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Coexistence of Microaerophilic, Nitrate-Reducing, and Phototrophic Fe(II) Oxidizers and Fe(III) Reducers in Coastal Marine Sediment

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Iron is abundant in sediments, where it can be biogeochemically cycled between its divalent and trivalent redox states. The neutrophilic microbiological Fe cycle involves Fe(III)-reducing and three different physiological groups of Fe(II)-oxidizing microorganisms, i.e., microaerophilic, anoxygenic phototrophic, and nitrate-reducing Fe(II) oxidizers. However, it is unknown whether all three groups coexist in one habitat and how they are spatially distributed in relation to gradients of O_2, light, nitrate, and Fe(II). We examined two coastal marine sediments in Aarhus Bay, Denmark, by cultivation and most probable number (MPN) studies for Fe(II) oxidizers and Fe(III) reducers and by quantitative-PCR (qPCR) assays for microaerophilic Fe(II) oxidizers. Our results demonstrate the coexistence of all three metabolic types of Fe(II) oxidizers and Fe(III) reducers. In qPCR, microaerophilic Fe(II) oxidizers (*Zetaproteobacteria*) were present with up to 3.2×10^6 cells g dry sediment^{-1}. In MPNs, nitrate-reducing Fe(II) oxidizers, anoxygenic phototrophic Fe(II) oxidizers, and Fe(III) reducers reached cell numbers of up to 3.5×10^4, 3.1×10^2, and 4.4×10^4 g dry sediment^{-1}, respectively. O_2 and light penetrated only a few millimeters, but the depth distribution of the different iron metabolizers did not correlate with the profile of O_2, Fe(II), or light. Instead, abundances were homogeneous within the upper 3 cm of the sediment, probably due to wave-induced sediment reworking and bioturbation. In microaerophilic Fe(II)-oxidizing enrichment cultures, strains belonging to the *Zetaproteobacteria* were identified. Photoferrotrophic enrichments contained strains related to *Chlorobium* and *Rhodobacter*; the nitrate-reducing Fe(II) enrichments contained strains related to *Hoeflea* and *Denitromonas*. This study shows the coexistence of all three types of Fe(II) oxidizers in two near-shore marine environments and the potential for competition and interrelationships between them.

Iron occurs in natural environments with the oxidation state of Fe(II) or Fe(III). In the biogeochemical Fe cycle at neutral pH, redox transformations of Fe are mediated by a variety of biotic and abiotic processes (1). In the biotic part of this cycle, different metabolic types of microorganisms are involved. Some microorganisms use Fe(III) as an electron acceptor for oxidation of organic carbon or hydrogen [Fe(III)-reducing bacteria] (2). Others use Fe(II) as an electron donor coupled with the reduction of O_2 [microaerophilic Fe(II) oxidizers] (3) or nitrate [nitrate-reducing Fe(II) oxidizers] (4). Still others use Fe(II) as an electron donor for photosynthesis and couple Fe(II) oxidation with reduction (fixation) of CO_2, with light as the energy source [anoxygenic phototrophic Fe(II) oxidizers; so-called photoferrotrophs] (5).

Fe(III)-reducing microorganisms were discovered in the late 1980s (6). Many of the better-known Fe(III)-reducing bacteria of the genera *Shewanella*, *Geobacter*, and *Geothrix* are found mainly in freshwater and more rarely in marine sediments (7, 8). Still, Fe(III) reduction can account for a large fraction of organic-matter mineralization in coastal marine sediments (up to 50%) (9).

Fe(II)-oxidizing bacteria were first described in 1836 by Ehrenberg (10) as so-called "iron bacteria." It took more than a century before the first microbes belonging to this group could be grown in the laboratory (11) and even longer until it was demonstrated that they grow autotrophically by oxidizing Fe(II) with O_2 (12). These bacteria face the problem that, at neutral pH, the abiotic reaction of oxygen and Fe(II) is fast. Therefore, bacterial Fe(II) oxidation with O_2 at neutral pH is limited to micro-oxic ($[O_2] < 50 \mu M$) conditions, where microbial iron oxidation can compete favorably with the $[O_2]$-limited abiotic reaction (13, 14). Favorable conditions for the growth of microaerophilic Fe(II) oxidizers are found in opposing gradients of Fe(II) and O_2, e.g., in the oxic-

anoxic transition zone in sediments or groundwater seeps (15). Today, there are at least four known groups of exclusively microaerophilic Fe(II) oxidizers, i.e., *Gallionella* (11, 12), *Sideroxydans* (3), *Leptothrix* (16, 17), and *Mariprofundus* (18). Furthermore, certain bacteria belonging to the genera *Marinobacter* and *Hyphomonas* are also described as growing under micro-oxic conditions by Fe(II) oxidation (19, 20).

The photoferrotrophs (5) are not only of interest for modern habitats, but may also be responsible for the formation of banded iron formations (BIFs) in the Precambrian (21, 22). Until now, only a few pure cultures have been known, and there is no known monophylogenetic group that specializes solely in anoxygenic phototrophic Fe(II) oxidation. Known strains are metabolically flexible and belong to various physiological groups of anoxygenic phototrophs, i.e., the purple sulfur, purple nonsulfur, and green anoxygenic phototrophic bacteria (5, 23–25).

For the nitrate-reducing Fe(II) oxidizers, most known strains cannot be maintained in culture over several transfers with nitrate

and Fe(II) alone but need an organic cosubstrate. For these mixotrophic strains, it is not yet known whether Fe(II) oxidation is just a chemical side reaction with nitrite, which is produced during heterotrophic denitrification (26, 27), or if it is an enzymatic reaction from which the bacteria can gain energy. Interestingly, Fe(II) oxidation seems to be a universal ability of all NO_3^--reducing bacteria when an organic substrate is provided (28). A unique feature of this group of Fe(II) oxidizers is that the cells become encrusted with Fe(III) minerals during Fe(II) oxidation (27), a process that is potentially harmful to the cells (29). A few strains have been proposed to be able to live autotrophically with only NO_3^- and Fe(II) (4, 30–35), although unambiguous evidence for this is missing. One enrichment culture, KS (4), can grow autotrophically and can be transferred and cultivated continuously with only Fe(II) and NO_3^-, but the mechanism of Fe(II) oxidation and the responsible strain in the coculture are still unknown.

For the three physiological types of Fe(II)-oxidizing bacteria, not much is known about their abundance and spatial distribution in marine sediments. It is known that microaerophilic Fe(II) oxidizers can reach numbers of up to 10^7 cells/ml in iron-rich marine environments, such as Fe(II)-containing hydrothermal vents (36, 37). While *Gallionella*, *Sideroxydans*, and *Leptothrix* seem to be restricted to limnic and terrestrial habitats, the genus *Mariprofundus* may be restricted to marine habitats (38–42). Information about the presence of photoferrotrophs in marine sediments is scarce, and there are only two isolates, *Rhodovulum iodosum* and *Rhodovulum robiginosum*, which were both isolated from a tidal mud flat in the German Wadden Sea (43, 44). Almost nothing is known about the abundance and activity of nitrate-reducing Fe(II) oxidizers in marine habitats. It was shown by Emmerich et al. (45) that nitrate-reducing Fe(II) oxidizers can be found in hypersaline lake sediments, and Rowe et al. (46) showed that microorganisms present in marine sediment have the capability to oxidize solid-phase Fe(II) with nitrate.

Until now, it has been unclear whether the three different metabolic types of Fe(II)-oxidizing bacteria coexist in one habitat and potentially compete or interact with each other. One of the reasons for this lack of knowledge is that there are no functional genes that are uniquely linked to Fe(II) oxidation. Thus, there are no general molecular markers available for Fe(II) oxidizers, and consequently, studies of the abundance and activity of Fe(II) oxidizers rely mostly on cultivation-dependent methods. Two exceptions are the marine group of microaerophilic Fe(II) oxidizers, the *Zeta-proteobacteria*, and the freshwater *Gallionella* spp., since for these groups, specific 16S rRNA primers are available for quantification. These two groups of microaerophilic Fe(II) oxidizers are monophyletic, and all known members are microaerophilic Fe(II) oxidizers.

A typical coastal marine sediment has pore water concentrations of Fe(II) of up to 150 μM, nitrate concentrations of around 10 μM, and O_2 and light penetration depths of only a few millimeters (47–51). Therefore, there is potential for all three metabolic types of Fe(II) oxidizers to be present and active within the same sediment, and it can be expected that they show the highest abundances and activities in certain zones where the conditions are favorable. These Fe(II) oxidizers may live in association with Fe(III) reducers that provide the Fe(II) (52). It has been suggested that each ion of Fe is cycled on average 100 to 300 times before it is finally buried in the sediment (53). Based on the knowledge gaps described above, the goals of the present study were (i) to deter-

mine the abundance and spatial distribution of the three metabolic types of Fe(II) oxidizers and the Fe(III) reducers in two typical but geochemically distinct marine coastal sediments; (ii) to determine how their spatial distribution relates to O_2, nitrate, and light; and (iii) to enrich and isolate representative cultures of each group of Fe(II) oxidizers and Fe(III) reducers.

MATERIALS AND METHODS

Field sites and sampling of sediment. Sediment was sampled at two field sites in the Aarhus Bay area on the east coast of the peninsula of Jutland (Denmark). The field site called Norsminde Fjord is located in a shallow, brackish estuary with a narrow entrance from the Baltic Sea. Samples were taken near the entrance in the southeastern part of the fjord (56°1.171′N, 10°15.390′E). The water depth at the sampling site was about 0.5 m, with no significant tidal variation, while the salinity varied from 14 to 23 depending on local wind and precipitation. The sediment was organic-rich black mud. The field site Kalø Vig is not influenced by local freshwater sources, and the salinity varies from 20 to 23. Samples were taken directly along the sandy beach (56°16.811′N, 10°28.056′E) at water depths ranging from 0.5 to 1 m. The sediment was fine sand and silt with low organic matter content.

