

# Anaerobic Sulfide Oxidation with Nitrate by a Freshwater *Beggiatoa* Enrichment Culture

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**A lithotrophic freshwater *Beggiatoa* strain was enriched in O<sub>2</sub>-H<sub>2</sub>S gradient tubes to investigate its ability to oxidize sulfide with NO<sub>3</sub><sup>-</sup> as an alternative electron acceptor. The gradient tubes contained different NO<sub>3</sub><sup>-</sup> concentrations, and the chemotactic response of the *Beggiatoa* mats was observed. The effects of the *Beggiatoa* sp. on vertical gradients of O<sub>2</sub>, H<sub>2</sub>S, pH, and NO<sub>3</sub><sup>-</sup> were determined with microsensors. The more NO<sub>3</sub><sup>-</sup> that was added to the agar, the deeper the *Beggiatoa* filaments glided into anoxic agar layers, suggesting that the *Beggiatoa* sp. used NO<sub>3</sub><sup>-</sup> to oxidize sulfide at depths below the depth that O<sub>2</sub> penetrated. In the presence of NO<sub>3</sub><sup>-</sup> *Beggiatoa* formed thick mats (>8 mm), compared to the thin mats (ca. 0.4 mm) that were formed when no NO<sub>3</sub><sup>-</sup> was added. These thick mats spatially separated O<sub>2</sub> and sulfide but not NO<sub>3</sub><sup>-</sup> and sulfide, and therefore NO<sub>3</sub><sup>-</sup> must have served as the electron acceptor for sulfide oxidation. This interpretation is consistent with a fourfold-lower O<sub>2</sub> flux and a twofold-higher sulfide flux into the NO<sub>3</sub><sup>-</sup>-exposed mats compared to the fluxes for controls without NO<sub>3</sub><sup>-</sup>. Additionally, a pronounced pH maximum was observed within the *Beggiatoa* mat; such a pH maximum is known to occur when sulfide is oxidized to S<sup>0</sup> with NO<sub>3</sub><sup>-</sup> as the electron acceptor.**

*Beggiatoa* spp. are gliding, filamentous, colorless sulfur bacteria (22). These multicellular bacteria can occur in dense mats at the surface of sulfide-rich sediments in many freshwater and marine habitats (2, 10, 11, 21). The filaments of bigger marine species of *Beggiatoa* can be more than 120 μm wide (2) and >1 cm long, are white, and are visible with the naked eye; even single filaments of narrow freshwater *Beggiatoa* species whose filaments are ca. 3 μm wide (14, 21) can be observed with a stereomicroscope. *Beggiatoa* spp. are sulfide-oxidizing bacteria that have an important effect on the benthic sulfur cycle (4, 6). The presence of *Beggiatoa* mats at the sediment surface prevents toxic sulfide from diffusing into the water column, because biological sulfide oxidation is much more rapid and efficient than chemical sulfide oxidation (13).

In addition, *Beggiatoa* spp. can have a great effect on the aquatic nitrogen cycle when they use NO<sub>3</sub><sup>-</sup> anaerobically as an alternative electron acceptor in place of O<sub>2</sub>. The ability of freshwater and marine *Beggiatoa* spp. to oxidize sulfide anaerobically with NO<sub>3</sub><sup>-</sup> has been studied for some time (11, 19, 20, 21), especially because large marine species contain a vacuole in which NO<sub>3</sub><sup>-</sup> can be stored at concentrations up to 160 mmol/liter (11). This enables the filaments to penetrate into anoxic sediment layers and perform anaerobic sulfide oxidation. However, anaerobic sulfide oxidation by freshwater *Beggiatoa* species has not been unequivocally documented, and the impact of freshwater *Beggiatoa* species on the nitrogen cycle is unclear (5, 11). Therefore, there is significant interest in ob-

taining more information about possible anaerobic sulfide oxidation with NO<sub>3</sub><sup>-</sup> by freshwater *Beggiatoa* species.

The freshwater *Beggiatoa* strain that was used in this study was sustained for more than 2 years in highly enriched O<sub>2</sub>-H<sub>2</sub>S gradient tubes (12). Using microsensors to measure changes in the O<sub>2</sub> contents, H<sub>2</sub>S contents, pH, and NO<sub>3</sub><sup>-</sup> contents in these gradient tubes, the position of the *Beggiatoa* filaments in the transparent agar could be optically related to high-resolution chemical gradients. This experimental approach was used to address the following questions. (i) Does the freshwater *Beggiatoa* sp. exhibit a chemotactic response to the presence of different NO<sub>3</sub><sup>-</sup> and H<sub>2</sub>S concentrations? (ii) Does a *Beggiatoa* mat use NO<sub>3</sub><sup>-</sup> as an alternative electron acceptor in place of O<sub>2</sub>? (iii) Do the *Beggiatoa* filaments alter the vertical O<sub>2</sub>, H<sub>2</sub>S, and pH gradients differently when they are exposed to NO<sub>3</sub><sup>-</sup> in addition to O<sub>2</sub>?

