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A Nitrite Microsensor for Profiling Environmental Biofilms

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A highly selective liquid membrane nitrite microsensor based on the hydrophobic ion-carrier aquocyanocobalt(III)-hepta(2-phenylethyl)-cobrynate is described. The sensor has a tip diameter of 10 to 15 μm. The response is log-linear in freshwater down to 1 μM NO_2^- and in seawater to 10 μM NO_2^-. A method is described for preparation of relatively large polyvinyl chloride (PVC)-gelled liquid membrane microsensors with a tip diameter of 5 to 15 μm, having a hydrophilic coating on the tip. The coating and increased tip diameter resulted in more sturdy sensors, with a lower detection limit and a more stable signal than uncoated nitrite sensors with a tip diameter of 1 to 3 μm. The coating protects the sensor membrane from detrimental direct contact with biomass and can be used for all PVC-gelled liquid membrane sensors meant for profiling microbial mats, biofilms, and sediments. Thanks to these improvements, liquid membrane sensors can now be used in complex environmental samples and in situ, e.g., in operating bioreactors. Examples of measurements in denitrifying, nitrifying, and nitrifying/denitrifying biofilms from wastewater treatment plants are shown. In all of these biofilms high nitrite concentrations were found in narrow zones of less than 1 mm.

Many intermediates and reactants of the nitrogen cycle can be measured with microelectrodes; however, nitrite is a notable exception. Microsensors for N_2O (28), NH_4^+ (8, 9), NO_3^- (16, 31), and O_2 (26) have been used for nitrification and denitrification studies in sediments and biofilms. Nitrite is an intermediate of both nitrification and denitrification. In sediments and biofilms with a close coupling between nitrification and denitrification, more than 50% of the nitrite formed by nitrification may be reduced by denitrification (25). Nitrite is a highly toxic compound for fish (10), benthic fauna (13), plants (35), bacterioplankton (12), nitrifiers (15), and methanogens (5). High nitrite concentrations favor accumulation of nitrous oxide (34), a greenhouse gas also involved in the destruction of the ozone layer. Nitrite is formed especially during disturbances, e.g., in nitrification bioreactors during oxygen depletion or ammonium overloading (15) or suddenly increased levels of biodegradable organics (21); by denitrification during electron donor deficits (32); or in the presence of oxygen (4). Nitrite can be present in high concentrations in sediments, as shown by pore water analysis (14) and from flux chamber measurements (2). Burrowing strongly increases efflux of nitrite from sediments (7, 19), possibly by stimulation of nitrite formation by the increased variation in oxygen conditions induced by animal activity.

Since nitrite is an intermediate, its presence can be very localized and temporary. This complicates sampling, e.g., by pore water analysis, as the nitrite concentration may change during sampling and storage. A nondisturbing measurement with microsensors would result in more reliable measurements of nitrite distributions, with high spatial resolution. Recently a microbiosensor equally sensitive for nitrate and nitrite was developed (20), but a useful microsensor selectively measuring nitrite has not been reported. A highly selective liquid ion-exchanging membrane (LIX) for nitrite was described (30). However, microsensors with a 1-μm tip based on this ion exchanger had a 10- to 100-fold higher detection limit and 10- to 100-fold lower selectivities than macrosensors. It was concluded that this ion exchanger could not be used to construct microsensors for measurement of nitrite in physiological concentrations (29). We used the same nitrite ion exchanger and, with a modified preparation procedure, constructed nitrite microsensors with a submicromolar detection limit that are sufficiently robust for profiling biofilms and sediments. The modified preparation procedure can be used for all LIX microsensors to improve their performance.

MATERIALS AND METHODS

LIX microsensors. By using a heating coil, green soda lime glass tubes (model 8516; Schott) were drawn to microcapillaries. The tip diameter was 3 to 5 μm for ammonium, nitrate, and pH microsensors and 10 to 15 μm for nitrite microsensors. After the tips were pulled, the glass surface of the capillaries was silanized to obtain a hydrophobic surface for optimal adherence of the LIX membrane. A previously described procedure was used (1), but with longer preincubation and reaction times. The capillaries were placed in a 1.5-liter glass container and baked for at least 3 h at 150°C to remove traces of water. Then, 0.25 ml of silanizing agent (*N*,*N*-dimethyltrimethylsilylamine) was added and the vessel was closed and left overnight at a temperature of 200°C. Excess silane vapor was released in a fume hood, and the remaining silane was baked off at 150°C for another 2 h.

