Microscale Biosensor for Measurement of Volatile Fatty Acids in Anoxic Environments

Rikke Louise Meyer,^{1*} Lars Hauer Larsen,² and Niels Peter Revsbech¹

Unisense A/S, Science Park, Aarhus,² and Department of Microbial Ecology, University of Aarhus,¹ 8000 Aarhus C, Denmark

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A microscale biosensor for acetate, propionate, isobutyrate, and lactate is described. The sensor is based on the bacterial respiration of low-molecular-weight, negatively charged species with a concomitant reduction of NO_3^- to N_2O . A culture of denitrifying bacteria deficient in N_2O reductase was immobilized in front of the tip of an electrochemical N_2O microsensor. The bacteria were separated from the outside environment by an ion-permeable membrane and supplied with nutrients (except for electron donors) from a medium reservoir behind the N_2O sensor. The signal of the sensor, which corresponded to the rate of N_2O production, was proportional to the supply of the electron donor to the bacterial mass. The selectivity for volatile fatty acids compared to other organic compounds was increased by selectively enhancing the transport of negatively charged compounds into the sensor by electrophoretic migration (electrophoretic sensitivity control). The sensor was susceptible to interference from O_2 , N_2O , NO_2^- , H_2S , and NO_3^- . Interference from NO_3^- was low and could be quantified and accounted for. The detection limit was equivalent to about 1 μM acetate, and the 90% response time was 30 to 90 s. The response of the sensor was not affected by changes in pH between 5.5 and 9 and was also unaffected by changes in salinity in the range of 2 to 32‰. The functioning of the sensor over a temperature span of 7 to 30°C was investigated. The concentration range for a linear response was increased five times by increasing the temperature from 7 to 19.5°C. The life span of the biosensor varied between 1 and 3 weeks after manufacturing.

The degradation of organic compounds by complex microbial communities is governed to a large extent by the electron acceptors available. The mineralization is thus generally fastest and most complete under oxic conditions, and common cell constituent polymers, such as starch and proteins, may be completely mineralized by only one kind of microorganism (3). Rather high-molecular-weight molecules, such as sugars, produced by the hydrolysis of polymers are thus taken up and mineralized to CO_2 . In contrast, the anaerobic degradation occurring in lake sediments or methanogenic reactors involves several types of bacteria and archaea. Methane is produced from H_2 plus CO_2 or by the cleavage of acetate, and fermenting and acetogenic bacteria work in concert to convert the organic matter to H_2 , CO_2 , and acetate (6, 7). The free intermediates in these anaerobic conversions, such as acetate and other volatile fatty acids (VFAs), may occur at considerable concentrations in methanogenic environments.

Information about the microscale distribution of VFAs within microbial communities may help researchers to describe and understand how the production and consumption of these species are regulated. We therefore decided to develop a microscale biosensor for the measurement of VFAs under anoxic conditions.

A wide range of large-scale biosensors for the measurement of available dissolved organic matter have been developed over the last 2 decades (11, 18, 19, 23, 24, 26, 29, 30). These sensors are used to measure short-term biological oxygen demand (BOD) as an estimate of 5-day biological oxygen demand in

wastewater. One microscale sensor for ecological studies was described by Neudörfer and Meyer-Reil (17). The principle of measurement is the same in all of these sensors. A culture of aerobic heterotrophic microorganisms is immobilized in front of an oxygen sensor. As the supply of organic electron donors increases, the rate of respiration of the bacteria increases correspondingly and results in a decreased flux of oxygen to the oxygen sensor. Oxygen is supplied from the medium in which the sensor is making measurements, and the measurements are therefore limited to environments with an oxygen concentration above a certain threshold level (80 μM for the microsensor described by Neudörfer and Meyer-Reil [17]). For ecological studies, however, the measurement of microgradients of available dissolved organic carbon is much more relevant in anoxic environments, as easily degradable monomers generally do not accumulate under oxic conditions.

The sensor described here is based on the bacterial respiration of NO_3^- to N_2O . The organism used in the sensor is a strain of denitrifying bacteria (*Stenotrophomonas* sp.), which produce N_2O from denitrification. The measure of the respiratory activity of the bacteria is therefore the production of N_2O as opposed to the consumption of O_2 , used in the BOD sensors described above. This sensor thus offers the first possibility of resolving microscale gradients of VFAs under anoxic conditions and is a new tool for describing the conversion of organic carbon compounds in anaerobic microbial communities.

