Nitric Oxide Microsensor for High Spatial Resolution Measurements in Biofilms and Sediments

Frank Schreiber,* Lubos Polerecky, and Dirk de Beer

Microsensor Research Group, Max-Planck-Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

Nitric oxide (NO) is a ubiquitous biomolecule that is known as a signaling compound in eukaryotes and prokaryotes. In addition, NO is involved in all conversions of the biogeochemical nitrogen cycle: denitrification, nitrification, and the anaerobic oxidation of ammonium (Anamox). Until now, NO has not been measured with high spatial resolution within microbial communities, such as biofilms, sediments, aggregates, or microbial mats, because the available sensors are not robust enough and their spatial resolution is insufficient. Here we describe the fabrication and application of a novel Clark-type NO microsensor with an internal reference electrode and a guard anode. The NO microsensor has a spatial resolution of 60–80 μm, a sensitivity of 2 pA μM−1, and a detection limit of ~30 nM. Hydrogen sulfide (H2S) was found to be a major interfering compound for the electrochemical detection of NO. The application of the novel NO microsensor to nitrifying biofilms and marine sediments revealed dynamic NO concentration profiles with peaks in theoxic parts of the samples. The local concentrations suggested that NO may be an important bioactive compound in natural environments. The consumption and production of NO occurs in separate regions of stratified microbial communities and indicates that it is linked to distinct biogeochemical cycles.

Nitric oxide (NO) is a gaseous compound that is present in various biological systems. In eukaryotes NO is synthesized by different cell types via the enzyme NO synthase (NOS) and acts as an important signaling molecule and as an antimicrobial agent.1 Similarly, in certain prokaryotes NO can be synthesized by NOS. In contrast to eukaryotes, NO synthesis in prokaryotes is not involved in cell signaling but was shown to be involved in the biosynthetic nitration of tryptophanyl moieties2 and the cytoprotection against oxidative stress.3

In addition, NO is produced and consumed during the microbial conversions of the nitrogen cycle. During denitrification NO is an intermediate within the sequential, respiratory reduction of NO−3 or NO2− to N2O or N2.4 In the respiratory chain NO is generated by the enzyme nitrite reductase (Nir) and further reduced by the enzyme nitric oxide reductase (Nor) to N2O. In contrast to the well-studied mechanisms of NO production and consumption during denitrification, the mechanisms of NO production during nitrification are still under debate. Nitrification is the oxygen-dependent two-step process of the oxidation of NH4+ by ammonia-oxidizing bacteria (AOB) to NO2− and the subsequent oxidation of NO2− to NO−3 by nitrite-oxidizing bacteria (NOB).

Studies on pure cultures, soils, and sewage sludge showed that considerable amounts of NO and N2O are produced by AOB in the presence of low O2 concentrations.5 Isotope-labeling studies attributed this N-loss to a denitrifying activity of AOB, termed nitrifier denitrification,6 that is carried out by Nir and Nor for which the genes were detected in several AOB.7 In addition, the recent first genome sequence of a bacterium that mediates the anaerobic oxidation of ammonia (Anamox) suggested NO as the most likely intermediate of this process because Nir was also detected in its genome.8 Understanding the liberation of microbial-derived NO into the atmosphere is of environmental concern since it is involved in a complex set of reactions in the troposphere that has effects on ozone distribution and global warming.9

Despite the lack of biochemical understanding of the conversions of the nitrogen cycle, the links and controls of the nitrogen conversions in natural settings are the subject of ongoing debate.10 The interactions of these conversions have been successfully studied by the use of microsensors for NO−3, NO2−, and N2O in stratified microbial communities, such as biofilms, aggregates, and sediments.11,12 Until now, the role of NO in the interaction of the...
different processes of the nitrogen cycle in stratified microbial communities has not been investigated because no suitable microsensors were available to date. Microsensors for environmental applications must fulfill four major requirements: (i) they must be sensitive enough to detect the concentration changes in the sample, (ii) the spatial resolution must be small enough to detect the concentration changes at the small scale (micrometers) at which they might occur in microbial communities, (iii) the sensors must be robust enough to withstand insertion into rough samples, e.g., sediments, and (iv) the sensor must be selective against possible interfering compounds that might be present in complex natural samples.

