

Reducing Fe(III) Using Geobacter

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Submitted to:
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Abstract

Geobacter sulfurreducens is an obligate anaerobe, it cannot survive in the presence of oxygen. This bacterium is unique for its ability to use ferric iron as an electron receptor at the end of the electron transport chain in cellular respiration, and for its known ability in the bioremediation of dangerous heavy metals such as uranium. However, the application of *G. sulfurreducens* in bioremediation and environmental characteristic adjustments is reliant upon several environmental factors. It is important that the bacteria have an electron donor (sodium acetate) in the environment as well as an electron receptor (ferric iron). Sediment samples from Great Meadows Marsh were spiked with exponentially varying amounts of ferric iron and sodium acetate in a matrix arrangement. After allowing the bacterium to reduce the iron in the samples for seven days, the remaining amounts of Fe(III) were determined using a colorimetric assay. By calculating the amount of ferric iron that was in each sample (the amount originally in the sediment and the amount added) and then determining the amount of Fe(III) remaining after the seven days, the amount of Fe(III) reduced to Fe(II) was determined. The data showed that as there was no distinct correlation between the amount of acetate added and the amount of iron reduced, meaning that the sediment already contained enough electron donors. Also, the data shows that as the amount of additional Fe(III) in each sample increased, so did the amount of Fe(III) reduced. This means that in the real-world application of *G. sulfurreducens* as a Fe(III)-reducing bacterium additional electron donors would not need to be added to sediment, and that the more Fe(III) in the environment there is, the more ferric iron the bacteria can reduce.

Introduction

Geobacter sulfurreducens is an anaerobic bacterium first isolated at the bottom of the Potomac River in 1987 by Dr. Derek Lovley and his associates at the University of Massachusetts (Lovley 2003). As it is an obligate anaerobe, it can live only where no oxygen is present. This makes underwater the perfect environment for it. *Geobacter sulfurreducens* is a member of the *Geobacteraceae* family along with other bacterium such as *Geobacter metallireducens*. These bacteria also have the distinct ability to reduce (add electrons to) metals including iron, manganese, and uranium. In many of the reactions that *Geobacter sulfurreducens* catalyzes, Fe(III) is used as an electron receptor. The focus of these reactions is the use of Fe(III) as an electron acceptor. These reactions are so important because of their geochemical applications.

In order to survive, all life must obtain energy through respiration. Bacteria are no different, and, through a series of processes, they obtain energy from the bonds of sugars. After glycolysis and the citric acid (or Krebs) cycle, the cell must get rid of electrons after their energy has been acquired in the electron transport chain. In most cases, the electrons travel through a series of five complexes and then meet up with oxygen. However, this is not the case with *G. sulfurreducens*. Instead of oxygen, Fe(III) is the electron acceptor. Therefore the used electron meets up with the ferric iron at the end of the electron transport chain where it reduces the ferric iron into ferrous iron. This makes the *G. sulfurreducens* unique, as it does not require oxygen to respire, instead it requires a environment without oxygen and with ferric iron. This environment occurs under water especially in places with deposits of Fe(III) such as estuaries (Lovley 1991).

The importance of *G. sulfurreducens* lies in that it has the ability to both bioremediate; or use biological agents including plants and bacteria to remove or neutralize environmental pollutants, dangerous metals such as uranium, and that it can reduce Fe(III) (Anderson 1999). The focus of this lab, however, is on its ability to reduce ferric iron. The process of reducing Fe(III) to Fe(II) is an extremely significant geochemical process, and is considered to be the most important redox change during the creation of anoxic soil conditions (Lovley 1991). It has been conclusively shown that ferric iron reduction is associated with the increase of pH; the release of trace metals, sulfate, and phosphate; the increase of ionic strength in soil; along with the displacement of elements including sodium, calcium, potassium, and magnesium (Lovley 1991). Along with having a significant impact on the elemental balance of soil, the reduction of ferric iron also affects the characteristics of clays. The reduction of Fe(III) to Fe(II) has been shown to greatly affect the porosity, friability, permeability, aggregate stability, and hydraulic conductivity of clays (Lovely 2003). It is clear that this geochemical process greatly affects the properties of soil, and thereby the life that inhabits it.

