



## Effect of iron bioavailability on dissolved hydrogen concentrations during microbial iron reduction

John Komlos & Peter R. Jaffé\*

Department of Civil and Environmental Engineering, Princeton University, E-205 Engineering Quad, Princeton, NJ 08544, USA (\*author for correspondence: e-mail: jaffe@princeton.edu)

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### Abstract

Dissolved hydrogen ( $H_2$ ) concentrations have been shown to correlate with specific terminal electron accepting processes (TEAPs) in aquifers. The research presented herein examined the effect of iron bioavailability on  $H_2$  concentrations during iron reduction in flow-through column experiments filled with soil obtained from the uncontaminated background area of the Field Research Center (FRC), Oak Ridge, TN and amended with acetate as the electron donor. The first column experiment measured  $H_2$  concentrations over 500 days of column operation that fluctuated within a substantial range around an average of 3.9 nM. Iron reduction was determined to be the dominant electron accepting process. AQDS (9,10-anthraquinone-2,6-disulfonic acid) was then used to determine if  $H_2$  concentrations during iron reduction were related to iron bioavailability. For this purpose, a 100-day flow-through column experiment was conducted that compared the effect of AQDS on iron reduction and subsequent  $H_2$  concentrations using two columns in parallel. Both columns were packed with FRC soil and inoculated with *Geobacter sulfurreducens* but only one was supplied with AQDS. The addition of AQDS increased the rate of iron reduction in the flow-through column and slightly decreased the steady-state  $H_2$  concentrations from an average of 4.0 nM for the column without AQDS to 2.0 nM for the column with AQDS. The results of this study therefore show that  $H_2$  can be used as an indicator to monitor rate and bioavailability changes during microbial iron reduction.

### Introduction

Iron reduction, along with denitrification, sulfate reduction and methanogenesis, is a very important anaerobic terminal electron accepting process (TEAP) frequently associated with the remediation of subsurface hazardous wastes ranging from the biodegradation of gasoline contaminated sites to the precipitation and subsequent containment of some trace metals and radionuclides. Many biological transformations that are important to achieve during a remediation scheme are specific to a given TEAP. For example, the reduction and subsequent removal of uranium from groundwater

has been observed to occur only after nitrate has been reduced and iron reduction has started (Istok et al. 2004). In addition, uranium precipitation from groundwater decreased when sulfate reduction replaced iron reduction as the dominant TEAP (Anderson et al. 2003). It is therefore important to monitor which TEAP is occurring at a particular time and space in order to understand and maximize the bioremediation/biotransformation processes. Dissolved  $H_2$  has been shown to be an indicator of TEAPs in the subsurface (Chapelle et al. 1996, 2002; Lovley et al. 1994) with specific  $H_2$  concentration ranges measured for different TEAPs; nitrate reduction (<0.1 nM), iron

reduction (0.2–0.8 nM), sulfate reduction (1–4 nM), methanogenesis (5–15 nM). The TEAP yielding the most energy (if that electron acceptor is present) proceeds before the TEAPs yielding less energy because the microbial population for the higher energy-yielding TEAP is able to utilize the  $H_2$  more efficiently, thus driving the  $H_2$  concentration to levels that cannot be utilized by lower energy-yielding TEAPs (Lovley & Goodwin 1988). The above statement assumes that  $H_2$  is the sole electron donor though additional electron donors may be present during the bioremediation of a contaminated aquifer such as the contaminant itself in the case of petroleum hydrocarbons (Chapelle et al. 1996; Lovley et al. 1994) or an electron donor added to stimulate bioremediation of a trace metal or radionuclide (Anderson et al. 2003). Theoretical analysis has shown that steady state  $H_2$  levels during the simultaneous utilization of  $H_2$  and a carbon source as electron donors will remain relatively constant for a wide range of co-electron donor concentrations (Brown et al., in review), thus providing an explanation as to how  $H_2$  concentrations can be used to determine TEAPs in the presence of additional electron donors.

The more energetically favorable TEAP outcompetes less energetically favorable TEAPs. For example, the presence of sulfate (>6–9 mg/l) has been shown to inhibit methanogenesis (Chapelle et al. 1995) and the presence of amorphous Fe(III) has been documented to inhibit both sulfate reduction and methanogenesis (Lovley & Phillips 1987b). Contrary to this, research has shown that iron reduction can occur simultaneously with sulfate reduction (Watson et al. 2003) even though more energy is gained from iron reduction. One possible explanation for both processes to occur simultaneously is that the limited availability of the iron to the iron reducing microorganisms (referred throughout this manuscript as “bioavailability”) decreases the potential rate of iron reduction, thus allowing other less energetically favorable TEAPs to occur. Iron bioavailability has been shown to be a limiting factor of Fe(III) reduction in soils (Chapelle et al. 2002). The extent of bioavailability is dependent on the chemical structure of the Fe(III) oxide; ranging from a crystalline structure (less bioavailable) to an amorphous structure (more bioavailable). Though the total amount of Fe(III) is an important parameter in characterizing the mineralogy of soil, the bioavailable Fe(III) is key in

predicting the extent of microbial iron reduction in soil. Therefore the bioavailability of the iron could limit iron reduction and even though iron is present, it cannot be reduced fast enough to outcompete lower energy yielding TEAPs. Electron shuttling compounds such as AQDS (9,10-anthraquinone-2,6-disulfonic acid) have been shown to increase iron bioavailability in soil (Hacherl et al. 2001; Lovley et al. 1996; Nevin & Lovley 2000) by acting as an electron shuttle between iron surfaces and iron reducing microorganisms that are not in direct contact with the surface bound iron, which significantly increases the rate of iron reduction in soils.