Each capillary was placed in a casing made from a Pasteur pipette, with the microcapillaries protruding ca. 2 cm. The casing was glued to the capillary with a silicon kit. After the preparation as described below, the casing was filled with 0.3 M KCl solution and connected to the reference with an Ag/AgCl wire. This is a highly effective way to protect the signal from electrical noise (16).

As LIX for nitrite sensors, we used a solution of 7% (wt/wt) aquocyanocobalt-(III)-hepta(2-phenylethyl)-cobrynate (nitrite ionophore-I) and 1% (wt/wt) sodium tetraphenyl borate in 2-nitrophenyl octyl ether. We used the Orion nitrate exchanger (model 92-07-01) for nitrate microsensors, 10% (wt/wt) of nonactin and 1% (wt/wt) sodium tetraphenyl borate in 2-nitrophenyl octyl ether for ammonium microsensors, and a solution of 6% (wt/wt) 4-nonadecylpyridine (H^+ Ionophore II, ETH1907) and 1% (wt/wt) potassium tetrakis(4-chlorophenyl)borate in 2-nitrophenyl octyl ether for pH microsensors. To a portion of each type of LIX, 10% (wt/wt) high-molecular-weight polyvinyl chloride (PVC) was added. Then, ca. 3 volumes of tetrahydrofuran (Selectophore quality) was added to the mixture. The PVC was dissolved within 24 h, after which the solution was mixed carefully. Addition with PVC improves the stability and performance of LIX microsensors, as described previously (33). The nitrate LIX was obtained from Orion, and all other LIX components, the tetrahydrofuran, and the silanizing agent were obtained from Fluka.

The filling electrolytes used were 10 mM sodium nitrite, 300 mM KCl, and 10 mM sodium phosphate adjusted to pH 7.0 for the nitrite sensor, 50 mM KNO_3 and 50 mM KCl for the nitrate sensor, 30 mM KCl for the ammonium sensors, and 300 mM KCl and 50 mM sodium phosphate adjusted to pH 7.0 for the pH

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sensors. The filling solutions were degassed under vacuum and filtered through a 0.2-μm-pore-size Millipore membrane. The silanized capillaries were filled with electrolyte by using a plastic syringe drawn in a flame to a 0.1-mm tip; the air pocket that typically was left in the tip was pushed out by applying pressure from the back. Then, under microscopic inspection the tips were dipped in LIX and suction was applied until a membrane with a thickness of ca. 300 μm was introduced. Additionally, 100 to 200 μm of the PVC containing LIX was sucked in. The capillaries were left for at least 2 h, during which the tetrahydrofuran evaporated and a solid ion-selective membrane was formed in the tip.

After hardening of the membrane, the sensors were dipped in a solution of 10% cellulose acetate in acetone. This was done as briefly as possible to avoid dissolution of the membrane in the acetone. If this treatment caused recessing of the membrane from the tip, PVC containing LIX was reapplied. To 1 ml of 10% (wt/vol) bovine serum albumin (BSA) in 50 mM sodium phosphate (pH 7.0) 10 μl of 25% glutaraldehyde was added and immediately thoroughly mixed. This mixture solidifies in a few minutes. Under microscopic guidance the tip of a microsensor was dipped ca. 400 μm deep in the protein solution and moved in and out slowly. As soon as the protein solution became syrupy, a protein layer formed on the microsensor tip. After drying, a cross-linked protein layer of ca. 1-μm thickness was formed, which was water insoluble and firmly fixed. Finally, the casing surrounding the capillary for shielding of the signal was filled with 0.3 M KCl and connected with a Ag/AgCl wire to the reference.

Nitrite, nitrate, and ammonium microsensors were calibrated in dilution series of nitrite, nitrate, or ammonium in the medium used for the experiment. Calibration of the pH microsensors was performed with standard pH solutions.

The selectivity constants were determined by using the fixed interference method (22), with 0.6 M NaCl, 10 mM $NaHCO_3$ (pH 8.0), and 10 mM $NaNO_3$.

O_2 microsensor. Clark-type O_2 microsensors with internal references and guard cathodes were prepared and calibrated as described previously (26). Their tip diameter was 10 μm, and their stirring sensitivity was <2%.

H_2S microsensor. Stirring-insensitive amperometric H_2S gas sensors with a tip diameter of 15 μm were prepared and used as described previously (17).