There have been several studies on the effects of ferric iron reduction on rice plants. While ferrous iron takes away large amounts of O₂ from soil, thereby impeding the growth of plants, the presence of Fe(II) has been shown to be beneficial to rice plants at certain levels (Nevin 2002). At high concentrations, Fe(II) is toxic to plants and animals (Nevin 2002). When there are phosphate or potassium deficiencies in the roots of these plants, the roots are damaged by the higher concentrations of Fe(II) (Nevin 2002). Through ferric iron reduction's connection to rice paddies, it indirectly has an impact on global methane fluxes.

Rice paddies are believed to be the cause for 40% of the annual atmospheric methane flux (Lovley 1991). When rice paddies are flooded and an anaerobic environment is created there is an increase in the activity of Fe(III) reducing bacteria. This increase in activity uses electrons that would have been used in the creation of methane. Therefore, the presence of Fe(III) reducing bacteria contributes to the decrease of methane production while rice paddies are flooded, while doing very little to effect methanogenesis (the process in which methane is created) when rice paddies are dry (Lovley 1991).

Possibly the most significant aspect of ferric iron reduction is its role in oxidizing organic matter. Oxidation of organic matter is extremely important to all environments as it assists in the decomposition of that matter (Lovley 2003). *G. sulfurreducens* is able to oxidize (remove electrons from) complex strands of organic matter into carbon dioxide and water (Lovley 1991). This oxidation of organic matter has been shown to occur in continental shelf sediment. It is extraordinarily important that this decomposition takes place in the continental shelves because this is where a large amount of marine and land matter comes together in the sediment (Lovley 1991). It is important that this matter is broken down and that CO₂ be created to promote plant growth.

The oxidation of organic matter in groundwater through the reduction of Fe(III) using iron-reducing bacteria is also important to the true significance of *G. sulfurreducens*. Often when groundwater is contaminated with organic matter, anaerobic conditions (conditions that include extremely little or no oxygen) are formed and iron-reducing bacteria can begin reducing iron and oxidizing those organic contaminants. These contaminants are oxidized and broken down into CO₂ and H₂O (Leang 2005).

This process of oxidizing organic contaminants in groundwater, coupled with the fact that Fe(III) is abundant in many soils, opens up the possibility of bioremediation of these groundwater contaminants using *G. sulfurreducens* and other iron-reducing bacteria (Leang 2005).

Materials

| Consumables | Supplies | Equipment |
|---|--|---|
| -Sediment from Great Meadows Marsh -Aluminum foil -Hydrous sodium acetate -Hydrous ferric chloride -Ethanol -1 mL syringes -Hypodermic needles -Deionized water -Plastic test tubes -FerroZine | -Small plastic cups -Marker -Metal scoop -Tank of anoxic gas -Plastic tubing -Glass tube filled with copper shavings -Glass tube with cotton filter -Large syringe -Rubber stopper -Test tubes containing stock Geobacter culture -Glass bottle and tops -Graduated cylinder -Cuvets | -Autoclave -Incubator -Spectrophotometer -Centrifuge |

Procedure

Firstly, about 700g of sediment from Great Meadows Marsh was collected. Then about 25 grams of the sediment was placed into 25 small plastic cups each. Each cup containing the sediment was then covered with aluminum foil as to keep in moisture, and then autoclaved to kill off any organisms in the sediment. Those cups, once autoclaved, were arranged in a five by five matrix and labeled so that each column would be assigned a letter (A, B, C, D, and E going from left to right), and each row would have a number (1, 2, 3, 4, and 5 going from bottom to top). Next, .034g of hydrous sodium acetate, meaning .026g of actual sodium acetate, was added to the each sample in the B column (second from the left). .051g of sodium acetate (calculated from .067g of hydrous sodium acetate) was added to each sample in the C column. .102g of sodium acetate (.134g of hydrous sodium acetate) was added to the D column. And .204g of sodium

acetate (.268g hydrous) was added to each sample in the E column. Then .28g of hydrous ferric chloride, meaning .17g of pure ferric iron was added to each cup in the 2-row. .34g of ferric iron (.56g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) was added to each cup in the 3-row. .65g of ferric iron (1.08g of hydrous ferric chloride) was added to each sample in the 4-row. And 1.37g of ferric iron (2.27g of hydrous ferric chloride) was added to each sample in the 5-row. All of the samples were then covered with aluminum foil and autoclaved.