It is unknown to what degree changes in iron bioavailability will affect steady-state  $H_2$  levels during iron reduction. Iron bioavailability may change (i) as the more bioavailable fraction of iron is being reduced and less available iron remains, (ii) as Fe(II) sorbs onto the Fe(III) phase, which may block Fe(III) reaction sites, and (iii) as the bioavailability of iron is increased in the presence of AQDS or other electron shuttling compounds. The objective of this study was to determine *via* flow-through column experiments how changes in iron bioavailability during long-term iron reduction and/or due to the addition of AQDS affect iron reduction rates and subsequent steady-state  $H_2$  levels. The results of this research will increase existing knowledge pertaining to the use of  $H_2$  as an indicator of redox processes, specifically iron reduction, during bioremediation.

## Material and methods

### *Soil composition*

The soil used for all experiments was obtained from the Field Research Center (FRC) which is located within the Y-12 Plant area on the Department of Energy's (DOE's) Oak Ridge Reservation in Oak Ridge, Tennessee. The soil was collected from the uncontaminated background area at depths between 36" and 72" and stored at 4°C until its use in microcosm or flow-through column experiments.

### *Bacterial strain*

*G. sulfurreducens* is an obligate anaerobic bacteria capable of iron reduction with acetate or hydrogen

as electron donors (Caccavo et al. 1994). This microorganism was chosen because microorganisms with 16S rDNA sequences closely related to *G. sulfurreducens* are the predominant microorganisms in a variety of subsurface environments in which Fe(III) reduction is the predominant TEAP.

#### *Growth culture preparation*

Growth cultures were prepared using a 50 mM ferric citrate media containing 13.7 g  $\text{FeC}_6\text{H}_5\text{O}_7$ , 2.5 g  $\text{NaHCO}_3$ , 0.25 g  $\text{NH}_4\text{Cl}$ , 0.6 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.1 g  $\text{KCl}$ , 10 ml vitamin solution (Lovley & Phillips 1988) and 10 ml trace minerals solution (Lovley & Phillips 1988) per liter of distilled water. The ingredients (except the vitamin and mineral solution) were combined and dissolved on a heated stir plate. The solution was then cooled and pH adjusted to  $\sim 7$ . Nine milliliter of solution was transferred to crimp-seal test tubes, purged with  $\text{CO}_2/\text{N}_2$  gas (20:80 ratio), sealed with a rubber stopper and autoclaved. The test tubes were inoculated with 1 ml of a previous *G. sulfurreducens* growth culture, supplied 0.1 ml of 1 M sodium acetate and statically incubated for 5 days at 30 °C.

#### *Batch experiment*

One gram of FRC soil and 9 ml of basal media (2.5 g  $\text{NaHCO}_3$ , 0.25 g  $\text{NH}_4\text{Cl}$ , 0.6 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.1 g  $\text{KCl}$ , 10 ml vitamin solution and 10 ml trace minerals solution per liter of distilled water) was added to crimp-seal test tubes and sealed with a butyl rubber stopper (Fisher Scientific). The test tubes were purged with  $\text{CO}_2/\text{N}_2$  gas (20:80 ratio) for 15 min. *G. sulfurreducens* growth culture, sodium acetate and AQDS (Sigma) were added to the appropriate test tubes to yield concentrations of 10% growth culture, 10 mM acetate, AQDS concentrations ranging from 0 to 500  $\mu\text{M}$  and a final volume of 10 ml. The test tubes were placed on a slow moving (7 rpm) rotator in a 30 °C incubator and Fe(II) concentrations were monitored over time.

#### *FRC column experiment*

A 30 cm long  $\times$  5 cm diameter polycarbonate column was fabricated and fitted with sampling ports every 2 cm along the length of the column. The sampling ports consisted of an SGE luer lock

stainless steel needle (50 mm, 23 ga., pt#2, Supelco) extending into the middle of the column with a 1-way male luer lock stopcock (Cole-Parmer) attached to the needle on the outside of the column. The column was filled with FRC soil and purged with  $\text{CO}_2$  for 30 min prior to continuously pumping a phosphate buffered (PB) solution (0.4 g  $\text{KH}_2\text{PO}_4$ , 1.23 g  $\text{K}_2\text{HPO}_4$ , 10 ml vitamin solution and 10 ml trace minerals solution per liter of distilled water) up-flow through the column at a rate of 0.5 ml min<sup>-1</sup>. A syringe pump (KD Scientific) supplied 1.13 M sodium acetate to the main flow line just before the column influent at a rate of 0.08 ml h<sup>-1</sup>, resulting in an acetate concentration entering the column of 3 mM. After 497 days of operation at 30 °C, the column was destructively sampled in an anaerobic hood for iron analysis along the length of the column.