MATERIALS AND METHODS

Isolation of a strain of denitrifying bacteria deficient in nitrous oxide reductase. Enrichment medium was inoculated with soil rich in nitrate. The medium (medium 1) contained the following compounds: tryptic soy broth (TSB), 0.1 g liter⁻¹; $\text{NH}_4\text{CH}_3\text{COOH}$, 3 g liter⁻¹; KH_2PO_4 , 2.5 g liter⁻¹; NaNO_2 , 5 mM;

* Corresponding author. Mailing address: Department of Microbial Ecology, University of Aarhus, Bd. 540, Ny Munkegade, 8000 Aarhus C, Denmark. Phone: 45 89423326. Fax: 45 86127191. E-mail: rikke.meyer@biology.au.dk.

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g liter⁻¹; CaCl_2 , 0.01 g liter⁻¹; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005 g liter⁻¹; trace metals, 2 ml liter⁻¹ (31); and mixed vitamins, 1 ml liter⁻¹ (31). The pH was adjusted to 7.

Enrichment cultures were incubated at room temperature for approximately 24 h before 10 μl of the supernatant was streaked on agar plates containing the same medium. The agar plates were incubated in anaerobic jars (Difco) under an N_2 atmosphere. When colonies appeared on the plates, these were used for inoculation of liquid medium in test tubes. The tubes were sealed with gas-tight rubber stoppers so that the only respiration process possible, after depletion of the available oxygen in the tubes, was nitrite reduction. The product of this reduction could be either N_2 , N_2O , or NH_4^+ , and the objective was to isolate a strain that produced only N_2O .

When a turbid cell culture appeared in the test tubes, we investigated whether a strain with the desired metabolic characteristics was obtained. The procedure was as follows: (i) check for N_2 production, (ii) check for NO_2^- depletion, and (iii) check for N_2O production.

To check for N_2 production, small glass tubes were placed upside down in the test tubes for the collection of gas bubbles. N_2 has a low solubility in water, and the lack of gas bubbles in the small tubes was recognized as an indication that no N_2 production had occurred. Complete depletion of NO_2^- from the medium was confirmed by use of nitrite sticks (E. Merck AG, Darmstadt, Germany), and the concentration of N_2O was measured by use of an N_2O sensor with an oxygen guard (1).

Cultures that did not produce N_2 and fully converted NO_2^- to N_2O were selected for further investigations. These cultures were tested for the ability to reduce NO_3^- as well as NO_2^- . All cultures were able to reduce NO_3^- , and as a final confirmation that N_2O was the end product of denitrification, these cultures were tested for the production of $^{15}\text{N}^{15}\text{N}$ following anaerobic growth in medium containing 100 μM $^{15}\text{NO}_3^-$. $^{15}\text{N}^{15}\text{N}$ was measured with a VG IRMS SIRA (series II) mass spectrometer. The culture used for the sensor described here converted less than 0.1% of the $^{15}\text{NO}_3^-$ to $^{15}\text{N}^{15}\text{N}$.

Phylogenetic analysis of the isolated denitrifying bacteria (*Stenotrophomonas* sp.). (i) **DNA extraction, amplification, and sequencing of the 16S ribosomal DNA (rDNA) gene.** An amount of bacterial biomass (several large colonies) about the size of a rice grain was scraped off an agar plate and used for DNA extraction. DNA was extracted by use of a Fast DNA MH kit (Bio 101, Vista, Calif.) according to the procedures described by the manufacturer.

The 16S rDNA gene was amplified by use of forward primer 26F and reverse primer 1390R. The numbers indicate the position of the 5' base of the primer according to the *Escherichia coli* numbering system (4). The amplification procedure included 1 min at 93°C followed by 25 cycles of 30 s at 92°C, 60 s at 57°C, and 45 s at 72°C. In the last cycle, the 72°C step was extended for 5 min, and the samples were finally cooled down to 4°C.

A Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for sequencing of the amplification product from the PCR. To obtain an almost complete sequence of the 16S rDNA gene, we used the following fluorescence-labeled primers for the sequencing reactions: 26F, 338F, 519F, 907F, 1492R, 907R, and 338R. The products were run on an ALFExpress automatic sequencer (Amersham).

