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Sulfide-Oxidizing Activity and Bacterial Community Structure in a Fluidized Bed Reactor from a Zero-Discharge Mariculture System

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In the present work we describe a comprehensive analysis of sulfide oxidation in a fluidized bed reactor (FBR) from an environmentally sustainable, zero-discharge mariculture system. The FBR received oxygen-depleted effluent from a digestion basin (DB) that is responsible for gasification of organic matter and nitrogen. The FBR is a crucial component in this recirculating system because it safeguards the fish from the toxic sulfide produced in the DB. Microscale sulfide oxidation potential and bacterial community composition within FBR biofilms were correlated to biofilter performance by integrating bulk chemical, microsensor (O_2, pH, and H_2S), and molecular microbial community analyses. The FBR consistently oxidized sulfide during two years of continuous operation, with an estimated average sulfide removal rate of 1.3 g of sulfide-S L_{FBR}^{-1} d^{-1}. Maximum sulfide oxidation rates within the FBR biofilms were 0.36 and 0.21 mg of sulfide-S cm^{-3} h^{-1} in the oxic and anoxic layers, respectively, indicating that both oxygen and nitrate serve as electron acceptors for sulfide oxidation. The estimated anoxic sulfide removal rate, as extrapolated from bench scale, autotrophic, nitrate-amended experiments, was 0.7 g of sulfide-S L_{FBR}^{-1} d^{-1}, which is approximately 50% of the total estimated sulfide removal in the FBR. Community composition analyses using denaturing gradient gel electrophoresis (DGGE) of bacterial 16S rRNA gene fragments from FBR samples taken at six-month intervals revealed several sequences that were closely affiliated with sulfide-oxidizing bacteria. These included the denitrifying, sulfide-oxidizing bacteria *Thiomicrospira denitrificans*, members of the filamentous *Thiothrix* genus, and sulfide-oxidizing symbionts from the Gammaproteobacteria. In addition, marine Alphaproteobacteria and Bacteroidetes species were present in all of the DGGE profiles examined. DGGE analyses showed significant shifts in the bacterial community composition between profiles over two years of sampling, indicating the presence of a diverse and dynamic microbial community within the functionally stable FBR. The FBR's combined capacity for both oxic and anoxic sulfide oxidation, as indicated by bulk chemical, microsensor, and molecular microbial analyses, gives it significant functional elasticity, which is crucial for proper performance in the dynamic environment of this mariculture system.

Introduction

Excessive fishing due to increased consumption, water pollution, and destruction of natural habitats has caused depletion of world fish stocks in natural aquatic environments. This phenomenon has generated a rising interest in the field of aquaculture. Culture of marine fish (mariculture) is conducted almost exclusively in cages and fenced ponds adjacent to the sea. Often these culture methods, based on a rapid exchange of the culture water with "unpolluted" seawater, do not include means for pollutant removal from their effluents. Discharge from mariculture facilities has been associated with eutrophication of adjacent coastal waters, alterations in the natural flora and fauna by contamination with heavy metals and antibiotics, and pollution of wild fish stocks by mariculture "escapees" (*1*, *2*). One approach to reduce the environmental impact of the fish farming industry is to shift operations to inland recirculating systems. Current recirculating systems primarily treat ammonia by use of ammonia-oxidizing biofilters. The largely untreated organic and nitrogen waste is discharged by daily water exchange (5−20%), which is either environmentally detrimental or expensive to treat. A unique, environmentally sustainable, zero-discharge intensive fish culture system (Figure 1) has been previously described for culture of both freshwater (*3*) and marine fish (*4*, *5*). Water in this system is treated by recirculation through two separate treatment loops, one aerobic and the other primarily anoxic. Ammonia is oxidized to nitrate in the aerobic loop by a trickling filter (TF). Concurrently, organic matter and nitrate are gasified by denitrification in the anoxic loop, which is comprised of a digestion/sedimentation basin followed by a fluidized bed reactor (FBR). Anoxic treatment leads to significant removal of both organic matter and nitrogen from the mariculture system, enabling long-term steady-state operation without water discharge.

A major drawback of anoxic treatment of organic-rich seawater is the reduction of sulfate to sulfide in nitrate-depleted zones (*6*) and consequently, in the case of mariculture systems, sulfide toxicity to the aquatic organisms (*7*). The ability to remove sulfide from wastewater has been previously demonstrated using a variety of biological reactors under highly diverse conditions (*8*−*12*). It was demonstrated that bioreactors implementing natural developing biofilms are more effective and more stable than those using pure cultures (*13*). Furthermore, heterogeneous biofilms are believed to be highly resilient under fluctuating environmental conditions due to their greater taxonomic and physiological diversity (*14*).

The present study focused on acquiring a comprehensive understanding of sulfide oxidation in the FBR. FBR perfor-

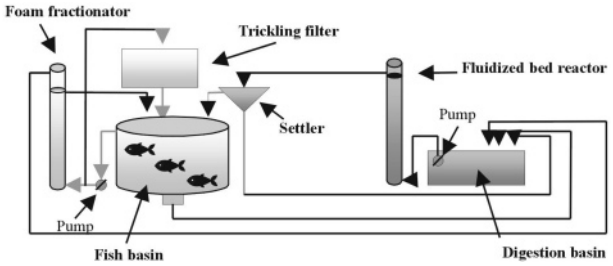

FIGURE 1. Schematic diagram of the zero-discharge mariculture system.

mance was evaluated in two pilot mariculture systems in Rehovot and in Eilat, Israel (3 and 11.5 m^3 total volume, respectively) by periodical chemical analyses of the reactor in- and outflow. Microsensors were used to measure local activities and pinpoint microenvironments of sulfide oxidation within the biofilm of selected FBR flocs, and bench-scale flow columns were used to assess the bulk anoxic sulfide oxidation potential of FBR flocs. Concomitant to chemical analyses, the bacterial community composition of the FBR floc biofilm was assessed at six-month intervals over two years using PCR-denaturing gradient gel electrophoresis (DGGE). Subsequently, phylogenetic analysis of excised DGGE bands was performed to identify key bacterial populations, specifically those potentially responsible for sulfide oxidation in the FBR.

