Use of an Oxygen-Insensitive Microscale Biosensor for Methane To Measure Methane Concentration Profiles in a Rice Paddy

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An oxygen-insensitive microscale biosensor for methane was constructed by furnishing a previously described biosensor with an oxygen guard. The guard consisted of a glass capillary containing heterotrophic bacteria, which consumed oxygen diffusing through the tip membrane, thus preventing it from diffusing into the methane-sensing unit. Oxygen microprofiles were measured through the oxygen guard capillary, demonstrating the principle and limitations of the method. When the tip of the guard capillary was exposed to 100% oxygen at 21°C, heterotrophic oxygen consumption prevented oxygen from diffusing further than 170 μm into the capillary, whereas atmospheric levels of oxygen were consumed within 50 μm . The capacity of the oxygen guard for scavenging oxygen decreased with decreasing temperature, and atmospheric levels of oxygen caused oxygen penetration to 200 μm at 5°C. The sensors could be manufactured with tip diameters as small as 25 μm , and response times were about 1 min at room temperature. Pore water profiles of methane concentrations in a rice paddy soil were measured, and a strong correlation between the depths of oxygen penetration and methane appearance was observed as a function of the light regimen; this finding confirmed the role of microbenthic photosynthesis in limiting methane emissions from surfaces of waterlogged sediments and soils.

Methane is an important intermediate in the global carbon cycle as a major product of the anaerobic breakdown of organic matter. Methane is an increasingly significant greenhouse gas (22); consequently, the detailed study of methane transformations in natural and man-made ecosystems is of great interest.

One method of studying the turnover dynamics of a compound is to measure its spatial distribution in a medium and then to make inferences about process rates for and fluxes of the compound in that medium (26). Methods for studying the spatial distribution of methane in sediments and rice paddy soils have been developed and used by several workers (2, 3, 9, 14, 17), yielding important information. With some of these methods, it has been possible to monitor several compounds simultaneously (3); however, all have suffered from a spatial resolution that is at best limited to 1 to 2 mm, which is not sufficient for studying the distribution of methane in, for instance, oxic surface layers of sediments that are a few millimeters thick. Furthermore, because of the size of the collecting devices and considerable analyte consumption by some of these methods, they have been very sensitive to stirring and to the diffusion characteristics of the medium (e.g., 28). Also, the roots of some aquatic macrophytes are surrounded by a thin oxic zone in which methane may be oxidized. Several experiments have been performed to quantify this methane oxidation activity by measuring whole-plant methane emission in air versus emission under an N_2 atmosphere (e.g., 8, 10, 12); however, this method may overestimate methane oxidation (7) and yields only an integrated measure for the whole root zone, without detailed information about roots of different ages and at different depths.

New information can be obtained about the turnover and transport of methane by studying the spatial distribution of

methane at the microscale level, and a true microsensor for methane is a prerequisite for such studies (25). We previously described a microscale biosensor for methane (6), but it had the disadvantage of being sensitive to oxygen, making it unsuitable for measurements in systems in which oxygen and methane coexist. Here we present an improved microscale biosensor for methane which was made insensitive to oxygen by the addition of an oxygen guard capillary containing heterotrophic bacteria. The sensor was used to measure methane concentration profiles in a rice paddy soil.

MATERIALS AND METHODS

Construction. As described previously (6), the methane biosensor without the oxygen guard was constructed from a slim oxygen microsensor (24) and two glass capillaries, which tapered from a diameter of 7 mm at the shaft ends to approximately 25 μm at the tip ends (Fig. 1). The oxygen microsensor was inserted into one of the capillaries—the gas capillary—and positioned so that its tip was aligned with or slightly protruded from the tip of the gas capillary. The two parts were fixed in that position with a drop of epoxy resin. After curing, a drop of uncured silicone rubber was brought in contact with the two tips. The capillary forces caused the silicone rubber to form a membrane in the gas capillary, penetrated by the oxygen microsensor tip. A silicone rubber membrane was likewise formed in the tip of the other capillary—the medium capillary. The gas capillary-oxygen microsensor assembly was inserted into the medium capillary and positioned to result in a distance of 80 to 300 μm between the tips of the gas capillary and the medium capillary. The medium capillary was fixed to the gas capillary at the shaft end with a drop of epoxy resin. Hereafter, the space between the two tips will be referred to as the reaction space.