#### *AQDS soil column experiment*

Two 1 cm diameter, 15 cm long glass columns (Kontes Glass Company) were filled with 14.7 g ( $\pm 0.1$  g) of FRC soil. The columns were purged with  $\text{CO}_2$  for 30 min. Phosphate buffered (PB) solution with or without 50  $\mu\text{M}$  AQDS was pumped up-flow using a peristaltic pump through the column at a rate of 0.5 ml min<sup>-1</sup> for 25 h before addition of *Geobacter sulfurreducens*. A *G. sulfurreducens* seed solution was prepared by centrifuging a growth culture at 5900  $\times g$  for 20 min, removing the supernatant *via* syringe, and resuspending the pellet with PB solution. This process was performed twice. One pore volume of seed solution was injected into each column and pumping was discontinued for 22 h. Flow to the column was resumed and acetate addition *via* syringe pump commenced (acetate concentration after mixing was 3 mM).  $T = 0$  is defined as when flow was turned on after inoculation. After 96 days of operation at 30 °C, the column was destructively sampled in an anaerobic hood for iron analysis along the length of the column.

#### *Hydrogen sampling in column experiments*

Dissolved  $\text{H}_2$  concentrations were measured using a miniature version of the  $\text{H}_2$  sampler used for the gas-strip method described by Chapelle et al. (1997). The hand blown glass sampler had a volume of 3.5 ml. The  $\text{H}_2$  sampler was purged with a

CO<sub>2</sub>/N<sub>2</sub> (20:80) mixture (BOC Gases) and attached to either the outflow or sampling ports of the column. Liquid from the column was allowed to flow through and fill the sampler until 1.5 ml of headspace remained. For relatively high H<sub>2</sub> levels, H<sub>2</sub> concentrations in the headspace reached equilibrium after 4 h, which was much longer than that required by Chapelle et al. (1995, 1997) due to the significantly slower flow rates in the column (0.5 ml min<sup>-1</sup>) compared to literature (>500 ml min<sup>-1</sup>). H<sub>2</sub> samples were obtained by removing 1.5 ml of headspace from the sampler and injecting into a RGA3 Reduction Gas Analyzer (Trace Analytical) equipped with a 0.5 ml sample loop. The H<sub>2</sub> detection limit of this method is approximately 0.05 nM and no background H<sub>2</sub> was detected in the CO<sub>2</sub>/N<sub>2</sub> gas mixture. The H<sub>2</sub> sampler was then supplied 1.5 ml of the CO<sub>2</sub>/N<sub>2</sub> gas mixture and allowed to come to equilibrium. For the long-term (500 day) column, H<sub>2</sub> measurements were first taken at the effluent followed by the sampling ports from the end to the beginning of the column. Samples were allowed to equilibrate in the H<sub>2</sub> sampler for 40 min to 2 h from the start of the experiment to day 181. After day 181, the H<sub>2</sub> sampler was connected to each sampling port for >4 h before a headspace sample was extracted. For the AQDS column experiment, a H<sub>2</sub> sampler was left in-line at the effluent of each column and sampled every 20–24 h. Dissolved H<sub>2</sub> concentrations were calculated using Equation (1).

$$H_{2(\text{dissolved})} = \frac{L \cdot P}{R \cdot T} \quad (1)$$

where  $H_{2(\text{dissolved})}$  is the dissolved H<sub>2</sub> concentration [mol l<sup>-1</sup>],  $L$  the Ostwald coefficient for H<sub>2</sub> solubility, 0.01895 at 30 °C (Wilhelm et al. 1977),  $P$  the H<sub>2</sub> pressure in headspace [atm],  $R$  the Universal Gas Constant [0.0821 l atm °K<sup>-1</sup> mol<sup>-1</sup>],  $T$  the temperature [°K].

#### Iron analysis

Iron was quantified using different variations of the ferrozine method previously described by Lovley & Phillips (1987a) and Anderson et al. (1998).

The Fe(II) concentration in the batch experiments and aqueous Fe(II) concentration in the column experiments was measured by removing 0.1 ml of sample and adding to 5 ml of 0.5 N HCl.

After 1 h at room temperature, 0.1 ml of sample/HCl solution was transferred to 5 ml ferrozine solution (Lovley & Phillips 1987a), mixed for 15 s, filtered using a 0.2 µm nylon filter, and analyzed using a Spectronic Genesys 2 spectrophotometer ( $\lambda = 562$  nm).

At the end of the column experiments, the columns were destructively sampled in an anaerobic glove box (3% H<sub>2</sub> balanced in N<sub>2</sub>) and Fe(II) quantified by adding a known weight of soil (~0.1 g) to 5 ml 0.5 N HCl and measured as described above. Moisture contents were obtained by weighing a soil sample before and after drying at 103 °C for 1 h. In addition to 1 h extraction, iron extractions in 0.5 N HCl were also performed after 22 h, 6, 21, or 50 days.

To measure the Fe(II) concentration sorbed to soil during column operation, the soil was locally disturbed at a sampling port by extracting 0.5 ml pore water *via* syringe and reinjecting it back into the column. A half milliliter of the now suspended soil/pore water solution was then extracted and added to 2.0 ml of 0.5 N HCl. After 1 h, 0.1 ml of sample/HCl was transferred to 5 ml ferrozine and analyzed as described above. Samples were collected in duplicate (up to day 93) and in triplicate (after day 96) for each sampling port and sample time. To obtain an estimate of the dry weight of soil in this mixture, the soil resuspending procedure was repeated in duplicate (up to day 93) and in triplicate (after day 96) with a separate 0.5 ml suspended soil/pore water sample added to a pre-weighed aluminum dish that was dried at 103 °C for 1 h and re-weighed. The results showed that, for the soil used here, this technique for the *in situ* quantification of sorbed iron concentrations during the column operation agreed well with iron concentrations quantified after the column was destructively sampled using the method described above.