Materials and Methods

Experimental Fish Culture System. The overall performance of the intensive recirculating aquaculture systems studied here (schematic diagram shown in Figure 1) has been previously described in detail (*3*, *5*). Two culture systems were examined in this study, one at the Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem in Rehovot, and the other at Israel Oceanographic and Limnological Research, National Center for Mariculture in Eilat (3 and 11.5 m^3 total volumes, respectively). In both systems, water from the upper part of a double-drain fish basin (2.3 m^3 in Rehovot and 10 m^3 in Eilat) was pump-recirculated at a rate of 1−2 fish basin volumes h^{-1} over a trickling filter. Simultaneously, water from the bottom center of the fish basin flowed into a digestion basin (DB) (working volumes of 0.4−0.5 and 1−2 m^3, respectively) at rates of 0.1−0.25 fish basin volumes h^{-1}. Water from the upper layers of the DB outlet was pumped (at rates of 5−7 and 15−40 L min^{-1}, respectively) into the FBR through a vertical pipe that extended into the water/sand slurry from the top center to approximately 3 cm above the base center of the FBR. The FBR (working volumes of 6.26 and 53 L, respectively) was filled with sand (average diameter 0.7 mm), which served as a carrier material for the biofilm. Flocs within the FBR were highly heterogeneous in both size and appearance, with diameters ranging between 0.7 and 5.0 mm. From the top of the FBR, water was drained back to the fish basin after passing through a settler for removal of particulate matter, which dripped back into the DB.

The system in Rehovot was monitored from December 1999 to December 2001 and the system in Eilat from May 2000 to August 2001, during which sulfide was monitored from May to August 2001. Artificial seawater (ASW) in the Rehovot system (salinity 20 ppt) was prepared by addition of sea salt (Red Sea Pharm Ltd., Eilat, Israel) to tap water and in Eilat (36 ppt) by mixing filtered seawater with tap water. Water for chemical analyses was taken from the inflow and from the outlet of the FBR. Flocs for molecular analyses and crude culture experiments were sampled via faucets positioned along the FBR, or by inserting a beaker from the top to the desired depth.

Physiochemical Measurements, Rehovot. Oxygen and temperature were measured with a YSI temperature/oxygen probe (model 57, Yellow Spring Instruments), and pH was measured using a Radiometer pH meter (model PHM92, Copenhagen, Denmark). Samples for nitrate analysis were filtered (0.2 μm) and kept frozen at −20 °C until analyzed. Nitrate was analyzed with the Szechrome Nas reagent provided by the Applied Research Institute, Ben-Gurion University of the Negev, Israel (*15*). For sulfide analysis, 2.5 mL samples were directly filtered (0.2 μm) into 1 mL of 5% zinc acetate solution, and the sulfide concentration was determined with the methylene blue method (*16*). Total sulfide measurements (H_2S, HS^-, and S^{2-}) are expressed throughout the text as S^{2-}.

Physiochemical Measurements, Eilat. Oxygen, temperature, and pH were measured continuously by an automatic monitoring/alarm system (Point Four, Port Moody, BC, Canada). Samples for nitrate and sulfide analysis were filtered through 1.2 μm glass fiber filters (Whatman GF/C) and were analyzed immediately or stored at 4 °C until analyzed, not later than 48 h afterward. Nitrate and sulfide were analyzed using a Technicon Autoanalyzer II (Bran and Luebbe GMBH, Norderstedt, Germany). Nitrate was measured as previously described (*17*); sulfide was instantly fixed and then measured by the dimethyl-*p*-phenylenediamine dihydrochloride and ferric chloride transformed to methylene blue method as described by the manufacturer.

Bench-Scale Analyses of FBR Sulfide Oxidation Potential. Controlled, bench-scale model FBR systems were constructed to assess potential anoxic sulfide oxidation by FBR flocs under autotrophic conditions. The experimental setup consisted of a 4.5 L vessel which contained degassed ASW supplemented with 10 mM nitrate (final concentration) connected via silicon tubing and a peristaltic pump to three 15 mL glass columns. Samples of 5 g wet weight of flocs were taken from the Rehovot FBR, washed twice in ASW, and immediately transferred to the glass columns. The medium vessel was constantly purged with N_2, and the system flow rate was 1−2 mL min^{-1}. The system was operated for 10 h to reach equilibrium, and samples for sulfide analysis were extracted using a 5 mL sterile syringe from designated inlet and outlet sampling stations at equal intervals over a 400 min period. The medium was autoclaved (121 °C, 20 min) and cooled for 1 h under N_2 flow. Two processes were assessed in triplicate analyses and in independent runs in the flow systems: (a) chemolithotrophic sulfide oxidation, ASW with 5 g wet weight of vital flocs was supplemented with sterile 1 M sodium sulfide solution to give a final concentration of 900 μM total sulfide; (b) chemical sulfide oxidation, the treatment was repeated with 5 g wet weight of autoclaved (121 °C, 20 min) flocs.

Microsensor Analysis. Microsensor measurements were performed in a separate aquarium setup as previously described (*18*). Briefly, samples were placed in a small flow cell accessible to microsensors from above. Filtered ASW (see above) was circulated through the flow cell from a 5 L reservoir by use of a submersible pump with a flow velocity of 1 cm^3 s^{-1}. Sulfide oxidation measurements were performed using ASW supplemented with nitrate (1 mM final concentration) and sulfide (40−80 μM total sulfide). Oxygen was kept at a level of 4% air saturation via purging with N_2. Replicate profiles of each parameter, i.e., O_2 (*19*) and pH and H_2S (*20*), were measured on several individual flocs.