(ii) **Phylogenetic analysis.** The nearly complete 16S rDNA sequence was aligned with sequences from the Ribosomal Database Project database. Phylogenetic analyses were performed by using the distance matrix algorithms in the computer program PAUP v. 4.0 (Sinauer Associates, Inc., Sunderland, Mass.).

DNA-DNA hybridization was performed with the closest relative according to the 16S rDNA sequence. The hybridization analysis was carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen in Braunschweig, Germany. Hybridization was carried out as described by De Ley et al. (5) with the modifications described by Huss et al. (9) and Escara and Hutton (8) by use of a Gifford System model 2600 spectrometer. Renaturation rates were computed with the TRANSFER.BAS program as described by Jahnke (10).

Physiological studies of *Stenotrophomonas* sp. A few relevant physiological parameters of the culture were investigated. These involved testing of the maximum temperature for growth, testing of tolerance to a range of salinities, investigation of growth by anaerobic respiration on a range of substrates as sole carbon sources, and testing of the ability to ferment.

Anaerobic respiration with various carbon sources was investigated by inoculating liquid medium containing 10 mM NO_3^- as the only electron acceptor and electron donor available in excess. Growth was detected by observing turbidity after 3 days of incubation at 30°C. As a rough estimate of the relative growth rates with the different carbon sources, the turbidities of the cultures were compared.

Growth at different temperatures was tested by streaking *Stenotrophomonas* sp. on agar plates containing 5 g of TSB liter⁻¹ and incubating them aerobically

FIG. 1. Biosensor for VFAs. Denitrifying bacteria producing N_2O are immobilized in front of an electrochemical N_2O sensor.

at different temperatures. The agar plates were checked for growth after 2 to 3 days of incubation. Tolerance to salinity was tested by observing growth in liquid medium in test tubes containing medium 1 plus 0 to 32 g of NaCl liter⁻¹.

A test to check for the ability to ferment was performed in order to eliminate the possibility of some substrates being fermented and not respired (with the consequent production of N_2O) and hence not detected by the biosensor. The fermentation test was carried out by inoculating anoxic medium containing only TSB and checking for growth.

Sensitivity to a range of antibiotics was also investigated to evaluate the possibility of adding antibiotics to the medium chamber of the sensor in order to decrease the risk of contaminating the pure culture in the tip of the sensor with bacteria entering the sensor through the medium chamber. The test was performed by streaking a dense culture of bacteria on 5 g of TSB liter⁻¹ on agar plates and placing a tablet (Neo-Sensi-Tab; Rosco Diagnostica, Taastrup, Denmark) containing a specific antibiotic on top of the agar. The sensitivity to each of the antibiotics was determined by measuring the size of the clearing zone around the tablet according to the instructions of the manufacturer. The following antibiotics were tested: ampicillin, 2.5 μg ; penicillin, 5 μg ; tetracycline, 10 μg ; gentamicin, 40 μg ; erythromycin, 78 μg ; kanamycin, 100 μg ; and streptomycin, 100 μg .

Construction of a biosensor for VFAs. The principle of the sensor was immobilization of a culture of denitrifying, N_2O -producing bacteria in front of an electrochemical N_2O sensor (Fig. 1). The bacteria were placed in the tip of a tapered glass casing (medium chamber; Fig. 1) containing growth medium without electron donors and with NO_3^- as the only available electron acceptor. Under anoxic conditions, the bacteria oxidized organic electron donors diffusing through an ion-permeable membrane in the tip of the biosensor into the reaction space in front of the N_2O sensor. The product of NO_3^- reduction was N_2O , which was detected by the electrochemical sensor (Fig. 2).

The N_2O sensor (described by Larsen et al. [14]) and a solution of membrane material (membrane dissolved in tetrahydrofuran) for the ion-permeable membrane were purchased from Unisense A/S. The medium chamber was constructed from a soda lime glass tube (5-mm outer diameter) which was tapered in two steps. In the first step, the glass was tapered by heating it over a gas flame. The second step involved heating the thin part of the tapered glass tube with a U-shaped Nichrome heating wire (21). This step was viewed at a magnification of $\times 25$ through a dissection microscope to be able to obtain the exact desired

FIG. 2. Theoretical concentration gradients through the reaction space of the sensor. Oxidation of VFAs results in the production of N₂O, which is detected by the transducer. The N₂O sensor measures the flux of N₂O to the cathode.

diameter of the glass. Excessive glass was cut off with a diamond knife (Amidia, Lohn, Switzerland) to achieve at the end of a 150- to 250- μ m-long parallel section a square-cut opening which could serve as a reaction space for bacterial processes (Fig. 1).