Total extracted iron was quantified similar to the above method for Fe(II) analysis except hydroxylamine hydrochloride was added to the 0.5 N HCl at a final concentration of 0.25 N prior to soil addition.

#### Supplemental analysis

Acetate and sulfate concentrations were analyzed using an ion chromatograph (Dionex) with a Biorad HPLC Fast Acid Analysis Column (100 × 7.8 mm) or a Dionex IonPac AS14-4mm

column, respectively. Methane was analyzed using a Fisher Gas Partitioner Model 1200 (column temperature, 50 °C). Statistical analysis was performed where indicated using paired *t*-tests. Data sets were considered significantly different if the *p*-values were lower than 0.05.

## Results

### FRC column experiment

A flow-through column experiment using FRC soil was performed to monitor iron reduction over time by indigenous microorganisms (no *G. sulfurreducens* added) and without the addition of AQDS. The bioavailable Fe(III) concentration of the FRC soil, as defined by 1 hr extraction in 0.5 N HCl with 0.25 N hydroxylamine (Anderson et al. 1998), prior to the start of the column experiment was  $10.9 \pm 2.9 \mu\text{mol g}^{-1}$  dry soil ( $n = 6$ ) and Fe(II) was not detected. For the first 22 days of the experiment, iron reduction was not detected in the column with Fe(II) concentrations below  $1.5 \mu\text{mol g}^{-1}$  dry soil. After 32 days of acetate addition, the Fe(II) concentration started to increase with the ratio of Fe(II) to total extracted iron steadily increasing throughout the column until leveling out after 115 days (Figure 1). Even though the Fe(II)/total extracted iron ratio leveled

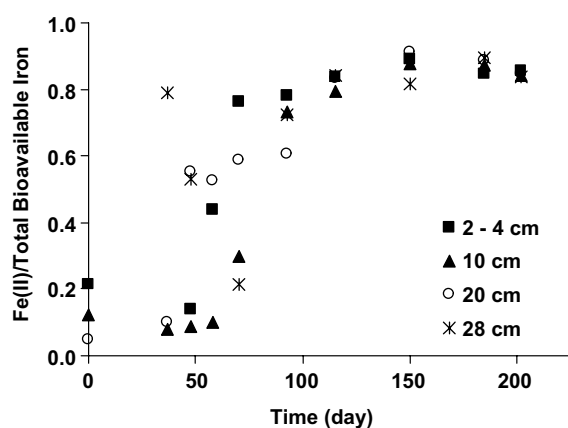


Figure 1. Ratio of Fe(II) to total bioavailable iron over time at different sampling points along the length of the 30 cm column filled with FRC soil. Fe(II) values are from 1 h extraction in 0.5 N HCl. After reading, 0.1 ml of 6.25 M hydroxylamine was added and allowed to extract for one additional hour for measurement of total iron.

off after 115 days, the Fe(II) concentration (Figure 2) and total extracted iron concentration continued to increase before leveling off between 200 and 300 days. An iron reduction rate of  $0.17 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$  was calculated using linear regression of Fe(II) production between day 70 and 254. The steady-state (days 254–490) Fe(II) and total extracted iron concentration throughout the column was  $37.3 \pm 7.8 \mu\text{mol g}^{-1}$  dry soil ( $n = 13$ ) and  $33.9 \pm 7.3 \mu\text{mol g}^{-1}$  dry soil ( $n = 13$ ), respectively. No Fe(II) was measured in the pore water of the column indicating that the majority of the Fe(II) produced was sorbed to the soil. The uniform accumulation of Fe(II) at each sampling point over time indicates similar rates of iron reduction throughout the column and coincided with a change in the soil color from a brown/orange color to a gray color.

Within the first five days of acetate addition to the column,  $\text{H}_2$  levels decreased below 0.3 nM before starting to increase after day 36 and leveling off after approximately 90 days (Figure 3). Throughout the duration of the experiment, steady-state  $\text{H}_2$  concentrations fluctuated between 0.3 and 25 nM with an average  $\text{H}_2$  concentration between day 92 and 422 of 3.9 nM. Steady-state acetate concentrations (day 36–453) decreased from 3.0 mM at the beginning of the column to 2.5 mM at the effluent ( $p$ -value < 0.001) with the majority of the acetate degradation occurring between the influent sampling port (just upstream of the acetate addition point) and the first sampling port (2 cm into the column) (Table 1). Sulfate was

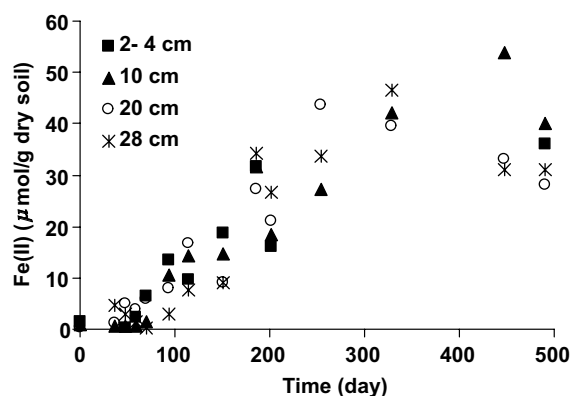


Figure 2. Fe(II) concentrations over time at different sampling points along the length of the 30 cm column filled with FRC soil. Samples were extracted for 1 h in 0.5 N HCl.

