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Investigation of an Iron-Oxidizing Microbial Mat Community Located near Aarhus, Denmark: Laboratory Studies

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We constructed a small flow chamber in which suboxic medium containing 60 to 120 μM $FeCl_2$ flowed up through a sample well into an aerated reservoir, thereby creating an suboxic-oxic interface similar to the physicochemical conditions that exist in natural iron seeps. When microbial mat material from the Marselisborg iron seep that contained up to 10^9 bacterial cells per cm^3 (D. Emerson and N. P. Revsbech, Appl. Environ. Microbiol. 60:4022–4031, 1994) was placed in the sample well of the chamber, essentially all of the Fe^{2+} flowing through the sample well was oxidized at rates of up to 1,200 nmol of Fe^{2+} oxidized per h per cm^3 of mat material. The oxidation rates of samples of the mat that were pasteurized prior to inoculation were only about 20 to 50% of the oxidation rates of unpasteurized samples. Sodium azide also significantly inhibited oxidation. These results suggest that at least 50% and up to 80% of the Fe oxidation in the chamber were actively mediated by the microbes in the mat. It also appeared that Fe stimulated the growth of the community since chambers fed with $FeCl_2$ accumulated masses of either filamentous or particulate growth, both in the sample well and attached to the walls of the chamber. Control chambers that did not receive $FeCl_2$ showed no sign of such growth. Furthermore, after 4 to 5 days the chambers fed with $FeCl_2$ contained 35 to 75% more protein than chambers not supplemented with $FeCl_2$. *Leptothrix ochracea* and, to a lesser extent, *Gallionella* spp. were responsible for the filamentous growth, and the sheaths and stalks, respectively, of these two organisms harbored large numbers of Fe-encrusted, nonappendaged unicellular bacteria. In chambers where particulate growth predominated, the unicellular bacteria alone appeared to be the primary agents of iron oxidation. These results provide the first clear evidence that the "iron bacteria" commonly found associated with neutral-pH iron seeps are responsible for most of the iron oxidation and that the presence of ferrous iron appears to stimulate the growth of these organisms.

It is well-known that *Thiobacillus ferrooxidans* and its acidophilic, metabolic relatives can grow lithoautotrophically by using ferrous iron as an energy source. However, the case for lithotrophic growth is less clear for the neutral-pH "iron bacteria" which commonly inhabit iron seeps or springs and other sites where sources of anoxic or suboxic Fe(II)-rich water flow into oxygenated zones. These bacteria, which are normally recognized by their unique morphologies (for example, the stalk-forming *Gallionella* spp., the sheath-forming organism *Leptothrix ochracea*, and members of the poorly defined family *Siderocapsaceae* that produce ferric iron-encrusted capsules), have generally proven to be difficult or impossible to maintain in the laboratory (15, 23). As a result, it has not been established whether these bacteria actually mediate the oxidation of iron and, if they do, whether they can conserve energy from the oxidation to grow lithotrophically.

The oxidation of Fe^{2+} to Fe^{3+} at neutral pH, $2Fe^{2+} + 0.5O_2 + 5H_2O \rightarrow 2Fe(OH)_3 + 4H^+$, is an exergonic reaction ($\Delta G^\circ = -8.6$ kcal [~ -36 kJ] $\cdot$ mol^{-1}) (6) that potentially should allow an organism to generate ATP and grow, albeit slowly. Since the 19th century, there has been circumstantial evidence, primarily from field observations, that the neutral-pH iron bacteria are lithotrophs; such speculation has been based on the apparent requirement of these organisms for Fe^{2+} and the difficulty of getting the organisms to grow heterotrophically in axenic culture. However, there have been few carefully controlled experiments, either in the field or in the laboratory, to back up this speculation or even to demonstrate that the bacteria associated with ferrous-iron-rich environments actively oxidize iron. For more discussion of these topics, see the recent reviews in references 7, 10, 11, 15, 16, and 18.

In addition to the problem of producing an insoluble product, ferric hydroxide, as a result of iron oxidation, another difficulty that the iron bacteria face is the rapid chemical oxidation of ferrous iron at neutral pH under fully oxygenated conditions. The short chemical half-life (12 to 15 min) of this abiotic reaction in aerated waters has been used to argue that Fe oxidation by microbes cannot hibit the abiotic reaction (5). In addition, bacterial surfaces, which often possess anionic capsular material, may serve as effective chelators of metal cations, such as Fe^{2+}, and thus sorb Fe^{2+} from solution (2), and this Fe^{2+} may subsequently oxidize on bacterial surfaces or sheaths. This provides a passive mechanism by which bacteria might be involved in Fe oxidation.