The tip of the medium chamber was coated with a solution of 1% aminosilane (Addid 900; Wacker, Munich, Germany) and dried for 10 h at room temperature and 1 h at 100°C. The aminosilane coating increased the adherence of the ion-permeable membrane to the glass surface.

A small amount of membrane solution was sucked into the medium chamber by dipping the tip very briefly (0.5 s) into the membrane solution. The tip was allowed to dry for a few minutes, and as tetrahydrofuran evaporated, a thin (10- to 30- μ m) membrane remained in the tip of the tapered glass casing. This membrane would only be ion permeable after resolubilization in fumes of tetrahydrofuran with the simultaneous addition of water directly on the membrane. The procedure for resolubilization was as follows. A test tube containing a headspace saturated with tetrahydrofuran fumes and a liquid phase of 1 ml of demineralized water and 1 ml of glycerol was constructed. The water was added carefully on top of the glycerol, so that the two liquids did not mix; hence, the test tube contained three phases—a tetrahydrofuran-saturated headspace above a volume of water, which was above a volume of glycerol. The tip of the medium chamber holding the membrane was first inserted through the tetrahydrofuran atmosphere and then through the water and the glycerol consecutively. The exposure time in each phase was 15 s. After this resolution procedure, the membrane structure was preserved by keeping the tip of the medium chamber submersed in 75% glycerol.

A thick suspension of bacteria in electron-donor free nutrient medium (medium 2; see below) was added to the medium chamber and forced into the tip by applying vacuum. Excess bacterial suspension was removed with a thin syringe, and a small volume of 40°C 0.5% agarose was injected behind the bacteria for immobilization of the mass of bacteria in the tip of the sensor.

The N₂O sensor was inserted into the medium chamber and glued to it with epoxy resin. The opening of the medium chamber was sealed off, except for a small glass capillary for insertion or exchange of nutrient medium. Medium 2 contained 25 mM KNO₃, 2 g of KH₂PO₄ liter⁻¹, 5 g of LiCl liter⁻¹, 0.2 g of MgSO₄ · 7H₂O liter⁻¹, 0.01 g of CaCl₂ liter⁻¹, 0.0005 g of FeSO₄ · 7H₂O liter⁻¹, 2 ml of trace metals liter⁻¹ (31), and 1 ml of mixed vitamins liter⁻¹ (31). The medium was saturated with hydrogen gas (see below), and the pH was adjusted to 7.

The denitrifying bacteria applied are facultative anaerobes, and oxygen would therefore interfere with the response of the sensor as oxygen replaces nitrate as an electron acceptor. The inside of the sensor therefore had to be kept strictly anoxic, and palladium on activated carbon (Pd content, 5%; Fluka, Copenhagen, Denmark) combined with H₂ was used as a very efficient catalyst for the reduction of O₂ in the growth medium.

A chlorinated silver wire was inserted into the medium chamber and polarized positively against an Ag-AgCl reference electrode (Radiometer, Copenhagen, Denmark) which was inserted into the sample to be analyzed. The positive tip potential created in this way facilitated the entry of negatively charged species as VFAs and other negatively charged ions (12).

Calibration of the sensor. Calibration was performed under strictly anoxic conditions by use of a glass container with tap water, which was flushed vigorously with N₂. The tip of the sensor and the reference electrode were inserted through two small holes in the lid of the container.

All calibrations used in this study were performed with a +0.5-V charge across the tip membrane, which caused negatively charged ions to be transported into the sensor by electrophoretic migration. This technique, which is referred to as electrophoretic sensitivity control (ESC) (12), made it possible to increase the sensitivity of the sensor to negatively charged compounds but not to uncharged or positively charged compounds. The sensitivity of the sensor to VFAs was thereby increased. A range of substrates were tested at various concentrations (from 100 μ M to 5 mM) with a number of sensors to investigate variations in responses to different substrates.

Test of interference. Interference from NO₃⁻, NO₂⁻, H₂S, and O₂ was investigated by adding various concentrations (from 100 μ M to 2 mM) of these compounds to a solution of 100 μ M acetate in tap water and observing changes in the signal of the sensor.