Samples were taken in different seasons from May 2013 until February 2015. For most geochemical and MPN data presented, sediment sampled in summer 2014 was used. Exceptions are the MPNs in deeper sediment layers (down to 18 cm), which were sampled in February 2015. Sediments for enrichment cultures of Fe(II) oxidizers and Fe(III) reducers were sampled at different times, as indicated below, where the methods used for the enrichment cultures are described. Sediment cores 15 to 20 cm long and with 2.5-cm inner diameters were taken with core liners made of 50-ml syringes with the front ends cut off. These core liners fit into a self-made slicing device, which enables sediment cores to be sliced with a depth resolution of 1 mm (54). Water samples for analysis of the overlying water were taken in 50-ml Falcon tubes. Processing of sediment samples for all measurements and analyses were performed as fast as possible after sampling of the sediment (a maximum of 2 days after sampling), and sediment was stored at 4°C until it was processed. Microsensor measurements were done directly after sampling.

Measurements in the field. The temperature, pH, salinity, and oxygen saturation of the water were measured directly in the field with a field multimeter (WTW; Multi 3430) equipped with a pH electrode with a temperature sensor (WTW; SenTrix), a conductivity electrode (WTW; TetraCon92), and an oxygen sensor (WTW; FDO925).

Microsensor measurements. Microsensor measurements were performed at *in situ* temperatures directly at the field site immediately after sampling. High-resolution profiles of dissolved O_2, pH, gaseous H_2S, and redox potential were measured with commercially available glass microelectrodes with a 100-μm tip diameter (Unisense, Denmark). Vertical profiles of oxygen were measured with a depth resolution of 200 μm and the remaining parameters with a resolution of 500 μm, using a manual micromanipulator. Data were recorded with the software Sensor Trace Pro (Unisense, Denmark). Before and during measurements, the overlying water in the sediment cores was aerated to prevent gradients in the overlying liquid phase. For each parameter, triplicate profiles were measured in each sediment core at different positions in the core. Concentrations of total sulfide (H_2S, HS^-, and S^{2-}) were calculated with the respective pH values at the same sediment depth by calculation of the pK_a.

Light profiles of scalar irradiance in the sediment cores were measured with self-made light sensors (55) connected to a spectrometer (USB4000; Ocean Optics, Germany). Profiles were measured with a resolution of 200 μm. For the measurements, sediment cores were illuminated with a halogen cold-light source (Euromex EK-1; Holland) from two sides. This sensor measured the scalar irradiance with wavelengths from 200 to 1,000 nm. Data were recorded with the software Spectra View (Ocean Optics, Germany). The scalar irradiance within the sediment was normalized to the irradiance in the supernatant of the core.

Pore water content and pore water geochemistry. The pore water content of the sediment was determined by weighing sediment slices (0.5-cm intervals from 0- to 3-cm depth) before and after drying the sediment for 3 days at 80°C. The dissolved organic carbon content (DOC) was measured in the water column and the sediment pore water. For pore water analysis of the DOC, nitrate, and nitrite, sediment from the upper 0 to 3 cm of a sediment core was centrifuged (15 min; $7,000 \times g$; Eppendorf 5430R). For DOC measurements after centrifugation, the supernatant was filtered through a 0.45-μm filter. The overlying water was also filtered through a 0.45-μm filter, and the DOC concentration was measured with a carbon analyzer (highTOC; Elementar, Germany). Nitrate and nitrite were quantified with a flow injection analyzer (FIA) equipped with a dialysis membrane that removed the iron to prevent side reactions during the measurement (Seal Analytical, Germany).

Fe content and Fe speciation in pore water and sediment. To determine the Fe concentration and speciation in pore water, sediment cores were sliced in an anoxic glove box (100% N_2 atmosphere) in 0.5-cm steps. Pore water was obtained by centrifugation of small amounts of sediment for 5 min at $12,000 \times g$ (Eppendorf MiniSpin) in spin filters (Costar Spin-X Centrifuge tube filter; 0.22-μm nylon). To maintain Fe redox speciation, the pore water was mixed in a 1:1 (vol/vol) ratio with anoxic 1 M HCl. For determination of the Fe content and speciation in the sediment, a sequential Fe extraction was performed. Fe was extracted from 0.25 g sediment, first for 1 h with 0.5 M HCl (bioavailable fraction) and then for 24 h at 70°C in 6 M HCl (crystalline fraction). All extraction steps were performed under anoxic conditions (100% N_2 atmosphere) to avoid changes in Fe redox speciation during extraction due to O_2 (56). After both extraction steps, the samples were centrifuged for 15 min at $5,000 \times g$ (Hermle 7300). Then, 200 μl of the supernatant was stabilized in 800 μl of 1 M anoxic HCl. Fe(II) and total Fe concentrations were determined by the spectrophotometric Ferrozine assay in triplicate (57) with a spectrophotometric plate reader (FlashScan 550; Analytic, Jena, Germany). For calculation of Fe(III) concentrations, the concentration of Fe(II) was subtracted from the total Fe concentration.

Enrichment and enumeration of Fe-metabolizing bacteria. For enrichment and enumeration of the different types of bacteria, anoxic artificial seawater (ASW) medium with a salinity of 23 was used. Per liter, it contained the following salts: 17.3 g NaCl, 8.6 g $MgCl_2 \cdot 6\ H_2O$, 0.025 g $MgSO_4 \cdot 7\ H_2O$, 0.99 g $CaCl_2 \cdot 2\ H_2O$, 0.39 g KCl, 0.059 g KBr, 0.25 g NH_4Cl, and 0.05 g KH_2PO_4. The medium was prepared anoxically with a headspace of N_2-CO_2 (90:10) in a Widdel flask and buffered with 22 mM bicarbonate buffer. Additionally, per liter, 1 ml of a vitamin solution (58), 1 ml of a trace element solution (59), and 1 ml of a selenite-tungstate solution (60) were added. The pH of the medium was adjusted to 7.1 with either anoxic 1 M HCl or anoxic 0.5 M Na_2CO_3. For enrichment and enumeration of microaerophilic Fe(II) oxidizers, the ASW medium was slightly altered. Instead of 0.05 g KH_2PO_4, 0.05 g K_2HPO_4 was used, and the concentration of the bicarbonate buffer was lowered to 10 mM.

Enrichment of Fe-metabolizing bacteria. Enrichment of microaerophilic Fe(II) oxidizers was done in petri dishes (55-mm diameter) with zero-valent iron (ZVI) powder (200 mesh; metal basis; Alfa Aesar, Ward Hill, MA). This method is similar to that described by McBeth et al. (38) and is referred to here as ZVI plates. All work with microaerophilic Fe(II) oxidizers was done under oxic and sterile conditions. For the initial inoculation, ca. 0.1 g homogenized sediment from the uppermost 3 cm of a sediment core was added to a petri dish containing ca. 60 mg ZVI and 8 ml anoxic ASW and mixed gently. The sediment for the enrichments presented in this paper was sampled in October 2013. By adding 100 μl of this first dilution to another petri dish containing ca. 60 mg ZVI and 8 ml ASW, the sample was further diluted. This dilution step was repeated 5 times. After preparation, the petri dishes were incubated in an acrylic anaerobic jar (Merck, Germany), together with a gas pack that produced a micro-oxic atmosphere with a 6 to 10% atmospheric O_2 concentration (BD GasPack EZ Campy; Becton, Dickinson and Co., NJ). After 2 to 3 days, the growth of microaerophilic Fe(II) oxidizers was checked by fluo-

rescence microscopy, for which cells were stained by LIVE/DEAD staining (BacLight; Invitrogen, Carlsbad, CA). The highest positive dilution at which growth of microaerophilic Fe(II) oxidizers was observed, i.e., where formation of characteristic twisted stalks was observed, was used for preparation of a new dilution series. The enrichments were subjected to several such dilution-to-extinction series. To test for aerobic heterotrophic bacteria in the enrichments, streakouts on oxic R2A-ASW plates were done.

For enrichments of anaerobic Fe(II) oxidizers and Fe(III) reducers in the first two dilution series, 2 mM Na_2MoO_4 was added to the medium in order to inhibit SO_4^{2-} reduction and production of H_2S (61, 62). All work with the anaerobic Fe(II) oxidizers and Fe(III) reducers was done under sterile and anoxic conditions. All enrichment cultures were grown in 15-ml Hungate tubes containing 9 ml ASW medium with different amendments. The headspace was N_2:CO_2 (90:10). Dilution series were prepared by adding 1 g of homogenized sediment from the uppermost 3 cm of a sediment core to a Hungate tube with 9 ml of medium. A 10-fold dilution series was then prepared up to a dilution of 10^{-6} in ASW medium. After 4 to 5 weeks of incubation, a new dilution series was set up with two dilution steps higher than the highest positive dilution of the previous dilution series. If Fe(II) oxidation activity was found up to a dilution of 10^{-6}, the new dilution series was prepared with dilutions up to 10^{-8} with the goal of reaching dilution to extinction so that in the highest dilutions no growth was observed. The growth and homogeneity of the enrichment cultures were checked before each transfer by fluorescence microscopy and LIVE/DEAD staining. Anoxic enrichments of photoferrotrophs were made in ASW medium amended with 10 mM $FeCl_2$. The tubes were then incubated in the light (15-W fluorescent tube; 5,500 K) at 20°C. The enrichments presented in this article were inoculated with sediment that was sampled in May 2013 (Norsminde Fjord) and August 2013 (Kalø Vig). Anoxic enrichments of autotrophic and mixotrophic nitrate-reducing Fe(II) oxidizers were set up in ASW medium amended with 10 mM $FeCl_2$ and 4 mM NO_3^- and with or without addition of 5 mM acetate. The headspace was N_2-CO_2 (90:10). The sediment for setting up the enrichments of nitrate-reducing Fe(II) oxidizers that are presented here was sampled in January 2014. Transfers of the highest positive dilution of anaerobic Fe(II) oxidizers were done after the development of an orange-brown rusty color, indicating the activity of Fe(II) oxidizers.

