Zero Valent Iron as an Electron-Donor for Methanogenesis and Sulfate Reduction in Anaerobic Sludge

Srilakshmi Karri, Reyes Sierra-Alvarez, Jim A. Field

Department of Chemical and Environmental Engineering, University of Arizona, P.O. Box 210011, Tucson, Arizona 85721-0011; telephone 520-626-5858; fax. 520-621-6048; e-mail: jimfield@email.arizona.edu

Received 15 April 2005; accepted 16 June 2005

Published online 31 August 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20623

Abstract: Zero valent iron (ZVI) is a reactive media commonly utilized in permeable reactive barriers (PRBs). Sulfate reducing bacteria are being considered for the immobilization of heavy metals in PRBs. The purpose of this study was to evaluate the potential of ZVI as an electron donor for sulfate reduction in natural mixed anaerobic cultures. The ability of methanogens to utilize ZVI as an electron-donor was also explored since these microorganisms often compete with sulfate reducers for common substrates. Four grades of ZVI of different particle sizes (1.120, 0.149, 0.044, and 0.010 mm diameter) were compared as electron donor in batch bioassays inoculated with anaerobic bioreactor sludge. Methanogenesis was evaluated in mineral media lacking sulfate. Sulfate reduction was evaluated in mineral media containing sulfate and the specific methanogenic inhibitor, 2-bromoethane sulfonate. ZVI contributed to significant increases in methane production and sulfate reduction-compared to endogenous substrate controls. The rates of methane formation or sulfate reduction were positively correlated with the surface area of ZVI. The highest rates of 0.310 mmol CH_4 formed/mol Fe^0·day and 0.804 mmol SO_4^{2-} reduced/ mol Fe^0·day were obtained with the finest grade of ZVI (0.01 mm). The results demonstrate that ZVI is readily utilized as a slow-release electron donor for methanogenesis and sulfate reduction in anaerobic sludge; and therefore, has a promising potential in bioremediation applications.
Keywords: anaerobic; metallic iron; Fe^0; elemental iron; elemental metal; bioremediation; permeable reactive barriers

INTRODUCTION

Permeable reactive barriers (PRB) are a relatively simple remediation technology in which a zone of semi permeable reactive media is placed in the flow path of a contaminant plume. As the plume moves through the media, the contaminants are either immobilized or transformed to nontoxic products through physical, chemical, or microbiological reactions or any combination thereof (Richardson and Nicklow, 2002; Scherer et al., 2000; USEPA, 1998). Zero valent iron (ZVI) is the most commonly used reactive media utilized in PRBs. A recent survey has shown the 45% of full scale PRBs utilize ZVI for the reactive media (Scherer et al., 2000). ZVI can catalyze the chemical conversion of a variety of pollutants such as chlorinated aliphatic, chlorinated aromatics, nitroaromatics, nitrates, and redox sensitive high valency toxic metals (e.g., Cr^{6+}) or radionuclides (e.g., U^{6+}) (Bigg and Judd, 2000; USEPA, 1998). In addition to chemical reactions, ZVI can also serve as an electron donor to support the reductive conversion of contaminants by microorganisms (Scherer et al., 2000). The best documented examples are microbial mediated dehalogenation of chlorinated aliphatics with the combined use of ZVI and anaerobic microorganisms (Novak et al., 1998; Parkin et al., 1998; Rosenthal et al., 2004; Weathers et al., 1997). Anaerobic mixed cultures were shown to improve the mineralization of the explosive, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in ZVI columns (Oh and Alvarez, 2002; Oh et al., 2001). Additionally, autotrophic denitrification is feasible utilizing ZVI as the electron donor (Kielemoes et al., 2000; Till et al., 1998).

The most accepted mechanism of electron transfer from ZVI to microorganisms is via cathodic hydrogen (Daniels et al., 1987; Till et al., 1998). Under anaerobic conditions cathodic H_2 is generated by the chemical reaction of H_2O with Fe^0 as indicated below in Eq. 1 (Liang et al., 2000):

$$Fe^0 + 2H_2O = Fe^{2+} + H_2 + 2OH^- \quad (1)$$

Field studies have revealed that the microbial density in the ZVI zone of PRBs are from 1 to 3 orders of magnitudes higher in comparison with background soil/groundwater samples (Gu et al., 2002). The presence of microorganisms in the reducing zone of the PRB contributes to the corrosion of ZVI. Microbial mediated corrosion results in the deterioration of the reactive media as well the formation of iron precipitates affecting the performance of ZVI (Furukawa et al., 2002; Liang et al., 2003; Phillips et al., 2003b). The major precipitates observed in reactive iron barriers include

Correspondence to: Jim A. Field

iron oxides, carbonates, iron sulfides, and elemental sulfur. The type and amount of these precipitates depends on the degree of iron oxidation, groundwater chemistry, and microbial activity (Roh et al., 2000). Corrosion and mineral precipitation alter surface composition of ZVI and cause cementation of ZVI particles. Pilot studies have demonstrated that such compositional changes may decrease the reactivity of the iron, and mineral precipitation may decrease flow through the barrier due to loss in porosity and eventually clogging (Gu et al., 1999; Liang et al., 2000; Wilkin et al., 2003).

PRB are presently being developed for the treatment of acid mine drainage (AMD) contaminated plumes (Benner et al., 1997; Ludwig et al., 2002; Waybrant et al., 2002). AMD is formed from the accelerated weathering of metal-sulfide rich rocks exposed to oxygen due to mining activities. The resulting acidic plumes contain sulfuric acid, dissolved iron and various toxic heavy metals (Benner et al., 2000; Fields, 2003). Sulfate reducing bacteria can be utilized to immobilize heavy metals (Gadd, 2001; Jong and Parry, 2003; Waybrant et al., 2002; White et al., 1997). Sulfides have low solubility products with heavy metals, favoring precipitation of metal sulfides. The reduction of sulfate to sulfide generates alkalinity, which is beneficial in correcting the pH of AMD (Benner et al., 1997; Waybrant et al., 2002). Sulfate reduction requires an electron donating substrate. The PRB applied to AMD have so far only considered organic substrates as electron donors (Benner et al., 1997; Ludwig et al., 2002; Waybrant et al., 2002). Potentially ZVI could be used as an inexpensive electron donating media in the PRB for sulfate reducing bacteria. However, this possibility has not yet been fully examined for heavy metal treatment.

