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[9] Analysis of Microbial Communities with Electrochemical Microsensors and Microscale Biosensors

By NIELS PETER REVSBECH

Abstract

Electrochemical microsensors for O_2, pH, H_2S, H_2, and N_2O are now available commercially, thus it has become a relatively simple task to analyze the microenvironment in stratified microbial communities for several chemical species. In addition, sensors are available for the physical parameters diffusivity and flow, and based on knowledge about both transport processes and microdistribution of chemistry, it becomes possible to calculate the spatial distribution and local rates of transformations, such as aerobic respiration or denitrification. As compared to other advanced techniques, microsensor equipment is inexpensive. For example, it is possible to start working with oxygen microsensors with an investment of only about $5000. Construction of one's own microsensors is only recommended for the very dedicated user, but the investment here is mainly in terms of man-hours as the equipment is simple and inexpensive. By establishing a microsensor construction facility, it is possible to work with short-lived

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sensors such as ion-selective microsensors for H^+, NO_2^-, NO_3^-, Ca^{2+}, and CO_3^- based on ion exchangers and with microscale biosensors for NO_x^-, NO_2^-, CH_4, and volatile fatty acids based on immobilized bacteria.

Introduction

By the introduction of few micrometer-thick microsensors for analysis of a wide range of chemical species in microbial ecology, it became possible to study small-scale chemical gradients and metabolism in stratified microbial communities (e.g., Revsbech and Jørgensen, 1986). It is even possible to analyze gradients and metabolism associated with individual larger microorganisms such as large colorless sulfur bacteria (Schulz and de Beer, 2002). A simple technique allowed for high-resolution determinations of oxygenic photosynthetic rates (Lassen *et al*., 1998; Revsbech and Jørgensen, 1986), and diffusion-reaction modeling of concentration profiles allows for the determination of respiration rates (Berg *et al*., 1998; Epping *et al*., 1999; Revsbech *et al*., 1986). Examples of stratified microbial communities that have been studied extensively using microsensors include hypersaline microbial mats (Canfield and DesMarais, 1991; Grotzschel and de Beer, 2002; Revsbech *et al*., 1983), hot spring microbial mats (Revsbech and Ward, 1984), deep-sea sediments (Glud *et al*., 1994; Gundersen *et al*., 1992; Reimers, 1987), soils (Meyer *et al*., 2002), the termite gut (Schmitt-Wagner and Brune, 1999), and various symbiotic associations such as corals (Kühl *et al*., 1995). Use of microsensors in microbial ecology requires some laboratory infrastructure, but if the sensors are obtained commercially, the infrastructure requirements are low and also low priced as compared to most other sophisticated analytical techniques. It is the author's experience that having an infrastructure of bench experience is much more essential than sophisticated equipment for the successful application of microsensors. It seems to be difficult for most scientists to start thinking in terms of small-scale diffusion gradients and diffusional transport and therefore also to design optimal experiments. For beginners in the field it is thus often worthwhile to visit a laboratory where microsensors are used routinely to learn about fundamental microsensor handling and to discuss experimental design.

It is the intention of this chapter to give the reader an impression about what sensors may be used for by the nonspecialist, and there is therefore relatively little emphasis on new and still experimental techniques that may only function in the hands of the specialist. Several types of microsensors only work for a few days or even shorter, and although some of these sensors have been in use for decades, they may be of limited interest as a routine tool. If the use of such techniques is crucial, it is usually worthwhile

to contact a specialist about joint experiments instead of allocating months to the learning of microsensor construction.

Commercially Available Microsensors and Their Characteristics

The nonfiber optic microsensors that, at present (2005) and to the author's knowledge, are available commercially for use in microbial ecology are listed in Table I, and this section gives a short description of each of them. They are mentioned in the sequence of estimated present use. Not all technical details, such as polarization voltage, are given, but the reader is given an impression about what these sensors may be used for. Technical details are given by the commercial supplier, and even more detail can be found in primary publications and extensive reviews listed later. All of the sensors for chemical analysis listed in Table I exhibit no or almost no sensitivity to flow in the medium if they are constructed correctly. Pressure-compensated versions for use in the deep sea may also be purchased.

Oxygen Microsensor

The oxygen microsensors of interest for microbial ecology are of the micro-Clark design (Clark *et al.*, 1953; Revsbech, 1989) (Fig. 1). For a very detailed description of the functioning of an oxygen microsensor, consult

TABLE I
COMMERCIALLY AVAILABLE MICROSENSORS OF HIGH IMPORTANCE TO MICROBIAL ECOLOGY

Sensor type	Minimum tip diameter	Serious interference	Meter type	Supplier[a]
O_2 micro-Clark	2 μm	—	Ammeter	Diamond, Unisense, AMT
pH glass	10 μm thick 100 μm long	—	Voltmeter	Unisense
pH exchanger	1 μm	—	Voltmeter	Diamond
H_2S micro-Clark	10 μm	—	Ammeter	AMT, Unisense
H_2 micro-Clark	2 μm	H_2S	Ammeter	AMT, Unisense
N_2O, O_2 insensitive	20 μm	H_2S	Ammeter	Unisense
Redox	20 μm	Drifting response	Voltmeter	Unisense
Diffusivity	20 μm	See text	Ammeter	Unisense
Flow	10 μm	See text	Ammeter	Unisense

[a] Diamond: *www.DiamondGeneral.com*; Unisense: *www.Unisense.com*; and AMT: *www.AMT-GMBH.com*.

