ENRICHMENT STRATEGIES FOR IRON AND SULFUR REDUCING BACTERIA FROM SIPPEWISSETT SALT MARSH

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ABSTRACT

Various parts of Sippewissett salt marsh contain iron and sulfur mats suggesting that iron and sulfur reducers can account for carbon mineralization. Two different strategies, liquid and solid, were attempted to enrich for iron reducers. Only liquid enrichments were performed with sulfur reducers. Primary enrichments show it is likely that iron and sulfur reducers are present at this marsh. Secondary enrichments still need to be done and eventual isolation is a future goal. These isolations can then be tested for metabolic versatility, i.e. ability to use multiple electron acceptors.

INTRODUCTION

The abundance of iron in the earth's crust is overwhelming. As a result, it becomes one of the more important redox active metals in the environment. Until recently, most of the reactions involving iron were thought to be abiological, even though it has been known for some time that certain bacteria are capable of reducing ferric iron [Fe(III)] to ferrous iron [Fe(II)]. The problem was that no organisms had been isolated which could couple organic compound oxidation to iron reduction and generate enough energy to grow. However, with proper culturing conditions, these microbes have recently been found in a variety of environments, like groundwater, freshwater sediments, and marine sediments. They have fallen into the genera of *Geobacter*, *Pelobacter*, *Desulfuromonas*, and *Shewanella* (4, 7).

Studies about iron reducing bacteria have mostly taken place at freshwater sites (5, 6), even though other research has shown that there is strong evidence for iron reducers in a marine environment (1, 3). These marine studies tend to look at deep cores from ocean

sediments, not salt marshes. Additionally, in marine systems, generally the sulfur cycle has been well characterized and thought to account for the largest portion of organic compound oxidation. One study of Sippewissett salt marsh dealt with this topic concluding that SO_4^{2-} reduction was responsible for 1/2 of the carbon mineralization. Iron is not considered at all in the anaerobic sediments only 1cm from the surface(3a). Incidentally, parts of Sippewissett salt marsh are loaded with iron. Therefore, it seems very likely that iron reducers in these high iron areas can account for a sizeable portion of carbon mineralization.

Some marine organisms typically known as sulfur reducers [*Desulfuromonas acetoxidans* (9), and *Pelobacter carbinolicus* (4a) and others] have recently been shown to have iron reducing ability. However, these strains were already present as pure culture and not enriched for under iron reducing conditions. As a result, there can be many more organisms in the marine environment, in which my focus is the salt marsh, with iron reducing or iron and sulfur reducing ability that remain to be discovered. This study therefore attempted to enrich for iron reducing and sulfur reducing bacteria from Sippewissett salt marsh on various carbon sources.

MATERIALS AND METHODS

Sample collection. Samples were collected from three separate areas of Sippewissett salt marsh. Sample #1 was from an iron rich area below the green layer of the mat. Sample #2 was from a very iron rich area due to ground water runoff. Sample #3 was from an area with no visible iron, consisting of grayish, blackish mud.

Making ferrihydrite. Dissolve 300mM $FeCl_3$ into 1 liter of ddH_2O. Neutralize pH with NaOH pellets. Filter through normal filter paper by

suction. Wash the filtrate several times with ddH_2O. Do not autoclave, otherwise it loses its amorphous nature (10).

Making polysulfide sulfur. Add 0.64g of elemental sulfur to 200mL of 2N NaOH. This will dissolve when autoclaved. The solution must be stored as an alkaline solution. Before use, neutralize solution with 2N HCl and let aggregate for 3-4hrs. This recipe is from Kurt Hanselmann.

Liquid iron or sulfur reducing enrichment. A basal medium modified from Zopfi for salt water was made with the following ingredients (g/L ddH_2O): NH_4Cl (0.268) ; $MgCl_2 \cdot 6H_2O$ (0.100) ; $MgSO_4 \cdot 7H_2O$ (0.375) ; NaCl (17.53) ; KCl (0.370) ; $CaCl_2 \cdot 2H_2O$ (0.150). After autoclaving, cool under N_2/CO_2 stream and add the following (mL) upon being separately autoclaved: 0.4M NaH_2PO_4 (4) ; 1M $NaHCO_3$ (30) ; acidic trace elements (.5)[a] ; basic trace elements (.5)[b] ; vitamin solution (10)[c] . Then add the colloidal sulfur or amorphous iron while stirring. The pH should be watched throughout the process and kept neutral. Dispense into 60mL bottles while gassing with N_2/CO_2, stopper the bottles, and crimp. The appropriate carbon source is then added from autoclaved, oxygen-free stocks. Finally, the bottles are inoculated with the desired sample. Some samples were sonicated as it is difficult to inoculate sand with a syringe. Incubate at 30°C.

[a] Acidic trace elements' stock solution contains (mg/L ddH_2O): $FeCl_2 \cdot 4H_2O$ (1491) ; H_3BO_3 (61.8) ; $ZnCl_2$ (68.1) ; $MnCl_2 \cdot 4H_2O$ (90) ; $CuCl_2 \cdot H_2O$ (17) ; $CoCl_2 \cdot 6H_2O$ (238) ; $NiCl_2 \cdot 6H_2O$ (261.8) (Zopfi).

[b] Basic trace elements' stock solution contains (mg/100mL 50mN NaOH): $Na_2MoO_4 \cdot 2H_2O$ (4.84) ; Na_2SeO_3 (0.26) ; $Na_2WO_4 \cdot 2H_2O$ (0.33) (Zopfi).

[c] Vitamin solution is made as follows from David Graham's recipe (mg/L ddH_2O): *p*-aminobenzoic acid (10) ; Nicotinic acid (10) ; Calcium pantothenate (10) ; Pyridoxine·HCl (10) ; Riboflavin (10) ; Thiamine·HCl (10) ; biotin (5) ; Folic acid (5) ; α-lipoic acid (5) ; vitamin B_{12} (5). Store in dark at 4°C.

Solid iron reducing enrichment. The above basal medium is made with the following additions: to final concentrations 0.75% agar, .01% peptone, and .02% yeast extract. Let cool in 42°C water bath. The additions are made after autoclaving just as above including 15mM K·acetate. (One may not need to use all of the iron). The additions are made at the temperature of 42°C while stirring so the agar does not solidify. Dispense 200mL into 250mL jars or fill a screwcap test tube 3/4 of the way, add sample, shake lightly to mix, and solidify in ice bath while agitating (Modified from ref. 6a, 6b, & 8). Incubate at room temperature. Others were given inocula from other areas, such as a sample from the bank where the ground water ran off and one from a canal.

Microelectrode analysis. Lars Damgaard's microelectrode was utilized to assess oxygen presence in the solid iron enrichments before and after 12 days incubation.

HPLC analysis. Those samples with obvious signs of growth were subjected to a Waters LC module 1 equipped with an RS Pak KC811 column refractive index detector using 0.2% H_3PO_4 as eluence.