The effect of changes in pH was tested by making consecutive calibration curves with acetate in citrate (20 mM) and phosphate (5 g of KH₂PO₄ liter⁻¹) buffers adjusted to different pHs in the range of 4.5 to 9. The same test was performed with different solutions of NaCl (2 to 32 g liter⁻¹) to evaluate tolerance to different salinities. The performance of the sensor at various temperatures was investigated by making calibration curves with acetate at different temperatures in a temperature-controlled container.

We examined whether the signal of the sensor was different in stirred and stagnant media by measuring the response to 100 μ M acetate in a stirred solution and in 1% agar, which was in diffusive equilibrium with the stirred solution.

Measurement of VFA gradients in anoxic biofilms. An approximately 2-mm-thick biofilm was collected from a pilot-scale wastewater treatment plant (Danish Hydraulic Institute, Aarhus, Denmark) which was fed with artificial wastewater. The wastewater contained sugar, meat extract, and milk powder with a total BOD of 1.98 g liter⁻¹ as carbon sources and ammonia (0.4 g liter⁻¹) as a nitrogen source. The piece of biofilm was about 2 by 2 cm and was fixed on a glass disk by embedding the edges of the biofilm in 1% agar attached to the glass surface. The glass disk with agar and biofilm was placed in a Plexiglas container with tap water constantly flushed with N₂ to keep the biofilm anoxic.

Profiles of VFAs were measured after 5 h of incubation in tap water under anoxic conditions. The VFA sensor was attached to a micromanipulator and advanced into the biofilm in steps of 200 μ m. The surface of the biofilm was determined by viewing at a magnification of 25 $\times$. The effect of NO₃⁻ in the overlying water phase on VFA production in the biofilm was investigated by measurement of a VFA profile in the anoxic biofilm before and after incubation with 2 mM NO₃⁻ in the overlying water for 2 h.

RESULTS AND DISCUSSION

Description of *Stenotrophomonas* sp. The phylogenetic analysis based on the 16S rRNA gene showed that our strain belonged to the *Xanthrophomonas-Stenotrophomonas* lineage. The distance matrix calculation resulted in differences between the 16S rDNA sequences of our strain and *Stenotrophomonas* sp. (GenBank accession no. Y13836), *Stenotrophomonas maltophilia* (GenBank accession no. AJ131904), and *Stenotrophomonas nitritireducens* (GenBank accession no. AJ012229) of 1.7, 2, and 1.5%, respectively. The only representative among the *Xanthrophomonas* genus which was less than 3% different from our strain was *Xanthrophomonas* sp. (GenBank accession no. Y13836). The analysis also showed only a 2.6% difference between our strain and *Pseudomonas pictorum* (GenBank ac-

FIG. 3. Example of calibration of the VFA sensor with acetate.

cession no. AB021392). This particular species has, however, also been suggested to belong to the *Xanthrophomonas-Stenotrophomonas* lineage (2) and should be renamed. DNA-DNA hybridization of our strain with *S. nitritireducens* showed only 61.7% homology; thus, it cannot be concluded that our strain belongs to this species. We therefore refer to the strain as *Stenotrophomonas* sp.

Physiological studies showed that our strain of *Stenotrophomonas* sp. was unable to ferment. It could grow by respiring on a number of substrates as sole carbon sources. These included acetate, propionate, butyrate, lactate, citrate, glucose, and glycerol. Growth on acetate was clearly faster than that on any of the other carbon sources. The strain grew very slowly on butyrate (the culture never became very turbid).

All the incubations with NaCl (up to 32 g liter⁻¹) were positive for growth, and the maximum temperature for growth on agar plates was 40°C. Tests of sensitivity to antibiotics showed that the strain was very sensitive to tetracycline, streptomycin, and kanamycin. It had intermediate resistance to ampicillin and gentamicin and high resistance to penicillin and erythromycin (no clearing zone at all). In liquid media, the bacteria could tolerate a concentration of erythromycin of 50 mg/liter.

Functioning of the sensor in tests of responses to different substrates. The response of the sensor is proportional to the flux of N₂O toward the electrochemical transducer (Fig. 2). An approximately linear calibration curve can be obtained when all the VFAs diffusing into the reaction space are oxidized in front of the transducer. The 90% response time and the linear concentration range of a sensor thus depend on the length of the reaction space and the amount and activity of the bacteria in front of the N₂O sensor. These three parameters vary from sensor to sensor; therefore, two different sensors will have different detection limits and 90% response times. When the flux of VFAs into the sensor exceeds the oxidation capacity of the bacteria in the reaction space, some of the VFAs will diffuse behind the N₂O sensor and will not be detected. The higher the capacity for VFA oxidation by the bacterial mass, the higher the concentrations of VFAs that can be measured.