The oxygen guard was a glass capillary similar in shape to the gas and medium capillaries, with a tip diameter as small as 25 μm and behind that a wider section, hereafter referred to as the electron donor reservoir. A silicone rubber membrane was formed in its tip as described above. The oxygen microsensor-gas capillary-medium capillary assembly was inserted into the guard capillary, and the tip of the medium capillary was placed 80 to 200 μm from the tip of the guard capillary and fixed in that position with a drop of epoxy resin at the shaft end.

Because of the small dimensions, all of the above-described manipulations were done with a microscope and micromanipulators. To ease handling, two steel tubes (length, 8 cm; inside diameter [i.d.], 0.5 mm) were inserted into the remaining openings in the shaft end of each capillary before these were completely sealed with epoxy resin. These steel tubes served as access channels for subsequent manipulations of the contents of the capillaries. Having two access tubes in each capillary facilitated injections through one tube, the other tube allowing displaced air to escape. A collar made of a 3- to 4-cm piece of 9-mm-i.d. glass tube was placed around the middle part of the oxygen microsensor shaft

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FIG. 1. Methane microsensor. (A) Entire sensor. (B) Transect through the sensor tip region.

and the steel tubes, and the remaining space inside the collar was filled with epoxy resin. This collar served as a physical support, enabling the sensor to be fitted in a hole in a rubber stopper during enrichment and calibration (see below).

After assembly, the sensor was left on a shelf for 2 to 3 days to allow complete curing of the silicone rubber and epoxy resin.

Bacteria. A culture of the methane-oxidizing bacterium *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. (29) but modified by increasing the concentration of phosphate buffer fourfold. Cells were harvested by centrifugation ($6,700 \times g$ for 10 min), and a few hundredths of a microliter of the resulting pellet was injected into the tip of the medium capillary through one of the steel access tubes by use of a 1-ml plastic syringe, the tip of which had been heated and pulled to a capillary. The sensor was subsequently subjected to vacuum treatment to remove air trapped in the medium 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 medium capillary for a few minutes. Then, the medium capillary was filled with the above-mentioned medium through one of the steel access tubes. Extra-strength buffer was applied to diminish 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 the inoculum.

The internal oxygen microsensor, hereafter referred to as the transducer, was connected to a picoammeter by conventional procedures (25); the picoammeter signal was recorded continuously on a strip-chart recorder.

Immediately after inoculation, some response of the sensor was present, but to achieve a full response, the methanotrophic bacteria in the reaction space were allowed to grow to a higher density by flushing a steady stream of methane gas through the empty guard capillary for 24 h.

The guard capillary was inoculated with cells of the heterotrophic bacterium *Agrobacterium radiobacter*, grown on tryptic soy broth agar plates; the capillary was filled with a medium consisting of 10 g of tryptic soy broth and 2 g of Na_2HPO_4 per liter and buffered to pH 7.0. This organism had previously been isolated and shown to have a high capacity for oxygen consumption (18). Enrichment of the heterotrophic cells in the tip of the sensor was performed by exposing the sensor tip to atmospheric levels of oxygen. This enrichment and subsequent treatments, in which a controlled gas phase around the sensor tip was necessary, were performed with an enrichment tube. The latter was a 12-cm glass tube (i.d., 20 mm), open at one end and closed at the other, which was furnished with inlets 2 and 6 cm from the closed end. The sensor tip was inserted through

FIG. 2. Section through the calibration setup. The drawing illustrates the situation after injection of both a calibration gas mixture and a few milliliters of water to force the gas in the rubber tube into the enrichment tube.

a hole in a rubber stopper, making a gastight fit between the sensor glass collar and the stopper. The stopper was placed in the open end of the enrichment tube, which was continuously flushed through one inlet with gas of the relevant composition.

To enrich for the heterotrophs in the guard capillary, the enrichment tube was flushed with atmospheric air. Within 24 h, the transducer signal decreased to a stable level independent of the oxygen content in the enrichment tube, indicating that the oxygen guard prevented oxygen in the enrichment tube from penetrating to the transducer.