On the other hand, *Leptothrix discophora*, a heterotrophic, sheath-forming Mn- and Fe-oxidizing bacterium, has been shown to possess both a Mn-oxidizing protein (1, 3) and an Fe-oxidizing protein (4) that appear to be responsible for accumulation of Mn oxides (8) and probably also iron oxides on the sheath. While the specific function(s) of the Fe-oxidizing protein remains to be elucidated, its existence demonstrates that enzymatic oxidation of Fe at neutral pH is possible even if it is not tied to lithotrophic growth. More importantly, *Gallionella ferruginea* has been obtained in highly purified culture, and recent evidence strongly suggests that this

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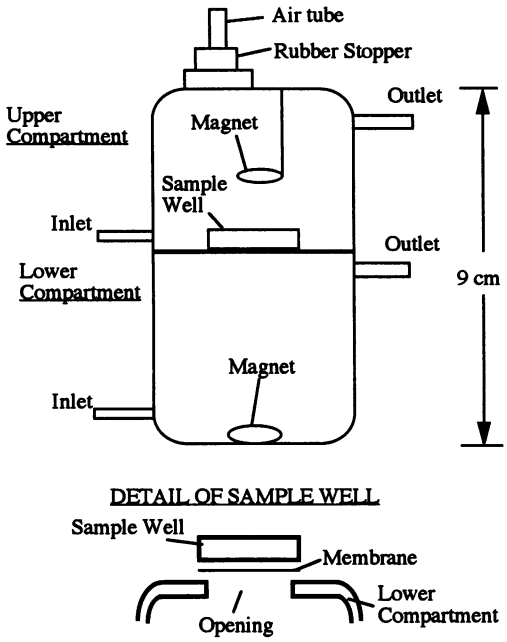

FIG. 1. Flow chamber, with a detailed view of the opening between the reservoirs and the sample well. See the text for details.

organism is capable of autotrophic or mixotrophic growth by using Fe^{2+} as an electron donor (13).

Our work on a natural iron seep, the Marselisborg site, described in the accompanying paper (9), showed that there was a large indigenous population of microorganisms, primarily bacteria, which were present at concentrations of up to 10^9 cells per cm^3 and that these microorganisms were tightly associated with the dense aggregations of Fe oxides that accumulated at this site. Because of difficulties in attempting controlled experiments at the field site, we developed a laboratory flow chamber that mimicked the field conditions by allowing medium containing $FeCl_2$ to flow across a suboxic-oxic interface containing samples of mat material. This made it possible to measure Fe oxidation rates under different conditions, to test the effects of inhibitors on the Fe oxidation rate, and to determine microscopically what type of bacterial community developed.

MATERIALS AND METHODS

Flow chamber. The chamber (Fig. 1) was constructed from a glass coupling joint. This chamber was originally designed to be operated either in flow mode, with bulk flow of medium between the reservoirs, or in diffusion mode, with simple diffusion of small molecules between the reservoirs. For all of the experiments described below the chamber was operated in flow mode. The chamber contained an upper reservoir (UR) (volume, ~35 ml) and a lower reservoir (LR) (volume, ~44 ml), which were separated by a glass plate with a 1.5-cm hole in the center. A polycarbonate membrane filter (diameter, 2.5 cm; pore size, 0.22 μm; Nuclepore) was placed over the hole and sealed with a thin bead of silicone. A polycarbonate ring (inside diameter, 1.5 cm; height, 0.5 cm) was placed on top of the membrane and was also sealed with silicone. This ring served as a well that held the inoculum directly in the flow path of Fe^{2+}-containing medium coming from the LR. The UR and the LR each had an inlet port (inside diameter, 0.15 cm) and an outlet port (inside diameter, 0.3 cm). The outlet port of the LR was sealed with a piece of tubing clamped at one end and was used only for sampling; water samples for iron analysis were obtained by piercing the tubing seal with a 25-gauge needle attached to a syringe. The inlet port of the UR was also sealed with a piece of clamped tubing. The UR had a 1.4-cm-diameter hole in the top, which was used for sampling or insertion of an oxygen microelectrode. This sampling port was sealed with a rubber stopper that had a 2-cm-long glass tube that was filled with glass wool inserted through it to provide a source of aeration for the UR. Magnets were placed in both reservoirs. The magnet in the UR was suspended on a thin metal rod, and a rotating U-shaped magnetic stirrer placed next to the chamber provided a magnetic field to rotate the magnets at approximately 50 rpm.

The feed source for each flow chamber was a 3-liter Erlenmeyer flask fitted with a special glass pressure cap that allowed a nearly constant gas composition and a nearly constant pressure to be maintained in the feed vessel during an experiment. The top of the pressure cap was fitted with two tubing ports that allowed medium to be pumped out. The cap also had two three-way valves incorporated into it which served as gas ports. One of the gas ports was used to gas the medium before an experiment was started, and the second gas port had a gas bladder attached (made from a mountain bicycle, butyl rubber inner tube) that was inflated with a gas mixture containing N_2 and CO_2 (95:5). The gas bladder was used to maintain the equilibrium of gas composition and pressure as medium was pumped out of the vessel during an experiment. The cap had fused into its side a short Balch tube with a butyl rubber stopper held in place with a crimp cap; this tube allowed $FeCl_2$ or other nutrients to be added to the feed solution with a syringe. The pressure cap and the feed reservoir were joined via identical flanges that sandwiched a butyl rubber gasket and were secured with a ring clamp.