Microsensor measurements. The electrodes were mounted on a micromanipulator and moved manually. The position relative to the biofilm surface was determined visually by using a dissection microscope. Due to irregularities, the surfaces of biofilms and aggregates are not always well defined. The surface was assumed to be reached if the tip disappeared.

Samples. A 2- to 3-cm-thick biofilm was removed from the walls of the outlet channel of the first activated sludge basin of the municipal wastewater treatment plant Seehausen (Bremen, Germany). After removal, it was transported to the laboratory and pieces of ca. 5 by 3 cm were incubated at 18°C in 0.5 liter of medium containing 25 mM K_2HPO_4, 380 μM $(NH_4)_2SO_4$, 100 μM Na-acetate, and 20 μM $MgSO_4$, adjusted to pH 7.0. The medium was mixed by sparging with air and refreshed twice per day. Nitrate and nitrite profiles were measured with 0.5 mM nitrate in the bulk medium.

Nitrifying aggregates were obtained from the inlet region of a lab-scale fluidized bed reactor as described previously (8). Aggregates with a diameter of ca. 2 mm were placed in a flow cell with insect needles and perfused with medium as described previously (8). The aggregates were left for 15 min before profile measurements started. Microelectrodes penetrated the aggregates at an angle of 120° with respect to the direction of flow. Microelectrodes could be positioned with an accuracy of 10 μm relative to the surface.

Nitrifying/denitrifying biofilms growing in a 22-liter pilot-scale membrane reactor were analyzed in situ. The reactor and its operating principle were described previously (24). Oxygen was supplied through permeable silicon tubing, with an exchange surface of 243 m^2/m^3, through which air was pumped under a pressure of 3 atm. The studied biofilms grew on the tubing. The reduced substrates ammonium and acetate were supplied from the bulk liquid. The reactor was fed continuously with synthetic wastewater (24) containing 34 mM ammonium and 17 mM acetate. The reactor liquid was recycled continuously with a rate of 2,000 liters/h, the liquid residence time was 4.2 h, the temperature was 26.5°C, and the pH was 7.7. The bulk liquid was anaerobic. The biofilms were 2 to 3 mm thick. The cylindrical reactor was placed horizontally with the sample ports on top, so that microsensors could be introduced through the sample ports.

RESULTS

The nitrite microsensor showed a log-linear response down to a nitrite concentration of 1 μM in 25 mM phosphate buffer and nitrite sensitivity to at least 0.1 μM (Fig. 1). Identical results were obtained in pure water (data not shown). The signal was stable, was not sensitive to noise, and drifted less than 1 to 2 mV/h. The signal was not influenced by stirring. The response time (t_{90}, the time needed to reach 90% of the end value upon a concentration change) was 10 to 15 s, allowing nitrite profiles with 25 measuring points to be measured within 10 min. Noncoated sensors showed the same calibration curve but had a response time of ca. 5 s (three sensors tested). The selectivity constants for Cl^-, NO_3^-, and HCO_3^- were $10^{-4.5}$, $10^{-4.5}$, and 10^{-4}, respectively. Sensors with a tip diameter of 3 μm consistently (six sensors tested) had a high detection limit (Fig. 1). To obtain a useful sensor, the tip diameter had to be at least 10 μm (15 sensors tested). The response in seawater was log-linear down to 10 μM NO_2^- and then bent off sharply due to chloride interference (Fig. 1). Addition of a pH buffer to the electrolyte resulted in more stable sensors. Upon exposure to more than 40 μM sulfide, the signal drifted in a negative direction and the sensitivity for nitrite was irreversibly lost. The damage could occur within a few seconds. Occasionally the sensitivity was not totally lost, but then the sensor became extremely slow, with response times measurable in minutes. Undamaged coated sensors could be used for months if stored dry and in the dark between experiments.