Pure cultures of sulfate reducing bacteria reducing bacteria such as *Desulfovibrio* strains and *Desulfobacterium* sp are known grow at the expense of utilizing ZVI as the sole electron donor (Dinh et al., 2004; Rajagopal and Legall, 1989). In two studies, ZVI columns were operated for long periods of time with sulfate in the feed. In one of these studies, evidence for sulfate reduction was observed (Köber et al., 2002). In the other study, enrichment of sulfate reducers was observed (Gu et al., 1999). Bioreactor biofilms operated under sulfate reducing conditions contain methanogens, which compete for electron donor (Omil et al., 1998; Raskin et al., 1996; Weijma et al., 2000). Methanogens are also known to utilize ZVI as the sole electron donor (Belay and Daniels, 1990; Daniels et al., 1987; Dinh et al., 2004; Lorowitz et al., 1992; Rajagopal and Legall, 1989) and would thus likely compete for electron donor in PRBs packed with ZVI.

The objective of this study was to evaluate various grades of ZVI as an electron donor to sulfate reduction in an anaerobic mixed culture obtained from a sulfate reducing bioreactor. The study is the first step in the evaluation of ZVI as an electron donor for a sulfate reducing based bioremediation of AMD in PRBs. Due to the anticipated competition with methanogens, the potential of ZVI as an electron donor for methane formation was also investigated.

MATERIALS AND METHODS

Microorganisms

A sulfate reducing anaerobic granular sludge was obtained from a full-scale upward sludge blanket reactor treating rayon fiber manufacturing wastewater (Twaron, Twente, The Netherlands). The sludge was washed and sieved to remove fine particles before using in the tests. The content of volatile suspended solids (VSS) in the Twaron sludge was 5.96% of the wet weight. The microbial cultures were stored under nitrogen gas at 4°C.

Media for Bioassays

The anaerobic basal mineral medium (pH 7.2) used in methanogenic bioassays (ABM-1) contained (in mg/L): NH_4Cl (280); $NaHCO_3$ (5,000); K_2HPO_4 (250); $CaCl_2 \cdot 2\ H_2O$ (10), $MgCl_2 \cdot 6\ H_2O$ (183), yeast extract (100), and 1 mL of trace element solution.

The basal medium (pH 7.2) utilized in the sulfate reducing bioassays (ABM-2) consisted of (in mg/L): NH_4Cl (280); $NaHCO_3$ (5,000); K_2HPO_4 (600); $NaH_2PO_4 \cdot 2\ H_2O$ (796), $CaCl_2 \cdot 2\ H_2O$ (10), $MgCl_2 \cdot 6\ H_2O$ (100), Na_2SO_4 (2,960); the specific methanogenic inhibitior 2-bromoethane sulfonate (BES) (6,330), yeast extract (20), and 1 mL of trace element solution.

The trace element solution contained (in mg/L): H_3BO_3 (50), $FeCl_2 \cdot 4\ H_2O$ (2,000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (50), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6\ H_2O$ (90), $CoCl_2 \cdot 6\ H_2O$ (2,000), $NiCl_2 \cdot 6\ H_2O$ (50), $CuCl_2 \cdot 2\ H_2O$ (30), $NaSeO_3 \cdot 5\ H_2O$ (100), EDTA (1,000), Resazurin (200), and 36% HCl (1 mL).

Different grades of ZVI were utilized in the bioassays as electron donors to test the slow release electron donating capacity. The various types of ZVI utilized were: <10 micron (0.010 mm diameter), 325 mesh (0.044 mm particle diameter), 100 mesh (0.149 mm particle diameter) and an industrial sample of sieve size $-8+50$ mesh (average particle diameter of 1.129 mm). Initial experiments of sulfate reduction and methanogenesis were conducted with a final assay concentration of 46.6 g/L of 325 mesh ZVI. Additional assays were later conducted to analyze the effect of particle diameter on the rate of electron releasing capability of ZVI for both sulfate reduction and methanogenesis. A final assay concentration of 18.64 g ZVI/L was utilized for these tests. Hydrogen was used as the electron donor in positive controls and was supplied as H_2/CO_2 gas (80/20, v/v) at 1.5 atm.

Various controls (uninoculated controls, no-substrate controls, positive controls with H_2 as electron-donor) were included, for all the experiments. All flasks were sealed with butyl rubber stoppers and aluminum crimp seals. All assays were conducted in triplicates. All bioassays were incubated in a climate-controlled chamber at 30 ± 2°C in an orbital shaker (75 rpm). Sulfate was added to the sulfate reducing bacteria (SRB) assays from sterile stocks to give final concentrations of 2,000 mg/L.

Methanogenic Test With 325 Mesh ZVI

Methanogenic activity measurements were conducted in 165 mL serum flasks. The anaerobic sludge (final assay concentration of 1.5 g VSS/L) was transferred to serum flasks with 80 mL of basal medium ABM-1. For serum flasks utilizing hydrogen as the electron-donor 25 mL of basal medium ABM-1 was used. ZVI was added at a final assay concentration of 46.6 g/L. The medium and the headspace were flushed with N_2/CO_2 gas (80:20, v/v) to exclude oxygen and bottles were sealed with butyl rubber septa. For assays utilizing H_2, serum flasks were first flushed with N_2/CO_2 and then pressurized with H_2/CO_2 (80/20, v/v, 1.5 atm) for 3 min. The flasks were incubated overnight at $30 \pm 2^{\circ}C$ to adapt the sludge to the medium conditions. On the following day, the flasks containing H_2, were reflushed with N_2/CO_2 and then pressurized with H_2/CO_2 (80/20, v/v, 1.5 atm) for 3 min. The methane content in the headspace was determined at regular intervals until the end of the experiment of 13.2 days duration. Samples were taken at the beginning and the end of the experiment for analyzing total and soluble iron and pH in the serum flasks containing ZVI.

