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A Microscale Biosensor for Methane Containing Methanotrophic Bacteria and an Internal Oxygen Reservoir

Lars R. Damgaard* and Niels P. Revsbech

Department of Microbial Ecology, University of Aarhus, Bygn. 540 Ny Munkegade, DK-8000 Aarhus C, Denmark

A microscale biosensor for continuous measurement of methane partial pressure based on a novel counterdiffusion principle is presented. Methane-oxidizing bacteria placed in the microsensor utilize oxygen from an internal oxygen reservoir when methane from the exterior diffuses through the tip membrane. The transducer is an internal oxygen microsensor with its tip positioned between the oxygen reservoir and the sensor tip membrane. The external partial pressure of methane determines the rate of bacterial oxygen consumption within the sensor, which in turn is reflected by the signal from the transducer. Tip diameters were down to 20 μm, enabling us to study methane distribution on a microscale. The microscale construction also results in a low stirring sensitivity and a 95% response time down to 20 s. By tailoring the geometry, sensors can be made to exhibit a linear response in the full range of 0−1 atm partial pressure of methane or, alternatively, to exhibit a linear response only at lower concentrations, improving the sensitivity to below 0.1 kPa, corresponding to ∼1 μM in aqueous solution. Temperature, oxygen, and H_2S interfere with the signal; no interferences were detected from H_2, NH_3, CO_2, or acetate.

Methane is a key component of the global carbon cycle. It has a 30-fold higher greenhouse effect per molecule than that of CO_2, and its atmospheric concentration has been increasing by approximately 1% per year.[1] Consequently, the dynamics of methanogenesis and methane oxidation in natural and seminatural ecosystems have attracted much attention. An obstacle in the study of these methane transformations has been a lack of methods for measuring concentrations of methane with sufficient spatial resolution in systems with steep gradients, for instance, the upper millimeters of freshwater sediment. The conventional method has been to transfer slices of the sediment to vessels where the methane dissolved in the pore-water equilibrates with a gas headspace, which subsequently is analyzed by gas chromatography.[2] As this method at best has a vertical resolution of several millimeters, attempts have been made to develop methods for finer scale measurements of pore-water methane.[3] Recently, Benstead and Lloyd[4] used membrane inlet mass spectrometry to determine the distribution of methane in peat cores, and Rothfuss and Conrad[5] also used a membrane-equipped sampling probe for in situ sampling of gases with subsequent gas chromatographic analysis. The spatial resolution of both of these methods was in the millimeter range and had the disadvantage of being very sensitive to stirring and to the diffusional characteristics of the medium, evidenced in the latter case by a 300% increase in signal when methane was measured in stirred water as compared to stagnant water.

A biosensor system for the measurement of methane based on methane-oxidizing bacteria has been described previously,[6] but this system was based on the injection of 40-mL gas samples and could thus not be used continuously or to resolve microscale gradients. Furthermore, as with all previous biosensors based on the microbial reduction of oxygen, oxygen had to be introduced with each sample.

Here we present a microscale biosensor for the continuous measurement of methane, which has excellent characteristics with respect to size, linearity, reproducibility, specificity, and stirring insensitivity, and which does not rely on an external supply of oxygen. With this microsensor, reproducible pore-water profiles of methane were measured with a spatial resolution of 100 μm.

EXPERIMENTAL SECTION

Construction of the Methane Microsensor. The biosensor was assembled from three parts: an oxygen microsensor and two glass capillaries (Figure 1). The oxygen microsensor (described by Revsbech[7]) was very slender, the diameter being less than 10 μm at the tip and not exceeding 30 μm for several hundred micrometers of its length. By means of a gas flame, a dissection microscope, and a heating loop (see Revsbech and Jørgensen[8]), a Pasteur pipet was carefully shaped into a 4-cm-long tapering glass capillary, 6 mm i.d. at one end—the shaft end—and only 15−25 μm at the other end—the tip end. The shape of this capillary, hereafter termed the gas capillary, allowed the oxygen microsensor to fit inside along the entire length of the gas capillary. A similar capillary with a larger shaft end opening, hereafter termed the media capillary, was made. The media capillary was made with dimensions that allowed the gas capillary to fit inside.