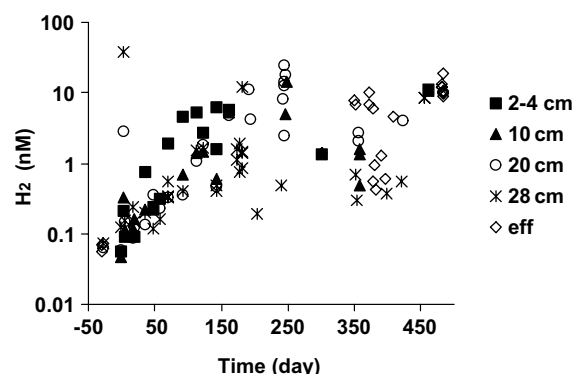


Figure 3. Dissolved hydrogen concentrations over time at different sampling points along the length of the 30 cm column filled with FRC soil.

present in the influent media as part of the trace mineral solution at a concentration of 0.28 mM ( $27 \text{ mg l}^{-1}$ ). Sulfate concentrations were first measured in the column after 100 days of column operation with sulfate reduction first observed on day 208. Between day 208 and 466, steady-state sulfate concentrations decreased from 0.28 mM at the influent to 0.24 mM at the effluent ( $p$ -value  $< 0.001$ ). In addition, qualitative evidence of sulfate reduction was observed through the presence of sulfide odor and the formation of a black precipitate throughout the column. Similar to the fate of acetate in the column, the majority of the sulfate removal occurred before the first sampling port of the column (Table 1). Methane was not detected throughout the column when analyzed for on day 146.

After 497 days of operation, the column was destructively sampled and the Fe(II) and total

extracted iron concentration profile along the length of the column was measured. The iron concentrations were relatively constant along the length of the column with measured Fe(II) and total extracted iron concentrations of  $38.6 \pm 8.6 \mu\text{mol g}^{-1}$  dry soil ( $n = 12$ ) and  $36.1 \pm 7.4 \mu\text{mol g}^{-1}$  dry soil ( $n = 12$ ), respectively, after 1 h extraction in 0.5 N HCl. The Fe(II) and total extracted iron concentrations measured after the column was destructively sampled agree well with the steady-state sorbed Fe(II) and total extracted iron concentrations ( $37.3 \pm 7.8 \mu\text{mol g}^{-1}$  dry soil and  $33.9 \pm 7.3 \mu\text{mol g}^{-1}$  dry soil, respectively). Increasing the length of extraction time significantly increased both the Fe(II) and total iron concentration in the destructively sampled soil. The average Fe(II) concentration throughout the column was  $76.8 \pm 12.0 \mu\text{mol g}^{-1}$  dry soil ( $n = 12$ ) and  $99.5 \pm 26.6 \mu\text{mol g}^{-1}$  dry soil ( $n = 12$ ) for 22 h and 21 days extraction, respectively, and the total iron concentration throughout the column for 22 h and 21 days extraction was  $77.2 \pm 17.4 \mu\text{mol g}^{-1}$  dry soil ( $n = 12$ ) and  $230.6 \pm 53.1 \mu\text{mol g}^{-1}$  dry soil ( $n = 12$ ), respectively. After 1 and 22 h extractions in 0.5 N HCl, the majority of the iron in the column was in the form of Fe(II) but after 21 days of extraction, the total iron concentration is significantly higher than the Fe(II) concentration resulting in an Fe(II)/Fe(III) ratio of 0.76.

#### AQDS experiments

Batch experiments were performed using FRC soil to determine if AQDS would increase iron reduction and what AQDS concentrations provided maximum iron reduction. A porous media flow-

Table 1. Steady-state acetate and sulfate concentrations along the length of the 30 cm column filled with FRC soil

	Acetate concentration (mM)	Sulfate concentration (mM)
Influent tee	$3.00 \pm 0.42$ (16)	$0.28 \pm 0.02$ (15)
Just before column		$0.26 \pm 0.02$ (3)
2 cm into column	$2.63 \pm 0.08$ (3)	$0.26 \pm 0.01$ (4)
4 cm into column	$2.63 \pm 0.32$ (8)	$0.24 \pm 0.01$ (2)
10 cm into column	$2.61 \pm 0.27$ (13)	$0.26 \pm 0.03$ (9)
20 cm into column	$2.57 \pm 0.22$ (14)	$0.23 \pm 0.03$ (8)
28 cm into column	$2.52 \pm 0.20$ (14)	$0.23 \pm 0.03$ (9)
Effluent	$2.50 \pm 0.22$ (10)	$0.24 \pm 0.03$ (15)

The acetate data is an average ( $\pm$ std) of data between days 36 and 456. The sulfate data is an average ( $\pm$ std) of data between days 208 and 466. ( ) indicates number of data points.