Methanogenic Test With Different Grades of ZVI

Batch bioassays were conducted in 165 mL serum flasks, to test the effect of particle diameter on the rate of production of methane. Four different grades of ZVI were utilized for this test with an experimental set up similar to that described above. Anaerobic sludge (final assay concentration of 3 g VSS/L) was transferred to serum flasks with 28 mL basal medium ABM-1. Only 25 mL of basal medium ABM-1 was used for the positive controls with H_2 as electron-donor and the no substrate controls. ZVI was added at a final assay concentration of 18.6 g/L. The flasks were incubated overnight at $30 \pm 2^{\circ}C$ to adapt the sludge to the medium conditions. On the following day, the flasks containing H_2, were reflushed with N_2/CO_2 and then pressurized with H_2/CO_2 (80/20, v/v, 1.5 atm), while all the other flasks where flushed with N_2/CO_2 for 3 min. All the flasks were incubated for 2 h. From thereon, methane, total iron, and soluble iron were monitored periodically for the subsequent 75 days. The controls containing H_2 as an electron donor were reflushed after 355 and 736 h, respectively, for 3 min (80/20, v/v, 1.5 atm), after flushing first with N_2/CO_2. At the same time periods, all the other flasks were reflushed with N_2/CO_2 to avoid build up of methane.

Sulfate Reduction Test With 325 Mesh ZVI

Sulfate reduction measurements were performed in 335 mL serum flasks. Anaerobic sludge (final assay concentration of 1.5 g VSS/L) was transferred to serum flasks containing 250 mL of basal medium ABM-2. In flasks containing H_2 as the electron-donor, 100 mL of basal medium was utilized instead. ZVI was added at a final assay concentration of 46.6 g/L. The medium and the headspace were flushed with N_2/CO_2 gas (80:20, v/v) to exclude oxygen and bottles were sealed with butyl rubber septa. Flasks containing H_2 as electron donor were first flushed with N_2/CO_2 and then pressurized with H_2/CO_2 (80/20, v/v, 1.5 atm) for 3 min. The flasks were incubated overnight at $30 \pm 2^{\circ}C$ to adapt the sludge to the medium conditions. On the following day, the flasks containing H_2, were reflushed with N_2/CO_2 and then pressurized with H_2/CO_2 (80/20, v/v, 1.5 atm) for 3 min. Sulfate and sulfide were monitored over the course of the experiment of 109 day duration. The controls containing H_2 as an electron donor were reflushed after 1,744 h for 3 min (80/20, v/v, 1.5 atm), after flushing first with N_2/CO_2. Samples for total and soluble iron were taken at the start and the end of the experiment. Samples for soluble iron were membrane filtered (0.45 μm).

Sulfate Reduction Test With Different Grades ZVI

Another experiment was performed in 335 mL serum flasks, to test the effect of particle diameter on the rate of sulfate reducing activity. Four different ZVI grades were used with a set up similar to that described above. The anaerobic twaron sludge (final assay concentration of 1.5 g VSS/L) was transferred to serum flasks with 200 mL basal medium ABM-2. ZVI was added at a final assay concentration of 18.64 g/L. All the serum flasks were incubated for 19 h in a $30^{\circ}C$ room. Sample analysis for sulfate, sulfide, total and soluble iron were measured periodically for the subsequent 80 days. The controls containing H_2 as an electron donor were reflushed after 902 h for 3 min (80/20, v/v, 1.5 atm), after flushing first with N_2/CO_2.

Analytical Methods

The methane content in the headspace of the serum flasks was determined by gas chromatography using an HP5290 Series II system (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector (GC-FID). The GC was fitted with a Nukol fused silica capillary column (30 m length $\times$ 0.53 mm ID, Supelco, St. Louis, MO). The temperature of the column, the injector port and the detector was 140, 180, and $275^{\circ}C$, respectively. The carrier gas was helium at a flow rate of 9.3 mL/min and a split flow of 32.4 mL/min. Samples for measuring methane content (100 μL) in the headspace were collected using a pressure-lock gas syringe. Sulfide was analyzed colorimetrically by the methylene blue method (Trüper, 1964). Sulfate was determined by ion chromatography with suppressed conductivity using a DIONEX system equipped with a Dionex AS11-HC4 column (Dionex, Sunnydale, CA) and a conductivity detector. The mobile phase was 15 mM KOH at a flow rate of 1.2 mL/min. The column temperature was maintained at room temperature. The injection volume was 25 μL. The pH was determined immediately with an Orion model

310 PerpHecT pH-meter with a PerpHecT ROSS glass combination electrode. Other parameter like volatile suspended solids, total iron, and soluble iron were determined according to Standard Methods for Examination of Water and Wastewater (APHA, 1998).

Chemicals

Iron powder, (−325 mesh; 97%; CAS 7439-89-6) and Iron powder, (<10 micron, CAS 7439-89-6, 99.9 + %) was obtained from Sigma Aldrich (St. Louis, MO); Iron powder (100 mesh; CAS 7439-89-6; 99.9%) was obtained from Mallinckrodt (Hazelwood, MO); Industrial Iron Sample (−8 + 50 mesh; 98%) was obtained from Conelly GPM, Inc., 3154 South California Ave, Chicago. Specialty gases N_2/CO_2 and H_2/CO_2 (80/20, v/v) were delivered from US Air weld (Phoenix, AZ) Sodium sulfate anhydrous (Na_2SO_4; 99%; CAS 7757-82-6) was obtained from Sigma Aldrich.

RESULTS

ZVI as an Electron Donor for Methanogenesis

ZVI was first tested as an electron donating substrate to support methanogenesis in anaerobic sludge. ZVI of 325 mesh was supplied at 46.6 g/L to an anaerobic culture containing 1.5 g VSS/L of sludge and incubated for approximately 2 weeks. Figure 1 illustrates the methane production in an uninoculated control; a control containing sludge but no ZVI and the complete treatment, containing ZVI and sludge. No methane production occurred in the uninoculated control. The methane production in the complete treatment was significantly greater than the sludge control, indicating the use of ZVI as an electron donor for methanogenesis. Table I provides data on the final cumulative methane production and methane production rates. At the end of the experiment, the net methane production attributed to the presence of ZVI accounted for only 0.7% of the electron equivalents supplied with 46.6 g/L of ZVI. The rate of methane production with ZVI was 9.4-fold lower than a positive control supplied with 1.5 atm of H_2/CO_2 (80:20 v:v). The lower rate indicates a lower bioavailability of ZVI compared to freely available H_2 as an electron donor.

