University of Alabama EMSP 96-10 Project

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"Advanced Experimental Analysis of Controls on Microbial Fe(III) Oxide Reduction"

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First Year Progress Report, July 1997

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1. Summary of major research accomplishments

We have made considerable progress toward a number of project objectives during the first several months of activity on the project. An exhaustive analysis was made of the growth rate and biomass yield (both derived from measurements of cell protein production) of two representative strains of Fe(III)-reducing bacteria ***(Shewanellaalga*** strain BrY and ***Geobactermetallireducens)*** growing with different forms of Fe(III) as an electron acceptor. These two fundamentally different types of Fe(III)-reducing bacteria (FeRB) showed comparable rates of Fe(III) reduction, cell growth, and biomass yield during reduction of soluble Fe(III)-citrate and solid-phase amorphous hydrous ferric oxide (HFO). Intrinsic growth rates of the two FeRB were strongly influenced by whether a soluble or a solid-phase source of Fe(III) was provided: growth rates on soluble Fe(III) were 10-20 times higher than those on solid-phase Fe(III) oxide. Intrinsic FeRB growth rates were comparable during reduction of HF0 and a synthetic crystalline Fe(III) oxide (goethite). A distinct lag phase for protein production was observed during the first several days of incubation in solid-phase Fe(III) oxide medium, even though Fe(III) reduction proceeded without any lag. No such lag between protein production and Fe(III) reduction was observed during growth with soluble Fe(III). This result suggested that protein synthesis coupled to solid-phase Fe(III) oxide reduction in batch culture requires an initial investment of energy (generated by Fe(III) reduction), which is probably needed for synthesis of materials (e.g. extracellular polysaccharides) required for attachment of the cells to oxide surfaces. This phenomenon may have important implications for modeling the growth of FeRB in subsurface sedimentary environments, where attachment and continued adhesion to solid-phase materials will be required for maintenance of Fe(III) reduction activity. Despite considerable differences in the rate and pattern of FeRB growth with different Fe(III) forms, a roughly consistent long-term biomass yield of 5 to 15 mg protein per mmol Fe(III) reduced was observed during growth on different forms of Fe(III). These results should prove useful for quantitative modeling of FeRB growth and metabolism in various types of experimental and ***in situ*** anaerobic sedimentary systems.

A preliminary examination was made of the potential utility of ^{3}H-leucine (^{3}H-Leu) incorporation as a method for measuring rates of FeRB growth, using the same two FeRB strains mentioned above. The results indicated that G. ***metallireducens*** lacks the ability to express a high rate of exogenous leucine uptake and/or incorporation, probably due to the lack of a cell membrane leucine transport system. In contrast, S. ***alga*** showed rapid uptake and incorporation of ^{3}H-Leu during short-term ($\leq$ 4 hr) experiments, with rates of incorporation being IO-1000 fold higher than those observed for G. ***metallireducens.*** An important implication of these findings is that ^{3}H-Leu incorporation is not likely to be an appropriate method for estimating growth rates of FeRB of the ***Geobacter*** lineage. Thus, all future experiments in this project which examine FeRB growth rate as a function of geochemical parameters/conditions will be conducted with S. ***alga as*** a test organism.

Rates of protein accumulation in cultures incubated in parallel to those use for ^{3}H-Leu incorporation were compared to those calculated from ^{3}H-Leu incorporation rates. As expected, protein production rates calculated from ^{3}H-Leu incorporation by ***G. metallireducens*** were far lower (10^{4}- 10^{5} fold) than gross rates of protein accumulation. Protein production rates calculated from ^{3}H-Leu incorporation by S. ***alga*** also greatly underestimated gross rates of protein production, in this case by a factor of $\approx 10^{3}$. A likelv explanation for this result is that the addition of unlabeled leucine in these experiments (10-700 nM) was much lower than what would have been required to completely stop ***de novo*** leucine biosynthesis; i.e. there was a high degree of isotope dilution by ***de novo*** leucine biosynthesis. This interpretation is supported by the fact that calculated rates of protein production based on ^{3}H-Leu incorporation showed a direct correlation with the concentration of total leucine added. These findings indicate that accurate evaluation of S. ***alga*** growth rates based on ^{3}H-Leu incorporation will likely require addition of mM levels of total leucine.

In an effort to gain a better understanding of nature of solid-phase Fe(II) compounds formed during Fe(III) oxide reduction, we attempted to develop a wet-chemical extraction procedure for quantifying Fe(II) 'sorbed' (adsorbed and/or surface precipitated) to Fe(III) oxide surfaces in Fe(III)-reducing cultures, in particular one that would liberate Fe(II) from discrete Fe(II)-bearing mineral phases such as siderite ($FeCO_3$), vivianite ($Fe_3(PO_4)_2$), or magnetite (Fe_3O_4). The need for such an extraction stems in part from previous studies in our laboratory which have suggested that formation of surface Fe(II) complexes and/or surface Fe(II) precipitates can impede or cause complete cessation of reduction of various types of synthetic Fe(III) oxides. It was therefore of interest to have a procedure for quantifying 'sorbed' Fe(II). A logical first step was to evaluate the ability of cations with a high affinity for Fe(III) oxide surfaces to displace (e.g. by mass action) surface-associated Fe(II). We tested salts of two transition metal cations (Cu^{2+} and Mn^{2+}) with an affinity for Fe(III) oxides similar to or greater than that of Fe(II) at circumneutral pH. M n^{2+} proved to be the best alternative as Cu^{2+} interfered substantially with the colorimetric (Ferrozine) assay for Fe(II). Extraction (under strictly anaerobic conditions) with 10 mM Mn^{2+} at pH 7.2 liberated ≈ 75% of Fe(II) preadsorbed to synthetic goethite, while liberating only a minor fraction (< 5%) of the Fe(II) associated with siderite, and somewhat greater amounts of the Fe(II) associated with vivianite (≈ 25%) and magnetite (≈ 20%). However, the 10 mM Mn^{2+} extraction liberated only ≈ 15% of total solid-phase Fe(II) in a carbonate-free, low-PO_4^{3-} (44 μmol L-1 total concentration) *S. **alga*** goethite reduction culture, in which very little of the solid-phase Fe(II) would have been expected to be bound in siderite or vivianite - i.e. in which 'sorbed' Fe(II) was likely the dominant form of solid-phase Fe(II). In addition, the 10 mM Mn^{2+} extraction liberated only ≈ 20% of solid-phase Fe(II) in a G. ***metallireducens*** HFO culture, in which only a minor fraction of the solid-phase Fe(II) could be accounted by carbonate and/or phosphate minerals. In general agreement with this negative result, virtually none of the Fe(II) presorbed to synthetic HFO was liberated by the 10 mM Mn^{2+} extraction. Greater concentrations (50 and 100 mM of Mn^{2+} did not yield a substantially higher degree of Fe(II) liberation from microbially-reduced goethite or amorphous Fe(III) oxide. These results cast doubt upon the efficacy of the Mn^{2+} extraction as a means of assaying 'sorbed' Fe(II) in our microbial Fe(III) oxide reduction systems. In contrast, extraction with pH 5 1M Na-acetate effectively liberated solid-phase Fe(II) in Fe(III) oxide-reducing cultures. However, because pH 5 1M Na-acetate was found to dissolve Fe(II)-bearing mineral phases as well as liberate 'sorbed' Fe(II), it appears that this extraction does not yield significantly different information than the 0.5 M HCI extraction. Our findings suggest that 'loosely sorbed' Fe(II), which would likely have been liberated by the Mn^{2+} extraction, did not constitute a large fraction of the solid-phase Fe(II). Rather, some phase(s) subject to dissolution under moderately acidic (pH 5) conditions appeared to dominate the solid-phase Fe(II) pool. It is likely that such phases represent surface Fe(II) precipitates and/or relatively tightly bound surface Fe(II) complexes. Further studies are required to characterize the precise nature of surface-associated Fe(II) produced in Fe(III) oxide-reducing systems.

Semicontinuous culturing was adopted as the first approach for assessing the potential effect that elimination of reaction products and periodic nutrient resupply may have on the time course and extent of Fe(III) oxide reduction in experiment systems. We initiated semicontinuous cultures with synthetic amorphous HFO or goethite as Fe(III) sources, and *S. **alga*** as the test organism. Four different residence times were established by replacing either 1, 5, 10 or 20 mL of the aqueous phase of the cultures (30 mL total volume) with fresh culture medium every three days, which corresponded to mean residence times of 90, 18, 9, or 4.5 days. Results showed that replacement of relatively high volumes (10 or 20 mL) of medium had a significant influence on the cumulative amount of Fe(II) produced in the cultures. These preliminary findings conform with our hypothesis that elimination of reaction products in open systems may promote Fe(III) oxide reduction in relation to that observed in typical batch culture experiments. However, oxide reduction was promoted to only a modest degree over the 3-4 week incubation period. One reason for this less than dramatic effect is the very strong tendency for Fe(II) generated during microbial Fe(III) oxide reduction to associate with the surface of the oxide being reduced.

2. Estimate of unexpended funds from first year budget period (9/15/96-9/15/97)

Salaries and Benefits	$35,2 18 (5 1% of total budgeted)
Equipment	$21,000 (35% of total budgeted)
Travel	$588 (24% of total budgeted)
Total	$46,028 (32% of total budgeted)

The considerable amount of unexpended funds in Salaries and Benefits is the result of two circumstances:

(i) Co-PI Urrutia's salarv was covered by funds leftover from a previous Battelle PNNL subcontract through April 1997.

(ii) there was an unavoidable delay in hiring the technician to work on the project; that individual began working on the project full-time as of 24 March 1997.

The unexpended equipment funds corresponds to those budgeted for the stirred flow-through reactor and column reactor systems, which have not yet been purchased. Some of these materials may in fact be acquired before the end of the first year budget period.

3. Description of completed work

There have been 5 main areas of activity (listed below) during this first 7 months of work on this project, noting (as communicated in a electronic-mail letter to Dr. Jay Grimes on 23 September 1996) that we did not effectively begin to work on the project until January 1997 due to previous research commitments (subcontract with Battelle PNNL). Each of these areas of activity are described in some detail in the remainder of the report.

(1) Purchase and set up of equipment

(2) Determination of biomass and growth yields of dissimilatory Fe(III)-reducing bacteria

(3) Preliminary examination of ^{3}H-Leucine incorporation as a method for measuring protein synthesis rates by Fe(III)-reducing bacteria

(4) Development of Fe(II) speciation procedure

(5) Semicontinuous culturing in comparison to batch work

(1) Equipment purchasing and set up

A freeze-dryer system (Labconco Freezone 6), a N_2-adsorption surface area analyzer (Micromeritics Gemini), and an anaerobic chamber (Bactron Environmental/Anaerobic Chamber) were purchased during this year as scheduled in the budget for the first year of the project. All of these items are currently set up and in use in our laboratory. We are currently developing plans for construction of the stirred flow-through reactor and column reactor systems. No purchase of materials for development of these systems has been made as of the completion of this report, but

may occur before the end of the first year budget period.

(2) Growth Rate and Biomass Yield of Dissimilatory Fe(III)-reducing bacteria

Background

Two parameters that will be critical for development of predictive models of subsurface Fe(III) oxide reduction (and associated bioremediative processes) are the growth rate and biomass yield of Fe(III)-reducing bacteria (FeRB). Although the ability of dissimilatory FeRB to obtain energy for growth from Fe(III) reduction has been clearly established (Lovley, 1991), few studies have examined the basic physiology of these organisms in terms of growth rate and biomass yield. Given the paucity of data on this subject and the importance of having some knowledge of these parameters for the modeling purposes, exhaustive time course studies of protein production coupled to Fe(III) reduction by two different FeRB, ***Geobactermetallireducens*** and ***Shewanella alga*** (strain BrY) were undertaken. These organisms are representative of two of the major recognized groups of dissimilatory FeRB: (i) the ***Geobacteracea*** (Lonergan et al., 1996), a group of strict anaerobes in the delta subdivision of the ***Froteobacteria*** which are capable of gaining energy for growth from the complete oxidation of organic carbon compounds coupled to Fe(III) reduction; and (ii) ***the Shewanella*** genus, a group of facultative organisms in the gamma subdivision of the *Proteobacteria* which are capable of gaining energy for growth from the oxidation of H_2 or from partial oxidation of organic compounds (e.g. lactate) to acetate, coupled to Fe(III) reduction. The major questions pursued were (1) the influence of soluble vs. solid-phase Fe(III) sources on growth patterns and rates; (2) the quantitative relationship between Fe(III) reduction and biomass (protein) production with the different Fe(III) oxides; (3) comparison of growth rates for the two organisms when grown with the same Fe(III) source; and (4) the impact of the type of inoculum (aerobic or anaerobically grown) on biomass production by the facultative ***Shewanella alga,*** the test organism used for numerous previous (Roden and Zachara, 1996, Urrutia et al., 1997a, Urrutia et al., 1997b) and ongoing Fe(III) reduction studies in our laboratory. A poster entitled "Growth rates and biomass production of dissimilatory Fe(III)-reducing bacteria with soluble and solid-phase Fe(III) based on this work was presented at the 97th General Meeting of the American Society for Microbiology held in Miami, FL, May 4-8 1997. The results of this work are summarized below.