An example of calibration with acetate is shown in Fig. 3. The detection limit of this particular sensor was about 1 μM acetate. The signal was enhanced (and could be enhanced further) by use of ESC. An example of how much ESC can

FIG. 4. Demonstration of calibration curves at different ESC voltages. The application of ESC could increase the sensitivity to acetate almost 40 times.

affect the response of a sensor is shown in Fig. 4. By increasing the ESC voltage from 0.2 to 0.6 V, the response to a certain acetate concentration was increased almost 40 times. The 90% response time was between 30 and 90 s and varied between individual sensors.

It should be noted that the calibration curve shown in Fig. 3 is slightly sigmoid. Some sensors had more or less sigmoid calibration curves. Linear calibration curves were obtained when the sensor was used without the application of ESC (data not shown). A condition for a linear response is that the ratio between the diffusion coefficients of acetate and N₂O does not change in the reaction space of the sensor (T. Kjær, L. H. Larsen, and N. B. Ramsing, submitted for publication). When ESC is applied, acetate is transported into the sensor by both diffusion and migration, whereas N₂O is transported by diffusion only. The condition for a linear response is therefore not fulfilled. In some cases, however, the sensors did exhibit a linear response despite the application of ESC. An explanation for this result can be very high transport resistance in the membrane. In such cases, the voltage drop is almost entirely across the membrane and not further into the reaction space (L. R. Damgaard, unpublished results). Acetate ions are thus transported by migration across the membrane, but transport within the reaction space is by diffusion. In this situation, ESC increases the flux of acetate into the sensor but not inside the sensor and therefore does not affect linearity. However, it cannot be expected that a sensor will have a perfectly linear response to acetate when ESC is applied.

The sensor responded to a range of substances, but the response per mole of substrate was different for each compound. The response is thus a function of both the quantity and the composition of organic compounds in a particular sample. Table 1 shows examples of the responses to a range of substrates as a percentage of the response to the same molar concentration of acetate. As mentioned above, the application of ESC increased the sensitivity to negatively charged compounds dramatically, thus increasing the selectivity for VFAs. The sensitivity to acetate, propionate, isobutyrate, and lactate was much higher than that to any other substrate tested. This result was caused by a combination of the increased transport of negative ions into the sensor, the substrate specificity of the

TABLE 1. Responses to a range of substrates as a percentage of the response to acetate^a

Substrate	Response (%)
Alcohols	
Methanol	0
Ethanol	20
Propanol	1
Isopropanol	1
Butanol	1
Glycerol	0
Amino acids	
L-Threonine	7
L-Proline	1
L-Lysine	0
Glutamate	3
Carboxylic acids	
Formate	0
Acetate	100
Propionate	170
Butyrate	7
Isobutyrate	200
Valerate	7
Lactate	120
Pyruvate	13
Citrate	0
Succinate	3
Benzoate	0
Mono- and disaccharides	
Glucose	2
Mannose	0
Galactose	0
Maltose	0
Sucrose	0

^a Measurements were performed by using a 0.5-V charge for ESC.

bacteria, and the molecular weight cutoff of the membrane (i.e., the maximum molecule size that can pass through the membrane). Taking the difference in diffusion coefficients and the number of electron transfers from lactate, isobutyrate, and propionate compared to acetate into account, the theoretical responses to these compounds should be approximately 140, 200, and 150% of the response to acetate, respectively. The values obtained were up to 14% different from the theoretical value. Thus, with respect to these three compounds, the response of the sensor was approximately proportional to the number of electrons oxidized. The VFA sensor should be calibrated with respect to acetate, propionate, isobutyrate, and lactate before use. To optimize the activity of the bacteria and thus the linear concentration range of the sensor, the sensor should be incubated overnight in a solution of acetate, propionate, isobutyrate, and lactate (each at 10 mM).