Medium was pumped from the feed vessel with a multichannel peristaltic pump (Watson-Marlow, Inc.) by using relatively oxygen-impermeable Iso-Versinic (Veneret) tubing and passed through a bubble trap before it entered the flow chamber. Medium leaving the flow chamber via the outlet in the UR was collected in an effluent container which was set at a height that maintained a nearly neutral pressure in the UR. A plastic three-way valve was placed in the exit tubing line and was used to obtain samples for determinations of flow rate and pH during an experiment. Each chamber was suspended by a jaw clamp attached to a ring stand; by using this setup it was possible to run up to four chambers simultaneously.

Media and set-up of the flow system. Bicarbonate-buffered synthetic medium MSV was prepared with deionized water and contained (per liter) 0.1 g of NH_4Cl, 0.06 g of $MgCl_2 \cdot 6H_2O$, 0.06 g of $CaCl_2 \cdot H_2O$, 0.02 g of KH_2PO_4, and 0.03 g of Na_2HPO_4. After this medium had been heat sterilized, filter-sterilized sodium bicarbonate was added to a final concentration of 2.5 mM, and 1 ml of a filter-sterilized vitamin solution per liter was also added (20). A medium that consisted of water collected from the field site (Marselisborg H_2O) was also used. To prepare this medium, water dripping from the Marselisborg seep was collected in a plastic carboy and transported back to the laboratory. After this water stood for 1 to 2 h, during which time it turned light brown because of oxidation of the Fe^{2+} present, it was successively filtered under a vacuum through a Whatman glass fiber filter and a Millipore filter (pore size, 0.2 μm) to remove the Fe oxides, as well as microorganisms. The final filtrate was stored at 5°C and remained clear of iron oxides. Marselisborg H_2O was used either undiluted or diluted with medium MSV.

The flow chambers were assembled and autoclaved separately from the feed vessels. During autoclaving the inlets and outlets of the flow chambers were sealed with short pieces of tubing clamped at one end. The feed vessels containing the appropriate medium and the pressure caps were each autoclaved separately. After autoclaving a pressure cap was attached to each feed vessel, and the medium was bubbled with N_2 for approximately 1 h and then with N_2-CO_2 (95:5) for 15 to 20 min. This resulted in the pH of the medium being between 6.9 and 7.2. Finally, the gas bladder was inflated, and the feed vessel was ready to be attached to the flow chamber.

For inoculation, several grams (wet weight) of ocherous material was collected at the Marselisborg field site from the top 1 cm of an actively accreting mat (9). After this material had settled, the overlaying water was removed, the mat material was mixed, and then 0.2 g of glass beads (average diameter, 50 μm) per ml of slurry was added. The glass beads provided bulk and surface area that helped stabilize the mat material when it was placed into the well of a chamber. After both reservoirs of a chamber were filled with medium, the pump and stirring magnets were turned off, and 1.0 ml of the slurry was carefully layered into the well of the chamber with a pipette. Following inoculation the chamber was left undisturbed for 2 h to allow the inoculum to settle before the pump and magnets were turned on again. The final depth of the inoculum in the well was 1 to 2 mm. After the pump was turned on, $FeCl_2$ from a 100 mM stock solution was added to the feed bottle with a syringe. The $FeCl_2$ stock solution was made in a serum bottle by adding $FeCl_2$ to deionized water that had been flushed for 20 min with N_2 and then capping the bottle with a butyl rubber stopper.

All of the experiments were done in an aquarium room where the temperatures ranged from 8 to 14°C, which were close to the ambient temperatures of the water at the field site. During an individual experiment the temperature rarely fluctuated more than ±1.5°C. Except during periods of sampling or inspection, the flow chambers were covered with a black plastic bag to prevent the growth of any photosynthetic microorganisms. To prevent the growth of nitrifying bacteria during some experiments, 50 μl of deionized H_2O that had been saturated with purified acetylene was added through the port in the LR at daily intervals. Appropriate controls were also included to demonstrate that acetylene treatment did not interfere with iron oxidation.

Azide inhibition. Sodium azide was added directly to the LR of a chamber with a syringe to a final concentration of 2.5 mM. Water samples from the UR were analyzed for Fe^{2+} just prior to addition of azide and at 30-min intervals thereafter. Appropriate controls indicated that azide neither bound Fe^{2+}, as determined by the ferrozine assay (see below), nor caused any release of Fe^{2+} when the preparation was incubated in the presence of the Fe oxides present in the mat.

Iron, oxygen, and protein measurements. The concentrations of ferrous iron in the LR and the UR were measured by using the ferrozine assay described previously (9). Water samples from the reservoirs were collected with a syringe, an aliquot from each sample was added directly to ferrozine with a pipette, and the A_{562} was determined.

The oxygen levels in the UR of the flow chamber and the mat suspension itself were determined by using oxygen microelectrodes (9). An oxygen microelectrode was mounted on a micromanipulator and was inserted into the UR via the sampling port in the top. Oxygen levels were also determined in the bubble traps upstream of the chamber with a microelectrode by briefly removing the stopper in each drip trap and inserting the microelectrode.