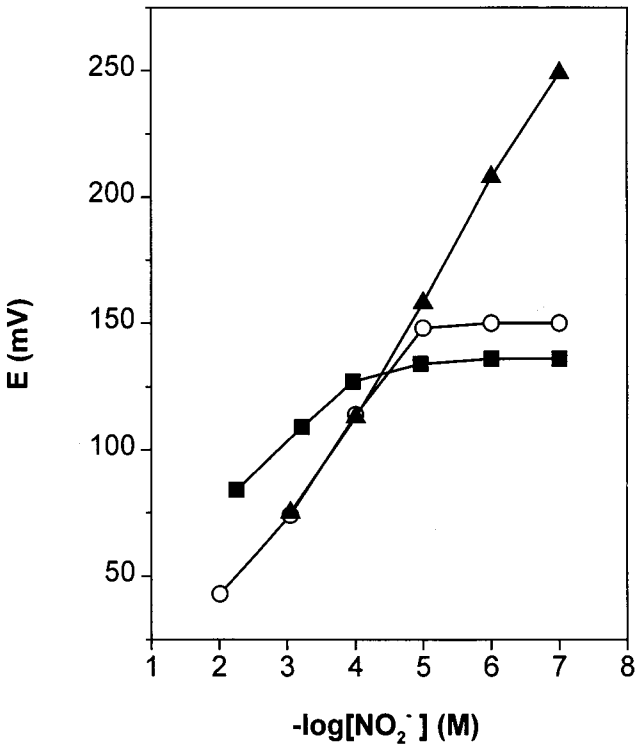

FIG. 1. Calibration curves of nitrite microsensors with a 15-μm tip in 25 mM phosphate buffer (▲), with a 15-μm tip in seawater (○), and with a 3-μm tip in 25 mM phosphate buffer (■).

LIX membranes completely gelled with PVC were not stable in larger microcapillaries. Short circuits between electrolyte and sample solution occurred, due to shrinking of the membrane, or the membrane expanded by an unknown process and was pushed through the tip. Only the combination of PVC-gelled and nongelled LIX in the tip resulted in a functional microsensor. Without priming with cellulose acetate, the protein coating did not stick to the tip. After drying, the protein coating cracked and the sensor was not protected. Good results were obtained only with the combined coating of cellulose acetate and cross-linked BSA. During drying the coating shrank so that the tips sometimes became slightly curved, without affecting the sensor characteristics.

The protein coating was used for ammonium (data not shown), pH (data not shown), nitrite, and nitrate sensors with satisfying results. Uncoated nitrate sensors often drifted at nitrate concentrations below 5 μM; with protein coating, this was not observed. The response time was 5 to 10 s, similar to that for the uncoated sensor. Coated sensors could be used for

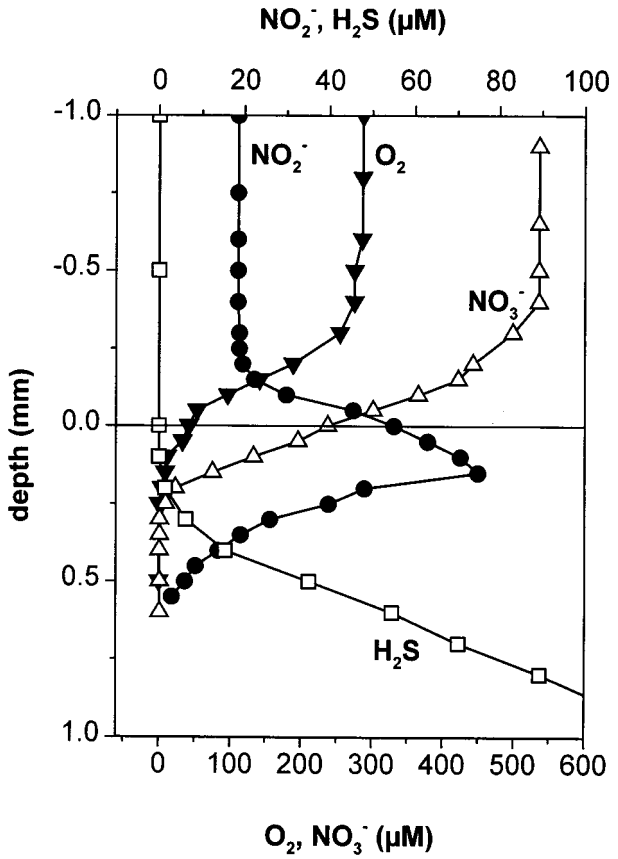

FIG. 2. Profiles of NO_2^- (●), NO_3 (△), O_2 (▼), and H_2S (□) in a thick denitrifying biofilm from a wastewater treatment plant. The biofilm surface is at a depth of 0.

a few days when stored dry between experiments, while the uncoated sensors had a lifetime of ca. 5 h. For ammonium and pH sensors the coating did not influence calibration or response times (<5 s). Coated pH and ammonium sensors could also be used for a few days.

