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Simple sensors that work in diverse natural environments: The micro-Clark sensor and biosensor family

Niels Peter Revsbech

Aarhus University Centre for Water Technology, Department of Biology, Aarhus University, Ny Munkegade 114-116, Aarhus C, 8000, Denmark

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ABSTRACT

In this review the developments made within the area of needle-shaped Clark-type gas sensors are summarized. Emphasis will be on design and functioning of the sensors, but examples of use in various environments are also included. Microscale Clark-type sensors have been constructed for O_2, N_2O, NO, H_2S and CO_2. The tip diameters are often 10−70 μm, but tip diameters can also be considerably larger, and O_2 and H_2S sensors can be made with tips down to < 2 μm. The excellent long term stability conferred by the enclosure of the electrochemistry behind a polymer membrane makes these sensors suited not only for very localized measurement including recording of concentration profiles, but also for long term environmental monitoring. The high level of stability and virtual absence of interferences make the gas microsensors ideal as transducers in biosensors. Biosensors for CH_4, NO_x^- (i.e., $NO_3^- + NO_2^-$), NO_2^-, Volatile Fatty Acids (VFAs), Urea, and SO_4^{2-} have thus been realized.

1. Introduction

The intention with this review is to demonstrate what can be accomplished by utilizing the Clark oxygen sensor design [1] to measure not only oxygen, but also a range of other gasses, and how these sensors can be used as sensing elements in biosensors. What Leland Clark did already in 1956 was to separate the electrochemistry (i.e., cathode, anode and electrolyte) from the external environment with a polymer membrane that is permeable only to small uncharged molecules such as O_2. By this approach most interference and fouling/electrode inactivation problems are avoided, and the sensors can thus be used for long term monitoring. Together with the glass pH electrode, the Clark O_2 sensor has thus become the most commonly used electrochemical sensor for monitoring of aquatic environments.

By constructing the Clark Oxygen sensor in a needle-shaped microscale design (Fig. 1) [2,3] it is possible to perform analysis with a spatial resolution of a few micrometers, and the short diffusion path within such sensors also ensures a very fast response. The general design of the needle-shaped Clark O_2 microsensor has subsequently been utilized to construct microsensors for H_2, H_2S, and NO, and equipped with O_2 traps also for N_2O and CO_2. The small scale makes it possible to have multiple electrodes within a sensor, or even to have a complete sensor within another sensor, thereby enabling simultaneous measurement of two species in the same point. The fast response time and short diffusion distances associated with microsensors have also made it possible to construct biosensors for a range of chemical species, where the gas sensor detects a product of a biological conversion, or, alternatively, the depletion of a reactant.

The sequence of presentation in this review is first description of O_2 microsensors, then sensors that are similar in design to O_2 microsensors (H_2, NO, and H_2S), followed by sensors that are equipped with a trap compartment to remove an interfering agent (microsensors for N_2O and CO_2, and H_2S insensitive H_2 microsensors). Sensors within sensors and biosensors are treated in the last sections. It is not possible to allocate space for details of construction and the interested reader is referred to original articles for such information. In terms of data obtained by use of microsensors a few examples will be mentioned, but it is not possible to give a comprehensive review of sensor use as especially the O_2 and H_2S microsensors have been used for a very large number of studies in multiple research fields. There are several reviews and book chapters describing use of needle-shaped Clark-type microsensors, but except for a review on botanical use [4], all are earlier than 2008 [5–11]. There are several publications on small Clark-type O_2 sensors based on silicon or similar micro-manufacturing technology and designed for implanting into tissue [12,13]. Such sensors will not be discussed further in this review.

2. General considerations when working with microsensors

Work with amperometric microsensors is associated with the

E-mail address: revsbech@biology.au.dk.

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measurement of very low currents, where reproducible resolution of current changes of 10^{-13} A may be essential. With contemporary electronics such resolution is not a problem, and it is not necessary to work in a Faraday cage, even in very noisy electrical environments such as next to the engine of a ship. Instead the sensors must be constructed so that signal conducting parts are efficiently shielded within the sensor. It is a good practice to have coaxial cables inserted deep into the sensors so that the electrolyte surrounds the otherwise exposed signal-conducting parts. Humidity may be a considerable problem as especially glass surfaces become conductive at even moderate air humidity. The solution is either to keep the environment dry (which may create problems with static electricity), or to coat critical glass surfaces with silicones and fill cavities with oil/silicone. We routinely fill the shaft for the sensing cathode/anode (cathode in the case of an O_2 sensor) with low viscosity silicone (Sylgard 184 from Dow Corning) and subsequently insert low noise coaxial cable into the still liquid silicone so that the exposed central conductor makes contact with the metal wire (platinum in Fig. 1) of the cathode/anode. A silver wire is often soldered to the central conductor to optimize the electrical contact with the sensing cathode/anode. Low noise coaxial cables have an additional graphite shielding to minimize electrical noise associated with cable movement, and use of such cables is an absolute requirement. After soldering wires to guard electrode and the counter electrode (Ag/AgCl electrode in Fig. 1), the whole upper part of the sensor may be imbedded into silicone, which also stabilizes the epoxy or UV-curing cement seal shown in Fig. 1. This embedding is usually done within a plastic or glass tube creating a nice handle at the upper end of the sensor. Sensors for use under high pressure should not contain any gas pockets, and for such sensors oil is used instead of silicone (see later). I have seen many examples of delicate sensors being handled with open alligator clip electrical connections or similar, and without appreciating the importance of possible leak currents along humid glass surfaces, resulting in much frustration.

It is essential to use the right glasses. There are enormous differences in the insulating ability and chemical resistance of glasses, and for metal coating they must have a heat extension coefficient close to the one for the metal. Use of poorly insulating glasses for platinum coating such as ordinary soda-lime glass may result in high baseline currents of about 10^{-10} A for O_2 sensors as those shown in Fig. 1, whereas use of the highly insulating glass Schott 8512 results in two orders of magnitude lower baseline. Glasses corrode in a normal laboratory atmosphere and should be acid-washed before use. Properties of various glasses can be found at the Schott homepage: https://www.schott.com/newsfiles/com/20100705105150_Technical_Glasses_final.pdf.

As the tips of needle-shaped microsensors are delicate, it is essential to use micromanipulators for most analyses. Most microsensors are designed to be used for detailed profiling or localized measurements in semi-solid matrices such as sediment and tissue, and they brake if inserted by hand. Several companies sell micromanipulators, and specialized sensor companies also supply software for micromanipulator positioning and data acquisition.

Some microsensors (example: N_2O) are mainly used for bulk liquid analysis and it may seem unnecessary to apply delicate microsensors for such use. By microscale construction it is, however, possible to apply reaction schemes that would not work at a macroscale. It is for example possible to supply reactants and remove products from reactions - such as chemical O_2 reduction within the sensor tip - by diffusion alone. Such O_2 removal has enabled construction of sensors for N_2O and CO_2 as described later, and for N_2O there are no other sensors available. It is