At the end of an experiment all of the material in the UR was harvested by removing most of the overlaying water from the UR and placing it in a centrifuge tube. The few milliliters of liquid that remained in the bottom of the UR and contained most of the biomass and ferric hydroxides was mixed thoroughly and pipetted into the centrifuge tube. Finally, the adherent material on the bottom and walls of the UR was washed off three times with some of the overlaying medium by using a 1-ml syringe equipped with a 25-gauge needle. The wash water was also placed in the centrifuge tube, and the entire contents were concentrated by centrifugation at 6,000 × *g* for 8 min. The amount of protein in the concentrated suspension was determined by using the microassay procedure and a Bradford method reagent kit (Bio-Rad) for proteins; bovine serum albumin (BSA) was used as the standard. Aliquots (100 μl) of the suspension were used directly in the assay; the sulfuric acid present in the dye reagent caused rapid solubilization of the ferric hydroxides present. The high iron concentrations present in the samples did not appear to interfere with the assay, as determined by controls that showed full recovery of known amounts of BSA when BSA was added to samples of the mat suspension and then assayed by the same procedure. The Fe^{3+} concentration in the material was determined by diluting an aliquot into a hydroxylamine hydrochloride solution to reduce Fe^{3+} to Fe^{2+} and then determining the Fe^{2+} concentration by the ferrozine assay (9).

Determination of flow rates and iron oxidation rates. The flow rates through the chambers were determined daily during each experiment. Water was collected from the three-way valve on the outlet tube from each UR in pretared vials. The difference in weight before and after collection divided by the time yielded the flow rate. The pH values of the same samples were also determined with a standard pH electrode. The iron oxidation rate was determined by taking the difference between the Fe^{2+} concentration in the LR and the Fe^{2+} concentration in the UR, multiplying this value by the flow rate, and dividing the result by the volume of the inoculum, which was approximately 1 cm^3.

Microscopy. Samples were removed from the UR of the flow chamber and placed directly on microscopes slides. A 1- to 2-μl drop of 0.01% (wt/vol) acridine orange was added to each slide, and the samples were viewed and photographed with a Zeiss epifluorescence photomicroscope.

RESULTS AND DISCUSSION

Operation of flow chamber. The flow chamber was designed to provide an oxic-anoxic interface within the sample well. In practice, however, it was not possible to remove all of the oxygen from the feed medium and the LR of the flow chamber without adding a reductant (other than the $FeCl_2$). As a result, there was usually a detectable concentration of oxygen in the feed vessel (approximately 5 μM), and, by the time the medium reached the LR, the O_2 concentration had increased to 10 to 25 μM. Presumably, these traces of O_2 were from leakage through the tubing or at joints and seals in the system.

Under these microaerobic conditions, the rates of chemical oxidation of Fe^{2+} were quite low in the feed reservoirs. The rate of chemical oxidation of Fe(II) is governed by the equation: $d[Fe(II)]/dt = -k[Fe(II)][O_2][OH^-]^2$ where k is the rate constant (21). While pH clearly has a profound effect on this reaction, dissolved O_2, especially at low concentrations, can also significantly decrease the rate of the reaction. Liang et al. (17) have shown that when the partial pressure of oxygen is low (≈10 μM), this reaction exhibits an apparent zero-order behavior, and the half-life increases from approximately 12

TABLE 1. Results of six separate Fe oxidation experiments performed under different conditions in the flow chambers

Expt	Medium	Flow rate (ml/h)	Fe concn (μM)	Amt of Fe^{2+} oxidized (nmol of Fe^{2+} oxidized/h/cm^3)[a]		% Microbial[b]	Protein concn[c]	Oxide morphotype[d]
				Viable inoculum	Pasteurized inoculum			
1	Marselisborg H_2O	15	90	701 (105)	ND[e]		ND	*L. ochracea* and *Gallionella*
2	Marselisborg H_2O	17	80	1,347 (142)	363 (137)	73	175 (36)	*L. ochracea*
3	Marselisborg H_2O-MSV (1:3)	12.5	105	1,144 (573)	232	80	ND	Particulate
4	MSV	9.5	70	658 (166)	ND		170 (39)	Particulate
5	MSV	17.5	50	891 (339)	490	45	138 (44)	*L. ochracea* and *Gallionella*
6	MSV	19.5	60	886 (118)	400 (83)	55	134 (41)	*L. ochracea* and *Gallionella*

[a] Calculated as described in Materials and Methods. The values shown are the averages for entire experiments. The numbers in parentheses are 1 standard deviation of the mean. The viable inoculum was the inoculum used for the sample chamber; the pasteurized inoculum was a similar inoculum that had been pasteurized at 70°C for 20 min.
[b] The percentage of iron oxidation thought to be of microbial origin, determined by dividing the viable inoculum oxidation rate by the pasteurized inoculum oxidation rate.
[c] The final total concentration of protein in the UR of the chamber, expressed as a percentage ([concentration in the viable inoculum chamber/concentration in the pasteurized inoculum control chamber] × 100). The numbers in parentheses are the protein concentrations (in micrograms).
[d] The oxide morphotypes were determined by examining samples from the flow chambers with a light microscope.
[e] ND, not determined.

min to 40 h under pH and temperature conditions similar to those described above. This may explain, in part, the relatively slow chemical oxidation observed in these experiments. The type of medium also appeared to influence the rate of chemical oxidation (see below).