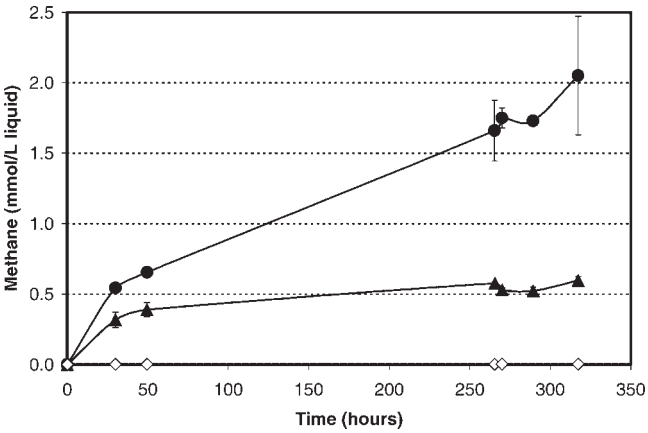

Figure 1. The production of methane with 46.6 g/L of ZVI (325 mesh) by 1.5 g VSS/L of anaerobic sludge. Legend: closed bullets, complete treatment with sludge and ZVI; closed triangles, endogenous sludge control; and open diamonds, uninoculated ZVI.

A longer term experiment was set up evaluating four different grades of ZVI, differing in particle size. Each ZVI was tested at 18.6 g/L as an electron donor for methane production with 3 g VSS/L of anaerobic sludge over a period of approximately 7 weeks. Figure 2A illustrates the time course of methane production in inoculated treatments. The three finest grades of ZVI (10 μm, 325 mesh and 100 mesh) permitted a significantly higher production of methane compared to the endogenous substrate control (sludge only). The result confirms that ZVI is utilized as an electron donor for methane production. The extent of the methane production as well as the methane production rate was inversely related to the particle size of the ZVI (Fig. 2A and Table II). The methane production obtained with finest ZVI (10 μm) far exceeded the methane production of the other treatments. Likewise, the next finest ZVI (325 mesh) provided a much higher methane production compared to 100 mesh. The methane production obtained with coarsest ZVI (industrial) was not significantly higher compared to the endogenous substrate control, probably due to limited surface area. Table II provides a summary of the net methane production at the end of the experiment. The values obtained correspond to 0.21, 0.94, 2.68, and 6.35% of the electron equivalents supplied with 18.6 g/L industrial, 100 mesh, 325 mesh and 10 μm ZVI, respectively. As in the previous experiment, H_2 was supplied to a positive control. The net rate of methane production in the positive control was 25-fold faster than that obtained with 10 μm ZVI. The result again illustrates limited bioavailability of ZVI compared to freely available H_2.

In the uninoculated controls, methane production was absent for the first 300 h (Fig. 2B). However, after that time significant methane production occurred in treatments with some of the ZVI samples. The production was most noteworthy with 325 mesh and 100 mesh ZVI. The occurrence of methane production in these treatments suggests growth of methanogens during the experiment. The uninoculated controls were not sterilized, thus growth of methanogens would have been feasible. The long lag phase prior to observing methane production is consistent with growth from a low-level of contamination of methanogens initially present in uninoculated cultures.

ZVI as an Electron Donor for Sulfate Reduction

ZVI was also tested as an electron donating substrate for sulfate in the anaerobic sludge. ZVI of 325 mesh was supplied at 46.6 g/L to the anaerobic culture containing 1.5 g VSS/L of sludge and incubated for approximately 15.5 weeks. Figure 3 illustrates the time-course of the sulfate concentration in an uninoculated control; a control containing sludge but no ZVI and the complete treatment, containing ZVI and sludge. Some sulfate was eliminated slowly from the two

Table I. Cumulative methane production and rate of methane formation in the experiment evaluating methanogenesis with 46.6 g/L of 325 mesh ZVI by 1.5 g VSS/L anaerobic sludge.

	Cumulative methane production[a]			Rate of methane formation[b]		
	Total		Net[c]	Total	Net[c]	Net rate/mol
	mmol CH_4/L_{liq}			mmol CH_4/L_{liq}·day		mmol CH_4/mol Fe^0·day
Treatment	Average	SD[d]	Average	Average	Average	Average
Sludge only	0.597	0.028		0.020		NA[d]
Sludge + ZVI	2.051	0.421	1.454	0.118	0.098	0.118
Sludge + H_2	9.277	1.199	8.681	0.945	0.925	NA

[a]Methane production at end of experiment 317 h for ZVI; and 266 h for H_2.
[b]Rates for ZVI between 30 and 317 h; rates for H_2 from 1 to 295 h; the rate in an uninoculated ZVI amended control was 0.000132 mmol CH_4/L_{liq}·day.
[c]Corrected for endogenous methane production or formation rate in sludge only control.
[d]SD, standard deviation; NA, not applicable.

controls; however the loss in sulfate concentration was distinctly greater and more rapid in the complete treatment. The results clearly indicate that ZVI was utilized by sulfate reducing bacteria. Table III provides data on the sulfate eliminated and the sulfate reduction rates. At the end of the experiment, the complete treatment removed all of the sulfate supplied to the medium. Based on the net removal of sulfate (corrected for endogenous sulfate reduction), the ZVI contributed to 72.5% of the sulfate reduced, which was equivalent to 7.56% of the electron equivalents supplied with 46.6 g/L of ZVI. The rate of sulfate removal in the complete treatment was 2.9 to 3.5-fold faster than in the controls. The occurrence of sulfate reduction in the uninoculated ZVI control would suggest some growth of sulfate reducers after prolonged incubations.

