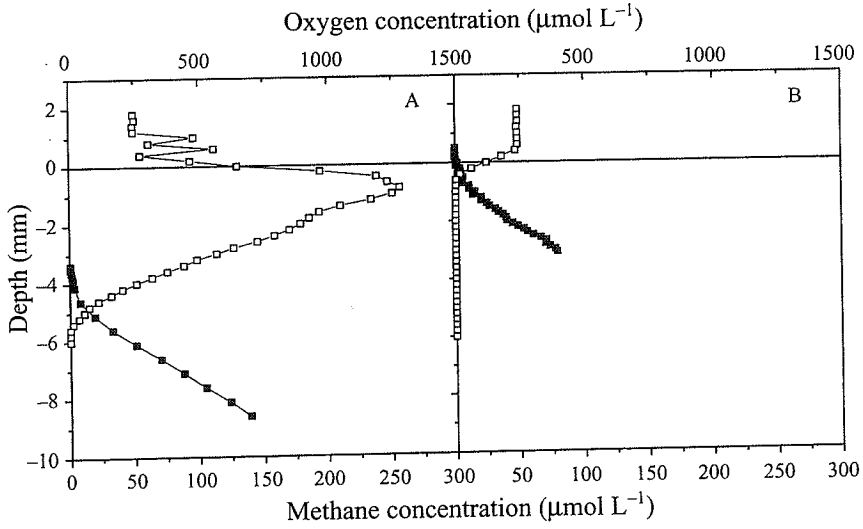

Figure 13. Oxygen (□) and CH_4 (■) profiles in a rice paddy soil as measured by microsensors. (A) Profiles during the day with deep O_2 penetration caused by cyanobacterial photosynthesis. (B) Profiles at night. The O_2 and CH_4 profiles were measured with different sensors, so they may not be perfectly aligned. Reproduced from Damgaard *et al.* [42] by permission of American Society for Microbiology

(Figure 13B) the O_2 penetration was less than 1 mm, but extensive CH_4 oxidation in this 1 mm led to almost full CH_4 depletion below the sediment surface. During the day (Figure 13A) illumination caused O_2 production by cyanobacteria living in the top soil layers so that the CH_4 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 O_2 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 NO_3^- biosensors a long-term stable NO_3^- biosensor for control of waste water treatment has been developed. At present this sensor and the O_2 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 NH_4^+ [12] and SO_4^{2-}. 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 NH_4^+ sensors exist, the niche for use of an NH_4^+ biosensor will be relatively narrow. Analysis of NH_4^+ in marine sediments could be done with such sensors, but a more interesting possibility is long-term monitoring of NH_4^+ 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 SO_4^{2-} biosensor would be of great scientific interest, as no reliable electrochemical sensor for SO_4^{2-} exists, and the principle of bacterial SO_4^{2-} reduction followed by electrochemical detection of the sulfide evolved should be tested in microscale biosensors.

By applying the proper microorganisms and membranes, the CH_4 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 CH_4, HS^-, and NH_4^+, 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 CH_4 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 O_2 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 Fe^{2+} or M_n^{2+} 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 NO_3^-, CH_4, O_2, etc.) [44] would also give a poor sensitivity. At present voltammetry (Buffle, Chapter 9) and dialysis methods (Davison, Chapter 11) seem to be the best *in situ* detection principles for Fe^{2+} and Mn^{2+}.

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 NO_3^- and CH_4 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 NO_3^- and NO_2^- whereby NO_3^- and NO_2^- are reduced to N_2 or N_2O.

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.

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 CO_2.

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 NH_4^+ to NO_2^- by one type of bacteria followed by further oxidation of NO_2^- to NO_3^- 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.

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