Equations. Lars Damgaard's Thermodyne program was used to calculate free energy changes with different chemical reactions which could be taking place in some of my enrichments. The calculations were made with the following boundary conditions for activity following the compound: ferric

hydroxide, 1 ; elemental sulfur, 1 ; water, 1 ; magnetite, 1 ; sulfide, 10^{-4}M ; bicarbonate, 10^{-2}M ; acetate, 2.5×10^{-3} ; ethanol, 2×10^{-3} ; formate, 10^{-2} ; propionate, 2×10^{-3} ; methane, 10^{-4}. The temperature is 25°C and the pH is 7. The ferric hydroxide was characterized as amorphous. The magnetite product was identified as a solid.

RESULTS

Growth from liquid enrichments. Growth from these enrichments after 18 days of incubation is summarized in Tables 1-3. In six of the liquid iron reducing enrichments growth was seen. These results were evenly distributed amongst the different samples. Formate was the most popular carbon source followed by ethanol and H_2/CO_2 respectively. There was no growth in the negative controls without inoculum or carbon source addition. The positive iron control MR-4 was slowly growing with this medium. Growth was identified by the obvious change of the red iron to a black color. Not all of the iron turned black yet. The blackness was observed only in parts of the iron settled at the bottom of the bottle. Microscopic examination of these cultures did not reveal a large amount of bacteria and not very many different morphologies. In the H_2/CO_2 culture, the major microbe was a plump rod with tapered ends as presented in Fig. 1 attached to the iron particle. There were also some cocci and curved rods moving around in the medium. In the ethanol grown culture with sample 1, rods were seen in small numbers. These were also found to be attached to iron particles just as in Fig. 1.

Fig. 1 ... liquid grown iron cultures w/ H_2/CO_2 to the iron.

