SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 26, No. 2, pp. 249–253, 2007

Printed in the USA
0730-7268/07 $12.00 + .00

BIOREMEDIATION OF BENZENE, ETHYLBENZENE, AND XYLENES IN GROUNDWATER UNDER IRON-AMENDED, SULFATE-REDUCING CONDITIONS

SONG JIN,*† PAUL H. FALLGREN,‡ A. AZRA BILGIN,§ JEFFREY M. MORRIS,‡ and PAUL W. BARNES§
†Hefei University of Technology, Tunxi Road, Hefei, Anhui 23009, People's Republic of China
‡Western Research Institute, 365 North 9th Street, Laramie, Wyoming 82072, USA
§Arcadis G&M, 630 Plaza Drive Suite 200, Highlands Ranch, Colorado 80129, USA

(*Received* 11 *May* 2006; *Accepted* 7 *September* 2006)

Abstract—Elevated concentrations of sulfide in groundwater (~63 mg S^{2-}/L in water and 500 mg dissolved H_2S/L dissipating from the wellhead) at a field site near South Lovedale (OK, USA) were inhibiting the activity of sulfate-reducing bacteria (SRB) that are known to degrade contaminants, including benzene, toluene, ethylbenzene, and xylenes. Elevated concentrations of these contaminants, except for toluene, also were present in this groundwater. Microcosms were established in the laboratory using groundwater and sediment collected from the field site and amended with various nutrient, substrate, and inhibitor treatments. All microcosms initially were amended with $FeCl_2$ to induce FeS precipitation and, thereby, to reduce aqueous sulfide concentrations. Complete removal of benzene, ethylbenzene, and *m*+*p*-xylenes (BEX; *o*-xylene not detected) was observed within 39 d in treatments with various combinations of nutrient and substrate amendments, including treatments with no amendments (other than $FeCl_2$). This indicates that the elevated concentration of sulfide is the only limiting factor to BEX biodegradation at this site under anaerobic conditions and that treating the groundwater with $FeCl_2$ may be a simple remedy to both facilitate and enhance BEX degradation by the indigenous SRB population.

Keywords—Benzene Ethylbenzene Groundwater bioremediation Sulfate-reducing bacteria Xylene

INTRODUCTION

Groundwater contaminated by aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylenes (BTEX), is a major environmental problem because of the recalcitrance and potential toxic effects of BTEX, including liver, kidney, and nervous system damage and cancer ([1]; http://www.epa.gov/OGWDW/mcl.html). Biodegradation of BTEX under aerobic conditions is possible; however, oxygen depletion resulting from microbial respiration usually drives the geochemical environment to reduced, anaerobic conditions. Studies conducted within the past two decades have demonstrated that BTEX can be biodegraded under anaerobic conditions by organisms such as denitrifying bacteria [2–4] and sulfate-reducing bacteria (SRB) [5–16].

One potential drawback to BTEX degradation by SRB is that SRB use sulfate as the terminal electron acceptor and, thus, produce hydrogen sulfide (H_2S) as a metabolic by-product. Hydrogen sulfide production is a concern, because high sulfide concentrations (>56 mg/L, depending on pH) are potentially toxic and inhibitory to various anaerobic, hydrocarbon-degrading microorganisms, including SRB [17–23]. Sulfide produced through SRB activity, however, can form complexes with metals in solution and precipitate as metal–sulfide compounds, thereby decreasing aqueous sulfide and metal concentrations [24–26]. This implies that adding divalent transition metal salts (e.g., ferrous salts) might be a possible remedy for removing sulfide in low-ionic-strength waters with SRB activity.

The present study was conducted to determine the optimal nutrient amendments required for complete benzene, ethylbenzene, and *m*+*p*-xylenes (BEX) biodegradation by SRB following a ferrous iron amendment to remove toxic sulfide from the system and to maintain low sulfide concentrations during the experiment. The present study serves as a verification of field remediation of BEX under sulfate-reducing conditions, both at sites where the environmental media samples were collected and at other sites exhibiting similar environmental characteristics. Microcosms in this work were established with groundwater and sediment collected from the field; therefore, the results were directly applicable to remediation of the field site.

MATERIALS AND METHODS

Site description

The field site is a former gas-compressor site near South Lovedale (OK, USA) challenged with elevated BEX and sulfide concentrations in the groundwater (Table 1). Site assessments have indicated that this site has been contaminated with BEX and other gasoline-range organics (GRO; i.e., compounds eluting with *n*-alkane markers ranging in size from C4 to C12) for more than a decade and that these elevated substrate concentrations have facilitated high aerobic microbial activity, which eventually lead to anaerobic conditions in the groundwater. Previous assessments indicated that hydrocarbon concentrations in this groundwater were declining, likely because of anaerobic hydrocarbon degradation, but concentrations eventually stabilized and have remained elevated. On-site gypsum beds, which theoretically could provide a source of sulfate as the electron acceptor, did not facilitate BEX biodegradation by SRB, initially suggesting that this system was either nitrogen and/or phosphorus limited. Toxic concentrations of sulfide, however, also were present in the groundwater (~63 mg dis-

* To whom correspondence may be addressed (sjin@uwyo.edu).
Any opinions, findings, conclusions, or recommendations expressed herein are those of the authors and do not reflect the view of the U.S. Department of Energy.