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

Calibration. Compared to solutions of nonvolatile compounds, gases are difficult to keep contained due to their ability to diffuse in both gas and liquid phases. For this reason, we describe our calibration setup in some detail.

For calibration, the enrichment setup was modified (Fig. 2). The upper inlet of the enrichment tube was sealed, and a hypodermic needle was inserted through the rubber stopper in such a way that the needle tip penetrated the rubber stopper very close to the glass wall of the enrichment tube. The needle was connected by a rubber tube (20-cm long; 1-mm i.d.) to a septum-covered injection port. The enrichment tube with the sensor was lowered into a water bath used in the experimental setup or a container with water of equal temperature. A 50-ml syringe was used to pull all air out of the enrichment tube through the injection port, the air being replaced by water from the water bath, entering through the lower inlet. Calibration was performed by injecting a known volume of methane gas followed by a known volume of N_2 gas, the two volumes adding up to a total volume sufficient to lower the water level in the enrichment tube to below the sensor tip. This step was followed by injection of a few milliliters of water to force the gas remaining in the rubber tube into the enrichment tube. The sensor signal was monitored on a strip-chart recorder (Fig. 3), and when it had stabilized, it was logged on a computer before the gas mixture was removed through the injection port. This procedure was repeated with a range of mixtures of methane and N_2 gas.

Interference and stirring sensitivity. Oxygen and CO_2 interferences were tested in the calibration setup by exposing the sensor to various mixtures of oxygen or CO_2 in N_2 and monitoring the signal. Stirring sensitivity was investigated by comparing the signal of the sensor immersed in a stagnant water phase to that of the sensor exposed to a gas phase. As the diffusion coefficient for methane in air is about 10^4 times larger than that for the same molecule dissolved in water, a gas phase is equivalent to a very vigorously stirred aqueous phase with respect to stirring sensitivity.

Model guard capillary. To explore the capacity of the oxygen guard, a stand-alone model was made. It was formed in glass as described above with an opening

FIG. 3. Redrawn strip-chart recorder trace from a calibration session. Arrows and numbers indicate point of exposure and partial pressures of methane (atmospheres), respectively, for each new calibration mixture. The inset shows a section enlarged to facilitate estimation of the response time. Due to the procedure for calibration (see the text), the sensor is exposed to methane-free water (0) before it is exposed to each calibration gas mixture. Note the apparent overshoot phenomenon immediately after the injection of gas, which is caused by the injection sequence of the gases, methane being injected before N_2 in this experiment. This sensor responded nonlinearly.

of 6 mm in one end and an opening of 160 μm in the other. The latter opening was closed by a 90- μm -thick silicone membrane. The silicone was allowed to cure for 6 h before a few microliters of a suspension containing approximately 10^7 cells of *A. radiobacter* ml^{-1} was injected into the capillary. Vacuum treatment was applied to remove any air in the capillary tip. About 0.3 ml of oxygen-free tryptic soy broth-phosphate medium (see above) was then injected into the capillary and, to seal off the medium from the atmosphere, 0.1 ml of liquid paraffin was added. The initial absence of oxygen in the medium ensured that conditions for heterotrophic growth in the capillary were most favorable near the tip membrane, through which oxygen could diffuse into the capillary. After incubation of the capillary in 100% O_2 for 3 days, inspection under a microscope revealed that the bacteria had grown in the medium adjacent to the silicone membrane, creating a 5- to 600- μm -long zone with a high cell density.

For measurements, the model guard was placed horizontally under a microscope, and the medium was replaced by new anoxic medium. An oxygen microsensor (5- μm tip diameter) was connected to a picoammeter and mounted on a computer-controlled motor-driven micromanipulator (25). The oxygen microsensor was introduced through the mineral oil film in the shaft end of the model guard, and oxygen partial pressure profiles were measured along the axis of the capillary. Due to its fine dimensions, the oxygen microsensor could penetrate both the bacterial cell mass and the silicone membrane nondestructively. At atmospheric oxygen levels, measurements were done both at room temperature (21°C) and in a 5°C cold room. Furthermore, measurements were done with the capillary tip exposed to 100% oxygen saturation at 21°C.