Methodology

Organisms and culture conditions. The two organisms listed above were grown anaerobically in a minimal, defined culture medium (Lovley and Phillips, 1988) at circumneutral pH with acetate ***(Geobacter)*** or H_2/malate (S. ***alga).*** as electron donors and C sources, and 50 mmol L^{-1} Fe(III) provided either as Fe(III)-citrate, amorphous hydrous ferric oxide (HFO) or goethite (Gt) as an electron acceptor.

For ***Shewanella,*** Fe(III)-reducing cultures were inoculated using either cells grown anaerobically with Fe(III) or aerobically in complex medium. In the latter case, cells were grown to late exponential phase in Tryptic Soy Broth (TSB), and washed once with 10 mM Pipes buffer at pH 6.8 before being used as inoculum. Culture tubes were incubated on their side, without shaking, and in the dark at 31 ºC. Standard anaerobic techniques were used for all procedures.

Synthesis of solid phase Fe(III) oxides. HFO and Gt were synthesized from $FeC1_3$ by the methods described in Schwertmann and Cornell (1991). The minerals were washed free of electrolytes by centrifugation. HFO was maintained as a slurry whereas Gt was freeze-dried and passed through a 100 µm (150 mesh) sieve. The total Fe content of the oxides was determined by dissolving the oxides in concentrated HCl and analyzing the Fe content of the extract with Ferrozine.

Fe(III) reduction. The total amount of Fe(II) produced during Fe(III) reduction experiments was quantified by extracting a small volume (0.25-0.5 mL) of culture in 5-10 mL of dilute (0.5 M) HCl, and measuring the Fe(II) content of the extract using the colorimetric reagent Ferrozine as previously described (Roden and Zachara, 1996).

Proteindetermination. Protein content was determined by the bicinchoninic acid (BCA) method after NaOH digestion. The BCA method is based in the reaction of proteins with Cu^{2+} in an alkaline medium to produce Cu^{+}, which subsequently reacts with two molecules of BCA to form a purple, water-soluble product with strong absorbance at 562 nm. Bovine serum albumin was used as protein standard. Because of the potential for Fe(III) oxides to adsorb organic molecules (e.g. proteins), and to possible interference of soluble iron (in the case of Fe citrate) with the colorimetric assay, protein standard curves were in all cases conducted in the same Fe(III)-containing medium as the culture samples. These standard curves were then used to calculate the protein content of the samples, thereby accounting for adsorption and/or Fe interference. Some examples of standard curves in Pipes buffer, Fe(III)-citrate, HFO, and Gt medium are shown in Fig. 1.

Both samples and standards were digested with 2.5 M NaOH for 15 minutes in a water bath at 100°C. Tubes were then cooled and neutralized with 2.5 M HCl. Duplicated 1-mL aliquots of the neutralized extract were mixed with an equal amount of freshly prepared BCA reagent (Pierce Chemical Co.) and incubated for 1 hour in a 60°C water bath. Absorbance at 562 nm was read when tubes were cool within an interval of 15 to 30 minutes, after filtering the colored extract through a 0.2-µm nylon filter to eliminate particulates. Protein contents are expressed in mg protein L^{-1} culture.

Results and Discussion

Protein production and Fe(III) reduction were tightly coupled during growth of S. ***alga*** and G. ***metallireducens*** in Fe(III)-citrate medium (Fig. 2). A clear linear relationship between Fe(II) and protein production was evident (Fig. 3). The age of the inoculum (2 or 10 d old culture) did not have a significant effect on this relationship. These results indicate that the FeRB have the capacity to generate energy for cell protein production immediately upon initiation of reduction of a soluble Fe(III) source.

In contrast to the growth patterns observed in soluble Fe(III) medium, protein production in cultures with solid-phase HFO as the electron acceptor showed a distinct lag phase during the first several days after inoculation, even though Fe(III) reduction proceeded without any lag (Fig. 4). Analogous patterns were observed for both ***S. alga*** and ***G. metallireducens.*** A similar lag in protein production was observed during growth of S. ***alga*** with synthetic Gt as an electron acceptor (Fig. 5). These results suggest that protein synthesis coupled to solid-phase Fe(III) oxide reduction in batch culture requires an initial investment of energy (generated by Fe(III) reduction), which is perhaps needed to allow for synthesis of materials (e.g. extracellular polysaccharides) required for attachment of the cells to oxide surfaces. This phenomenon (which is currently being investigated further in our laboratory) may have important implications for modeling the growth of FeRB in subsurface sedimentary environments, where attachment and continued adhesion to solid-phase materials will be required for maintenance of Fe(III) reduction activity.

Growth rates of the two FeRB with different Fe(III) sources were estimated from semilog plots of protein concentration vs. time during the period of most active protein synthesis in the cultures. Growth rates with Fe(III)-citrate as the electron acceptor were comparable for the two organisms ($4.2\ d^{-1}$ and $3.5\ d^{-1}$ for *S. alga* and *G. metallireducens*, respectively; Fig. 6), and 10-20 times higher than those observed with solid-phase HF0 (Fig. 4D,E) or Gt (Fig. 5C) as electron acceptors (note that this comparison refers to growth rates of S. ***alga*** in cultures inoculated with cells pregrown anaerobically in Fe(III) oxide-containing medium as opposed to cells pregrown

aerobically in TSB; see further discussion below). Growth rates of *G. metallireducens* and S. ***alga*** were similar with HF0 as the electron acceptor (0.32 d^{-1} and 0.35 d^{-1}, respectively; Figs. 4D,E). S. ***alga*** showed comparable growth rates with HFO and Gt as electron acceptors (oxide-grown inoculum; Figs. 4D and SC), although the total amount of Fe(II) and protein produced in medium containing 50 mmol Gt L^{-1} was much lower than that produced in medium with an equimolar amount of HFO (compare Figs. 4A and 5A). The reason for the large difference in the extent of HFO vs. Gt reduction is related to, among other things, the large difference in the surface area (and reduction site density) of the two oxides (Roden and Zachara, 1996, Urrutia et al., 1997a).

Biomass yields (mg protein produced per mmol Fe(III) reduced) during the period of active protein synthesis did not differ systematically for ***G. metal1ireducens*** and *S.* ***alga*** with HFO as the electron acceptor (Fig. 7A). The biomass yield during growth of S. ***alga*** with Gt as the electron acceptor was ≈ 3-fold lower than that with HFO. Interestingly, the biomass yield calculated for the period of active protein synthesis in the HFO cultures (Fig. 7A) was 2-fold higher than that observed during growth with Fe(III)-citrate (Fig. 3). However, when biomass yields were calculated as the ratio of the maximum amount of protein produced divided by the amount of Fe(II) produced during the corresponding time interval, higher yields were generally calculated for the Fe(III) citrate cultures (Fig. 8). This result can be attributed to the quite significant amount of Fe(III) reduction which occurred prior to initiation of high rates of protein synthesis in HFO and Gt cultures (shown clearly in Fig. 7). Despite the observed variations in overall growth yield shown in Fig. 8, our results are encouraging in that they suggest that a roughly comparable amount of protein biomass (≈ 5-15 mg per mmol Fe(II) produced) is generating during growth of FeRB coupled to Fe(III) reduction. This general result should prove useful for developing quantitative models of FeRB growth and metabolism in various types of experimental and ***in situ*** anaerobic sedimentary systems.

A comparison of Fe(III) reduction and protein production patterns by *S. alga* as a function of the type of inoculum (aerobically or anaerobically grown cells) showed important differences in both the total amounts of protein generated and the growth rates of the cells during HFO and Gt reduction (Figs. 4 and 5). Total amounts of protein produced were ≈ 50% lower, and growth rates during the period of active protein synthesis were ≈ 3 and 10-fold greater in HF0 and Gt cultures, respectively, inoculated with oxide-grown versus TSB-grown cells. The use of anaerobically pregrown cells of ***Shewanella putrefaciens*** as inoculum of Fe(III)-citrate media had previously been shown to result in substantially higher maximal growth yields relative to those obtained with aerobically pregrown cells (Myers and Meyers, 1994). Interestingly, growth rates were once again approximately equal in the HFO and Gt cultures (0.088 d^{-1} and 0.081 d^{-1}, respectively). These results reinforce previous the previous suggestion (Lovley, 1991) that quantitative studies of FeRB growth rates, at least in batch culture systems, should whenever possible be conducted using cells pregrown in medium with Fe(III) as the electron acceptor.

Conclusions

From the results presented above, we can drawn the following general conclusions:

- Two fundamentally different types of FeRB showed comparable rates of Fe(III) reduction, cell growth, and biomass yield during reduction of soluble Fe(III)-citrate and solid-phase amorphous HFO.
- Intrinsic growth rates of the two FeRB were strongly influenced by whether a soluble VS. solid-phase source of Fe(III) was provided.
- Intrinsic growth rates of S. ***alga*** were comparable during reduction of amorphous (HFO) and crystalline Fe(III) oxide (Gt).

- A roughly consistent biomass yield of 5 to 15 mg protein per mmol Fe(III) reduced was observed during growth of the two FeRB during reduction of different forms of Fe(III).

- The source of inoculum (aerobically vs. anaerobically grown) significantly affected biomass yield and growth rate of *S.* ***alga*** during reduction of solid-phase Fe(III) oxides, with faster growth rates and higher protein yields being obtained with anaerobically-grown inocula.

3. ^{3}H-Leucine Incorporation by Fe(III)-reducing bacteria.

Background

An important goal of this project is to develop a radiotracer approach for making short-term (time scale of a few hours) measurements of FeRB growth rates so that variations in FeRB growth can be related to key geochemical parameters with high temporal resolution. Our initial work toward this goal has focused on the use of the ^{3}H-labeled amino acid leucine (^{3}H-Leu) to estimate rates of bacterial protein synthesis. The basis for this approach is that during growth bacteria will take up exogenous ^{3}H-Leu and incorporate it into their cellular protein pool, and that the rate at which the ^{3}H appears in that pool during short-term incubations can be used to estimate the total rate of bacterial protein synthesis (Kirchman et al., 1985). Unlabeled ("cold") leucine is added together with ^{3}H-Leu to repress ***de novo*** leucine biosynthesis by the cells, which if it occurred would lead to isotope dilution of the added ^{3}H-Leu and thereby complicate estimation of the protein synthesis rate based on the rate of ^{3}H-Leu incorporation (Kirchman, 1993). As discussed in more detail below, accounting for this phenomenon is apparently going to be critical to developing an accurate protocol for estimating FeRB growth rates based on ^{3}H-Leu incorporation rates in our experimental systems.

Methodology

A portion of a ^{3}H-Leu stock solution (0.05-0.1 mL) was added to Fe(III)-reducing cultures (5-10 mL total volume) to yield a final ^{3}H activity of $\approx 2\ \mu$Ci mL^{-1}. The ^{3}H-Leu stock solutions also included variable amounts of unlabeled leucine such that a range of final total leucine concentrations of ≈ 10 to 1000 nM were achieved. The specific activity of the original concentrated ^{3}H-Leu solution (purchased from Amersham Radiochemicals) was 150-200 Ci mmol^{-1}, so that addition of the radiotracer leucine solution itself corresponded to a baseline addition of ≈ 10 nM total leucine. After incubation for 0.5-24 hrs, a 0.5-mL subsample of culture was added to 0.5 mL of 10% trichloroacetic acid (TCA) in a microcentrifuge tube to achieve a final TCA concentration of 5% (Kirchman, 1993). The TCA stops biological activity and precipitates cellular macromolecules, including proteins. Killed controls consisted of cultures amended with concentrated TCA to a final concentration of 5%. After fixation in TCA for 1-16 hrs, a 0.25 or 0.5-mL aliquot of the fixed culture was filtered through a 0.2 µm nylon filter. The filters were then washed twice with ice-cold 5% TCA to remove unincorporated ^{3}H-Leu from material on the filter, and twice with ice-cold 80% to remove any ^{3}H-Leu that may have become incorporated into cellular lipids (Kirchman, 1993). The filters were placed into scintillation vials and 10 mL of scintillation cocktail (Aquasol, Packard Radiochemicals) plus 5 mL of distilled H_20 were added (the addition of H_20 caused formation of a gel which holds the filter in suspension). The ^{3}H activity on the filters was measured by liquid scintillation counting (Beckman model LS 5801). In all cases, appropriate portions of the ^{3}H-Leu stock solutions were counted in the same matrix as the filters in order to allow calculation of the fraction of added ^{3}H-Leu that was incorporated into cell protein per unit time during the incubation period.