Until now, NO microsensors were mainly produced for the sensitive detection of NO release from mammalian cells in cultures or for the implantation on a fixed position in mammalian tissues. The two different sensor designs that were described can be divided into (i) single-anode electrodes with an external reference electrode placed in the sample and (ii) a Clark-type combined sensor design, whereby the sensing anode and the reference electrode are placed in an internal electrolyte that is separated from the sample by a gas-permeable membrane. To achieve adequate sensitivity to NO and prevent interference with NO₂⁻, most single-anode-type NO microsensors rely on exposed carbon fiber electrodes coated with a selective Nafion membrane. Due to the length of the exposed electrode (up to several millimeters), this design is not suitable for most environmental applications, where a spatial resolution in the micrometer range is required. Furthermore, the sensitive Nafion membrane is easily destroyed when inserting the sensor into environmental samples, such as coarse sediments. In contrast, most Clark-type NO microsensors would be robust enough to withstand penetration of such samples, but to achieve high sensitivity for the design of these sensors, wide tip openings have to be employed. However, microbial conversions in biofilms occur within very small scales, and confined sensor openings need to be employed in order to study the fluxes and conversion rates of the compounds in these systems.

In this study, we describe the construction of a robust, Clark-type NO microsensor that can measure at high spatial resolution and that is yet sensitive enough to detect concentration changes of NO that are relevant in environmental settings. Furthermore, we report on the sensitivity of the sensor toward interfering compounds that might be present in natural systems and that have not been considered in previous studies by medical physiologists. Subsequently, the novel Clark-type NO microsensor was applied in natural stratified microbial communities, i.e., a nitrifying biofilm and marine sediments.

**EXPERIMENTAL SECTION**

**Chemicals and Preparation of NO Solutions.** KI, NaNO₂, H₂SO₄, NaOH, KMnO₄, HCl, KCl, Na₂HPO₄, NaH₂PO₄, NH₄Cl, ascorbate, NH₂OH, CS₂, dimethylsulfide (DMS), sodium methanethiolate, Na₂S, and H₂O₂ were obtained from Sigma or Merck. N₂, N₂O, H₂, and CH₄ were obtained from Messer (Sulzbach, Germany). NO solutions were prepared as described previously. Briefly, concentrated H₂SO₄ was slowly added to a N₂-flushed 1/1 mixture of saturated NaNO₂ and KI. Evolved NO gas was passed through 10 M NaOH to remove traces of NO₂ and captured in a Hungate tube fitted with a butyl rubber stopper containing 3 mL of double-distilled H₂O. The excess NO that escaped from this reservoir was led into a wash bottle containing 0.4 M KMnO₄ in 1.2 M NaOH for neutralization. Saturation of the NO solution was verified by mass spectrometry of the headspace gas that was completely comprised of NO.

**NO Microsensor Fabrication.** The sensor was composed of a carbon fiber sensing anode, an Ag/AgCl reference electrode, and a Pt guard anode placed inside an outer glass casing filled with an internal electrolyte (Figure 1). The carbon fiber sensing anode consisted of a carbon fiber that was sealed with epoxy resin into a pulled glass capillary. The glass capillary (Schott 8533, Schott AG, Mainz, Germany) was pulled in a propane flame to an o.d. of 80 μm. The carbon fiber (d ≈ 30 μm, World Precision Instruments, Inc., Sarasota, FL) was inserted from the back and protruded slightly from the tip of the glass capillary. The tip of the glass capillary was dipped into freshly mixed, liquid epoxy resin (105 resin and 206 hardener, West System Inc., Bay City, MI). The resin went up between the carbon fiber and glass by capillary force and was then left to cure for 2 days to seal the carbon fiber in the glass. Then the carbon fiber was polished with a diamond lapping...
film (Ultra prep, Buehler, Lake Bluff, IL) which was attached to a rotary table in successive steps from 9 to 3 μm to achieve a planar sensing surface.

