

# Perchlorate Reduction by Autotrophic Bacteria in the Presence of Zero-Valent Iron

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A series of batch experiments were performed to study the combination of zero-valent iron (ZVI) with perchlorate-reducing microorganisms (PRMs) to remove perchlorate from groundwater. In this method, H<sub>2</sub> produced during the process of iron corrosion by water is used by PRMs as an electron donor to reduce perchlorate to chloride. Perchlorate degradation rates followed Monod kinetics, with a normalized maximum utilization rate ( $r_{max}$ ) of 9200  $\mu\text{g g}^{-1}$  (dry wt)  $\text{h}^{-1}$  and a half-velocity constant ( $K_s$ ) of 8900  $\mu\text{g L}^{-1}$ . The overall rate of perchlorate reduction was affected by the biomass density within the system. An increase in the OD<sub>600</sub> from 0.025 to 0.08 led to a corresponding 4-fold increase of perchlorate reduction rate. PRM adaptation to the local environment and initiation of perchlorate reduction was rapid under neutral pH conditions. At the initial OD<sub>600</sub> of 0.015, perchlorate reduction followed pseudo-first-order reaction rates with constants of 0.059 and 0.033  $\text{h}^{-1}$  at initial pH 7 and 8, respectively. Once perchlorate reduction was established, the bioreductive process was insensitive to the increases of pH from near neutral to 9.0. In the presence of nitrate, perchlorate reduction rate was reduced, but not inhibited completely.

## Introduction

The discovery of perchlorate, ClO<sub>4</sub><sup>-</sup>, in a large number of ground and surface water supplies, coupled with its disruption of the production of thyroid hormones, resulted in perchlorate being added to the U.S. EPA's candidate contaminant list (1, 2). In Riverside and San Bernardino, CA, drinking water wells have been found with up to 216 ppb ClO<sub>4</sub><sup>-</sup>, and nine wells have been closed. Further concern has developed from the discovery of perchlorate in milk and other dairy products (3) and lettuce (4). The EPA has established a perchlorate reference dose of 0.0007  $\text{mg kg}^{-1} \text{d}^{-1}$  (5), which translates to a drinking water level of 24.5 ppb. In contrast, the California Department of Health Services set an Action Level for perchlorate at 6 ppb (6).

Biological perchlorate remediation is preferred over ion exchange because ClO<sub>4</sub><sup>-</sup> is completely transformed into chloride and other nontoxic end products (2). Microbiological degradation of perchlorate is via the sequence: ClO<sub>4</sub><sup>-</sup> → ClO<sub>3</sub><sup>-</sup> → ClO<sub>2</sub><sup>-</sup> → Cl<sup>-</sup> + O<sub>2</sub>. Transformation of perchlorate to chlorate,

ClO<sub>3</sub><sup>-</sup>, is the rate-limiting step with complete conversion to chloride (7).

Perchlorate reducing microorganisms (PRMs) are ubiquitous in the natural environment, and they can use a variety of organic substrates as electron donors including ethanol, methanol, acetate, and lactate. Bioreactors with organic substrate feedings have been successfully used in the field (8). For ex situ application, the possible release of unoxidized organic substrate and subsequent stimulation of microbiological growth in water distribution systems is a concern (2). For in situ application, injection of a carbon source such as corn syrup may lead to biofouling as the result of growth of heterotrophic microbes in the aquifer, not just PRMs (9). Thus, an important factor in perchlorate biotreatment is the selection of an electron donor that favors the relevant bacteria (10).

As an alternative, H<sub>2</sub> gas has significant advantages as an electron donor; it minimizes biomass clogging and can be more cost-effective than acetate, ethanol, or methanol (11). Strains of *Dechloromonas* sp. are able to reduce perchlorate to chloride using H<sub>2</sub> as the electron donor and carbon dioxide or dissolved carbonates as the carbon source (2). Steady removal of perchlorate by H<sub>2</sub>-fed bioreactors has already been demonstrated (10, 12). However, the low solubility of H<sub>2</sub> in water and its hazardous (explosive) properties during handling and storage may hinder large-scale application.

Zero-valent iron (ZVI) has also shown great versatility in treating contaminants such as halogenated organic compounds, chromate, and uranyl via chemical reduction. For this reason, ZVI is used widely in permeable reactive barriers (PRB) (13). Thermodynamically, ZVI can reduce perchlorate ( $\Delta G^\circ = -596.27 \text{ kcal mol}^{-1}$ ). However, due to the large activation energy barrier, chemical reduction is too slow for ZVI to be used in situ for remediation (14).

As iron corrodes in water, H<sub>2</sub> is released (see reaction 1). Weather et al. (15) first demonstrated that a mixed methanogenic culture increased chloroform reduction rate by using H<sub>2</sub> produced from ZVI corrosion. Similar improved performances with integrated ZVI-microbial systems have been observed for reduction of RDX and TCE, as compared to the treatment with ZVI alone (16, 17). Recently, ZVI was employed together with PRMs and successfully reduced perchlorate without the need for an external H<sub>2</sub> or organic carbon source (18).



In a recent report by Shrouf et al. (19), perchlorate removal was achieved using iron and a mixed culture obtained from the anaerobic digester sludge of a wastewater treatment plant in Iowa. However, they concluded that ZVI was not a suitable energy source for PRMs due to the increase of pH and encapsulation of bacteria by iron precipitates (19). While the environment in an iron wall is nonoptimal for bacteria (20), as compared to the qualities of typical groundwater, the HCO<sub>3</sub><sup>-</sup> (1260  $\text{mg L}^{-1}$ ) and HPO<sub>4</sub><sup>-</sup> (430  $\text{mg L}^{-1}$ ) used in Shrouf et al.'s research were much higher than is commonly found in groundwater. The adverse effects of encapsulation of bacteria by vivianite (Fe<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>) and siderite (FeCO<sub>3</sub>), the major iron precipitates identified in their research, are not likely to occur in typical groundwater conditions. Precipitation of vivianite has not been found to be significant in full-scale ZVI PRBs (21). The concentration of perchlorate used in Shrouf et al.'s research was also high, ranging from 5 to 120  $\text{mg L}^{-1}$ , whereas the level commonly found in contaminated groundwater is 50–2000  $\mu\text{g L}^{-1}$ . In a recent study, under