In most experiments, the Fe^{2+} concentration could be maintained between 50 and 100 μM, values which were similar to the Fe^{2+} concentrations found in the bulk of the Marselisborg Fe mat (9). Flow rates of up to 25 ml/h could be maintained in the chamber without any visible disturbance of the sample in the well, and these flow rates provided enough iron so that appreciable Fe oxidation rates were observed. It should be noted that these flow rates were significantly lower than the rates that occurred at the Marselisborg site, where the lowest flow rate was approximately 40 ml/min. The oxygen concentrations in the UR ranged from 5 to 40 μM, values which were similar to values measured in the Marselisborg mat (9). There was a small oxygen gradient, decreasing from top to bottom, within the mat in the sample well.

Iron oxidation by the microbial community. Table 1 shows the results of six separate experiments conducted in the flow chambers by using different medium compositions and flow rates. The most significant result was that in all cases, when the iron oxidation rates of viable samples and samples pasteurized at 70°C for 20 min were compared, the viable samples had much higher rates, suggesting that the microbes mediated between 45 and 80% of the iron oxidation observed. Each of the iron oxidation rates shown in Table 1 is the average of the values obtained in an entire experiment (usually six to eight time points). In cases where the standard deviation was large (e.g., experiments 3 and 5), there was significant loss of Fe in the source reservoir because of chemical oxidation during the course of the experiment. In all of these experiments the pH of the medium remained in the range from 6.8 to 7.1. In an experiment in which no inoculum was present in a chamber fed with medium MSV containing 100 μM $FeCl_2$, more than 90% of the Fe^{2+} passed from the LR through the UR, suggesting there was little binding or oxidation of the Fe^{2+} in the absence of mat material. In other experiments, the amounts of Fe^{3+} (measured as Fe^{2+} after reduction with hydroxylamine-hydrochloric acid) in the UR at the end of the experiments exhibited increases of 45 to 90% compared with the amount of Fe^{3+} present in the original inoculum.

The dynamics of Fe oxidation during three separate experiments are shown in Fig. 2. The data in Fig. 2A and B are data from experiments 2 and 6, respectively. In these two experiments the Fe^{2+} concentrations in the LR decreased by 15 and 20%, respectively, during the experiment. In experiment 5, (Fig. 2C), the Fe^{2+} concentration in the LR decreased more rapidly, possibly as a result of oxygen leaking into the feed vessel; as a result, $FeCl_2$ had to be added to the feed vessel to maintain the supply of Fe^{2+}. Because of rapid microbial oxidation, Fe^{2+} often became limiting in the UR of the chambers that contained viable mat material (Fig. 2, right panels), even when the flow rate was comparatively high (17.5 ml/h) (Fig. 2C). It did appear that when Marselisborg water was used undiluted or diluted 1:1 with medium MSV, higher Fe^{2+} concentrations could be maintained in the feed vessels. One possible explanation for this is that complexation of Fe^{2+} with natural organic material in the Marselisborg water may help stabilize Fe^{2+} against chemical oxidation. However, the interactions of Fe^{2+} and organic matter are complex; in some cases natural organic matter has been shown to increase the Fe oxidation rate (17), while in other cases it may hinder the reaction (22). In some experiments, addition of 30 μM Mn, a concentration similar to the concentration of Mn^{2+} present at the field site (9), had no noticeable effect on iron oxidation or cell growth (data not shown).

Sodium azide significantly inhibited Fe oxidation (Fig. 3). In the presence of 2.5 mM azide the rate of Fe oxidation decreased from 765 to 468 nmol/h/cm^3, a 39% decrease in the Fe oxidation rate. Addition of azide to a pasteurized sample was accompanied by a small (12%) increase in the Fe oxidation rate, from 342 to 391 nmol/h/cm^3. Azide has been shown to be an effective poison for demonstrating catalysis of Mn oxidation by bacteria (19), but to our knowledge, it has not been used previously to demonstrate poisoning of iron oxidation by microbes.

Additional evidence that Fe oxidation and microbial growth were related came from the fact that chambers supplied with Fe^{2+} had visible accumulations of bacteria and iron oxides in the sample wells and on the walls of the chambers (Fig. 4), while control chambers that contained viable samples but were not supplied with Fe^{2+} did not. Furthermore, the protein concentrations in Fe-amended chambers were consistently higher than the protein concentrations in controls that lacked Fe (Table 1). However, it was not possible to show any stoichiometric relationship between the Fe concentration and the protein content. In some experiments, otherwise identical chambers received Fe^{2+} concentrations that differed by a factor of