Afterward, the planar carbon fiber surface was modified by deposition of nickel(II) tetakis(3-methoxy-4-hydroxyphenyl)porphyrin (Ni–TMPP; Frontier Scientific Europe Ltd., Lancashire, U.K.) using differential pulse amperometry (DPA). The electrochemical cell comprised the carbon fiber working electrode, a Ag/AgCl reference electrode (Metrohm AG, Herisau, Switzerland), and a platinum counter electrode connected to the control unit (μAutolab Type III, Eco Chemie B.V., Utrecht, The Netherlands). Before each plating session 0.5 mM Ni–TMPP was freshly prepared in 0.1 M NaOH. A resting potential of 0 V for 1 s and a polymerization potential of 1 V for 1 s was applied for DPA plating. Currents were recorded at the end of each potential pulse to monitor Ni–TMPP plating. Plating was performed for 30 s because after that no change in current could be observed which indicated the end of film growth.

The guard anode was prepared from a Pt wire (d = 100 μm, stretched Pt; Ögussa, Vienna, Austria). The Pt wire was inserted into a slim glass capillary with the tip protruding ~10 cm and was electrochemically etched in concentrated KCN (1–7 V) to a diameter of ~30 μm. The Ag/AgCl reference electrode was prepared by electroplating of a Ag wire (d = 300 μm, Gold- und Silberschneideranstalt AG, Pforzheim, Germany) in a 1 M HCl solution. The outer casing was prepared by pulling glass (Schott AG, Mainz, Germany) to a conical shape. The glass was opened at the tip with fine tweezers to obtain an opening of 30–40 μm. The tip was closed with a 5 μm thick gas-permeable silicone membrane (Dow Corning 92-009, Dow Corning Corp., Midland, MI) that cured for 1 day. The tip of the sensing anode was placed at approximately 100–200 μm distance from the silicone membrane. The tip of the guard anode was placed ~200 μm behind the tip of the sensing anode. Glass beads (30–60 μm) were added from the back to avoid sedimentation of particles between the sensing anode and the silicone membrane. Thereafter, the casing was filled with an internal electrolyte (100 mM KCl buffered with 100 mM sodium phosphate pH 7.2) and the reference electrode was inserted into the electrolyte.

**Characterization of NO Microsensor Performance.** The NO microsensor was connected to a sensitive picameter and polarized at +750 mV (sensing anode vs reference and guard anode vs reference) until a stable zero current was obtained. Amperometric responses were recorded with a data acquisition system (DAQCard-AI-16XE-50; National Instruments, Austin, TX) connected to a computer with a data acquisition software (μ-Profilier, Max-Planck-Innovation GmbH, München, Germany). Electrode signals at specific NO concentrations were recorded in 1 s intervals, whereas every single recording was an average of 2000 readings obtained at a sampling frequency of 10 kHz. Calibrations were obtained by adding increasing amounts of NO stock solutions to 100 mL of deoxygenated 100 mM sodium phosphate buffer (pH 7.4) under weak continuous stirring. Sensor noise was calculated as the standard deviation of 10 subsequent recordings of the sensor signal.

The selectivity of the NO microsensor was assayed by exposing it to common interfering compounds (NaNO₂, ascorbate, H₂O₂) and a variety of oxidizable compounds that are able to penetrate a silicone membrane and may occur in nitrifying biofilms or marine sediments (H₂S, NO₂⁻, NH₂OH, NH₄Cl, H₂, CH₄, sodium methanethiolate, C₅H₅DMS). H₂S was introduced as Na₂S. The actual H₂S concentration was calculated based on its equilibrium constant and pH.21

**Application of NO Microsensors.** NO microsensors were used to study nitrifying biofilms and marine sediments. Nitrifying biofilms were grown in a gently aerated flow cell (~800 mL) using Tygon tubing as a substratum for biofilm growth. The inoculum was obtained from a sewage treatment plant (Seehausen, Bremen) and was fed with nitrifying media at a flow rate of 1 mL min⁻¹. The medium for nitrifying bacteria consisted of 10 mM NH₄Cl and trace elements at final concentrations of 3 μM Na₂EDTA, 1.5 μM FeSO₄·7H₂O, 77 nM H₂BO₃, 100 mM MnCl₂, 160 mM CoCl₂, 20 mM NiCl₂·6H₂O, 100 mM ZnSO₄, and 30 mM Na₂MoO₄ in tap water at pH 7.4. For microsensor measurements, small pieces of the biofilm-covered substratum were transferred into a smaller flow cell (~80 mL) that was placed in an aquarium. The aquarium served as a reservoir for medium that was recirculated through the small flow cell at a flow rate of 3 mL s⁻¹ to create a constant flow of ~0.2 cm s⁻¹ above the biofilm. Biofilm samples adjusted for the flow conditions for at least 2 h.