Measurements on the surface of rice paddy soil. In January 1997, an intact core of water-covered rice paddy soil from a deep-water rice field at the International Rice Research Institute, Los Baños, Philippines, was collected in a Plexiglas tube and brought into the nearby laboratory. The core was placed in tap water at 27°C in a dark-incubated water bath. The water in the water bath was continuously bubbled with atmospheric air, serving the double purpose of providing stirring of the water while saturating it with atmospheric levels of oxygen. To ensure a steady state, the soil core was maintained for 24 h before measurements were performed.

The methane microsensor was mounted on a motor-driven micromanipulator, and methane concentration profiles were made by computer-controlled sensor propagation in depth steps down to 100 μm . Data acquisition was also computer controlled. Oxygen concentration profiles were measured with oxygen microsensors (24) and the same automated procedure. The position of the sensors relative to the surface was determined visually through the sides of the Plexiglas tube by use of a dissection microscope. Replicate concentration profiles were measured in different locations within a soil core.

Before analysis, the soil was illuminated with a halogen lamp for 8 h at an intensity of 2,000 μmol of photons $\text{m}^{-2} \text{s}^{-1}$. After concentration profiles of methane and oxygen were measured in the light, light was excluded by covering the setup with black plastic. After 12 h, measurements of steady-state dark profiles were performed.

Calculations. A steady-state version of Fick's second law of diffusion extended to include rates of consumption (R) and production (P) per volume of pore water

describes the second derivative of the concentration (C) as a function of depth (x) (26):

$$D_s \frac{d^2 C(x)}{dx^2} = R(x) - P(x) \quad (1)$$

where D_s is the effective diffusion coefficient. Nielsen et al. (23) noted that for depth intervals of constant R , P , and D_s , double integration of equation 1 yields

$$C(x) = \frac{R - P}{2D_s} x^2 + bx + c \quad (2)$$

(although Nielsen et al. accidentally omitted the number 2 in the denominator) where b and c are integration constants. Thus, if a parabola can be fitted to such intervals of a concentration profile, the first coefficient of the parabola is equal to $(R - P)/2D_s$. Knowing or assuming a value for D_s thus makes it possible to calculate the net consumption ($R - P$).

If a reference diffusion coefficient is available, diffusion coefficients can be calculated (11) as a function of temperature and salinity by use of the relationship of diffusion coefficient versus temperature and viscosity (19) and the relationship of viscosity versus temperature and salinity (21). With a reference diffusion coefficient of $1.57 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ in pure water at 10°C (5), the oxygen diffusion coefficients in the bacterial cell mass in the model guard capillary were calculated to be $1.2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 5°C and $1.9 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 21°C, assuming the diffusion coefficient in the bacterial cell mass to be 90% of the molecular diffusion coefficient in 12‰ salt water (10 g of tryptic soy broth liter $^{-1}$ plus 2 g of Na_2HPO_4 liter $^{-1}$) and assuming a porosity in the bacterial cell mass of 1. For paddy soil collected in Italy, dried, sieved through a 1-mm pore-size sieve, and reflowed, Rothfuss and Conrad (28) found the porosity to be 0.542 and the effective diffusion coefficient for methane in pore water at 25°C to be $0.51 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$. As diffusion coefficients of gases increase approximately 2.5% per degree Celsius at 25°C (5), the effective diffusion coefficient for methane in paddy soil can be estimated to be $0.54 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 27°C, and the diffusion coefficient for oxygen in paddy soil at 27°C can be estimated to be $0.63 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ by use of the ratio between the diffusion coefficients of oxygen and methane of 1:0.85 (5).

The actual fitting of the parabola to the data was done visually with a computer spreadsheet program. Multiplying the pore water volume-specific rate by the porosity and the thickness of the consumption zone yielded the area-specific rate of consumption.

RESULTS

Methane sensor performance. When the construction of the glass parts and the injection of *M. trichosporium* cells were successful, more than 90% of the sensors achieved a functional response to methane within 24 h after inoculation. When most cells in the medium capillary were deposited behind the reaction space, however, some sensors did not become functional, possibly due to nutrient competition between cells in and behind the reaction space. No effect was seen due to the age of the inoculation culture.