Enrichments for Fe(III) reducers were set up in ASW medium amended with 5 mM ferrihydrite, prepared according to the method of Straub et al. (63), plus 5 mM acetate, 5 mM lactate, and 2 mM Na_2MoO_4. The sediment for the enrichments that are presented here was sampled in October 2013. Activity of Fe(III) reducers in these enrichments was obvious when the red color of the ferrihydrite changed to black.

SEM. Scanning electron microscopy (SEM) was used to characterize the structure of the stalks formed in microaerophilic enrichment cultures at high resolution. Culture samples from ZVI plates were washed in sterile anoxic distilled water and filtered onto 0.2-mm-pore-size polycarbonate (GTTP) filters (Isopore, Merck, Germany) and air dried as described previously (64). Filters were mounted onto aluminum stubs with carbon tape and coated with ~8-nm Pt with a BalTec SCD 005 sputter coater (BalTec, Liechtenstein). The samples were examined with a Leo 1450 VP (Zeiss, Germany) at 5 kV and 5-mm working distance using an Everhart-Thornley secondary electron detector.

MPN enumerations of Fe-metabolizing bacteria. For enumeration of Fe-metabolizing microorganisms in summer 2014, sediment was sliced in 0.5-cm slices from 0- to 3-cm sediment depth. In February 2015, sediment was sliced in 1-cm slices from 0 to 3 cm, in 2-cm slices from 3 to 7 cm, and in a 3-cm slice from 15 to 18 cm. Each slice was homogenized and used for preparing a dilution series. Each tube of the dilution series contained 9 ml nonamended ASW. The headspace was N_2:CO_2 (90:10). To the first tube of a dilution series, 1 g of sediment was added, and a $10\times$ dilution series up to a dilution of 10^{-12} was prepared.

MPN enumerations of anaerobic bacteria were performed in 96-well deep-well plates (each well had a volume of 1 ml) with 7 replicates for each dilution. To prevent sulfate reduction, 2 mM Na_2MoO_4 was added to the

medium. All work was done in an anoxic glove box (N_2 atmosphere). For the different types of microbial metabolism, different media were prepared. The medium for photoferrotrophs was amended with 10 mM $FeCl_2$ and filtered through a 0.22-μm filter after 48 h to remove the Fe(II) minerals that formed with the phosphate and bicarbonate present in the medium. After filtration, the concentration of Fe(II) in the medium was approximately 6 mM, and the PO_4^{3-} concentration was typically lowered to 40 μM (65). For quantification of mixotrophic nitrate-reducing Fe(II) oxidizers, the Fe(II)-containing medium was amended with 4 mM NO_3^- and 0.5 mM acetate. For Fe(III)-reducing bacteria, the medium contained 5 mM ferrihydrite, 5 mM acetate, and 5 mM lactate. To each well, 900 μl of the respective medium was added, and then 100 μl of the respective dilution of the sediment was added. Each dilution was inoculated into 7 wells, while 1 well served as a sterile control and remained uninoculated. For anoxic incubation, the Anaerocult system (Merck, Germany) was used, together with an O_2 indicator stick (Merck, Germany). Incubation was done for 8 weeks at 20°C either in light for photoferrotrophs (the same light source as for enrichments) or in the dark for nitrate-reducing Fe(II) oxidizers and Fe(III) reducers. Evaluation of positive wells was done visually, as the rusty-orange Fe(III) minerals formed were easily detectable. To check, whether Fe(II) was oxidized by anoxygenic phototrophic Fe(II) oxidizers and not by oxygenic phototrophs, control experiments were performed in which MPN plates for photoferrotrophs were incubated in infrared (IR) light (wavelength > 730 nm) by placing an IR bandpass filter (infrared filter 87; Lee, United Kingdom) between the MPN plates and a normal 40-W halogen light bulb (following references 66 and 67).

MPN enumerations for microaerophilic Fe(II) oxidizers were performed in gel-stabilized gradient tubes with a bottom layer containing 1% (wt/vol) agarose with a 1:1 mixture of FeS and ASW medium overlaid by ASW medium with 0.15% (wt/vol) agarose and air in the headspace (3, 68). These tubes provide opposing gradients of Fe(II) and O_2 and thus optimal conditions for growth of microaerophilic Fe(II) oxidizers. Duplicate tubes were inoculated with 100 μl of the respective dilution. Control tubes remained uninoculated. Incubation was done at 20°C in the dark. Growth of microaerophilic Fe(II) oxidizers was evaluated visually by the formation of a distinct rusty-orange growth band and was confirmed by fluorescence microscopy and LIVE/DEAD staining. In order to confirm the results from gradient tubes, some experiments were performed in which the dilution series was inoculated into ZVI plates, where the presence of Fe(II) oxidizers was later evaluated by fluorescence microscopy and LIVE/DEAD staining (based on the presence of cells and twisted stalks).

For all MPN incubations, positive wells/tubes per dilution were counted, and the most probable number was calculated with the software Klee (69).

DNA extraction. From environmental samples, DNA was extracted from 0.25 g sediment with the PowerSoil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). For the enrichment cultures, DNA was extracted from 1.8 ml of the liquid cultures with the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) or with the PowerSoil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) if DNA isolation with the UltraClean kit was not successful or DNA concentrations were too low.

qPCR for *Zetaproteobacteria* and total bacteria. To confirm the results of the MPN studies for microaerophilic Fe(II) oxidizers, a quantitative PCR (qPCR) for *Zetaproteobacteria* was performed. Copy numbers of 16S rRNA genes in the environmental DNA extracts were quantified on an iQ5 Real-Time PCR cycler (Bio-Rad Laboratories GmbH, Germany) using SsoAdvanced Universals SYBR Green Supermix (Bio-Rad). The primers Zeta672F (5′-CGGAATTCCGTGTGTAGCAGT-3′) and Zeta837R (5′-GCCACWGYAGGGGTCGATACC-3′) (36) were used at a concentration of 250 nM. In addition, reaction mixtures contained 5 μl SYBR green master mix, 1 μl of template (between 10 and 100 ng of DNA), and DNase-free water in a final volume of 10 μl. The qPCR program was as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at

60°C. At the end, a melting curve from 60 to 99°C (in 0.5°C steps) was recorded.

To be able to calculate the percentages of Fe(II) oxidizers and Fe(III) reducers relative to the total bacterial community, qPCR for total bacteria was done using DNA extracts from sediment samples. The same cycler and master mix as for the *Zetaproteobacteria* qPCR was used. The primers 341F (5′-CCTACGGGAGGCAGCAG-3′) (70) and 797R (5′-GGACTAC CAGGGTATCTAATCCTGTT-3′) (71) were used at concentrations of 75 and 225 nM, respectively. The reaction mixture further contained 5 μl SYBR green master mix, 1 μl of template, and DNase-free water in a final volume of 10 μl. The qPCR program was as follows: 2 min at 98°C, followed by 40 cycles of 98°C for 5 s and 60°C for 12 s. The thermal program was completed by a melting curve from 60 to 99°C (in 0.5°C steps).

After qPCR, 2% agarose gels were run with the qPCR products to ensure amplification specificity and that the amplicons had the expected length. Cell numbers per gram dry sediment were calculated from the qPCR 16S rRNA gene copy numbers, considering the average rRNA operon numbers of the respective taxa for bacteria as listed in the rRNA Operon Copy Number Database (http://rrndb.mmg.msu.edu/index .php). For *Zetaproteobacteria*, a 16S rRNA gene copy number of 2 was assumed, according to Kato et al. (36).

Phylogenetic affiliation of enrichment cultures; DGGE, sequencing, and sequence analysis. A PCR to obtain 16S rRNA gene fragments from the enrichment cultures for denaturing gradient gel electrophoresis (DGGE) (70) was performed with the general bacterial 16S rRNA gene group-specific primers 341GC F (70) and 907R (72). Depending on the concentration of the PCR product, 5 to 20 μl was loaded on an acrylamide gel with a urea gradient ranging from 35% at the top to 60% at the bottom of the gel. After 16 h of electrophoresis, the gel was stained with ethidium bromide. Under UV light, the most prominent bands were excised from the DGGE gel, diluted with 200 μl water, and incubated for 2 h at 4°C. After this incubation, a PCR was run with the primers 341F and 907R, with the same PCR program as for the DGGE. The resulting PCR products were loaded on a 1% agarose gel, and the bands were excised and cleaned with the Wizard PCR cleanup system (Promega Laboratories, WI) and cloned using a Qiagen PCR cloning kit (Qiagen, Germany). Vectors were transformed into competent cells (*Escherichia coli* DH5α) and plated onto LB medium containing ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for blue/white screening. White colonies were picked and tested for the correct insert size. Overnight cultures were prepared from colonies with the correct inserts in 5 ml liquid LB medium containing ampicillin. Plasmid DNA was isolated from these cultures and sent to GATC Biotech (Constance, Germany) for sequencing. For each band excised from the DGGE gel, at least three clones were picked and sequenced. The resulting sequences were trimmed using the software program Geneious R6 (Biomatters). Then, 16S rRNA gene sequences were identified and classified using the Basic Local Alignment Search Tool (BLAST) (73).

Nucleotide sequence accession numbers. Sequences for enrichment culture clones of the Fe(II) oxidizers and Fe(III) reducers were deposited in the ENA and GenBank databases under accession numbers LN986435 to LN986485 (see Table S2 in the supplemental material).