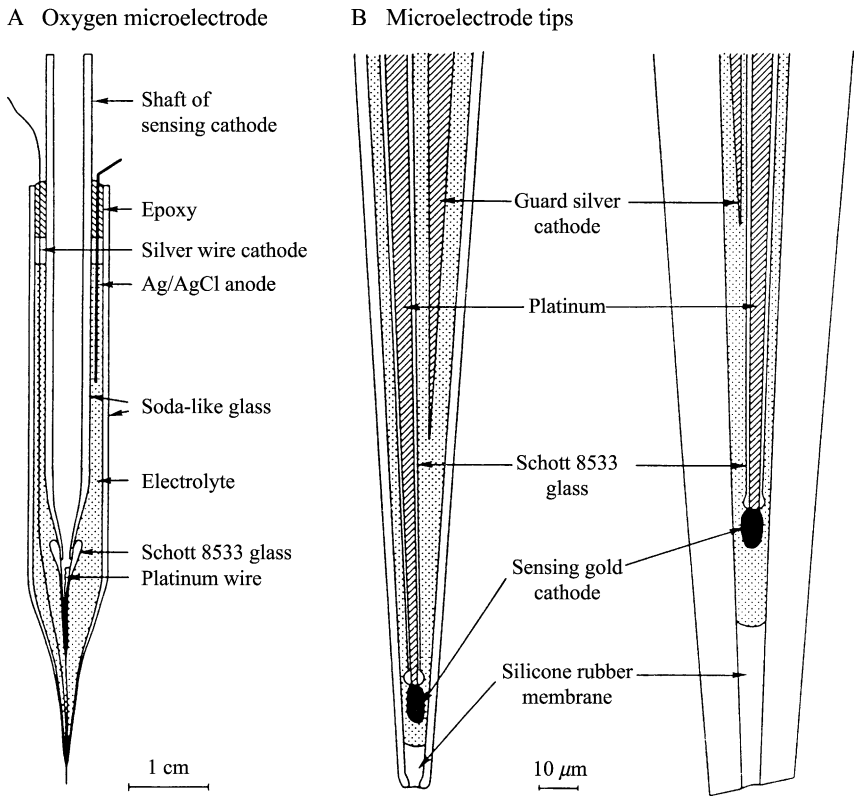

FIG. 1. Oxygen microsensor of the micro-Clark type shown with both a thin tip, which can be used for analysis where minimum sample disturbance is essential, and a sturdy tip for use in media that might otherwise cause sensor damage. The internal guard cathode removes oxygen from the internal electrolyte that might otherwise interfere. From Revsbech (1989), with permission from Limnology and Oceanography.

Glud *et al.* (2000). They can be made with diameters as small as 1–2 μm, but these are fragile (Fig. 1A), or they can be made with very thick glass walls (Fig. 1B) with total diameters up to about 1 mm, in which case they are extremely robust. The oxygen microsensor is at present the microsensor with the overall best performance. It is sensitive with a detection limit <1 μM, fast responding with 90% response times down to 0.1 s, long-term stable with minimal drift during a day, and it does not suffer from pronounced interferences from environmentally relevant chemical species. Due to the availability of very thin sensors, it is possible to perform experiments that cause minimal disturbance to the analyzed medium. The very fast response time makes it possible to record detailed concentration profiles over distances

of millimeters within a few minutes, and it also makes it possible to record detailed photosynthesis profiles by the light/dark shift technique (Lassen *et al.*, 1998; Revsbech and Jørgensen, 1986).

Usually there are no problems with interfering agents. Short-term exposure to low concentrations of hydrogen sulfide has little or no effect, but exposure to high concentrations should be avoided, as the zero current of the sensor may be affected. It is possible to block the entrance of oxygen into the microsensor by gas-impermeable matter, resulting in erratic low readings. Usually, such effects are not observed by analysis in microbial ecology, but problems have been encountered by analysis of animal tissue. Plant waxes may also be very efficient in blocking the sensor tip. Where such physical clogging of the sensor tips takes place it may be advantageous to use oxygen optodes (e.g., Kühl and Revsbech, 2001), which are also described elsewhere in this volume. For most profiling purposes, however, electrochemical oxygen sensors are superior to optodes because of the rapid response and extreme range in total diameter from the very thin and delicate to the thick and extremely sturdy. If the tip of an oxygen microsensor or any other electrochemical gas sensor gets coated with sticky material, it is possible to clean it by deep introduction (using a micromanipulator) into, for example, 4% agar gel, and washing in nonpolar and polar solvents such as hexane and ethanol is also possible. Oxygen microsensors of the Clark type copied from the design of Revsbech (1989) are available from Diamond General (DiamondGeneral.com) and Unisense (Unisense.com). The average lifetime of these oxygen microsensors is about 1 year. There is, however, a pronounced tendency to a change in the calibration curve against lower currents by the aging of oxygen microsensors. Apparently, the membrane gets less and less permeable to oxygen. Some sensors may end up with such a low current (e.g., less than 20 pA difference between anoxic and fully aerobic conditions) that electrical noise starts to be a problem, and they must then be discarded. A similar oxygen microsensor is sold by the German company AMT (AMT-GMBH.com). A limited amount of detail is available about this microsensor, and the author has not been able to get any information from the company about prices on sensors and meters. According to the published material, it has an internal reference element (probably lead in an alkaline electrolyte) that causes a self-polarization of the sensor so that no external polarization source is needed. It should have an operational lifetime of about 2 months.