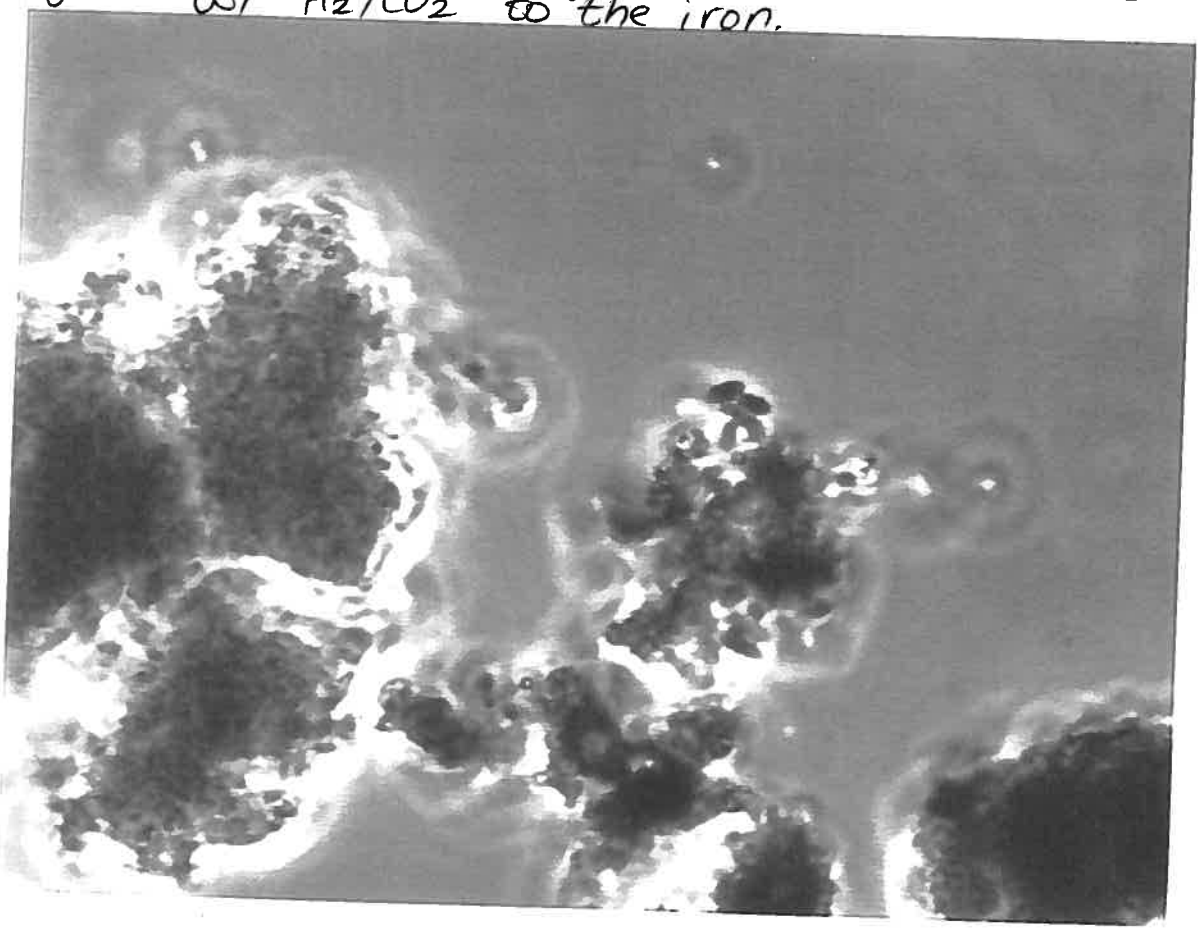


Fig. 2 Attachment of microbes to sulfur particles with acetate as carbon source

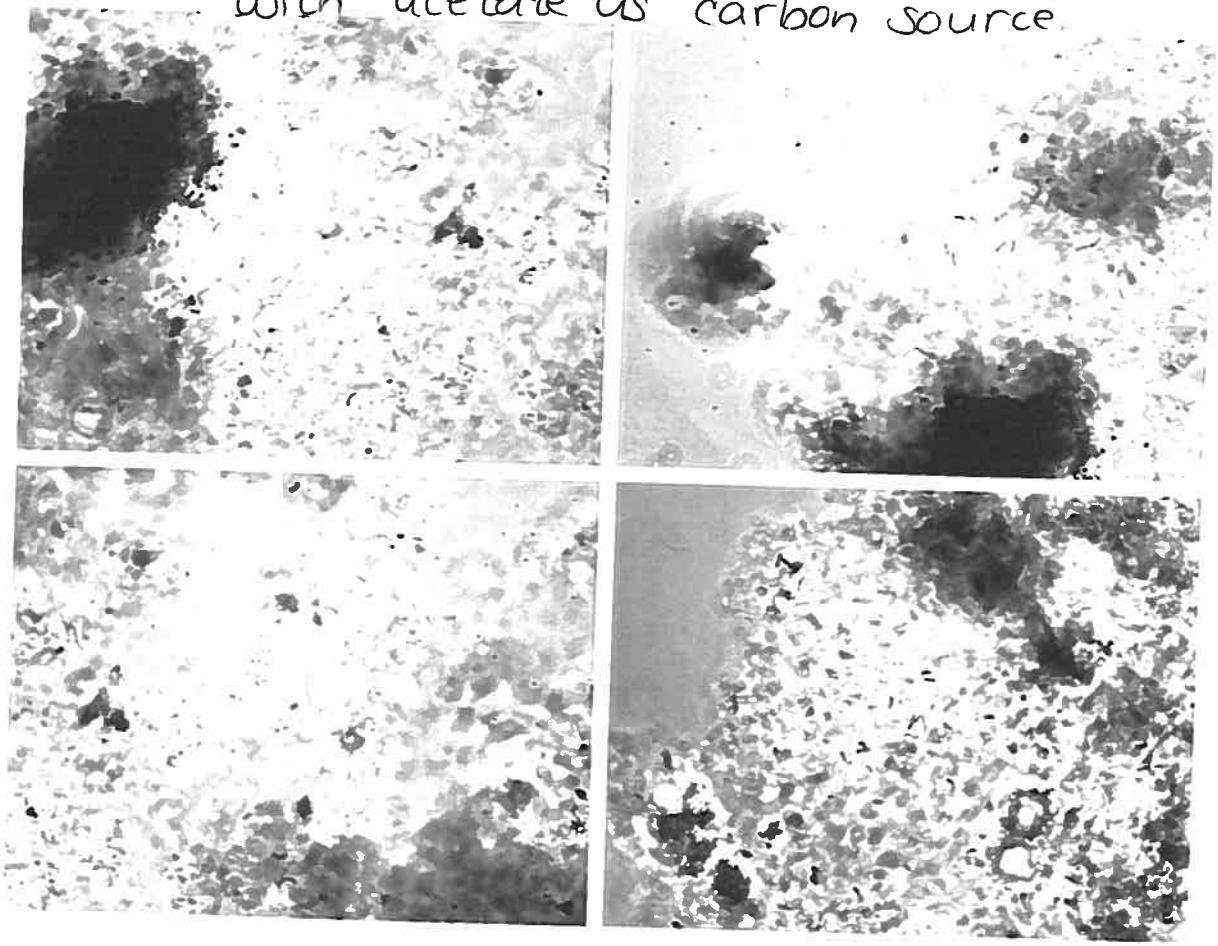


TABLE 1. Observed growth from sample 1 as carbon source (rows) vs. electron acceptor (columns).

	IRON	SULFUR	NONE*
H_2/CO_2	-	+	+
METHANE	-	-	+
FORMATE	+	+	+
METHANOL	-	-	+
METHYLAMINE	-	-	-
ACETATE	-	-	+
ETHANOL	+	-	+
PROPIONATE	-	-	+
PALMITATE	-	-	-
NONE	-	-	-

* It is possible that CO_2 can serve as an electron acceptor. Also, there is a very small amount of sulfate in the medium.

For the sulfur enrichments, only sample 2 showed the most positive growth results in which the white colloidal sulfur turned to black clumps. Blackness was observed only for two, H_2/CO_2 and formate, of the enrichments with sample 1. No color change occurred with sample 3. These cultures exhibited more growth than those on iron as seen in Fig. 2. These particular pictures were from sample #2 with acetate. There was a lot more diversity of cell morphology and an obvious attachment of cells to sulfur particles just as with the iron enrichments. Rods, spirochetes, curved rods, and cocci were observed under the microscope.

Those enrichments with no additional electron acceptor displayed the most growth collectively as compared to the iron and sulfur enrichments. Except for those incubated with ethanol, these bottles were not strikingly turbid. However, if one gently swirled

the bottle, a thin white veil came up from the bottom of the bottom indicative of biomass. These were not examined microscopically.

TABLE 2. Observed growth from sample 2 as carbon source (rows) vs. electron acceptor (columns).

	IRON	SULFUR	NONE*
H_2/CO_2	-	-	+
METHANE	-	+	+
FORMATE	+	+	+
METHANOL	-	-	+
METHYLAMINE	-	-	+
ACETATE	-	+	-
ETHANOL	-	+	+
PROPIONATE	-	+	-
PALMITATE	-	-	-
NONE	-	-	-

* It is possible that CO_2 can serve as an electron acceptor. Also, there is a very small amount of sulfate in the medium.

TABLE 3. Observed growth from sample 3 as carbon source (rows) vs. electron acceptor (columns).

	IRON	SULFUR	NONE*
H_2/CO_2	+	-	+
METHANE	-	-	-
FORMATE	+	-	-
METHANOL	-	-	-
METHYLAMINE	-	-	-
ACETATE	-	-	+
ETHANOL	+	-	+
PROPIONATE	-	-	-
PALMITATE	-	-	-
NONE	-	-	-

* It is possible that CO_2 can serve as an electron acceptor. Also, there is a very small amount of sulfate in the medium.

Growth from solid iron enrichments. All of the jars and test tubes displayed a color change from red to black even though they had different inocula. There were those inoculated with the samples from the liquid enrichments. Samples taken from the jars at various layers were crawling with life after 11 days of incubation. All different kinds of microbes grew in these cultures. They ranged from cocci, short rods (Fig. 3), curved rods, long rods (Fig. 4), spirilla, to spirochetes (Fig. 5). Some of the cocci were grouped as tetrads and triads (Fig. 6). Many of these were swimming rapidly (Fig. 5), gliding (Fig. 4), or remaining in place (Fig. 6). Also, there was an obvious association of many of these cells with the iron particles (Fig. 7).

Microelectrode analysis. The results from this test demonstrated that the solid iron enrichments were indeed anaerobic starting at a depth of 1.5mm on the day of inoculation (Fig. 8). After 12 days, the anaerobic zone became larger as oxygen was not detected below 0.8mm (Fig. 9).

HPLC analysis. Those cultures containing ferric iron and sulfur which showed growth were tested to determine which end products accumulated. These results are summarized in Table 4. Acetate was detected in five of the twelve samples. Formate was only detected in one sample.

Fig. 3 Short rods from solid iron enrichment near iron.