Generally, the methane sensors functioned for at least several days. However, there was great variability, and some sensors have been operational for 6 months when regularly exposed to methane. Exposure to methane for a less than 1 day every week is sufficient to maintain the metabolic apparatus of the cells in the reaction space and to ensure sensor functionality. The variability in life span may be due to different times needed for contaminating organisms to change the chemistry of the reaction space.

Each sensor was handmade, and the resulting geometry of the different parts was variable. Consequently, sensor response with regard to current at zero methane, slope of calibration, and linear response range varied.

The sensors used here did not respond linearly in the full range of 0 to 1 atm of partial pressure of methane. However, they responded linearly in the range of methane concentrations that they were exposed to during measurements (Fig. 4), simplifying calibration calculations. Analytical resolution, as judged by the signal-to-noise ratio, is as low as 1 μM for some sensors, but for the sensors used in the present work, the resolution was about 5 μM . No deviation of the signal was

FIG. 4. Calibration curve for methane microsensor. The linear correlation coefficient r^2 is 0.9998.

detected within the resolution of the sensors as a function of stirring.

Oxygen and CO₂ interference. All sensors with oxygen guards were insensitive to exposure to atmospheric concentrations of oxygen, but when sensors with short oxygen guards were exposed to concentrations of oxygen above the atmospheric level, oxygen interference was seen as an increase in signal.

Carbon dioxide interfered by increasing the transducer current in one sensor. At exposure to 0.1 atm of CO₂ the magnitude of this increase was about 20% of the decrease in trans-

ducer current caused by exposure to 0.1 atm of methane. This sensor was characterized by having an extremely slim oxygen microsensor as the transducer. Sensors with less slim transducers exhibited no or only very little interference from CO₂.

Model guard capillary. The oxygen profiles in the model guard capillary (Fig. 5) showed that at room temperature and at atmospheric oxygen partial pressure, heterotrophic oxygen consumption prevented oxygen from diffusing further than 60 to 70 μm into the capillary; at 5°C, however, the oxygen penetrated to 190 to 200 μm . At 21°C with exposure to 100% oxygen, penetration occurred to approximately 165 μm .

Estimated volume-specific oxygen consumption rates at atmospheric oxygen levels were 190 $\text{nmol cm}^{-3} \text{s}^{-1}$ at 21°C and 20 $\text{nmol cm}^{-3} \text{s}^{-1}$ at 5°C. The difference in rates at 5 and 21°C corresponds to a temperature coefficient (Q_{10}) of 4.1. At 21°C and 100% oxygen saturation, the oxygen concentration was best fitted by dividing the oxidized zone into a zone from 0 to 70 μm with a rate of 65 $\text{nmol cm}^{-3} \text{s}^{-1}$ and a zone from 70 to 160 μm with a rate of 192 $\text{nmol cm}^{-3} \text{s}^{-1}$.

Micropore measurements in rice paddy soil. The methane sensors used for the paddy soil measurements were sensitive to oxygen levels above 150 to 200% atmospheric saturation; due to the high levels of oxygen in the photosynthetic layer during the light treatment, oxygen interfered with the methane measurements in this layer during illumination. Thus, reliable methane measurements could be made only in the parts of the soil which had oxygen concentrations below these levels.

During the light treatment, oxygen penetration was 5.3 ± 0.9 mm (mean $\pm$ standard deviation; $n = 3$), while methane was detected below a depth of 4.0 ± 0.8 mm. The area-specific oxygen consumption rate below the photosynthetic zone was 0.69 ± 0.31 $\text{mmol m}^{-2} \text{h}^{-1}$; the area-specific methane consumption rate was 0.043 ± 0.011 $\text{mmol m}^{-2} \text{h}^{-1}$. Representative concentration profiles during illumination are shown in Fig. 6A. In the dark, oxygen penetrated only 0.8 ± 0.4 mm, while methane was detected below 0.2 ± 0.4 mm. The area-specific consumption rates were 0.36 ± 0.10 $\text{mmol m}^{-2} \text{h}^{-1}$ for

FIG. 5. Oxygen partial pressure profiles through a model guard capillary. Symbols: $\times$, tip exposed to atmospheric oxygen level at 21°C; $*$, tip exposed to atmospheric oxygen level at 5°C; $+$, tip exposed to 100% oxygen at 21°C. The width-to-height ratio of the schematic drawing of the capillary tip is smaller than that for the actual capillary used in the experiment.