pH Microsensor

The glass pH microsensor (Fig. 2) is, in principle, identical to larger commercial pH sensors, and except for its small size and need for a higher resistance voltmeter, it also has the same basic characteristics. Due to high

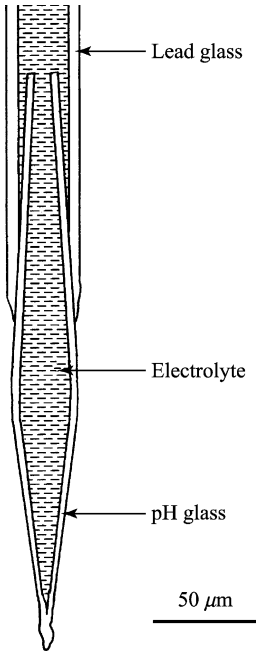

FIG. 2. Tip of glass pH microsensor. The cone made from pH-sensitive (i.e., hydrogen ion exchange) glass will integrate ambient pH over the entire length.

resistance in the circuit of about 10^{10} Ω, the 90% response time is often in the 1-min range when ordinary coaxial shielding of electrical connections is used. Faster responses may be obtained by placing the amplifier in close proximity to the sensor, and presumably also by using a voltmeter with triaxial input wire (Keithley, www.Keithley.com), which alleviates capacity problems associated with the charging of electrical shielding. Because of the very thin glass layers, there is a more pronounced tendency to over-hydration as compared to macroscale pH sensors, causing a lower than Nernstian response and interference from sodium ions. However, a good glass pH microsensor exhibits a response of close to 59 mV per pH unit and very little sodium interference. The minimum dimensions for routinely well-functioning glass pH sensors are, however, diameters of about 10 μm and lengths of exposed pH-sensitive glass of about 100 μm. Such sensors thus integrate pH values over a length of minimum 100 μm, whereas it is possible to get a spatial resolution down in the 1- to 2-μm range by an oxygen microsensor. One problem with microscale glass pH sensors is fragility. It is possible to protect the tip by building the sensors into hypodermic needles (available commercially from Unisense), but this

results in a very slow response. Due to high electrical resistance in a small pH sensor, the measurement may be disturbed easily by external magnetic fields. It is therefore highly recommendable to use coaxially shielded sensors; such shielded sensors are sold by the commercial supplier. The lifetime of microscale glass pH sensors is usually about 6 months, and up to 1 year if some non-Nernstin (i.e., less slopes of less than 59 mV per pH unit) response can be accepted. Very small sensors have a shorter lifetime than larger (e.g., 250-μm exposed tip) sensors due to the very thin pH-sensitive glass layer.

LIX-type pH sensors (LIX sensors are described in more general terms later) may have tips in the 1-μm range, and such sensors should be used when the $\sim$100-μm resolution of glass microsensors is insufficient. Diamond General sells down to 1-μm-thick pH sensors that seem to be LIX based. The shelf-life of these sensors by dry storage is probably about 1 month (Cai and Reimers, 2000), which is far more than for most other LIX-type sensors. It has, however, not been possible for the author to get information about the actual lifetime from the company.

H_2S Sensor

The Clark-type H_2S microsensors (Fig. 3) sold by AMT and Unisense are, in appearance, almost identical to the Clark O_2 microsensor. However, H_2S is oxidized indirectly through a ferri-ferrocyanide shunt (Jeroschewski *et al.*, 1996), and they thus contain an oxidizing measuring electrode (an anode), where the measuring electrode in an O_2 sensor is a cathode. H_2S sensors are usually painted black to avoid destruction of the inner electrolyte by light. Light may also result in erratic readings, as the outermost 1 mm or so will be uncoated. The main characteristics for a H_2S microsensor are almost the same as for the O_2 microsensor, and the detection limit may be <1 μM H_2S. There are apparently no major interfering agents in natural environments. The response is usually linear between 0 and 500–2000 μM, but this should be tested for each sensor. The linear range is affected by sensor age, and linearity should thus be tested repeatedly for the same sensor. The shelf-life is usually about 1 year, but prolonged exposure to high sulfide lowers the lifetime, probably by precipitation of elemental sulfur within the sensor. As the fraction of dissolved sulfide present as H_2S varies with pH, it is necessary to run parallel pH determinations with pH microsensors. A procedure for calibration and a very detailed description of the H_2S sensor in general are found in Kühl and Steuckart (2000). Only about 2% of the dissolved sulfide is found as H_2S at pH 8.5, and determinations of low sulfide concentrations are generally not possible at pH values above 8–8.5.