For all experiments, flocs sampled from the Rehovot FBR were transferred to the flow-through system and equilibrated for a minimum of 2 h for adaptation to the experimental conditions before measurements were initiated. Local volumetric conversion rates of sulfide oxidation were calculated from measured concentration microprofiles for different layers within the biomass. For this purpose, a stepwise

procedure was applied to each individual profile as described earlier (*21*). Molecular diffusion coefficients (D_o) in water of 2.16×10^{-5} and 2.2×10^{-5} cm^2 s^{-1} were used for H_2S. The ratio between the effective diffusion coefficients and the molecular diffusion coefficients (D_{eff}/D_o) was assumed to be 0.9 for diffusion within the biofilm.

DNA Extraction and PCR. DNA was extracted from 1 g wet weight of FBR flocs by a modified bead-beating method described previously (*22*). Pooling of DNA from multiple flocs (ca. 10−20 flocs) was performed to reduce the potential for variations within specific sampling time points. The extracted DNA was stored at −20 °C until it was further processed. PCR amplifications for DGGE were performed with the previously described bacterial primer pair 341F with a GC clamp at its 5′ end and 907R (*23*) using a T-gradient thermocycler (Biometra, Goettingen, Germany). Each 50 μL reaction contained the following components: 1.5 U of *Taq* DNA polymerase (Red Taq; Sigma, St. Louis, MO), *Taq* buffer containing a final magnesium concentration of 1.5 mM, dNTPs, 20 nmol each, 12.5 μg of bovine serum albumin, 25 pmol of each primer, and 1 μL of DNA template. The PCR program was carried out with an initial denaturation step of 95 °C for 30 s followed by 33 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s. Cycling was completed with a final elongation stage at 72 °C for 2 min. PCR of species from the *Thiomicrospira denitrificans* cluster from the Epsilonproteobacteria was performed with the forward primer epsilon257, 5′-ATGAC-GGGTAGCGGGTTT-3′, and the reverse primer epsilon1437, 5′-CTTACGGTGGAATTAGCG-3′, as described above, with an annealing step of 60 °C for 30 s and an elongation step of 72 °C for 1 min. The presence and size of the PCR fragments were determined by agarose gel electrophoresis (1−2%) and staining with ethidium bromide.

DGGE Analysis. Denaturating gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments was performed using a D-gene system (BioRad, Hercules, CA), as previously described (*23*). In this analysis, 1 mm thick 6% (w/v) polyacrylamide gels with a 20−55% denaturing gradient were run for 16 h in 1× TAE buffer at a constant voltage of 100 V. Following electrophoresis, the gels were stained with GelStar nucleic acid stain (Bio Whittaker Molecular Applications, Inc., Rockland, ME) and photographed on a UV transillumination table (302 nm) with a KDS digital camera (Kodak Co., New Haven, CT) using imaging software provided by the supplier. Prevalent DGGE bands were carefully excised on a Dark Reader transilluminator (Clare Chemical Research, Inc., Dolores, CO) and purified as described previously (*23*). Purified bands were reamplified and verified by a second DGGE analysis.

Cloning of PCR Products and Sequencing. The pGEM-T Easy vector system (Promega, Madison, WI) was used to clone PCR products amplified from excised DGGE bands. Ligation and transformation reactions were performed according to the protocol described by the manufacturer. Clones were screened by colony PCR amplification and verified by DGGE (as described above). Plasmids from selected colonies were purified with the Concert Rapid Miniprep plasmid purification system (Gibco BRL Products, Rockville, MD) according to the protocol provided by the manufacturer.

Clones were sequenced using the Applied Biosystems PRISM Dye Terminator cycle sequencing ready reaction kit with Ampli Taq DNA polymerase and the T7 primer as suggested by the manufacturer. The sequencing products were analyzed with an Applied Biosystems 377 DNA sequencer.

Phylogenetic Analysis. Nucleotide sequences (530 bp) were incorporated into a prealigned database of 16S rRNA sequences using the aligning tool supplied by the ARB phylogenetic program package (*24*). Phylogenetic trees were generated with neighbor-joining and maximum likelihood methods provided with the ARB program package using the Felsenstein correction method and applying a 50% cutoff filter to remove highly variable positions (*24*). The topologies of the resulting trees were identical for both methods.

Nucleotide Sequence Accession Numbers. The 16S rRNA sequences determined in this study have been deposited in the GenBank database under accession numbers AY263712 to AY263737.

Results and Discussion

Digestion of particulate matter is an efficient method for organic and nitrogen removal from mariculture effluent. However, organic matter digestion in low-redox zones of accumulating sludge can generate hydrogen sulfide, which is highly toxic to fish. The FBR deployed in this system was found to facilitate sulfide oxidation of DB effluent, thus safeguarding the fish from toxic sulfide concentrations. To improve understanding of the sulfide-oxidizing processes in the FBR, bulk chemical, microsensor, and molecular-microbial community analyses were combined to (a) evaluate the long-term sulfide oxidation capacity of the FBR, (b) determine the spatial distribution of sulfide oxidation within individual FBR flocs, and (c) analyze bacterial community composition with the aim of identifying bacterial communities responsible for sulfide oxidation in the FBR.