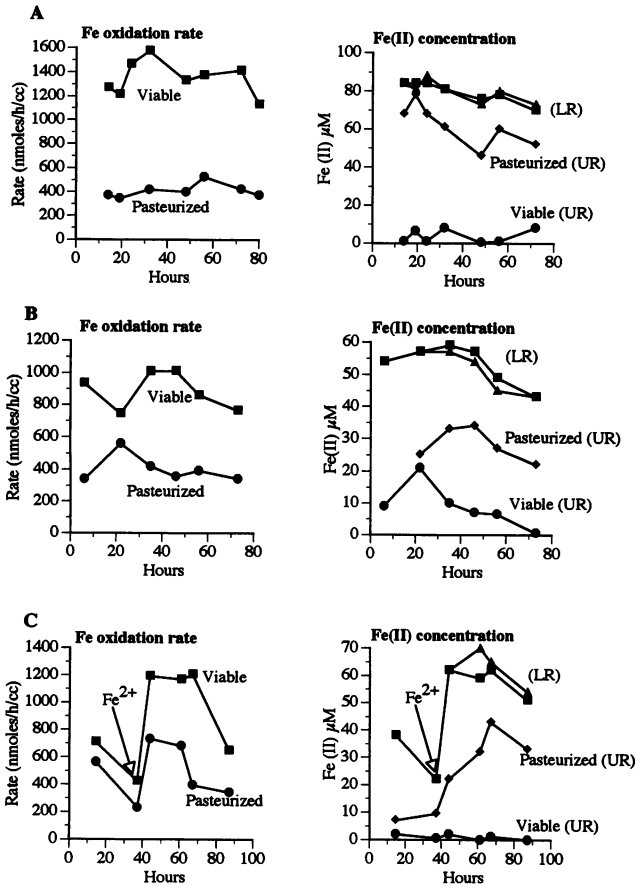

FIG. 2. Iron oxidation rates and iron concentrations in the UR and LR in three separate experiments. The panels on the left show the rates of Fe oxidation for viable and pasteurized mat samples. The panels on the right show the corresponding Fe^{2+} concentrations in the UR and LR for the separate chambers in each experiment. In panel C, the arrows indicate when $FeCl_2$ was added to the source reservoir to replenish the Fe^{2+} concentration, which had decreased significantly because of chemical oxidation. The experiments shown in panels A, B, and C correspond to experiments 2, 6, and 5, respectively (Table 1).

two, and although there was a corresponding difference in the Fe oxidation rates, there was only a small difference (<10%) in the total protein concentrations between the chambers. Although the reason for this is unknown, one difficulty with interpreting these data is that there was probably significant washout of bacteria in the chambers. The flow rates necessary to provide a sufficient supply of Fe^{2+} to the UR (10 to 20 ml/h) resulted in retention times in the UR of approximately 1.5 to 3 h. In any case, the retention time was probably much shorter than the doubling time of the microorganisms. Washout, together with the periods of Fe^{2+} limitation that occur in the UR (see above), would make it difficult to measure any stoichiometric relationships. Despite these problems, the trend toward increased protein concentrations in chambers amended with Fe^{2+} suggests that iron stimulated the growth of the microorganisms.

Field investigations of the microbial iron mat at the Marselisborg site (9) revealed that there were three primary oxide morphotypes present in the mat. These included oxides associated with the sheaths of *L. ochracea*, oxides associated with the stalks of *Gallionella* spp., and amorphously shaped

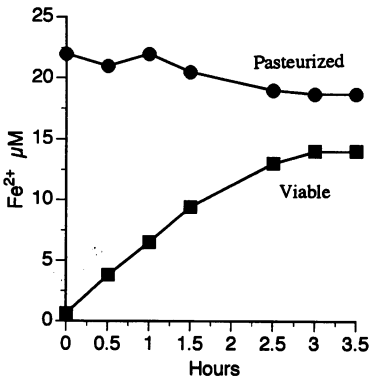

FIG. 3. Azide inhibition of Fe oxidation. Sodium azide (final concentration, 2.5 mM) was added to the LR of the chamber at time zero, and samples were removed from the UR and analyzed for Fe^{2+} concentration at different times. The Fe^{2+} concentrations in the UR of a chamber containing a pasteurized sample that also received the azide treatment are also shown.

particulate oxides which had large numbers of nonappendaged, unicellular bacteria associated with them. All three of these morphotypes were seen in the flow chambers (Table 1). Filamentous growth indicating the presence of *L. ochracea* and/or *Gallionella* sp. oxide morphotypes predominated in some experiments (Fig. 4A and 5A), while in other experiments particulate oxides tended to predominate (Fig. 4B and 5B). There was no obvious relationship between the type of medium and the oxide morphotype, nor did Fe concentration appear to have an effect (Table 1). The source of inoculum from the Marselisborg iron seep also did not appear to have an

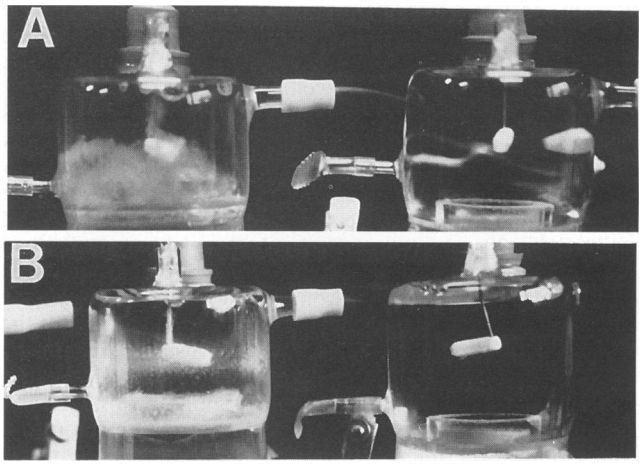

FIG. 4. Macroscopic effects of iron on the growth of microorganisms in flow chambers. (A) The chamber on the left received approximately 80 μM Fe^{2+} in full-strength Marselisborg H_2O at a flow rate of 17 ml/h (Table 1, experiment 2), and the chamber on the right contained an identical mat sample but received no Fe^{2+}. The UR of the chamber on the left is nearly filled with filamentous growth, primarily *L. ochracea* growth; in the chamber on the right the sample remained virtually unchanged in the well. (B) The chamber on the left received 105 μM Fe at a flow rate of 12.5 ml/h and the chamber on the right received no Fe. There was a significant amount of particulate growth on the wall and in the sample well of this chamber. The chamber on the right contained identical sample material but received no Fe^{2+} and exhibited little or no visible growth.