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**TABLE 1. Summary of Experimental Conditions**

experiment	number	iron to solution ratio (g:mL)	perchlorate concentration ( $\mu\text{g/L}$ )	initial pH <sup>b</sup>	initial nitrate concentration ( $\mu\text{g/L-N}$ )	initial OD <sub>600</sub>
perchlorate reduction by ZVI-PRM	A-1	4:42	1000	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
	A-2	4:42 <sup>a</sup>	1000	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
	A-3	4:42	1000	6.5 $\pm$ 0.2	0	0
	A-4	25:268	10 000	6.5 $\pm$ 0.2	0	0.08 $\pm$ 0.005
	A-5 <sup>d</sup>	25:268	1000	8.5 $\pm$ 0.2	0	$\geq$ 0.025
	A-6 <sup>d</sup>	0:268	1000	8.5 $\pm$ 0.2	0	$\geq$ 0.025
	A-7 <sup>d</sup>	25:268	10 000	8.5 $\pm$ 0.2	0	$\geq$ 0.025
	A-8 <sup>d</sup>	0:268	10 000	8.5 $\pm$ 0.2	0	$\geq$ 0.025
	A-9 <sup>d</sup>	25:268	10 000	8.5 $\pm$ 0.2	0	$\geq$ 0.063
	A-10 <sup>d</sup>	0:268	10 000	8.5 $\pm$ 0.2	0	$\geq$ 0.063
effect of perchlorate concentration	B-1	4:42	200	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
	B-2	4:42	500	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
	B-3	4:42	1000	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
	B-4	4:42	2500	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
	B-5	4:42	5000	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
	B-6	4:42	15 000	6.5 $\pm$ 0.2	0	0.015 $\pm$ 0.005
effect of pH	C-1	4:42	500	6.0	0	0.015 $\pm$ 0.005
	C-2	4:42	500	7.0	0	0.015 $\pm$ 0.005
	C-3	4:42	500	8.0	0	0.015 $\pm$ 0.005
	C-4	4:42	500	9.0	0	0.015 $\pm$ 0.005
effect of nitrate	D-1	25:268	1000	7–8.5 <sup>c</sup>	5000	0.015 $\pm$ 0.005
	D-2	25:268	1000	7–8.5 <sup>c</sup>	10 000	0.015 $\pm$ 0.005
	D-3	25:268	1000	7–8.5 <sup>c</sup>	20 000	0.015 $\pm$ 0.005
	D-4	25:268	1000	7–8.5 <sup>c</sup>	6000 <sup>e</sup>	0.015 $\pm$ 0.005

<sup>a</sup> 4 g of sand was used. <sup>b</sup> Initial pH was adjusted by purging with CO<sub>2</sub> and N<sub>2</sub>. <sup>c</sup> pH was maintained by adjusting with 2 N H<sub>2</sub>SO<sub>4</sub> once every 2–4 days. <sup>d</sup> Combined H<sub>2</sub>/N<sub>2</sub>/CO<sub>2</sub> at a ratio of 1:92:7 (volume) was purged during the experiment. <sup>e</sup> Nitrate was added after four cycles of perchlorate addition–reduction.

flow-through condition with an influent perchlorate concentration of 1000  $\mu\text{g L}^{-1}$ , successive perchlorate removal was achieved up to 4600 pore volumes (22). A combined ZVI-PRM system may have significant potential for field-scale perchlorate remediation.

To promote and enhance this technology for perchlorate remediation, the objectives of this research were to confirm the feasibility of reducing perchlorate to chloride by combining PRMs with ZVI and to quantify the effects of basic operational variables, such as perchlorate concentration, pH, nitrate concentration, cell density, and mass transfer efficiency, on the reduction process. Efforts were also made to improve the efficiency of the reduction process by trying to enhance the ZVI H<sub>2</sub> generation rate, and by increasing the density of PRMs.

## Materials and Methods

**Bacteria and Solutions.** *Dechloromonas* sp. HZ (ATCCBAA-563) was chosen for this study as it was shown to use H<sub>2</sub> as an electron source and CO<sub>2</sub> as a carbon source for perchlorate reduction (2). Bacteria were first grown in Luria-Bertani (LB) solution and washed with mineral solution (MS) multiple times prior to each experiment. The MS used in this study (please see Supporting Information) was modified from the one used by Miller and Logan (12).

Experimental solutions (ESs) were prepared by mixing appropriate amounts of sodium perchlorate solution (5 g L<sup>-1</sup>) with MS to make up the desired concentration. The resultant ESs were purged with a mixture of N<sub>2</sub> and CO<sub>2</sub> overnight to maintain the initial solution pH at 6.5  $\pm$  0.2, unless specified otherwise.

**Experimental Protocol. Perchlorate Reduction Using PRMs and ZVI.** Experimental Series A focused on confirming the feasibility of the process (see Table 1). At an initial perchlorate concentration of 1000  $\mu\text{g/L}$ , three batch experiments using ZVI-PRM, sand-PRM, and ZVI alone were performed to test the benefit of combined ZVI-PRMs in perchlorate reduction (see Table 1, A-1–3). For the first set,

30–36 tubes were prepared as follows: 4 g of ZVI (28–35 mesh, Peerless; Detroit, MI) was placed into 42-mL glass tubes. One mL of washed cells was added into each tube (OD<sub>600</sub> = 0.01–0.02), followed by the addition of ES, headspace free, and then the tubes were capped. All tubes were then placed in an orbital shaker (120 rpm). At selected times, three tubes were sacrificed for analysis. From each tube, 2 mL was removed with a syringe and passed through a 0.2  $\mu\text{m}$  filter (Fisher). Measurements of pH and Eh on the filtrate were made (Orion, model 720). The filtrate was also analyzed for perchlorate concentration using a DIONEX 120 ion chromatograph. Whenever >80% perchlorate removal was observed, new concentrated perchlorate solution was spiked into the tubes. The pH was not controlled during the experiments. The second and the third sets of tubes were used as controls. ZVI was replaced by sterile sand in the second set, and only ZVI, without washed cells, was added to the tubes in the third set, while the other conditions remained the same. To facilitate measurement of Cl<sup>-</sup>, chloride mass balances were performed at a relatively high concentration of perchlorate, 10 000  $\mu\text{g L}^{-1}$  (Table 1, A-4).

During experiments A-5–10, H<sub>2</sub> gas was continuously sparged (0.3 mL min<sup>-1</sup>) into the solution, and perchlorate reduction was studied with and without the presence of ZVI. The setup of these experiments was similar to that employed in the study of nitrate effects (discussed later).

**Relevance of pH Conditions.** Using conditions similar to those of experiments A1–3, perchlorate reduction kinetics were studied at six different initial concentrations of perchlorate, 200–15 000  $\mu\text{g L}^{-1}$  (see Table 1, B1–6) and four different initial pH's, 6–9 (see Table 1, C1–4). Overall, kinetic parameters assuming Monod kinetics were obtained using a nonlinear fitting routine (Sigma Plot 9.0, Jandel Scientific).