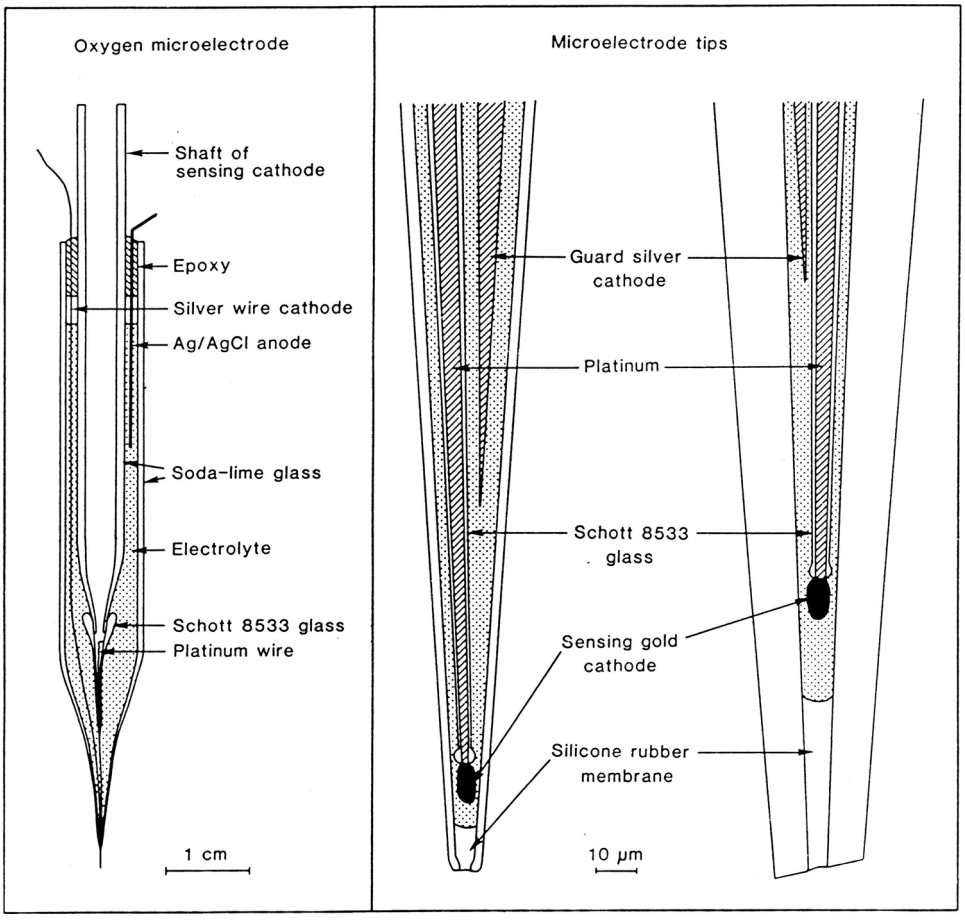

Fig. 1. Clark-type O_2 microsensor. Left: Overview of sensor components. Right: Two alternative geometries of sensor tips with a thin and fast responding and a thicker and slower responding sensor. Reproduced from Limnology and Oceanography [3].

evident that the delicate tip should be protected by a surrounding sleeve when microsensors are used for bulk monitoring in for example wastewater.

3. O_2 microsensors

3.1. Simple Clark-type O_2 microsensors

Two designs of O_2 microsensor tips are shown in the right panel of Fig. 1, with a thin fast-responding version to the left and a relatively robust but slower-responding sensor to the right. The sensors are made by inserting a gold cathode into a glass microcapillary equipped with a silicone membrane in the tip. A silver guard cathode is placed about 100 µm behind the gold cathode to prevent O_2 from the bulk electrolyte reservoir (about 1 mL of 0.5 M HCO_3^- / CO_3^{2-} buffer in 0.5 M KCl) from entering the tip region. Both cathodes are usually polarized at -0.8 V against the internal Ag/AgCl anode shown in the left panel of Fig. 1 [3]. The sensing electrode used in the sensors developed in our laboratory is generally made from glass-insulated platinum [5], where the exposed platinum tip is plated with the desired metal, but for sensors with organic electrolytes it is also possible to use tungsten coated with a suitable glass such as aluminum silicate glass [14]. An alternative to the O_2 microsensor design presented in Fig. 1 is to apply a less noble anode such as Pb or Cd in combination with a strongly alkaline electrolyte [15], and in this case no external polarization is needed. The Clark-type O_2 microsensor marketed by AMT (www.amt-gmbh.com) is apparently such a galvanic cell.

Oxygen microsensors can be constructed so that they have tip diameters down to < 2 µm and response time (t_{90}) of < 0.2 s. Often they are made as a hybrid of the two designs shown in Fig. 1 and have outside diameters of about 10 µm, but with an only 1−2 µm opening for the membrane. By such a design it is possible to make relatively sturdy but still fast responding sensors. The stirring sensitivity should be kept as low as possible, and by 10 µm thick microsensors with a 100 pA signal for 21 % O_2 saturation it is usually $< 2\%$. A small diameter and long distance between exterior and cathode results in low stirring sensitivity, whereas a large diameter and short distance results in high current and high stirring sensitivity. If a sturdy sensor with relatively large diameter is wanted, there should thus be a considerable distance between tip membrane and cathode to lower the stirring sensitivity – but at the expense of slow response. The relationship between sensor signal and sensor geometry has earlier been described in detail [8]. The calibration curve of O_2 microsensors is perfectly linear from zero and up to saturation with pure O_2 gas. The spatial resolution of measurements is the same as the tip diameter. No change in current was thus recorded when a 5 µm tip diameter sensor was advanced at steps of 1 µm towards a glass plate – until the sensor broke by hitting the plate (L.H. Larsen, personal communication). This is in accordance with the usual $< 2\%$ stirring sensitivity, indicating that by far the largest transport resistance towards the cathode is within the sensor. Oxygen microsensors may function for >12 months, but often with a gradual decrease in sensitivity that must be due to lowered membrane permeability. Alternative membrane materials such as Teflon AF have apparently not been tested in needle shaped Clark-type O_2 microsensors, and it would be interesting to see if better long-term stability can be achieved with such alternative membranes.

The construction of needle-shaped Clark-type sensors is largely done by hand, resulting in a wide scatter of characteristics in a batch of constructed sensors. Several attempts have been made to construct micro-scale Clark-type sensors by micro-machining/chip technology [12,13], that could result in cheaper and more reproducible sensors [16], but until now needle shaped sensors with few micrometer tips have not been constructed by such techniques.

3.2. Application of O_2 microsensors

By the introduction of O_2 microsensors in environmental research [17,18] the steep and dynamic O_2 gradients in aquatic environments became documented. These first investigations from 1980 were actually based on simple gold-tipped microelectrodes, that did not have the stability of the micro Clark oxygen sensor that was developed in 1983 [2]. An example of O_2 profiles in a cyanobacterial mat during dark and light incubation [19] illustrating the kind of data that can be obtained by analysis with microsensors is shown in Fig. 2. Also shown in Fig. 2 are distributions of net O_2 production and consumption rates obtained by diffusion-reaction modelling of the experimental data.

The oxygen microsensors have been used in many environmental and physiological studies ranging from in situ measurements at 10,800 m water depth in the Mariana Trench [20] to metabolism of embryos [21]. The microsensor analyses in the sediments of the deep ocean have been particularly interesting, as in situ rates and vertical distribution of metabolism can be calculated from the data. The deep ocean covers about half of our globe, but due to the high hydrostatic pressure and its effect on biological processes we know relatively little about the biogeochemistry at great depth. The best way to obtain reliable data is to measure in situ as enabled by microsensors (Fig. 3), so that problems associated with decompression and temperature changes during sample retrieval can be avoided. Another field where O_2 microsensors have been very useful is in the study of photosynthesis in sediments [22,23], biofilms and algal mats [24], and symbiotic associations like corals and foraminifera [25]. The studies of photosynthesis are often conducted in combination with microsensors for other variables, including sensors for spherical and scalar irradiance [24,26].

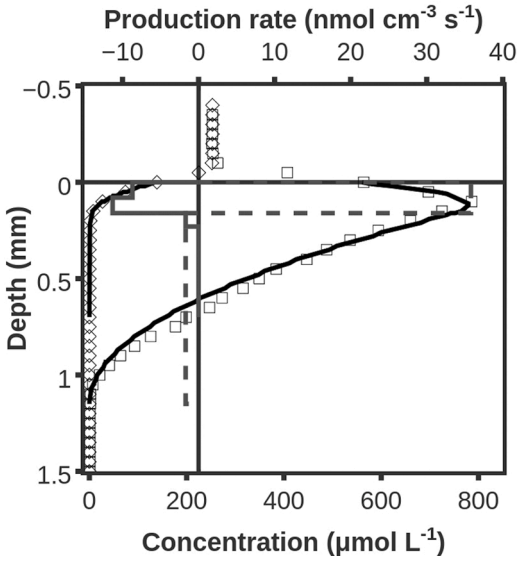

Fig. 2. Depth profiles of O_2 in a cyanobacterial mat during darkness (diamonds) and illumination (squares). The data were inserted in a diffusion-reaction model that calculated the production rates of O_2 (positive or negative) shown with broken line box diagram for illumination, and solid line box diagram for darkness. The theoretical O_2 profiles that would give perfect matches with the calculated rates are shown with solid lines. More variation of reaction rates with depth could have resulted in better curve fit with the experimental data (especially for the profile under illumination), but the applied statistical approach did not allow for such detailed curve-fitting. Notice the diffusive boundary layer above the mat resulting in O_2 gradients extending 0.1 mm into the water. Reproduced from Frontiers in Microbiology [19].