NO measurements in marine sediments were performed in marine sediments from Janssand, an intertidal sand flat situated landward of the island Spiekeroog, North Sea, Germany (53° 44′ 07″ N, 007° 41′ 57″ E).21 For laboratory measurements, intact sediment cores were retrieved approximately 25 m away from the low water line toward the upper sand flat in July 2006 and stored with overlying seawater from the site for 3 days at 4 °C. Measurements were performed within 1 day at 19 °C with a continuous air flow directed on the overlying water in the core to induce a constant water flow above the sediment surface. In addition, NO microprofiles were measured during low tide directly on the exposed sand flat during a field campaign in October 2006. The investigated area was similar to the area where the cores were taken for laboratory measurements. During the measurements the sediment was completely exposed to air but was saturated with seawater.

Calibrations of the NO microsensor were performed as described above, at the same temperatures and in the same media as used for subsequent measurements. Vertical NO concentration profiles were measured with the NO microsensor mounted on a three-axis micromanipulator (MM 33; Märzhäuser, Wetzlar, Germany). The vertical axis was motorized for micropositioning (VT-80 linear stage, Micos, Germany, equipped with a 3564-K-80 MHz controller by μ-Profilier software). The microsensor tip was adjusted manually to the sample surface by using a dissection microscope (Stemi SV 6; Carl Zeiss AG, Oberkochen, Germany). In addition, microprofiles of O₂ and H₂S concentrations were measured.
The microprofiles were fitted and analyzed with a diffusion-reaction model\(^2^7\) to calculate the local conversion rates, which was done with a program written in Matlab. The NO diffusion coefficient \(D\) was\(^2^8\) \(2.21 \times 10^{-9} \text{ m}^2 \text{s}^{-1}\). The effective diffusion coefficient \(D_{\text{eff}}\) in biofilms was calculated according to \(D_{\text{eff}} = \varphi^2 D\), where \(\varphi = 0.9\) is the porosity of biofilms.\(^2^1\)

### RESULTS AND DISCUSSION

#### Sensor Performance and Characteristics.

The NO microsensor responded to increasing amounts of NO that have been added to a deoxygenated buffer solution (Figure 2A). The response was linear within the investigated range between 40 nM and 4 \(\mu\)M NO, with a sensitivity of 2.22 pA \(\mu\)M\(^{-1}\) (Figure 2B). The sensor signal was very stable, with fluctuations of \(\sim 0.022\) pA. Accepting a signal-to-noise ratio of not less than 3, the sensor had a lower detection limit of \(\sim 30\) nM. The response time (\(t_{90}\)) was \(\sim 1\) s upon a change of 60 nM NO (Figure 2A, inset), the temperature sensitivity was 5–10 °C\(^{-1}\), and the sensor was not sensitive to stirring.

In the constructed NO microsensor the sensing anode and reference electrode are situated in an internal electrolyte (Clark-type design) and, hence, are separated from the sample by a gas-permeable membrane. This feature makes the novel NO microsensor ideal for the application in environmental samples, as the sensing surface is protected from mechanical disturbances by hard sample particles, and the confined tip opening of the sensor casing of 30–40 \(\mu\)m allows measurements with high spatial resolution (60–80 \(\mu\m\)), estimated as approximately twice the outer diameter of the sensor tip.\(^2^2\) Although the sensor signal is limited by transport through the confined tip and the silicone membrane, the detection limit of the NO microsensor is still very low (\(\sim 30\) nM). These characteristics compare favorably with those reported previously for different sensor designs. For example, previous designs optimized for small spatial resolution and high sensitivity employed coated sensing anodes that were in direct contact with the sample and, thus, not sufficiently robust for environmental applications.\(^2^9^–^3^0\) On the other hand, the reported Clark-type NO sensors, optimized for robustness, had tip openings that were too wide (>100 \(\mu\m\)) and decreased the spatial resolution to a level that is not sufficient for the measurement in biofilms.\(^1^8^–^2^0\)