RESULTS

Characterization of sediments. The results of sediment analyses and geochemical measurements for both field sites, Norsminde Fjord and Kalø Vig, from summer 2014 are shown in Table 1 and Fig. 1. The sediments were visually very different. Sediment from Norsminde Fjord was fine grained and muddy. Its color was brownish for approximately the upper 0.5 cm, suggesting oxidized conditions. Below 0.5 cm, the sediment was dark and blackish, suggesting the presence of FeS (see Fig. S1 in the supplemental material for an image of the core). The water content of Norsminde Fjord sediment was 80% by weight (80 weight%) in the top 0.5 cm and 75% below that. The sediment TOC was 3.31 ± 0.42

Applied and Environmental Microbiology

TABLE 1 Geochemical parameters of sediments and water from the two field sites, Norsminde Fjord and Kalø Vig, Aarhus Bay, Denmark

Parameter[a]	Value	
	Norsminde Fjord	Kalø Vig
Water content by wt (%)	71 ± 3	19 ± 4
DOC water column (mg liter^{-1})	5.28 ± 0.06	4.21 ± 0.09
DOC porewater (mg liter^{-1})	8.24 ± 0.12	6.56 ± 0.06
TOC sediment (%)	3.31 ± 0.42	0.38 ± 0.05
Pore water NO_3^- (μM)	18.3 ± 8.2	4.2 ± 2.4
Pore water NO_2^- (μM)	BDL[b]	BDL
water column pH	7.9	8.1
Water column O_2 saturation (%)	100	100
Fe(II) in pore water (μM)	104 ± 64	70 ± 52

[a] All parameters were determined in summer 2014.
[b] BDL, below detection limit.

weight%, and DOC in the pore water was 8.24 ± 0.12 mg liter^{-1}. In contrast, sediment from Kalø Vig had a larger grain size and a brighter color. It was light brown in the uppermost 2 mm. Below this presumed oxidized layer, a 1- to 2-mm-thick purple layer was found, suggesting the presence of anoxygenic phototrophic bacteria. Below the purple layer, the sediment was grayish, again indicating the presence of FeS (see Fig. S1 in the supplemental material for an image of the core). The purple layer and the grayish color of the sediment were observed only in summer. In winter, the sediment was a light-brown color throughout the sampled depth. The water content of Kalø Vig sediment was 40% in the top 0.5 cm of the sediment and 18% below that. The TOC of the sediment was 0.38 ± 0.05 weight%, and the DOC in the pore water was 6.56 ± 0.06 mg liter^{-1}. The concentrations of nitrate in the pore water of both sediments were very low, <20 μM (Table 1), and nitrate was present only in the upper 3 mm. The Fe(II) concentrations in Norsminde Fjord sediment were up to 207 ± 22.21 μmol per g dry sediment in 6 M HCl extractions and thus about 10 times higher than in Kalø Vig sediments, where only a maximum of 21 ± 2.42 μmol Fe(II) per g dry sediment could be extracted with 6 M HCl (Fig. 2). Pore water concentrations of Fe(II) were also higher in Norsminde Fjord sediment (104 ± 64 μM) than in Kalø Vig sediment (70 ± 52 μM). Light penetrated
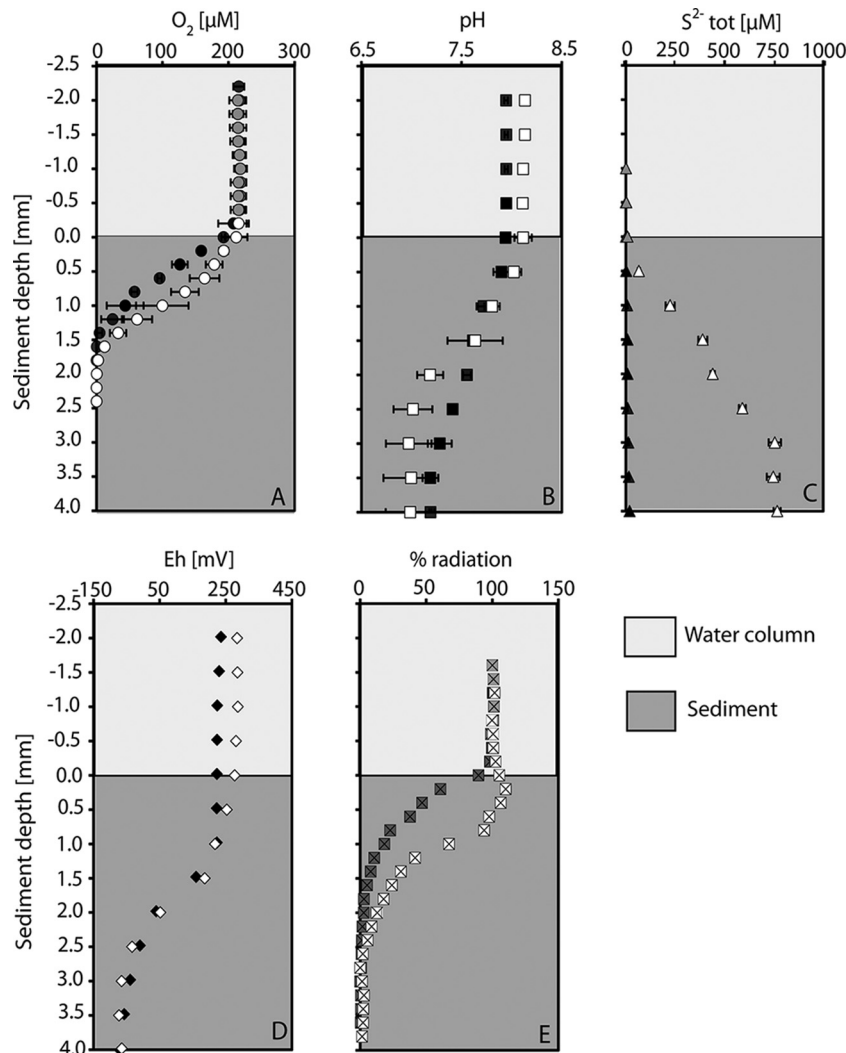

FIG 1 Microsensor data from summer 2014 for sediment from Norsminde Fjord (black symbols) and Kalø Vig (white symbols). (A) Oxygen. (B) pH. (C) Total sulfide. (D) Redox potential. (E) Scalar irradiance. The error bars indicate standard deviations.
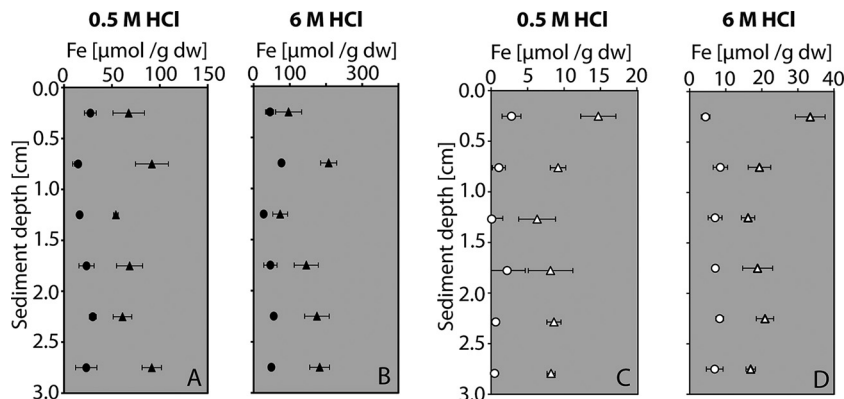
FIG 2 Sedimentary Fe content determined by sequential Fe extractions given in micromoles of Fe extracted per gram dry sediment (dw, dry weight) with 0.5 M HCl (A and C) and 6 M HCl (B and D). The black symbols show data for Norsminde Fjord and the white symbols for Kalø Vig. Triangles, Fe(II); circles, Fe(III). The sediment for these measurements was sampled in summer 2014. The error bars indicate standard deviations.

more deeply into the sandy sediment of Kalø Vig (3-mm penetration depth) than into the Norsminde Fjord sediment (2-mm penetration depth) (Fig. 1).

The Kalø Vig site was primarily barren sand with patches of sea grass (*Zostera* spp.) and brown algae (*Fucus* spp.). Roots of seagrass occasionally were found in the sediment cores. At both field sites, the presence of bioturbating/bioirrigating fauna was observed but not quantified (mainly different bristle worms—*Nereis* spp. at Norsminde Fjord and *Arenicola* sp. at Kalø Vig—together with different crustaceans). The abundance of burrows of *Arenicola* at the Kalø Vig site can be seen in Fig. S2 in the supplemental material.

Oxygen, pH, sulfide, redox potential, and scalar irradiance. Microelectrode profiles revealed that oxygen penetrated less than 2 mm in both sediments. The pHs in the water column were 7.9 and 8.1 for Norsminde Fjord and Kalø Vig, respectively, decreasing to 7.1 along the depth gradient in both sediments. Sulfide was not detectable in pore water of Norsminde Fjord sediment. In Kalø Vig sediment, sulfide was detected in the pore water already at 0.5 mm sediment depth with a concentration of 65 μM and increased to a maximum of 750 μM at 3-mm sediment depth. Measurements with redox microelectrodes showed that in both cases the redox potential was positive in the water column and in the pore water of the oxygenated layer of the sediment ($+235$ mV and $+285$ mV for Norsminde Fjord and Kalø Vig sediments, respectively). As soon as the anoxic part of the pore water was reached, the redox potential decreased to -65 mV and -75 mV for Norsminde Fjord and Kalø Vig sediments, respectively (Fig. 1). Interestingly, light penetrated deeper than oxygen in both sediments during our summer sampling campaign. This was different in winter, when oxygen penetrated much deeper than light (5 to 6 mm) (data not shown).