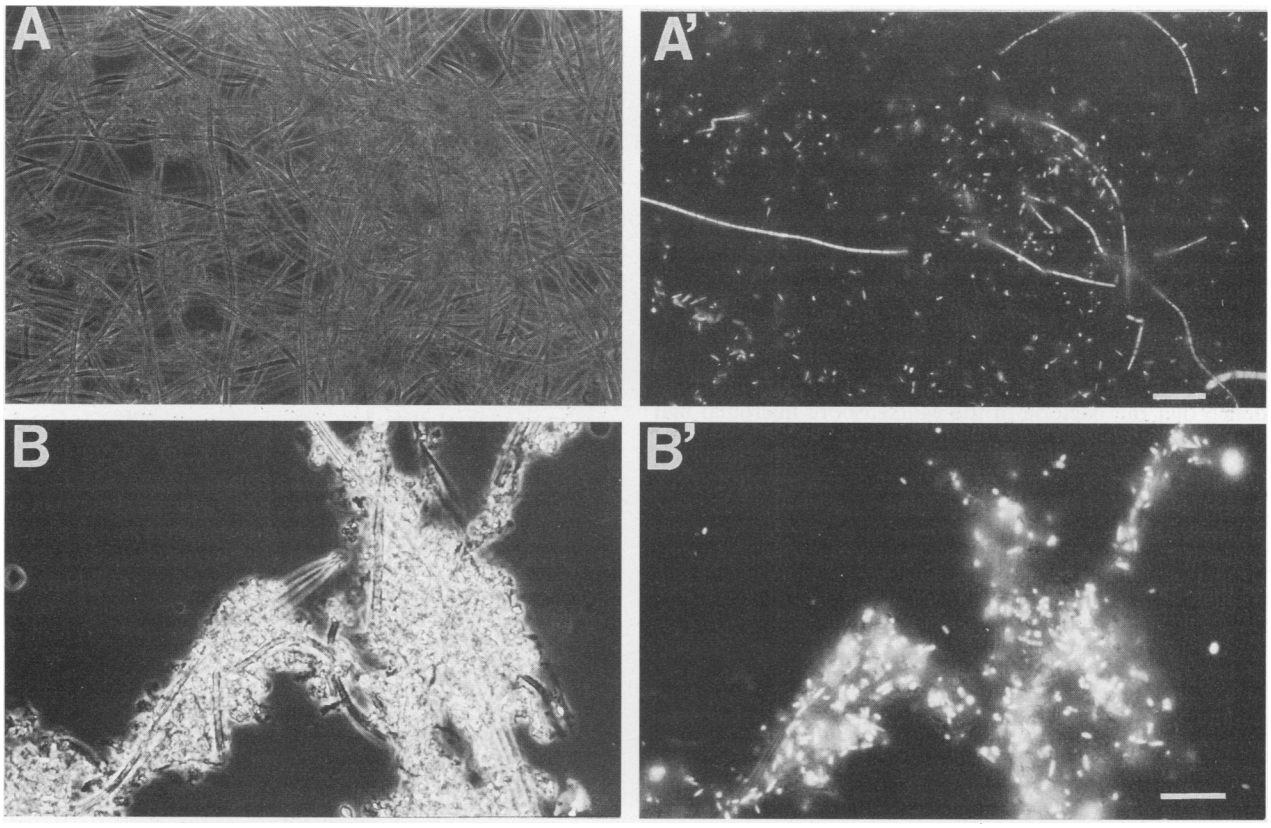

FIG. 5. Microscopic effects of Fe on growth. (A) Sample of filamentous material taken from a chamber similar to that shown in Fig. 4A, which was dominated by newly formed *L. ochracea* sheaths. (A′) Epifluorescence microscopy of the field shown in panel A revealed numerous unicellular bacteria that were associated with the iron-encrusted sheaths, as well as filaments of *L. ochracea* cells inside the sheaths. Bar = 15 μm. (B) Sample of particulate oxide material taken from a chamber similar to that shown in Fig. 4B. The amorphous particulate oxides appeared to have formed on a matrix of old sheath material. (B′) Epifluorescence microscopy of the field shown in panel B revealed large numbers of unicellular bacteria that were associated with the oxides but relatively few chains of cells inside the sheaths. Bar = 10 μm.

effect on the morphotype (data not shown). However, there did appear to be a positive relationship between the flow rate during an experiment and the presence of filamentous bacteria. When the flow rate was ≥15 ml/h, filamentous forms were visible to the naked eye (Fig. 4A). At lower flow rates (Table 1, experiments 3 and 4), the particulate oxides were predominant (Fig. 4B). This same trend was seen in three other experiments whose results are not shown in Table 1.