through column experiment was then performed to determine the effect of AQDS addition on steady-state  $\text{H}_2$  concentrations. Fe(II) concentrations over time for batch microcosm experiments with different AQDS concentrations are presented in Figure 4. Iron reduction was first detected in microcosms with AQDS after 10 days of incubation. After 34 days of incubation, significant Fe(II) concentrations were measured in all microcosms with AQDS ( $41.1 \pm 8.2 \mu\text{mol g}^{-1}$  soil,  $n = 5$ ). More Fe(II) was produced in the two higher AQDS concentration microcosms (200 and  $500 \mu\text{M}$ ) than in the lower AQDS concentration microcosms (5 and  $50 \mu\text{M}$ ). Microcosms without AQDS did not show evidence of Fe(II) production. The microcosms were again sampled after 320 days of incubation and the amount of Fe(II) produced increased for all AQDS concentrations to  $116.0 \pm 24.8 \mu\text{mol g}^{-1}$  soil ( $n = 5$ ). The amount of Fe(II) produced increased with increasing AQDS concentration with lower Fe(II) concentrations measured in microcosms supplied 5 and  $50 \mu\text{M}$  AQDS ( $83.4$  and  $111.3 \mu\text{mol g}^{-1}$  soil, respectively) compared to microcosms supplied 200 and  $500 \mu\text{M}$  AQDS ( $142.2$  and  $131.9 \mu\text{mol g}^{-1}$  soil, respectively). The Fe(II) concentrations in batch microcosms inoculated with *G. sulfurreducens* (but not supplied AQDS) also increased between day 34 ( $3.1 \pm 0.6 \mu\text{mol g}^{-1}$  soil) and day 320 ( $40.1 \pm 5.1 \mu\text{mol g}^{-1}$  soil). No iron reduction was observed in a control microcosm with  $50 \mu\text{M}$  AQDS but not inoculated with *G. sulfurreducens*. Three additional microcosm experiments were performed repeating this scenario ( $50 \mu\text{M}$  AQDS

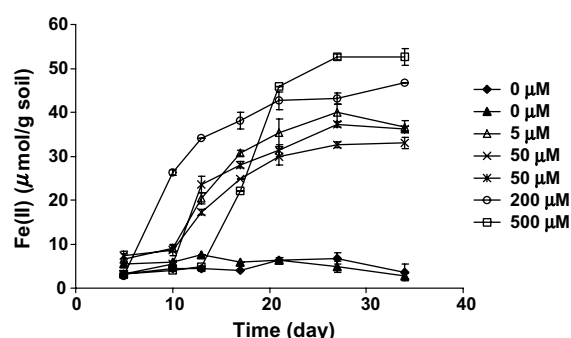


Figure 4. Fe(II) production over time for microcosms inoculated with *G. sulfurreducens*. AQDS concentrations ranged from 0 to  $500 \mu\text{M}$ . Values are average of duplicate samples taken from the same microcosm ( $\pm$ std).

but not inoculated with *G. sulfurreducens*). When the experiment was terminated after 73 days,  $43.7 \mu\text{mol g}^{-1}$  soil of Fe(II) was detected in one of the three microcosms, while the other two remained below detection levels ( $0.4 \mu\text{mol g}^{-1}$  soil). It is not known if Fe(II) production would have been detected in the other microcosms after a longer incubation time.

Two soil columns were run in parallel to measure the effect of iron bioavailability on steady-state dissolved  $\text{H}_2$  concentrations. Both columns were filled with FRC soil, inoculated with *G. sulfurreducens*, and fed a phosphate buffered solution amended with 3 mM acetate. The influent media of one of the columns contained  $50 \mu\text{M}$  AQDS, for which the batch experiments showed a significant enhancement in the iron reduction rate. Both columns started at approximately the same  $\text{H}_2$  concentration (Figure 5), which correlated with atmospheric  $\text{H}_2$  in equilibrium with water (this was expected since the influent media to the column was open to the atmosphere). An increase in  $\text{H}_2$  concentration is observed over time with the column supplied AQDS reaching steady-state conditions after 20 days and the column without AQDS reaching steady-state conditions after 30 days. The steady-state  $\text{H}_2$  concentration in the column without and with AQDS was  $4.0 \text{ nM}$  ( $\pm 3.1 \text{ nM}$ ,  $n = 23$ ) and  $2.0 \text{ nM}$  ( $\pm 1.2 \text{ nM}$ ,  $n = 29$ ), respectively. The steady-state  $\text{H}_2$  concentration measured at the effluent of the column supplied  $50 \mu\text{M}$  AQDS was significantly lower ( $p < 0.005$ ,  $n = 23$ ) than the column without AQDS between day 30

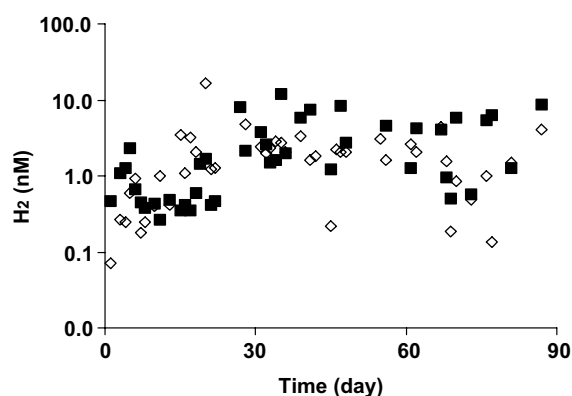


Figure 5. Dissolved  $\text{H}_2$  concentrations over time from the effluent of flow-through column experiments without (■) and with (◇)  $50 \mu\text{M}$  AQDS addition.