The oxygen microsensor was inserted into the gas capillary and carefully manipulated so that its tip was aligned with or slightly protruding through the tip of the gas capillary. In this position, the shaft end of the gas capillary was fixed to the shaft of the oxygen microsensor with a drop of epoxy resin (Super Epoxy,

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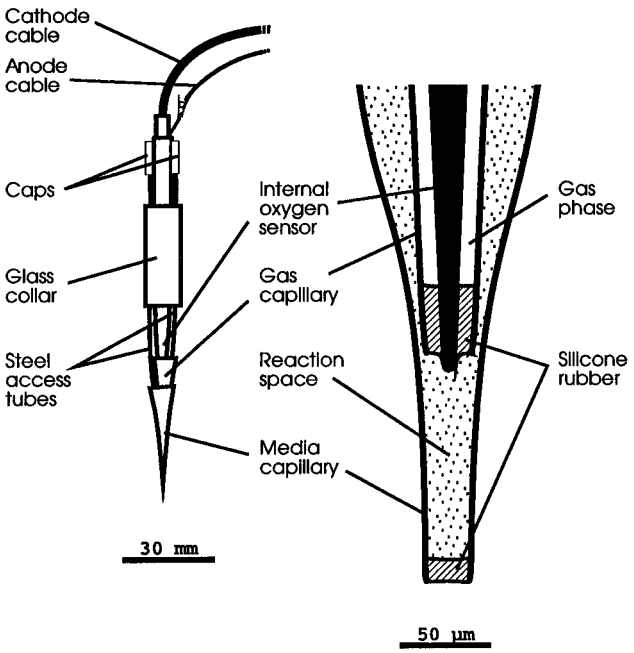

Figure 1. Schematic drawings of a methane microsensor. Left: the entire sensor. Right: section through the tip region.

Hisingeplast AB, Göteborg, Sweden). A drop of uncured silicone rubber (Silicone 732, Dow Corning, Midland, MI) was brought in contact with the tips of the gas capillary and the oxygen microsensor, and the capillary forces were allowed to pull 30−60 μm of silicone into the gas capillary tip. Thus, the silicone rubber formed a membrane penetrated by the oxygen microsensor but otherwise sealing the gas capillary tip. The tip of the media capillary was likewise brought in contact with a drop of uncured silicone rubber, and a 10−20-μm-thick membrane was allowed to close its tip. The combined oxygen microsensor and gas capillary was inserted into the media capillary and positioned so that the distance between the tip of the gas capillary and the tip of the media capillary was 80−300 μm. The space in front of the tip of the gas capillary and behind the silicone rubber membrane of the media capillary is hereafter termed the reaction space (Figure 1). The media capillary was fixed to the gas capillary at the shaft end with a drop of epoxy resin.

Because of the small dimensions, all manipulations were performed under a microscope (320× magnification) by means of micromanipulators.

To ease later access, two steel tubes (i.d. 0.5 mm, 8 cm long) were inserted through the remaining orifice in the shaft end openings of both the media capillary and the gas capillary before the openings were completely sealed with epoxy resin. A glass collar (3−4 cm of a 9-mm-i.d. glass tube) was placed to encompass the shaft of the oxygen microsensor and the middle part of the steel tubes, and the remaining space inside the glass collar was filled with epoxy resin. The purpose of the glass collar was to serve as a physical support that could be fitted into a hole in a rubber stopper during sensor enrichment (see below) and other procedures.

After each use of epoxy resin or silicone rubber, a period of at least 30 min was allowed for curing before further manipulations were done. The complete assembly was left on the shelf for 2−3 days for total curing.