A contraption (used later on) is needed to provide anoxic gas. First a tank of anoxic gas was placed on the floor with about one meter of plastic tubing connecting it to a glass tube filled with copper shavings. This tube was placed inside an incubator set at 30°C. Then about one meter of plastic tubing connected the opposite end of the glass tube to another glass tube with a cotton filter at one end. The end of that glass tube was then stuck into a rubber stopper that was stuck into the back end of a large syringe with the plunger removed. All parts of this contraption were sterile either originally or autoclaved. Anoxic gas then flows through this contraption, which removes all oxygen.

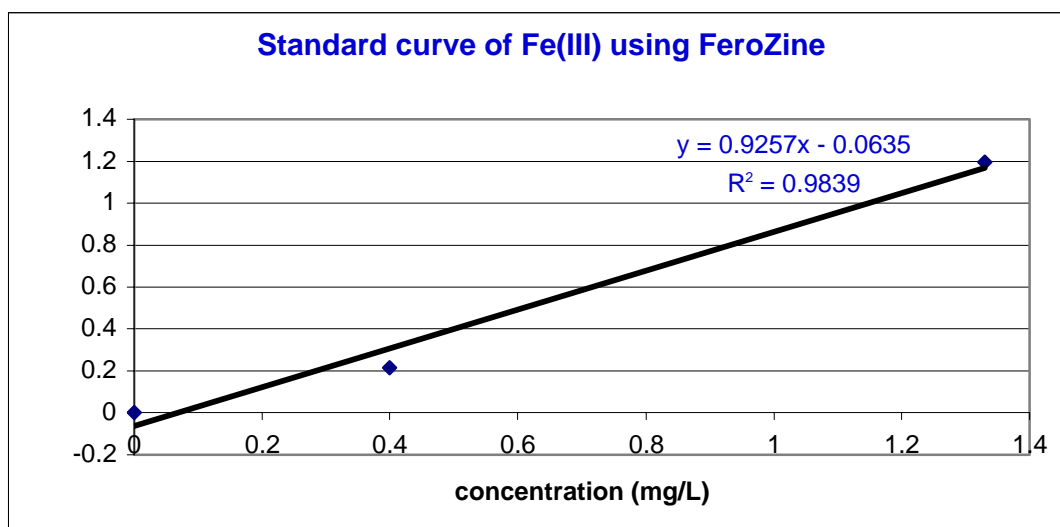
Once autoclaved, each sample was inoculated with .5mL of *G. sulfurreducens* culture. In order to do this, a sterile field must be kept. So, about 2 drops of ethanol was placed on the top of the rubber stopper of each test tube containing the Geobacter culture. That ethanol was then lit on fire. Then sterile 1mL syringes were taken out and attached to sterile hypodermic needles. The needle was then inserted into the tip of the sterile syringe at the end of the contraption described above. The needle was inserted just far enough to form a seal between the two syringes. Then the transferring syringe's plunger was pulled back, to take in the anoxic gas. Then the entire syringe was pulled back just enough to lose the seal, and the plunger was pushed back down. This process was then

repeated about twelve times to rid the syringe and needle of any trace amounts of oxygen and the plunger was pulled back so that the syringe contained .5mL of anoxic gas. The needle and syringe then were containing only .5mL of anoxic gas and then were inserted through the rubber stopper of the test tube containing the bacteria culture. The gas was injected into the test tube as a replacement for the .5mL of culture that was then removed so that a vacuum was not formed. When taking culture into the syringe, the plunger was pulled back to the .5mL mark, then the syringe was flicked a few times so that the gas bubbles rose to the top, the culture was then pushed back into the test tube, and then .5mL of culture were pulled back. The .5mL of culture were then injected into each of the 25 cups containing sediment. Those cups were then covered with aluminum foil and placed in an incubator set at 25°C. The samples sat for seven days.