Initially well functioning microsensors (pH, ammonium, nitrate, and nitrite) without coating were immediately destroyed by touching the biofilm from the activated sludge plant. During penetration, sudden signal changes, drift, and increase or decrease of the offset potential were observed. After touching the biofilm, ammonium, nitrate, and nitrite microsensors no longer responded to substrate changes. The pH microsensor became extremely slow, with response times measurable in minutes. Microscopic inspection did not reveal any visual damage to the microsensor tip or LIX membrane. Microsensors coated with only cellulose acetate were damaged in the same way. However, the sensors coated with cellulose acetate and protein were insensitive for the destructive effect of the biofilm, even after exposure of several hours. Nitrate sensors were also sensitive to sulfide, causing a signal drift in the negative direction, but the effect was reversible. Ammonium and pH sensors were insensitive to sulfide.

The thick biofilm from the activated sludge plant showed high nitrate consumption rates. In the absence of nitrate in the bulk, no nitrate or nitrite could be detected in the oxic zone of the biofilm, indicating that nitrification was not significant. If present in the bulk, nitrate penetrated the biofilm more deeply than oxygen (Fig. 2). At a nitrate concentration of 0.5 mM, nitrite profiles showed a peak of 80 µM at the depth where nitrate was consumed. Nitrite profiles were measured to a depth of 0.5 mm, below which sulfide induced signal drift.

The nitrifying aggregates were rather irregularly shaped conglomerates of spheroids. The spheroids had a diameter of ca. 50 µm and were clustered into solid aggregates of ca. 2 mm in diameter. In these aggregates, ammonium was not completely converted to nitrate. The nitrite profile showed an increase to a maximum concentration of 105 µM, whereas nitrate increased only from 100 to 135 µM (Fig. 3). Oxygen penetrated 150 µm into the aggregate, and nitrate and nitrite were formed in the outer 200 µm.

Nitrite, nitrate, ammonium (not shown), and pH (not shown) profiles could be measured without noise problems in the operating pilot-scale membrane reactor placed in a hall with numerous other operating units. In the nitrifying/denitrifying biofilms the oxygen and nitrate concentration decreased in the direction from the membrane to the bulk liquid (Fig. 4). Oxygen concentration at the membrane surface was 800 µM, corresponding with the partial pressure in air under 3 atm, and decreased to zero within ca. 1.5 mm. Nitrate reached a concentration of 2 mM at the membrane and penetrated the whole biofilm, extending into the reactor liquid outside the biofilm. In the oxic zone a 300 µM nitrite peak was measured. Nitrite did not reach the anoxic zone.

DISCUSSION

Liquid membrane microsensors are relatively easy to prepare, and the great variety of ion exchangers allows measurement of many different compounds. However, their use in bioreactors and in the environment has been frustrated by three main problems: their noise sensitivity, which required working in a Faraday cage; their sensitivity to other ions; and

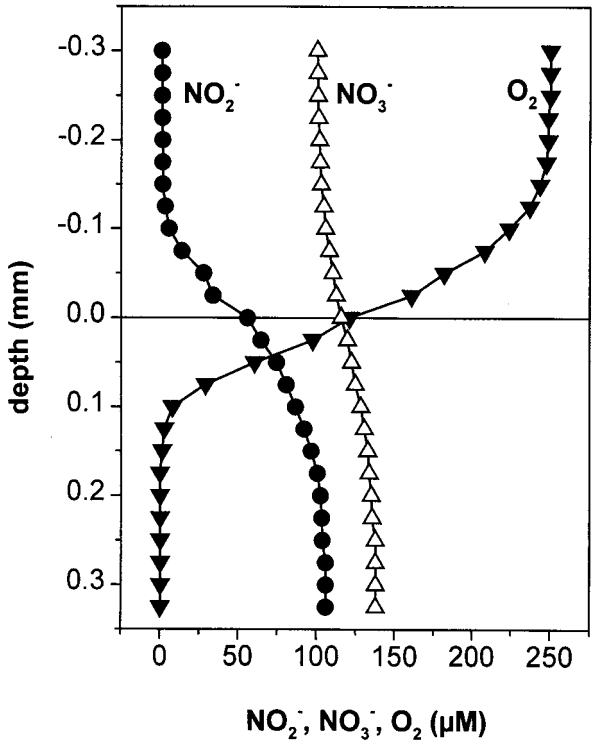

FIG. 3. Profiles of NO_2^- (●), NO_3 (△), and O_2 (▼) in a nitrifying aggregate from a fluidized bed reactor. The aggregate surface is at a depth of 0.