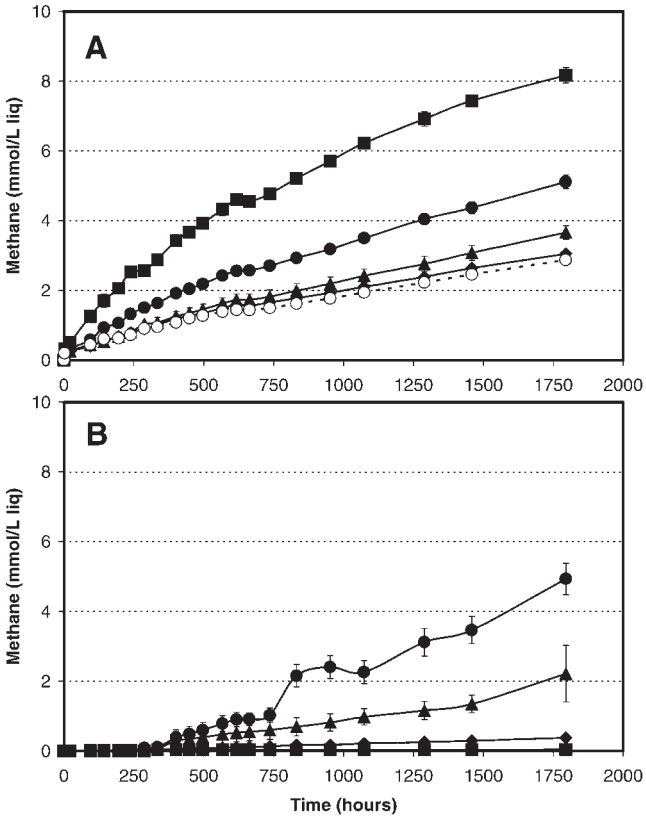

Figure 2. The production of methane with 18.6 g/L of ZVI of different particle sizes. **A**: Treatments inoculated with 3 g VSS/L of anaerobic sludge. **B**: uninoculated controls. Legend: closed sqaures, ZVI of 0.01 mm; closed bullets, ZVI 325 mesh (0.044 mm); closed triangles, ZVI of 100 mesh (0.149 mm); closed diamonds, industrial ZVI (1.12 mm); open bullets, endogenous substrate control.

A second sulfate reducing experiment was set up evaluating the four different grades of ZVI as an electron donors, each supplied at 18.6 g/L together with 1.5 g VSS/L of anaerobic sludge. The experiment was incubated for approximately 11 weeks. Figure 4A illustrates the time-course of sulfate concentration in the treatments inoculated with anaerobic sludge. In this experiment all grades of ZVI eventually permitted a significantly higher removal of sulfate compared to the removal achieved with endogenous substrate control. The result confirms that ZVI can be utilized as an electron donor for sulfate reduction. The extent of the sulfate removal as well as the sulfate reduction rate was inversely related to the particle size of the ZVI (Fig. 3A and Table IV). As was the case in the methanogenic experiment, the sulfate removal in the treatment containing the finest ZVI (10 μm) greatly outperformed the other treatments. The next highest removal of sulfate was obtained in the treatments containing either 325 mesh ZVI and 100 mesh ZVI, both of which performed similarly in terms of total sulfate removed at the end of the experiment. However, rates of sulfate removal were higher in the 325 mesh ZVI treatment compared to the 100 mesh ZVI treatment (Table IV), since the sulfate concentrations at the beginning of the period considered for evaluating the rates were higher in the former. The treatment with the coarsest ZVI (industrial) provided the lowest net sulfate removal of all the ZVI treatments as would be expected due to its low specific surface area. Table IV provides a summary of the net sulfate removal at the end of the experiment. The treatment with the finest ZVI (10 μm) had removed almost all of the sulfate supplied (96.4%). In terms of electron equivalence,

Table II. Cumulative methane production and rate of methane formation in the experiment evaluating methanogenesis with 18.6 g/L of ZVI of different particle sizes by 3.0 g VSS/L anaerobic sludge.

		Cumulative methane production[a]			Rate methane formation[b]		
		Total		Net[c]	Total	Net[c]	Net rate/mol
		mmol CH_4/ L_{liq}			mmol CH_4/ L_{liq}·day		
Treatment	Particle diameter (mm)	Average	SD[d]	Average	Average	Average	mmol CH_4/ mol Fe^0·day
Sludge Only		2.87	0.05	NA	0.0465	NA	NA[d]
ZVI Industrial + Sludge	1.120	3.05	0.09	0.17	0.0543	0.0078	0.0232
ZVI 100 mesh + Sludge	0.149	3.66	0.20	0.79	0.0601	0.0136	0.0406
ZVI 325 mesh + Sludge	0.044	5.11	0.19	2.24	0.0879	0.0414	0.1241
ZVI 10 μm + Sludge	0.010	8.17	0.22	5.30	0.1499	0.1034	0.3098
H_2 + Sludge		14.54	1.22	11.67	2.6349	2.5884	NA

[a]Methane production at end of experiment; 1794 h for ZVI; and 335 h for H_2.
[b]Rates for ZVI between 94.3 and 616.7h; for H_2 between 6 and 143 h; rates in uninoculated ZVI amended controls measured from 0 to 335 h, ranged from 0.0012 to 0.0060 mmol CH_4/L_{liq}·day.
[c]Corrected for endogenous methane production or formation rate in sludge only control.
[d]SD, standard deviation; NA, not applicable.

the net sulfate removal obtained at the end of the experiment corresponded to 19.0, 28.4, 25.4 and 83.4% of the sulfate supplied and 4.7, 7.1, 6.3, and 20.5% of the iron supplied for industrial, 100 mesh, 325 mesh and 10 μm grades of ZVI, respectively. H_2 was supplied to a positive control. The net rate of sulfate removal in the positive control was 1.4- and 2.7-fold faster than the net rate obtained with 10 μm ZVI and 325 mesh ZVI, respectively (Table IV). The relative difference in rates between H_2 and ZVI are noticeably smaller in the case of sulfate reduction compared to methanogenesis. The sulfate reduction rates on H_2 were lower than the rates of methanogenesis on H_2.

In the uninoculated controls, a slow but significant trend in sulfate reduction was observed in the period after 739 h (Fig. 4B), due to contaminating sulfate reducing bacteria as explained previously. The sulfate removal was greatest in the treatment with with the finest ZVI.

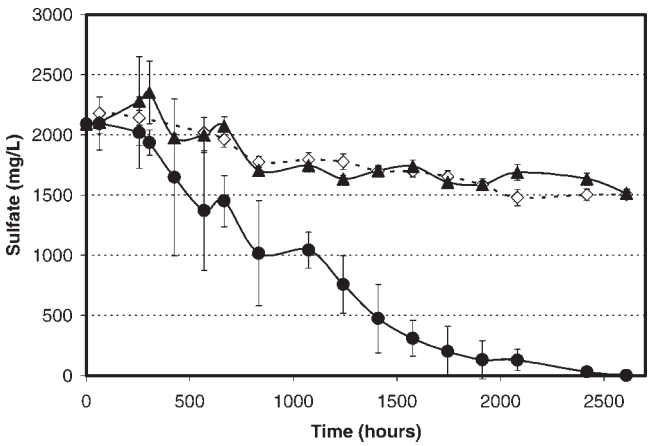

Figure 3. The time course of the sulfate concentration with 46.6 g/L of ZVI (325 mesh) and 1.5 g VSS/L of anaerobic sludge. Legend: closed bullets, complete treatment with sludge and ZVI; closed triangles, endogenous sludge control; and open diamonds, uninoculated ZVI.