Fig. 3. Left: Recovery of deep sea lander for microprofiling after a completed mission. The electronics cylinder in the lower center of the yellow frame is mounted with an array of microsensors that were introduced stepwise into the sediment. Right: Close-up of electronics cylinder mounted with microsensors. The transparent bulbs are filled with oil that is squeezed into all sensor cavities when the pressure increases. After a 12-24 h long operation at the bottom, the ascent is initiated by release of ballast. Photos by S. Rysgaard & R.N. Glud, with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Ultra sensitive O_2 sensors and their use in oceanic oxygen minimum zones

Until 2007 it was the general perception that the so called Oxygen Minimum Zones (OMZs) in the oceans contained 1−2 μM O_2, as such concentrations were suggested by the available sensor techniques. The OMZs are very important for the global nutrient cycles, which are very much affected by the presence or absence of O_2. A sensor with the ability to detect low nanomolar O_2 concentrations by in situ analysis down to >1000 m depth in the ocean was therefore developed [27,28]. The STOX (Switchable Trace OXygen) sensor functions by having an oxygen sensor inside another oxygen sensor (Fig. 4). When the front cathode is polarized very little O_2 reaches the cathode of the internal O_2 sensor, whereas there is free diffusion to this cathode when the front cathode is depolarized. The calibration of this sensor is not based on an absolute current for zero O_2, but on the amplitude in current during front cathode on-off cycles. There is thus no requirement for a 100 % efficiency of the front cathode in blocking the O_2 entry when polarized. An amplitude in current can be measured at very high resolution, and the best sensors have detection limits about 1 nM – or 3 orders of magnitude better than the conventional techniques used for oceanic in situ analysis before 2007. Interesting outcomes of the work was not only to document

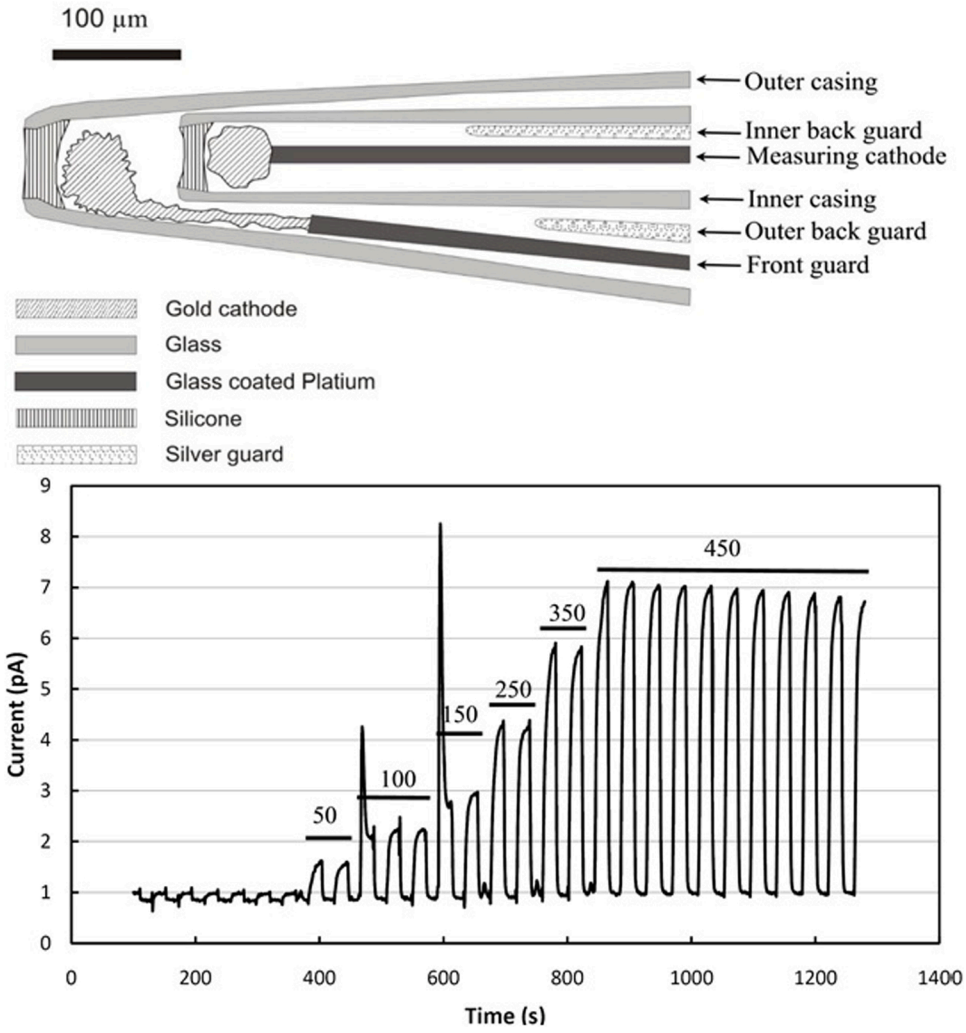

Fig. 4. The STOX sensor. Above: Components of the STOX sensor tip. The illustrated glass coated platinum wire making contact to the front porous gold cathode is now replaced with Teflon coated platinum-iridium, making the sensors more shock resistant. Below: Calibration of sensor in dilute ascorbate with added O_2 concentration in nM shown above sensor trace (initial concentration 10 nM). The amplitude of the signal is proportional to the O_2 concentration. Reproduced from Limnology and Oceanography Methods [27].

absolute anoxia (i.e., < 1 nM O_2) in the OMZs off Chile, Peru and Equador [27,29] but also the finding of substantial O_2 contamination by conventional procedures used for handling of anoxic environmental waters [30], and development of new procedures to measure respiration in water samples at low rates [31]. For laboratory purposes analysis of ultra-low O_2 concentrations is now easier managed by specially designed O_2 optodes [32], but for in situ oceanic analysis the STOX sensors still yield the most reliable data.

4. Microsensors for H_2, NO and H_2S using the basic design of the O_2 microsensor

4.1. H_2 microsensors

The first attempt to use the Clark-microsensor design for analysis of other chemical species was done by Witty [33] who made a H_2 microsensor to study the H_2 formation in legume root nodules. The sensor was based on a platinum anode in an acidic aqueous electrolyte. However, H_2 is only found in low nanomolar concentrations in most aquatic environments, and furthermore interfering H_2S is usually present at high concentrations in the relevant anoxic environments. Successful H_2 analysis with simple H_2 sensors has therefore been limited to very special environments such as legume root nodules [33] and termite gut [34].

4.2. NO microsensors

There is a large interest in NO as signaling molecule in human physiology [35]. However, NO is also an important intermediate in environmental nitrogen metabolism, and the microsensor research group at Max Planck Institute for Marine Microbiology in Germany therefore decided to construct an environmentally useful NO microsensor [36]. In contrast to the other Clark-type gas microsensors described in this review, the sensing electrode (anode) was based on a carbon fiber, and not on platinum or tungsten. The tip diameter of the epoxy- and glass-insulated carbon fiber was 80 µm to give sufficient signal for the expected nanomolar NO concentrations. A layer of a Ni-porphyrin was deposited on the end of the carbon fiber to catalyze the oxidation of NO. The silicone membrane in the 30−50 µm glass tip was only about 10 µm thick to maximize sensitivity.

The NO microsensor had a detection limit of 30 nM, and it has been used to analyze NO profiles in environmental biofilms and sediments [36–38] and dental biofilm [39]. Even in the most active microbial communities (nitrifying biofilms) the concentrations were below 2 µM, and concentrations in sediments were much lower. A complicating factor by use of the NO microsensor is a very high cross-sensitivity to H_2S.