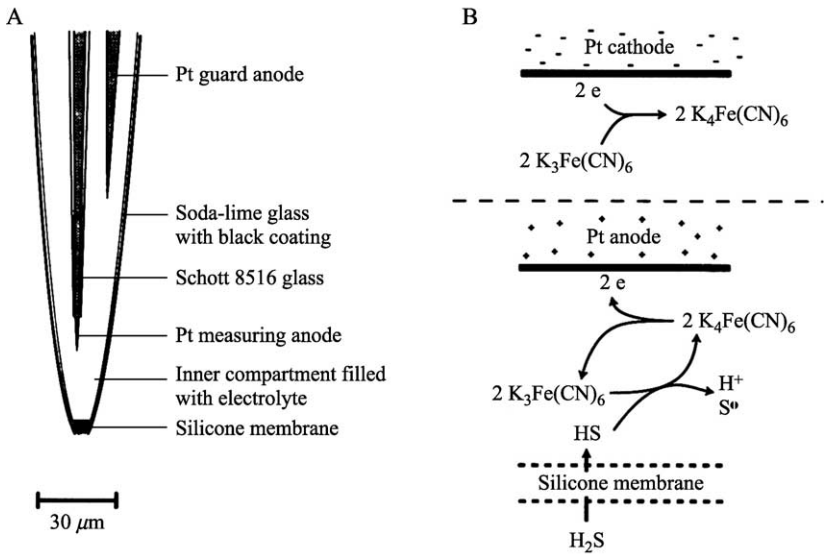

FIG. 3. Tip of H_2S microsensor. Hydrogen sulfide is oxidized by ferricyanide, which is subsequently reoxidized by the platinum anode. An internal guard anode keeps the ferricyanide along the shaft of the measuring anode fully oxidized, although ferrocyanide is produced continuously by the reference cathode (shown to the right). From Kühl *et al.* (1998), with permission from Aquatic Microbial Ecology.

The first environmental sensor determinations of dissolved sulfide were done with Ag/Ag_2S electrodes (Berner, 1963; Kühl and Steuckart, 2000; Revsbech *et al*., 1983). The relatively recently developed H_2S sensor is much more reliable and easier to use and, in contrast to the solid-state Ag/Ag_2S electrodes, it does not suffer from a pronounced O_2 interference observed with Ag/Ag_2S microelectrodes.

H_2 Microsensor

A Clark-type H_2 microsensor was first described by Witty (1991). It was basically identical to the oxygen microsensor, but operated with a platinum anode in an acidic electrolyte instead of a gold cathode in an alkaline electrolyte. These sensors were, however, extremely unstable, and the sensitivity to H_2 could disappear within hours. A new version of a Clark-type H_2 microsensor with a different chemistry has been marketed by Unisense. They do not disclose how it is made, but it has long-term stability. However, like the previously developed H_2 sensors, it is extremely sensitive to H_2S, and there may also be other interfering agents. The detection limit is about 0.2 μM. The lifetime is supposed to be about 1

year. A hydrogen microsensor is also sold by AMT, which should have a similar stability as the one from Unisense. The drift during an operational period of 1 week is thus negligible. The AMT sensor also exhibits a pronounced interference from H_2S.

N_2O Microsensor

A Clark-type, oxygen-insensitive N_2O microsensor was developed by Revsbech *et al*. (1988). It only had a lifetime of a few days, and only a small fraction of the sensors manufactured worked satisfactorily. Lately a long-term stable N_2O sensor chemistry has been developed by Unisense, and this new sensor chemistry has been the basis for a new type of oxygen-insensitive N_2O microsensor (Andersen *et al*. 2001) (Fig. 4). The tip diameter of these oxygen-insensitive sensors may be down to about 20 μm, and the detection limit is <1 μM. The linear range is very large (up to several m*M*), but prolonged exposure of the sensor to only moderately high N_2O may lead to sensor drift. The sensor should thus not be used for long-term monitoring of high N_2O. These sensors are quite slow responding, with 90% response times of about 10–20 s. The most disturbing interference is H_2S, which reversibly inactivates the sensor. N_2O sensors without a front O_2-removing compartment are much simpler to make and are much faster responding and more sensitive to N_2O. Sensitivities approaching 10 n*M* N_2O may be obtained by such sensors, but oxygen should then be absolutely absent. Most experimental conditions do, however, not allow use of such oxygen-sensitive N_2O sensors.

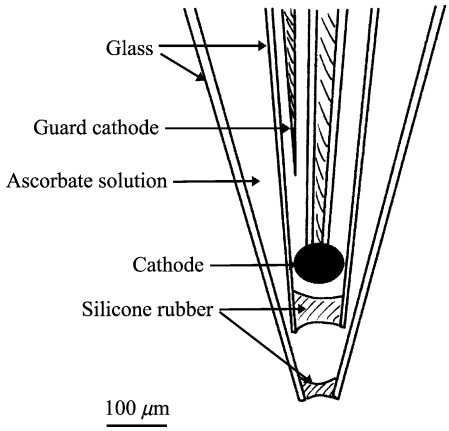

FIG. 4. Tip of oxygen-insensitive N_2O sensor. The front compartment with 1–2 *M* alkaline ascorbate removes all oxygen so that only N_2O reaches the electrochemical N_2O sensor.

Redox Microsensors

Redox potential is, in principle, a well-defined parameter, but it is difficult to perform accurate experimental determinations in microbiological media. This will not be gone into detail here, as these problems have been discussed elsewhere (e.g., Kühl and Revsbech, 2001). However, the reading as such is very simple to perform, and sensors with tips down to 10 μm are available commercially from Unisense.