**Effects of Nitrate Presence.** Two sets of experiments were performed to study the effects of nitrate, in which concentrated NaNO<sub>3</sub> solution (1 g L<sup>-1</sup>-N) was added to the batch cultures in ZVI-PRM before and during perchlorate reduction. In these experiments, pH control was employed (see

**TABLE 2. Experimental Conditions in Process Modification<sup>a</sup>**

experiment	number	ZVI type			pH <sup>b</sup>	initial OD <sub>600</sub>
		pretreatment	size (mesh)	source		
effect of ZVI surface modification	E-1	none	28–35	Peerless	7.0–8.3	0.025 ± 0.005
	E-2	acid washed	28–35	Peerless	7.0–8.3	0.025 ± 0.005
	E-3	sonicated	28–35	Peerless	7.0–8.3	0.025 ± 0.005
	E-4	Fe <sup>2+</sup> amended <sup>c</sup>	28–35	Peerless	7.0–8.3	0.025 ± 0.005
	E-5	none	100–150	Peerless	7.0–8.3	0.025 ± 0.005
	E-6	none	100–150	Fisher	7.0–8.3	0.025 ± 0.005
effect of cell density	F-1	none	28–35	Peerless	7.0–8.3	0.08 ± 0.005
	F-2	acid washed	28–35	Peerless	7.0–8.3	0.08 ± 0.005
	F-3	sonicated	28–35	Peerless	7.0–8.3	0.08 ± 0.005
	F-4	Fe <sup>2+</sup> amended <sup>c</sup>	28–35	Peerless	7.0–8.3	0.08 ± 0.005
	F-5	none	100–150	Peerless	7.0–8.3	0.08 ± 0.005
	F-6	none	100–150	Fisher	7.0–8.3	0.08 ± 0.005

<sup>a</sup> In these experiments, the iron-to-solution ratio was 25 g:250 mL, and the initial perchlorate concentration was 1000 µg/L. <sup>b</sup> pH's were maintained by the addition of 2 N H<sub>2</sub>SO<sub>4</sub> every 2–4 days. <sup>c</sup> Three initial Fe<sup>2+</sup> concentrations, 20, 50, 100 mg/L, were tested.

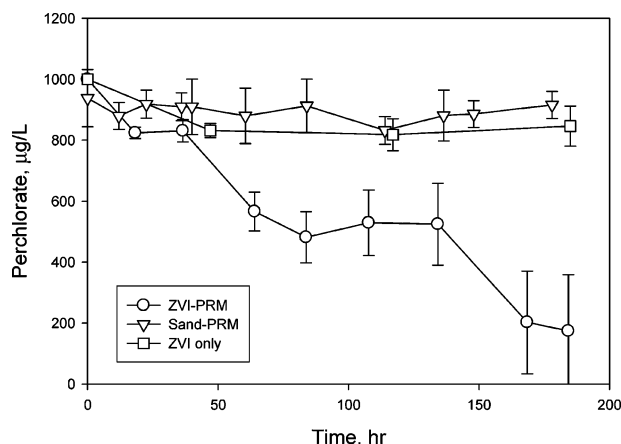
Table 1, footnote). In the first set (Table 1, D1–3), three different nitrate concentrations, 5, 10, and 20 mg L<sup>-1</sup>-N, were tested with an initial perchlorate concentration of 1000 µg L<sup>-1</sup>. Experimental methods were similar to those in experiment series A, except that 250 mL flasks (maximum volume was 268 mL) were used and the ratio of ZVI to solution was 25:268 (g:mL). In addition, the headspace in the flask created after sampling was refilled with DI water. Both perchlorate and nitrate concentrations in the samples were monitored with time. Triplicates were used in the experiment. The experimental protocol of the second set (Table 1, D1–4) was similar to that of the first set, except that the initial nitrate and perchlorate concentrations were 0 and 500 µg/L, respectively. Nitrate (6 mg L<sup>-1</sup> as N) was added to the flask only after four successive perchlorate reduction cycles occurred.

**Process Modification.** A series of experiments were performed to discern whether modification of ZVI surface to increase H<sub>2</sub> generation rates would improve the efficiency of ZVI-PRM. The methods of modification included: (1) pretreatment of ZVI by acid wash (0.1 N HCl), sonication, or amendment with Fe<sup>2+</sup> (20, 50, 100 mg L<sup>-1</sup>); (2) decrease of ZVI particle size (100 mesh); and (3) a different ZVI source (electrolytic iron from Fisher Scientific). Acid wash and sonication are commonly used methods to improve the reactivity of ZVI surface. In a recent study (23), bathing ZVI in an Fe<sup>2+</sup> solution significantly increased the nitrate reduction rate at near-neutral pH. Besides the modification on ZVI, experiments with greater cell density were conducted with both unpretreated and pretreated ZVI to determine whether the process was limited by the amount of bacteria. Conditions for these experiments are summarized in Table 2. Detailed experimental procedures can be found in Yu's dissertation (28).

**Analyses.** The concentration of perchlorate was analyzed with an AS16 column, while nitrate and chloride were analyzed with AS14 column on a DIONEX 120 ion chromatograph. The detection limits were 4, 100, and 200 µg L<sup>-1</sup>, for perchlorate, nitrate (as N), and chloride, respectively. The concentration of soluble iron was measured with an atomic absorption spectrophotometer (Shimadzu, model 6701) with a detection limit of 0.05 mg L<sup>-1</sup>.