Bacteria. A culture of *Methylosinus trichosporium* OB3b was grown at 30 °C to an optical density of 0.180 (600 nm) in an ammonium mineral salts medium as described by Whittenbury et al.,[9] modified by increasing the concentration of phosphate buffer 4-fold. Cells were harvested by centrifugation (6700*g* for 10 min), and a few hundredths of a microliter of the resulting pellet was injected into the tip of the media capillary through one of the steel access tubes using a 1-mL plastic syringe, of which the tip had been heated and pulled to a capillary. The sensor was subsequently subjected to vacuum to remove air trapped in the media capillary between the tip and the cells. If an air bubble persisted in the reaction space after the vacuum treatment, it was removed by pressurizing the media capillary for a few minutes. Afterward, the media capillary was filled with the above-mentioned medium through one of the steel access tubes, the other allowing the displaced air to escape. The extra-strength buffer was applied to ameliorate steep pH gradients in the narrow reaction space as a result of bacterial metabolism. As an alternative to a pure culture, an enrichment of methanotrophic bacteria could be used for inoculum.

Small pieces of rubber tubing closed with silicone at one end served as caps on the steel access tubes of the media capillary to prevent desiccation. Similarly, small glass tubes with air-tight plugs of dental wax were glued with epoxy resin onto the access steel capillaries of the gas capillary to seal off the gas phase in the gas capillary (Figure 1). This gas phase could be of atmospheric composition, but depending on the desired sensor characteristics, other partial pressures of oxygen could be chosen.

The internal oxygen microsensor, hereafter termed the transducer, was connected to a picoammeter by conventional procedures,[8] and the picoammeter signal was recorded continuously on a strip-chart recorder. Typically, the sensor would exhibit a response to methane immediately after inoculation, but to achieve maximal response, the sensor bacteria were further enriched by exposing them to a constant supply of methane. This was done by mounting the glass collar of the sensor in a hole in a rubber stopper which was fitted in the neck of a glass vessel constantly flushed with methane gas. After approximately 24 h, the signal would stabilize, indicating that the maximal response was achieved.

Calibration. A novel agar profiling method was used for calibration of the methane microsensor (Figure 2). A 5-mm-thick Plexiglas disk with agar-filled 3-mm holes was placed between two cylindrical chambers. The lower chamber was closed except for two openings in its side wall, through one of which it was continuously flushed with humidified methane gas. The gas could escape through the other opening, and the flow was adjusted to a rate high enough to keep other gases out of the lower chamber but too low to create significant pressure gradients that could displace the agar plugs. The top of the upper chamber was a Plexiglas disk with a diameter somewhat larger than that of the chambers and with a central hole. The top disk was not fixed and could thus be moved horizontally. The upper chamber had one opening in its side wall, through which it was continuously flushed with humidified N_2 gas which could escape through the hole in the top disk. Again, the flow rate was high enough to keep other gases out of the upper chamber but too low to displace the agar plugs. After about 1.5 h of flushing, a linear concentration gradient of methane was established through the agar plugs as

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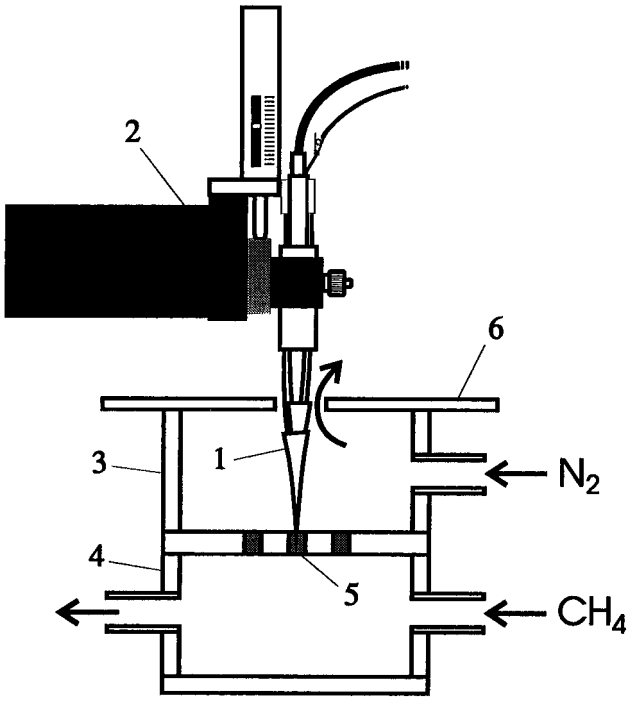