## MATERIALS AND METHODS

**Sampling site and cultivation.** Samples of *Beggiatoa* sp. with a filament width of 3 μm were collected in 2003 from the NO<sub>3</sub><sup>-</sup>-rich stream Giber Aa, south of Aarhus, Denmark. Here, mats of *Beggiatoa* were found on the mud around outlets for primary treated sewage.

The *Beggiatoa* filaments were enriched in lithotrophic agar gradient tubes, modified as described by Nelson and Jannasch (12). These gradient tubes contained two layers of agar, a layer of dense bottom agar (1.5% Bacto Agar [Difco Laboratories]) containing a high ΣH<sub>2</sub>S concentration ([ΣH<sub>2</sub>S] = [H<sub>2</sub>S] + [HS<sup>-</sup>] + [S<sup>2-</sup>]) overlaid by a layer of softer top agar (0.25%) without ΣH<sub>2</sub>S, which led to opposing gradients of ΣH<sub>2</sub>S and O<sub>2</sub> in the top agar. The composition of the medium is shown in Table 1. The pH was adjusted to approximately 7.0 with NaOH. The gradients were prepared in screw-cap tubes (length, 150 mm; inside diameter, 14 mm). The tubes were filled with 4 ml of autoclaved bottom agar and 8 ml of top agar. Unless indicated otherwise, the bottom agar was prepared with 4 mmol/liter Na<sub>2</sub>S. The top agar also contained 150 μl of a sterile vitamin solution (Table 1), 4 mmol/liter NaHCO<sub>3</sub>, and, unless indicated otherwise, 50 μmol/liter NaNO<sub>3</sub>, 50 μmol/liter NH<sub>4</sub>Cl, and 50 μmol/liter sodium acetate. The screw caps on the tubes were left loose to permit exchange of the headspace gas

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TABLE 1. Compositions of medium, micronutrient solution, and vitamin solution

Medium or solution	Composition
Medium	0.01 g EDTA, 0.12 g CaSO <sub>4</sub> · 2H <sub>2</sub> O, 0.2 g MgSO <sub>4</sub> · 7H <sub>2</sub> O, 0.016 g NaCl, 0.14 g Na <sub>2</sub> HPO <sub>4</sub> , 0.138 g NaH <sub>2</sub> PO <sub>4</sub> , 0.264 g CaCl <sub>2</sub> · 2H <sub>2</sub> O, 2 ml FeCl <sub>3</sub> solution (0.29 g/liter), 1 ml micronutrient solution, 1,000 ml distilled water
Micronutrient solution	0.5 ml H <sub>2</sub> SO <sub>4</sub> (>98%), 2.28 g MnSO <sub>4</sub> · H <sub>2</sub> O, 0.5 g ZnSO <sub>4</sub> · 7H <sub>2</sub> O, 0.5 g H <sub>3</sub> BO <sub>3</sub> , 0.025 g CuSO <sub>4</sub> · 5H <sub>2</sub> O, 0.025 g Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O, 0.045 g CoCl <sub>2</sub> · 6H <sub>2</sub> O, 1,000 ml distilled water
Vitamin stock solution <sup>a</sup>	1 mg vitamin B <sub>12</sub> , 1 mg inositol, 1 mg biotin, 1 mg folic acid, 10 mg <i>p</i> -aminobenzoic acid, 100 mg nicotinic acid, 100 mg D-pantothenate, 200 mg thiamine (each vitamin was dissolved in 10 ml distilled water)

<sup>a</sup> For the final vitamin solution 1 ml of each vitamin stock solution was added to 100 ml (final volume) of distilled water.

with the atmosphere. To allow gradient development, the agar was aged for at least 2 days before inoculation. For the different experiments, *Beggiatoa* filaments were taken from existing gradient tubes, pooled, and mixed, and identical subsamples of enriched *Beggiatoa* biomass were inoculated approximately 5 mm below the agar surface. All cultures were grown at room temperature in the dark.

**Vertical position of the *Beggiatoa* mats.** For determination of the NO<sub>3</sub><sup>-</sup> and ΣH<sub>2</sub>S-dependent vertical positions of the *Beggiatoa* mats, the agar was prepared with 0, 100, 200, 400, and 600 μmol/liter NaNO<sub>3</sub> and with 4 and 8 mmol/liter Na<sub>2</sub>S, respectively (*n* = 3). The mat positions within the gradient system were determined using the tip of a microsensors dummy as a pointer. The dummy was mounted vertically on a micromanipulator, which was attached to a heavy stand. Via its motor drive, the micromanipulator allowed slow, small-scale insertion of the microsensors dummy into the agar down to the *Beggiatoa* mat, while the tip was viewed through the side of the gradient tube with a stereomicroscope (magnification, ×10 to ×20). The meniscus of the agar surface was defined as a depth of 0 μm, from which the position of the clearly visible upper boundary of the *Beggiatoa* mat was measured. The mat position was determined 1 to 6 days after inoculation.

**Chemical microgradients.** The O<sub>2</sub> concentrations, H<sub>2</sub>S concentrations, pH values, and NO<sub>3</sub><sup>-</sup> concentrations in the gradient tubes were measured with microsensors. Agar was prepared with 0 and 600 μmol/liter NaNO<sub>3</sub>, and profiles were determined 2 and 4 days after inoculation; profiles in uninoculated tubes that were the same age were also determined.