Rates of protein synthesis were estimated from rates of ^{3}H-Leu incorporation according to the following equation (Kirchman, 1993):

$$\text{Protein synthesis} = \frac{^{3}\text{H - Leu incorporation rate}}{\text{Total DPM } ^{3}\text{H - Leu added}} \times [\text{Leu}]_{\text{Tot}} \times 131 \times (0.07)^{-1} \qquad (1)$$

where protein synthesis is expressed in units of ng $L^{-1} hr^{-1}$, ^{3}H-Leu incorporation rate is expressed in units of DPM $mL^{-1} hr^{-1}$, total DPM ^{3}H-Leu added is expressed in units of DPM mL^{-1}, $[Leu]_{Tot}$ is the total concentration of leucine added (radiotracer plus unlabeled) in units of nmol L^{-1} (nM), 131 is the molecular weight of leucine (ng $nmol^{-1}$), and 0.07 is the assumed fraction (mass basis) of cellular protein accounted for by leucine (Kirchman, 1993). The assumed conversion factor of 0.07 may not be exactly correct for the FeRB strains used in this study, but is likely to be reasonably close and thus useful for making first approximations of protein synthesis rates from ^{3}H-Leu incorporation rate measurements. As is revealed below, minor variations in this parameter would not change the conclusions drawn from the experiments described in this report.

Results and Discussion

A preliminary experiment with S. ***alga*** growing in synthetic HFO medium with 30 mM lactate as an carbon/energy source showed that an ample amount of ^{3}H-Leu was incorporated into cellular protein during a 3-hr incubation period with a 10-700 nM range of total added leucine (Table 1). A time course experiment with ***G. metallireducens*** growing in acetate/FIFO medium (Fig. 9) showed considerably lower (5-10 fold) rates of ^{3}H-Leu incorporation during a comparable incubation period (Table 1). Relatively low rates of ^{3}H-Leu incorporation by ***G. metallireducens*** growing in acetate/HF0 medium were also observed in a second time course experiment with three different levels of total added leucine (Fig. 11; Table 1). Each of these experiments was conducted with 4-d old cultures, in which (based on results shown in section 2 above) protein synthesis should have been at least starting to become well-coupled to Fe(III) reduction.

The rate of Fe(III) oxide reduction during the time period in which that the second ^{3}H-Leu incorporation experiment was conducted with *G.* ***metallireducens*** ($\approx$ 4.3 mmol $L^{-1} d^{-1}$; Fig. 11A) was comparable to that occurring during the time period in which the ^{3}H-Leu incorporation experiment was conducted with *S.* ***alga*** (Fig. 11B). Assuming that protein synthesis was well-coupled to Fe(III) reduction during the ^{3}H-Leu incorporation experiments, the fact that G. ***metallireducens*** showed much lower rates of ^{3}H-Leu incorporation ***than S. alga,*** while reducing Fe(III) at a comparable rate, suggested that ***G. metallireducens*** has an intrinsically lower capacity for uptake and/or assimilation of ^{3}H-Leu than ***S. alga.*** This question was examined in a subsequent experiment with both organisms growing in soluble Fe(III)-citrate medium, with acetate as the electron donor and carbon ***source*** for ***G. metallireducens,*** and H_2/malate as the electron donor/carbon source for ***S. alga.*** This experiment was conducted with cultures in the middle of their growth cycle ($\approx$ 13 hrs after inoculation), so that there was no question that Fe(III) reduction was closely coupled to protein production (see Fig. 6). S. ***alga*** incorporated ^{3}H-Leu at a rate approximately 600-fold higher than ***G. metallireducens,*** despite the fact that rates of Fe(III) reduction were similar (Fig. 12).

Taken together the above results suggest that ***G. metallireducens*** lacks the ability to express a high rate of exogenous leucine uptake and/or incorporation, perhaps due to the lack of a cell membrane leucine transport system. This suggestion is consistent with the fact that rates of ^{3}H-Leu incorporation by this organism were not fundamentally different with soluble Fe(III)-citrate vs. solid-phase HF0 as an electron acceptor (Table l), even though rates of Fe(III) reduction (and cell growth; compare Figs. 3B and 4E) were vastly higher ($\approx$ 20-fold) in the former, which likely indicates that simple diffusion was the mechanism for ^{3}H-Leu uptake by *G.* ***metallireducens.*** In contrast, rates of ^{3}H-Leu incorporation by ***S. alga*** were $\approx$ 75 times higher with Fe(III) citrate vs. HFO as the electron acceptor (100- 150 nM total leucine concentration; Table l), likely due to the fact the cells were reducing Fe(III) and growing much more rapidly in Fe(III) citrate medium

(compare Figs. 3A and 4D) and thus capable of expressing a much higher rate of exogenous leucine uptake and incorporation. The conclusion that *G.* ***metallireducens*** lacks, and S. ***alga*** possesses, an active leucine import system is not surprising considering that ***G. metallireducens*** is a strict anaerobe known only to inhabit anaerobic soils and sediments (Lovley et al., 1993), whereas S. ***alga*** is a facultative organism originally isolated from spoiled food and clinical samples (Rossello-Mora et al., 1994). That is, *S.* ***alga*** is known to commonly inhabit highly organic-rich environments where free amino acids are likely to be readily available, whereas this is clearly not the case for *G.* ***metallireducens*** or other recently isolated strains of the genus ***Geobacter*** (Coates et al., 1996).

An important implication of the above findings is that ^{3}H-Leu incorporation is not likely to be an appropriate method for estimating growth rates of FeRB of the ***Geobacter*** lineage. Thus, all future experiments in this project which examine FeRB growth rate as a function of geochemical parameters/conditions will be conducted with *S.alga* as a test organism. An additional implication of this finding is that ^{3}H-Leu incorporation may not be an appropriate means of estimating bacterial growth rates in samples of natural Fe(III)-reducing soil or sediment.

It was of interest whether protein synthesis rates calculated from ^{3}H-Leu incorporation rates were in the same range as rates of gross protein accumulation in the cultures. Rates of gross protein accumulation were estimated from measured Fe(III) reduction rates and appropriate conversion factors for protein production coupled to Fe(III) reduction (see Table 2 for details). As expected from the results just presented, protein production rates calculated from ^{3}H-Leu incorporation by G. ***metallireducens*** were far lower (10^{4}- 10^{5} fold) than gross rates of protein accumulation (Table 2). Protein production rates calculated from ^{3}H-Leu incorporation by S. ***alga also*** greatly underestimated gross rates of protein production (Table 2), in this case by a factor of $\approx 10^{3}$. The most likely explanation for this latter result is that the addition of unlabeled leucine in these experiments was much lower than what would have been required to completely stop *de* ***novo*** leucine biosynthesis; i.e., there was a high degree of isotope dilution (cf. Kirchman, 1993) caused by ***de*** *nova* leucine biosynthesis. This argument is supported by the fact that calculated rates of protein production based on ^{3}H-Leu incorporation showed a direct correlation with the concentration of total leucine added (Fig. 13). These findings indicate that accurate evaluation of S. ***alga*** growth rates in Fe(III)-reducing media based on ^{3}H-Leu incorporation will likely require addition of mM levels of total leucine. This represents a current line of investigation by the project PI.

(4) Fe(II) speciation

Background

At present two wet-chemical procedures are typically used to speciate Fe(II) produced during microbial Fe(III) oxide reduction experiments: (i) 0.5 M HCl extraction to recover total Fe(II), and (ii) direct determination of soluble Fe(II) after centrifugation or filtration to remove particulates. These procedures allow for a simple separation of aqueous and solid-phase-associated Fe(II). However, they do not allow for differentiation of various types of solid-phase-associated Fe(II), e.g. 'sorbed' (adsorbed and/or surface precipitated) Fe(II) vs. Fe(II) associated with discrete Fe(II)-bearing mineral phases [e.g. $Fe_3(PO_4)_2$ (vivianite), $FeCO_3$ (siderite) or Fe_3O_4 (magnetite)]. The nature of solid-phase-associated Fe(II) compounds formed during Fe(III) oxide reduction may have an important influence on the long-term rate and extent of oxide reduction in Fe(III) reduction experiments. For example, previous studies in our laboratory have suggested that formation of surface Fe(II) complexes and/or surface Fe(II) precipitates can impede or cause complete cessation of reduction of various types of synthetic Fe(III) oxides (Roden and Zachara, 1996, Urrutia et al., 1997a). In contrast, formation of discrete Fe(II)-bearing mineral phases separate from the oxides being reduced might be expected to have little effect on oxide reduction. It was therefore our

intention to search for an extraction which would allow us to obtain an estimate of 'sorbed' Fe(II) (adsorbed and/or surface precipitated) without affecting discrete Fe(II)-bearing mineral constituents.

Approach and Methodology

A logical first step in the development of an extraction to liberate 'sorbed' Fe(II) from Fe(III) oxide surfaces was to test the ability of cations with a high affinity for Fe(III) oxide surfaces to displace (e.g. by mass action) surface-associated Fe(II). This approach is analogous to the use of Mg^{2+} ions to displace loosely adsorbed trace metals from soils and sediments (Tessier et al., 1979), with the exception that transition cations with a much higher affinity for Fe(III) oxides than Mg^{2+} (Stumm, 1992) would likely be required to disrupt the very stable Fe(III)-O-Fe(II) interaction (Sherman, 1987). Extraction with pH 5 1M Na-acetate has been used to liberate 'specifically adsorbed' trace metals in soils and sediments (Tessier et al., 1979), and testing in our laboratory (Roden and Urrutia, unpubl data) has shown that the pH 5 1M Na-acetate efficiently liberates 'sorbed' Fe(II) from Fe(III) oxide surfaces. However, we knew from previous tests that pH 5 1M Na-acetate completed dissolves freshly synthesized $FeCO_3$, $Fe_3(PO_4)_2$, and Fe_3O_4 - i.e., materials likely to be similar to formed during microbial Fe(III) oxide reduction in carbonate-buffered medium. Thus we did not feel this extraction was appropriate as a general means of quantifying 'sorbed' Fe(II) because of possible interference with carbonate-associated Fe(II).

Based on the above considerations, preliminary experiments therefore tested salts of two transition metal cations (Cu^{2+} and Mna+) with an affinity for Fe(III) oxides similar to or greater than that of Fe(II) at circumneutral pH. The salts were dissolved in anaerobic (N_2-bubbled) Pipes (10 mM) or Hepes (100 mM) buffer at pH 7.2, and small quantities of culture material or pure phase minerals were added to 5-10 mL of salt solution under strictly anaerobic conditions. After a 0.5-2 hr extraction period, an aliquot of the extract was centrifuged inside the anaerobic chamber, and 1 mL of supernatant was acidified with 0.1 mL of 1N HCl to stabilize the Fe(II). The Fe(II) content of the extract was then analyzed with Ferrozine as described above.

Results and Discussion

Preliminary experiments with synthetic Gt preadsorbed with Fe(II) (180 µmol g^{-1}, ≈ 80% of the maximum Fe(II) sorption capacity; Urrutia et al., 1997) showed that extraction with 10 mM M-$6O_4$ offered better results than 10 mM $CuSO_4$, since the Cu^{2+} caused considerable interference with the Ferrozine assay of Fe(II) in the extract. Subsequent tests showed that the presence of 10 mM Mn^{2+} did not interfere at all with the Ferrozine determination of Fe(II) (Fig. 14). The extraction with 10 mM Mn^{2+} extraction (in 10 mM Pipes buffer) liberated ≈ 80% of the Fe(II) adsorbed to synthetic Gt.