Diffusivity Sensor

A diffusivity sensor based on the diffusion of a gas away from a sensor tip was described by Revsbech *et al*. (1998) (Fig. 5). A built-in gas sensor monitors the gas concentration near the tip of the sensor, and this concentration is a function of the relative resistance against diffusion away from the sensor tip versus the diffusional resistance inside the sensor. The diffusional resistance inside the sensor is constant, and the signal from the sensor is therefore a simple function of the outside diffusivity. The diffusivity sensors currently sold by Unisense use H_2 as tracer gas, but the author personally prefers the less reactive N_2O. Such sensors may be used to determine diffusivity in biofilms, tissue, sediments, and so on, but

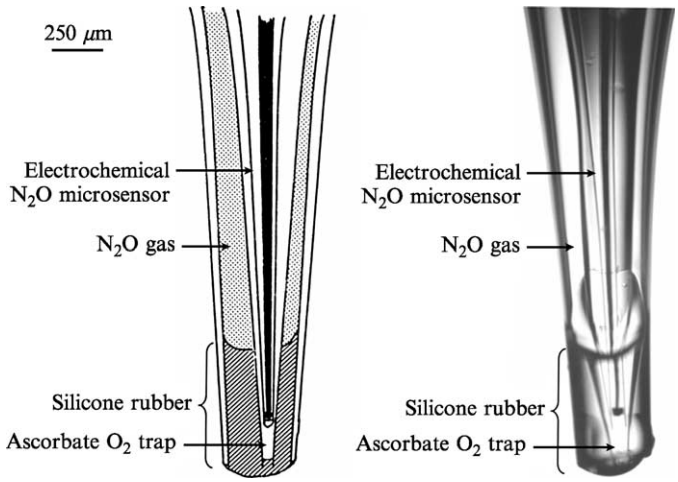

FIG. 5. Tip (drawing and photograph) of diffusivity sensor showing the internal N_2O sensor, the N_2O reservoir, and the thick silicone plug that constitutes the internal diffusional barrier for gas diffusion. The N_2O concentration, and thus the sensor signal, is a function of the quotient between internal and external diffusional barriers. As the internal barrier is constant, the sensor signal is a function of the external diffusivity. The internal oxygen-insensitive N_2O sensor is shown in more detail in Fig. 4.

inhibitors should be added to prevent consumption of the tracer gas in the medium. In the case of H_2, all biological activity should be stopped by addition of a heavy metal salt such as Hg^{2+}, whereas addition of high concentrations of acetylene should be sufficient by use of N_2O. The diffusivity given by the signal of the sensor corresponds to an integrated value close to the sensor tip. The strongest contribution by far comes from the volume within about two sensor diameters from the tip. This also means that there is a limit to how thin diffusivity sensors should be, as a very local measurement may not be representative for the whole sediment (e.g., a measurement between two sand grains with a sensor so thin that the sand grains themselves are not included in the resulting diffusivity estimate). As a rule of thumb, the sensor should have a diameter of at least twice the average grain diameter in the investigated sediment, and preferably three to four times as large. There is, however, a problem with the response time of very thick sensors, as even a sensor with a diameter of only 300 μm needs a response time of about 10 min to approach a stable signal. It is, however, possible to use nonsteady state signals for determination of diffusivity, in which case even sensors of 1-mm diameter may be read after less than 10 min (N.P. Revsbech, unpublished results).

Flow Microsensors

The diffusivity sensors described earlier may also be used as flow sensors, and they can then be made with tip diameters down to about 10 μm when using H_2 as tracer gas (Unisense). With calibration, such sensors may exhibit an extreme sensitivity in the detection of low rates of flow— down to a few micrometers per second. It is, however, not possible to make flow sensors with an ideal symmetrical sensor tip, and flow sensors may therefore show different responses to different directions of flow. Such sensors could, however, be helpful by investigation of flow fields around small objects, such as meiofauna.

General Equipment for Microsensor Analysis

There are three major suppliers of equipment for microsensor use: Diamond General, World Precision Instruments (WPI), and Unisense. AMT sells a combined volt/ammeter for microsensor use. For use of commercially available microsensors in the environment, Unisense offers the most complete package of equipment, whereas WPI offers a wide variety of equipment for microsensor construction and also a variety of meters, micromanipulators, wires, etc. The equipment essential for use of microsensors is basically a few sensors (the first one may break), a meter (for ammeters with a built-in polarization source), a micromanipulator with a sturdy stand, and either a computer with built-in AD converter or

a strip-chart recorder to record data. A dissection microscope on a boom stand (an inexpensive one is sold by WPI) is usually also essential so that the microenvironment in which the microsensor is inserted can be observed. The total cost (2005 prices according to Unisense and Diamond General price lists) of starting to work with microsensors (assuming that a computer with AD converter and a dissection microscope were already present in the laboratory) amounts to about $5000. The more advanced user may prefer to have a motor-controlled micromanipulator and a data acquisition program that can both control the position of the micromanipulator and read the AD converter. The cost of getting started may then increase to about $12,000. The author also advises the advanced user to invest a few hours of work time in the construction of a vibration-protected workplace. The easiest way to do this is to make a table plate of plywood attached to a ca. 5-cm-deep frame that is subsequently filled with concrete. Weighing about 100–150 kg, if the table plate is rested on strips of sturdy foam rubber, most vibrations originating from elevators in the building, outside traffic, and so on will be dampened.