FIG. 6. Pore water concentration profiles for oxygen (□) and methane (■) in rice paddy soil after 8 h of incubation in the light (A) or after 12 h of incubation in the dark (B). Note that methane and oxygen profiles were not measured at exactly the same locations, so the exact extent of overlap between oxygen and methane cannot be determined.

oxygen and $0.031 \pm 0.007 \text{ mmol m}^{-2} \text{ h}^{-1}$ for methane. Representative concentration profiles in the dark are shown in Fig. 6B. The thicknesses of the methane oxidation zone (estimated as the nonlinear parts of the methane concentration profiles) were not significantly different in the light ($1.3 \pm 1.1 \text{ mm}$) and in the dark ($0.8 \pm 0.4 \text{ mm}$). Below the oxic zone, methane concentration profiles were linear both during illumination and in the dark.

When methane microsensors without oxygen guards were used, such an overlap of oxygen- and methane-containing layers resulted in a continuous signal ranging from above the zero-methane level in the overlying water, decreasing with depth with decreasing oxygen concentration, to below the zero-methane level with increasing methane concentration. Thus, for a sensor without an oxygen guard, it is impossible to distinguish the effect of oxygen from the effect of methane in the overlap zone.

DISCUSSION

Functioning of the sensor and oxygen guard. The sensor response to methane is produced by the methane-oxidizing bacteria in the medium capillary. When the sensor tip is exposed to methane, methane diffuses into the sensor, passing through the guard capillary tip membrane, the heterotrophic bacterial culture, and the medium capillary tip membrane to end up in the reaction space, where it is consumed by the methanotrophic bacteria, with a concomitant consumption of oxygen diffusing out from the gas capillary. This oxygen consumption results in a displacement of the oxygen concentration gradient within the reaction space, which in turn is reflected by the signal of the transducer. If a sensor without an oxygen guard is exposed to oxygen, oxygen diffuses through the medium capillary tip membrane and displaces the oxygen concentration gradient within the reaction space, regardless of whether the sensor is simultaneously exposed to methane or

not. In a sensor with an efficient oxygen guard, according to the principle described here, oxygen is intercepted by the heterotrophic bacteria and does not interfere with the oxygen concentration gradient within the reaction space, so the signal is not affected.

In principle, an oxygen guard can be constructed with a chemical reaction as the oxygen-scavenging component. We have used titanium citrate, which is a potent reductant (30), but atmospheric levels of oxygen penetrated more than $200 \mu\text{m}$ into the guard at room temperature.

Interference. Apart from the influence of temperature on the oxygen guard, there is a separate effect of temperature on the methane-sensing aspect caused by two different mechanisms (6). First, the rates of diffusion of oxygen and methane within the reaction space and in the transducer electrolyte are temperature dependent. Second, the catalytic capacity of the methanotrophic bacteria increases with temperature, increasing the amount of methane to which the sensor can respond. The sensor signal has been observed to deteriorate at 35°C , probably due to cell death. This maximal temperature limit is lower than expected, and the choice of a more thermotolerant methanotrophic bacterium may enable measurements at higher temperatures.

The interference of CO_2 in sensors with extremely slim transducers is probably due to a pH decrease in the transducer electrolyte caused by the hydration of CO_2 to carbonic acid. This pH shift increases the electrochemical reduction of electrolyte water at the transducer cathode, causing an increase in transducer current. In normal transducers, the casing containing the electrolyte is made less slim behind the tip region to allow the buffering components of the electrolyte (0.5 M bicarbonate buffered to pH 10.3) to maintain a constant pH. In one sensor, the selectivity coefficient for methane was -0.2 for 0.1 atm of CO_2 versus 0.1 atm of methane in terms of partial pressure. At pH 7, water equilibrated with CO_2 at a partial pressure of 0.1 atm contains approximately 20 mM total dis-

solved inorganic carbon, whereas water equilibrated with 0.1 atm of methane contains about 150 μM methane. Thus, in terms of total concentration, the selectivity coefficient was only approximately -0.0016 .