Based on the above results, the 10 mM Mn^{2+} extraction procedure was evaluated for its ability to liberate Fe(II) from various materials commonly associated with our experimental Fe(III) oxide reduction systems: Fe(II) presorbed to goethite (Fe(II)-Gt; 180 µmol g^{-1}), or to HFO (Fe(II)-HFO; 0.02-0.4 mM Fe(II) sorbed to a 50 mmol L^{-1} HFO suspension), siderite, vivianite, and magnetite [1]. Extractions were also performed with microbially-reduced and synthetic HFO and Gt. In all cases Fe(II) liberated by extraction with Mn^{2+} was corrected for soluble Fe^{2+} in the medium. In addition, the amount of Fe(II) liberated in a control extraction with Pipes or Hepes buffer alone

[1] Siderite was synthesized by reaction of a solution of $FeCl_2$ (300 nM) with Na_2CO_3 (300 mM) under anaerobic conditions. Vivianite was obtained by reaction of a $FeC1_2$ solution (200 mM) with a $KHPO_4$ solution (600 mM) for 4 days inside an anaerobic chamber. Both minerals were washed twice with anaerobic distilled water and resuspended to a thick slurry in which total Fe(II) concentration was determined by 0.5 M HCl extraction. The magnetite tested in this procedure was obtained from old *S. alga* HFO cultures after sterilization and washing in sterile buffer. Fe(II)-Gt and Fe(II)-HFO were prepared as described in Urrutia et al. 1997.

was determined to account for possible dissolution of solid Fe(II) phases during extraction of relatively small volumes of medium in relatively large volumes of extractant. The amount of Fe(II) liberated was expressed as % of 0.5 M HCl-extractable Fe(II) (Fig. 15).

Extraction with 10 mM Mn^{2+} at pH 7.2 liberated ≈ 75% of Fe(II) preadsorbed to synthetic Gt, while liberating only a minor fraction (< 5%) of the Fe(II) associated with siderite, and somewhat greater amounts of the Fe(II) associated with vivianite (≈ 25%) and magnetite (≈ 20%) (Fig. 15). However, the 10 mM Mn^{2+} extraction liberated only ≈ 15% of total solid-phase Fe(II) in an old carbonate-free, low-PO_4^{3-} (44 µmol L^{-1} total concentration) *S.* ***alga*** Gt culture, in which very little of the solid-phase Fe(II) would have been expected to be bound in siderite or vivianite - i.e. in which 'sorbed' Fe(II) was likely the dominant form of solid-phase Fe(II). In addition, the 10 mM Mn^{2+} extraction liberated only ≈ 20% of solid-phase Fe(II) in an old *G.* ***metallireducens*** HFO culture, in which only a minor fraction of the solid-phase Fe(II) could be accounted by carbonate (based on a wet chemical assay for solid-phase vs. aqueous dissolved inorganic carbon; Roden, unpubl method) and/or phosphate (based on the relatively low total PO_4^{3-} concentration - 0.44 mmol L^{-1} - in the medium) minerals. Note that in the case of this HFO culture, approximately 13% of solid-phase Fe(II) was dissolved in buffer alone, compared to 35% in the 10 mM Mn^{2+} extraction, with the difference yielding a value of 22% for the fraction of 'sorbed' Fe(II) displaced by Mn^{2+} ions. In general agreement with this negative result, virtually none of the Fe(II) presorbed to synthetic HFO was liberated by the 10 mM Mn^{2+} extraction. Greater concentrations (50 and 100 mM) of Mn^{2+} did not yield a substantially higher degree of Fe(II) liberation from microbially-reduced Gt or HF0 (data not shown).

The above results cast doubt upon the efficacy of the Mn^{2+} extraction as a means of assaying 'sorbed' Fe(II) in our microbial Fe(III) oxide reduction systems. In contrast, the pH 5 1M Na-acetate extraction effectively liberated solid-phase Fe(II) in Fe(III) oxide-reducing cultures in which discrete Fe(II)-bearing mineral phases could not have accounted for a large fraction of the solid-phase Fe(II) pool. In this regard, the Na-acetate extraction functioned in manner essentially identical to 0.5 M HCl. Since pH 5 1M Na-acetate is likely to dissolve Fe(II)-bearing mineral phases as well as liberate 'sorbed' Fe(II), it appears that this extraction does not yield significantly different information than the 0.5 M HCI extraction.

Our findings suggest that 'loosely sorbed' Fe(II), which would likely have been liberated by the Mn^{2+} extraction, did not constitute a large fraction of the solid-phase Fe(II). Rather, some phase(s) subject to dissolution under moderately acidic (pH 5) conditions appeared to dominate the solid-phase Fe(II) pool. It is likely that such phases represent surface Fe(II) precipitates and/or relatively tightly bound surface Fe(II) complexes. Because the cultures tested were not likely to contain large amounts of Fe(II)-bearing mineral phases, it is possible that surface or discrete Fe(II) hydroxide precipitates (e.g. $Fe(OH)_2$) constituted the dominant form of solid-phase Fe(II). Further studies are required to characterize the precise nature of surface-associated Fe(II) produced in Fe(III) oxide-reducing systems.

(5) Semicontinuous culturing

Background

To date laboratory studies of the controls on microbial Fe(III) oxide reduction have been conducted almost exclusively in batch cultures, usually with pure phase synthetic Fe(III) oxides. In contrast, the subsurface represents an open system in which removal of reaction end-products (e.g. Fe(II)) can occur. Semicontinuous culturing was adopted as the first approach to assess the potential effect that the elimination of reaction products and periodic nutrient resupply may have on the time course and extent of Fe(III) oxide reduction. These goal of these studies and future studies with stir-flow reactor and column systems is to generate quantitative insight into how

chemical and microbiological components interact to control rates of FeRB growth and activity in open experimental systems.

Approach and Methodology

We initiated semicontinuous cultures in 60 mL serum bottles with 50 mmol Fe(III) L^{-1} in the form of synthetic HFO or Gt. *S.* ***alga*** was used as the test organism. The growth medium (30 mL per bottle) contained a relatively low level of PO_4^{3-} (44 µmol L^{-1}), with 8 mM malate as a carbon source and H_2 an electron donor. Four different residence times were established by replacing either 1, 5, 10 or 20 mL of the aqueous phase of the cultures with fresh culture medium every three days. These levels of medium replacement corresponded to mean dilution rates of 0.011,0.056, 0.11, or 0.22 d^{-1}, equivalent to mean residence times of 90, 18, 9, or 4.5 days. A batch culture which received no medium replacement was run in parallel with the semicontinuous cultures.

The solid-phase materials in the culture bottles was allowed to settle overnight before sampling. The appropriate volume of spent medium was extracted with a 4" 18G sterile stainless steel needle. An aliquot of this spent medium was acidified with 0.5 N HCI for subsequent analysis of Fe(II) concentration. An equal amount of fresh sterile medium was then added in the bottles, after which the bottles were thoroughly mixed an a sample removed for total Fe(II) determination by 0.5 M HCl extraction. An amount of H_2 gas equal to the amount of medium was then added before returning the cultures to the 30 ^oC incubator.

Results and Discussion

Our results showed that replacement of relatively high volumes (10 or 20 mL) of spent medium had a significant influence on the cumulative amount of Fe(II) produced in the semicontinuous cultures (Fig. 16). In the experiment with Gt, replacement of 10 mL of medium every three days increased the total amount of Fe(II) produced by ≈ 35%. Replacement of 20 mL of medium every three days increased total Fe(II) production in the HFO experiment by a comparable amount. These preliminary results conform with our hypothesis that elimination of reaction products in open systems may promote Fe(III) oxide reduction in relation to that observed in typical batch culture experiments. However, oxide reduction was promoted to only a modest degree over the 34 week incubation period. One likely reason for this less than dramatic effect is the very strong tendency for Fe(II) generated during microbial Fe(III) oxide reduction to associate with the surface of the oxide being reduced (see section 4 above). In upcoming experiments, the growth medium will be amended with soluble compounds capable of complexing Fe(II) in order to evaluate the extent to which the presence of such compounds may enhance the long-term reducibility of Fe(III) oxides in open culture systems.

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Table 1. ^{3}H-Leucine incorporation rates by *G. **metallireducens** **(G.m.)*** and *S. **alga*** strain BrY ***(S.a.)*** during reduction of amorphous HFO and Fe(III)-citrate.

Organism	Fe(III) Form	Total [Leu] Added (nM)	^{3}H-Leu Incorporation Pate (DPM mL^{-1} hr^{-1})
S.a.	***HFO***	10	14147a
S.a.	***HFO***	50	29178[a]
S.a.	***HFO***	150	9096[a]
S.a.	***HFO***	400	9176[a]
S.a.	***HFO***	700	21424[a]
S.a.	Fe(III)-citrate	100	682130[b]
G.m.	***HFO***	100	2810[C]
G.m.	***HFO***	9.6	353[d]
G.m.	***HFO***	101	265[d]
G.m.	***HFO***	829	1184[e]
G.m.	Fe(III)-citrate	100	

[a] average of triplicate samples, 3-hr incubation time

[b] slope of DPM ^{3}H-Leu incorporated mL^{-1} vs. time for 3 hr time course (Pig. 12).

[c] slope of DPM ^{3}H-Leu incorporated mL^{-1} vs. time for 24 hr time course (Fig. 9).

[d] slope of DPM ^{3}H-Leu incorporated mL^{-1} vs. time for 4 hr time course (Fig. 10).

[e] slope of DPM ^{3}H-Leu incorporated mL-1 vs. time for 6 hr time course (Fig. 12).

Table 2. Protein production rates by *G. **metallireducens*** and *S. **alga*** estimated from ^{3}H-Ieucine incorporation vs. gross increase in culture protein content[a].

Organism	Fe(III) Form	Total [Leu] Added (nM)	Protein Production Rate (ng $L^{-1} hr^{-1}$) ^{3}H-Leu Incorp	Gross
S.a.	HFO	700	7.60×10^{3}	[b]2.84×10^{6}
S.a.	Fe(III)-citrate	100	19.9×10^{3}	[c]35.41×10^{6}
G.m.	HFO	100	0.97×10^{2}	ND
G.m.	HFO	829	1.68×10^{2}	[d]6.53×10^{6}
G.m.	Fe(III)-citrate	100	0.35×10^{2}	[e]24.7×10^{6}

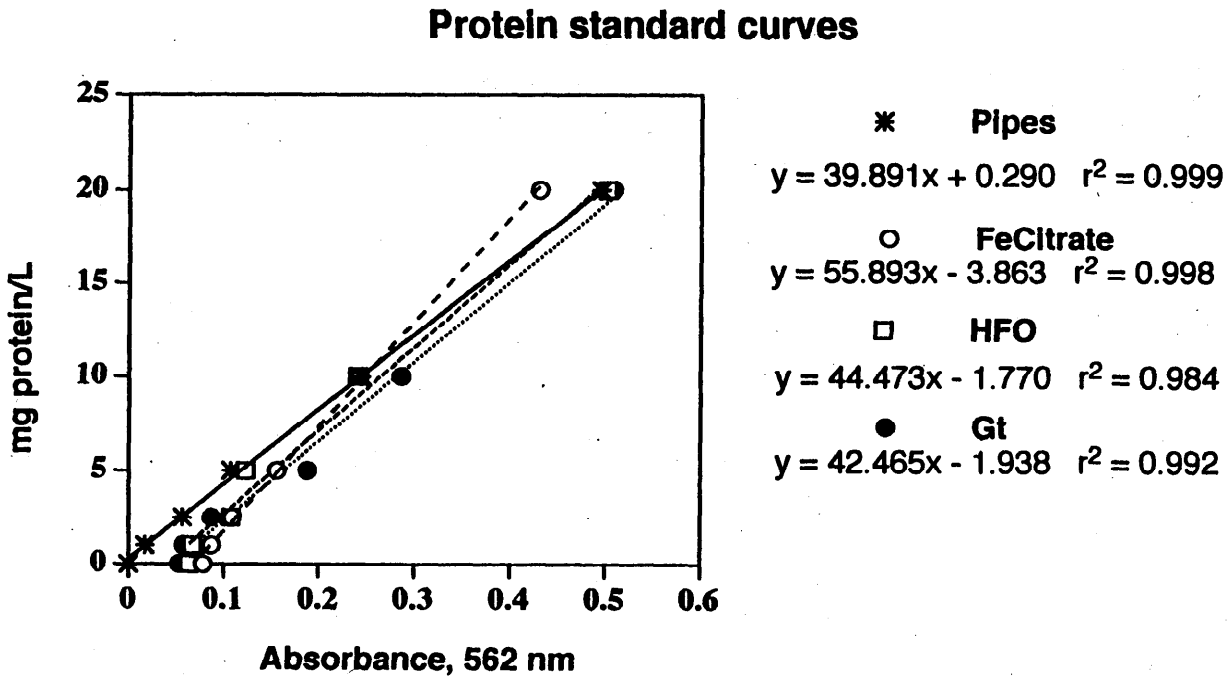
[a] Gross protein production was estimated from rates of Fe(III) reduction (measured in parallel cultures contemporaneously with the ^{3}H-Leucine incorporation studies) multiplied by previously conversion factors for protein production coupled to HFO and Fe(III)-citrate reduction.