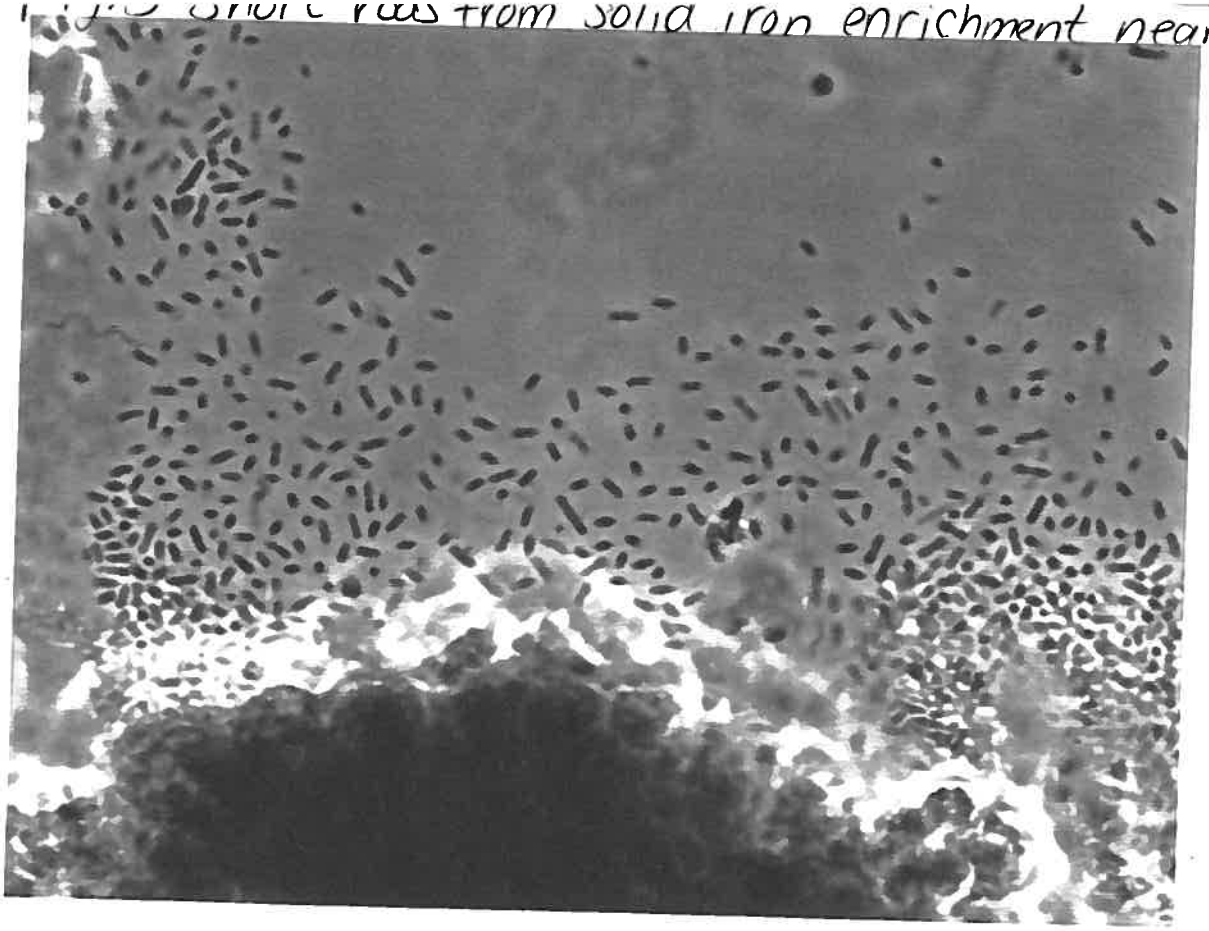


Fig. 4 Long, gliding rods observed in solid iron enrichments.

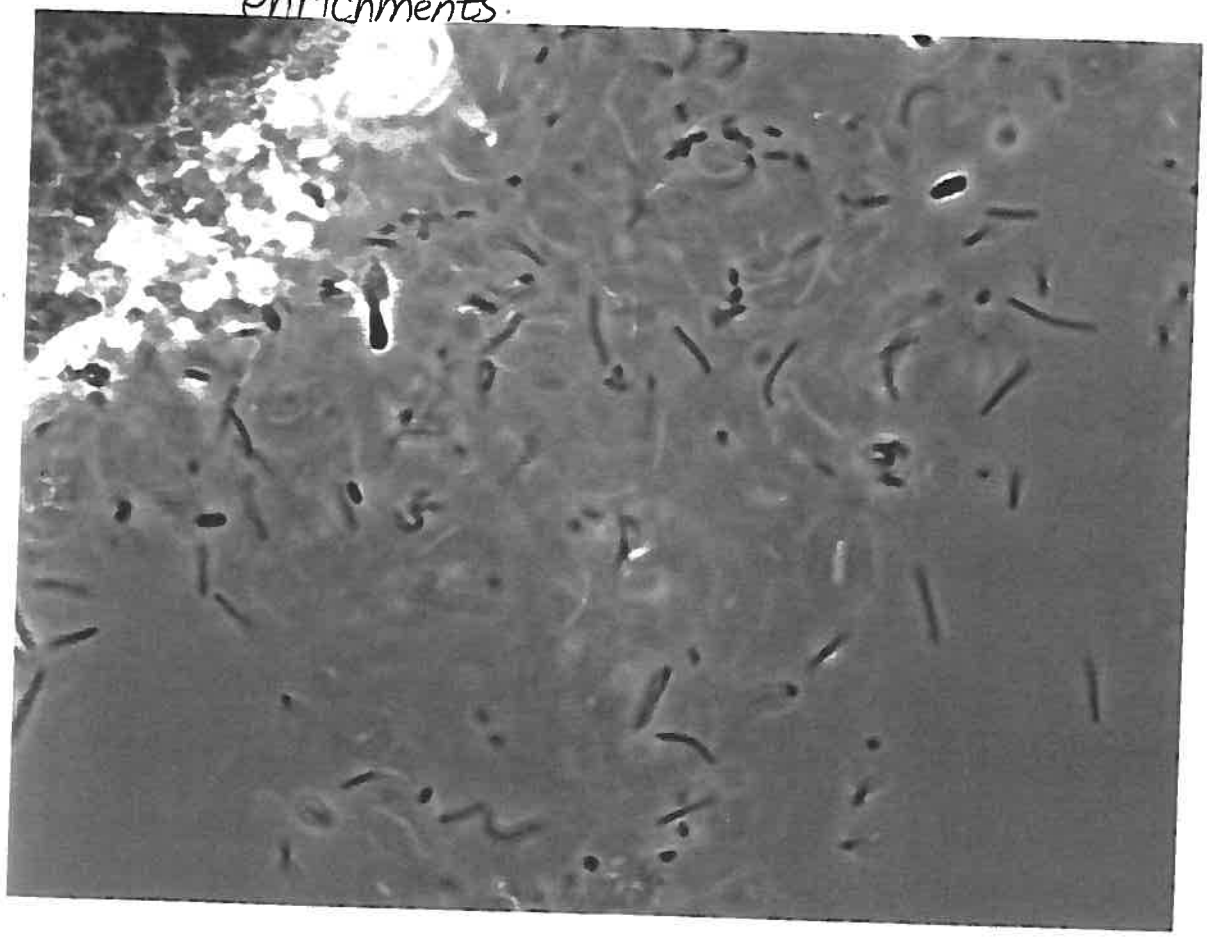


Fig. 5 Spirochetes in solid iron enrichments.