Sensitivity of the constructed NO microsensor was \(\sim 2.22\) pA \(\mu\m\)^{-1}. This is considerably lower than that reported for sensors using long single anodes exposed directly to the solution\(^1^5^,^1^7\) or for Clark-type microsensors with wide tip openings and wide sensing surfaces,\(^2^9^,^3^0\) which had sensitivities in the nanoampere per micromolar range. However, as reported by Kitamura et al.,\(^3^1\) the use of a planar sensing surface with a tip diameter of 10 \(\mu\m\) leads to reduced sensitivity of 0.8 pA \(\mu\m\)^{-1}, which is in the same range as that reported here for a sensing surface with a diameter of 30 \(\mu\m\). In contrast, Patel et al.\(^2^9\) and Malinski and Taha\(^3^0\) reported sensitivities in the nanoampere per micromolar range even though the sensors had sensing surfaces similar in size to the NO microsensor of this study.

To understand the wide range of sensitivities reported for sensing surfaces that are similar in size but arranged in different sensor designs (combined vs bare single anode), we developed a simple model describing the physical transport of NO molecules toward the sensing surfaces in these geometrical arrangements (see the Supporting Information). For a combined sensor, the sensing surface is enclosed in a protective casing with a confined opening (Figure 1), and the NO flux toward the electrochemically active sensing surface is limited by one-dimensional diffusion through the membrane and electrolyte. Considering the dimensions of the constructed sensor (Figure 1), maximum sensitivity of 2.28 pA \(\mu\m\)^{-1} is calculated by the model, which is similar to the actually measured sensitivity. On the other hand, the physical transport toward a bare single anode is three-dimensional, which allows for greater NO fluxes toward the sensing surface. Indeed, assuming diffusive transport, sensitivity in the range of \(\sim 120\) pA \(\mu\m\)^{-1} was calculated for a sensing surface with the same diameter.

According to the model the sensitivity of bare single anodes might be further increased to the nanoampere per micromolar range by stirring the medium, as flow enhances transport of the NO molecules by decreasing the diffusive boundary around the sensing surface. However, this stirring sensitivity is undesirable.

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\(^{27}\) Berg, P.; Risgaard-Petersen, N.; Rysgaard, S. Limnol. Oceanogr. 1998, 43, 1500–1510.


because the flow may vary between calibration and measurement.

The use of an outer casing leads to increased robustness and a lower sensitivity. In addition, it shields the sensing electrode from electromagnetic interferences. Thus, the combined sensor design results in a superior signal-to-noise ratio, leading to increased resolution and decreased lower detection limit. The use of carbon instead of Pt as the sensing electrode material might contribute to improved stability. In contrast to metal surfaces, the carbon surface does not adsorb oxygen and does not form oxides\(^{32}\) that may lead to fluctuations of background currents due to unstable polarization of the sensing surface. Furthermore, the use of a guard anode helped to reduce fluctuations in the background currents. We detected ~2-fold increased fluctuations of the background current upon disconnecting the guard anode after it was polarized for several hours (see the Supporting Information). A possible explanation is the occurrence of inorganic and organic micropollutants in the electrolyte that may be oxidizable at the high oxidation potential of 750 mV. The guard anode might oxidize these compounds and thus prevent the compounds from reaching the sensing anode.

**Interference with NO Detection.** The interference with NO detection was investigated at environmentally relevant or higher concentrations of substances that have been already reported to interfere with NO detection. Since the silicone membrane would allow any gas to enter the sensor, the silicone membrane effectively selects for in situ NO detection. However, H\(_2\)S is a seriously interfering substance, leading to increased currents (Figure 3). The slope of the signal versus concentration plot at H\(_2\)S concentrations between 0.6 and 2.44 \(\mu\)M was 3.53 pA \(\mu\)M\(^{-1}\), which gives an interference of 158% (interference = [H\(_2\)S sensitivity/NO sensitivity] \times 100); however, at lower H\(_2\)S concentrations (0–0.61 \(\mu\)M) the interference was only 85%.