The microsensors were either purchased from Unisense A/S (Aarhus, Denmark) or manufactured at the Max Planck Institute for Marine Microbiology (Bremen, Germany). The O<sub>2</sub> microsensors with a guard cathode (17) had tip diameters of 10 to 15 μm and 90% response times of <5 s. They were calibrated with air- and N<sub>2</sub>-flushed medium used for agar preparation (100 and 0% air saturation, respectively). The glass-type pH microsensors (18) had tip diameters of <12 μm and 90% response times of <20 s and were calibrated with commercial buffer solutions (pH 4.0, 7.0, and 9.2; Mettler-Toledo, Switzerland). The pH microsensors were used together with homemade reference electrodes, which consisted of a chlorinated Ag wire (length, 30 mm; diameter, 0.5 mm) that was inserted into one end of a glass capillary. The capillaries (length, 100 mm; inside diameter, 1 mm) were filled with 1% agar prepared in 3-mol/liter KCl and thus served as a salt bridge. The H<sub>2</sub>S microsensors (3) had tip diameters of 10 μm and 90% response times of <10 s. They were calibrated with deoxygenated PO<sub>4</sub> buffer (200 mmol/liter K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.5) to which Na<sub>2</sub>S was added stepwise to obtain final concentrations of approximately 0 to 400 μmol/liter (9). The precise ΣH<sub>2</sub>S concentration of each calibration solution was determined spectrophotometrically by the method of Pachmeyer (16). The concentrations of free H<sub>2</sub>S in the calibration solutions were calculated as follows:

$$[\text{H}_2\text{S}] = [\Sigma\text{H}_2\text{S}]/[1 + (10^{\text{pH}}/10^{\text{pK}_1})] \quad (1)$$

where pK<sub>1</sub> = 7.027 is the negative logarithm of K<sub>1</sub>, the first dissociation constant of the sulfide equilibrium system (pK<sub>2</sub> can be neglected at pH <9). From these data, the calibration curve for the H<sub>2</sub>S microsensors was plotted. ΣH<sub>2</sub>S gradients in the tubes were calculated as follows:

$$[\Sigma\text{H}_2\text{S}] = [\text{H}_2\text{S}] \times [1 + (10^{\text{pH}}/10^{\text{pK}_1})] \quad (2)$$

using the [H<sub>2</sub>S] and the pH gradients measured with microsensors.

LIX-type NO<sub>3</sub><sup>-</sup> microsensors (1) with tip diameters of 5 to 10 μm and 90% response times of <30 s were prepared on the day before use to improve the signal stability. NO<sub>3</sub><sup>-</sup> microsensors were used together with homemade reference electrodes (see above). Calibration was performed using uninoculated gradient tubes in which the NaNO<sub>3</sub> concentration was adjusted to 0, 15, 30, 60, 150, 300, or 600 μM. All sensors were calibrated before and after measurement at room temperature. One microsensor at a time was mounted on a motorized micromanipulator that was operated by the software *Profix* (Unisense A/S, Aarhus, Denmark). The microsensor was positioned in the center of the tube cross section and then lowered toward the agar surface (depth, 0 μm [see above]). Starting at this depth, vertical profiles were recorded at increments of 100, 200, or 400 μm down to 30 mm. The O<sub>2</sub>, pH, H<sub>2</sub>S, and NO<sub>3</sub><sup>-</sup> profiles were determined at the same spot of the same tube whenever possible and were related to the position and thickness of the *Beggiatoa* mat in the inoculated enrichment culture (for mat position designations see above). The lower boundary of the mat was defined as the position where filaments were present more than just sporadically.

**Flux calculations.** The amounts of O<sub>2</sub> and ΣH<sub>2</sub>S that flowed across a unit of area per unit of time (flux) were determined for uninoculated controls as well as for the tubes that were inoculated with the *Beggiatoa* enrichment. Assuming steady-state conditions, Fick's first law of diffusion was used:

$$J = -D(\delta C/\delta x) \quad (3)$$

where *J* is the flux (in nmol cm<sup>-2</sup> s<sup>-1</sup>), *D* is the diffusion coefficient (in cm<sup>2</sup> s<sup>-1</sup>), *C* is the concentration (in nmol cm<sup>-3</sup>), and *x* is the depth (in cm). The diffusion coefficients for O<sub>2</sub> and ΣH<sub>2</sub>S (in agar at room temperature) were 2.03 × 10<sup>-5</sup> and 1.57 × 10<sup>-5</sup> cm<sup>2</sup> s<sup>-1</sup>, respectively (13). For the uninoculated controls, the linear regions of the concentration gradients above and below the O<sub>2</sub>-ΣH<sub>2</sub>S overlap zone were used for δ*C*/δ*x* (13); for the *Beggiatoa*-containing gradient tubes, the linear regions above and below the *Beggiatoa* mat were used.