Of acetate, ammonia, and sulfide, only concentrations of sulfide above 100 μM had an effect on a sensor without an oxygen guard (6). Compounds that could interfere specifically with the oxygen guard should be compounds having a toxic effect on the heterotrophic bacteria. However, as the tip membrane is silicone rubber, only nonionic substances can diffuse from the environment to the bacteria in the guard capillary.

Methane sensors without guard capillaries have been shown to have a stirring sensitivity of less than 2% of the total signal (6). This stirring sensitivity has been observed even in the absence of methane due to the diffusion of oxygen from the internal oxygen reservoir through the sensor tip at a rate dependent on the stirring. In sensors with an oxygen guard, this stirring effect at low or zero concentrations of methane was absent, as the oxygen from the internal oxygen reservoir was scavenged by the guard and thus did not diffuse through the sensor tip.

Calibration. Several procedures for calibration have been described (6), but for calibrations at other than an ambient temperature and when a very detailed calibration curve is not necessary, the procedure described here is fast and convenient and consumes only a small amount of gas. The volumes of methane and N_2 gases can be injected with a precision of approximately 1%. The subsequent injection of water forces all of the injected gases into the enrichment tube, where the gases mix rapidly by diffusion. This volume of water must be as large as or larger than the volume of the rubber tube and needle. Larger volumes will not change the gas volume, as excess water will percolate down the glass wall and out of the bottom inlet of the enrichment tube.

In principle, an error is introduced by gas exchange between the calibration gas mixture and the water in the enrichment tube. However, due to the relatively low solubility of the relevant gases—methane, oxygen, and N_2 (20)—a 10-ml aqueous phase can change the partial pressures of these gases in a 25-ml gas phase by only 1 to 2%, given complete equilibration between the two phases. As water is easily forced out of the tube almost nonturbulently and as a calibration procedure takes much less time than the diffusion equilibration time, such a worst-case scenario will not likely be observed in practice.

As a gas phase is analogous to a vigorously stirred water phase with regard to a stirring effect on sensors, the calibration setup used here cannot be used for sensors with a high stirring sensitivity (i.e., a high analyte consumption) if subsequent measurements are to be made in media other than gas.

Although some sensor calibrations were constant over many hours or even days, calibrations were performed at regular intervals when sensors were used over extended periods of time, as the zero current occasionally showed some drift, possibly due to increasing reaction space respiration by heterotrophic contaminants. The sensors used in this investigation responded linearly only in the range of 0 to 0.25 atm of partial pressure of methane due to relatively small reaction spaces. However, by tailoring the physical dimensions, one can construct sensors to respond linearly in the whole range of 0 to 1 atm of methane (6).

Model guard capillary. We found volume-specific oxygen consumption rates of up to 192 $\text{nmol cm}^{-3} \text{ s}^{-1}$ in the model guard capillary. The dry weight of bacteria is normally in the range of 30% (4). If one assumes that the density of bacterial cells is 1.0 and that the cells take up 30% of the space, this volume-specific rate corresponds to approximately 8 mmol of

O_2 g of dry weight $^{-1} \text{ h}^{-1}$. Higher specific oxygen consumption rates have been reported for other bacteria (e.g., 1), and more efficient oxygen guards may be made with organisms other than *A. radiobacter*.

The tip diameter and consequently the ratio of the tip volume to the volume of the electron donor reservoir were larger for the model guard than for the oxygen guards used on actual methane sensors. This means that the oxygen consumption in the model guard was more prone to be limited by the diffusive supply of electron donor than was that in the oxygen guards used on actual sensors with tip diameters as small as 25 μm . A diffusive limitation was seen in the 100% oxygen treatment, in which bacterial oxidation was concentrated in a zone 70 to 160 μm from the membrane—and thus closer to the electron donor reservoir—as opposed to the atmospheric oxygen treatment, in which oxygen consumption was concentrated immediately adjacent to the membrane. Apparently, at 100% oxygen, the diffusive supply of electron donor could match the supply of oxygen only at a certain distance from the membrane. Consequently, the results of the model guard experiment are likely to underestimate the oxygen consumption capacity of oxygen guards used on sensors.

The Q_{10} for heterotrophic activity was 4.1, which is somewhat higher than the factor of 2 often cited. This difference probably reflects the fact that 5°C is below the range in which the process rate grows exponentially with temperature.