DISCUSSION

ZVI as Electron Donor of Methanogenesis and Sulfate Reduction

The results of this study indicate that ZVI is utilized as an electron for methanogenesis and sulfate reduction by natural mixed cultures present in anaerobic sludge. The findings are consistent with previous observation that selected pure cultures of methanogens and sulfate reducing bacteria utilize cathodic H_2 from iron corrosion as an electron donor. The conversion of ZVI and CO_2 to methane has been observed with the following species of hydrogenotrophic methanogens: *Methanococcus thermolithotrophicus* (Belay and Daniels, 1990; Daniels et al., 1987), *Methanococcus deltae* (Belay and Daniels, 1990), *Methanococcus maripaludis* (Dinh et al., 2004), *Methanobacterium thermoautotrophicum* (Belay and Daniels 1990; Daniels et al., 1987; Deckena and Blotevogel, 1990; Lorowitz et al., 1992), *Methanosarcina barkeri* (Belay and Daniels, 1990; Daniels et al., 1987), *Methanobacterium bryantii* (Daniels et al., 1987) and *Methanospirillium hungatei* (Rajagopal and Legall, 1989) (Daniels et al., 1987). Growth of *M. thermolithotrophicus* and *M. barkeri* linked to the use of ZVI as the electron donor was demonstrated by increases in cell counts and protein concentration (Belay and Daniels, 1990; Daniels et al., 1987; Lorowitz et al., 1992). Reduction of sulfate by ZVI has been reported for the following sulfate reducing bacteria: *Desulfovibrio desulfuricans* (Deckena and Blotevogel, 1990; Rajagopal and Legall, 1989), *Desulfovibrio vulgaris* (Rajagopal and Legall, 1989), *Desulfovibrio multispirans* (Rajagopal and Legall, 1989), *Desulfovibrio salexigens* (Dinh et al., 2004), and *Desulfobacterium* sp. strain IS4 (Dinh et al., 2004). Evidence of growth linked to the use of ZVI for sulfate reduction has been demonstrated for *Desulfovibrio desulfuricans*, based on increases in optical density and protein content (Deckena and Blotevogel, 1990; Rajagopal and Legall, 1989).

Table III. Elimination of sulfate and rate of sulfate removal in the experiment evaluating sulfate reduction with 46.6 g/L of 325 mesh ZVI by 1.5 g VSS/L anaerobic sludge.

	Sulfate removed[a]			Rate of sulfate removal[b]		
	Total		Net[c]	Total	Net[c]	Net rate/mol
	mM SO_4^{2-}				mM SO_4^{2-}/day	mmol SO_4^{2-}/mol Fe^0·day
Treatment	Average	SD[d]	Average	Average	Average	Average
ZVI only	6.09	0.43		0.086	NA[d]	NA
Sludge only	5.99	0.35		0.104	NA	NA
Sludge + ZVI	21.8	0.06	15.8	0.302	0.198	0.2371

[a]Sulfate removed after 2609 h (the initial SO_4^{2-} concentration was 21.8 mM).
[b]Rates between 63 and 1912 h except for the rate of the "ZVI only" treatment which was determined between 63 and 1576 h.
[c]Corrected for endogenous sulfate removal or removal rate in sludge only control.
[d]SD, standard deviation; NA, not applicable.

In addition to methanogenesis and sulfate reduction, *Desulfovibrio desulfuricans* (Rajagopal and Legall, 1989) catalyzed dissimilatory reduction of nitrate to ammonia (DNRA) and *Paracoccus denitrificans* (Till et al., 1998), catalyzed denitrification (to N_2), utilizing ZVI as the electron donor. Evidence is also available, indicating that acetogenesis by Acetobacterium woodii can convert CO_2 to acetate with ZVI as electron donor (Rajagopal and Legall, 1989).

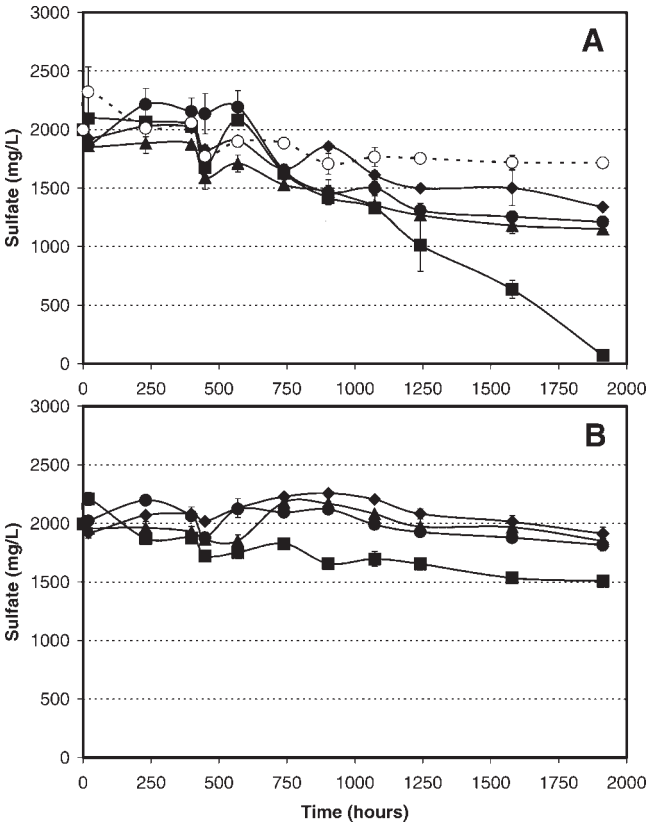

Figure 4. The time course of the sulfate concentration with 18.6 g/L of ZVI of different particle sizes. **A**: Treatments inoculated with 1.5 g VSS/L of anaerobic sludge. **B**: uninoculated controls. Legend: closed squares, ZVI of 0.01 mm; closed bullets, ZVI 325 mesh (0.044 mm); closed triangles, ZVI of 100 mesh (0.149 mm); closed diamonds, industrial ZVI (1.12 mm); open bullets, endogenous substrate control.