## RESULTS

**Mat position experiments.** The experiments showed that the mat position depended on three factors: the concentrations of NO<sub>3</sub><sup>-</sup> and ΣH<sub>2</sub>S and the length of incubation (Fig. 1). Generally, the mat position was deeper when the NO<sub>3</sub><sup>-</sup> concentration was higher. This effect was less pronounced when 8 mmol/liter Na<sub>2</sub>S was used instead of 4 mmol/liter Na<sub>2</sub>S. In all treatments *Beggiatoa* mats moved upward with time (12). Three-way analysis of variance with NO<sub>3</sub><sup>-</sup> and ΣH<sub>2</sub>S concentrations as between-subject factors and with time as a within-subject factor revealed that the dependence of the mat position on all three factors (for NO<sub>3</sub><sup>-</sup>, *F*<sub>4,19</sub> = 478 and *P* < 0.001; for ΣH<sub>2</sub>S, *F*<sub>1,19</sub> = 529 and *P* < 0.001; and for time, *F* = 1,229, *df* = 5, and *P* < 0.001) was highly significant.

**O<sub>2</sub> and ΣH<sub>2</sub>S microgradients.** Without NO<sub>3</sub><sup>-</sup> addition, the vertical O<sub>2</sub> and ΣH<sub>2</sub>S gradients were steeper in the *Beggiatoa* gradient tubes than they were in the uninoculated controls (Fig. 2A to D). Correspondingly, the O<sub>2</sub> and ΣH<sub>2</sub>S fluxes into the *Beggiatoa* mats were greater than those into the O<sub>2</sub>-ΣH<sub>2</sub>S overlap zone (Table 2). Furthermore, the O<sub>2</sub> and ΣH<sub>2</sub>S gradients became steeper with time, which resulted in upward movement of both the O<sub>2</sub>-ΣH<sub>2</sub>S overlap zone (uninoculated controls) and the *Beggiatoa* mat (Fig. 2A to D; cf. Fig. 1). The *Beggiatoa* mat in the experiment without added NO<sub>3</sub><sup>-</sup> was approximately 0.4 mm thick and was slightly above the O<sub>2</sub>-ΣH<sub>2</sub>S

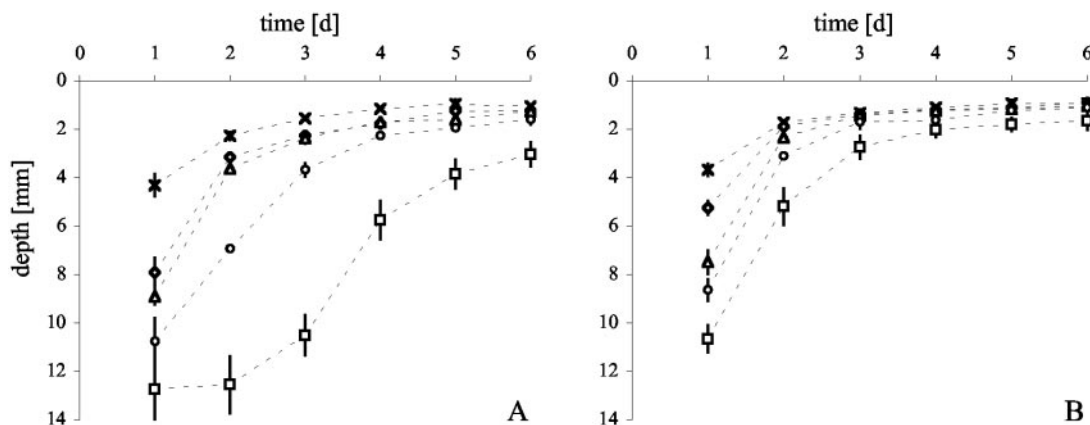


FIG. 1. Mean depth (in mm) of the upper boundary of the *Beggiatoa* mat, depending on the  $\text{NO}_3^-$  and  $\Sigma\text{H}_2\text{S}$  concentrations in the gradient tubes over time (days [d]). (A) Bottom agar prepared with 4 mmol/liter  $\text{Na}_2\text{S}$ . (B) Bottom agar prepared with 8 mmol/liter  $\text{Na}_2\text{S}$ . Symbols:  $\times$ , no  $\text{NO}_3^-$ ;  $\diamond$ , 100  $\mu\text{mol/liter}$   $\text{NO}_3^-$ ;  $\triangle$ , 200  $\mu\text{mol/liter}$   $\text{NO}_3^-$ ;  $\circ$ , 400  $\mu\text{mol/liter}$   $\text{NO}_3^-$ ;  $\square$ , 600  $\mu\text{mol/liter}$   $\text{NO}_3^-$ . Some of the error bars, which indicate standard deviations ( $n = 3$ ), are smaller than the symbols.