## Results and Discussion

**Perchlorate Reduction Using PRMs and ZVI.** The proof of concept of perchlorate reduction by bacteria supported by ZVI was originally demonstrated by Sanchez et al. (18) and was confirmed in this research as shown in Figure 1. Little reduction of perchlorate was observed in the systems containing either sand-PRMs or ZVI alone, while a steady decrease of perchlorate concentration was observed in the

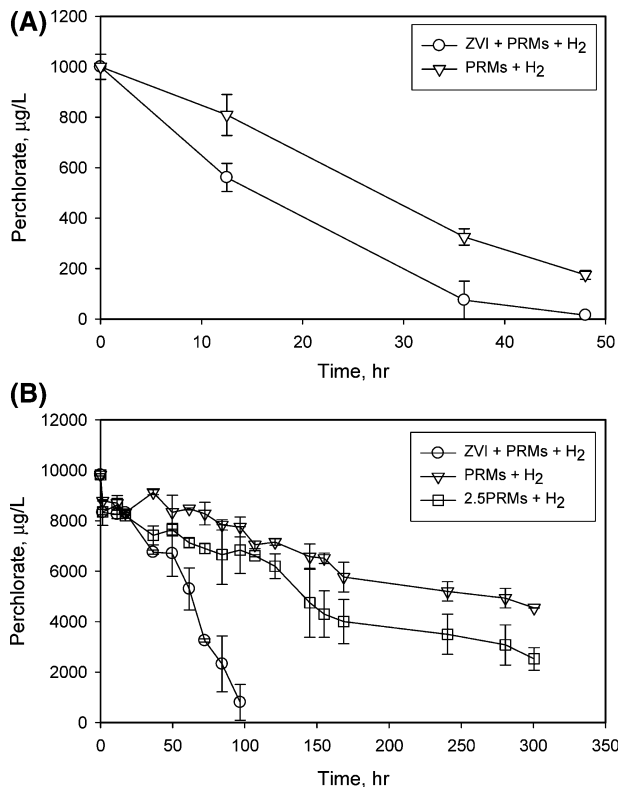


**FIGURE 1. Reduction of perchlorate by ZVI-PRM, sand-PRM, and ZVI only. Error bars show the standard deviation from triplicates.**

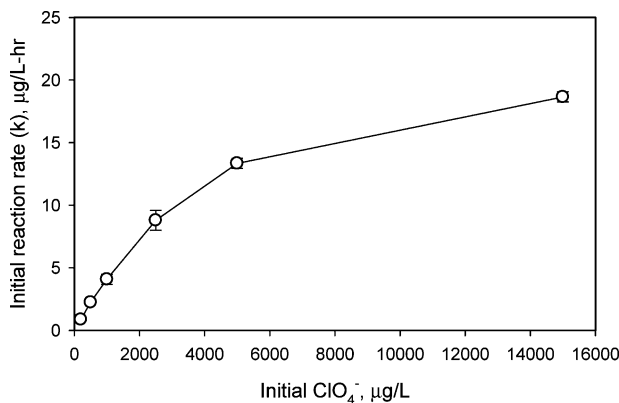
flasks containing combined ZVI-PRMs. When H<sub>2</sub> was purged in the solution in the absence of ZVI, perchlorate reduction was also observed, however, at a slower rate than when ZVI was present (see Figure 2). These data provide strong evidence that ZVI provides the electron donor, H<sub>2</sub>, via the corrosion reaction.

Nearly 100% recovery of chloride was achieved (please see Supporting Information), confirming that perchlorate was reduced to chloride. In both biochemical and chemical studies, it has been pointed out that the initial transformation step of perchlorate to chlorate is rate-limiting in the sequential reduction of perchlorate to chloride (7, 24). The reduction of perchlorate in the ZVI-only control was negligible (see Figure 1) because of the large activation energy for the chemical reduction of perchlorate. Thus, by utilizing H<sub>2</sub> and driving ZVI toward corrosion (reaction 1), the *Dechloromonas* sp. HZ bacteria were able to catalyze the reduction effectively and carry out the initial step of perchlorate reduction to chlorate. Although chlorate can be rapidly reduced to chloride abiotically by ZVI (25), perchlorate reduction was most probably biological. This assertion is supported by the absence of detection of any partially reduced metabolite (ClO<sub>3</sub><sup>-</sup> or ClO<sub>2</sub><sup>-</sup>) when PRMs reduced perchlorate with H<sub>2</sub> only, in the absence of ZVI (discussed later).

At low concentrations of perchlorate, when H<sub>2</sub> gas was bubbled into the cultures in the absence of ZVI, the rate of perchlorate removal was slightly slower than when ZVI was present (see Figure 2A). This is because degradation rate was probably limited by perchlorate concentration, as the concentration during the experiment was well below the K<sub>s</sub> value (see below). However, at high initial perchlorate



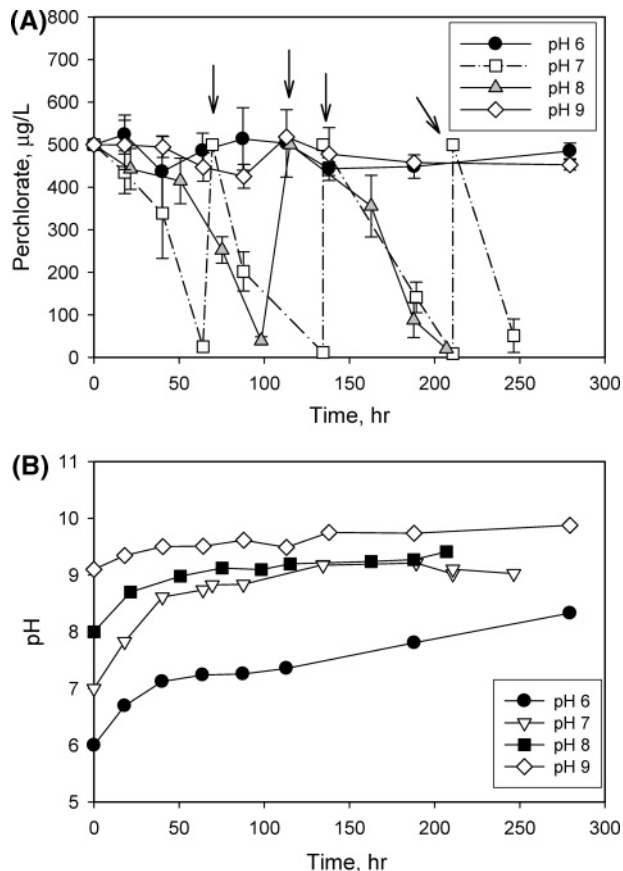
**FIGURE 2.** Perchlorate reduction by H<sub>2</sub> with/without the presence of ZVI. Error bars show the standard deviation from triplicates. (A) Perchlorate reduction at the initial concentration of 1000 µg/L. (B) Perchlorate reduction at the initial concentration of 10 000 µg/L. 2.5 PRMs in (B) indicates a 2.5-fold increase in initial bacteria density.