4.3. H_2S microsensors

When a Clark-type amperometric hydrogen sulfide sensor was introduced [6,40] it revolutionized our ability to measure H_2S in the environment (Fig. 5). Until then microscale sulfide analysis was relying on Ag/Ag_2S microelectrodes [5] which exhibited O_2 interference and were difficult to calibrate. The oxidation of sulfide by the internal platinum anode (Fig. 5) is catalyzed by $Fe(CN)_6^{3-}$ that oxidizes H_2S entering through the tip membrane. The $Fe(CN)_6^{4-}$ formed by this process is subsequently re-oxidized by the measuring anode. The counter electrode is a platinum wire, where $Fe(CN)_6^{4-}$ is formed when there is a current flow in the circuit. A platinum guard anode is oxidizing ferrocyanide from the electrolyte reservoir (originating from the cathodic reaction) before it reaches the tip region. For new sensors the recommended polarization of measuring and guard anodes is +0.085 V versus the platinum counter electrode, but this potential should be adjusted when $Fe(CN)_6^{4-}$ accumulates in the electrolyte and changes the redox potential. The accumulation of $Fe(CN)_6^{4-}$ in the electrolyte results in a limited operational lifetime of a few weeks. The ferricyanide based sensors are light sensitive, and even with black coating they are difficult to use in bright sunlight. Application of organic electrolytes has been crucial in the development of for example N_2O and CO_2 microsensors (see later), and it may be possible to find organic electrolytes enabling direct oxidation of H_2S and thereby obtain improved long-term stability. Sensors that work by direct oxidation are actually already commercially available [41].

H_2S microsensors have been used for a large variety of environmental [6,41,42] and technical [43] analyses, and there is also a

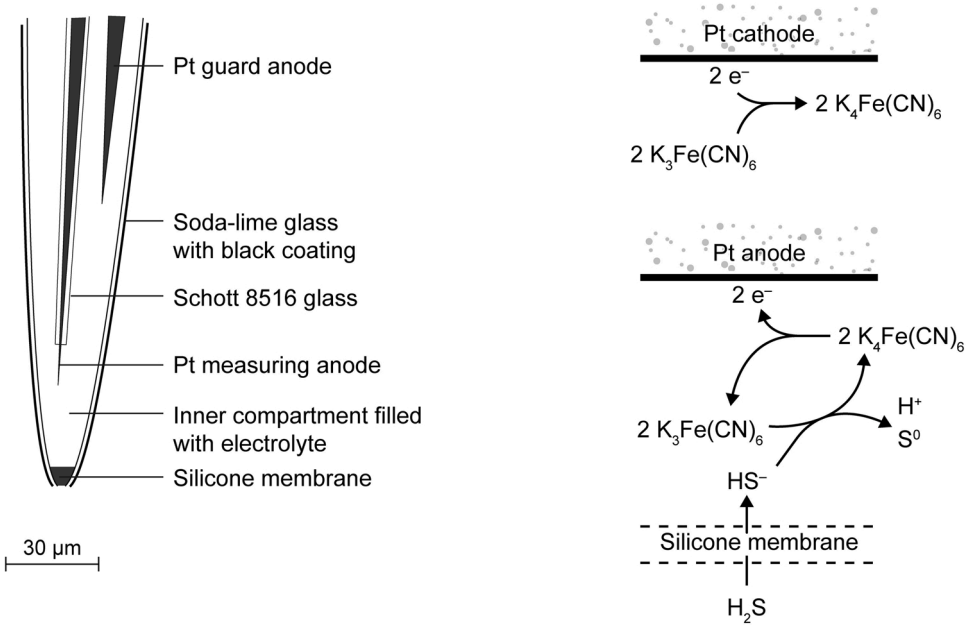

Fig. 5. Microsensor for H_2S. Left. Drawing of microsensor tip. The platinum counter electrode is placed far away from the tip and is not shown. Right: Mechanism of H_2S detection. Ferricyanide is the primary oxidant for H_2S and is reduced at the counter electrode. The measuring anode is reoxidizing the ferrocyanide formed by H_2S oxidation. Reproduced from from Aquatic Microbial Ecology [40] with permission from Inter-Research Science Publisher.

considerable interest in H_2S as a signaling molecule in physiology, where sensor analysis is feasible [44].

5. Microsensors with front trap for interfering species

5.1. N_2O microsensors

Nitrous oxide (N_2O) is a key intermediate in the biological reduction of NO_3^- to N_2 gas, and due to an anthropogenic input of combined N into the environment that is of the same order as the natural input by nitrogen fixation [45], we see increasing levels of N_2O in the atmosphere. N_2O is almost 300 times more potent than CO_2 as a greenhouse gas [46], and it is thus important to understand the N_2O transformations in nature. It is possible to reduce N_2O at various metal cathodes, but at the substantial negative potentials needed, O_2 is also reduced. The problem was solved by placing a porous gold cathode polarized at -1.0 V (against internal Ag/AgCl electrode) in front of a Ag cathode polarized at -1.18 V [47]. Both cathodes and the anode were bathed in a pH 13 electrolyte. The porous gold cathode reduced all O_2, but allowed N_2O to pass on to the Ag cathode. By this approach it was possible to get simultaneous data for O_2 and N_2O in the same point, although the relatively large diameter (about 20 μm) needed to get sufficient N_2O sensitivity resulted in a large stirring artifact on the O_2 signal. The application of this sensor resulted in a much improved understanding of denitrification in sediments [48] and biofilms [49].

The combined O_2/N_2O microsensor was a potent tool for N-cycle research, but it was difficult to make and usually only worked for a few days. A much simpler nitrous oxide sensor has now replaced the combined sensor. It is based on an indium cathode in organic electrolyte, and an O_2-removing compartment [50] is added onto the tip of the Clark-type N_2O transducer (Fig. 6). It produces linear calibration curves over a large concentration range and has a detection limit of about 0.2 μM. The lifetime, even by continuous use, is several months. The sensor has been used for analysis of N_2O production in sediments [36], biofilms [37] and soils [51] and is now used extensively for analysis and control of N_2O formation in wastewater treatment [52].

For the N_2O sensors a 90 % response time of about 30 s is normal. There is, however, an issue with the 100 % response for all sensors based on the architecture shown in Fig. 6 – or rather the 99 % response. For the sensors to work there needs to be diffusional exchange between the bulk