overlap zone.  $\text{NO}_3^-$  addition to *Beggiatoa* tubes had a strong effect on the  $\text{O}_2$  and  $\Sigma\text{H}_2\text{S}$  microgradients, on the mat position, and on the thickness of the mat, which increased to  $>8$  mm (Fig. 2E and F). The  $\text{NO}_3^-$  effect was most pronounced 2 days after inoculation. An approximately 4-mm gap appeared be-

tween the  $\text{O}_2$  and  $\Sigma\text{H}_2\text{S}$  profiles (Fig. 2E). Additionally, the corresponding  $\text{O}_2$  microgradient was considerably less steep, resulting in a flux of  $3.6 \text{ pmol cm}^{-2} \text{ s}^{-1}$ , which was only one-half the value obtained for the uninoculated control and less than one-fourth the value obtained for the treatment without

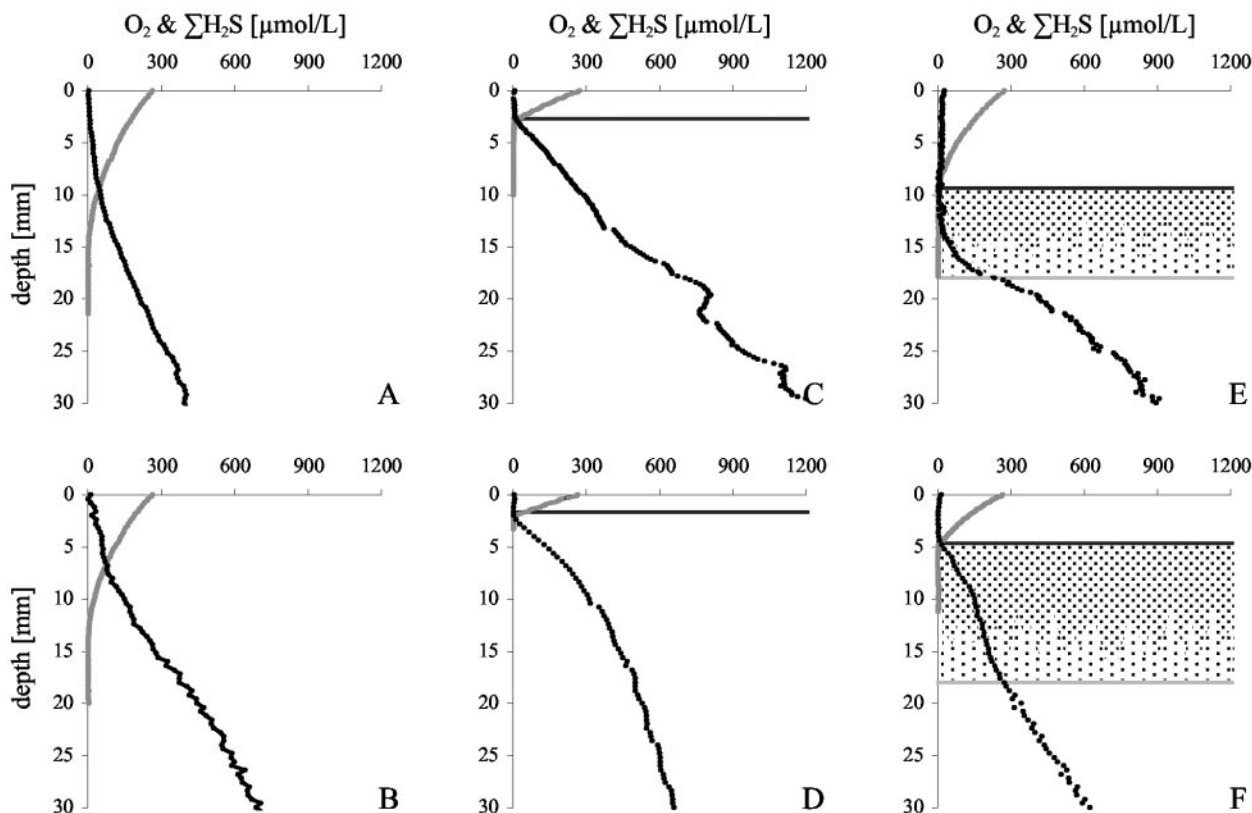


FIG. 2. Microprofiles of  $\text{O}_2$  (gray circles) and  $\Sigma\text{H}_2\text{S}$  (black circles) and positions of the upper (dark gray lines) and, where applicable, lower (light gray lines) boundaries of the *Beggiatoa* mats. (A and B) Uninoculated gradient tubes. (C and D) *Beggiatoa* gradient tubes without  $\text{NO}_3^-$ . (E and F) *Beggiatoa* gradient tubes with an initial  $\text{NO}_3^-$  concentration of 600  $\mu\text{M}$ . The incubation times were 2 days (A, C, and E) and 4 days (B, D, and F) after inoculation. The shaded areas within the boundaries of the *Beggiatoa* mats (E and F) indicate that filaments were more abundant in the upper mat regions. Gray and black circles overlap in some panels.