Bulk Chemical Analyses. FBR performance was evaluated by periodical measurements of inlet and outlet oxygen, nitrate, and sulfide concentrations. Oxygen concentrations in both pilot systems ranged from 20 to 40 μM at the FBR inlet and from 0 to 30 μM at the FBR outlet. Nitrate concentrations ranged between 1000 and 4000 μM in Rehovot and between 40 and 4150 μM in Eilat with average nitrate removal rates by the FBRs of 68 and 43 μM min^{-1}, respectively. Sulfide oxidation was monitored for 25 months in the Rehovot FBR (Figure 2a) and 3.5 months in the Eilat FBR (Figure 2b). Sulfide concentrations and removal rates in the FBR were dictated by daily feeding regimes and thus fluctuated on a diurnal basis (Jaap van Rijn, unpublished results). Furthermore, sulfide production in the DB was influenced by the level of organic loading of the DB (determined by the feed/feces ratio). Inlet sulfide concentrations in the Rehovot FBR fluctuated between 0 and 80 μM with an average FBR sulfide removal rate of 30 μM min^{-1} (8.3 g of S^{2-} d^{-1}, 1.3 g of S^{2-} L_{FBR}^{-1} d^{-1}). For the first two months of analysis, sulfide oxidation rates in the Eilat FBR averaged 12 μM min^{-1} (19.3 g of S^{2-} d^{-1}, 0.36 g of S^{2-} L_{FBR}^{-1} d^{-1}), with inlet values of 25−50 μM and outlet values normally below the detection limit. In mid-July 2001, with nitrate concentrations dropping below 1500 μM, sulfide concentrations entering the FBR increased dramatically and reached 500 μM (Figure 2b). The increase in sulfide at the FBR inlet generated a proportional increase in the FBR's sulfide oxidation rate, which reached a maximum of 186 μM min^{-1} [300 g of S^{2-} d^{-1}, 5.7 g of S^{2-} L_{FBR}^{-1} d^{-1}, 40 mg of S^{2-} (g of organic matter)$^{-1}$ d^{-1}]. This demonstrates the high sulfide-oxidizing capacity and exceptional quantitative resilience of this biofilter.

Microsensor Assessment of Sulfide Oxidation. To identify chemical microenvironments and pinpoint local sulfide oxidation activities within the FBR floc biofilm, microsensors for O_2, pH, and H_2S were applied. Analyses were performed on two floc types, distinguished by their macroscopic appearance. F-type flocs (average diameter of 3.0 mm) were generally large and densely coated with white filamentous bacteria, with intracellular sulfur globules phylogenetically affiliated with the genus *Thiothrix* (see below). The smaller P-type flocs (average diameter of 1.2 mm) had a plain surface and a light brown color. The microscale sulfide oxidation potential was assessed by incubating FBR flocs in nonorganic

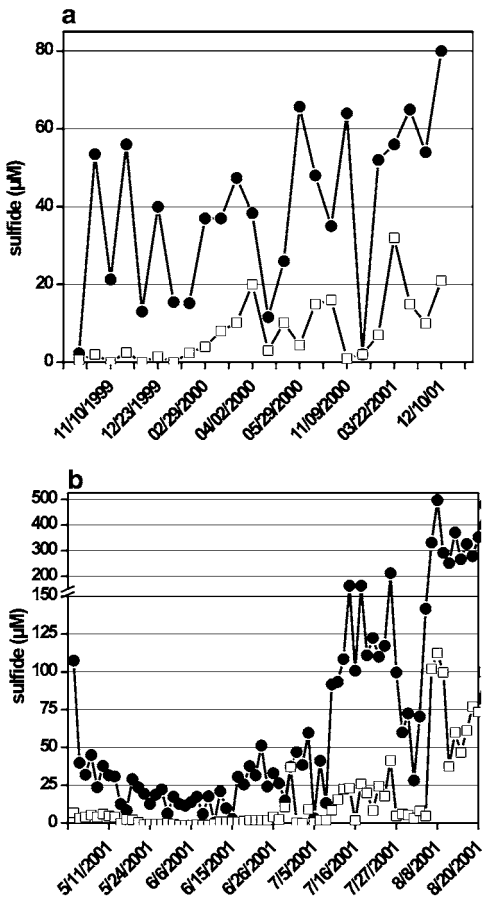

FIGURE 2. Sulfide concentrations in the Rehovot (a) and Eilat (b) fluidized bed reactors, over 25- and 3-month periods, respectively. Symbols: inlet (●) and outlet (□) total sulfide concentrations.

sulfide- and nitrate-amended ASW. To mimic in situ system conditions, oxygen in the medium was kept at 12 μM (4.3% air saturation) and was below 3 μM (1% air saturation) in the biofilm. Under these conditions, white filaments developed on the surface of F-type and certain P-type flocs after several hours of incubation. Although the range of activity differed between individual flocs, consumption of sulfide (sum of H_2S, HS^-, and S^{2-}) was observed close to the surface for both biofilm types (Figure 3). Maximum sulfide oxidation rates of 11.2 and 7.6 μmol of S^{2-} cm^{-3} h^{-1} were detected for F- and P-type flocs, respectively. The pH decreased less in the P-type flocs (about 0.1 unit h^{-1}) than in the F-type flocs (about 0.6 unit h^{-1}), seemingly in proportion to the sulfide oxidation rates. Sulfide oxidation was also detected in the anoxic layer of the floc biofilm, suggestive of sulfide oxidation coupled to nitrate reduction. In these anoxic zones maximum sulfide oxidation rates of 6.5 and 1.6 μmol of S^{2-} cm^{-3} h^{-1} were measured for the F- and the P-type flocs, respectively. The extrapolation of microsensor data measured on single flocs to the macroscopic performance of the FBR system only gives a rough estimate of the ratio of oxic versus anoxic sulfide oxidation. Local rates of sulfide oxidation integrated over the oxic and the anoxic layers of the floc indicated that the anoxic sulfide oxidation represents 30% (P-type flocs) and 60% (F-type flocs) of the total sulfide oxidation.