The formation of cathodic hydrogen from the chemical corrosion of iron under anaerobic conditions (Eq. 1) is based on two half reactions which have reduction potentials that are close in values, H^+/H_2 ($E'^\circ = -0.414$ V) and Fe^{2+}/Fe^0 ($E'^\circ = -0.44$ V) (Dinh et al., 2004). Therefore the thermodynamic favorability of cathodic H_2 formation is low with a $\Delta G^{\circ\prime}$ of only -5.02 kJ/mol Fe^0. The build-up of H_2 would eventually make the reaction unfavorable. However, cathodic H_2 formation could continue indefinitely if coupled with an exorgenic biological reaction such as methanogenesis (CO_2/CH_4 $E'^\circ = -0.245$ V) or sulfate reduction (SO_4^{2-}/S^{2-} $E'^\circ = -0.217$ V) (Madigen et al., 2003), as shown in Eqs. 2 and 3below:

$$8H^+ + 4Fe^0 + CO_2 \rightarrow CH_4 + 4Fe^{2+} + 2H_2O \qquad \Delta G^{\circ\prime} = -150.5 \text{ kJ/mol } CH_4 \tag{2}$$

$$8H^+ + 4Fe^0 + SO_4^{2-} \rightarrow S^{2-} + 4Fe^{2+} + 2H_2O \qquad \Delta G^{\circ\prime} = -172.2 \text{ kJ/mol } SO_4^{2-} \tag{3}$$

The coupled reactions would effectively maintain a low steady state H_2 concentration, ensuring the feasibility of Eq. 1. The rate and amount of cathodic hydrogen equivalents formed was shown to be greatly increased when comparing chemical corrosion with corrosion assisted by sulfate reducing bacteria (Daniels et al., 1987) or methanogens (Lorowitz et al., 1992). Likewise inclusion of high concentrations of hydrogen in the headspace would be expected to effectively halt cathodic hydrogen formation. In agreement with this expectation, the rate of methanogenesis with H_2/CO_2 was not increased with Fe° additions, even though Fe^0 added alone supported significant methane production (Daniels et al., 1987).

There is compelling evidence for the involvement of cathodic hydrogen in the biological reactions utilizing ZVI. Several studies have utilized a "two-bottle" set up, in which

Table IV. Elimination of sulfate and rate of sulfate removal in the experiment evaluating sulfate reduction with 18.6 g/L of ZVI of different particle sizes by 1.5 g VSS/L anaerobic sludge.[a]

		Sulfate removed[b]			Rate of sulfate removal[b]		
		Total		Net[c]	Total	Net[c]	Net rate/mol
		mM SO_4^{2-}			mM SO_4^{2-}/day		
Treatment	Particle diameter (mm)	Average	SD[d]	Average	Average	Average	mmol SO_4^{2-}/mol Fe^0·day
Sludge Only		2.92	0.27	NA[d]	0.0381	NA	NA
ZVI Industrial + Sludge	1.120	6.87	0.31	3.95	0.0991	0.0610	0.1827
ZVI 100 mesh + Sludge	0.149	8.83	0.30	5.91	0.1097	0.0716	0.2144
ZVI 325 mesh + Sludge	0.044	8.20	0.20	5.28	0.1749	0.1368	0.4097
ZVI 10 μm + Sludge	0.010	20.05	0.45	17.13	0.3065	0.2685	0.8038
H_2 + Sludge		15.73	0.53	12.81	0.4131	0.3750	NA

[a]Sulfate removed after 1913 h (the initial SO_4^{2-} concentration was 20.8 mM).
[b]Rates for ZVI between 398 and 1912; for H_2 between 398 and 1092; rates in uninoculated ZVI amended controls measured from 398.4 to 1912 h, ranged from 0.0045 to 0.0526 mM SO_4^{2-}/day.
[c]Corrected for endogenous sulfate removal or removal rate in sludge only control.
[d]SD, standard deviation; NA, not applicable.

liquid medium of the two bottles is not in contact but the gas phases are confluent (Daniels et al., 1987). The ZVI is placed in one bottle and the microorganisms are placed in the other bottle with the electron acceptor. The two bottle set up has been used to confirm that H_2 generated from cathodic depolarization of ZVI in one bottle passes with the gas phase to the other bottle and is utilized by methanogens (Belay and Daniels, 1990; Daniels et al., 1987), sulfate reducers (Rajagopal and Legall, 1989), or denitrifers (Till et al., 1998). An alternative mechanisms, in which microorganisms directly obtain electrons from ZVI, has been proposed for a sulfate reducing isolate, *Desulfobacterium* sp. strain IS4, and a methanogenic isolates, strain IM1 (Dinh et al., 2004). These isolates were observed to reduce sulfate and produce methane at rates significantly faster than other strains known to utilize cathodic H_2.

Role of Specific Surface Area on Kinetics

In the present study, rates of methanogenesis and sulfate reduction in positive controls supplied with H_2 as electron donor were greatly higher compared to treatments containing ZVI as the sole electron donor. Similar findings were observed with pure methanogenic cultures (Daniels et al., 1987). The discrepancy in rates clearly suggests a rate-limitation in the release of cathodic H_2 from the surfaces of ZVI particles. Increased surface area would be expected to be associated with greater rates of cathodic H_2 formation. Therefore, the surface area was calculated from the mean particle diameters, mass of ZVI, and particle density of ZVI (7.874 g/mL) in order to plot the net rates of the methane formation and sulfate reduction as a function of surface area as shown in Figure 5A and B, respectively. The graphs clearly show that the rates were highly correlated with the surface area. At any given surface area, the molar rates of sulfate reduction were approximately two-fold higher than the molar rates of methane formation. The rates are directly comparable, since both sulfate and methane involve 8 electron equivalents/mol. In one previous study, ''fine-grained'' and ''coarse-grained'' ZVI were compared, and the methane formation rate by *Methanobacterium thermoautotrophicum* was approximately two-fold higher in the treatment with the