**FIGURE 3.** Initial perchlorate reduction rate at different initial concentrations. Monod equation can be used to describe the reduction kinetics. Error bars show the standard deviation from triplicates.

concentration, 10 000 µg L<sup>-1</sup>, and initial OD<sub>600</sub> of greater than 0.025, the rate of perchlorate degradation was probably limited by the H<sub>2</sub> gas solubility (2 mg L<sup>-1</sup> at 1 atm H<sub>2</sub> at ambient temperature) (10) (see Figure 2B). Because the bacteria adhere to the ZVI surface, where H<sub>2</sub> is generated due to corrosion, perchlorate degradation rate is expected to be enhanced by the higher H<sub>2</sub> local concentrations. As seen in Figure 2B, perchlorate degradation rate increased from 0.0033 ± 0.0009 to 0.0758 h<sup>-1</sup> in the presence of ZVI. Similar enhancement has been noted (19) at a perchlorate concentration of about 100 mg L<sup>-1</sup> and a constant pH of 7.1.

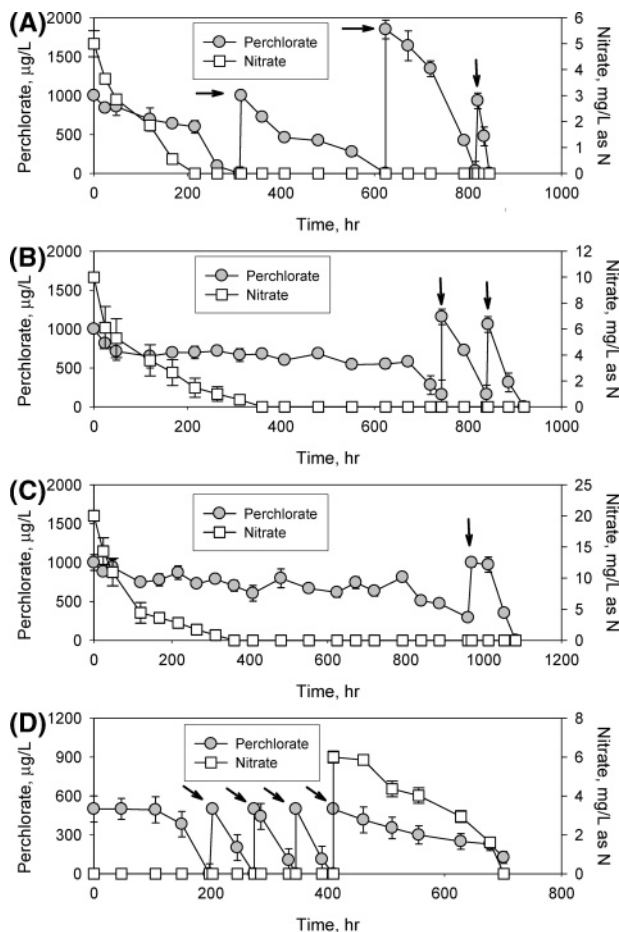
The kinetics of perchlorate reduction by combined ZVI-PRM followed the Monod equation (see Figure 3) and is consistent with the findings of Miller et al. (12), in which they reported Monod kinetics for a hydrogen-oxidizing



**FIGURE 4.** Effects of pH on the reduction of perchlorate by ZVI-PRM. (A) Perchlorate reduction at initial pH 7, 8, 6, and 9. (B) pH increase with time. Error bars show the standard deviation from triplicates. Arrows indicate respiking of perchlorate.

heterotroph (*Dechloromonas* sp. JM). In this research, at an initial OD<sub>600</sub> of 0.015 (~4 µg dry wt mL<sup>-1</sup>), a normalized maximum utilization rate ( $r_{max}$ ) of 9200 µg g<sup>-1</sup> (dry wt)·h<sup>-1</sup> and half-velocity constant of 8900 µg L<sup>-1</sup> were obtained. In the study of Sanchez et al. (18), the kinetics of perchlorate reduction by a mixture of autotrophs, enriched from wastewater samples from the Walnut Creek Wastewater treatment Plant (Austin, TX), was investigated under H<sub>2</sub>-deficit and perchlorate-deficit conditions, respectively. Values of  $K_s$  were found in the range of 16–72 µg L<sup>-1</sup>, and  $r_{MAX}$  was from 1300 to 3900 µg (g·h)<sup>-1</sup>. The values of  $r_{MAX}$  were on the same order of magnitude as those obtained in this research using *Dechlorimonas* sp. HZ. The value of  $K_s$  for the mixed culture was significantly lower, however, suggesting better affinity of perchlorate by the reductase enzyme produced by the mixed culture (18).

**Relevance of pH Conditions.** The impact of system pH is shown in Figure 4. After an initial adaptation period, successive perchlorate reduction cycles were monitored at an initial pH of 7 and 8. As shown in Figure 4, the fastest initial perchlorate reduction occurred when the initial pH was 7 (0.059 h<sup>-1</sup>,  $r^2 = 0.99$ , for the second addition of perchlorate). This result is consistent with those of Zhang et al. (2) in which they found optimum perchlorate reduction activity of *Dechloromonas* sp. HZ at pH 6.8. At the initial pH of 8, the reduction rate was about one-half of that at pH 7 (0.033 h<sup>-1</sup>,  $r^2 = 0.81$ ). At initial pH values of 6 and 9, little, if any, reduction of perchlorate was observed in the solution. Because of the iron corrosion process and generation of OH<sup>-</sup>, a steady increase of pH was observed in all solutions with the amount of increase followed the order of pH 6 > 7 > 8 > 9.



**FIGURE 5. Effects of nitrate on the reduction of perchlorate by ZVI-PRM. (A-C) Perchlorate reduction at initial  $\text{NO}_3^-$  concentrations ranging from 5 to 20 mg/L as N. (D) Nitrate was added into the solution after consecutive perchlorate reduction. Error bars show the standard deviation from triplicates. Arrows indicate respiking of perchlorate or nitrate.**

However, once perchlorate bioreduction was established, no decrease in the perchlorate reduction rate was observed despite the pH increasing up to 9 due to iron corrosion; successive perchlorate reductions were maintained without significant change.

In contrast, at an initial pH of 6, although the pH had steadily increased to 7, the optimum pH for perchlorate reduction, little or no reduction of perchlorate was observed. Further, although continuous perchlorate reduction can occur at pH 9 or above as noted before, little reduction occurred when the initial pH was 9. Thus, the initial pH appears to be important for the bacteria to initiate perchlorate reduction.

**Impacts of Nitrate Presence.** In the presence of nitrate, microbiological perchlorate reduction has been found to be reduced or inhibited (11). In a set of experiments to determine the impact of nitrate on the initiation of perchlorate reduction by ZVI-PRM, nitrate was added in increasing concentrations to the culture flasks. As can be seen in Figure 5A-C, perchlorate reduction was not initiated until most, if not all, of the nitrate was degraded by the ZVI-PRM. Thereafter, perchlorate reduction was initiated. The time needed for initiating perchlorate reduction followed the order of  $20\ 000 > 10\ 000 > 5\ 000\ \mu\text{g L}^{-1}\text{-N}$ . After initiation, the perchlorate reduction rates were similar in all solutions.