The model guard silicone membrane was thicker than the membranes actually used in methane sensors, but as evidenced by the slope of the oxygen partial pressures in silicone (Fig. 5), the permeability of oxygen in silicone is so great that the membrane thickness has only a small effect on the supply of oxygen to the bacteria.

The oxygen concentration profile measurements in the model guard illustrate that the oxygen guard has to be designed according to the application. The colder the experimental setup and the more that oxygen is expected to be encountered, the longer the oxygen guard must be. However, as the response time is a square function of the length of the total diffusive path for methane and as sensitivity decreases with length, oxygen guards should not be made unnecessarily long. One methane microsensor was insensitive to 100% oxygen, with a 95% response time of about 1 min and with a sensitivity to methane of -26 pA/atm at low concentrations (Fig. 3); sensors without a guard capillary have had a 95% response time of less than 20 s and a maximum sensitivity of up to -350 pA/atm . The electron donor is transported from the reservoir to the heterotrophic bacteria by diffusion through the gap between the tip of the medium capillary and the inner walls of the guard capillary. Thus, the larger this gap, the higher the oxygen guard capacity. On the other hand, the gap has to be minimized to limit the amount of methane that can diffuse through the gap to the electron donor reservoir, where an internal methane pool may build up. The consumption of such an internal pool by the methanotrophic bacteria can take a long time following a change from a high to a low concentration and may prolong the 95% response time to several minutes, as shown in Fig. 3 after the shift from 1.0 to 0 atm of methane.

Measurements in rice paddy soil. There was a strong correlation between the depths of oxygen penetration and methane appearance as a function of light regimen. We did not measure methane flux to the overlying water directly, but in some profiles in the dark, methane appeared so close to the surface (Fig. 6B) that a little methane was likely to have escaped oxidation and to have diffused to the water phase. Other dark methane profiles exhibited a distinct methane-free zone in the top of the

oxic layer. As the oxygen guard of the methane microsensor used for this experiment was unable to scavenge all oxygen at concentrations above 1.5 times atmospheric saturation, valid methane concentration measurements could not be made in the most oxygenated part of the photosynthetic layer during illumination. However, as the methane concentration was zero on both sides of the interval in which oxygen interfered with the methane measurements, it is reasonable to assume that no methane was present in the hyperoxic layer. Thus, the experiment confirms the finding of King (15) that photosynthetic microorganisms can regulate methane oxidation in sediments by supplying oxygen for methane oxidation in the light.

The thicknesses of the methane oxidation zone did not differ significantly between dark and light, indicating that the capacity of the methane-oxidizing bacteria was of the same magnitude in the top layers in the dark and in the deeper layers during illumination. Methanotrophic bacteria in sediments have been shown to survive prolonged periods of anoxia (16, 27), and the activity in the deeper layers may thus be due to a nonmotile population which is metabolically inactive during dark periods. Alternatively, all or some of the methanotrophic population in the soil may be motile and may move with the oxic boundary. In both treatments, methane consumption amounted to 8 to 9% of oxygen consumption. Assuming a stoichiometric ratio of methane to oxygen of 1:1.7 (13), methane oxidation thus accounted for approximately 14.5% of soil oxygen consumption.

We did not measure the porosity or the effective diffusion coefficients in this soil, a fact which introduces some potential errors in our calculation of consumption rates. Furthermore, the diffusion coefficient may decrease with depth due to compaction, but as the diffusivities of oxygen and methane should be equally affected by differences in diffusivities between this soil and the soil used by Rothfuss and Conrad (28), as well as by changes in diffusivity with depth, the ratio of the calculated consumption rates for oxygen and methane at any particular layer of the soil should be valid.

The micropore measurements in rice paddy soil demonstrated that the oxygen-insensitive methane microsensor described here can be used to resolve microscale gradients in systems in which methane and oxygen coexist, yielding information about the process of aerobic methane oxidation. Future developments will comprise the use of a sulfide-tolerant alkalophilic heterotrophic microorganism in the oxygen guard to allow the use of a high-pH medium, ionizing any H_2S entering the guard and thus preventing its entry into the reaction space. Preventing both oxygen and sulfide from entering the reaction space should render the methane microsensor free from interference from any chemical factor of natural environments.

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