Oxygen microsensors are often used for the determination of photosynthesis in sediments, biofilms, etc. In such work it is necessary to monitor the rate of decrease in oxygen concentration at any particular depth within the first few seconds after darkening. Unisense supplies a version of their data acquisition and micromanipulator control program that can do such operations automatically.

The most common factor causing problems during microsensor analyses is air humidity. In most cases the microsensors sold commercially are sealed so that humidity does not cause problems with short circuits. The user should, however, still be aware that high and variable zero currents from amperometric sensors and calibrations curves exhibiting non-Nernstian responses from potentiometric sensors such as the pH sensors may be due to humidity-caused short circuits. The glass surfaces of the microsensors are especially subject to electrical leaks. At very high air humidities there may also be problems with leaks within the electronics. Problems with static electricity may be a problem at low air humidities, such as those indoors during a cold winter day. The most disturbing effect of static electricity is the puncture of silicone membranes in Clark-type gas sensors. A person can easily get charged with several thousand volts when walking on a synthetic floor covering, and when subsequently touching the equipment, the effect may be a similar potential difference between the sensor interior and the analyzed medium. It is no wonder that about 10 μm of silicone can be punctured by 10,000 V! The best way to avoid ruining sensors by static electricity is to have a ground-connected metal plate under the setup and to touch this plate before touching the equipment. It is also

recommended to insert a ground-connected reference electrode into the analyzed medium.

Many *in situ* measurements can be made with the equipment listed previously. The majority of the work the author has done at the hot springs of Yellowstone has been carried out using standard battery-operated equipment and ordinary humidity-sealed microsensors. The instruments have been enclosed in transparent polyethylene plastic bags together with some silica gel to avoid problems with humidity. By *in situ* work at water depths exceeding about 50 cm it is, however, best to have dedicated underwater equipment. Unisense supplies benthic landers for use with microsensors that can be used at water depths up to about 5000 m, as well as pressure-compensated sensors for use with these landers, In addition to the relatively expensive deep-water equipment with built-in micromanipulation and data acquisition, Unisense also supplies shallow-water profilers that can be used down to water depths of about 50 m. These profilers are relatively simple and need cable connection to an above-water unit. Unisense also provides less expensive equipment for scuba-diver operation.

Microsensor Construction

General Procedures and Equipment

Construction of microsensors for one's own use can, as also mentioned earlier, only be recommended for those users willing to spend considerable time and effort on the project. For most short-term applications of noncommercial sensors, collaboration with one of the laboratories that master the construction would be more productive. A possible exception of this is Liquid Ion eXchanger (LIX) sensors, which are based on commercially available ion exchangers from companies such as Fluka and WPI. The construction of these sensors is quite simple and is described in detail by de Beer (2000). It should be emphasized that new and better ion-exchange compounds continue to be synthesized and that there thus is reason to expect more and better LIX sensors in the future. Good LIXes for Mn^{2+} and Fe^{2+} would, for example, be highly welcome! Table II gives a list of sensors that may be relevant for microbial ecology . An in-depth description of the different types of sensors and also references to primary literature can be found in Kühl and Revsbech (2001), de Beer (2000), Revsbech *et al*. (2000), Glud *et al*. (2000), and Cai and Reimers (2000). In addition, general concepts of metal ion detection by *in situ* voltametry are described in Buffle and Tercier-Waeber (2000).

The equipment necessary for the construction of microsensors is actually not very sophisticated. It was described in some detail by Revsbech and

TABLE II
MICROSENSORS WITH ELECTROCHEMICAL SIGNAL GENERATION OF POTENTIAL INTEREST TO MICROBIAL ECOLOGY[a]

Microsensor type	Minimum tip	Lifetime	Interference	Recent review or primary reference
O_2, Clark	1 μm	1 year	H_2S	Glud *et al.* (2000)
H_2, Clark	1 μm	1 year	H_2S	*www.Unisense.com*, www.AMT-gmbh.com
N_2O, Clark	1 μm	1 year	H_2S	www.unisense.com
N_2O, Clark, O_2 insensitive	20 μm	1 year	H_2S	Andersen *et al.* (2001)
H_2S, Clark	2 μm	1 year	Light	Kühl and Steuckart (2000)
CO_2, Severinghaus	10 μm	Days	H_2S	Cai and Reimers (2000)
pH, glass	10 μm	1 year	No serious	Revsbech and Jørgensen (1986)
pH, LIX	1 μm	Weeks	No serious	de Beer (2000)
NO_2^-, LIX	10 μm	Weeks (dry storage)	H_2S, high Cl^-	de Beer (2000)
NO_3^-, LIX	1 μm	Hours	H_2S, HCO_3^-, Cl^-	de Beer (2000)
CO_3^{2-}, LIX	1 μm	Days	No serious	Choi *et al.* (2002)
NH_4^+, LIX	1 μm	Days	K^+, Na^+	de Beer (2000)
Ca^{2+}, LIX	1 μm	Days	No serious	de Beer (2000)
Redox, bare metal	10 μm	Years	See text	Kühl and Revsbech (2001)
Fe^{2+}, Mn^{2+}, Hg^{2+}, etc., voltametry	100 μm	?	Humic matter	Luther *et al.* (1998)
Diffusivity	20 μm	1 year	See text	Revsbech *et al.* (1998)
Flow	10 μm	1 year	See text	*www.Unisense.com*
Temperature	20 μm	Years	—	www.Unisense.com
NO_2^-, biosensor	20 μm	Days	H_2S, NO, N_2O	Nielsen *et al.* (2004)
NO_x^-, biosensor	20 μm	Days–weeks	H_2S, NO, N_2O	Revsbech *et al.* (2000)
CH_4, biosensor	20 μm	Weeks	H_2, H_2S	Revsbech *et al.* (2000)
VFA, biosensor	20 μm	Days	H_2S, ethanol	Meyer *et al.* (2002)
Glucose, enzyme	10 μm	Days	H_2S	Cronenberg (1991)