[b] Factor for converting Fe(III) reduction rate to protein production rate = 32.0 mg protein mmol $Fe(II)^{-1}$ (Fig. 7A).

[c] Factor for converting Fe(III) reduction rate to protein production rate = 17.2 mg protein mmoI $Fe(II)^{-1}$ (Fig. 3A).

[d] Factor for converting Fe(III) reduction rate to protein production rate = 32.0 mg protein mmol $Fe(II)^{-1}$ (Fig. 7A).

[e] Factor for converting Fe(III) reduction rate to protein production rate = 15.2 mg protein mmol $Fe(II)^{-1}$ (Fig. 3B).



gure 1. Bovin serum albumin (BSA) standard curves in Pipes buf
e citrate, HFO and Gt media for the BCA protein determination
ocedure.

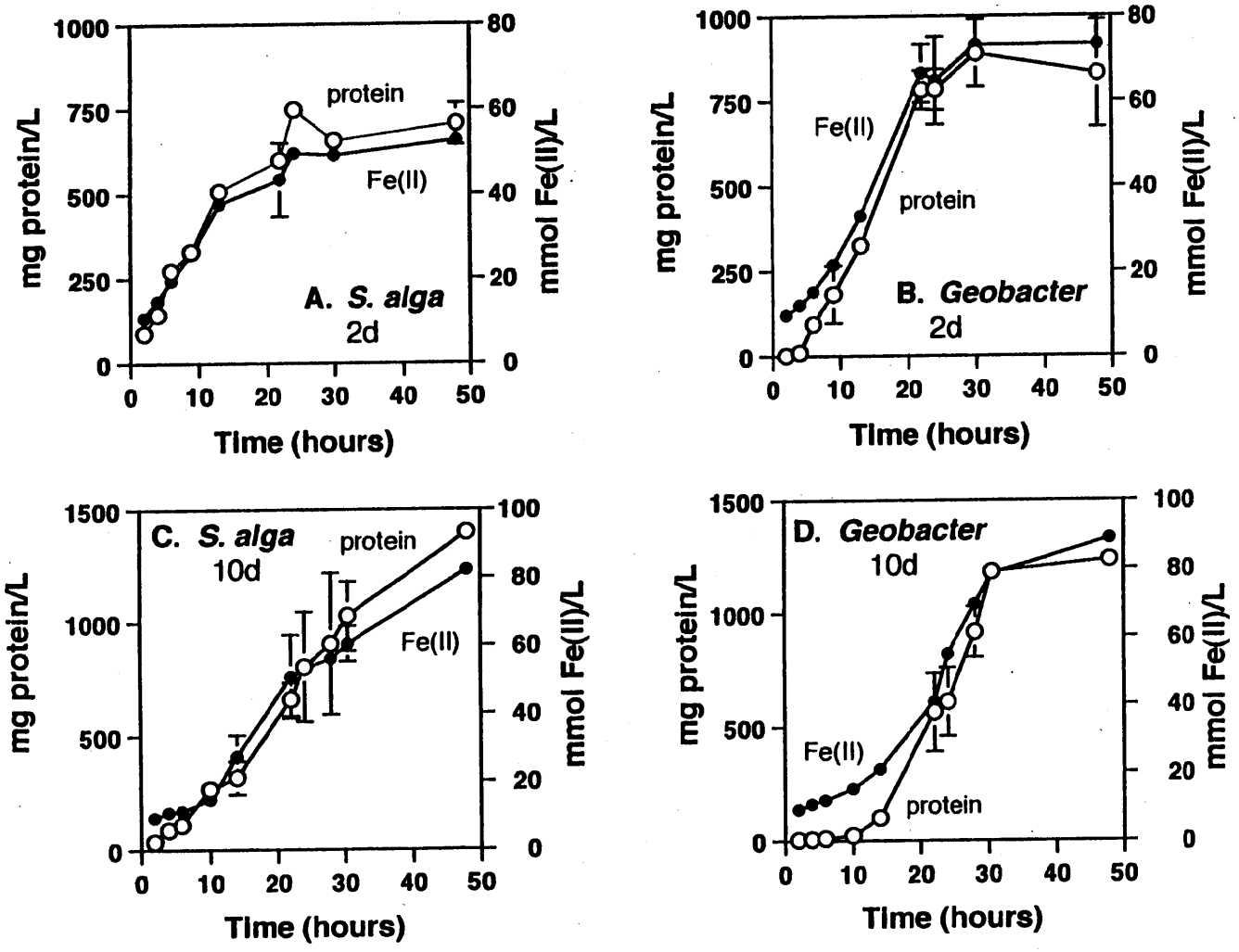


Figure 2. Protein and Fe(II) production by *S. alga* (A, C) and *G. metallireducens* (B,D) during reduction of Fe(III) citrate. Panels A and B show results for a 2d-old inoculum and C and D for a 10d-old inoculum.

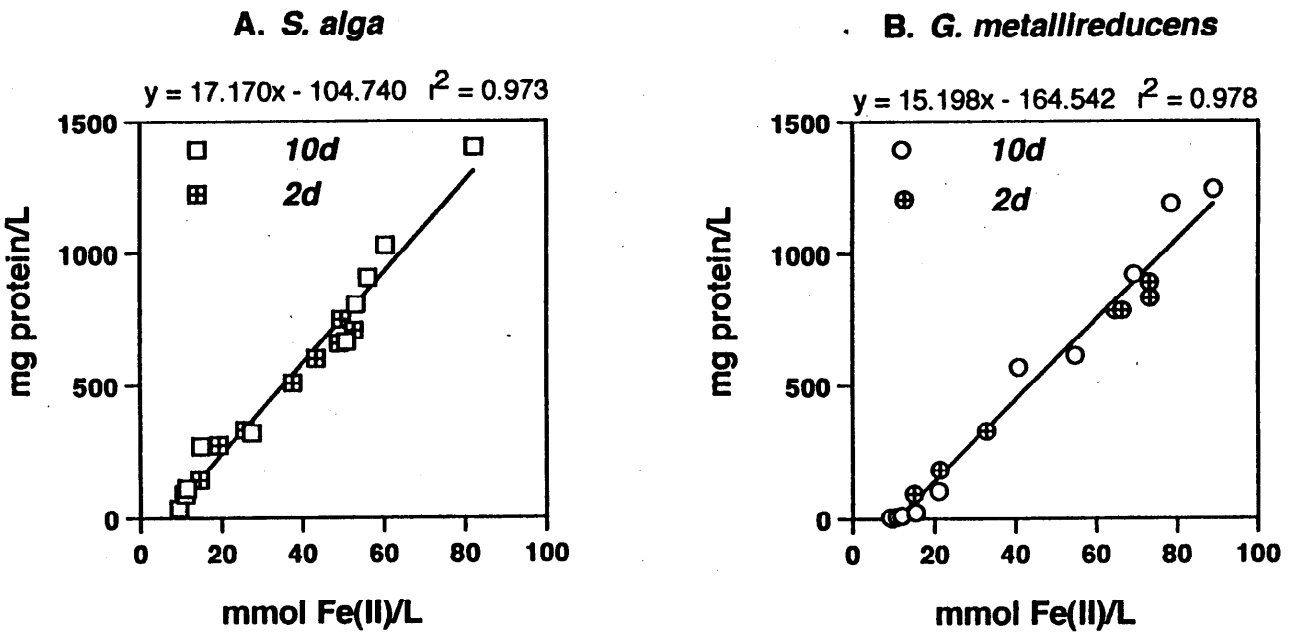
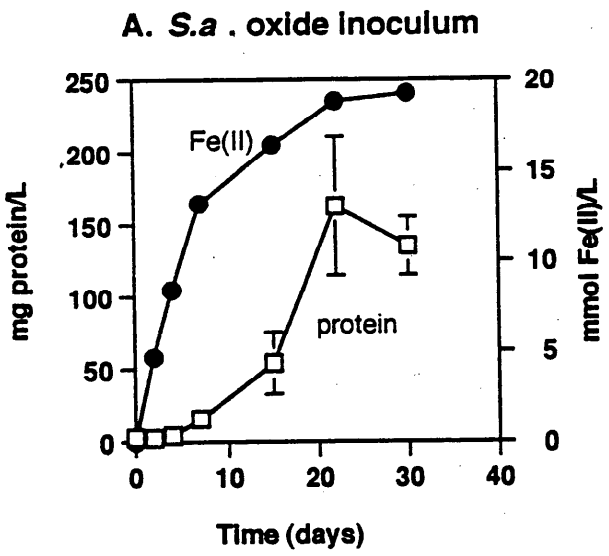
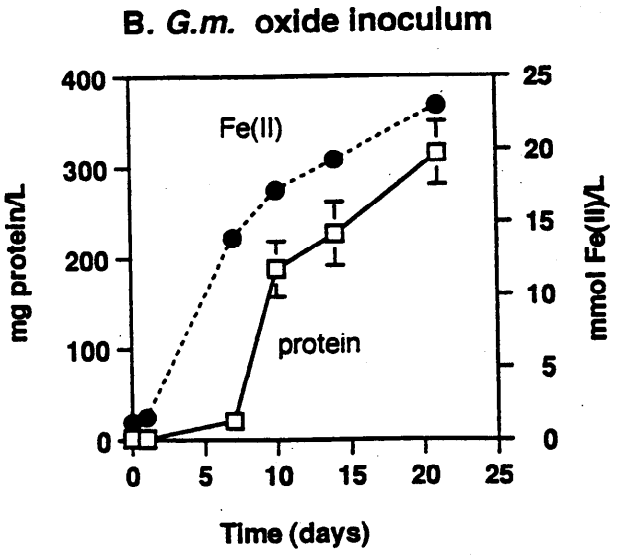


Figure 3 . Plot of Fe reduced (mmol/L) versus protein produced by *S. alga* (A) and *G. metallireducens* (B) during a two day period in Fe citrate medium. 10d and 2d in the legend refer to the age of the inoculum used in each case.





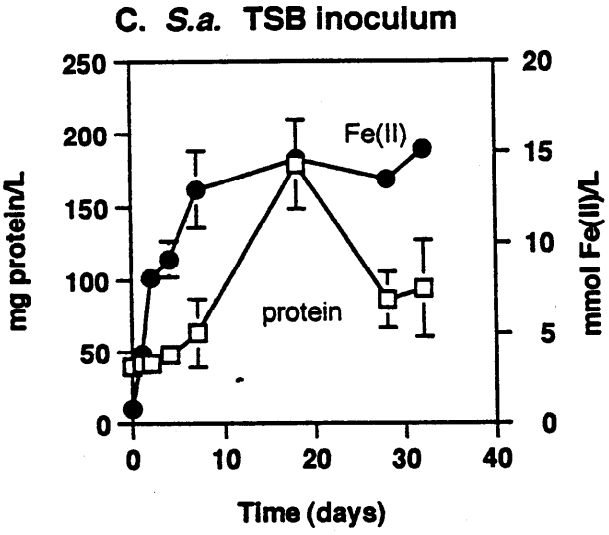
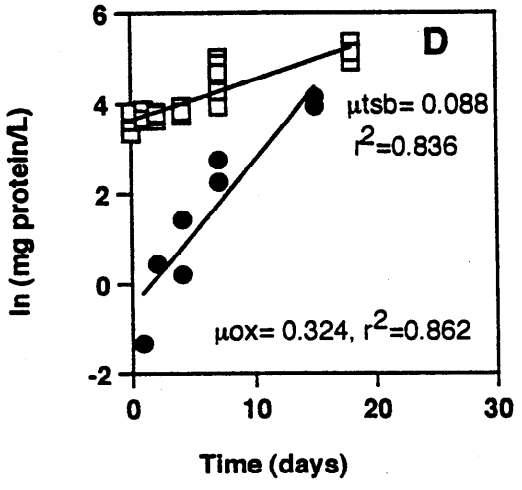
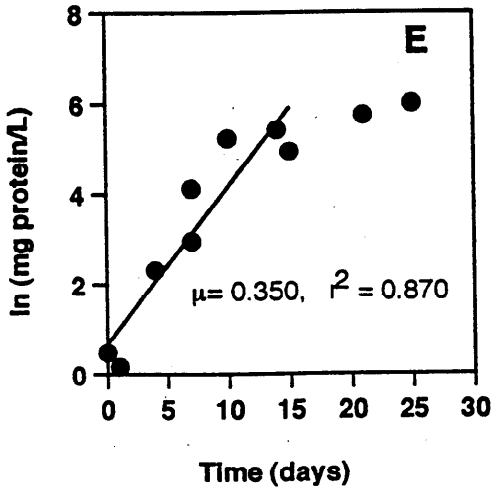


Figure 4 . Protein and Fe(II) production by *S. alga* (*S.a.*) or *G. metallireducens (G.m.)* in HFO (A-C) and respective growth rates (D,E). For *S.a.* results from cultures inoculated with oxide-grown inoculum (A) or TSB-grown inoculum (C) are presented.