Figure 2. Schematic drawing of calibration setup. 1, Methane microsensor; 2, motor-driven micromanipulator; 3, upper chamber flushed with humidified N_2 gas; 4, lower chamber flushed with humidified methane gas; 5, agar plug in Plexiglas disk separating upper and lower chamber; 6, upper Plexiglas disk with central hole.

no production or consumption of methane occurred in the agar.[10] While the gas flushing was continued in both chambers, a methane microsensor mounted on a motor-driven (Oriel Corp., Stratford, CT) micromanipulator (Märzhäuser Wetzlar GmbH, Wetzlar, Germany) was inserted through the hole in the top disk. Calibration could now be performed by lowering the sensor tip stepwise into an agar plug, recording the signal after each step. The linearity of the concentration gradient through the agar allowed the partial pressure of methane at any point in the agar to be calculated as the ratio between the distance from the upper surface and the total agar plug thickness. As the top disk was not fixed, the sensor could be moved horizontally after retraction, and the calibration could be replicated in a different agar plug.

Alternatively, calibration was performed by exposing the sensor to various partial pressures of methane and logging the signal, but the procedure described here could produce a very detailed calibration curve with small effort.

Stirring Sensitivity Measurements. The sensor tip was placed in stirred water saturated with a certain partial pressure of methane. The signal was logged, and stirring was terminated to get a reading of the signal under nonstirred conditions. This was performed with a range of methane partial pressures from 0 to 1 atm.

Response Time and Sensor Life Span. Response time was measured as the time taken for the sensor to exhibit 95% of the final change in response after being exposed to a different partial pressure of methane. Sensor life span was determined as the age at which a previously functioning sensor would cease to respond to changes in methane partial pressure.

Interference Tests. Five substances relevant to microbial ecology were tested for interference: H_2S, acetic acid, NH_3, CO_2, and H_2. Oxygen-free 1 M stock solutions of Na_2S, sodium acetate, and NH_4Cl were prepared. The sensor tip was placed in an oxygen-free pH buffer, and known amounts of the stock solutions were added. A change in signal was awaited for 5 min. H_2 was tested by reading the signal in water saturated with N_2 gas versus H_2 (saturation with H_2 corresponds to approximately 800 μM[11]). CO_2 was tested by reading the signal in different mixtures of N_2 and CO_2 gas.

Temperature Effect. The effect of temperature on the sensor performance was tested by calibrating a sensor in a water bath at different temperatures from 2 to 35 °C.

Measurements in Sediment and Biofilm. During the summer, undisturbed sediment cores were taken from the eutrophic Lake Wilhelmsborg in 53-mm-diameter Plexiglas tubes. A large methane production was evidenced by vigorous bubbling when the sediment was disturbed, and many gas bubbles in the sediment were visible through the side of the Plexiglas tubes below a depth of approximately 1.5 cm. The sediment was heavily infested with tubificid worms. Biofilm was collected from a small sewage outflow to a creek. The sediment cores and biofilm were brought into the laboratory and incubated in tap water for 24−72 h before measurements were made. The methane microsensor was mounted on a motor-driven micromanipulator together with an oxygen microsensor.[7] The tips of the sensors were positioned in the same vertical position and horizontally spaced approximately 2 mm apart. Both microsensors were connected to picoammeters, and a computer was used to control data logging and sensor positioning.

RESULTS AND DISCUSSION

Functioning of the Methane Microsensor. The methane microsensor principle is based on a counter diffusion principle where methane-oxidizing bacteria in the reaction space consume oxygen from the internal reservoir in the gas capillary when the sensor tip is exposed to methane. The change in oxygen concentration associated with this consumption is reflected by the transducer.