TABLE 2. O<sub>2</sub> and ΣH<sub>2</sub>S fluxes in uninoculated controls and in *Beggiatoa*-enriched gradient tubes without NO<sub>3</sub><sup>-</sup> and with an initial NO<sub>3</sub><sup>-</sup> concentration of 600 μmol/liter<sup>a</sup>

Time (days)	Flux (pmol cm <sup>-2</sup> s <sup>-1</sup> )					
	Controls		<i>Beggiatoa</i> enrichments without NO <sub>3</sub> <sup>-</sup>		<i>Beggiatoa</i> enrichments with 600 μmol/liter NO <sub>3</sub> <sup>-</sup>	
	O <sub>2</sub>	ΣH <sub>2</sub> S	O <sub>2</sub>	ΣH <sub>2</sub> S	O <sub>2</sub>	ΣH <sub>2</sub> S
2	7.2	2.8	16.7	5.9	3.6	11.9
4	7.8	3.9	23.4	7.4	7.7	4.3 <sup>b</sup>

<sup>a</sup> The data correspond to profiles shown in Fig. 2.

<sup>b</sup> The flux may have been underestimated because there were no long-term steady-state conditions for ΣH<sub>2</sub>S.

NO<sub>3</sub><sup>-</sup> (Table 2). In contrast, the ΣH<sub>2</sub>S flux was about twofold higher than that in the *Beggiatoa* gradient tube without NO<sub>3</sub><sup>-</sup> and about fourfold higher than that in the uninoculated control (Table 2). The NO<sub>3</sub><sup>-</sup> effect was less pronounced after 4 days; the O<sub>2</sub> profile in the NO<sub>3</sub><sup>-</sup>-containing *Beggiatoa* enrichment culture became steeper, and the ΣH<sub>2</sub>S profile became less steep (Fig. 2F).

**NO<sub>3</sub><sup>-</sup> microgradients.** The NO<sub>3</sub><sup>-</sup> microsensor measurements for the uninoculated control (Fig. 3A) and the *Beggiatoa* enrichment culture after 2 and 4 days (Fig. 3B and C) illustrate that the NO<sub>3</sub><sup>-</sup> concentrations decreased in the presence of *Beggiatoa* sp. during incubation. The mean NO<sub>3</sub><sup>-</sup> concentration in the upper 30-mm agar layer decreased from the initial concentration (600 μmol/liter) to 86 μmol/liter after 2 days and to 54 μmol/liter after 4 days. Furthermore, the profiles show that all of the NO<sub>3</sub><sup>-</sup> diffused from the small upper agar volume into the mat, whereas some NO<sub>3</sub><sup>-</sup> was still diffusing upward from the much larger volume of agar below the mat that also contained a larger total amount of NO<sub>3</sub><sup>-</sup>. In contrast to O<sub>2</sub> and ΣH<sub>2</sub>S, which were spatially separated after 2 days in the NO<sub>3</sub><sup>-</sup>-containing treatment, NO<sub>3</sub><sup>-</sup> and ΣH<sub>2</sub>S overlapped in the *Beggiatoa* mat (Fig. 2E and 3B).

**pH microgradients.** In the uninoculated control, the pH was 7.8 at the agar surface and increased to 8.3 at a depth of 30 mm due to the increasing ΣH<sub>2</sub>S concentration (Fig. 4A). In the *Beggiatoa* enrichment culture without NO<sub>3</sub><sup>-</sup>, the pH profile showed that the minimum pH was close to the *Beggiatoa* mat (Fig. 4B). In contrast, in the *Beggiatoa* enrichment culture with NO<sub>3</sub><sup>-</sup> the pH profile had a completely different shape and there was a pronounced maximum pH in the *Beggiatoa* mat (Fig. 4C).

## DISCUSSION

The hypothesis that the freshwater *Beggiatoa* strain investigated is able to oxidize ΣH<sub>2</sub>S anaerobically with the alternative electron acceptor NO<sub>3</sub><sup>-</sup> originated from observations made during the mat position experiments; at higher NO<sub>3</sub><sup>-</sup> concentrations the *Beggiatoa* mats moved deeper into the agar toward the electron donor ΣH<sub>2</sub>S (Fig. 1). This hypothesis was supported by microsensor profiles and flux calculations, which demonstrated that the *Beggiatoa* filaments indeed moved into anoxic, NO<sub>3</sub><sup>-</sup>-rich agar layers and could oxidize even more ΣH<sub>2</sub>S if NO<sub>3</sub> was available (Fig. 2C to F and Table 2). Fur-

thermore, the O<sub>2</sub> flux into the *Beggiatoa* mat exposed to NO<sub>3</sub><sup>-</sup> was much lower than the O<sub>2</sub> fluxes in the tubes without NO<sub>3</sub><sup>-</sup> and the uninoculated control tubes after 2 days (Table 2). This finding can be explained by the missing O<sub>2</sub>-ΣH<sub>2</sub>S overlap zone in the NO<sub>3</sub><sup>-</sup>-amended *Beggiatoa* tubes (Fig. 2E). Because of the spatial separation of O<sub>2</sub> and ΣH<sub>2</sub>S, neither chemical nor biological ΣH<sub>2</sub>S oxidation with O<sub>2</sub> could take place. The effect of the initial NO<sub>3</sub><sup>-</sup> concentration on *Beggiatoa* sp. became less pronounced over time (Fig. 1 and 2C to F), which is explained by the finding that NO<sub>3</sub><sup>-</sup> limitation occurred as incubation progressed (Fig. 3). It is likely that not all NO<sub>3</sub><sup>-</sup> was immediately used for anaerobic ΣH<sub>2</sub>S oxidation and that an unknown