In a separate experiment in which nitrate was not added until after consecutive perchlorate reduction cycles had occurred, although perchlorate degradation did not cease,

there was a notable decrease of perchlorate reduction rate concurrent with the spiking of nitrate into the solution (see Figure 5D).

There are two possible phenomena that may have occurred in these experiments and are related to the results shown in Figure 5. The first one is direct competition between nitrate and perchlorate biodegradation. It is well known that most PRMs can also use nitrate as an electron acceptor for autotrophic denitrification. Hence, when nitrate concentration is high relative to the perchlorate concentration, perchlorate reduction slows due to the repression of (per)chlorate reductase (11). Till et al. (26) studied nitrate reduction by steel wool in the presence of a denitrifier (*Paracoccus*) and noted that  $\text{N}_2$  was the main end product of the nitrate reduction. Although not measured, nitrate appeared to increase the amount of gas bubbles on the ZVI surfaces, suggesting the formation of  $\text{N}_2$  gas.

The other possible explanation for the results of Figure 5 is that nitrate was chemically reduced by the ZVI to ammonia as described by Siantar et al. (27). It was hypothesized that chemical reduction of nitrate was faster than the biological reduction of perchlorate to take place, thereby depriving PRMs of hydrogen required for perchlorate reduction. Therefore, long lag phases (50–400 h) were observed, during which time nitrate was depleted and the culture underwent regrowth. Further detailed studies are needed to elucidate the cause for the observed effects of nitrate. Irrespective of the mechanism, a longer reaction time will be necessary to ensure complete removal of perchlorate when nitrate nitrogen is greater than  $5\ \text{mg L}^{-1}$ .

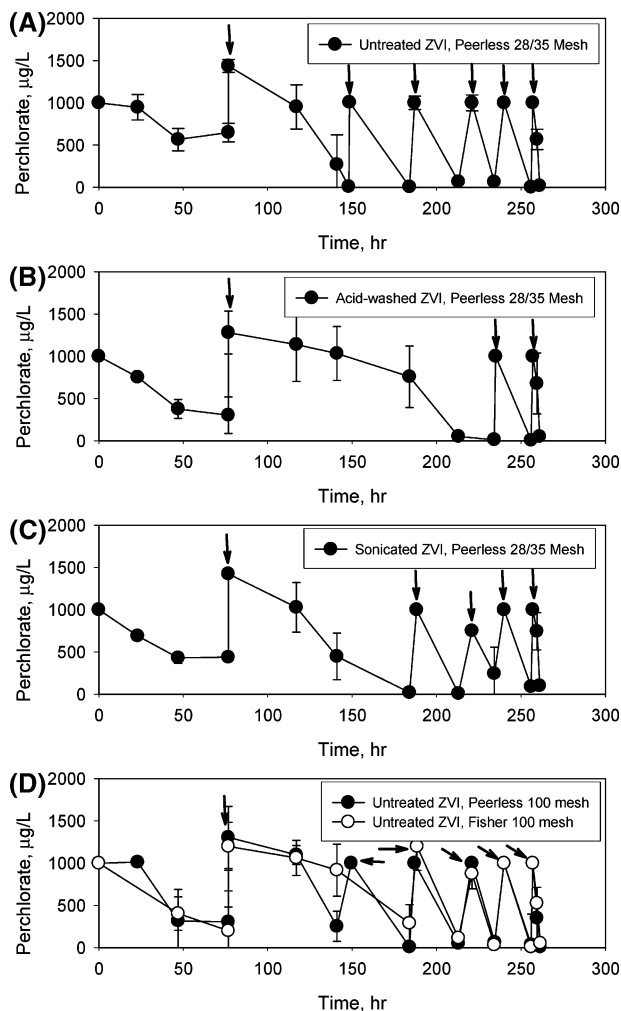
**Process Modification.** The reduction of perchlorate by combined ZVI-PRMs can be simplified into three steps: (1)  $\text{H}^+$  is reduced to  $\text{H}_2$  by ZVI at the surface; (2)  $\text{H}_2$  diffuses into bacteria that adhere onto the iron surface or into bulk solution and then into bacteria suspended in solution; and (3) perchlorate is reduced by bacteria using  $\text{H}_2$ . On the basis of this simplified reasoning, a series of experiments were performed to test if perchlorate reduction rates could be improved by (1) increasing the reactivity of iron surface and  $\text{H}_2$  production; (2) enhancing mass transfer; and (3) increasing the cell density.

Methods of modification used to try to enhance  $\text{H}_2$  generation rates were pretreatment of ZVI by acid wash, sonication, or amendment with  $\text{Fe}^{2+}$ ; decrease of ZVI particle size; and different ZVI source.

The results of perchlorate reduction by combined PRMs ( $\text{OD}_{600} = 0.025$ ) with either untreated, acid washed, or sonicated iron filings are shown in Figure 6. As compared to the untreated ZVI, acid-washing and sonication did not improve perchlorate reduction appreciably, either during the adaptation period or once the PRM acclimated. The minimal enhancement of perchlorate reduction rate on pretreated ZVI at reaction initiation was probably due to increased adsorption of perchlorate rather than enhanced  $\text{H}_2$  generation. Moore et al. (25) found a similar rapid adsorption of perchlorate to acid washed ZVI, which was followed by a slow reduction of perchlorate. However, as shown in Figure 6, any improvement is very limited.

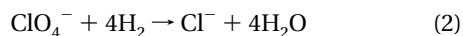
There was little difference in the rate of perchlorate reduction when ZVI of different size (100 mesh) was used, nor did iron from Fisher Scientific, which has been reported to degrade perchlorate faster and more complete than Peerless ZVI (25), improve perchlorate reduction efficiency (see Figure 6). Hence, the perchlorate reduction process was probably not limited by the corrosion of ZVI.