H\(_2\)S is a highly reduced gaseous compound that can pass the silicone membrane and is oxidized at the anode to elemental sulfur. The sulfur deposits did not seem to interfere with NO detection, since the interference of H\(_2\)S with the sensor was reversible and did not permanently damage the sensor. After short-term exposure to H\(_2\)S the sensor was still able to detect NO without loss of sensitivity (data not shown). Furthermore, we investigated H\(_2\)S interference at Ni–TMPP-plated and nonplated bare carbon fiber anodes without glass casing. Both plated and nonplated anodes were sensitive to H\(_2\)S. Plating with Ni–TMPP led to increased H\(_2\)S sensitivity (1.9–2.9-fold) and to slightly increased interference with H\(_2\)S (1.2–3.3-fold). However, regardless of the Ni–TMPP plating, H\(_2\)S sensitivity was always higher than NO sensitivity (3–40-fold). Further investigations are needed to clarify how commonly used electrode materials and sensing surface modifications in NO microsensors influence H\(_2\)S interference with NO detection. To our knowledge, for all NO sensors that have been reported for the application of NO detection in medical physiology, H\(_2\)S was never considered as an interfering compound. However, H\(_2\)S has been a fairly well-established messenger molecule for mammalian cells.\(^{34}\) Thus, our findings may have important implications in this research field.

The interference of NO detection with H\(_2\)S restricts the use of the sensor in environmental samples toward oxidized parts of the microbial communities. In addition, H\(_2\)S should be separately monitored with the H\(_2\)S microsensor. Since the conventional H\(_2\)S microsensor for environmental application has a lower detection limit of ~1 \(\mu\)M\(^{29}\), only the occurrence of elevated H\(_2\)S concentra-


Lipschultz, F.; Zafiriou, O. C.; Wofsy, S. C.; McElroy, M. B.; Valois, F. W.; for NO2 reduction. This nitrifier denitrification was discussed to be a way as an electron acceptor. In this metabolism NH4 consumption was located within the upper 300 μm of the biofilm.

NO Microprofiles in Nitrifying Biofilms. Vertical NO microprofiles were measured in nitrifying biofilms (Figure 4). The concentration measurements in a certain spot of the biofilm were reproducible; however, the maximum NO concentrations varied considerably across the biofilm (ranging from 1 to 2.3 μM; Figure 4, parts A and B), reflecting the heterogeneity of a natural, multispecies biofilm. In addition to NO, O2 microprofiles were measured in nitrifying biofilms (Figure 4C). The zone of O2 consumption was located within the upper 300 μm of the biofilm, and O2 became depleted below 300 μm. NO was produced in the micro-oxic zone of the biofilm between 150–300 μm depth to concentrations of ~1 μM at a rate of ~136 pmol cm⁻³ s⁻¹, as calculated from the fitted profile with the diffusion–reaction model (Figure 4C). In the top, oxic zone of the biofilm the produced NO is only insignificantly consumed and leaves the biofilm matrix by diffusion, which is indicated by the linear decrease of NO concentrations away from the surface of the biofilm. Furthermore, NO diffuses downward inside the anoxic zone where the fit of the profile indicated a consumption activity of ~31 pmol cm⁻³ s⁻¹. The switch of AOB at micro-oxic conditions to denitrification with concomitant NO production has been reported earlier.35,36 AOB that are present in the micro-oxic part of the biofilm can use NO3⁻ as an electron acceptor. In this metabolism NH₃⁺ is activated in the presence of O₂ by the enzyme ammonia monooxygenase to hydroxylamine, which serves as the electron donor for NO3⁻ reduction. This nitrifier denitrification was discussed to be a way for NO3⁻ detoxification35 or involved in energy metabolism38,39 and is accompanied by the production of NO.

The NO microprofile in the biofilm shows a change in concentration of ~400 nM within 50 μm (depth from 50 to 100 μm). Due to its small spatial resolution and sensitivity to NO concentrations in the nanomolar range, the novel NO microsensor could resolve these concentration changes in microbial biofilms. The low amount of sulfate in the medium excludes H2S as an interfering species during NO detection.

NO Microprofiles in Marine Sediments. The vertical microprofiles of NO and the simultaneous occurrence of O2 in marine sediments are shown in Figure 5. NO and O2 distributions showed differences when measured directly on the field site (in situ; Figure 5A) and when measured in sediment cores stored for 3 days (ex situ; Figure 5B). In both systems a NO peak could be observed within the oxic zone of the sediment. The maximum concentrations were ~900 nM in collected cores and ~500 nM in the field.