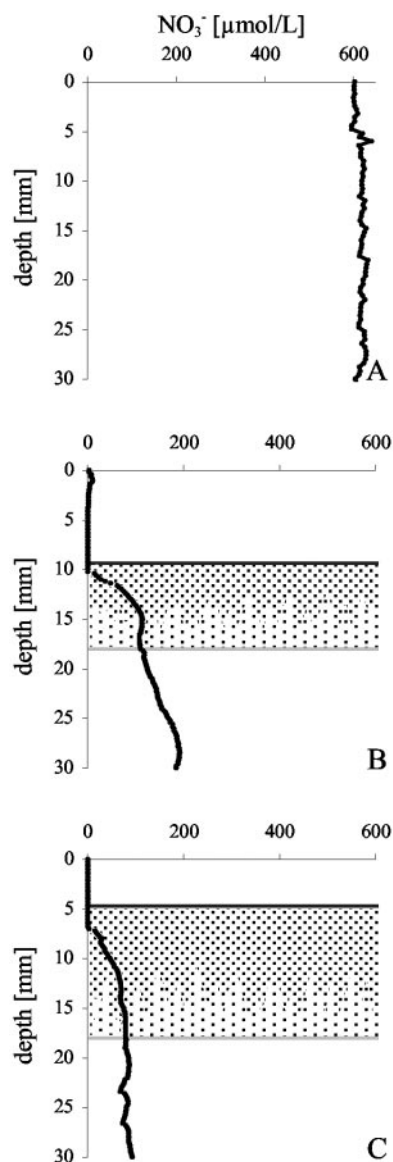


FIG. 3. Microprofiles of NO<sub>3</sub><sup>-</sup> (circles) and positions of the upper (dark gray lines) and lower (light gray lines) boundaries of the *Beggiatoa* mats. (A) Uninoculated gradient tube. (B and C) *Beggiatoa* gradient tubes 2 days (B) and 4 days (C) after inoculation. The initial NO<sub>3</sub><sup>-</sup> concentration was 600 μM. The shaded areas within the boundaries of the *Beggiatoa* mats (B and C) indicate that filaments were more abundant in the upper mat regions. Circles overlap in some panels.

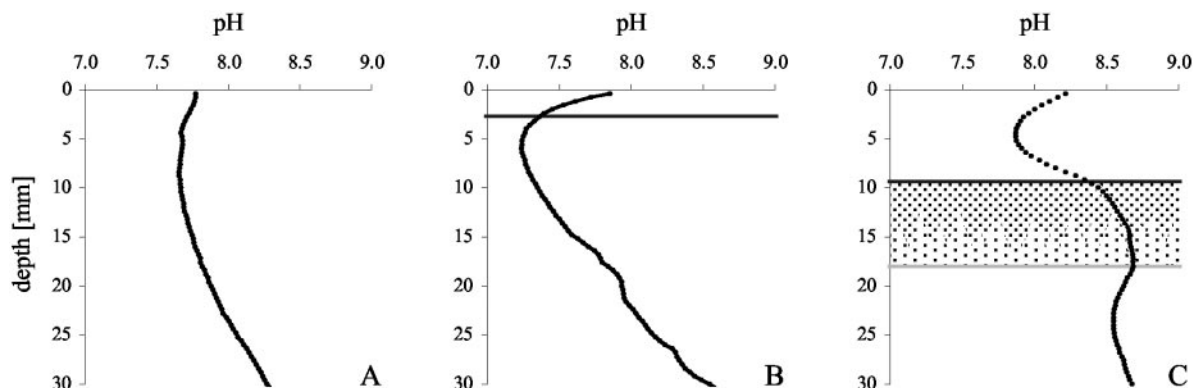


FIG. 4. Microprofiles of pH (circles) and positions of the upper (dark gray line) and, where applicable, lower (light gray line) boundaries of the *Beggiatoa* mats. (A) Uninoculated gradient tube. (B and C) *Beggiatoa* gradient tubes without  $\text{NO}_3^-$  (B) and with an initial  $\text{NO}_3^-$  concentration of  $600 \mu\text{M}$  (C). The incubation time was 2 days. The shaded area within the boundaries of the *Beggiatoa* mat (C) indicates that filaments were more abundant in the upper mat regions. Circles overlap in some panels.

fraction of  $\text{NO}_3^-$  was assimilated or stored intracellularly (11, 23). Vacuoles in freshwater *Beggiatoa* have not been detected so far (22), but cytoplasmic storage of  $\text{NO}_3^-$  is another possibility. This could explain the finding that more  $\text{NO}_3^-$  was taken up during the first 2 days of incubation than during the second 2 days (Fig. 3).