Depth distribution of Fe(II)-oxidizing and Fe(III)-reducing bacteria. Depth profiles of viable counts for Fe(II) oxidizers and Fe(III) reducers determined by the MPN method in sediments that were collected in summer 2014 are shown in Fig. 3. Surprisingly, the abundances of the different Fe metabolizers did not change significantly with depth throughout the upper 3 cm of the sediment. In a further MPN enumeration in February 2015, where viable counts were made at 0- to 3-, 3- to 7-, and 15- to 18-cm sediment depths, similar cell numbers were found over the entire

depth interval. Cell numbers were highest in the organic-rich Norsminde Fjord, except for photoferrotrophs, which were most prominent at the sandy Kalø Vig (Fig. 3). The lowest cell numbers were found for photoferrotrophs, which at different sediment depths reached from 1.5×10^2 to 3.1×10^2 cells per g (dry weight) for Norsminde Fjord and 2.92×10^1 to 7.7×10^1 cells g^{-1} (dry weight) for Kalø Vig sediment. MPNs for photoferrotrophs in full-spectrum light and in IR light alone yielded comparable cell numbers, demonstrating that the observed Fe(II) oxidation indeed stemmed from phototrophic Fe(II) oxidizers and was not caused by O_2 produced by cyanobacteria (data not shown). Nitrate-reducing Fe(II) oxidizers had abundances of 7.9×10^3 to 3.5×10^4 cells g^{-1} (dry weight) in Norsminde Fjord and 6.6×10^2 to 7.7×10^3 cells g^{-1} (dry weight) in Kalø Vig sediments. Among all Fe-metabolizing microorganisms, the highest cell numbers were found for microaerophilic Fe(II) oxidizers. Both cultivation-based methods used for quantifying microaerophilic Fe(II) oxidizers, i.e., the gradient tubes and ZVI plates, showed comparable results (data for ZVI plates are not shown). The cell numbers of microaerophilic Fe(II) oxidizers determined by the gradient tube method were 1.4×10^5 to 1.0×10^6 cells g^{-1} (dry weight) for Norsminde Fjord and 2.1×10^3 to 2.4×10^4 cells g^{-1} (dry weight) for Kalø Vig. Cell numbers of Fe(III) reducers ranged from 1.1×10^3 to 4.4×10^4 cells g^{-1} (dry weight) in Norsminde Fjord and from 2.9×10^2 to 5.1×10^2 cells g^{-1} (dry weight) in Kalø Vig sediments. The total bacterial cell numbers determined by qPCR were 2.87×10^9 ($\pm 2.27 \times 10^9$) to 5.77×10^{10} ($\pm 4.48 \times 10^{10}$) cells g^{-1} (dry weight) for Norsminde Fjord sediment and 2.12×10^8 ($\pm 1.89 \times 10^8$) to 8.16×10^9 ($\pm 3.34 \times 10^9$) cells g^{-1} (dry weight) for Kalø Vig sediment (see Table S1 in the supplemental material).

Zetaproteobacteria. The cell numbers of *Zetaproteobacteria* determined by qPCR for Norsminde Fjord sediment (1.95×10^6 to 3.21×10^6 cells g^{-1} [dry weight]) were only slightly higher than the MPNs, while for Kalø Vig, the cell numbers determined by qPCR (2.03×10^5 to 7.35×10^5 cells g^{-1} [dry weight]) were higher by up to 2 orders of magnitude (Fig. 4). Neither MPN nor qPCR cell numbers changed significantly with depth.

Enrichment cultures of microaerophilic Fe(II) oxidizers. Enrichment cultures for microaerophilic Fe(II) oxidizers on ZVI plates from both field sites showed Fe(II) oxidation and growth of
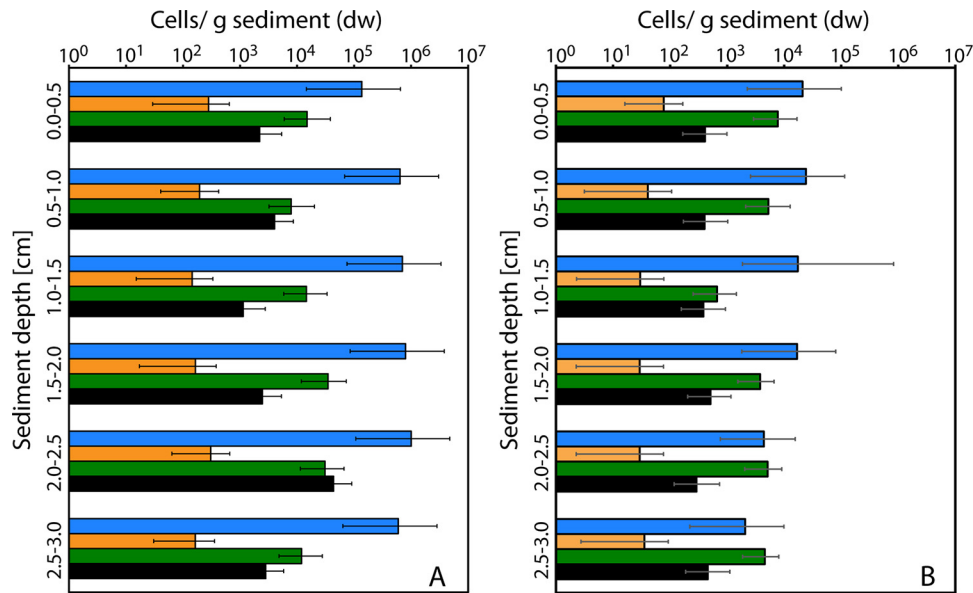
FIG 3 MPN counts of Fe(II)-oxidizing and Fe(III)-reducing microorganisms over sediment depth for Norsminde Fjord (A) and Kalø Vig (B). Blue bars, microaerophilic Fe(II) oxidizers; orange bars, photoferrotrophs; green bars, nitrate-reducing Fe(II) oxidizers (mixotrophic); black bars, Fe(III)-reducing bacteria. The sediment for MPN incubations was sampled in summer 2014. The error bars indicate standard deviations.

cells already after 2 to 3 days, as indicated by large numbers of twisted stalks visible under the light microscope (Fig. 5). After 3 or 4 transfers from dilution-to-extinction series, the microaerophilic enrichments from both field sites showed homogeneous cell mor-
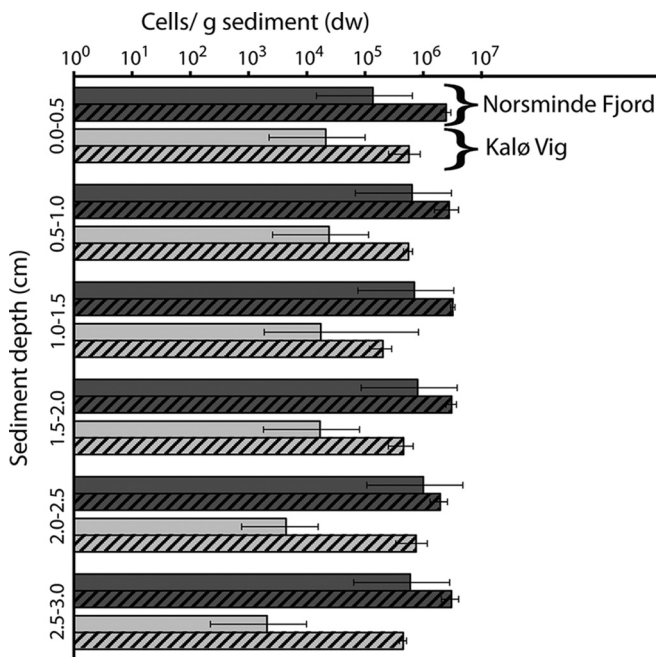

FIG 4 Comparison of cell numbers found by MPNs for microaerophilic Fe(II) oxidizers from Norsminde Fjord (dark-gray bars) and Kalø Vig (light-gray bars) and qPCR data for *Zetaproteobacteria* from Norsminde Fjord (dark-gray hatched bars) and Kalø Vig (light-gray hatched bars). Cell numbers were calculated from qPCR-based 16S rRNA gene copy numbers using a correction of 2 gene copies per cell, according to the method of Kato et al. (36). Sediment for these analyses was sampled in summer 2014. The error bars indicate standard deviations.

phologies (Fig. 5). Microscopic analyses showed that some cells were attached to stalks while others were motile, as described previously (74). Motile cells were found in lower dilutions and cultures that were grown for a longer time (>7 days). In the microaerophilic Fe(II)-oxidizing enrichment cultures from both field sites, the cells were rod and bean shaped (ca. 1 μm long and 0.4 μm wide). For each enrichment culture from the two field sites, two distinct bands were found on the DGGE gel and excised. After sequencing, the phylogenetic investigation showed that all the sequences of the enrichment cultures from both field sites belonged to the class *Zetaproteobacteria*. All the sequenced clones (6 for each field site) had 98% sequence similarity to *Mariprofundus* sp. strain M34. The sequenced clones were 99.7% and 99.6% identical to each other for the microaerophilic Norsminde Fjord and Kalø Vig enrichments, respectively. The sequences were uploaded to the ENA database and accession numbers are shown in Table S2 in the supplemental material. No heterotrophic growth was observed on R2A-ASW plates.