[a] For sensor types, ''Clark'' (Clark *et al.*, 1953) designates amperometric gas sensors with a ion-impermeable membrane; ''Severinghaus'' (Severinghaus and Bradley, 1958) designates gas sensors based on pH change in a buffer behind an ion-impermeable membrane; ''LIX'' designates sensors based on a liquid ion exchanger; ''biosensor'' designates sensors based on immobilized microorganisms; and ''enzyme'' designates sensors based on immobilized enzyme. The lifetimes indicated are for microsensors; macroscale analogs of, for example, biosensors and sensors based on ion exchangers may have much longer lifetimes. Sensors with minimum tip diameters are usually less sensitive and/or less durable than somewhat larger sensors.

Jørgensen (1986), where the key steps in construction are also described. A microscope with a 10× objective and also, preferably, a higher magnification (20–40×) long-working distance objective is essential, as is a dissection microscope on a boom stand. The inexpensive microscopes sold by WPI are fine for construction work but should be fitted with a graded ocular. In addition to the micromanipulator essential for analysis with microsensors, one more manually operated micromanipulator should be acquired. The only other essential piece of larger equipment is a variable transformer that can supply an output of 0–20 V and 20 A. In addition to the equipment, materials for microsensor construction should, of course, also be bought: glasses, epoxy cement, platinum and silver wires, etc. Again, this will not be gone into detail, as such information can be obtained from the reviews mentioned earlier and with more detail in the publications describing each specific sensor.

Construction of Microscale Biosensors Based on Immobilized Bacteria

Microscale sensors containing immobilized bacteria offer some possibilities for environmental analysis not offered by other classes of sensors, but due to their short lifetimes of days to weeks, these sensors are not sold commercially. The methane biosensor (Damgaard and Revsbech, 1997) is the only sensor currently available for methane, although the possibility of membrane-inlet mass spectrometry (Lloyd *et al*., 1996) might be investigated as a future alternative. The microscale biosensors for NO_x^- (Larsen *et al*., 1997) and NO_2^- (Nielsen *et al*., 2004) based on the design shown in Fig. 6 allow for sensitive analysis of marine systems. The microscale NO_x^- and NO_2^- biosensors are short-lived, but their macroscale analogs (Unisense) have lifetimes of months. The biosensor for volatile fatty acids (VFAs), published by Meyer *et al*. (2002), is currently the only microsensor for VFAs, which are key compounds in anaerobic degradation, so use of such a sensor may be attractive in many systems.

Construction of the biosensors is based on the same techniques as described by Revsbech and Jørgensen (1986), but for those sensors based on N_2O detection it is probably worthwhile to buy the N_2O sensors from Unisense, as the published N_2O sensors are inferior to those with new sensor chemistry that Unisense has developed. Additionally, the membrane solution for casting ion-permeable membranes should be obtained from Unisense, as the author's own experiences with alternatives has been negative. Although the microscale biosensors based on immobilized bacteria have been developed in the author's laboratory, development has been a difficult learning process. There are two main problems with bacteria-based biosensors: to keep the bacterial population dense and active and for

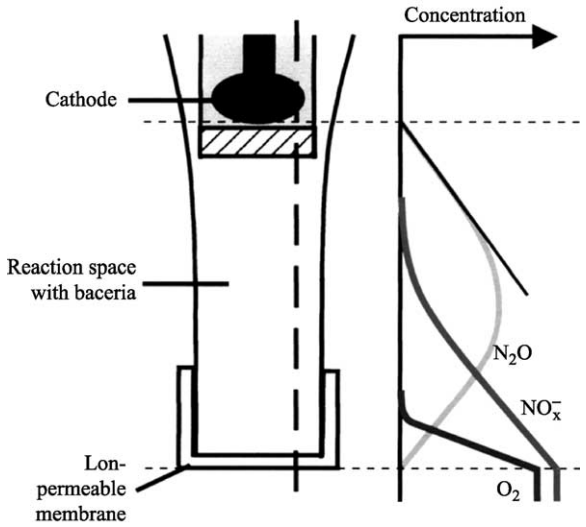

FIG. 6. Microscale biosensor for NO_2^- or NO_x^-. Bacteria in the tip convert incoming NO_2^- or nitrite plus nitrate (NO_x^-) to N_2O, which is subsequently monitored by the internal N_2O microsensor.

the ion sensors to cast ion-permeable membranes that are sufficiently permeable. The first of these problems has been solved by injecting the bacterial biomass obtained by centrifugation or from young agar plate colonies directly into the sensor tips, i.e., introduced via thin glass capillaries, subsequently flushing away excess bacterial biomass with a 0.4% agar growth medium so that only an about 300-μm length of the tip is filled with bacteria. The agar efficiently locks the bacteria in place while at the same time allowing growth of the bacterial population. The membrane is made permeable by resolubilization of the cast membrane in tetrahydrofurane vapor while the inner side of the membrane is in contact with water. This has to be done under the microscope in a humid atmosphere (e.g., in a cold-room), and solubilization has to continue until the membrane almost collapses.