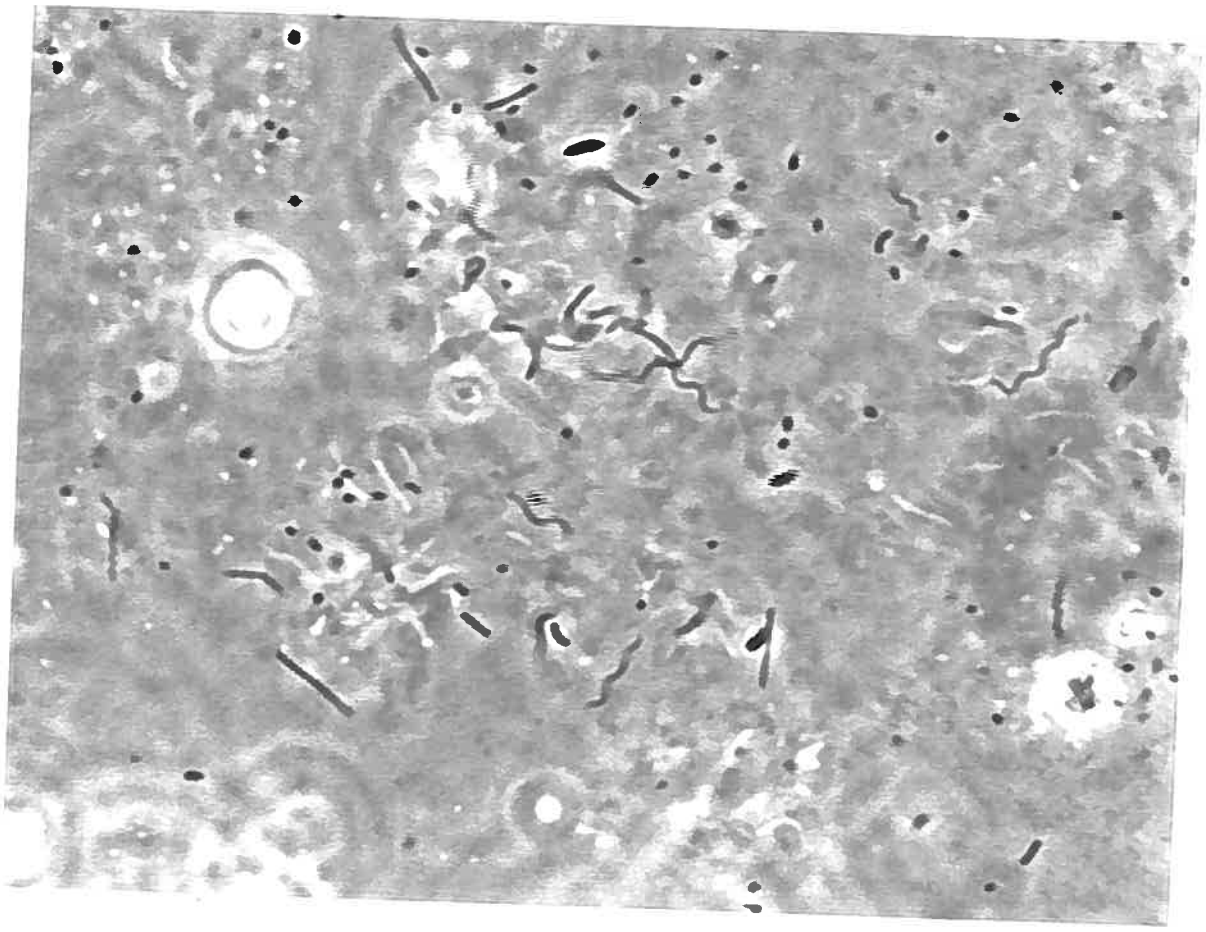


Fig. 6 Coccal packages in solid iron enrichments.

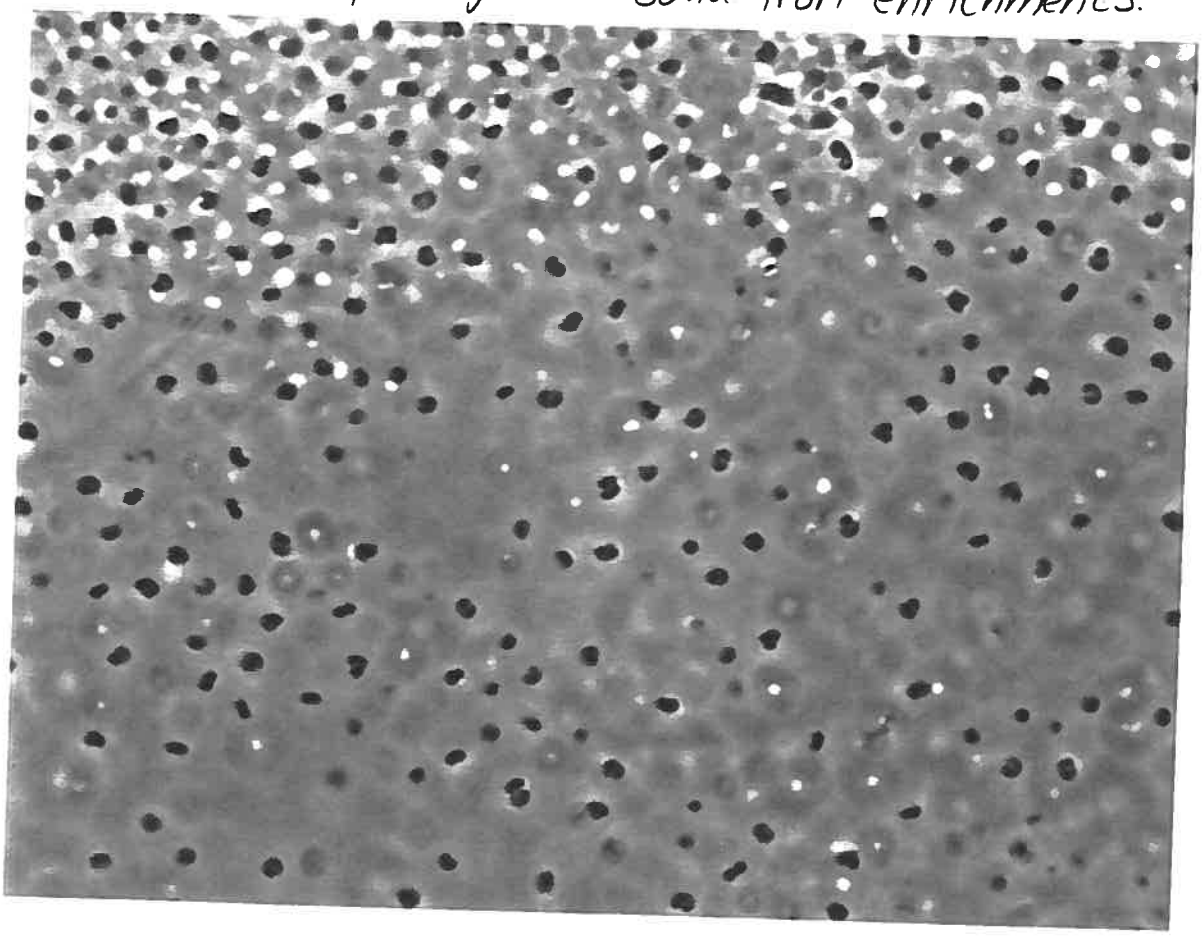


Fig 7 Attachment to iron particles from solid iron enrichments.