Figure 3 represents a simplified model of the partial pressure profiles of oxygen and methane inside the sensor. At steady state, the fluxes to the zone of reaction of the two compounds must match the stochiometry of the methane oxidation reaction, if the capacity of the bacteria is not exceeded. When the sensor is exposed to high partial pressures of methane, the flux of methane will be higher and the zone of reaction will be located closer to the transducer tip (Figure 3A) than at low partial pressures of methane (Figure 3B), resulting in a lower transducer signal. In the model illustrated in Figure 3, it is assumed that (1) there are equal diffusivities of oxygen and methane in both silicone rubber and media, (2) the system can be considered a one-dimensional diffusion reaction system, (3) the bacteria can concentrate their activity into a very narrow zone, and (4) the oxygen consumption by the transducer is negliglible. Although none of these assumptions are strictly valid, the model demonstrates the basic working principle of the sensor.

Calibration. By varying the geometric dimensions of the sensor and the composition of the gas in the gas capillary, methane microsensors with different characteristics could be produced. As can be seen from the calibration curves in Figure

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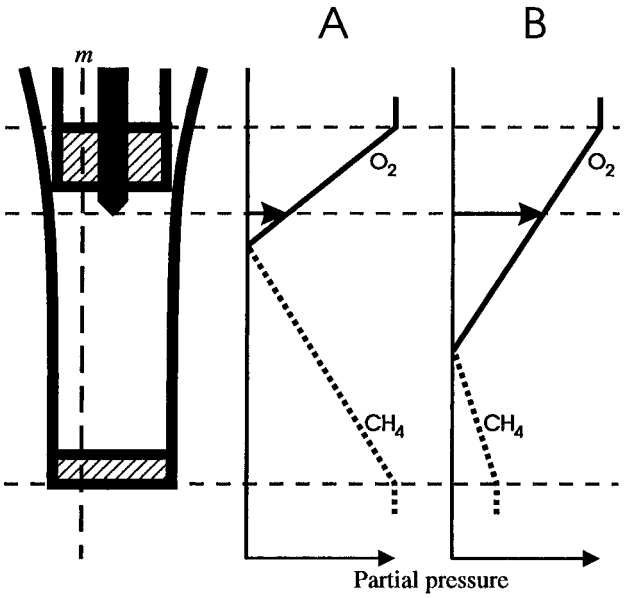

Figure 3. Schematic model of the gradients of oxygen (—) and methane (- - -) partial pressures inside the tip of a methane microsensor along a vertical axis indicated by the line *m*. The partial pressure of oxygen sensed by the transducer in the two situations is indicated by the arrows. (A) The sensor is exposed to a high partial pressure of methane. (B) The sensor is exposed to a low partial pressure of methane.

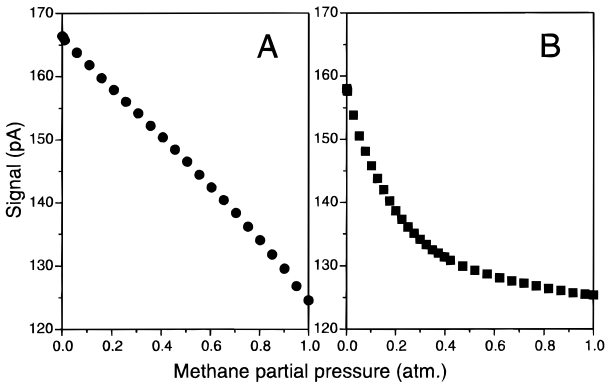

Figure 4. Calibration curves for two different methane microsensors obtained using an agar profiling method (see text). (A) A linearly responding sensor. The linear coefficient of determination, r^2 = 0.9988. (B) A nonlinearly responding sensor.

4A, a sensor could be constructed to exhibit a linear response over the range of 0−1 atm partial pressure of methane (coefficient of determination, $r^2 = 0.9988$). Alternatively, a different geometry allowed for a higher sensitivity to low methane activities at the cost of linearity, as shown in Figure 4B. Such sensitive sensors are constructed with a relatively long distance between the oxygen reservoir and the tip of the transducer.