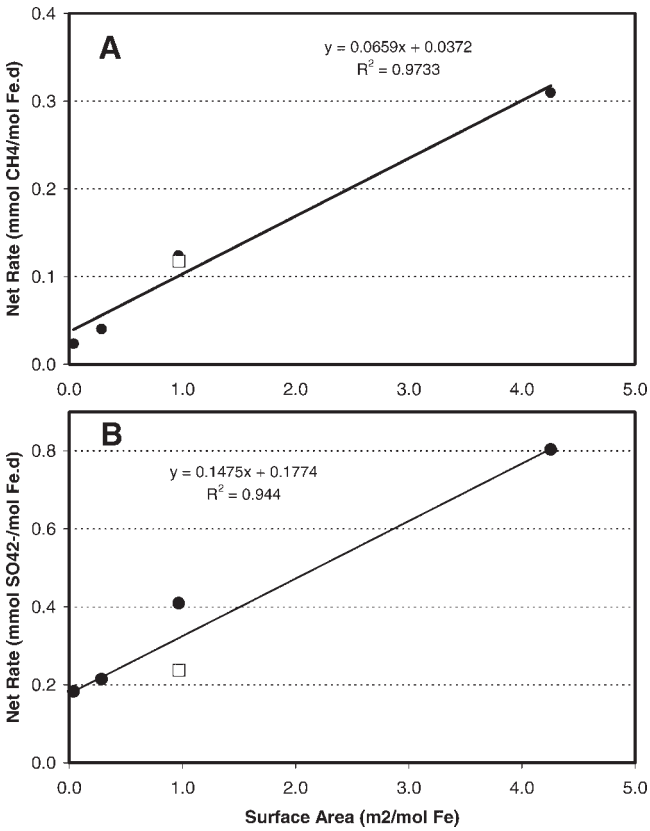

Figure 5. Correlation of net molar rates (corrected for endogenous substrate control rates) with the surface area of ZVI. **A**: Rate of methane formation. **B**: Rate of sulfate reduction. Legend: closed bullets, results from experiments evaluating different particle sizes; open square, results from experiments evaluating ZVI of 325 mesh.

''fine-grained'' ZVI (Deckena and Blotevogel, 1990). Based on the slopes in Figure 5, ZVI is estimated to sustain approximately 0.066 mmol/m^2·day of methane formation and 0.148 mmol/m^2·day of sulfate reduction. Based on the arithmetic average of the calculated surface area specific molar rates, ZVI is estimated to sustain 0.215 mmol/m^2·day of methane formation and 1.327 mmol/m^2·day of sulfate reduction. These values are in the same order of magnitude from a previous study evaluating methanogenesis and sulfate reduction by pure cultures incubated with 2mm granular ZVI (Dinh et al., 2004). The surface area specific molar rates that were calculated from the study's data were found to range from 0.656 to 2.625 mmol/m^2·day for methane formation and 0.656 to 3.675 mmol/m^2·day for sulfate reduction.

Comparison Rates in Pure- and Mixed Cultures

In studies utilizing Fe^0 in ''powder'' form, the rates of methane formation by the various pure culture studies ranged from 4.1 to 40.2 mmol CH_4/mol Fe^0·day (Belay and Daniels, 1990; Daniels et al., 1987; Lorowitz et al., 1992). These values are 10 to 100-fold higher than that observed in this study with the mixed culture utilizing ZVI of 10 μm (Table II). The rates observed with pure methanogenic cultures supplied with granular ZVI or steel coupons ranged from 0.014 to 0.46 mmol CH_4/mol Fe°·day (Daniels et al., 1987; Dinh et al., 2004), which ranged from being comparable to 20-fold higher than the rates in this study with industrial ZVI.

The discrepancies in rates are attributable to several factors. Firstly, the particle size of powder ZVI used in the literature was not defined, and it may have contained significantly more surface area per mol Fe^0 than the ZVI of 10 μm used here. Secondly, methanogens in natural mixed cultures are expected to have significantly lower specific activities compared to pure cultures. The lower activity of mixed cultures would be associated with a higher steady-state cathodic H_2 concentration in the gas phase. A higher steady state H_2 concentrations could effect the thermodynamic favorability of chemical H_2 formation from ZVI (Daniels et al., 1987) and thus possibly also the rate. Thirdly, the media used in this study was less corrosive than media utilized in the pure culture studies which tended to contain aggressive reducing agents such as sulfide (Belay and Daniels, 1990; Daniels et al., 1987), diothinite (Rajagopal and Legall, 1989), or Ti(III) (Deckena and Blotevogel, 1990) to reduce the anaerobic medium. It should be noted that abiotic rates of H_2 formation from ZVI is measurably faster in the presence of sulfide (Dinh et al., 2004) and rates of methanogenesis increased several-fold with increasing concentrations of sulfide (Lorowitz et al., 1992). Also seawater medium was utilized in some of the pure culture studies (Dinh et al., 2004). Thirdly, the time scale used in the experiments reported here was much greater than those used in previous studies, which would allow for more passivation of ZVI surfaces with precipitates (Liang et al., 2003; Phillips et al., 2003a). Lastly, the fastest rates were usually associated with lower concentrations of ZVI of 10 g/L or less (Daniels et al., 1987; Lorowitz et al., 1992), which would be least likely to encounter problems of cementation.

There is limited information on rates of sulfate reduction in the literature when ZVI is used as the electron donor. However, in one study, granular ZVI of 2 mm sustained rates of sulfate reduction by pure cultures that ranged from 0.014 to 0.078 mmol CH_4/mol Fe^0·day (Dinh et al., 2004), which were somewhat lower than rates observed in this study with the mixed culture and industrial ZVI (Table II).

Implications

Taken as a whole, the present study demonstrates that ZVI is readily utilized by anaerobic mixed cultures to support methanogenesis and sulfate reduction. Therefore, ZVI should be considered as a slow release electron donor for PRBs. PRB systems with ZVI were shown to be effective in the reductive dechlorination of chloromethanes and chloroethanes and methanogens were implicated as playing a key role in the process (Novak et al., 1998; Parkin et al., 1998). Sulfate reducing PRBs for the treatment of metals in AMD have previously been stimulated with slowly biodegradable organic matter (Benner et al., 1997, 2002; Blowes et al., 2000; Kim et al., 1999; Ludwig et al., 2002). Based on the findings in this study, ZVI could also be used as the electron donor by natural mixed cultures of sulfate reducers developing in PRBs. This conclusion is supported by the occurrence of sulfate reduction and enrichment of sulfate reducers in flow through ZVI columns (Gu et al., 1999; Köber et al., 2002). The present study also establishes that the rate of methane formation and sulfate reduction with ZVI is kinetically controlled by the specific surface area of the ZVI particles. Therefore fine grained ZVI should be considered to improve kinetically limited PRB systems.

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