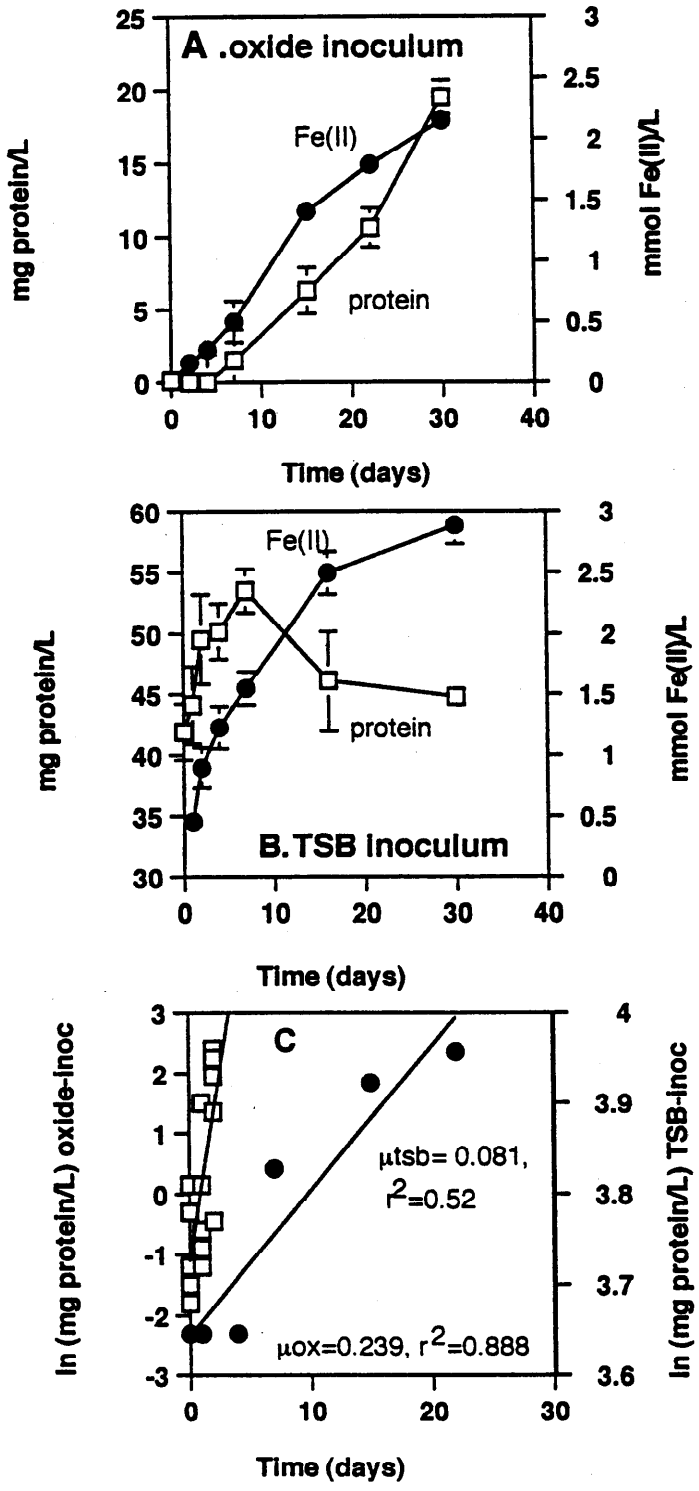


Fig. 5 . Protein and Fe(II) production by *S. alga* in Gt from cultures inoculated with oxide-grown (A) or TSB-grown (B) inoculum and respective growth rates (C).

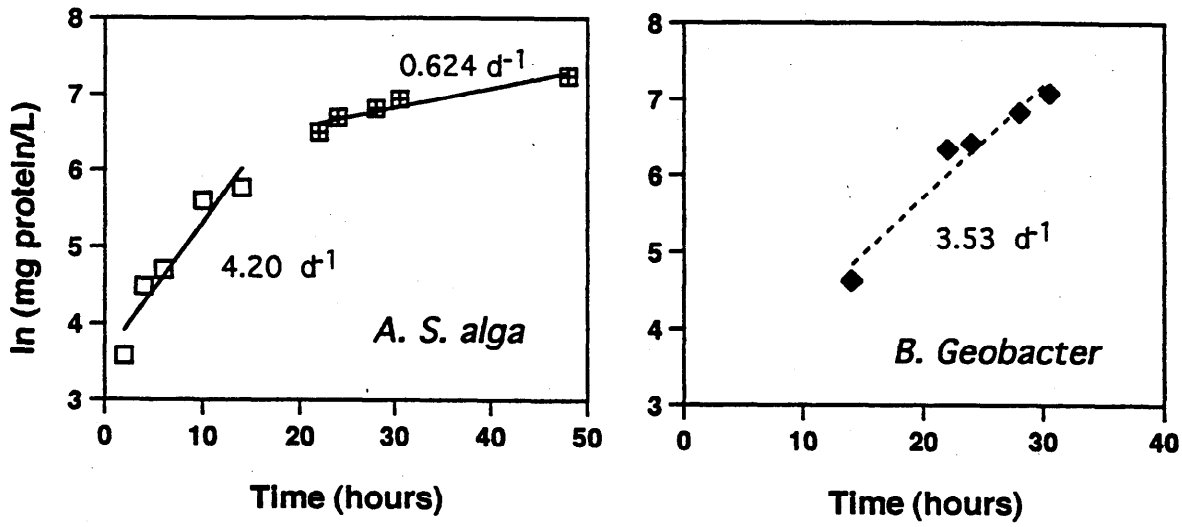


Figure 6 . Growth rates of *S. alga* (A) and *Geobacter* (B) in Fe citrate during the first 1-2 days of growth (10d-old, oxide-grown inocula).

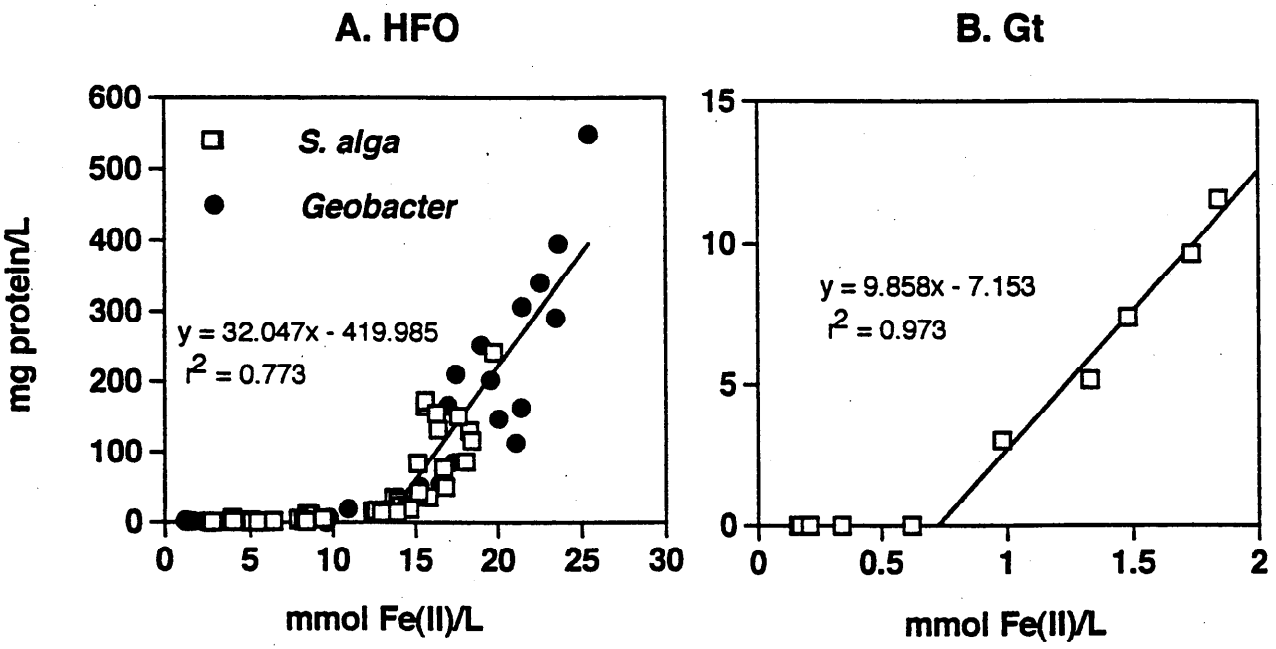


Fig. 7 . Plot of Fe reduced (mmol/L) versus protein produced (mg/L) by *S. alga* and *G. metallireducens* during a 4 week period in HFO (A) or Gt (B)

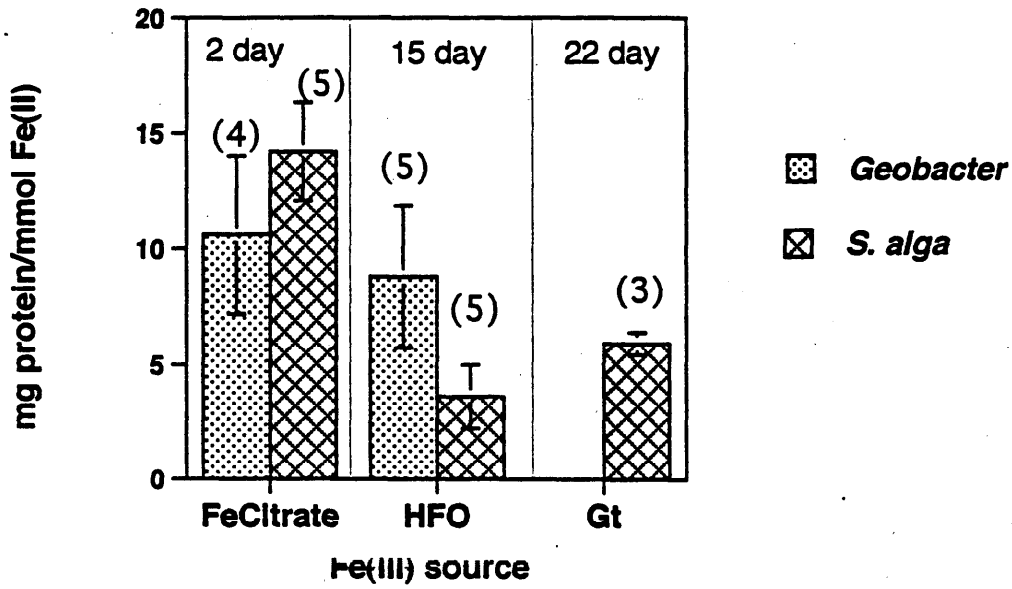


Figure 8 . Comparison of growth yield (mg protein produced per mmol of Fe(II) generated) of *Geobacter* and *S. alga* from soluble (FeCitrate) or solid phase (HFO, Gt) Fe(III) during the active growth phase. Numbers in brackets indicate averaged number of samples.