Many factors influence the size and the range of the signal, so each methane sensor has to be calibrated individually. Figure 5 shows the result of two calibrations of a linearly responding sensor made with an 18-h interval. In this case, calibration was performed by exposing the sensor to different methane gas concentrations and logging the signal with subsequent gas chromatography analysis of the gas. Linear regressions for the two curves are not significantly different (time 0, $r^2 = 0.9966$, slope = −42.87 ± 2.23, intercept = 60.66 ± 0.918; after 18 h, $r^2 = 0.9971$, slope = −43.24 ± 1.75, intercept = 61.18 ± 1.09, confidence level 95%). Thus, sensors are often very stable from day to day, but

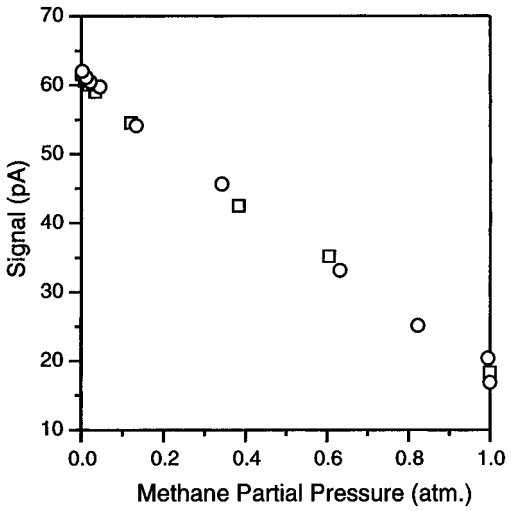

Figure 5. Two calibration curves produced with an 18-h interval. □, Calibration at time 0. ○, Calibration after 18 h.

over longer periods of time some drift may occur, and calibrations should be performed at regular intervals to ensure valid measurements.

Stirring Sensitivity. The stirring effect measured as the change in signal between stirred and unstirred conditions was below 2% at all concentrations. At a methane partial pressure of 0, the stirring effect was about 1% of the sensor signal, corresponding to a maximal error of measurement of approximately 10 μM methane. The reason for a detectable stirring effect even in the absence of methane is that, if not all the oxygen diffusing from the gas capillary is consumed in the reaction space, the oxygen will diffuse out of the tip of the sensor at a rate dependent on the degree of stirring, which will be reflected by the transducer. The overall low stirring sensitivity is stressed by the fact that, for all sensors, the signal is almost continuous at the gas−agar interfaces during agar profiling calibration (see above). Due to the high diffusivity of gases in gas, a gas phase is equivalent to an extremely stirred aqueous medium with respect to stirring sensitivity.

Response Time and Sensor Life Span. Response time relied on sensor geometry, and the 95% response time was in the range of 20−100 s. Maximal sensor life span is at least in the range of months if the sensor is regularly exposed to methane. This long lifetime is due to the fact that the catalytic capacity is maintained in living cells and not just in trapped molecules of methane monooxygenase which rapidly lose activity when isolated from whole cells.[12,13] Sensors that have lost activity have usually done so after being subjected to stressful manipulations (e.g., evaporation of the medium). In several cases, sensor activity resumed when normal conditions were restored.

Interference. Due to the physicochemical properties of the silicone rubber constituting the membrane in the media capillary tip, only small, nonionic molecules can diffuse into the reaction space from the tip. According to the functioning principle of the sensor, oxygen is a major interferent, causing a signal in the opposite direction to the signal caused by methane. It would be possible to measure the signals caused by combinations of oxygen and methane and countercalibrate for oxygen by comeasuring with