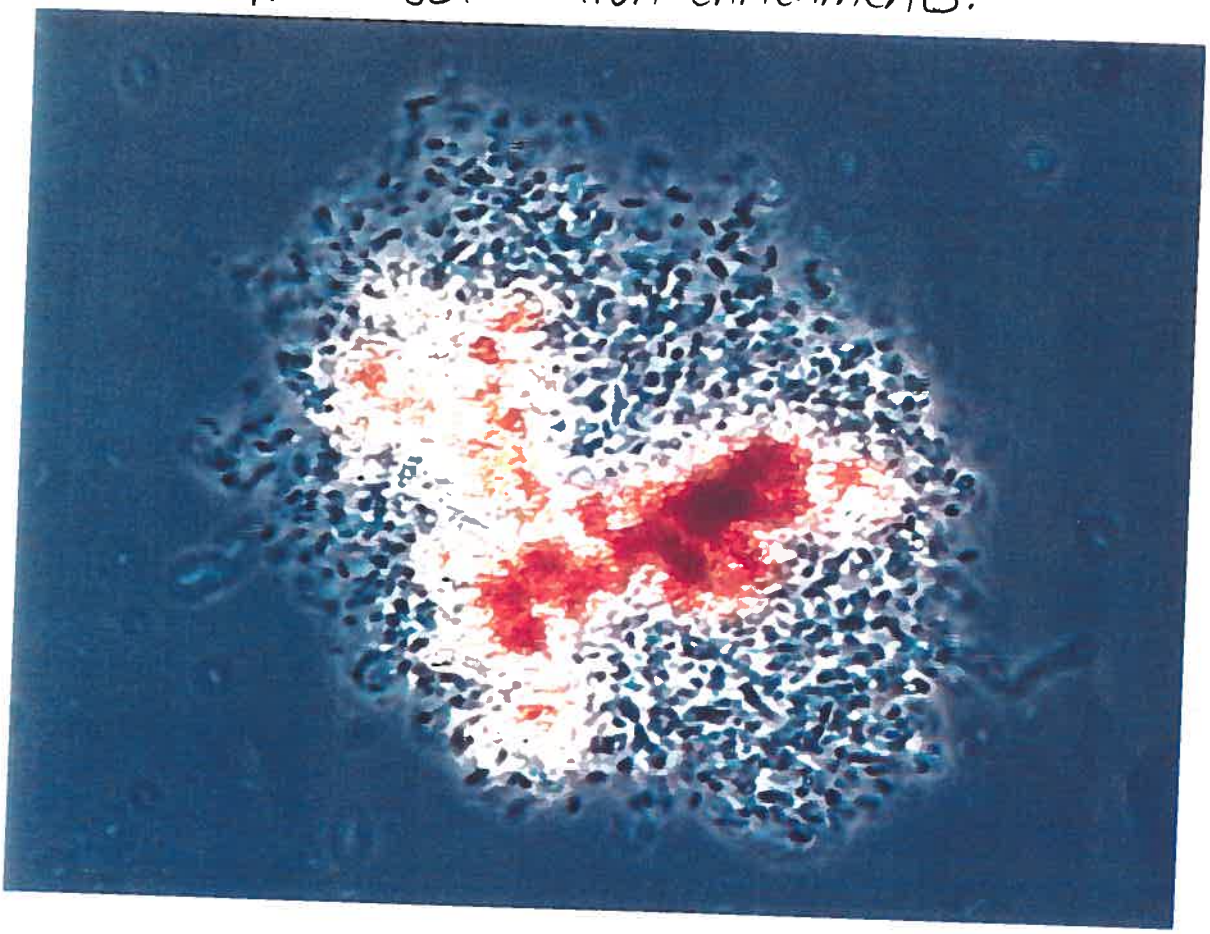


Fig. 8

Oxygen profile of solid (0.75% agar) iron enrichment @ start

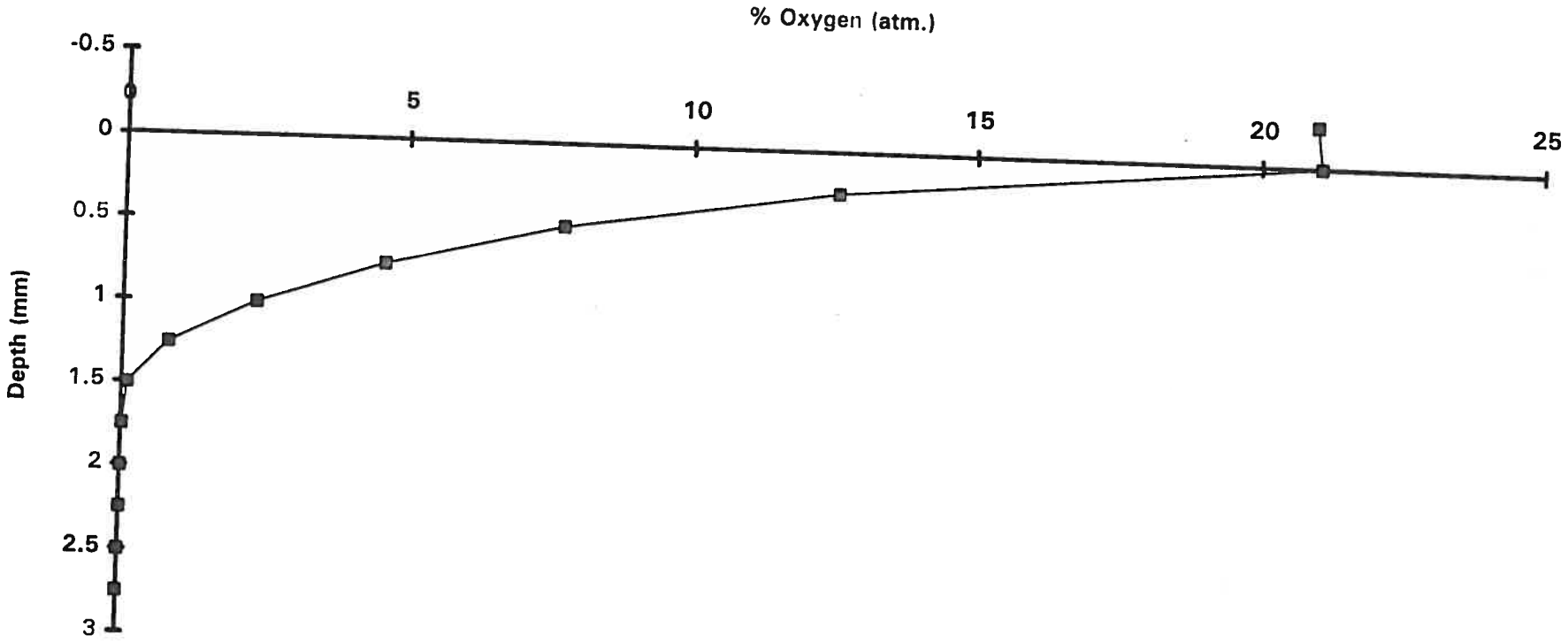


Fig. 9 **Oxygen profile, groundwater run-off after 2 weeks**

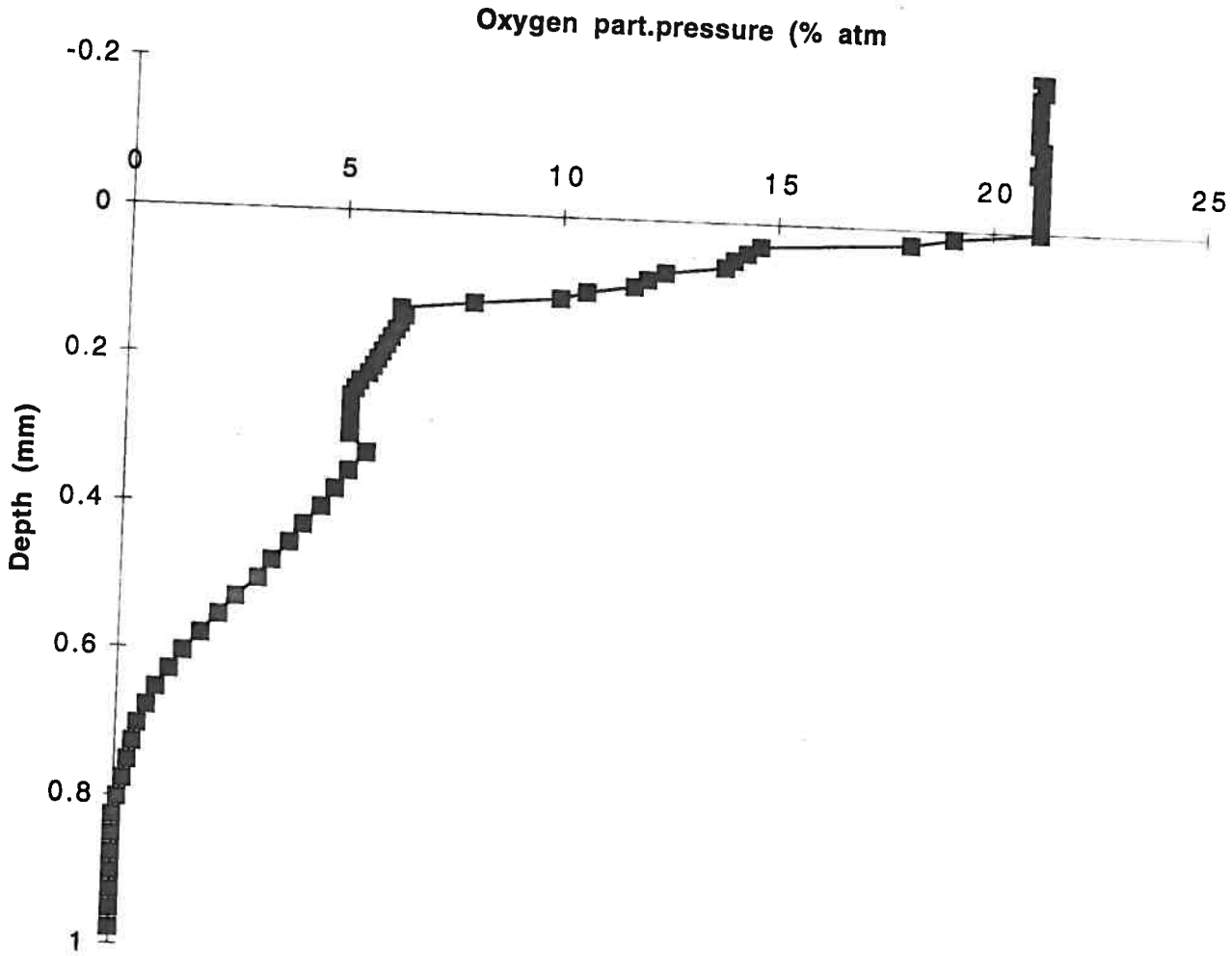


TABLE 4. HPLC of cultures showing growth.

SAMPLE #	FINAL e^- ACCEPTOR	C-SOURCE	COMPOUND DETECTED
1	-*	CH_4	-
2	-*	CH_4	-
2	sulfur	H_2/CO_2	-
2	sulfur	formate	-
2	sulfur	ethanol	acetate
1	sulfur	H_2/CO_2	-
1	sulfur	formate	-
2	sulfur	acetate	acetate
2	sulfur	propionate	acetate
1	ferric iron	ethanol	acetate
3	ferric iron	H_2/CO_2	formate and acetate
-	-	-	-

*In these enrichments, there was no additional ferric iron or sulfur. It is possible that CO_2 from the headspace or the small amount of sulfate could have supported growth by acting as terminal electron acceptor. Sample 12 was just the medium with no C-source, inoculum, or added final electron acceptor.

DISCUSSION

There were large differences amongst the diversity and amount of microbes observed between the liquid and solid enrichment strategies with iron. There are a few explanations for this. First, the iron used in the liquid enrichment was autoclaved which changed the form from an amorphous nature to a very crystalline type. The highly crystalline form of iron seems to be more difficult for the microbes to access, hence the low numbers seen in the enrichments and the low amount of positive results from

these enrichments. These enrichments will certainly take much longer for growth to occur, however the stringent conditions will only allow primarily for true iron reducers to grow. One must be patient and observe periodically for a change from red to black. Upon writing this report, there is evidence that many of these enrichments are just starting due to the sudden appearance of some black particles. This was not a failed attempt to enrich for iron reducers. It just takes more time than 3 weeks.

Another reason for the strikingly different results between liquid and solid enrichments is that a small amount of yeast extract and peptone was used for the solid enrichments. This can serve to jump start the growth of iron reducing bacteria and as a result, these enrichments grow much faster. However, by adding these substrates, one runs the risk of obtaining fermenters as well as iron reducers. To lower the numbers of potential fermenters, secondary enrichments should be more stringent as far as substrate additions are concerned.