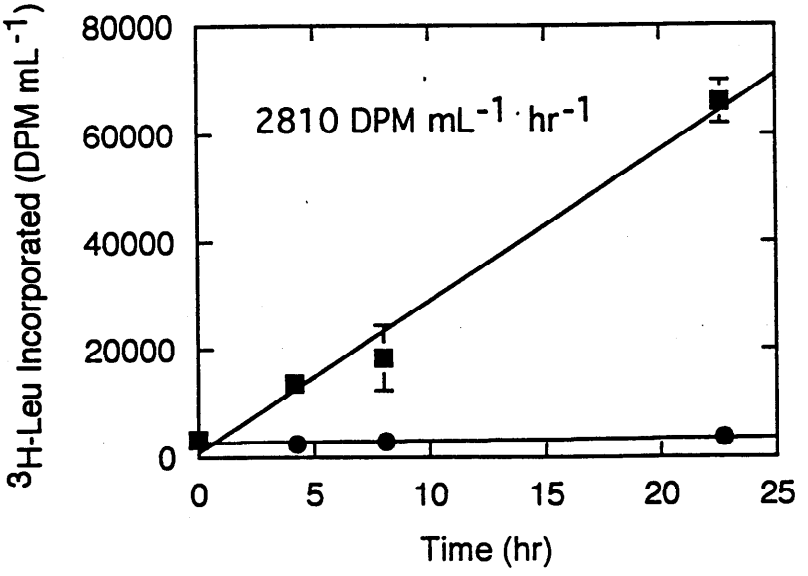


Figure. 9. 3H-Leu incorporation by G. metallireducens in acetate/HFO medium. The total concentration of leucine added was 100 nM. Squares and circles represent averages of triplicate live samples and TCA-killed controls, respectively. Error bars show standard deviation of the mean. The solid line shows the result of a linear least squares regression analysis, yielding the specified 3H- Leu incorporation rate.

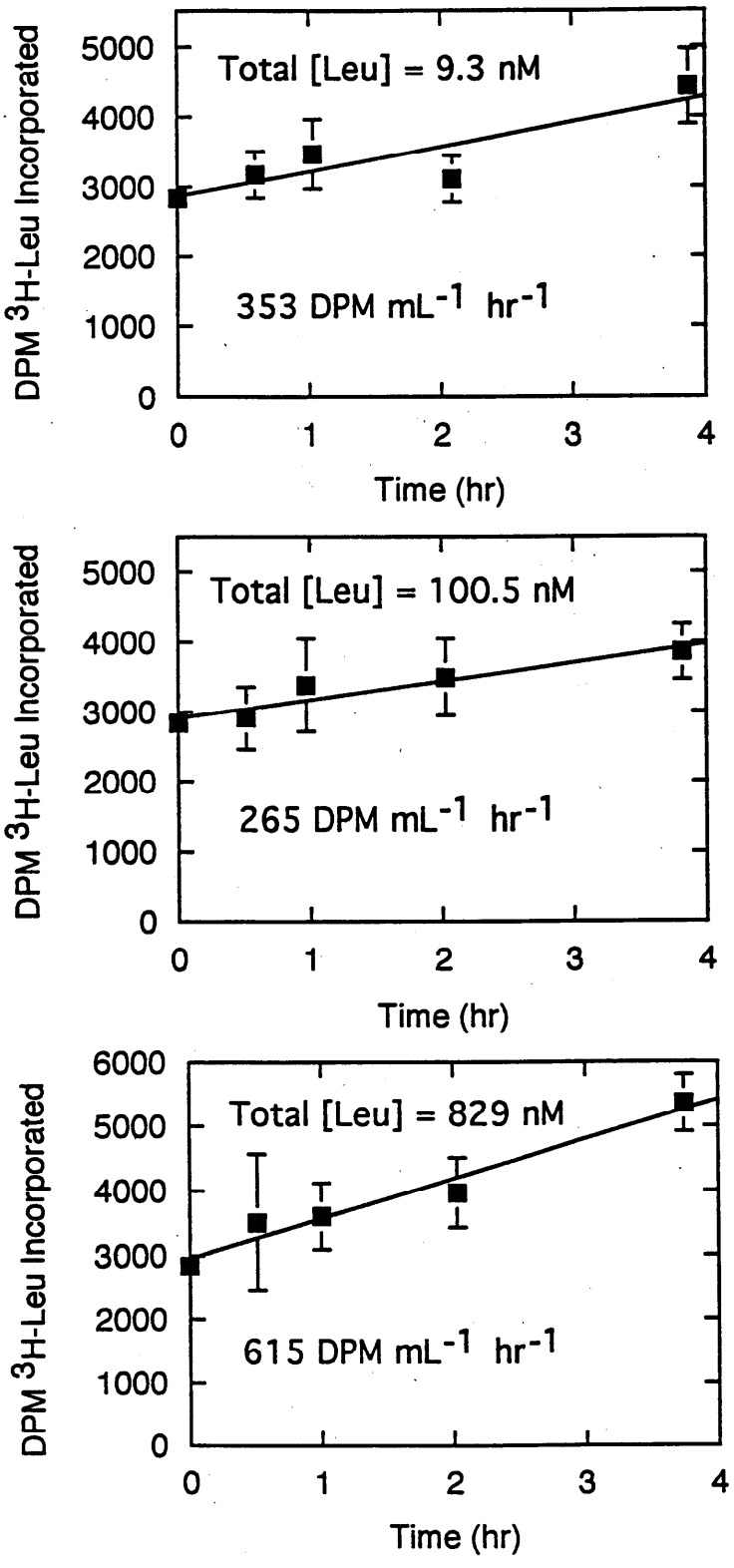


Figure. 10. 3H-Leu incorporation by G. metallireducens in acetate/HFO medium with total added leucine concentrations of ≈ 10, 100, and 700 nM. Symbols show the average of triplicate live samples. Error bars show standard deviation of the mean. The solid lines show the results of linear least squares regression analyses, yielding the specified 3H-Leu incorporation rates.

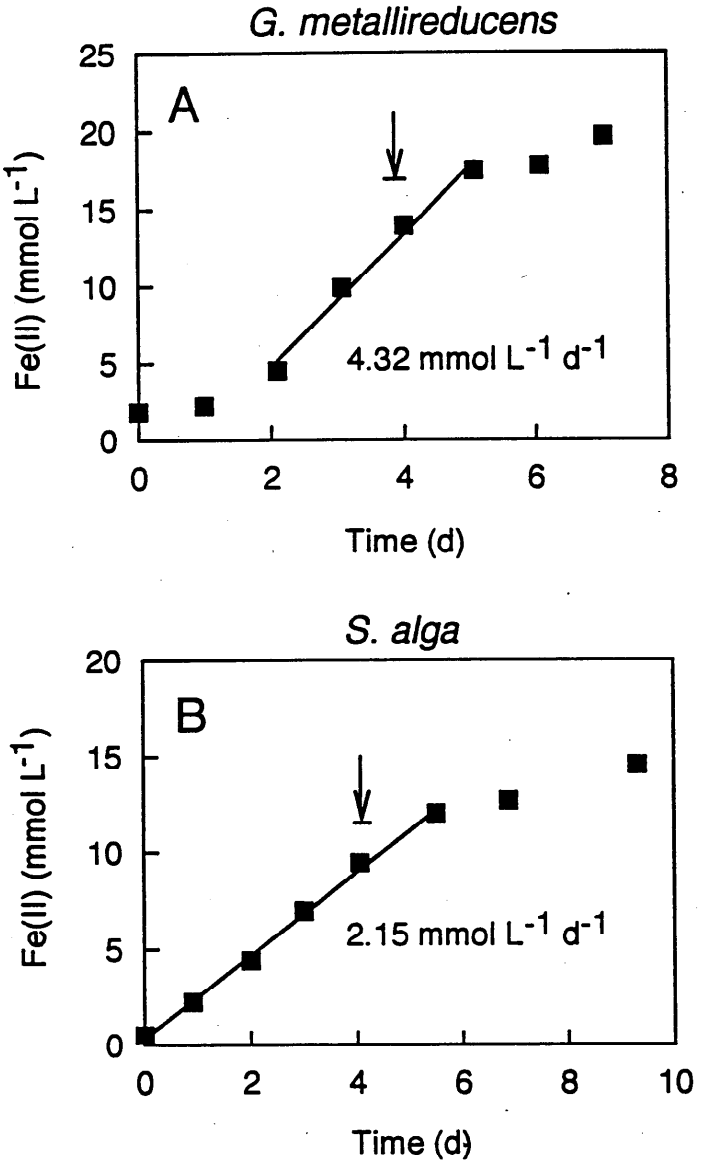


Figure. 11. Reduction of HFO by G. metallireducens and S. alga in cultures incubated in parallel with 3H-Leu incorporation experiments. The arrows point to horizontal bars whose length represent the duration of the 3H-Leu incorporation measurements. The solid lines show the results of linear least squares regression analyses, yielding the specified Fe(III) reduction rates.

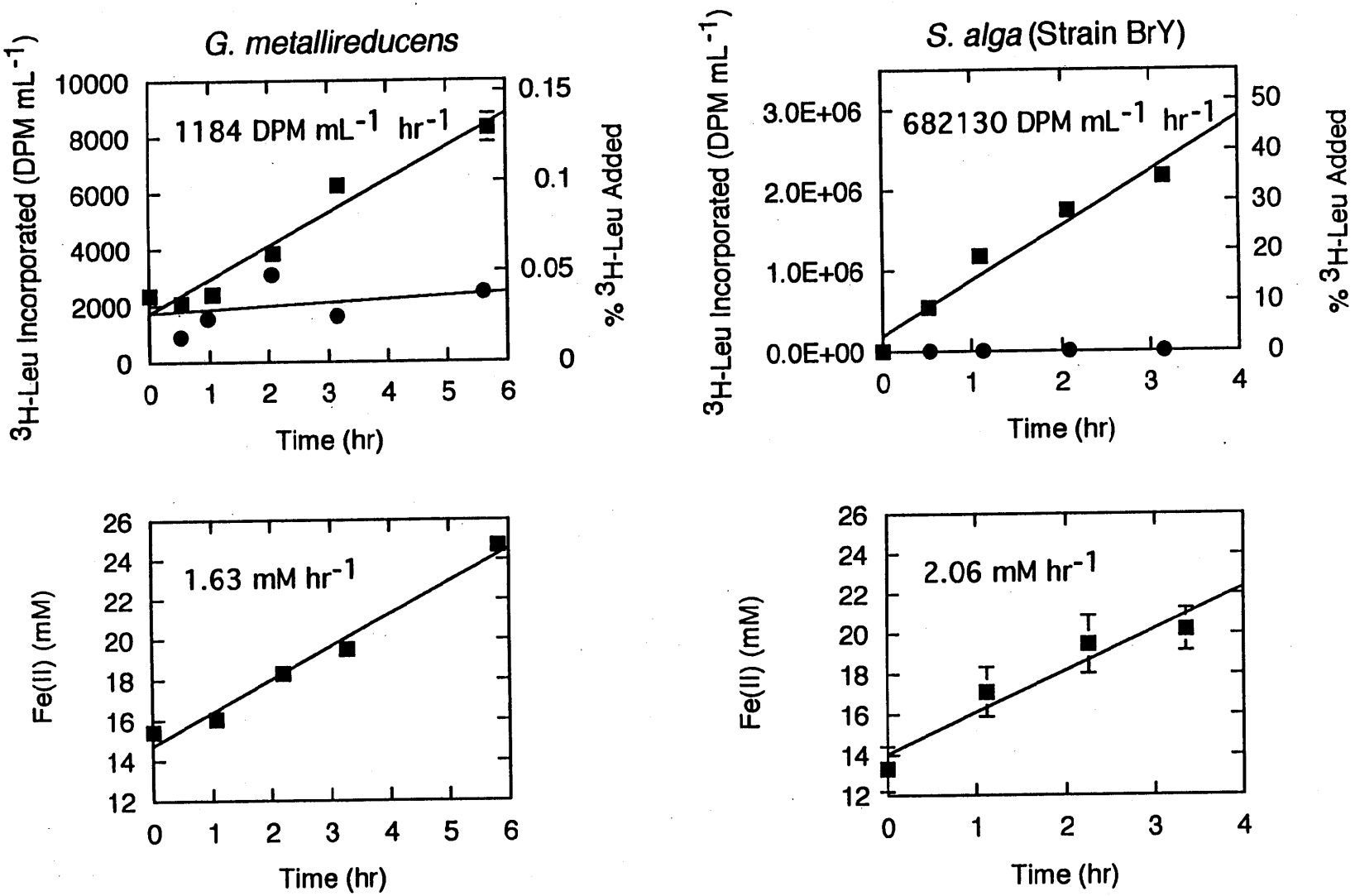


Figure. 12. Upper panels: 3H-Leu incorporation by G. metallireducens and S. alga in Fe(III)-citrate medium. The total concentration of leucine added was 100 nM. Squares and circles represent averages of triplicate live samples and TCA-killed controls, respectively. The solid lines show the results of linear least squares regression analyses, yielding the specified 3H-Leu incorporation rates. Lower panels: Reduction of Fe(III)-citrate in cultures incubated in parallel with 3H-Leu incorporation experiments. The solid lines show the results of linear least squares regression analyses, yielding the specified Fe(III) reduction rates.