and the end of the experiment. After 20 days of column operation, the soil in the column supplied a steady concentration of AQDS turned from an orange/rust color to a gray color, indicating iron reduction. Similar to the 500 day FRC column experiment, no Fe(II) was measured in the pore water flowing out of the column. After 50 days of column operation, the column without AQDS still retained its orange/rust color. For both columns, sulfate concentrations remained constant between the influent and effluent of the column ( $0.28 \pm 0.01$  mM,  $n = 19$ ) and acetate removal was  $<0.5$  mM for the duration of the experiment. The columns were taken off-line after 96 days of operation when the soil in the column without AQDS changed from an orange/rust color to a gray color. The columns were destructively sampled and Fe(II) and total extracted iron concentrations were quantified after 1 h, 6 and 50 days extraction in 0.5 N HCl (Table 2). Less iron reduction was measured at the beginning of both columns due to the introduction of dissolved oxygen present in the influent media, which was in contact with the atmosphere. Therefore, measurements from the first two cm of each column were not considered in the average iron concentrations presented in Table 2. For the 1 h extraction, all of the iron measured was in the form of Fe(II). In addition, the Fe(II) concentration in the column supplied 50  $\mu$ M AQDS was double the Fe(II) concentration in the column without AQDS (Table 2). Using the initial and final Fe(II) concentrations (1 h extraction), the rate of iron reduction for the column without and with AQDS is  $0.16 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$  and  $0.35 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$ , respectively. Note that the iron reduction rate in this shorter time column experiment without AQDS compares well to that measured in the long-term column experiment. The Fe(II) and total iron concentrations both increased

with increasing extraction time. After 6 days of extraction, the Fe(II) concentration doubled and the total extracted iron concentration more than tripled compared to the 1 h extraction, resulting in an Fe(II)/Fe(III) ratio of 0.70 and 1.98 for the column without and with AQDS, respectively. The Fe(II) concentration leveled off in the column supplied AQDS between 6 and 50 days extraction while the column without AQDS increased slightly. However, the total extracted iron concentration increased significantly for both columns, resulting in an Fe(II)/Fe(III) ratio of 0.18 and 0.33 for the column without and with AQDS, respectively, after 50 days of extraction (Table 2). At each extraction time, the Fe(II) concentration in the column supplied AQDS was greater than the column without AQDS while comparable total extracted iron concentrations for columns with and without AQDS were measured after 6 and 50 days extraction (Table 2).

## Discussion

In the long-term (500 days) column experiment, steady-state  $\text{H}_2$  concentrations fluctuated between 0.3 and 25 nM. Fe(II) production was first detected in the column on day 32 and Fe(II) concentrations increased until day 250 when the sorbed Fe(II) concentrations leveled out at  $37.3 \pm 7.8 \mu\text{mol g}^{-1}$  dry soil ( $n = 13$ ) (Figure 2). Fe(III) reduction and subsequent Fe(II) adsorption/precipitation may have occurred after day 250 because more Fe(II) was detected when the extraction time was increased in soil samples analyzed at the end of the experiment, indicating that measured Fe(II) concentrations remained constant after day 250 at least partly due to insufficient time of extraction. Increased Fe(II) concentrations were measured

Table 2. Fe(II) and total extracted iron concentrations after 96 days of column operation without and with 50  $\mu$ M AQDS

Extraction time	Column without AQDS		Column with 50 $\mu$ M AQDS	
	Fe(II) $\mu\text{mol (g soil)}^{-1}$	Total extracted Iron $\mu\text{mol (g soil)}^{-1}$	Fe(II) $\mu\text{mol (g soil)}^{-1}$	Total extracted Iron $\mu\text{mol (g soil)}^{-1}$
1 h	$15.2 \pm 1.6$	$16.0 \pm 1.6$	$33.8 \pm 3.9$	$32.5 \pm 5.8$
6 day	$30.8 \pm 8.0$	$74.8 \pm 9.9$	$66.8 \pm 8.0$	$100.6 \pm 14.7$
50 day	$42.0 \pm 3.8$	$275.0 \pm 35.5$	$64.6 \pm 4.6$	$259.2 \pm 55.4$

Samples are an average ( $\pm$ std) taken from the middle and end of each column ( $n = 6$ ).



with increased extraction time between extraction times of 1 h, 22 h and 21 days, with the majority of the Fe(II) (77.2%) measured after 22 h of extraction. After 497 days of column operation, the Fe(II) concentration was an order of magnitude higher than the initial bioavailable Fe(III) concentration (as defined by 1 h extraction in 0.5 N HCl) in FRC soil ( $10.9 \pm 2.9 \mu\text{mol g}^{-1}$  dry soil). In addition, quantification of Fe(II) using only 1 h of extraction time in 0.5 N HCl would have significantly underestimated the total amount of Fe(II) produced after 500 days of iron reduction. A decrease in sulfate concentration, indicating sulfate reduction, was observed after day 200 but only a small amount of sulfate was removed from the column (0.04 mM) and the majority of the removal occurred before the first sampling port inside the column (Table 1). Methane was not detected when analyzed for on day 146. Sulfate concentrations throughout the experiment ranged from 15 to  $30 \text{ mg l}^{-1}$ , which is above the minimum sulfate concentration reported to inhibit methanogenesis in aquifers ( $4\text{--}6 \text{ mg l}^{-1}$ ) (Chapelle et al. 1995, 2002). Therefore iron reduction appears to have been the dominant TEAP in this system throughout the duration of the experiment, though the average  $\text{H}_2$  concentration (3.9 nM) was higher than reported in the literature for iron reducing zones (0.2–0.8 nM) (Chapelle et al. 1996, 2002; Lovley et al. 1994).