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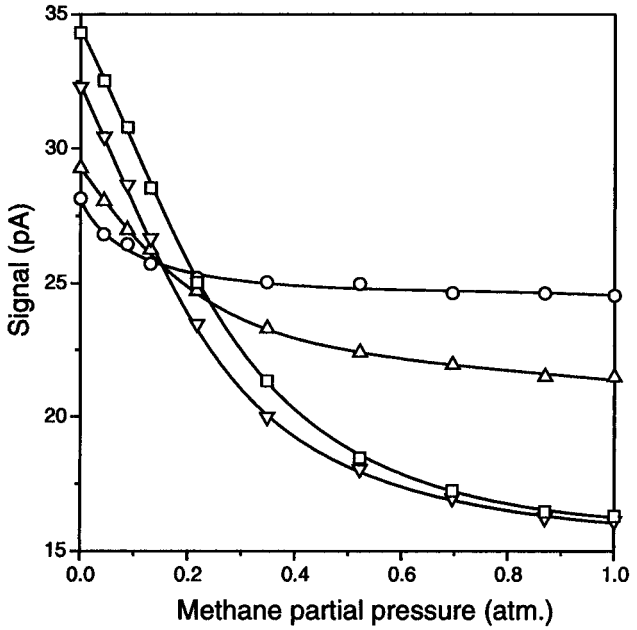

Figure 6. Calibration curves of a single sensor at different temperatures: ○, 10; △, 15; ▽, 20; and □, 30 °C.

an oxygen microsensor. This procedure would, however, introduce some inaccuracies, especially when measuring low methane concentrations. For this reason, in its present design the sensor is mostly useful for measurements in anoxic environments. Other compounds may interfere by increasing or decreasing the oxygen consumption in the reaction space. Of the compounds tested here, only hydrogen sulfide had a detectable interference and decreased the sensor signal at stochiometric concentrations above 100 μM at pH 7. Methane-oxidizing bacteria are not reported to be able to oxidize sulfide, so the interference is probably due to a purely chemical oxidation. Chemical oxidation of sulfide is normally a relatively slow process, but it is possible that particles or cell surfaces in the reaction space catalyzed the reaction. The interference was reversible and not due to a poisoning effect on the transducer, which was made with a large cathode surface to minimize problems with poisoning and signal drift. For H_2S, the sensitivity was 33% of the sensitivity to methane in the presence of no methane and 7% in the presence of 1 atm partial pressure of methane. Methane-oxidizing bacteria have been reported to oxidize NH_3 by several workers,[14] but no interference was detected here, probably due to the extremely low partial pressure of even 1 mM NH_3 in aqueous solution. Carbon dioxide was found to increase the signal of a sensor characterized by an extremely slim transducer. Carbon dioxide causes a decrease in the pH of the transducer electrolyte, which in turn causes an increased electrochemical reduction of electrolyte water by the cathode. In less slim transducers, the diffusive conditions allow the buffered electrolyte (0.5 M bicarbonate buffer, pH 10.3) to effectively neutralize the CO_2 and thus eliminate the problem.

The experiment shows that the methane microsensor is unlikely to respond to interfering agents (except for oxygen) in freshwater sediments, which usually do not contain dissolved hydrogen sulfide in high concentrations, whereas use in anoxic marine systems should be performed with simultaneous monitoring of the hydrogen sulfide concentration. Of compounds other than those mentioned above, especially acetylene is expected to exert a strong interference on the methane microsensor, as acetylene is a "suicide substrate" for methane-oxidizing bacteria.[15]

Temperature Effect. Temperature interferes with the sensor signal by two different mechanisms. First, the diffusivities of methane and oxygen in the sensor components increase with increasing temperature. Second, the methane oxidation capacity of the bacteria increases with temperature until the enzymes begin to denature at high temperatures. The temperature effect on the diffusivities results in an increase with temperature in signal at low partial pressures of methane (Figure 6). The increase was 0.6−2% of the sensor signal per degree Celsius, which is somewhat lower than that reported for oxygen microsensors.[16,17] To minimize this effect relative to the sensitivity to methane, the oxygen concentration in the gas capillary can be lowered, lowering the overall signal, but this may be at the expense of sensor response to high partial pressures of methane due to oxygen limitation. The effect of temperature on the catalytic capacity is seen as an increase with temperature in the range of methane partial pressures which the sensor can respond to. At 2 and 5 °C, there was only a small response to methane in this sensor, and at 35 °C, the sensor ceased to function, probably due to enzyme denaturation and cell death (data not shown). At 10 °C, the sensor responded linearly in the range of 0−0.1 atm partial pressures of methane with no response above 0.2 atm, whereas the sensor responded linearly from 0 to 0.35 atm at 30 °C with some response up to 1 atm (Figure 6). It should be possible to optimize sensors to work at low temperatures by constructing them with a long reaction space whereby the total catalytic capacity is increased or by inoculating with a more psycrophilic organism.