*Beggiatoa* oxidizes  $\Sigma\text{H}_2\text{S}$  first to  $\text{S}^0$ , which can be stored as intracellular globules, and subsequently to  $\text{SO}_4^{2-}$  (22, 24). When  $\text{O}_2$  is used as the electron acceptor, the oxidation of  $\text{H}_2\text{S}$  to  $\text{S}^0$  is pH neutral (if  $\text{HS}^-$  is used as the electron donor, its oxidation to  $\text{S}^0$  is moderately alkaline;  $\text{S}^{2-}$  can be neglected at  $\text{pH} < 9$ ), whereas the oxidation of  $\text{S}^0$  to  $\text{SO}_4^{2-}$  is acidogenic. In total, the aerobic oxidation of  $\Sigma\text{H}_2\text{S}$  to  $\text{SO}_4^{2-}$  is acidogenic, which explains the pH profile found in the *Beggiatoa* enrichment culture without  $\text{NO}_3^-$ , in which the minimum pH largely coincided with the position of the *Beggiatoa* mat (Fig. 4B) (7, 13). When  $\text{NO}_3^-$  is used as the electron acceptor, the oxidation of  $\Sigma\text{H}_2\text{S}$  to  $\text{S}^0$  increases the pH, while the oxidation of  $\text{S}^0$  to  $\text{SO}_4^{2-}$  decreases the pH (20). This was visible in the pH profiles that were determined for the  $\text{NO}_3^-$ -containing treatments; after 2 days of incubation, the maximum pH was 8.7 in the lower region of the *Beggiatoa* mat (Fig. 4C), which must have resulted from the oxidation of  $\Sigma\text{H}_2\text{S}$  to  $\text{S}^0$  with  $\text{NO}_3^-$ . Toward the upper region of the *Beggiatoa* mat, where less  $\Sigma\text{H}_2\text{S}$  was available, the pH decreased. However, the pH in this layer did not decrease to values lower than those in the uninoculated control (Fig. 4A and C). Therefore, there was no indication that oxidation of  $\text{S}^0$  to  $\text{SO}_4^{2-}$  took place in the upper region of the *Beggiatoa* mat. However, if oxidation of  $\text{S}^0$  to  $\text{SO}_4^{2-}$  occurred at all,  $\text{NO}_3^-$  rather than  $\text{O}_2$  must have been used as the electron acceptor, because the  $\text{O}_2$  flux into the *Beggiatoa* mat was extremely low. The measured pH profiles are consistent with the results of a recent study of Sayama et al. (20), in which these authors found similar pH profiles in marine sediment colonized with *Beggiatoa* spp. It was hypothesized that the oxidation of  $\text{H}_2\text{S}$  to  $\text{S}^0$  occurred with  $\text{NO}_3^-$  and was not necessarily spatially coupled to the oxidation of  $\text{S}^0$  to  $\text{SO}_4^{2-}$ .

Furthermore, Sayama et al. (20) demonstrated that the marine *Beggiatoa* spp. investigated reduce  $\text{NO}_3^-$  to  $\text{NH}_4^+$  under anoxic conditions (dissimilatory nitrate reduction to ammo-

nium). This metabolic pathway was also hypothesized to occur in other marine sulfur bacteria (19) and is known to occur in large marine *Thioploca* spp. (15) that are close relatives of large marine *Beggiatoa* spp. (22). Another possibility for anaerobic  $\Sigma\text{H}_2\text{S}$  oxidation with  $\text{NO}_3^-$  is denitrification, which was discussed by Sweerts et al. (21) for freshwater *Beggiatoa* spp. To date, this study is the only study in which anaerobic  $\Sigma\text{H}_2\text{S}$  oxidation with  $\text{NO}_3^-$  was postulated for freshwater *Beggiatoa* spp., but questions about contamination of the *Beggiatoa* filaments with unicellular denitrifying bacteria have been raised by other authors (5, 11). The *Beggiatoa* enrichment culture used in our study also contained unicellular bacteria. Despite numerous trials, a pure culture could not be obtained, suggesting that this *Beggiatoa* strain is not able to grow without associated bacteria, which is a well-known phenomenon for other bacteria (8). However, the visibility of the *Beggiatoa* filaments in the transparent agar can be used. Using a stereomicroscope, it was observed that  $\text{NO}_3^-$  had an effect on the filaments because the *Beggiatoa* mat position and thus the chemotactic response of the filaments to  $\text{O}_2$  and  $\Sigma\text{H}_2\text{S}$  were indeed changed. Alternatively, the movement of the *Beggiatoa* filaments may have resulted from an intimate association with unicellular  $\text{NO}_3^-$  reducers, which were directly responsible for the  $\Sigma\text{H}_2\text{S}$  oxidation, and because of an absolute dependence of the *Beggiatoa* sp. on these reducers, the *Beggiatoa* sp. followed the movement of the  $\text{NO}_3^-$  reducers in the gradient tubes. However, this seems unlikely because in this case the *Beggiatoa* sp. would have had to disassociate from the energetically favorable electron acceptor  $\text{O}_2$ . Hence, the changed chemotactic response of the *Beggiatoa* sp. strongly suggests that the freshwater *Beggiatoa* filaments themselves were chiefly responsible for the anaerobic  $\Sigma\text{H}_2\text{S}$  oxidation with  $\text{NO}_3^-$ .

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