Anoxic Sulfide Oxidation Potential of FBR Flocs. To further explore anoxic sulfide oxidation in the fluidized bed reactor and assess the FBR's anoxic sulfide oxidation potential, flocs from the Rehovot system were transferred to 15 mL flow columns fed with oxygen-free nitrate- and sulfide-amended ASW. Sulfide was oxidized at an average rate of 3.23 μmol (g of biofilm dry weight)$^{-1}$ min^{-1} for the entire duration of the experiment (400 min) in columns containing FBR flocs with no sulfide oxidation in columns containing autoclaved flocs (Figure 4). This implies that sulfide oxidation detected in anoxic zones of the biofilm is a microbial-mediated process, with nitrate potentially serving as an electron acceptor. Normalizing this analysis to the FBR working volume in the Rehovot mariculture system, we calculated a potential anoxic sulfide oxidation removal rate of approximately 4.6 g of S^{2-} d^{-1} (0.7 g of S^{2-} L_{FBR}^{-1} d^{-1}). This value is approximately half of the total sulfide oxidation rate (8.3 g of S^{2-} d^{-1}) derived from the bulk analyses on the same reactor (see above). Coherent with these values are the results obtained with microsensor analyses (see above), which pointed to anoxic sulfide oxidation rates between 30% and 60% of the total sulfide oxidation in the FBR flocs.

Bacterial Community Composition. PCR−DGGE analysis of partial 16S rRNA gene sequences was employed to study and monitor changes in the bacterial community structure in the FBR (Figure 5). The DGGE method restricts the length of the analyzed PCR products (530 bp), which may limit the

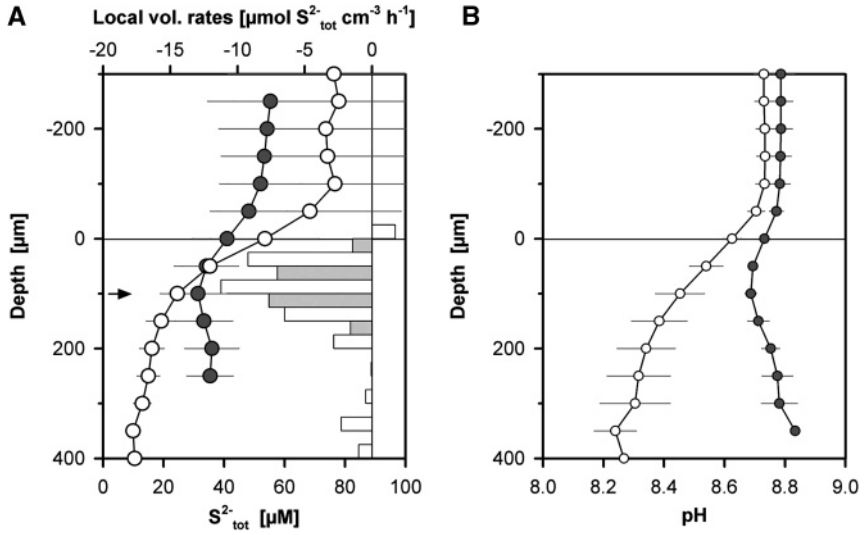

FIGURE 3. Sulfide oxidation within FBR flocs as measured with microsensors. (A, left) Averaged total reduced sulfide profiles (white, $n = 6$ profiles; gray, $n = 4$ profiles) and local sulfide oxidation rates (bars). Errors are 95% confidence limits of the mean. (B) Averaged pH profiles (white, $n = 4$ profiles; gray, $n = 3$ profiles). White color refers to F-type flocs, gray to P-type flocs; the arrow indicates the oxygen penetration depth.

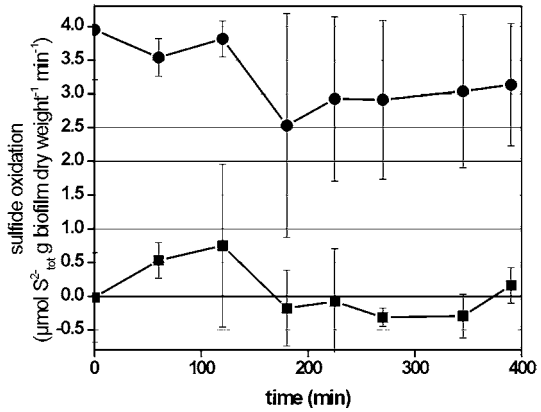

FIGURE 4. Anoxic sulfide oxidation rates in bench-scale experimental columns containing FBR flocs. Columns were incubated with ASW containing 900 μM total sulfide and 10 mM nitrate. Symbols: (●) vital flocs and (■) autoclaved flocs (control). Error bars represent standard deviations in sulfide concentrations between triplicate samples.