Effects of pH, salinity, and temperature. The effect of pH was investigated by making calibrations with acetate in buffers of different pHs. The response did not change in the interval between pH 5.5 and pH 9 (data not shown). At pH 4.5, the response to acetate was 11% lower than that to the other pHs (data not shown). Underestimation of the VFA concentration may thus occur if measurements are performed within steep pH gradients where the pH drops below 5.5. The dissociation constant for acetate is 4.74, and the reduced response at pH 4.5 could therefore be explained by the pH being below the dis-

FIG. 5. Effect of variations in temperature on the signal from the VFA sensor. Changes in temperature affected both the size of the response and the concentration range of the sensor.

sociation constant for acetate, a situation which would affect the molar concentration of ionized acetate.

Changes in salinity of between 0 and 32 g of NaCl liter⁻¹ did not affect the response or linear range of the sensor significantly. We recommend, however, that the sensor be calibrated in water of the same salinity as the sample to be analyzed, as variations in ionic strength may affect the current of the ESC circuit and thereby the transport of VFAs into the sensor.

Variations in temperature had a substantial effect on the signal of the sensor (Fig. 5). Both the response and the concentration range that could be measured increased with increasing temperature. At 7°C, the bacteria became saturated with substrate at 200 μM acetate, which corresponded to a response of about 20 pA. At 19.5°C, the response of the sensor was linear at up to 1 mM acetate, corresponding to a response of 120 pA. The zero current was also severely affected by changes in temperature. The zero current depends on the endogenous respiration rate of the bacteria in the absence of externally supplied substrate. The change in this respiration as a function of increasing temperature resulted in an increase in the zero current from 35 pA at 7°C to 240 pA at 30°C. We therefore stress the importance of maintaining a constant temperature in the sample being analyzed.

Interfering substances. It is important to consider the possibility of interfering substances when using microsensors in natural systems. Substances that interfere with the activity of the bacteria will reduce the linear range of the sensor but will not interfere with the response to a certain concentration. Compounds that are likely to interfere with the response of the sensor are chemical species that interfere directly with the N₂O sensor or in some way change the stoichiometry of bacterial processes.

The type of N₂O sensor applied in this study is susceptible to interference from hydrogen sulfide (22), and application of the sensor in reduced marine sediments is therefore limited. The biosensor was susceptible to interference from concentrations of more than 100 μM H₂S. The N₂O transducer measures the flux of N₂O to the cathode, and N₂O diffusing into the reaction space from the environment is measured together with N₂O

produced by the bacteria. The interference from N_2O in the sample being analyzed should therefore be quantified and accounted for.

Substances that affect the processes of bacteria include oxygen and nitrite. *Stenotrophomonas* spp. are facultative anaerobic bacteria, and in the presence of oxygen they will use oxygen rather than nitrate as an electron acceptor for respiration. The signal of the sensor thus decreases rapidly when the addition of oxygen stops the production of N_2O .

Nitrite was also expected to interfere with the signal of the sensor. The bacteria can use both NO_3^- and NO_2^- as electron acceptors. The use of NO_2^- , however, results in the production of twice as much N_2O per mole of acetate as the use of NO_3^- . The presence of NO_2^- was therefore expected to increase the signal of the sensor at a given acetate concentration, and this finding was also observed. The signal increased dramatically (approximately fourfold) when high concentrations (1 mM) of NO_2^- were added to the acetate solution being analyzed. The signal increased much more than the stoichiometry would suggest, and this result could indicate that the bacteria reduce NO_2^- to either N_2O or NO as a detoxification mechanism.

NO_3^- affected the zero current but not the response of the biosensor. However, the effect was observed only when ESC was applied. The concentration of NO_3^- in the medium was 25 mM, and we therefore expected that the bacteria were fully saturated with NO_3^- diffusing from the medium chamber into the reaction space. It is possible that ESC caused such a high level of migration of NO_3^- away from the reaction space that the bacteria closest to the ion-permeable membrane experienced an NO_3^- -free environment. Supply of NO_3^- from both sides of the reaction space ensured the availability of NO_3^- for all parts of the bacterial mass and thereby caused a slight increase in the endogenous respiration rate for the bacteria. We therefore recommend that calibration of the sensor be performed at the ambient NO_3^- concentration.