Once the samples sat for seven days, the sediment of cup 1A was emptied into a glass bottle. Then 200mL of deionized water was added to the bottle and sediment. The bottle was covered and shaken for about one minute. Next two plastic 14 mL test tubes were filled with the sediment and water mixture and run in a centrifuge for three minutes. Then 25mL of the top liquid from test tubes was poured into a graduated cylinder. One solution pillow of FerroZine (a colorimetric indicator of ferric iron) was then added to that graduated cylinder and shaken for about one minute. That solution was then used to fill up a plastic cuvet. The cuvet filled with the solution was then placed into a previously blanked spectrophotometer with a cuvet filled with deionized water acting as the reference. The spectrophotometer reading was recorded, and this process was repeated for each of the remaining 24 samples.

To create a standard curve for the FerroZine assay a 1.33 mg/L solution of ferric iron (using hydrous ferric chloride) was created. 25mL of this solution was then added with one solution pillow of FerroZine and shaken for one minute. That solution was then used to fill up a plastic cuvet. The cuvet filled with the solution was then placed into a previously blanked spectrophotometer with a cuvet filled with deionized water acting as the reference. The data was recorded. Then this same process was followed for a .4mg/L solution of ferric iron. These points were graphed and used to create a standard curve (See Figure 1).

Figure 1:



Results

Table 1

| | | Sodium acetate (grams) | | | | |
|-----------------|-------|------------------------|--------|--------|--------|--------|
| | | 0g | .026g | .051g | .102g | .204g |
| Fe(III) (grams) | 1.37g | 69.51% | 71.07% | 68.52% | 67.16% | 71.14% |
| | .65g | 58.24% | 66.36% | 68.09% | 59.81% | 57.87% |
| | .34g | 57.52% | 65.78% | 62.88% | 64.74% | 65.65% |
| | .17g | 59.04% | 61.78% | 63.34% | 63.92% | 66.64% |
| | 0g | 0.00% | 0.00% | 0.00% | 0.00% | 0.00% |