Enrichment cultures of phototrophic Fe(II) oxidizers. Enrichment cultures from both field sites incubated with Fe(II) amendment under anoxic conditions in the light showed the first rusty patches after 4 to 5 weeks, indicating activity of phototrophic Fe(II) oxidizers. The presence of cells was confirmed by fluorescence microscopy, and the highest dilution showing the presence of cells and Fe(II) oxidation was subjected to a further dilution series. After transfer through more than 10 dilution series, cell morphologies in the enrichments from Norsminde Fjord were very homogeneous. Cells in Norsminde Fjord enrichments were small and rod shaped (1.5 to 1.8 μm long and 0.5 to 0.6 μm wide) (Fig. 5). In the enrichment from Kalø Vig, in which cells were growing much more slowly, two distinct morphologies were observed after nine dilution series, namely, short (ca. 1-μm-long and 0.2- to 0.3-μm-wide) and long (ca. 2- to 3-μm-long and 0.5-μm-wide) rod-shaped cells (Fig. 5). Both enrichment cultures oxidized Fe(II) in infrared light (wavelength > 730 nm) (data not shown)
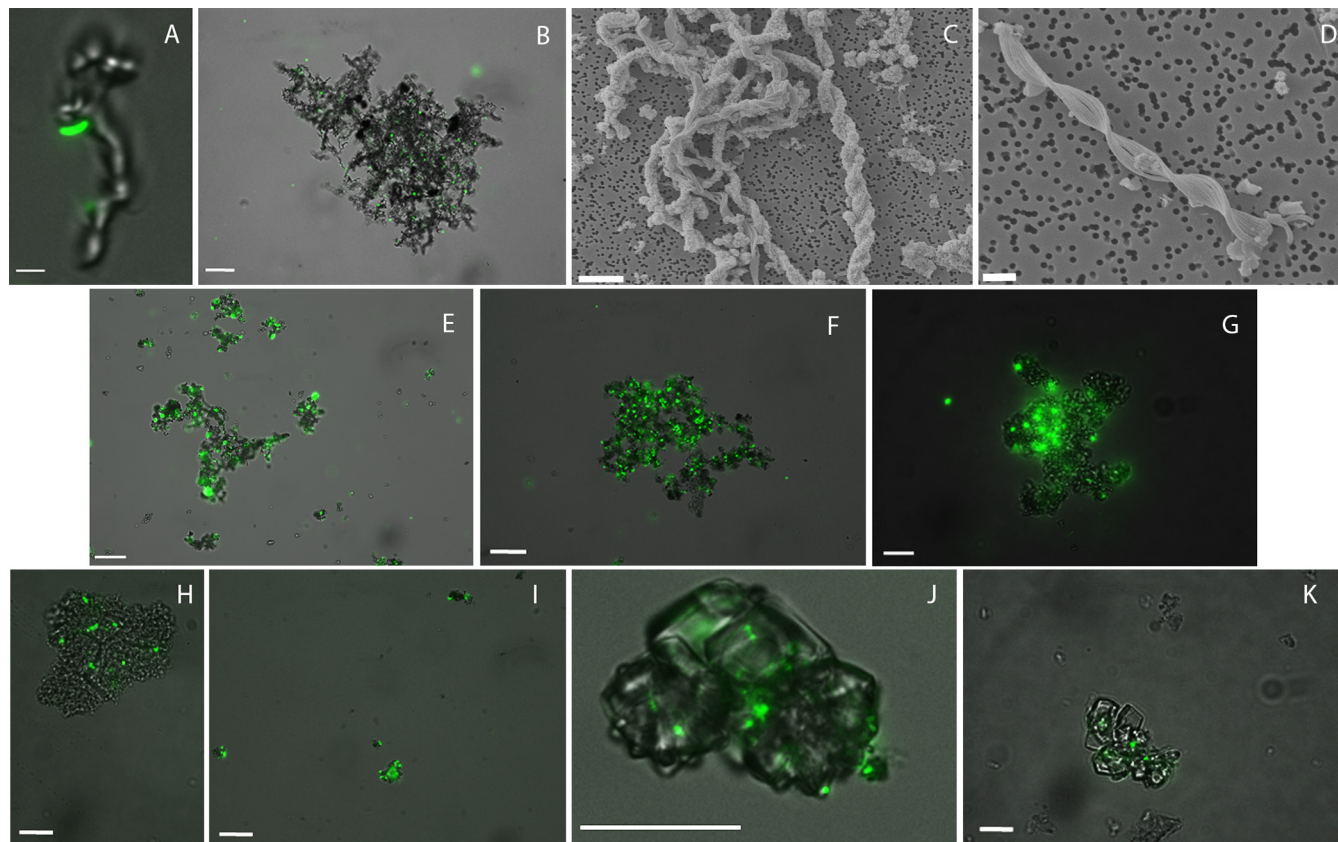
FIG 5 Fluorescence microscopy (A, B, and E to K) and scanning electron microscopy (C and D) images of the Fe(II) oxidizers and Fe(III) reducers enriched from sediments from Aarhus Bay. (A, D, and E) Microaerophilic enrichments from Norsminde Fjord. (B and C) Microaerophilic enrichments from Kalø Vig. (F) Photoferrotrophic enrichment from Norsminde Fjord. (G) Photoferrotrophic enrichment from Kalø Vig. (H and I) Nitrate-reducing Fe(II)-oxidizing enrichments from Norsminde Fjord and Kalø Vig, respectively. (J and K) Fe(III)-reducing enrichments from Kalø Vig and Norsminde Fjord, respectively. Scale bars, 25 μm (B, E, F, and H to K), 1 μm (A and D), 3 μm (C), and 10 μm (G).

at rates similar to those under full-spectrum light, indicating that anoxygenic photosynthesis was responsible for Fe(II) oxidation (66, 67). For the anoxygenic phototrophic Fe(II)-oxidizing enrichment from Norsminde Fjord, three distinct bands were identified on the DGGE gel. All the bands were excised and sequenced. All the sequences (a total of 9) of the enrichment from Norsminde Fjord had 99% sequence similarity to that of *Chlorobium luteolum*. The 9 sequences of the Norsminde Fjord photoferrotroph were 99.6% identical to each other. For the Kalø Vig enrichment, two distinct bands were identified on the DGGE gel, and both bands were excised. The sequences of the enrichment from Kalø Vig confirmed the microscopic investigation showing that different species were present. The sequences were found to be 99% homologous to that of *Rhodobacter* sp. strain SW2 (2 of 6 sequences) or 98% homologous to that of *Marichromatium purpuratum* (4 of 6 sequences). The sequences were uploaded to the ENA database, and accession numbers are shown in Table S2 in the supplemental material. Detailed results and information on the physiological characteristics of the identified closest relatives are shown in Table 2.

Enrichment cultures of nitrate-reducing Fe(II) oxidizers. Within 1 week of incubation of sediments from both field sites in medium either amended with Fe(II), nitrate, and acetate or amended with Fe(II) and nitrate, a color change of the Fe(II)-containing medium (from grayish-blue to orange) was ob-

served, indicating the growth and activity of nitrate-reducing Fe(II) oxidizers. The presence of cells was confirmed by fluorescence microscopy, and the highest dilution showing growth or Fe(II) oxidation was subjected to a further dilution series. For enrichments from both field sites, growth was not observed after 3 or 4 transfers in dilution series to which no acetate was added, although growth initially was as fast as in the enrichments to which acetate was added. The enrichments to which acetate was added showed continuous Fe(II) oxidation and growth over more than 10 dilution series. Therefore, only the enrichments to which acetate was added were further transferred so that all enrichment cultures that remained were mixotrophic nitrate-reducing Fe(II) oxidizers. For enrichments from both field sites, cell morphologies after several transfers in enrichments containing nitrate, Fe(II), and acetate were homogeneous. Fluorescence microscopy showed that cells were closely associated with Fe minerals and also appeared to be located within the minerals. The cells from the enrichments from Norsminde Fjord appeared to be slightly larger (ca. 2.5 to 3 μm long and 0.5 μm wide) than the cells in enrichments from Kalø Vig (ca. 2 μm long and 0.5 μm wide). Both were rod-shaped cells (Fig. 5). On the DGGE gel, two distinct bands were found for both enrichments. Both bands were excised, and sequence analysis revealed that the sequences of the enrichment from Norsminde Fjord had 99% sequence simi-

TABLE 2 Identification of sequences from DGGE bands of enrichment cultures and physiological characteristics of closest relatives

Name and sampling site	Enrichment medium and technique	Characteristics and morphology	Closest cultured relative and origin of relative	Closest known relative able to oxidize/reduce Fe
Microaerophilic Fe(II) oxidizer S1 Oct. c; Norsminde Fjord	ZVI plates with ASW; dilution to extinction	Bean-shaped cells ca. 0.4 μm wide and 1 μm long; makes filigree stalks with several filaments; motile	*Mariprofundus* sp. M34 (98% sequence similarity); isolated from an iron mat at Loihi Seamount (HI) (76)	*Mariprofundus* sp. M34 (98% sequence similarity); isolated from an iron mat at Loihi Seamount (HI) (76)
Microaerophilic Fe(II) oxidizer S2.5; Kalø Vig	ZVI plates with ASW; dilution to extinction	Bean-shaped cells ca. 0.4 μm wide and 1 μm long; makes rigid stalks; motile	*Mariprofundus* sp. M34 (98% sequence similarity); isolated from an iron mat at Loihi Seamount (HI) (76)	*Mariprofundus* sp. M34 (98% sequence similarity); isolated from an iron mat at Loihi Seamount (HI) (76)
Photoferrotroph S1.1; Norsminde Fjord	ASW with Fe(II) in light; dilution to extinction	Rod-shaped cells ca. 0.5–0.6 μm wide and 1.6–1.5 μm long; closely associated with iron minerals; can also grow photolithotrophically with hydrogen or photoheterotrophically with organic acids like lactate	*C. luteolum* (99% sequence similarity); isolated from a freshwater ditch close to the river Rhine (100, 111)	*C. ferrooxidans* KoFox (93% sequence similarity); isolated from a freshwater ditch close to Constance (24)
Photoferrotroph S2.2; Kalø Vig	ASW with Fe(II) in light; dilution to extinction	Two different morphotypes: smaller rod-shaped cells ca. 0.2–0.3 μm wide and 1 μm long and larger rod-shaped cells 0.5 μm wide and 2–3 μm long; closely associated with iron minerals; can also grow aerobic on R2A ASW-agar plates, where red and yellow colonies form	*Rhodobacter* sp. SW2 (99% sequence similarity); isolated from a freshwater pond close to Hannover (5) / *M. purpuratum* (98% sequence similarity); isolated from a marine sponge (112)	*Rhodobacter* sp. SW2 / *Rhodobacter* sp. SW2 (84% sequence similarity)
Nitrate-reducing Fe(II) oxidizer S1.5; Norsminde Fjord	ASW with Fe(II), NO_3^-, and acetate; dilution to extinction and agar shakes	Long rod-shaped cells ca. 0.5 μm wide and 2.5–3 μm long; tightly associated with iron minerals; encrusted with iron minerals	*H. marina* (99% sequence similarity); isolated from the Baltic Sea (113)	*H. siderophila* (99% sequence similarity); isolated from an iron-rich spring in Russia (97)
Nitrate-reducing Fe(II) oxidizer S2.5; Kalø Vig	ASW with Fe(II), NO_3^-, and acetate; dilution to extinction and agar shakes	Long rod-shaped cells ca. 0.5 μm wide and 2 μm long; tightly associated with iron minerals; encrusted with iron minerals	*D. indolicum* strain MPKc (99% sequence similarity) isolated from sediment from Bay of Hong Kong (C. W. Yip and J. D. Gu, unpublished data)	*D. aromatica* RCB (91% sequence similarity); isolated from sediments of the Potomac River, USA (114)
Fe(III) reducer S1.3; Norsminde Fjord	ASW with Fe(III), acetate, and lactate; dilution to extinction	Rod-shaped cells ca. 0.5 μm wide and 2 μm long; grow associated with iron minerals	*S. colwelliana* (99% sequence similarity); isolated from an estuarine aquaculture of oysters (111)	*S. colwelliana* (99% sequence similarity); isolated from an estuarine aquaculture of oysters (111)
Fe(III) reducer S2.3; Kalø Vig	ASW with Fe(III), acetate, and lactate; dilution to extinction	Rod-shaped cells ca. 0.5 μm wide and 2 μm long; grow associated with iron minerals	*S. colwelliana* (99% sequence similarity); isolated from an estuarine aquaculture of oysters (115)	*S. colwelliana* (99% sequence similarity); isolated from an estuarine aquaculture of oysters (115)

larity to that of *Hoeflea marina* (6 sequences). For the enrichment from Kalø Vig, it was found that the sequences had 99% sequence similarity to that of *Denitromonas indolicum* strain MPKc (6 sequences). The sequenced clones from the Nors-

minde Fjord and the Kalø Vig enrichments were 99.0% and 99.6% similar to each other. The sequences were uploaded to the ENA database, and accession numbers are shown in Table S2 in the supplemental material.