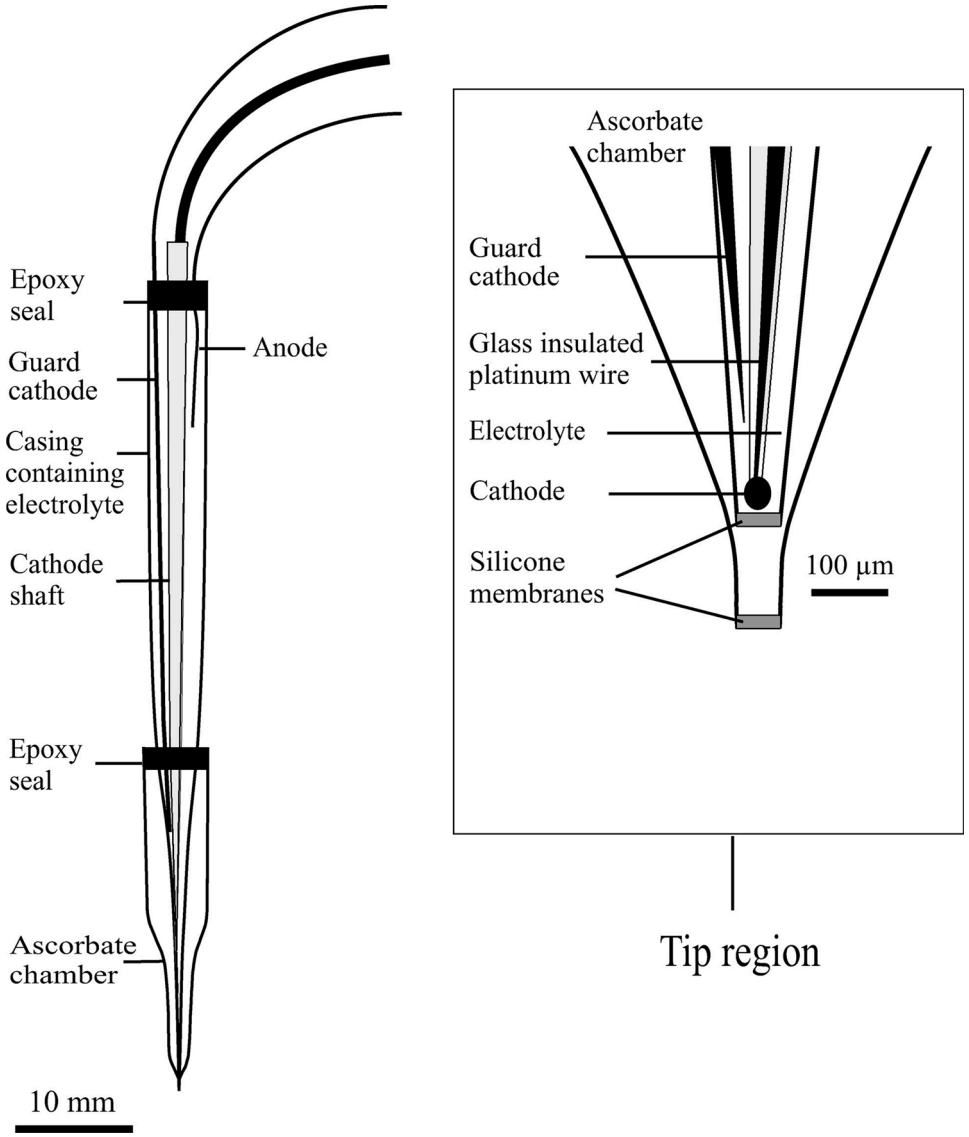

Fig. 6. Components of N_2O microsensor. The same design, but with different cathode material, electrolyte, and with use of acidic Cr^{2+} solution instead of alkaline ascorbate to remove O_2, is used for a CO_2 microsensor. The solution in the outer capillary may also be used to trap H_2S in other gas sensors. Reproduced from Sensors and Actuators B [50].

O_2 (or other chemical species) trap solution and the solution between the two membranes. The applied concentration of the O_2 consuming species is usually around 1 M, so the gap between internal sensor and casing of O_2 trap compartment can be very narrow, but it has to be present! That means that there is also an exchange of analyte between bulk trap solution and the solution in the tip. This constitutes a problem if low concentrations should be quantified immediately after analysis of very high concentrations, and in this case it may be necessary to leave the sensor in analyte free medium for hours to get a sufficiently stable baseline current. It is actually best never to expose sensors designed for analysis of low N_2O concentrations to millimolar levels.

As there is a pronounced interest for N_2O transformations in nature, and as water in equilibrium with the atmosphere only contains 8 nM N_2O, it would be valuable if a sensor with low nanomolar resolution could be constructed. This was accomplished by using the design of the STOX sensor shown in Fig. 4, but with application of indium cathodes and organic electrolytes [53]. For such a sensor to be environmentally useful it was, however, necessary to avoid O_2 interference as with the normal N_2O sensors. Aqueous ascorbate solution could not be applied in the front electrolyte compartment, as the surface of the indium cathode became inactivated after a short time of polarization. It turned out that 1 M diphenylphosphine and 0.3 M tetrabutylammonium iodide (TBAI) in propylene carbonate was a suitable combined electrolyte and O_2 scrubber [54], and detection limits of around 2 nM could be achieved [53]. Use of an organic electrolyte in the front compartment also reduced the transport of H_2O to the sensing cathode and thereby lowered the baseline current caused by H_2O reduction. The aggressiveness of the phosphine towards the membranes led, unfortunately, to rapid sensor deterioration, and already after a week the detection limit had increased significantly. The sensor was used for measurement of N_2O profiles in the Pacific Ocean water column off Mexico [53], but more extensive use would require better long-term stability. Longer lifetimes might be obtained by use of less aggressive O_2 reducing agent or by use of more resistant membranes. Also for the "normal" N_2O sensors it would be an advantage to apply a O_2 reducing compartment based on aprotic solvent, as the following reduction in baseline current would improve the detection limit.

5.2. CO_2 microsensor

The design shown in Fig. 6 has also been used for construction of a carbon dioxide sensor. CO_2 can be reduced at gold or silver cathodes charged at highly negative potentials, but at these potentials both O_2 and H_2O are also reduced. The issue by developing an amperometric CO_2 microsensor was thus to remove O_2 as had been accomplished for the N_2O sensor, and also to suppress H_2O reduction by finding a suitable catalysis to reduce the overpotential so that less negative potentials could be applied. The evident choice was to use an organic electrolyte and also an organic O_2 trap solution, so that only little H_2O would diffuse to the cathode. Until now no suitable organic trap solution has been identified. Diphenylphosphine turned out to be very efficient, but as mentioned above it is also very aggressive towards membranes [54]. Surprisingly, 1 M $CrCl_2$ in 0.1 M HCl turned out to do the job. It was expected that H_2O diffusing through the membrane from such an aqueous solution would result in high currents from H_2O reduction, as such high currents were seen in preliminary tests with TBAI in propylene carbonate as electrolyte. However, an electrolyte consisting of 100 % 1-Ethyl-3-methylimidazolium dicyanamide [14] or 80 % 1-Ethyl-3-methylimidazolium dicyanamide and 20 % dimethylformamide [55] suppressed H_2O reduction while catalyzing CO_2 reduction.

After addition of 20 % dimethylformamide to the electrolyte [55], the CO_2 microsensor has characteristics very similar to the N_2O microsensor. Although calibration curves often have linear regression coefficients of >0.99 in the range of 0–4 kPa, the linearity is somewhat inferior to the perfectly linear ones recorded for N_2O. Many sensors thus exhibit lower sensitivity for CO_2 partial pressures < 100 Pa (the atmosphere partial pressure is 40 Pa). The resolution (and detection limit) at these low concentrations is about 0.5 Pa. There is also lower sensitivity at very high CO_2 concentrations, but it is easy to tailor sensors for high concentration ranges by constricting the tip membrane-filled opening so that less CO_2 enters the sensor [55]. It should be stressed that the CO_2 microsensor is a very recent invention, and that the characteristics most probably are going to be improved. Some sensors have functioned continuously for > 5 months with excellent linearity of response and low baseline currents, while others deteriorate after few days. A key issue for improvement is the adhesion to the glass wall of the applied solvent-resistant Teflon AF membrane in front of the sensing cathode [55]. A membrane-friendly O_2 scrubber solution based on aprotic solvent is very high on the wish-list, as it would substantially lower the flux of H_2O to the sensing cathode and thereby decrease the baseline current.

The CO_2 microsensor can be used for environmental and physiological studies. Until now studies have been performed on carbon fixation in aquatic plants [56], biofilm CO_2 metabolism [57] and root respiration [58]. A very promising field for application of the CO_2 microsensor is as sensing element in decarboxylase-based biosensors. Until now an enzymatic biosensor for urea based on CO_2 detection has been realized [59] as described later.

5.3. H_2S insensitive H_2 sensor

Hydrogen sulfide corrupts just about any sensor-based measurement, but the sensor design shown in Fig. 6 can be utilized to prevent H_2S entry – simply by replacing the O_2 trap solution with a H_2S trap. Various solutions were tested for avoiding H_2S interference on a H_2 microsensor [60]. The most efficient was 0.3 M ferricyanide, as ferricyanide oxidizes H_2S very efficiently. However, alkaline ascorbate (pH 12.5) was also quite efficient, and it simultaneously removed atmospheric O_2 that turned out to have a small interference corresponding to about 1 μM H_2 on the signal of a H_2 microsensor with ferricyanide trap. The O_2 interference is probably due to platinum oxide formation. The perhaps most interesting trap solution was 100 g L^{-1} of $ZnCl_2$ in propylene carbonate. This solution was not very efficient in trapping H_2S, but after addition of a tiny amount of saturated aqueous NaOH solution it had a good trapping efficiency and prevented interference from 5 mM H_2S by 6 h exposure. As the trap contained only little water it resulted in a very low baseline current from the applied H_2 sensor obtained from Unisense A/S (Denmark), that is based on organic electrolyte. The low baseline current resulted in a detection limit of only 20 nM at room temperature. The non-aquatic H_2S trap enabled measurement of detailed H_2 profiles in a 57 °C Yellowstone hot spring microbial mat [61]. Such high temperatures result in excessive baseline currents by sensors without an organic front trap compartment.