Table 2

ANOVA

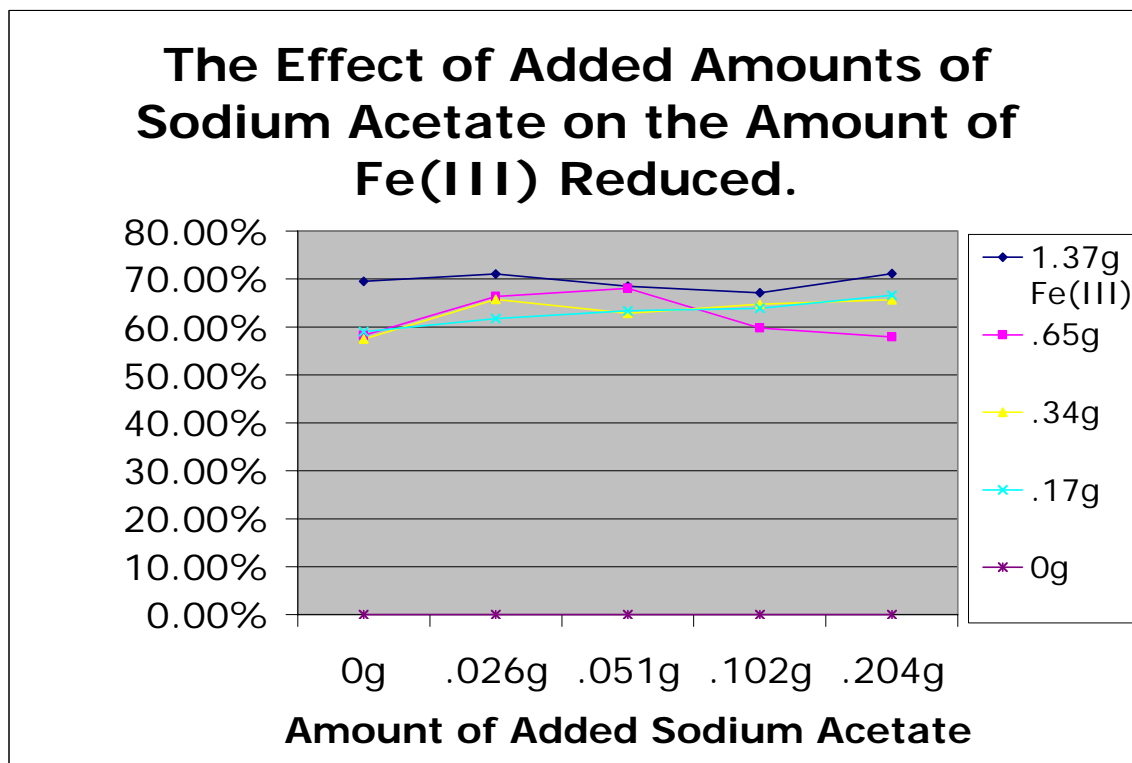
IV: Concentration of sodium acetate

| | Sum of Squares | df | Mean Square | F | Significance |
|----------------|----------------|----|-------------|------|--------------|
| Between Groups | .007 | 4 | .002 | .904 | .486 |
| Within Groups | .029 | 15 | .002 | | |
| Total | .035 | 19 | | | |

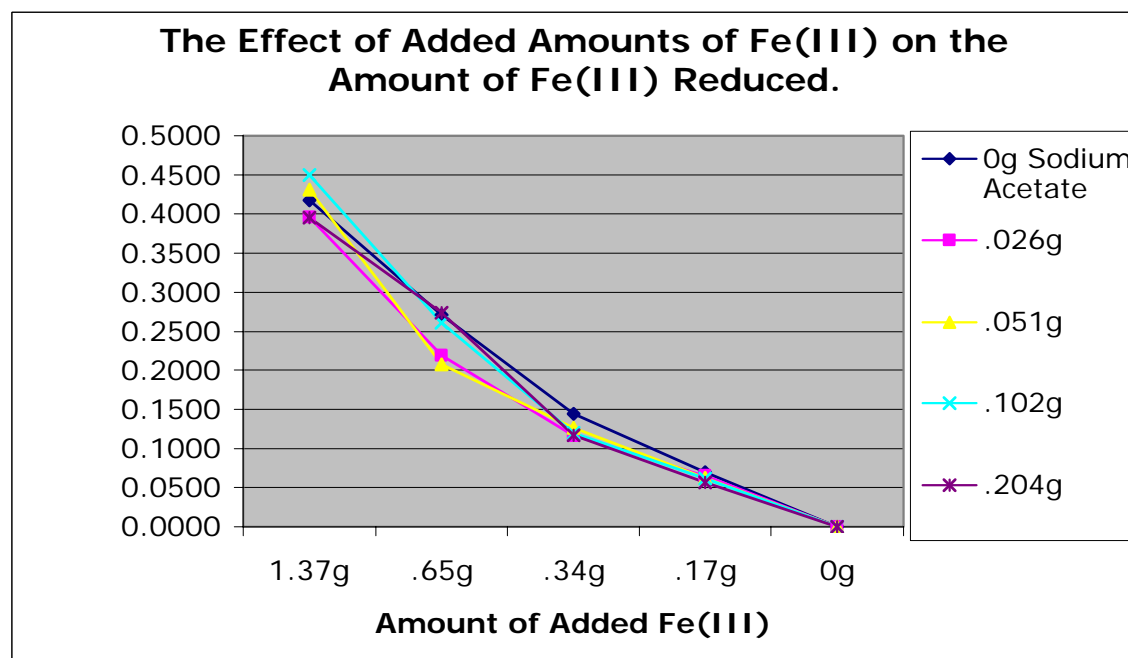
Table 3

| | | Amount of Fe(III) Remaining After Seven Days (In Grams) | | | | |
|-----------------|-------|---|--------|--------|--------|--------|
| | | Sodium acetate (grams) | | | | |
| Fe(III) (grams) | | 0g | .026g | .051g | .102g | .204g |
| | 1.37g | 0.4176 | 0.3963 | 0.4313 | 0.4499 | 0.3954 |
| | .65g | 0.2715 | 0.2187 | 0.2074 | 0.2612 | 0.2738 |
| | .34g | 0.1444 | 0.1164 | 0.1262 | 0.1199 | 0.1168 |
| | .17g | 0.0696 | 0.0650 | 0.0623 | 0.0613 | 0.0567 |
| | 0g | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |

Graph 1



Graph 2



Geobacter sulfurreducens was able to reduce ferric iron into ferrous iron in sediment from Great Meadows Marsh of Long Island Sound when samples were inoculated and incubated. There was an average of about 65% reduction of Fe(III) to Fe(II) (See Table 1).

The sediment proved to be a particularly hospitable habitat for the bacteria as additional electron donors were unnecessary. This is shown by the insignificant effect of added sodium acetate to sediment samples. The sodium acetate acts as an electron donor for the bacteria. But because there was no effect of added amounts of acetate at any level as compared to without any acetate added, it can be concluded that the sediment contained enough electrons to perform reduce the iron. Notice the horizontal orientation of the lines in Graph 1.

There was a steady relationship between the amount of Fe(III) (which was in the form of ferric chloride) added to a sample and the amount of Fe(III) reduced. The more iron that was added, the more iron that was reduced. This was proportional so that about 65% of the ferric iron in the sample was reduced no matter the amount of iron added (See Graph 2).

Conclusions

As shown in Graph 1, there was no significant effect of any added amounts of sodium acetate on the amount of iron reduced. Because the addition of electron donors was inconsequential (an ANOVA (Table 3) evaluation showed the significance at .486), it can be concluded that any amount of electrons needed by the bacteria to reduce Fe(III)

was already present in the sediment. This means that the sediment in Great Meadows Marsh had the nutrients and electron donors available to sustain *Geobacter*. Had there been a significant change depending on the amount of sodium acetate then it could be concluded that the *Geobacter* was being limited by the amount of electrons present. This was not the case.

When more Fe(III) was added to the sediment, more of the ferric iron was reduced. This could be because the *Geobacter* came in contact with more iron in the samples where more iron was present. So, this proportionate amount of iron reduced as shown in Table 1 could be because the bacteria came into contact with a proportional amount of the actual ferric iron.

In none of the samples was all of the ferric iron reduced. This could be because the *Geobacter* was unable to come in direct contact with some of the iron. Another possibility is that the *Geobacter* simply needed more time to reduce the iron. Because the samples of sediment were left untouched over seven days various gradients in the amount of ferric iron and other nutrients in the sediment could have formed. Since the samples were not agitated the *Geobacter* may well have not come in contact with all of the ferric iron. Another possibility is that the *Geobacter* did not have enough time to reduce all of the iron present. Time was a constant in the experiments, so the effect of time on the amount of ferric iron reduced is not known.

While temperature was not taken into account as a variable in this experiment (the samples were incubated at 30° C), the results from other variables suggest that *Geobacter* could reduce iron in Long Island Sound. *G. sulfurreducens* could be used in the adjustment of environmental characteristics of Long Island Sound. These characteristics

include: soil characteristics, methane production, and the release of trace elements (refer to introduction for applications of Geobacter). Also, the success of *G. sulfurreducens* encourages future research as a means to control uranium in ground water, and other aspects of bioremediation.

Areas where future study could be conducted is the effect of time and water currents on *G. sulfurreducens*'s ability to reduce Fe(III) to Fe(II). Geobacter is not a particularly transient bacterium and it can only reduce iron that it comes into physical contact with. Therefore, more time might allow the bacterium to spread so that it comes in contact with more iron. Also, by introducing water currents to the sediment, ferric iron may be moved into contact with the bacteria, allowing for it to be reduced. However, the data collected does show that Geobacter was effective in reducing Fe(III) which is important because of its implications enumerated in the introduction. These include Geobacter's ability in changing soil characteristic, methane production, oxidizing organic contaminants, and reducing other heavy metals such as uranium.

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