NO is produced in the oxic part and consumed in the anoxic parts of the sediments. Other studies attributed the production of NO to pure cultures of ammonia oxidizers and to the process of nitrification in batch incubations of sediments and soils.35,36,40 Together, this suggests that NO is produced by AOB that thrive in oxic or micro-oxic zones of the upper sediment layers. However, an alternative source of NO at the sediment surface might be NO synthesis from NOS by diatoms. NO synthesis has been shown to be involved in cell–cell signaling by bloom-forming, pelagic diatoms41 and might also occur in benthic diatoms that inhibit the sediment surface. In addition, NO was shown to be produced by green algae and cyanobacteria, when inhibited photosynthesis resulted in the production of NO by the enzyme assimilatory nitrate reductase.42 Furthermore, the NO microprofiles indicate that NO produced in the oxic sediment layers is channeled into the respiratory chain of denitrifying bacteria that occur below the

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Figure 4. Vertical NO (filled and open circles) and O2 (open triangles) microprofiles measured in a nitrifying biofilm using NO and O2 microsensors, respectively. The dashed line represents the biofilm surface. Panels A and B show NO microprofiles measured in different spots of the biofilm, whereby filled and open circles in each graph represent repeated measurements in the same spot. Panel C shows the NO microprofile (filled circles) presented in (A) together with the best fit by a diffusion model (dotted line). The solid line represents the rate of NO production (rNO). Note the different x-axis scaling in (C).

oxic–anoxic interface. The denitrifying bacteria will subsequently reduce NO to N\textsubscript{2}O and N\textsubscript{2}. This is supported by the view that denitrifying bacteria effectively control the ambient NO concentrations to levels in the low nanomolar range.\textsuperscript{43} In cores, H\textsubscript{2}S developed in the anoxic parts of the sediment, as confirmed with the H\textsubscript{2}S microsensor, resulting in a drastic rise of signals of the NO sensor below the oxic zone. In contrast, due to the more powerful hydrodynamics in the field, H\textsubscript{2}S is absent in the top 1 cm of the sediment. Nevertheless, care should be taken when analyzing the results because it cannot be excluded that submicromolar amounts of H\textsubscript{2}S might contribute to the signals obtained with the NO microsensor.

The novel NO microsensor was suitable to measure NO concentration changes in marine sediments. The sensitivity in the nanomolar range was sufficient to resolve the ambient NO concentration changes. Concentration changes in sediments are not as confined as in biofilms, and the step size during profiling was 250 \textmu m and 1 mm for ex situ and in situ measurements, respectively. Thus, the spatial resolution of the NO microsensor was more than sufficient to study these sediments. In addition, the Clark-type NO microsensor was robust enough to penetrate the coarse sediments. The robust glass casing protected the sensing anode against big and hard sediments particles and was sturdy enough not to break upon inserting the sensor into the sediment.

**CONCLUSIONS**

The Clark-type design is a useful design for NO microsensors, especially if high spatial resolution and robustness against rough samples are required. The Clark-type design in combination with a guard anode leads to low background currents. The reduced noise contributed to lowering the detection limit and sensor resolution to sufficient levels, even though the sensitivity achieved was relatively low.

H\textsubscript{2}S was shown to interfere with NO detection. As H\textsubscript{2}S is an established signaling compound in mammalian systems, the application of NO microsensors in biomedical research should regard H\textsubscript{2}S as a potential interference for NO detection. This interference should be investigated for each specific sensor design employed.

The Clark-type NO microsensor made it possible for the first time to measure NO microprofiles in nitrifying biofilms and marine sediments. NO concentrations can be confidently quantified in areas were H\textsubscript{2}S is not present, namely, in oxic parts of the sample or in anoxic, yet oxidized regions. The NO microprofiles showed dynamic changes of NO concentrations, indicating an active involvement of NO in the cycling of nitrogen in stratified microbial communities.

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**SUPPORTING INFORMATION AVAILABLE**

Theoretical maximum of sensitivity for a combined NO microsensor operating at a constant potential, theoretical maximum of sensitivity for a bare single-anode NO microsensor operating at constant potential, and influence of a guard anode on fluctuations of the background current. This material is available free of charge via the Internet at http://pubs.acs.org.

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