The results described above require some explanation, since the opposite phenomenon was observed in the mat itself, where low flow rates resulted in the growth of filamentous organisms but high flow rates appeared to be correlated with the presence of particulate oxides. However, it is difficult to compare the laboratory system with the field environment in this regard, because the flow rates in the chamber were so much lower than the flow rates in situ. While the high flow rates at some of the field sites probably resulted in large shear or drag forces that prohibited filamentous organisms from growing, in the chamber even the highest flow rates (23 ml/h) probably had little physical effect on the growth of filamentous organisms. In contrast, the overall much lower flow rates in the flow chambers may have resulted in more competition for Fe between the unicellular bacteria, which grew in dense aggregates in the sample well and attached to the wall, and the filamentous bacteria, which tended to grow out into the bulk liquid in the UR. At a lower flow rate (<15 ml/h) most of the Fe^{2+} was probably oxidized by bacteria aggregated in the mat sample and never became available to the filamentous organisms, which by the nature of their growth move up and away from more densely aggregated oxides.

The presence of the unicellular bacteria associated with the amorphously shaped particulate oxides (Fig. 5B and B′), as well as attached to Fe-encrusted sheaths (Fig. 5A and A′) and stalks, is consistent with the finding that unicellular bacteria were numerically dominant members of the in situ mat community (9). Of course, without axenic cultures and/or phylogenetic probes it is not possible to assess the relationship between the unicellular bacteria present in the flow chambers and the bacteria found in situ. However, qualitative microscopic observations revealed that the unicellular bacteria associated with fresh Fe oxide deposition in the flow chambers were less morphologically diverse than the unicellular organisms observed in in situ samples. This suggests that the bacteria in the chambers probably represented a limited number of species. The prevalence of these organisms, especially in experiments in which particulate oxides predominated, indicated that they were probably the principal microbes involved in microbial Fe oxidation.

Interestingly, *L. ochracea* and *Gallionella* spp. did appear to coexist in the flow chambers. In most cases where filamentous growth was observed, *L. ochracea* appeared to be the dominant organism, as determined by light microscopy; however, in all experiments fresh stalk material with attached apical *Gallionella* cells was also observed. The results of our field studies

suggested that the active growth zones of these two organisms did not overlap, although they could be found in very close proximity to one another (9). A possible explanation for this is that *Gallionella* spp. can grow at lower O_2 concentrations than *L. ochracea* and thus dominate in regions of the mat with very low O_2 concentrations ($\leq$10% of the ambient concentration). During some experiments in the flow chambers the O_2 concentration in the UR ranged from 5 to 40 μM. Thus, it is possible that oxic conditions favorable to *L. ochracea* and oxic conditions favorable to *Gallionella* spp. could have occurred during the same experiment. It is also possible that the bulk sampling techniques used in this study did not distinguish between different microsites that the two organisms might have occupied in the flow chamber.

Conclusions. As a whole, our results provide compelling evidence that the bacteria in the Marselisborg microbial mat community play a major role in catalyzing the oxidation of iron. On the basis of reaction kinetics alone it has been argued that iron oxidation in some Fe(II)-rich freshwater environments is an entirely abiological process (5). This does not appear to be the case at the Marselisborg iron seep, since pasteurization of the microbial community significantly inhibited Fe oxidation and azide also poisoned the process, suggesting that enzymatic catalysis of Fe oxidation probably occurs. When the microbial community was allowed to stabilize for an appreciable amount of time (several hours) and the oxygen and Fe^{2+} concentrations, as well as the flow conditions, that exist in the natural system were faithfully mimicked, it appeared that microbially mediated Fe^{2+} oxidation significantly inhibited the chemical oxidation of iron.

While our results suggest that bacteria play a significant role in Fe oxidation, proof of chemolithoautotrophic growth on iron by members of the community which we studied must wait until axenic cultures of its major members are obtained and/or workers perform more detailed laboratory studies that show stoichiometric relationships between growth of the community, CO_2 fixation, and iron oxidation. Some of this work has already been done, and evidence is mounting that *G. ferruginea* is a chemolithoautotroph that is capable of coupling oxidation of Fe^{2+} to growth (13). Of particular interest at the Marselisborg site are the unicellular bacteria that were the numerically dominant members of the microbial community both in flow chamber experiments and in situ. Although we cannot rule out the possibility that these organisms are *Gallionella* cells growing without stalks (12), this seems unlikely, since the bacteria in the flow chambers were predominantly long thin rods rather than the short bean-shaped cells characteristic of *Gallionella* spp. An intriguing possibility is that these bacteria are members of the family *Siderocapsaceae*, which are generally described as unicellular bacteria that encase themselves in capsules of Fe oxide but about which little else is known (14). The apparent stimulation of the growth of these organisms in response to iron leaves open the enticing possibility that they represent a group of lithoautotrophic iron-oxidizing bacteria that remain to be discovered and characterized.

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