Concentration Profiles from a Freshwater Sediment. Due to the shape of the methane microsensor used for these measurements, the tip could not be horizontally aligned to the tip of an oxygen microsensor closer than 2 mm, and consequently precise vertical alignment of the two sensors relative to the somewhat uneven sediment surface was difficult to achieve. During data processing, the methane concentration profiles were, therefore, vertically adjusted to the corresponding oxygen concentration profiles, aligning the depth of oxygen interference on the methane microsensor with the oxic−anoxic boundary as measured with the oxygen microsensor. The methane concentration profiles in the sediment cores (Figure 7A) have a rightward convex shape from 8 to 15 mm, which would indicate that methane was produced in that zone if the sediment were strictly to be considered a diffusion reaction system. As ebullition is a major transport factor in this sediment, the curvature rather reflects the minimum depth where sediment density and texture allow gas bubbles to be trapped. The concentration profile converges toward methane saturation, which is approximately 1600 μM at the ambient temperature of 18 °C. This corresponded well with gas bubbles visible below that depth. If only diffusion accounted for all transport, N_2 would make up a large part of the gas in the sediment bubbles, but due to ebullition, this gas apparently was flushed out of the sediment. A substantial amount of CO_2 would be expected to be produced along with the methane, but due to the high solubility of CO_2 and the conversion of CO_2 to bicarbon-

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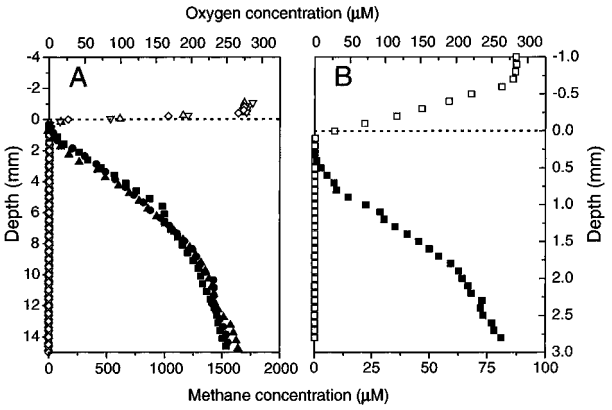

Figure 7. Depth profiles of pore-water concentrations of methane and oxygen. Open symbols, oxygen; closed symbols, methane. Sediment and biofilm surfaces are indicated by dashed lines. (A) Three sets of concentration profiles from sediment from a eutrophic lake. (B) Data from a biofilm collected at a sewage outflow. Note differences in scale for depth and methane concentration.

ate, this production results in a negligible partial pressure contribution. One of the methane concentration profiles in Figure 7A has a vertical section at a depth of 6 mm, which probably indicates that the sensor penetrated a gas bubble with homogeneous gas composition at that depth.

Concentration Profiles from a Sewage Outflow Biofilm. Profiles were measured in 100-μm depth steps, a spatial resolution which is unprecedented for measurements of methane concentration profiles (Figure 7B). Again, the oxygen and methane profiles were measured with electrode tips 2 mm apart, and vertical adjustment of the profiles was performed during data processing using the oxygen interference on the methane sensor as a reference. The rightward convex methane profile from 1.5- to 2.2-mm depth indicates methane production in that zone. The rightward concave methane profile below the oxic zone from 0.4 to 1.2 mm may indicate an anaerobic methane consumption in the biofilm by some unknown mechanism.

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