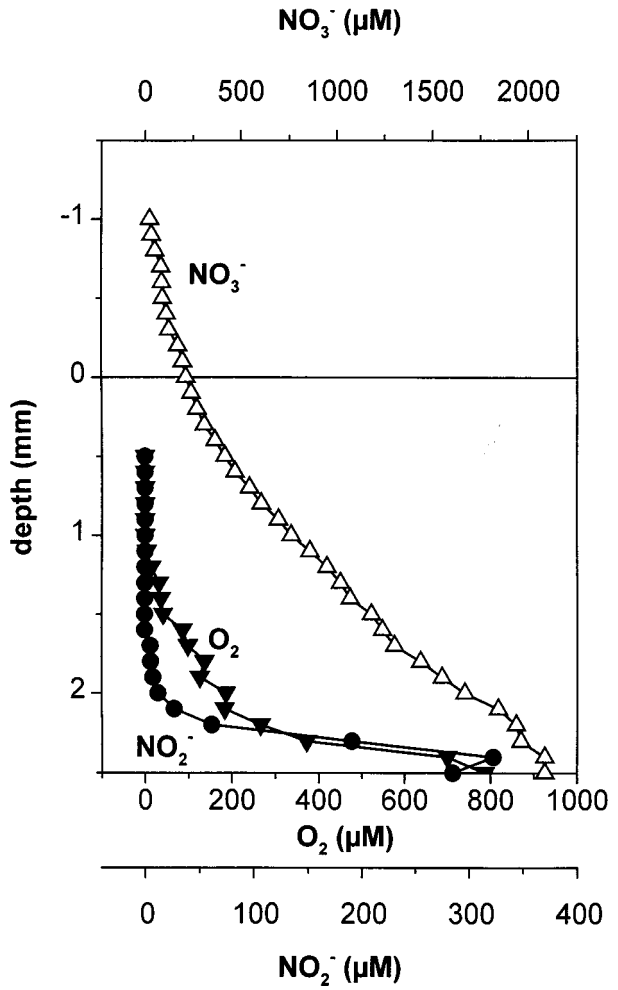

FIG. 4. Profiles of NO_2^- (●), NO_3 (△), and O_2 (▼) in a nitrifying/denitrifying biofilm from a membrane reactor. The biofilm surface is at a depth of 0; the membrane surface is at a depth of 2.5 mm.

their signal instability after and during contact with biomass. The noise sensitivity was effectively cured by shielding (16), which functions much better and is more convenient than a Faraday cage. The latter two problems will be discussed below.

The interference of other ions is partly caused by the need for an extremely small sensor tip to stabilize the liquid membrane in the tip by capillary force. The contribution to the signal of ion shunts through the glass wall and at the glass-LIX interface becomes increasingly important with smaller tip diameter and can dominate over the ion exchange through the LIX membrane (6). Most likely, this phenomenon was responsible for the poor behavior of 3-μm nitrite microelectrodes. The ion shunts can to some extent be countered by good silanization (23). The problem was solved by increasing the tip diameter. However, by increasing the tip size, the capillary force was reduced and, therefore, the membrane had to be stabilized in the tip by gelation with PVC. This resulted in a nitrite microsensor with a response similar to that of a macroelectrode. Enlarging the tip also increased the mechanical strength of the microsensor. A tip diameter of ca. 10 μm excludes use for intracellular studies but is excellent for probing microbial mats and biofilms. With exception of the pH LIX, the limited selectivity of ionophores requires calibration of LIX sensors in the experimental medium.

Irregular behavior of LIX sensors, such as sudden potential shifts, loss of signal, drift, and strong increase of response time, is observed especially during measurements in environmental samples with high cell densities, such as biofilms from wastewater treatment plants and microbial mats (27). This phenomenon is unpredictable—no problems occurred in sediments (9, 16, 31) or in methanogenic and nitrifying aggregates (8)—but when it occurs it affects all types of LIX electrodes. Sensor damage occurs during contact with the biomass and not during penetration of the boundary layer, indicating that the damage on the LIX membrane is caused by direct interaction with a water-insoluble biomass component. Since no physical damage was observed, we suspect a chemical change of the LIX membrane by a hydrophobic substance. The thin protein coating shields the hydrophobic LIX membrane surface effectively from interaction with hydrophobic surfaces in the environmental sample matrix. Cross-linked BSA forms dense layers in which the diffusion coefficient is 3 orders of magnitude lower than in water (18). The unknown damaging substance cannot penetrate the protein layer, either because its pore size is too small or because the damaging substance is too hydrophobic to penetrate the hydrophilic coating. The extra diffusional resistance does not significantly increase the response time as shown by the pH and nitrate sensors. Possibly, the slower response of the nitrite sensor is caused by reversible binding of nitrite to the coating.