In a previous study on iron corrosion, an iron corrosion rate of  $1.85\ \text{mmol kg}^{-1}\ \text{d}^{-1}$  was obtained for the type of iron used in this research (28). Based on this rate and the stoichiometry between  $\text{Fe}^0$  and  $\text{H}_2$  (reaction 1),  $1.93\ \mu\text{mol}$  of  $\text{H}_2$  will be produced by 25 g of iron in 1 h, which ideally can



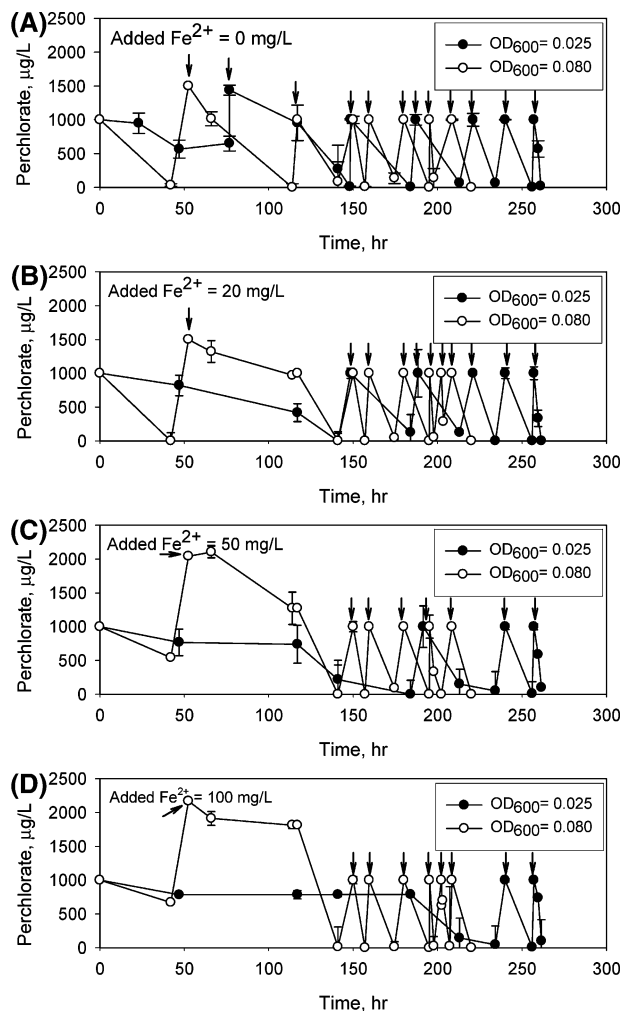
**FIGURE 6.** Perchlorate reduction by PRMs with untreated, acid washed, sonicated ZVIs (Peerless 25/38 mesh), and reduced-sized Peerless and Fisher ZVI. Error bars show the standard deviation from triplicates.  $\blacktriangledown$ Arrows indicate respiking of perchlorate.

reduce  $0.47 \mu\text{mol}$  of  $\text{ClO}_4^-$  (see reaction 2).



In this research,  $1.08 \mu\text{mol}$  of  $\text{ClO}_4^-$  was reduced within 1 h at an initial  $\text{OD}_{600}$  of 0.08. This result indicates the possibility that more than  $1.93 \mu\text{mol h}^{-1}$  of  $\text{H}_2$  was generated and that the rate of iron corrosion increased due to the presence of bacteria. This result is not surprising. Sanchez reported an increased rate of  $\text{H}_2$  generation when PRMs were added to ZVIs (22). In addition,  $\text{H}_2$  production was enhanced when methanogenic cultures were introduced to ZVI to assist the removal of chlorinated aliphatic hydrocarbons (17).  $\text{Cl}^-$  and  $\text{O}_2$ , the two products of perchlorate biodegradation, are also well-known corrosion stimulants (29). These factors make the ZVI corrosion a process influenced by the  $\text{H}_2$ -utilizing bacteria.

In the study of abiotic nitrate reduction, greatly enhanced nitrate reduction rate has been reported when  $\text{Fe}^{2+}$  was amended in solutions containing ZVI (23). In this research, however, when  $\text{Fe}^{2+}$  was added, the adaptation period increased with increasing  $\text{Fe}^{2+}$  concentration (see Figure 7), after which perchlorate reduction rates were similar. The inhibitory effect of  $\text{Fe}^{2+}$  on bacterial reactivity is consistent with the results of Andrews and Novak (30) and ShROUT et al. (19). In addition, formation of iron oxide precipitates due to the addition of high concentrations of  $\text{Fe}^{2+}$  probably also



**FIGURE 7.** Perchlorate reduction by ZVI-PRM when  $\text{Fe}^{2+}$  was added in the solutions at initial concentrations of 0, 20, 50, and 100 mg/L. Error bars show the standard deviation from triplicates. Arrows indicate respiking of perchlorate.

contributed to the prolonged acclimation period. In the experiment, white precipitates immediately formed after amendment with  $\text{Fe}^{2+}$  and the amount of precipitate was proportional to the concentration of  $\text{Fe}^{2+}$  added. However, it should be noted that high concentrations of  $\text{Fe}^{2+}$  (i.e.,  $\geq 20 \text{ mg L}^{-1}$ ) are not common in field-scale PRBs (21).

The effect of bacteria concentration on perchlorate reduction is shown in Figure 7. The negative impact of  $\text{Fe}^{2+}$  was partially offset by increasing the initial number of cells. As compared to the initial lower  $\text{OD}_{600}$  of 0.025 (see Figure 7), the adaptation periods were notably smaller when the initial  $\text{OD}_{600}$  was increased to 0.08, suggesting that the process is limited by the biological kinetics. A similar decrease in the adaptation period was also observed for the untreated, acid washed, and sonicated ZVI when the initial  $\text{OD}_{600}$  was increased from 0.025 to 0.08 (data not shown). Greater biomass also improved the perchlorate reduction rate. As the systems matured and biomass grew at an initial  $\text{OD}_{600}$  of 0.025, the reduction rate increased from  $0.012$  to  $0.23 \text{ h}^{-1}$  after five cycles of perchlorate reduction and increased from  $0.056$  to  $0.79 \text{ h}^{-1}$  at the initial  $\text{OD}_{600}$  of 0.08. The fact that the systems were limited by biological kinetics explains why chemical/physical modification of the ZVI, discussed earlier in this section, had little effect. Instead, process intensification should focus on growing dense cultures at the surface of ZVI. In a recent study of perchlorate bioremediation, it was also proposed that an increase in cell density would be an

important optimization measure (31). However, it should be noted that a certain level of biomass is expected to exist, above which a further increase of biomass will not enhance the performance of perchlorate reduction, because the bacterial-stimulated corrosion of iron is not infinite. In addition, excessive rates of iron corrosion may also lead to an increase of pH that is intolerant to the perchlorate reducing bacteria.