6. Sensors within sensors

The STOX sensor described in section 3.3 consists of an oxygen sensor within another oxygen sensor (Fig. 4). By use of the STOX sensor the signal from the front cathode is not collected, as the cathode just serves to intersect O_2 entry to the inner cathode. It is, however, also possible to insert a complete sensor for one chemical species inside a sensor for another species. As mentioned above ferricyanide is a very efficient H_2S oxidizer, and is used in the type of amperometric H_2S microsensor illustrated in Fig. 5. That opens for the possibility of inserting a H_2 sensor inside a ferricyanide-based H_2S sensor (Fig. 7), and thereby obtain a sensor that measures H_2S and H_2 in the same spot [62]. Data from a dark incubated cyanobacterial mat, obtained with such a sensor, is shown in Fig. 8 [19]. The H_2 is formed by fermentation after the surface mat layers change from oxic to anoxic by transition from illumination to darkness (O_2 data from same mat shown in Fig. 2). Instead of a H_2 sensor, an O_2 microsensor could also be inserted inside the H_2S sensor, or a H_2 sensor could be inserted into an O_2 sensor,

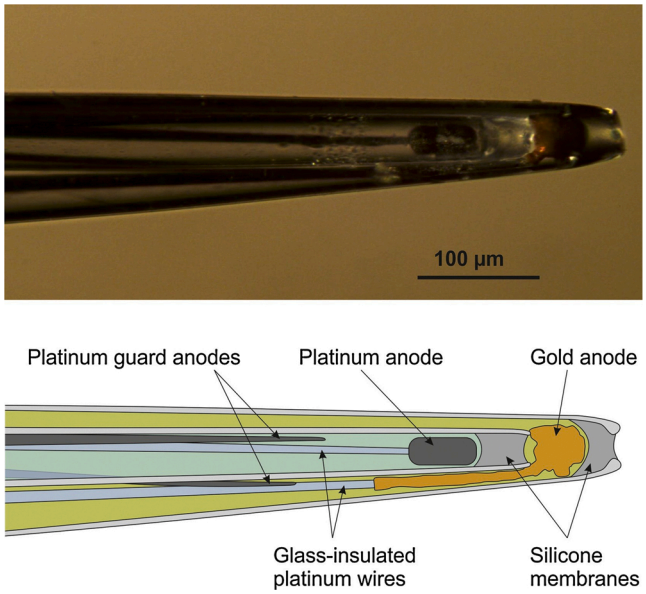

Fig. 7. Combined H_2-H_2S sensor. The outer compartment with the H_2S sensing anode has a ferricyanide-containing electrolyte as also shown in Fig. 5. Note that the platinum anode of the original H_2S sensor [6] is replaced with gold, as H_2 would bind to the surface of platinum. Reproduced from Sensors and Actuators B [62].

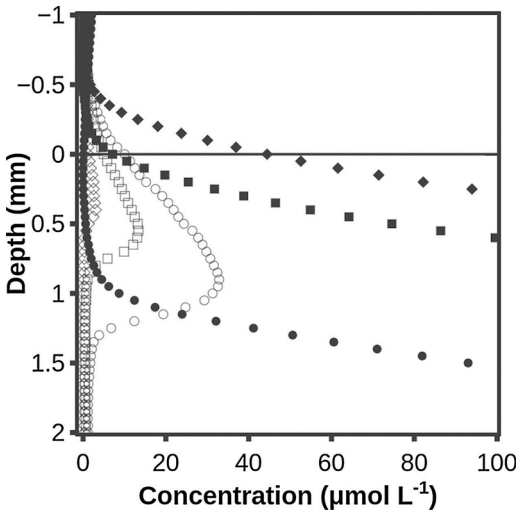

Fig. 8. Profiles of H_2 (open symbols) and H_2S (dark symbols) in a cyanobacterial mat obtained with a combined H_2/H_2S sensor. The circles illustrate data 40 min, squares 140 min and diamonds 160 min after stop of illumination, respectively. Reproduced from Sensors and Actuators B [62].

resulting in signals for both O_2 and H_2 [19].

7. Biosensors based on Clark type microsensors

As demonstrated for the purely electrochemical sensors, it is possible to place a gas microsensor behind a compartment where some chemical or electrochemical reaction is taking place. So why not construct biosensors by placing a compartment with a biologically mediated reaction in front of the gas microsensor? The biological conversion in such biosensors may be mediated by intact cells, but it can also be catalyzed by a purified enzyme. In the following it is described how O_2, N_2O, CO_2, and H_2S microsensors can be used as transducers in biosensors.

7.1. Methane biosensor

It is possible to quantify the concentration of degradable dissolved organics by placing a layer of microorganisms (bacteria or yeast) in front of an oxygen sensor [63,64]. The microorganisms have high respiration rates when the concentration of organic matter is high, and less oxygen will therefore reach the oxygen sensor. Such sensors are marketed as BOD (Biological Oxygen Demand) sensors [63,64], and are primarily used for characterization of wastewater. A needle-shaped microscale BOD sensor based on an oxygen microsensor has also been described [65]. Placing CH_4 oxidizing bacteria in front of the oxygen sensor should thus result in a biosensor for CH_4. The problem with this approach is that CH_4 primarily is produced and found in anoxic environments. The solution to enable measurement in an anoxic environment was to supply O_2 to the methanotropic bacteria from an internal gas reservoir [66]. An oxygen microsensor with its tip fixed at the surface of the membrane to the internal gas reservoir measures a high O_2 concentration when little CH_4 is present, and a low signal at high CH_4, as illustrated with arrows in Fig. 9 [66].

The methane microsensor produces surprisingly reproducible calibration curves, but has an “inverted signal” with high signal for low CH_4 and low signal for high CH_4. The high “zero” signal is affected by changes in temperature and by stirring/diffusivity in the external medium, making it difficult to resolve concentrations below 10 µM. The concentration range with linear response depends on the geometry of the sensor, but it is possible to construct sensors with linear response from zero to CH_4 saturation. The sensor requires regular or constant feeding with CH_4 to keep the culture (*Methylosinus trichosporium*) active, but they can then be used for months.

The methane sensor has been used to characterize methanogensis in sediment [66] and biofilm [67]. As with the other gas sensors it is possible to add an extra compartment in front, for example to trap O_2 so that the sensor can be used in oxic environments, and even in the transition from oxic to anoxic environments [68]. H_2S interferes with the signal [69], and a H_2S trap would thus also be relevant.

In principle it is possible to construct biosensors for other species with the design shown in Fig. 9, just using a dialysis membrane or nuclepore filter in the tip instead of silicone (see description of ammonium biosensor below). The sensor is, however, difficult to construct, and the “inverted” signal is not optimal, although BOD (Biological Oxygen Demand) sensors giving a similar inverted signal have been widely applied in waste water analysis [63,64]. Furthermore heterotrophic microorganisms exhibit slower response in respiration to increases or decreases in substrate supply than the methanotrophic bacteria used for

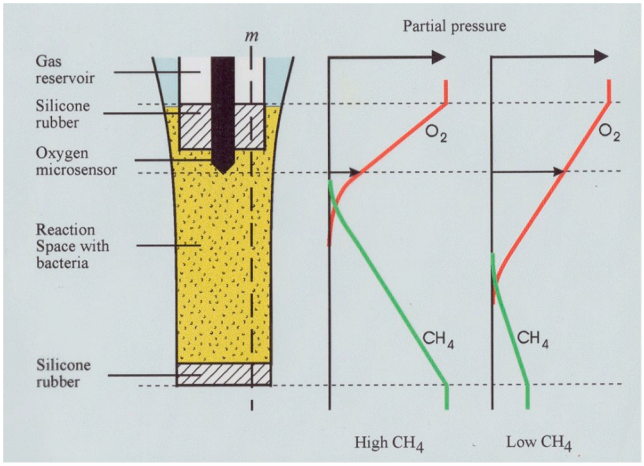

Fig. 9. Biosensor for measurement of methane in anoxic environments. Arrows illustrate magnitude of signal from internal O_2 microsensor at high and low CH_4. Original drawing by L.R. Damgaard, with permission.

the methane sensor (personal observation), where the 95 % response time to changes in CH_4 concentration was about 1 min..