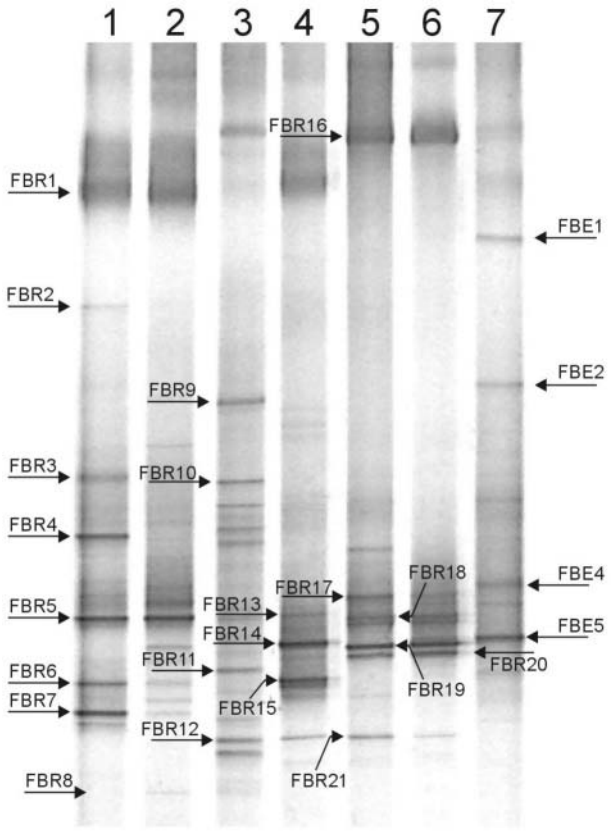

FIGURE 5. DGGE analysis of 341f-907r PCR-amplified 16S rRNA gene fragments from the fluidized bed reactor. Arrows indicate excised bands. Lanes 1−5, FBR samples from the Rehovot system, December 1999, April 2000, November 2000, April 2001, and December 2001; lane 6, white filaments from the December 2001 sample; lane 7, FBR sample from the Eilat system, August 2001.

phylogenetic placement of the analyzed bacterial strains. Nonetheless, it is believed that these sequences are adequate for inferring phylogenetic affiliation above the species level and that they can provide information regarding the physiological and metabolic characteristics of these strains (*25*). Prevalent DGGE bands were sequenced to identify significant bacterial populations, specifically those potentially responsible for sulfide oxidation. Five samples (December 1999, April 2000, November 2000, April 2001, and December 2001) were analyzed together with a single sample from the FBR of the Eilat system (August 2001). DGGE band patterns of identical sampling time points were highly similar, and therefore, one profile of each is presented. A total of 25 prevalent DGGE bands from all of the samples were excised, sequenced, and phylogenetically analyzed together with related bacterial sequences (Figures 6 and 7).

A majority of the identified bacteria (14 out of 25) were from the Proteobacteria phylum (Figure 6). Several of these proteobacterial populations were highly similar to cultured sulfide-oxidizing bacteria, consistent with the chemical evidence of sulfide oxidation in the FBR. Strain FBR3 (Figure 6) was closely related (98% sequence identity) to the obligatory chemolithotrophic nitrate-reducing, sulfide oxidizer *T. denitrificans* sp. CVO (*26*). While most prevalent in the December 1999 sample, identical species were detected in all Rehovot FBR samples following amplification with specific primers (data not shown). Significant occurrence of *T. denitrificans* related species concurs with data from the flow column experiments and from sulfide oxidation detected in anoxic layers of the FBR floc biofilm via microsensor analyses.

Increased FBR inlet sulfide concentrations (December 2001, Figure 6) often resulted in the formation of dense white filament-coated (F-type) flocs containing bacteria with intracellular sulfur globules. Phylogenetic analysis revealed that the major constituents of these filaments were *Thiothrix* species (bands FBR 19 and FBR 20) most similar (95%) to *Thiothrix disciformis* (Figure 6). Fluorescent in situ hybridization analyses using both gammaproteobacterial (*27*) and *Thiothrix*-specific (*28*) probes verified that these filamentous bacteria were members of the *Thiothrix* genus (Eddie Cytryn, unpublished results). *Thiothrix* are filamentous sulfide-oxidizing bacteria that have been previously detected in wastewater treatment facilities (*29*), in sulfidic marsh waters (*30*), in deep-sea vents (*31*), and recently in a high-diversity biofilm for the oxidation of sulfide-containing effluents (*13*). *Thiothrix* are metabolically versatile, capable of autotrophic, mixotrophic, and heterotrophic growth (*29*, *32*). They are generally considered to be obligatory aerobes, although some strains showed metabolic activity under denitrifying conditions where intercellular sulfur globules were formed (*29*). *Thiothrix* species have been shown to attach to solid surfaces by producing gonidial holdfast material (*32*), which may be essential for endurance and might increase transport of substances into the biofilm via advection in the high-flow environment of the FBR. The potential significance of *Thiothrix* in sulfide oxidation in the FBR was demonstrated by proliferation of *Thiothrix* filaments following sulfide amendment and by the higher sulfide oxidation rates in *Thiothrix*-containing F-type flocs, compared to P-type flocs, in microsensor analyses.

The gammaproteobacterial strain FBR5 (Figure 6), dominant in sequential samples from December 1999 and April 2000, was affiliated (94%) with a cluster of symbiotic sulfide-oxidizing bacteria found in invertebrates in sulfide-rich marine environments (*33*). An identical sequence (DBS6I) was previously detected in the digestion basin (DB) of the Rehovot system (*6*), and highly similar sequences (BT4A and BT3J) were previously identified in the Eilat DB. RNA profiles using RT-PCR−DGGE showed that the activity of these strains proliferated following incubation of DB sludge and FBR flocs in sulfide- and nitrate-amended ASW (Eddie Cytryn, unpublished results), supporting the notion that these species are capable of nitrate-mediated sulfide oxidation.

Eight sequences were phylogenetically associated with four clusters from the Rhodobacteraceae family with high sequence similarity to *Paracoccus denitrificans* (FBE5), *Rhodovulum sulfidophilum* (FBR6), *Roseobacter denitrificans* (FBR13), and *Ruegeria gelatinovorum* (FBR17, FBR18) (Figures