In this research, it was confirmed that without external H<sub>2</sub> supply, ZVI can serve as electron donor providing H<sub>2</sub> in situ to assist the reduction of perchlorate by PRMs. Cell density and pH are two factors that will affect the success of the technology. Favorable pH conditions, between 7 and 8, are important for the adaptation of the bacteria to the local environment and their establishment of perchlorate reduction pathway. A high PRM density will facilitate the startup of perchlorate reduction and enhance the reduction rate. Even though buildup of a biofilm on the ZVI surface might cause limitations related to mass transport, such limitations were not observed in this research (data not shown). The success of this technology will also be influenced by other parameters such as the presence of nitrate and other oxidative contaminants, competition for H<sub>2</sub> between introduced PRMs and indigenous microbes, long-term passivation of ZVI surface by minerals, etc. Further research on these aspects is needed to ascertain the successful long-term application of this methodology for perchlorate remediation.

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### Supporting Information Available

Composition of mineral solution, mass balance between perchlorate and chloride, and SEM and EDS pictures of ZVI surface. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## SUPPORTING INFORMATION

### A. Composition of mineral solution (g/L):

$\text{K}_2\text{HPO}_4$  (0.1875);  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (0.10625);  $\text{NH}_4\text{H}_2\text{PO}_4$  (0.0625);  $\text{NaHCO}_3$  (0.5);  
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , (0.003);  $\text{Na}_2\text{EDTA}$  (0.006);  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.001);  $\text{Na}_2\text{MoO}_4$  (0.0002);  
 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.0004),  $\text{Na}_2\text{SeO}_3$  (0.000066);  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$  (0.0001);  $\text{ZnSO}_4$ , (0.0014);  
 $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  (0.0002);  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , (0.00085); and  $\text{H}_3\text{BO}_3$ , (0.0006).

### B. Mass balance between perchlorate and chloride:

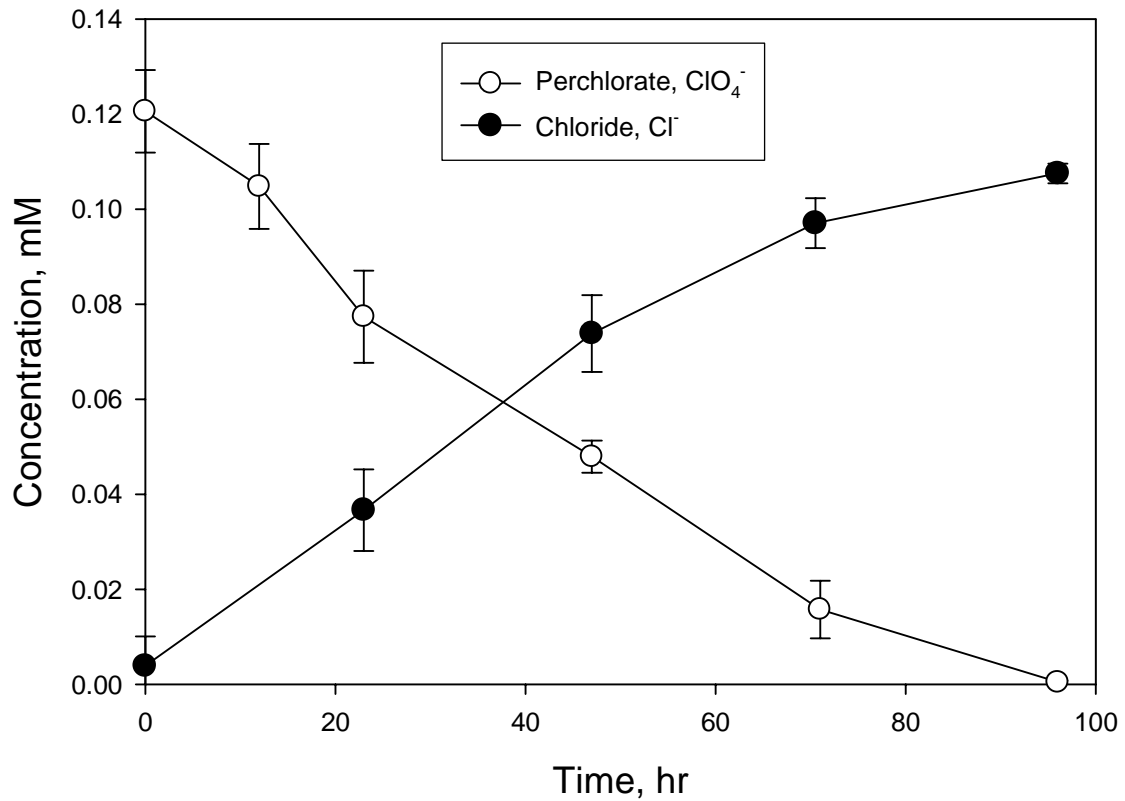
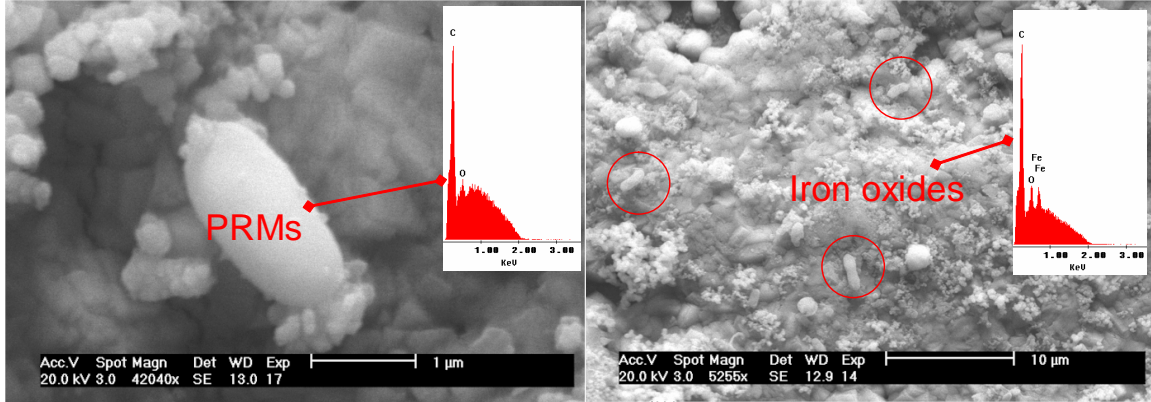


Figure B. Mass balance on perchlorate reduction at an initial concentration of 10 mg/L. Error bars show the standard deviation from triplicates



### C. SEM and EDS pictures of ZVI surface.



**Figure C. SEM and EDS pictures of ZVI surface.**

**Left:** Bacteria were found on ZVI surface

**Right:** Bacteria and iron oxides were differentiated by EDS, in which iron element could be detected for iron oxides.