7.2. Biosensors for NO_2^- and NO_x^-

Many bacteria can respire by reducing NO_3^- and NO_2^- to N_2 gas, with N_2O as an intermediate. This so-called denitrification process is a major component of the global nitrogen cycle. Some denitrifying bacteria do not have the enzyme nitrous oxide reductase, and therefore have N_2O as final product. It is thus possible to make a nitrate biosensor (actually a NO_x^- (i.e., NO_3- + NO_2^-) biosensor) by placing such bacteria in front of a N_2O sensor [70]. Some bacteria neither have nitrous oxide reductase nor nitrate reductase, making it possible to construct nitrite biosensors [71]. The function of these sensors is illustrated in Fig. 10.

The bacteria are supplied by diffusion with dissolved organics from a huge (1 mL) reservoir of bacterial growth medium that has access to the tip region through a narrow passage between N_2O micosensor and inner wall of the glass casing housing the bacteria. Macroscale versions (diameter of N_2O sensor tip up to 0.5 mm) may have a polymer (e.g. PET) based bacterial chamber [71]. The amount of nutrients in the medium reservoir should in theory be able to keep the bacteria active for several months, but accidental contamination with bacteria having a N_2O reductase has often limited the lifetime to days or weeks. There is thus still some optimization to be done on these biosensors in terms of lifetime. A problem to be solved is an apparently active migration of bacteria away from the tip region. Immobilization in polymers has until now failed to result in better long-term stability, but application of non-motile bacteria may solve the problem.

The NO_x^- and NO_2^- biosensors produce perfectly linear calibration curves, but the dynamic range depends on factors such as temperature and distance between tip membrane and tip of N_2O sensor. By application of cold-resistant bacteria isolated from a Greenland spring it was possible to measure NO_x^- at 2 °C, but with a rather narrow dynamic range. Such a sensor exhibited a dynamic (linear) range in seawater of 0–130 µM at 4 °C but a range of 0–600 µM at 20 °C [72]. In some environments N_2O may accumulate, and to account for interference from N_2O separate N_2O measurements may be necessary.

The NO_x^- and NO_2^- biosensors have been used for many environmental studies, and they have given new insights into the nitrogen cycle. One unexpected finding was a discrepancy between NO_x^- in a sediment measured by the sensor, and NO_x^- measured by extraction. The finding resulted in the discovery of nitrate accumulation (up to 100 mM) and nitrate respiration by sediment-inhabiting foraminifera [73]. The perhaps most extreme environment where the sensors have been successfully applied was in Sagami Bay off Tokyo at 1450 m water depth [74] where the bottom temperature was only 2.5 °C. The sensors were introduced into the sediment by robotics equipment (Fig. 3) together with microsensors for O_2 and H_2S, so that detailed profiles of all 3 parameters could be obtained. Rates of nitrate production in the 3−10 mm thick oxic layer and nitrate reduction in deeper anoxic layers could be calculated from the data, that showed pronounced variability between sites. The sensors have subsequently been tested at even larger depths, but the combination of low temperature and high hydrostatic pressure was apparently too challenging for the bacteria in the biosensors (R.N. Glud, personal communication).

Detailed microsensor measurement of NO_x^- and NO_2^- may be of high scientific interest, but a potentially much larger field of application is environmental and wastewater monitoring [75]. Unisense A/S (Denmark) was marketing a NO_x^- biosensor for such use, but marketing stopped in 2019 due to sales that were too low for profitable production. The biosensors can yield data of higher quality in for example wastewater treatment plants than alternative methods (Fig. 11), but the present lifetime of the sensors before biochamber replacement is presently too short. There should, however, be a high probability that future modifications can result in much improved lifetimes.

7.3. Electrophoretic sensitivity control (ESC) of NO_x^- and NO_2^- biosensors

When Clark-type gas microsensors are used as sensing elements in biosensors, the biological conversion and the gas sensing occur in two compartments that are electrically insulated from each other. For biosensors like the ones for NO_x^- and NO_2^- it is therefore possible to regulate the transport of ions into the biosensor by applying a charge between a Ag/AgCl electrode inserted into the nutrient medium and an external counter electrode (Fig. 12). The applied potential causes migration of ions in the electric field near the sensor tip, and can cause much enhanced transport as compared to diffusion, or with inverted polarity inhibit transport into the sensor. In the published papers [76,77] on application of the ESC technique a commercial Ag/AgCl reference electrode was used as counter electrode. Reference electrodes may not maintain stable potentials under substantial current loads, but stable currents over hours were actually observed [77] when a constant charge was applied. It would, however, be better to apply a galvanostat to ensure a constant current in the ESC circuit. The application of a

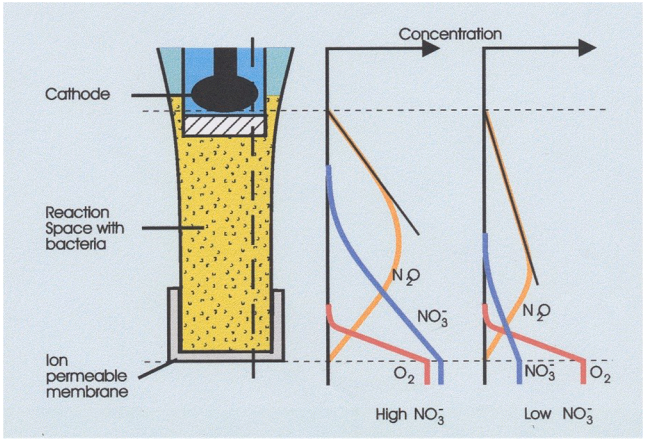

Fig. 10. Schematic representation of NO_x^- and NO_2^- biosensor funtioning. The bacteria are facultative anaerobes and use O_2 until it is depleted. Part of the N_2O produced diffuse into the N_2O sensor (transducer) and result in a current. The black lines illustrate the gradients in partial pressure resulting in N_2O diffusion towards the N_2O reducing cathode. Original drawing by L.R. Damgaard, with permission.

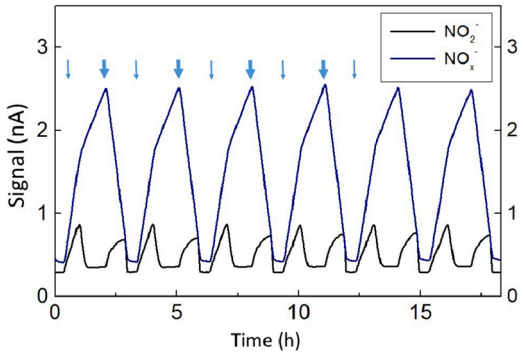

Fig. 11. Raw data for NO_x^- (large peaks) and NO_2^- (small peaks) during aeration/anoxic cycles in a pilot scale wastewater treatment plant fed with synthetic wastewater. NO_x^- is increasing during oxic conditions and decreasing during anoxic conditions. The times of transition to oxic conditions are shown with thin arrows and transitions to anoxic conditions with fat arrows. Nitrite exhibited a more complex dependence of aeration/anoxia. The lowest signals recorded for both sensors are for zero concentration, and the sensitivities were 217 µM/nA for NO_x^- and 89 µM/nA for NO_2^-. Reproduced from T.M. Nielsen et al. 2002 Water Science & Technology 45(4-5) 69-76, with permission from the copyright holders, IWA Publishing.