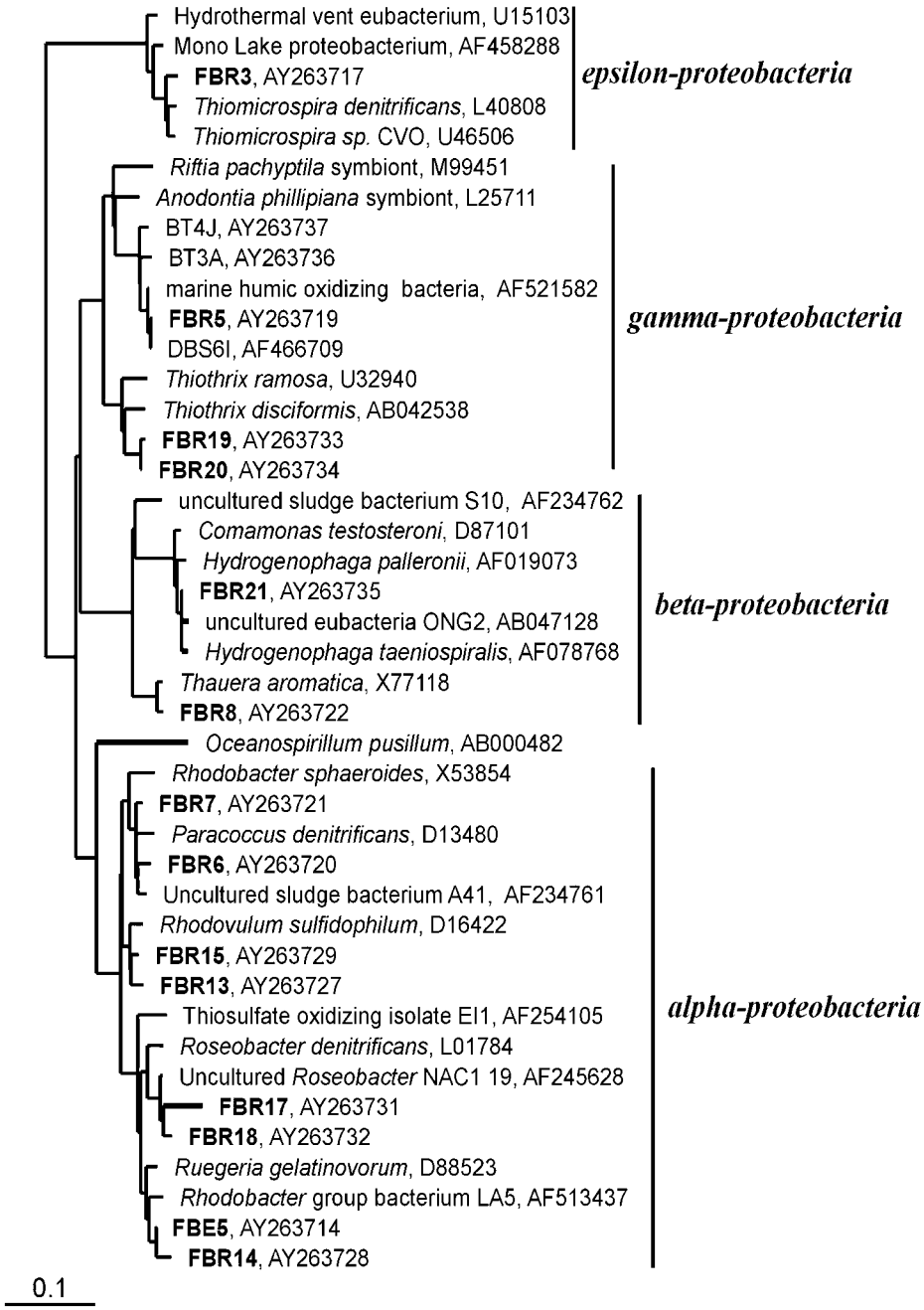

FIGURE 6. Proteobacterial phylogenetic tree based on partial 16S rRNA sequences showing the relation of excised DGGE band sequences from the FBR in relation to previously documented sequences. The tree was inferred with the neighbor-joining method using a 50% similarity cutoff filter and the Felsenstein correction method. The scale bar represents a 10% estimated difference in nucleotide sequence positions.

5 and 6). Previous studies have demonstrated that Rhodobacteraceae are highly abundant and widely distributed in various marine environments (*34*−*36*) and they have been found to be numerically dominant in the early colonization of submerged surfaces in marine waters (*37*, *38*). The ability to effectively colonize surfaces may give the Rhodobacteraceae a competitive advantage in the FBR where high flow rates, low retention time, and strong sheering forces may restrict colonization of surfaces. Rhodobacteraceae are known to exhibit a diverse range of metabolic activity. Various species can oxidize reduced sulfur species including sulfide under autotrophic, heterotrophic, or mixotrophic conditions (*39*−*43*), and recently, Rhodobacteraceae were found to be highly prevalent in a high-diversity biofilm for the oxidation of sulfide-containing effluents (*13*). The prevalence of Rhodobacteraceae in the FBR may stem from the metabolic elasticity of certain species from this group, giving them a competitive advantage in the FBR where diurnal and periodical fluctuations in organic matter, sulfide, and oxygen concentrations have been shown to exist (*5*). Further research will need to be carried out in an attempt to correlate among the phylogenetic affiliation of these species, their position in the biofilm and specific microenvironment, and their metabolic activity in the FBR.

Several nonproteobacterial sequences were identified (Figure 7). Seven sequences (FBE1,2, FBR1,2, FBR4, FBR10,11, and FBR16) belonged to the Bacteroidetes phylum, and several of these were closely related to species identified in marine environments. Bacteroidetes are a widely diverse group capable of degrading a variety of complex organic substances, and certain Bacteroidetes isolates have the ability to oxidize reduced sulfur compounds (*43*).