Even though the morphology and numbers were quite heterogeneous between the two types of enrichments, there was one underlying similarity. Many of the organisms examined microscopically displayed a direct attachment to iron or sulfur. This indicates that in order to utilize these solids for their metabolism, they must directly attach to the desired final electron acceptor. Other organisms have the luxury of reducing soluble final electron acceptors and don't have to worry about direct attachment. The gliders seen in the iron enrichments therefore make perfect sense if they are actually growing anaerobically and reducing the iron to gain

energy for growth. In order to glide in the first place, there must be some kind of attachment to the substratum. This correlates well with the seeming necessity of iron reducers to attach to the insoluble ferric iron.

With those liquid iron and sulfur cultures showing positive growth at that time, HPLC analysis was performed. From the compounds detected, it is possible to develop possible thermodynamic reactions and free energy changes using the boundary conditions given in the Materials and Methods section(Table 5). It is evident that using iron as the final electron acceptor generates the most thermodynamically favorable results. Those enrichments grown on ethanol and propionate displayed acetate accumulation, therefore this was used as the product. Nothing was detected on those grown with acetate and formate indicating potential mineralization. (Of course, some acetate was detected in the acetate grown cultures, but this is not indicative of break down). It is hard to explain why the iron enrichment on H_2/CO_2 produced formate and acetate. This needs to be retested.

Future directions of this work involve making secondary enrichments for the solid iron enrichments and liquid sulfur reducer cultures. The conditions for the secondary solid iron enrichments must be a bit more stringent to eliminate fermenters. Tertiary enrichments can be done in liquid with ferric citrate or ferric pyrophosphate in which ferrous iron production can be measured. One must give the liquid iron enrichments more time before secondary enrichments are made. Eventually these microbes from all of these enrichments can be isolated with the use of roll tubes,

agar shakes, and then dishes. Upon isolation their metabolic versatility can be studied in more detail.

TABLE 5. Thermodynamics of certain reactions.