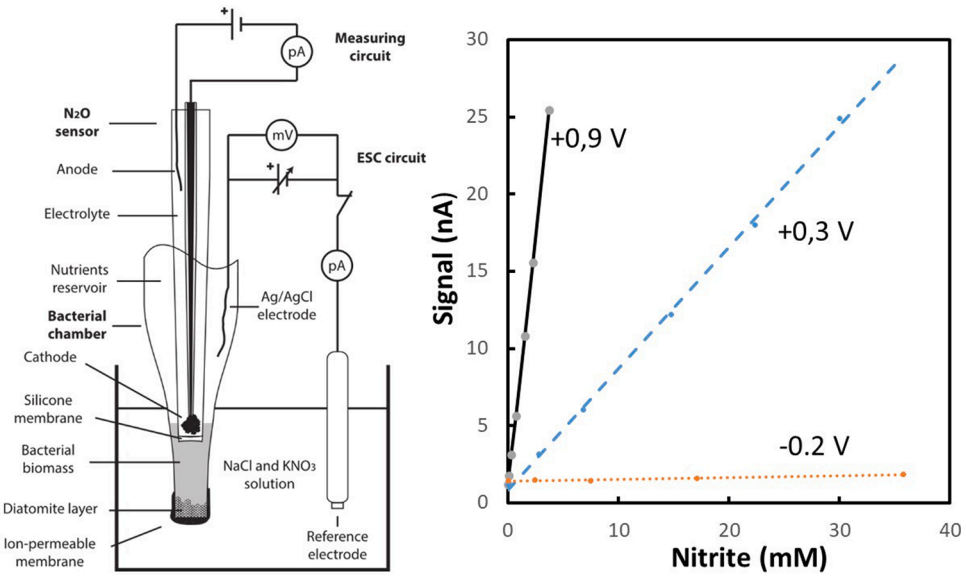

Fig. 12. Illustration of the ESC technique for ion detection by biosensors based on micro-Clark gas sensors. Left: Illustration of ESC circuit (Reproduced from Sensors and Actuators B [77]). Right: Calibration using 3 different ESC potentials. The sensor used for recording the data was not particularly micro, as it was based on a 0.5-mm tip N_2O sensor. Higher sensitivity could be obtained by use of more permeable membranes, and a galvanostat should then be used to assure a constant current in the ESC circuit. Data was obtained from the laboratory of the author.

galvanostat would also enable use of very simple counter electrodes such as coils of chlorinated silver wire.

The calibration curves were linear when ESC polarizations of -0.2 to + 0.9 V were applied (Fig. 12, right). For the recording of the data shown in Fig. 12, a "macro" sensor with 0.5 mm diameter of the membrane-covered tip opening was applied, and the currents are therefore in nA instead of the usual pA currents measured for microsensors. The membrane was a 0.015 µm pore size Nuclepore polycarbonate membrane, which has a low permeability suitable for application of ESC. Similar results have been obtained with 50 µm tip microsensors equipped with casted dialysis membranes [77].

The efficiency of the ESC technique is highly dependent on the ionic strength of the analyzed medium. In the example shown in Fig. 12, water with 10 g/L of NaCl was analyzed. Very low salinities are not suited for ESC application as the signal becomes very sensitive to stirring [77]. There was thus a 55 % decrease in sensitivity by vigorous stirring at 0.1 g/L NaCl [77], whereas the decrease was only 3% at 5 g/L. The effect of ESC is relatively small at very high salinities such as seawater, as the high concentration of Cl^- means that only a very small fraction of the anions transported by migration are NO_3^- or NO_2^-.

The ESC technique has been used routinely for environmental analysis with NO_x^- and NO_2^- microscale biosensors, and it allows the same sensor to be used for very low and very high concentrations [78]. It has not been applied for long term monitoring using macrosensors (as used to record the data in Fig. 12), although it would present advantages such as a possibility for in situ baseline current calibration (by application of negative ESC potentials).

7.4. Other biosensors

It is evidently possible to construct more biosensors based on Clark-type gas microsensors. One realized sensor is a biosensor for Volatile Fatty Acids (VFAs) [79] to be used under anoxic conditions where VFAs often accumulate. It is identical to the NO_x^- biosensor shown in Fig. 10, but with a purely inorganic medium containing NO_3^- in the bacterial compartment. The bacterial respiration is thus dependent on the supply of dissolved organics through the front membrane, and ideally this respiration results in the formation of N_2O. It turned out to be difficult to make sensors that actually worked, probably because the bacteria often stopped NO_3^- reduction at the stage of NO_2^- and did not proceed to N_2O when NO_3^- was available in excess. However, some sensors worked as planned [79]. By application of positive ESC potentials and poorly permeable membranes, the sensors could be made fairly specific for negatively charged organic ions, i.e. VFAs. The relative sensitivity for each VFA (acetate, propionate, isobutyrate) corresponded to the number of electrons liberated by full oxidation of the compounds. The only major organic interference was from lactate (120 % sensitivity compared to acetate) and ethanol (20 % sensitivity), whereas other alcohols, sugars and amino acids showed no or very little interference. Surprisingly there was almost no sensitivity to butyrate.

The VFA microsensors were used for detailed analysis of VFAs in biofilms [79], but no follow up studies were made using this sensor. It would be highly relevant to have such a simple sensor for in situ VFA analysis in anoxic media, as the concentration of VFAs is of high importance in for example biogas reactors and by biological phosphate removal in wastewater treatment plants. A major obstacle to be solved is high and variable baseline currents. The heterotrophic bacteria applied until now do not fully adjust their metabolism to the immediate supply of electron donors, but rely on internal storages when the external supply decreases or stops. It may be possible to use NO_2^- instead of NO_3^- inside the sensor if the internal medium is buffered at sufficiently high pH to prevent interference from free HNO_2 that can pass the silicone membrane.

Ammonium is a key species in the nitrogen cycle, and ion-selective ammonium electrodes are thus marketed by several companies and are widely applied in wastewater treatment [80]. Compensation for K^+ interference is, however, necessary by use of ion-selective ammonium electrodes, and they cannot be used in seawater. An ammonium biosensor based on ammonium-oxidizing bacteria was therefore tested [81]. It was based on the design shown in Fig. 9, but with a dialysis membrane in the tip. Under anoxic conditions and 20 °C, linear calibrations in the 0–300 µM range could be obtained. However, contamination of the bacterial culture inside the sensor resulted in cross-sensitivity to organics, and as also mentioned for the methane sensor, the "inverted signal" gives a low accuracy by determination of low concentrations. It was thus concluded that construction of an ammonium biosensors based on ammonium oxidizing bacteria is possible, but with the tested design it is not suited for general application.

Many biological reactions result in either production or consumption of CO_2, and biosensors can thus be based on use of a CO_2 microsensor as transducer. The first one to be realized is a biosensor for urea, where urease enzyme is placed in front of the CO_2 microsensor [59]. In contrast to earlier described urea biosensors the detection mechanism (i.e., the CO_2 microsensor) is shielded from the analyzed medium and thus not affected by fouling and other inactivation. The sensor thus has better

long-term stability (70 % of initial response after 2 weeks) by continuous use than other published approaches. A convenient method for CO_2 removal prior to analysis needs, however, to be developed before the new urea sensor has any chance of general application. It will also be necessary to invent a technique allowing for replacement of the compartment with enzymes about every second week.

The H_2S sensor can also be used as a transducer for the construction of biosensors. By use of the same general scheme as shown in Fig. 10, but applying a mixture of sulfate reducing bacteria and aerobic heterotrophs, it is possible to measure sulfate (N.P. Revsbech, unpublished data). H_2S will obviously interfere with the SO_4^{2-} measurement, so measurement is limited to relatively oxidized environments.

8. Conclusion

The micro-Clark type gas sensors are excellent for analysis at high spatial resolution, but O_2 and N_2O microsensors are also extensively used for measurement in bulk liquid. A special feature by the microscale construction is that sensors can be equipped with traps for interfering chemical species like O_2 and H_2S, where the reactants in the traps are constantly renewed by diffusional transport. The Clark-type microsensors are ideally suited for application as sensing elements in biosensors where enzymes or cells are placed in front of the sensor, and all reactants are supplied by diffusion. Several types of biosensors based on micro-Clark gas sensors have been realized and some have been used to elucidate processes in nature and in bioreactors. The major factor preventing more extensive use of such biosensors is a limited long term stability of the biological components. Future improvements in microorganism-based sensors may enable general use for environmental monitoring.

Authorship statement

NPR defined the content of the review and wrote the manuscript. The contributions by others in terms of previously published figures and data are fully acknowledged.

Declaration of Competing Interest

Niels Peter Revsbech has part of the ownership of the sensor company Unisense A/S.

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Niels Peter Revsbech received his PhD in microbial ecology in 1981. He became professor of microbiology at Aarhus University in 1997. From 2017–2020 he was leading the Aarhus University Centre for Water Technology. Together with his technicians, students and postdocs he has developed several novel microscale (2–100 μm tip diameter) sensors including biosensors for nitrate + nitrite, nitrite, methane, urea, and volatile fatty acids. He has used these sensors extensively for the study of microbial metabolism in sediments, soils, sea- and wastewater, and biofilms. According to Institute for Scientific Information he has published more than 200 scientific papers that have been cited more than 17.000 times. His work has been recognized with several awards.