Construction of microscale biosensors so definitely requires specialist instruction. However, the procedures can be demonstrated within a few hours, and new users should, for example, visit the author's laboratory for a day or two to avoid too many frustrations. An illustration of the potential offered by microscale biosensors is shown in Fig. 7, which shows oxygen and NO_x^- profiles analyzed in a diatom-covered sediment during dark and light incubation regimes. Also shown in Fig. 7 are reaction rates calculated

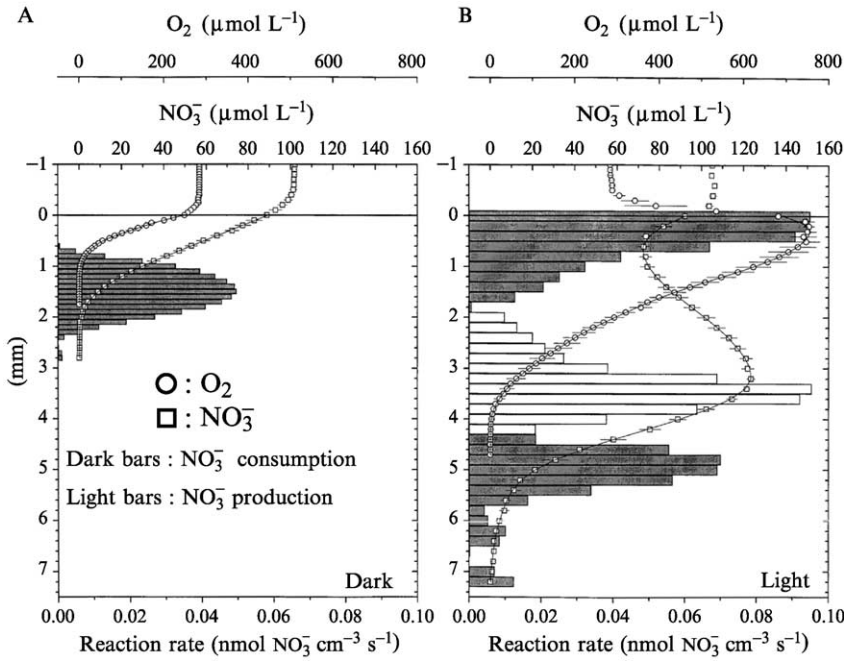

FIG. 7. Profiles of O_2, NO_3^-, and computer-modeled reaction rates in a diatom-covered sediment. The sensor used for analysis was a NO_x^- biosensor, but for simplicity, this is shown as NO_3^-. (A) Data recorded during darkness where the only NO_3^- transforming process was nitrate reduction (presumably denitrification) in the anoxic layers. (B) Data during illumination when algal photosynthesis caused a peak of oxygen in the surface layer and an oxygen penetration down to about 4.3 mm. Nitrate was assimilated in the 0- to 2-mm surface layer, causing a local minimum in NO_3^- concentration, followed by a subsurface peak at the 3-mm depth due to nitrification in the 2- to 4-mm layer. In the anoxic layers below 4.3 mm, NO_3^- was reduced by respiratory processes, and all NO_3^- was depleted at the 6- to 7-mm depth. The slow response of the sensor used (about 1 min for 90% response) causes some inaccuracy in the estimation of exactly where NO_3^- goes to zero.

from the concentration profiles by a diffusion-reaction model. An extensive discussion of similar data and the methods used can be found in Lorenzen *et al.* (1998).

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[10] Optical Microsensors for Analysis of Microbial Communities

By MICHAEL KÜHL

Abstract

Fiber-optic microprobes connected to sensitive light meters are ideal tools to resolve the steep gradients of light intensity and spectral composition that prevail in aggregates and surface-associated microbial communities in sediments, biofilms, and microbial mats. They allow for a detailed mapping of light fields and enable insights to the complex optical properties of such highly light-scattering and -absorbing microbial systems. Used in combination with microsensors for chemical species, fiber-optic irradiance microprobes allow for detailed studies of photosynthesis regulation and of the photobiology of microbial phototrophs in intact samples under ambient microenvironmental conditions of the natural habitat. Fiber-optic microprobes connected to sensitive fluorometers enable microscale fluorescence measurements, which can be used to map (i) diffusivity and flow; (ii) distribution of photosynthetic microbes, via their photopigment autofluorescence; and (iii) activity of oxygenic photosynthesis via variable chlorophyll fluorescence measurements. Furthermore, by immobilizing optical indicator dyes on the end of optical fibers, fiber-optic

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