EQUATION	ΔGr° (kJ/rxn)	ΔGr (kJ/rxn)
$CH_3CH_2OH + 2S° + H_2O \rightarrow CH_3COO^- + 3H^+ + 2HS^-$	+73.8	-87.74
$2HCOO^- + 2S° + 2H_2O \rightarrow 2HCO_3^- + 2H^+ + 2HS^-$	+26.8	-98.78
$0.5CH_3COO^- + 2S° + H_2O \rightarrow HCO_3^- + 2.5H^+ + 2HS^-$	-140.8	-290.3
$CH_3CH_2COO^- + 2S° + 2H_2O \rightarrow 1.25CH_3COO^- + 0.5HCO_3^- + 2.75H^+ + 2HS^-$	+104.5	-59.91
$0.5CH_4 + 2S° + 1.5H_2O \rightarrow 0.5HCO_3^- + 2.5H^+ + 2HS^-$	+103.75	-36.1
$CH_3CH_2OH + 12FeO(OH) \rightarrow CH_3COO^- + H^+ + 4Fe_3O_4 + 7H_2O$	-366	-405.4
$2HCOO^- + 12FeO(OH) \rightarrow 2HCO_3^- + 4Fe_3O_4 + 6H_2O$	-413	-419.9
$0.5CH_3COO^- + 12FeO(OH) \rightarrow HCO_3^- + 0.5H^+ + 4Fe_3O_4 + 6H_2O$	-343.4	-367.4
$CH_3CH_2COO^- + 12FeO(OH) \rightarrow 1.25CH_3COO^- + 0.75H^+ + 0.5HCO_3^- + 4Fe_3O_4 + 6H_2O$	-335.3	-379.8
$0.5CH_4 + 12FeO(OH) \rightarrow 0.5HCO_3^- + 0.5H^+ + 4Fe_3O_4 + 6.5H_2O$	-336.1	-350.3

It is curious that so much growth occurred with the enrichments with no added electron acceptor. These were supposed to serve as negative controls. However, at the time of media making, it was not considered that carbon dioxide could be reduced or that the little bit of sulfate in the basal medium could possibly be utilized. There was not a ton of growth as most of these cultures were not very turbid. Only after gentle agitation was it seen that some biomass had settled on the bottom. Certainly, in those enrichments with formate, H_2/CO_2, methanol, acetate, methylamine, and ethanol,

2) Coates, JD, et al. 1995. *Desulfuromonas palmitatis* sp. nov, a marine dissimilatory Fe(III) reducer that can oxidize long-chain fatty acids. *Arch Microbiol.* **164**:406-413.

3) Hines, ME, et al. 1991. Anaerobic microbial biogeochemistry in s sediments from two basins in the gulf of Maine: evidence for iron and manganese reduction. *Estuarine, Coastal and Shelf Science*. **32**:313-324.

3a) Howes, BL ; Dacey, JW and GM King. 1984. Carbon flow through oxygen and sulfate reduction pathways in salt marsh sediments. *Limnol. Oceanogr.* **29**:1037-1051.

4) Lovley, DR and DJ Lonegran. 1990. Dissimilatory Fe(III) and Mn (IV) reduction. *Microbiological Reviews*. **55**:259-287.

4a) Lovley, DR ; Phillips, EJP ; Lonegran, DJ and PK Widman. 1995. Fe(III) reduction and S° reduction by *Pelobacter carbinolicus*. **61**: 2132-2138.

5) Lovley, et al. 1993. *Geobacter metallireducens* gen. nov. sp. nov, a microorganism capable of coupling the complete oxidation of organic compounds to the reduction or iron and other metals. *Arch Microbiol.* **159**: 336-344.

6) Lovley, DR and EJP Phillips. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl. Environ. Micro*. **54**:1472-1480.

6a) Myers, CR and KH Nealson. 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science*. **240**:1319-1321.

6b) Myers, CR and KH Nealson. 1988. Microbial reduction of manganese oxides: interactions with iron and sulfur. *Geochimica et Cosmochimica Acta*. **52**:2727-2732.

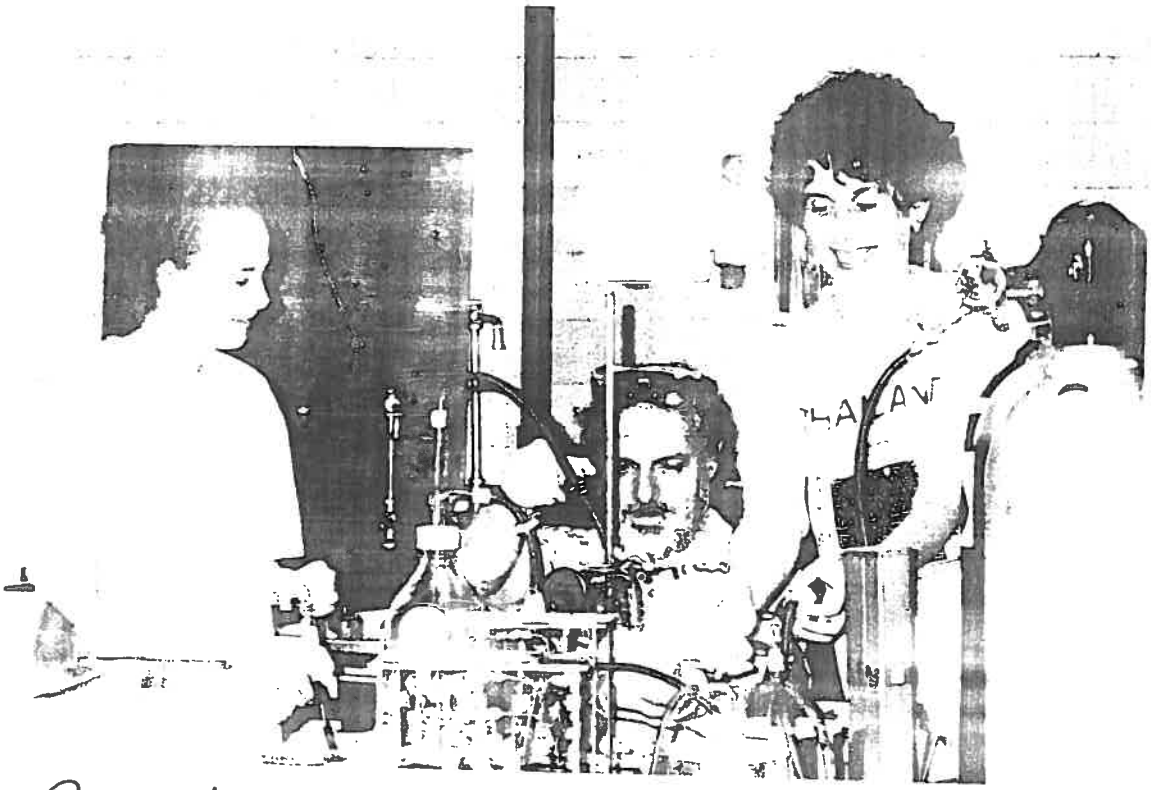
7) Nealson, KH and Daad Saffarini. 1994. Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. *Annu Rev Microbiol.* **48**:311-343.

8) Nealson, KH ; Meyers, CR ; and BB Wimpee. 1991. Isolation and identification of manganese-reducing bacteria and estimates of microbial Mn(IV)-reducing potential in the Black Sea. *Deep-Sea Research*. **38**:S907-S920.

9) Roden, ER and DR Lovley. 1993. Dissimilatory Fe(III) reduction by the marine microorganism *Desulfuromonas acetoxidans*. *Appl Environ Micro*. **59**:734-742.

10) Zopfi, Jakob. 1995. Anaerobe Mineralisierung unter ferrischen Bedingungen und Optimierung einer Anreicherungsstrategie für dissimilative Eisen(III)reduzenten. Diplomarbeit.

Kurt, Rachel & Annick making media.



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