Stirring sensitivity. Stirring sensitivity is usually not a problem for microsensors because of their small size and small rate of consumption of analyte compared to the rate of diffusion of analyte to the sensing tip of the electrode (20). In biosensor-type microsensors like the one described in this study, however, conditions for zero stirring sensitivity are the same as those for a linear response described above (a constant ratio between the diffusion coefficients of acetate and N_2O through the reaction space). If acetate is transported into the sensor by diffusion only, the sensor is expected to be unaffected by the degree of stirring. Increased stirring makes the diffusive boundary layer around the tip of the sensor smaller. This situation leads to an increased flux of acetate into the sensor, but it also increases the flux of N_2O out of the sensor. Hence, the increased production of N_2O when measurements are made with a stirred solution compared to a stagnant solution is balanced by the increased flux of N_2O out of the sensor, and the signal is therefore not affected.

When ESC is applied, acetate is transported into the sensor by both diffusion and migration. Migration may not be affected significantly by the degree of stirring. When the solution being analyzed is stirred as opposed to stagnant, the signal may decrease as the flux of N_2O out of the sensor is increased (leading to less of the produced N_2O being detected by the

FIG. 6. Profiles of VFAs in anoxic biofilm with and without the application of ESC. The profile without ESC confirmed that the compounds contributing to the measured profile were negatively charged VFAs.

transducer), but the flux of acetate into the sensor remains constant.

We observed an approximate 10% decrease of the signal in stirred media for sensors which exhibited a sigmoid calibration curve with the application of ESC. Stirring sensitivity was observed only when ESC was applied. Sensors exhibiting a linear response to acetate with the application of ESC did not show stirring sensitivity, even with the application of ESC.

Measurement of VFAs in anoxic biofilms. The VFA sensor described here has potential for use in many studies of carbon mineralization in anoxic microenvironments. We demonstrated the use of the sensor for anoxic biofilm from a pilot-scale wastewater treatment plant. A VFA profile showed that VFAs accumulated in the biofilm to a concentration corresponding to almost 70 μM acetate (Fig. 6). The shape of the profile indicated that most of the VFA production was taking place at about a 1-mm depth in the biofilm. This profile was obtained by applying an ESC voltage of 0.5 V. We confirmed that the signal originated from VFAs by measuring a profile without the use of ESC. The response of the sensor at a 1.2-mm depth was only 2% of the response at the same depth but with ESC. It can therefore be concluded that almost all of the signal originated from negatively charged species, such as VFAs and lactate.

VFA profiles were also measured before and after the addition of NO_3^- . The presence of NO_3^- led to a much lower level of accumulation of VFAs in the biofilm and a reduced flux of VFAs out of the biofilm (Fig. 7). The signal of the sensor at a 1.2-mm depth was 39% lower in the presence of 2 mM nitrate in the water phase than in the absence of nitrate. The results thus show that the net production of VFAs in the biofilm decreased in the presence of such high nitrate concentrations. It is likely that VFAs were consumed by bacteria within the biofilm during anaerobic respiration with NO_3^- as an electron acceptor. This assumption could have been confirmed by measuring microprofiles of nitrate.

Future improvements and perspectives. The life span of a VFA biosensor was typically 1 to 3 weeks. The cause of ceased

FIG. 7. Profiles of VFAs before and after incubation with NO_3^- under anoxic conditions. The presence of NO_3^- decreased the accumulation of VFAs in the biofilm and the flux of VFAs out of the biofilm.

functioning of the sensors was usually loss of activity of the microbial biomass or decreased permeability of the ion-permeable membrane. Optimizing the nutrient medium and identifying a different type of membrane which is more stable over time may increase the life span of the sensors.

It was very difficult to control the permeability of the ion-permeable membrane in the resolution process. The procedure for resolubilization could be optimized, aiming for a degree of permeability that results in sensors with a linear response and a lack of stirring sensitivity.

This type of sensor also has the prospect of being manufactured in macroscale design for bulk measurements. As an example, VFAs play an important role as electron donors in enhanced biological phosphorous removal, where acetate is the key substrate for the polyphosphate-accumulating bacteria (28). Optimization of these processes would therefore benefit from online monitoring of VFAs.

The microscale biosensor for VFAs described here represents the first possibility of resolving microgradients of VFAs under anoxic conditions, leading to new ways to describe the processes involved in anaerobic carbon metabolism. In anoxic systems such as rice paddies, wetlands, or aquatic sediments, the organic compounds known to accumulate are acetate and propionate and, in some cases, butyrate, formate, and lactate (13, 15, 16, 25, 27). The high sensitivity of the biosensor to acetate especially and propionate therefore makes it a very useful tool for the study of such environments.

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