Simultaneous iron and sulfate reduction has also been measured in a long-term batch experiment using aquifer sediments (Watson et al. 2003) in which  $\text{H}_2$  concentrations during iron reduction were not always in the range typically reported for iron reducing conditions. Though amorphous iron has been shown to inhibit sulfate reduction and methanogenesis (Lovley & Phillips 1987b), significant increases in spatial and temporal Fe(II) concentrations are measured in aquifers where other TEAPs that are less energetically favorable (sulfate reduction, methanogenesis) are active and considered to be the dominant TEAP due to higher  $\text{H}_2$  concentrations (Lovley et al. 1994; Chapelle et al. 1995, 1996, 1997). One possible reason for less energetically favorable TEAPs occurring in iron reducing zones is that, due to the crystalline (less bioavailable) fraction present in aquifer sediments, the bioavailability of Fe(III) limits competitive exclusion of  $\text{H}_2$  concentrations. In an attempt to increase iron bioavailability, AQDS was added as

part of the feed media supplied to a soil column inoculated with *Geobacter sulfurreducens* while a parallel control column was not supplied AQDS. Enhancement of iron bioavailability through AQDS addition not only increased the rate of iron reduction from  $0.16 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$  to  $0.35 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$ , but also decreased steady-state  $\text{H}_2$  concentrations from 4.0 nM ( $\pm 3.1$  nM,  $n = 23$ ) to 2.0 nM ( $\pm 1.2$  nM,  $n = 29$ ), which is a statistically significant decrease. In addition, less variability in  $\text{H}_2$  concentrations over time was measured in the column supplied with AQDS. Steady state  $\text{H}_2$  concentrations in the column supplied with AQDS, though lower than the column without AQDS, were still higher than reported in the literature for iron reducing conditions (0.2–0.8 nM) (Chapelle et al. 1996, 2002; Lovley et al. 1994). It is important to note that all column experiments were performed at 30 °C, which is significantly higher than field conditions (15 °C or less, for which  $\text{H}_2$  levels are reported) and supplied a variety of trace vitamins and minerals that may be limiting in an aquifer and which would affect the biokinetic coefficients. Hoehler et al. (1998) demonstrated that a decrease in temperature from 30 to 15 °C decreased  $\text{H}_2$  concentrations in batch experiments by a factor of 5.0 and 5.4 for sulfate reducing and methanogenic conditions, respectively. Applying this temperature dependency to the  $\text{H}_2$  concentrations reported here decreases the steady-state  $\text{H}_2$  concentrations for all column experiments in this study to within the range reported for iron-reducing conditions in the field. Model analysis using biokinetic coefficients obtained from more ideal growth conditions (soluble Fe(III), 30 °C, trace vitamins and minerals addition) also predicted higher steady-state  $\text{H}_2$  values compared to field data under iron reducing conditions (Brown et al., in review).

The use of  $\text{H}_2$  as an indicator of TEAPs assumes that the availability of the electron donor(s) is limiting. This is the case when  $\text{H}_2$  is the sole electron donor (Lovley & Goodwin 1988) and when an additional carbon source is present at relatively low concentration (Brown et al. in review). Electron donor limiting conditions would result in competition between different TEAPs with the more energetically favorable TEAP having an advantage over less energetically favorable TEAPs. However, research has shown that when the electron donor becomes less limiting, more

than one TEAP can occur simultaneously (Vrobletsky et al. 1996). This is a possible explanation as to why both iron and sulfate reduction occurred simultaneously in the long-term column experiment. Sulfate concentrations did decrease modestly, but statistically, along the length of the column suggesting that a small part of the electron flow went to sulfate, which would be expected if the electron donor limitation was not severe. The assumption that the electron donor was not limiting appears valid because only a small amount (0.5 mM) of the 3 mM influent acetate was consumed throughout the column.

Steady-state  $H_2$  concentrations were similar between the long-term (500 day) column experiment utilizing only the indigenous iron-reducing populations and the short-term column experiment that was supplemented with *Geobacter sulfurreducens*, though the column supplied *G. sulfurreducens* reached steady-state conditions significantly faster. In addition, more Fe(II) was produced in columns with *G. sulfurreducens* when destructively sampled after 96 days compared to the Fe(II) concentration in the long-term column without *G. sulfurreducens* at the same time point. Though the column inoculated with *G. sulfurreducens* (but not supplied AQDS) (Figure 5) reached steady-state conditions faster than the column not supplied *G. sulfurreducens* (Figure 3), the rate of iron reduction calculated in the column with *G. sulfurreducens* ( $0.16 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$ ) was similar to the rate of iron reduction calculated for the column without *G. sulfurreducens* ( $0.17 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$ ). The addition of AQDS increased the rate of iron reduction from  $0.16 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$  to  $0.35 \mu\text{mol (g soil)}^{-1} \text{ day}^{-1}$  and decreased the steady-state  $H_2$  levels from 4.0 to 2.0 nM.  $H_2$  concentrations have been shown to be solely dependent upon the physiological parameters of the hydrogen-consuming microorganisms (Lovley & Goodwin 1988) with the steady-state  $H_2$  concentration inversely proportional to the rate of  $H_2$  consumption. Accordingly, a doubling of the iron reduction rate through the addition of AQDS decreased the steady-state  $H_2$  concentration by half. The long-term column experiment showed that a steady-state  $H_2$  level was achieved when the Fe(II) production rate was constant, indicating that, as expected, pseudo-steady state conditions had been reached. The results of this study have shown that although steady-state  $H_2$  levels during iron reduction are not

unique and depend on factors such as the iron bioavailability, they remain relatively constant for particular conditions over extended time periods, and may be used to monitor changes in iron reduction rates. It should be cautioned that though average  $H_2$  values compared well for different rates of iron reduction,  $H_2$  levels fluctuated within a substantial range over time as measured in these small columns allowing only small sample sizes and low flow rates.

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