Enrichment cultures of Fe(III) reducers. Within 10 days, a color change from orange to dark brown to blackish was observed in enrichments from both field sites amended with Fe(III) oxyhydroxide ferrihydrite as the electron acceptor, suggesting microbial Fe(III) reduction. Growth was investigated by fluorescence microscopy, and the highest dilution showing growth (color change and cells) was subjected to a further dilution series. After 5 such dilution-to-extinction series, cell morphologies were homogeneous. There were no visible differences in cell morphologies between the enrichments from the two field sites. In both cases, the cells were rod shaped (ca. 2 μm long and 0.5 μm wide) and closely associated with the Fe minerals (Fig. 5). On the DGGE gel, two distinct bands were found for both enrichments. All the bands were excised from the gel, and the two enrichment cultures were found to be phylogenetically identical. All sequences from both enrichments had 99% sequence similarity to that of *Shewanella colwelliana* (6 sequences of each of the enrichments). The sequences of the clones from the Norsminde Fjord were 99.7% similar to each other, and those from Kalø Vig were 99.5% similar to each other. The sequences were uploaded to the ENA database, and accession numbers are shown in Table S2 in the supplemental material.

DISCUSSION

Coexistence of different physiological types of Fe(II) oxidizers. Based on typical geochemical characteristics of marine sediments, it can be expected that autotrophic microaerophilic and nitrate-reducing Fe(II) oxidizers are widely distributed in marine sediments while phototrophic Fe(II) oxidizers are probably restricted to shallow coastal sediments, where sunlight reaches sediments that contain significant amounts of Fe(II) (47, 50, 51). This is why we conducted our study in shallow coastal sediments, where we expected the possible coexistence of all three types of Fe(II) oxidizers. Geochemical analysis of the two sediments studied here suggested that they could potentially harbor microaerophilic, nitrate-reducing, and anoxygenic phototrophic Fe(II)-oxidizing bacteria. MPN enumerations and enrichment cultures indeed showed that all three types of Fe(II) oxidizers, and even Fe(III) reducers, coexist in these two typical but geochemically distinct marine sediments. This suggests that in both sediments the prerequisites for a complete biogeochemical Fe cycle, as described by Schmidt et al. (52), are present. This is the first time that the coexistence of all three metabolic types of Fe(II) oxidizers in the same habitat has been demonstrated, and we even identified members of these groups in enrichment cultures (see below). Marine microaerophilic Fe(II) oxidizers have so far been isolated only from deep-sea hydrothermal-vent systems or other marine habitats with elevated Fe(II) concentrations (18, 38, 75, 76). Marine photoferrotrophs have been isolated from only one field site, i.e., a tidal mud flat in the German Wadden Sea by Straub et al. (43). So far, only two marine cultures of nitrate-reducing Fe(II) oxidizers have been described; they were isolated from a deep-sea hydrothermal vent and a brackish lagoon (4, 31). Thus, there are only a very limited number of isolates of marine Fe(II)-oxidizing microorganisms, illustrating the need for further isolation attempts.

Autotrophic and mixotrophic nitrate-reducing Fe(II) oxidizers. While we were able to detect and also identify mixotrophic nitrate-reducing Fe(II) oxidizers in sediments from both studied field sites, we were unable to cultivate autotrophic nitrate-reducing Fe(II) oxidizers that could be transferred continuously without the addition of an organic cosubstrate. The rapid oxidation of Fe(II) in the first autotrophic transfers could be explained by organic compounds that were present in the sediment used for inoculation. The subsequent autotrophic dilution series still showed Fe(II) oxidation, but the cells were probably running out of stored organic material and were transiently using organic C from extracellular polymeric substances or from dead cell biomass for mixotrophic Fe(II) oxidation. Alternatively, other growth factors, such as vitamins, trace metals, or other bacteria, needed by the autotrophic nitrate-reducing Fe(II) oxidizers were no longer present after several dilutions, and the organisms were therefore not able to grow. Dependence of autotrophic nitrate-reducing Fe(II) oxidation on coculture strains is known for the autotrophic enrichment culture KS (4), for which organisms such as *Bradyrhizobium*, *Rhodanobacter*, or *Nocardioides* (77) are necessary for continuous autotrophic Fe(II) oxidation. Additionally, it was recently suggested that complexation of Fe(II) by humic substances could be important for autotrophic nitrate-reducing Fe(II) oxidizers (78), although that study failed to show that the bacteria can grow autotrophically over several transfers. Despite these shortcomings, it would be worthwhile to set up enrichment cultures for autotrophic Fe(II) oxidizers from the two sediments investigated here in the presence of humic substances or other organic Fe ligands, in particular for the Norsminde Fjord site, which contained the largest amount of organic matter. Such experiments will allow us to determine whether organically complexed Fe(II) is a suitable substrate for nitrate-reducing Fe(II) oxidizers, first, because its redox potential is more favorable for Fe(II) oxidation than dissolved Fe(II), and second, because after oxidation, the Fe(III) formed can potentially be complexed by the organic ligand as well, thus preventing cell encrustation by Fe(III) minerals (29).

Homogeneous depth distribution of Fe(II) oxidizers. Our MPN analyses showed that the different Fe-metabolizing bacteria constituted only a small percentage of the total bacterial community, with 5×10^{-3}% [microaerophilic Fe(II) oxidizers] to 3×10^{-7}% (photoferrotrophs) in Norsminde Fjord sediment and 8×10^{-3}% [microaerophilic Fe(II) oxidizers] to 6×10^{-7}% (photoferrotrophs) in Kalø Vig sediment. Similar low abundances for nitrate-reducing Fe(II) oxidizers and microaerophilic Fe(II) oxidizers were reported in previous studies on Fe(II) oxidation in soil and freshwater sediments (79, 80). These cultivation-based MPNs may strongly underestimate the true number of Fe(II)-oxidizing bacteria in the sediment. Since in our calculations we compare the cultivation-based MPNs with the total cell numbers based on qPCR, the actual percentage of Fe(II) oxidizers is expected to be much higher, as can also be seen in our comparison of MPN and qPCR data for microaerophilic Fe(II) oxidizers, where cell numbers determined by qPCR were always higher than MPN counts. For comparison, MPN counts of sulfate-reducing bacteria in Norsminde Fjord (also called Kysing Fjord) obtained by a highly sensitive radiotracer MPN method were up to 10^3-fold higher than standard MPN counts (81, 82). Based on the calculated mean cell-specific rates of sulfate reduction, those radiotracer MPN counts were probably still at least 2 orders of magnitude lower than the actual number of sulfate-reducing bacteria *in situ* (83).

Surprisingly, we found that the abundances of the different Fe-metabolizing bacteria did not significantly change with depth within the top 3 cm of the sediment. This was not expected, as profiles of O_2 and light suggested that the different Fe(II) oxidizers could be active only in thin sediment layers with appropriate en-

vironmental conditions. For example, O_2 was available only in the uppermost 2 mm of the sediment, and consequently, we expected to find the highest abundances of microaerophilic Fe(II) oxidizers in this zone. However, abundances of microaerophilic Fe(II) oxidizers determined by two different cultivation-dependent MPN approaches (gradient tubes and ZVI plates) and by qPCR were constant down to a 3-cm sediment depth. We repeated the MPNs for the three different types of Fe(II) oxidizers at a higher spatial resolution of 0.2 cm instead of 0.5 cm as shown here (data not shown) but still found no distinct trend of depth distribution of the physiological groups of Fe(II) oxidizers. It has to be noted, however, that the activities of the different Fe(II) oxidizers at the different sediment depths are unknown, and we do not know whether, as could be expected, the activities of the Fe(II) oxidizers correlate with geochemical profiles in our sediments.