As a result of the improvements (increased tip diameter, gelation with PVC, protein coating, and shielding), LIX microsensors can now be used in a variety of environmental samples, outside the laboratory and even in operating reactors, as illustrated by the profiles in Fig. 4.

The irreversible damage of the nitrite sensor by sulfide is caused by reduction of the Co(III) in the porphyrin ring (11). Sulfite will have the same effect. This phenomenon is a problem for measurements in anaerobic biofilms and sediments, as nitrite and sulfide may be present simultaneously. However, from the continuous drift induced by sulfide it can easily be recognized when the nitrite signal becomes unreliable.

In both the denitrifying and nitrifying/denitrifying biofilms the nitrite and nitrate profiles were shaped differently. The straight profiles of nitrate and nitrite showed that in the aerobic zone no significant nitrogen conversions occurred. No nitrite formation was observed in the aerobic zone, which was reported to indicate aerobic denitrification (4). The nitrite accumulation was the highest where nitrate consumption occurred, just below the oxic zone. Nitrite diffused from this zone into the deeper layers of the biofilm, where it was further reduced, and it diffused out of the biofilm into the bulk liquid. The nitrite peak showed that the reduction rate of nitrate is locally higher than that of nitrite. Denitrification does not always lead to nitrite accumulation, since in the nitrifying/denitrifying biofilm a nitrite peak was observed in the nitrifying zone but not in the anaerobic denitrifying zone.

In the nitrifying aggregates the ammonium oxidation was faster than the nitrite oxidation. From the interfacial gradients it can be calculated that about six times more nitrite is produced than nitrate. No nitrite peak was observed. A previous study on aggregates from the same reactor showed complete ammonium conversion to nitrate (8). Also, the appearance of the aggregates was changed from smooth spheres into a structure resembling a bunch of grapes. This might have been caused by a population change due to a reactor breakdown 8 months prior to the measurements. The 200-μm-thick zone where nitrite and nitrate were formed did not perfectly match with the oxygen penetration of 150 μm. This may be attributed to the rather irregular surface, which made it difficult to match

the different profiles. Alternatively, the 15-min incubation time before the start of the measurements may have been too short to develop steady state.

Nitrite profiles often show a peak, as can be expected from an intermediate that is both produced and consumed. Therefore, nitrite efflux from biofilms, sediments, and aggregates can be minimal, even when considerable concentrations are present. The maximum nitrite concentrations measured in the biofilms ranged from 80 to 400 μM. Although nitrite is generally known to be toxic, consistent data are hard to find. The effect of nitrite is strongly species dependent. Nitrite is highly toxic for *Nitrobacter agilis* (K_i for HNO_2, 10 to 20 μM) but not very toxic for *Nitrosomonas europaea* (K_i HNO_2, >50 mM) (15). A concentration of 140 μM nitrite reduced methanogenesis in sewage sludge by 60%, and 700 μM totally blocked methanogenesis (5). A concentration of 700 μM nitrite killed 50% of a crayfish population in 48 h (13), 5 μM nitrite increased the susceptibility of trout to pathogens by 50% (3), and 100 μM nitrite decreased ion uptake by plant roots (35). This list is certainly not comprehensive, but it can be concluded that the measured nitrite concentrations will have a variety of physiological effects on plants, animals, and bacteria.

These preliminary studies showed that considerable nitrite concentrations are present in nitrifying and denitrifying biofilms, as well as in biofilms where both nitrification and denitrification occur simultaneously. The nitrite-containing zones were narrow, in the order of 0.5 to 1 mm in thickness. Since alternative methods such as pore water extraction or slicing do not have sufficient spatial resolution, accurate profiles could be detected only with microsensors. The nitrite microsensor is expected to be highly useful for further studies to elucidate which factors regulate the nitrite concentration in biofilms and sediments.

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