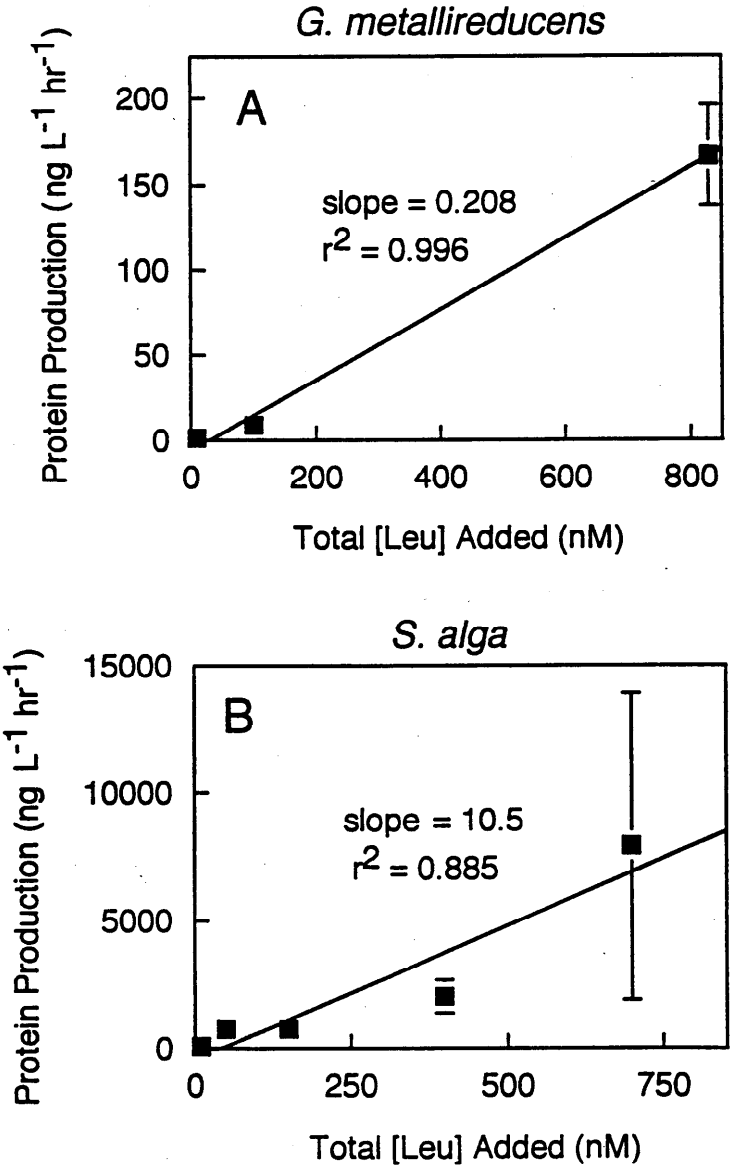


Figure. 13. Rates of protein synthesis by G. metallireducens (A) and S. alga (B) in HFO medium calculated from measured 3H-Leu incorporation rates (see text) with a range of total added leucine concentrations. The solid lines show the results of linear least squares regression analyses.

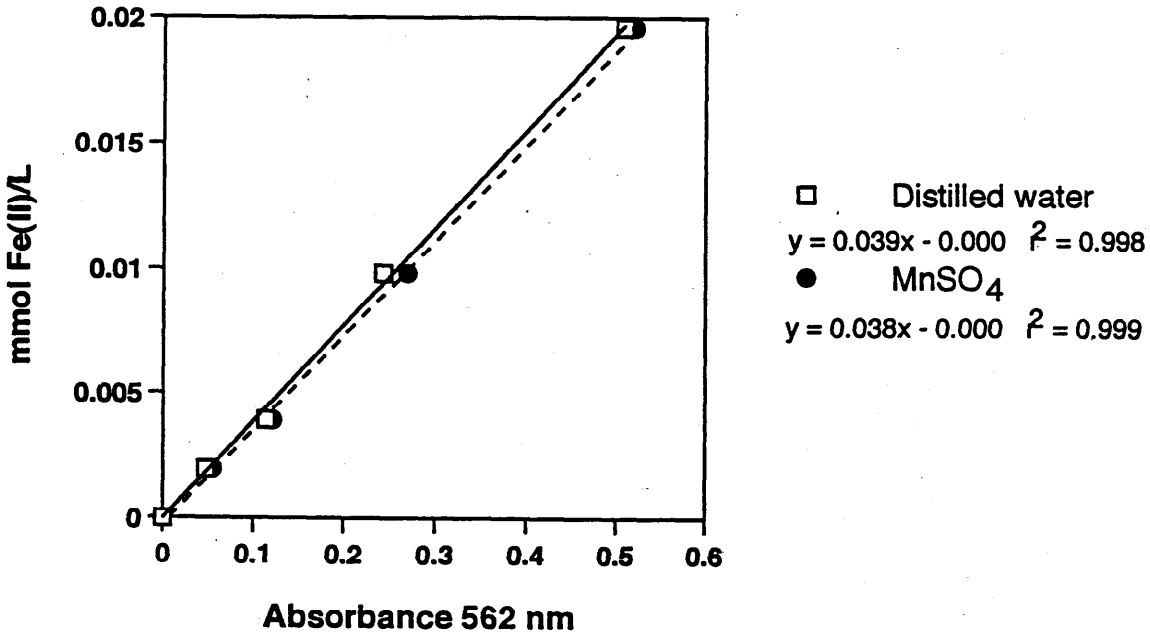


Figure 14 . Fe(II) standard curve for the ferrozine assay in presence of 10 mM $MnSO_4$ in Pipes buffer at pH 7.2

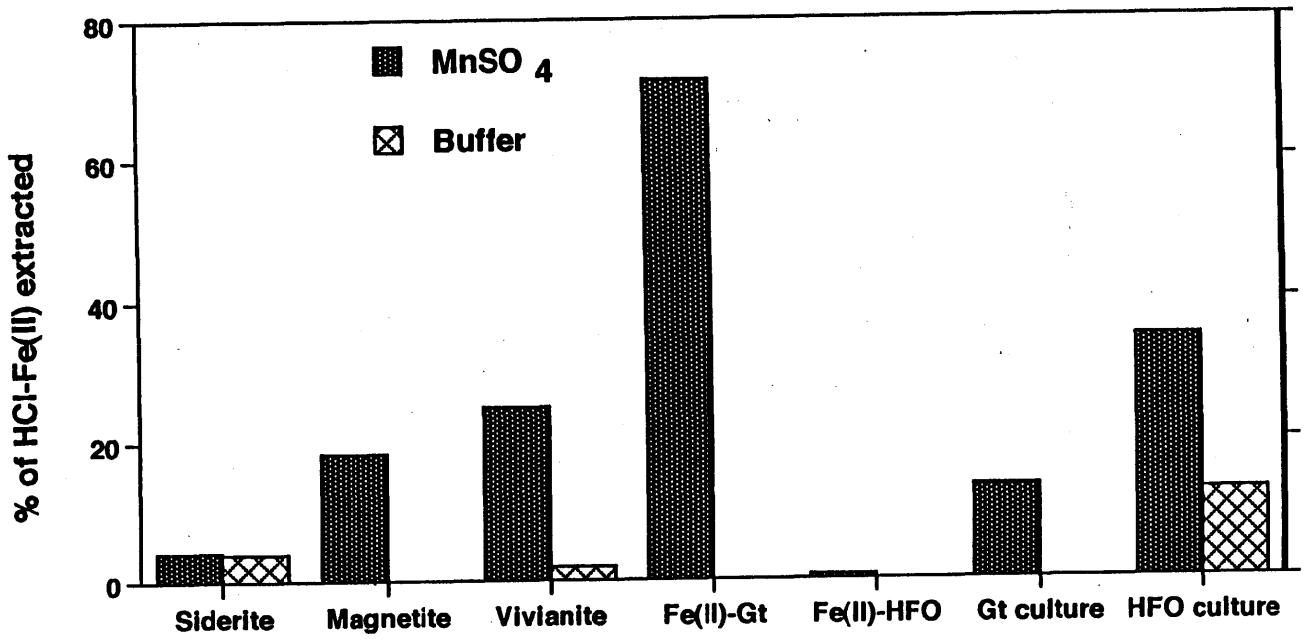


Figure 15. Fe(II) extracted in 10 mM $MnSO_4$ at pH 7.2 from several solid-phase sources in comparison to buffer (Pipes or Hepes) alone at equal pH. Results are expressed as percentage of Fe(II) extracted by 0.5 M HCl after substracting the amount soluble Fe(II).

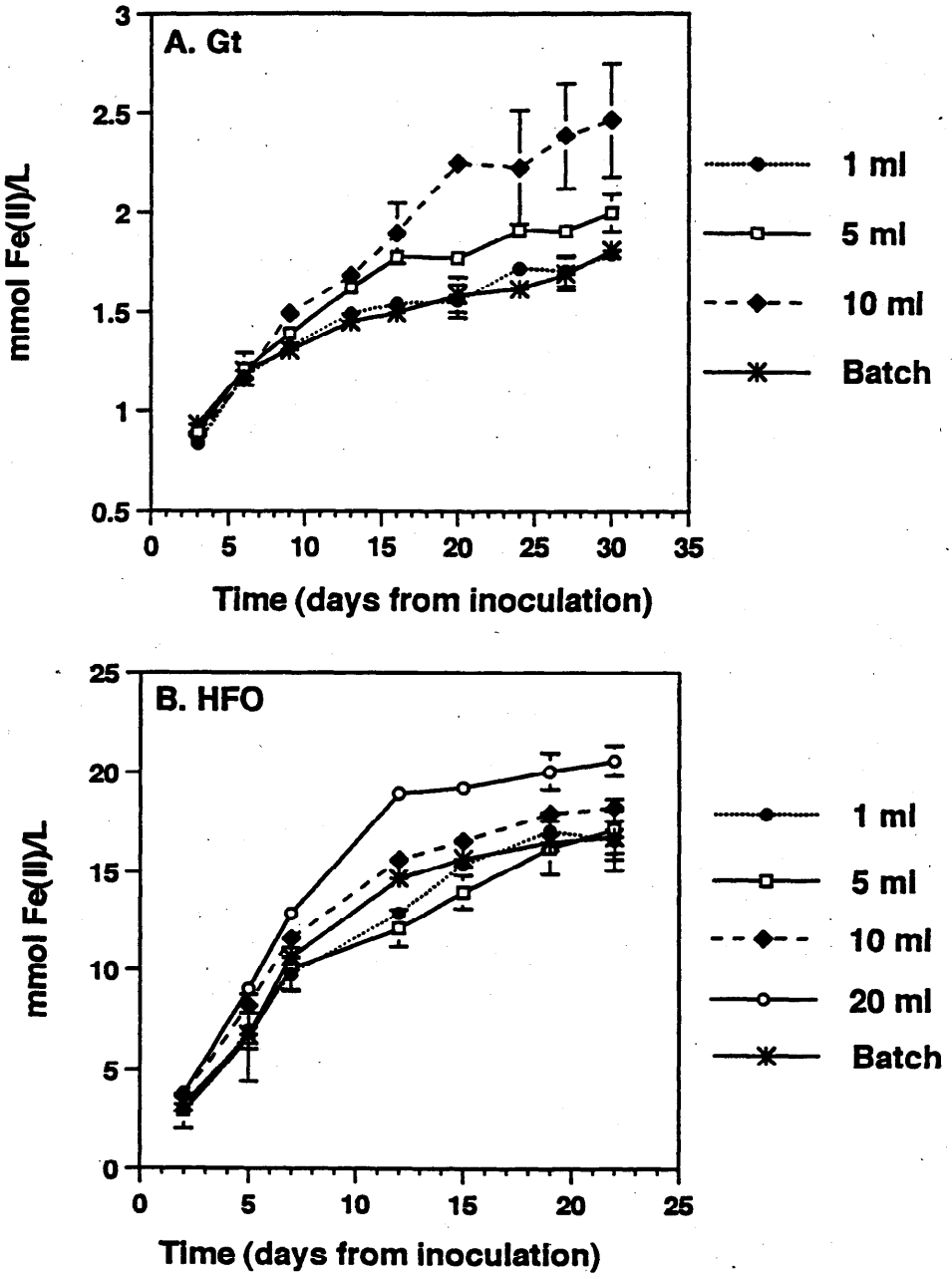


Figure 16. Reduction of Gt (A) and HFO (B) in semicontinuous cultures at several medium replacement levels in comparison to simultaneous batch experiments. 1ml, 5ml, 10ml and 20ml indicate the volume of medium replaced every three days .