The homogeneous distribution of Fe(II) oxidizers could have different reasons. First, it could be that the Fe(II) oxidizers were distributed evenly in the sediment due to mixing by frequent wave action (84) or by bioturbating fauna, i.e., burrowing fauna that move surrounding sediment and pore fluid around in the upper layers of the sediment (85, 86). As a consequence, cells might be transiently dormant when conditions are not favorable for Fe(II) oxidation, and only the cells that are in sediment layers with favorable geochemical conditions at any given time would be actively oxidizing Fe(II). Bioturbation by burrowing fauna is well known from the upper 6 to 8 cm of Aarhus Bay sediments, where it was found to stimulate iron cycling by enhancing pore water exchange and by supplying solid-phase Fe(III) down to the Fe reduction zone and simultaneously transporting Fe(II) upward toward the sediment surface (47). Together with the mixing of sediment material, bacteria are also passively moved up and down in the sediment. Bioturbation in these sediments, and particularly the ventilation of worm burrows, probably takes place at a low rate to even greater depths of 10 to 20 cm and more (87). Another explanation for the more or less even distribution of these metabolic groups could be that O_2- and/or nitrate-rich microenvironments are created in different sediment layers due to the activity of the bioturbating and bioirrigating fauna, which we observed visually at both field sites. The activity of the animals creates microniches where oxygen and nitrate are locally available (88–90), potentially also allowing Fe(II) oxidation with oxygen or nitrate. The different polychaetes observed (*Nereis* sp. and *Arenicola* sp. at Norsminde Fjord and Kalø Vig, respectively) make 10- to 30-cm-deep burrows into the sediment. Also, crustaceans, such as the amphipod *Corophium* sp., burrow to 6-cm depth into the sediment (91). Such bioirrigation is dynamic and not easily picked up by microsensor measurements or pore water extraction. The walls of the holes of burrowing worms can be seen as an extension of the sediment surface, because the overlying water is actively flushed through the burrows (92–94). This means that the geochemical gradients extend laterally from the sides of the burrow. Oxygen or nitrate penetration from the burrow wall into the surrounding sediment can be similar to the penetration from the overlying water into the sediment surface (89). These changes in geochemical conditions have also been shown to influence microbial communities, e.g., the general biodiversity of a microbial community (95) or, more specifically, the microbial community linked to nitrogen cycling (88). In Kalø Vig sediment, the presence of seagrass could also be involved in O_2 transport through plant roots into deeper sediment layers (96) and could facilitate the activity of

microaerophilic Fe(II) oxidizers in sediment layers below the oxygen penetration depth. Although worm burrows were frequently observed, and although at the Norsminde Fjord site small polychaete worms were observed in almost every sediment core, we were not able to specifically sample the burrows, as they were either too small (<1 mm wide in Norsminde Fjord) or not included (in the case of the large *Arenicola* sp. in Kalø Vig) when we sliced the cores.

Identity of the Fe(II)-oxidizing bacteria. We were able to cultivate highly enriched cultures of all three types of Fe(II) oxidizers and of Fe(III) reducers from both field sites. The DGGE gels showed more than one band from most of the enrichments. However, all the sequences from these different bands appeared to be the same, according to the BLAST results, except for the photoferrotrophic enrichment culture from Kalø Vig, where we indeed found two different strains. Although from microscopy and sequencing of DGGE bands the enrichment cultures seem to be nearly pure, it still remains to be determined, by simultaneous hybridization with a specific and a general catalyzed reporter deposition (CARD)-fluorescence *in situ* hybridization (FISH) probe, how pure the enrichment cultures are.

Some close relatives of our enriched cultures are also known Fe(II) oxidizers. The 16S rRNA sequence of our mixotrophic nitrate-reducing Fe(II)-oxidizing enrichment culture from Norsminde Fjord is 99% similar to that of *Hoeflea siderophila*, which was reported by Sorokina et al. (97) to be capable of mixototrophic nitrate-reducing Fe(II) oxidation, as well as lithoautotrophic Fe(II) oxidation, under micro-oxic conditions. For the other nitrate-reducing enrichment from Kalø Vig, the closest-relative Fe(II) oxidizer was *Dechloromonas aromatica* strain RCB (91% sequence similarity), which is also capable of mixotrophic nitrate-reducing Fe(II) oxidation (98). The closest relatives of the anoxygenic phototrophic Fe(II)-oxidizing *Chlorobium* strain, which we enriched from Norsminde Fjord, are not known to be able to oxidize Fe(II) but oxidize H_2S (99, 100). However, another known *Chlorobium* strain that is phylogenetically more distantly related, i.e., *Chlorobium ferrooxidans* strain KoFox (93% sequence similarity), which is a freshwater strain that lives in coculture with a *Geospirillum* strain (KoFum), is also able to oxidize Fe(II) photoautotrophically (24, 101). For most isolated and published *Chlorobium* strains, unfortunately, no information exists about whether they can oxidize iron, probably because this was not tested. In the photoferrotrophic enrichment culture from Kalø Vig, two different sequences were found. One was 98% similar to that of *M. purpuratum*, whose ability to oxidize Fe(II) is not known. The closest known relative able to oxidize Fe(II) is the freshwater photoferrotroph *Rhodobacter* sp. SW2, with only 84% sequence similarity. The second DGGE band in the enrichment culture showed 98% similarity to that of *Rhodobacter* sp. SW2, which was the first cultivated Fe(II)-oxidizing anoxygenic phototroph isolated from a freshwater pond (5). It is surprising that the isolated photoferrotrophs are more closely related to known freshwater photoferrotrophic strains than to the known marine representatives of this physiological group. However, so far, only two marine species of anoxygenic photoferrotrophs are known (43, 44), and there has been only a small amount of research on anoxygenic phototrophic Fe(II) oxidation in marine sediments.

The microaerophilic Fe(II)-oxidizing enrichments from Aarhus Bay were all closely related to *Mariprofundus* sp. M34, which was isolated as a microaerophilic Fe(II) oxidizer from a microbial

mat at a deep-sea hydrothermal vent at Loihi Seamount (Hawaii) by McAllister et al. (76). Our study thus shows that microaerophilic Fe(II)-oxidizing *Zetaproteobacteria* are also naturally present in coastal marine sediments.

Microbial Fe(II) oxidation in marine coastal sediments. Our findings raise the question of the extent to which these three metabolic types of Fe(II)-oxidizing bacteria contribute to Fe(II) oxidation *in situ*. Ideally, activity measurements in microcosms or sediment cores, in combination with RNA-based molecular analyses, are needed to answer this question. However, we can draw several conclusions based on the cell abundances and the geochemical profiles. For the photoferrotrophs, we determined very low abundances, and these organisms can also live on other types of metabolism than Fe(II) oxidation (23, 24, 43, 102). They may even prefer reduced sulfur species or organic acids over Fe(II). Based on microelectrode measurements taken at different times throughout the year (data not shown), we suggest that the photoferrotrophs mainly contribute to iron cycling in summer, since in winter, oxygen penetrated deeper into the sediment than light. In summer, however, the phototrophs would have a distinct advantage, since oxygen penetration was shallow, and the nitrate-reducing Fe(II) oxidizers would compete for the sparse nitrate from the water column with microphytobenthos on the sediment surface. Although the nitrate-reducing Fe(II) oxidizers were more abundant than the photoferrotrophs, we were unable to isolate autotrophic representatives. For the cultivated mixotrophic nitrate-reducing Fe(II) oxidizers, it could be that Fe(II) oxidation is not an enzymatic process but an abiotic side reaction caused by nitrite that is formed during heterotrophic denitrification (27). It could even be that this side reaction is unavoidable during denitrification in the presence of Fe(II) (28). Thus, such indirect Fe(II) oxidation could be important in nitrate- and organic-rich marine sediments, such as in Norsminde Fjord. The highest abundances of Fe(II) oxidizers in both sediments were those of the microaerophilic Fe(II) oxidizers, which we could assign to the *Zetaproteobacteria*. In contrast to the nitrate-reducing and phototrophic Fe(II) oxidizers, marine microaerophilic Fe(II) oxidizers belong to the *Zetaproteobacteria*, as the ones that we found in the two different sediments from Aarhus Bay appear to be metabolically very restricted. All currently known isolates originate from iron-rich environments and are obligate microaerophilic Fe(II) oxidizers (37, 38, 40, 76). Although it was found that they carry the genes for nitrate reduction (75, 103), there is so far no evidence from growth experiments that they can actually live by that type of metabolism. Other microaerophilic Fe(II) oxidizers, e.g., different *Leptothrix* spp., are known to grow either by solely oxidizing organic carbon or by cometabolizing organic carbon and Fe(II) coupled with O_2 reduction (104, 105). Therefore, it is particularly interesting to investigate the physiology of newly isolated strains of *Zetaproteobacteria*, and we expect that the microaerophilic Fe(II) oxidizers are the most important group of microbial Fe(II) oxidizers in Aarhus Bay sediments.

Microbial iron cycling in marine coastal sediments. Our data showed that Fe(III) reducers coexist with the three physiological types of Fe(II) oxidizers, even within the same sediment layers, thus allowing small-scale Fe cycling in microniches. Fe(III) reduction and sulfate reduction are the most important carbon mineralization pathways in the upper anoxic layers in marine sediments, and both lead to the production of Fe(II), either directly [enzymatic Fe(III) reduction] or indirectly [via abiotic Fe(III) reduc-

tion by sulfide] (9, 106). Therefore, Fe(II) is readily available and constantly supplied in these marine sediments. The low concentrations of dissolved sulfide, the black sediment color, and the high Fe concentrations in Norsminde Fjord sediments suggest that the Fe(II) reacted effectively with sulfide and formed FeS. It is known that some Fe(II) oxidizers can use FeS as an Fe(II) source, e.g., photoferrotrophs, which can also oxidize other solid-phase substrates (4, 24, 107–109). For microaerophilic Fe(II) oxidizers, FeS is commonly used for cultivation in gradient tubes (3, 68), and they can also oxidize solid-phase substrates (110). Therefore, we expect that the FeS in the sediments investigated here is also bioavailable for microbial Fe(II) oxidation.

Conclusions and outlook. Based on the data presented in this study, we conclude that in coastal marine sediments, all three known metabolic types of Fe(II) oxidizers coexist together with Fe(III) reducers, and they are expected to contribute to Fe cycling in these sediments. This coexistence, however, could be caused simply by bioturbation and physical mixing, and it does not necessarily mean that the microbes are in direct competition or interact with each other. The Fe(II)-oxidizing activity of these bacteria should be clearly restricted to certain sediment layers by geochemical gradients, i.e., gradients of O_2, light, NO_3^-, and Fe(II), with the exception of microniches, as they are, e.g., produced by burrowing animals. The next steps will be (i) to quantify the activities of the different Fe(II) oxidizers in natural marine sediments, (ii) to identify the most active members of the Fe(II)-oxidizing and Fe(III)-reducing community, and (iii) to determine how the different metabolic types of Fe(II) oxidation interact and compete, e.g., when geochemical gradients are changing, for example, in a diurnal cycle.

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