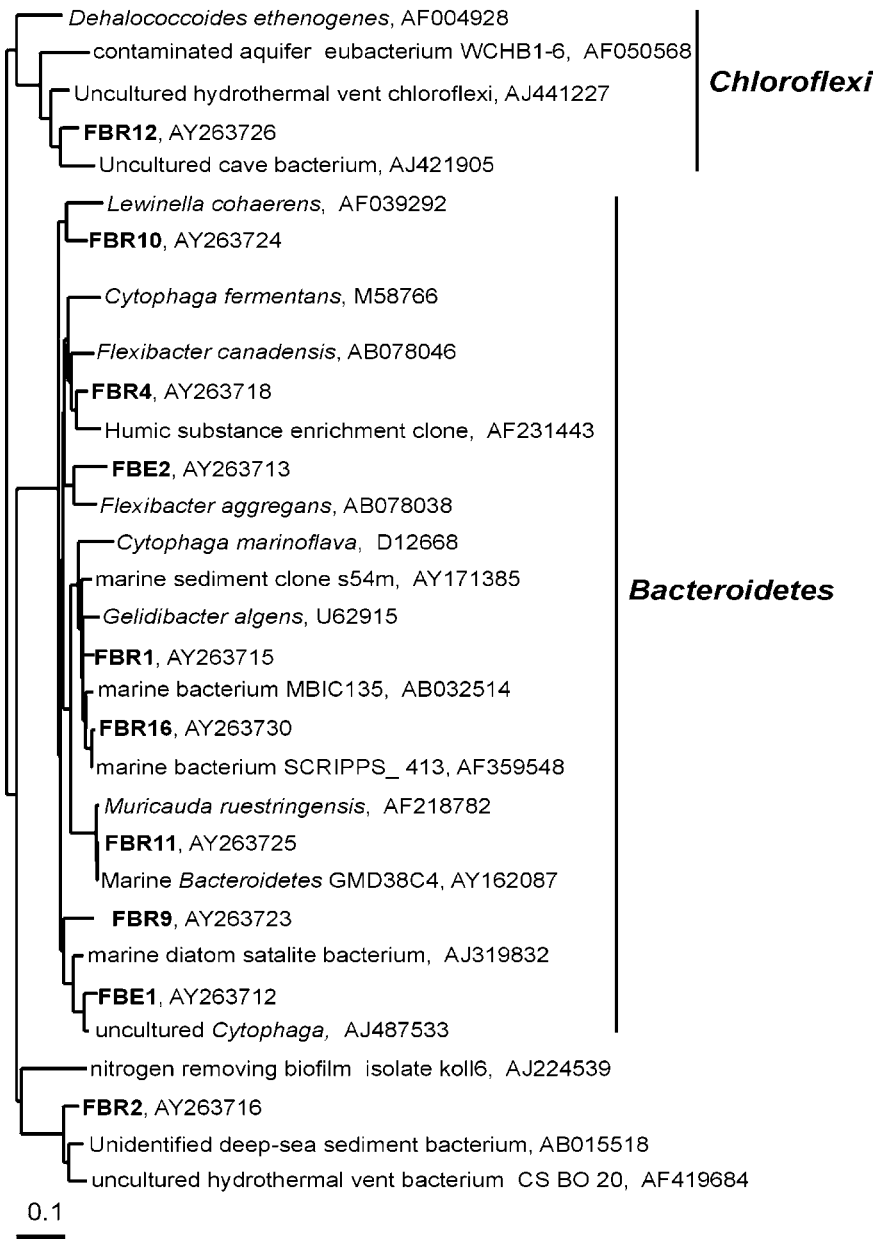

FIGURE 7. Nonproteobacterial phylogenetic tree based on partial 16S rRNA sequences showing the relation of excised DGGE band sequences from the FBR to previously documented sequences. The tree was constructed as described in Figure 6.

DGGE analysis of bacterial communities colonizing FBR flocs sampled over a two-year period (Figure 5) indicated substantial time-related shifts in the dominant consortia of the FBR biofilm despite the FBR's relatively high overall functional stability. Comparison of DGGE band patterns from successive sampling times (six-month intervals) showed only 19−24% similarity and between nonsuccessive sampling times, less than 14% similarity. In contrast DGGE band patterns from samples analyzed from identical time points showed over 90% similarity. Despite the significant change in the FBR bacterial consortia over time, strains belonging to the Rhodobacteraceae family and the Bacteroidetes phylum were significantly represented in all profiles from both the Rehovot and Eilat systems, implying the potential importance of these groups in the FBR. Previous studies have suggested that microbial diversity can provide a reservoir of closely related strains adapted to complementary ecological niches, resulting in superior bioreactor performance under changing environmental conditions (*14*). Bacteria not detected in specific DGGE profiles may have been below the PCR−DGGE detection limit, and these bacteria may still have been metabolically significant in the biofilm, possessing the potential to proliferate under optimal conditions. *T. denitrificans*-like species detected using group-specific primers but not in the general bacterial DGGE analysis demonstrated that this phenomenon existed in the FBR biofilm. Variations in bacterial community structure have been previously described in a variety of functionally stable bioreactors, indicating that a dynamic community can sustain a functionally stable ecosystem (*44*−*46*). In addition, modeling demonstrated that complex communities growing under multiple limiting substrate conditions can show chaotic community dynamics (*47*).

The comprehensive analysis provided in this study demonstrates the FBR's ability to efficiently oxidize sulfide produced during digestion of organic waste. The integration of microsensors and molecular microbial analyses enabled us to pinpoint microenviroments of sulfide oxidation in both oxic and anoxic zones and identify a diverse selection of bacterial communities that were likely responsible for oxic

and anoxic sulfide oxidation within the FBR flocs' biofilm. Future research will attempt to isolate potential sulfide-oxidizing bacteria detected in this study for a better understanding and control of their physiology and activity. Furthermore, combined use of microsensors and specific fluorescently labeled oligonucleotide probes will link the spatial distribution of specific sulfide oxidizers to oxic and anoxic zones of the FBR biofilm in an attempt to enhance understanding of oxic vs anoxic sulfide oxidation within the FBR biofilm. It is anticipated that a comprehensive understanding of the environmental factors and organisms underlying sulfide oxidation in these reactors will be instrumental in the design of sulfide-oxidizing FBRs to be used in future commercial land-based mariculture systems.

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