Table 1. Concentrations of various chemicals in groundwater collected from a representative monitoring well near South Lovedale (OK, USA) on December 17, 2004

Chemical	Groundwater	Method detection limit
Benzene	700 μg/L	20 μg/L
Toluene	BDL[a]	5 μg/L
Ethylbenzene	220 μg/L	20 μg/L
m+*p*-Xylenes	310 μg/L	20 μg/L
GRO[b]	3.07[b] mg/L	1.0 mg/L
Sulfate	1,730.0 mg/L	8.0 mg/L
Sulfide	63 mg/L	0.012 mg/L
Ammonia-N	2.0 mg/L	0.05 mg/L
Nitrate-N	4.3 mg/L	0.1 mg/L

[a] BDL = below the method detection limit.
[b] Gasoline-range organic hydrocarbons (n = 5–10 carbons per chain). GRO = total GRO − BTEX (benzene, toluene, ethylbenzene, and *m*+*p*-xylenes).

solved S^{2-}/L in the water and 500 mg dissolved H_2S/L dissipating from the wellhead) and likely inhibited SRB degradation of BEX regardless of the available nutrient concentrations.

Groundwater was collected with a peristaltic pump from a selected monitoring well at this site for chemical analyses and to fill microcosms in the laboratory. Groundwater samples were collected in glass/Teflon® containers with zero headspace. A soil core was removed near the well, and sediment was collected from the uppermost portion of the phreatic zone of this core. This sediment and groundwater were used to establish microcosms in the laboratory.

Groundwater analyses

Groundwater samples from the field site were transported on ice to the Western Research Institute. Groundwater was analyzed for BEX, GRO, sulfate, sulfide, ammonia nitrogen, and nitrate nitrogen to establish baseline concentrations before addition to the microcosms. The pH of the groundwater also was measured at the field site during sample collection using an Orion® model 720A+ pH meter (Thermo Electron Corporation, Waltham, MA, USA) equipped with an Orion Ag/AgCl combination electrode.

Nitrate nitrogen, nitrite, phosphate, and sulfate concentrations were measured with a DX-100 Ion Chromatograph (Dionex Corporation, Sunnyvale, CA, USA) equipped with a 4-m × 250-mm IonPac AS14 anion-exchange column. Ammonium-nitrogen was measured by the indophenol blue method and ultraviolet–visible light spectroscopy [27]. Sulfide was analyzed using a modified version of the colorimetric/methylene blue method ([28]; http://www.epa.gov/region01/info/testmethods/pdfs/testmeth.pdf) using a ultraviolet–visible light spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). Aqueous BEX was analyzed by injecting aliquots into organics-free, distilled water mixed with 250 μl of methanol in 40-ml volatile organic analysis vials. The BEX concentrations were measured with a gas chromatograph–mass spectrometer (model 6890/5973; Agilent Technologies, Palo Alto, CA, USA) equipped with a model 3100 purge-and-trap concentrator (Tekmar Control Systems, Spokane, WA, USA). Aqueous GRO was analyzed with a gas chromatograph–flame ionization detector equipped with a Tekmar number-8 trap and a DB-5 column (30 m × 0.53 mm; Agilent Technologies) ([29]; http://www.epa.gov/region01/info/testmethods/pdfs/testmeth.pdf). The method detection limits (MDLs) for all analyses are listed in Table 1.

Microcosms

Anaerobic microcosms were established in 125-ml serum bottles (which actually hold >125 ml when filled to zero headspace) using groundwater and sediment collected from the field site. All microcosms were prepared under a N_2 (O_2-free) atmosphere within a glove box. Each microcosm was filled with approximately 150 ml of groundwater to minimize headspace and contained 5% sediment (dry wt) based on the mass of water in the microcosms (~7.5 g of sediment). Ferrous iron (Fe^{2+}) was added at a molar ratio of 2:1 Fe^{2+}:S^{2-} to ensure the complexation of all sulfide that was present and could potentially be produced in the groundwater (based on the sulfate concentration; a molar ratio of 1:1 Fe^{2+}:S^{2-} is required for complete complexation). The Fe^{2+} was added to each microcosm as ferrous chloride tetrahydrate ($FeCl_2 4H_2O$; reagent grade; Sigma-Aldrich, St. Louis, MO, USA). The purpose of this $FeCl_2$ addition was to remove sulfide in the groundwater through iron sulfide (FeS) precipitation as well as to remove sulfide that could potentially be produced through sulfate reduction during the experiment. The concentration of sulfate in the groundwater was approximately 100-fold the stoichiometric concentration required by SRB to fully degrade the BEX in the groundwater (a molar SO_4^{2-} to BEX- and GRO-C ratio of 1:2 is required); therefore, no sulfate was added to the microcosms.

Various combinations of nutrients, substrate, and inhibitors were added to the microcosms to determine the optimal amendments for complete BEX degradation as well as to identify the most efficient biodegrading group in the microbial consortia present in the groundwater. The following amendment combinations were applied to microcosms set up in triplicate: No amendments; nitrogen and phosphorus; nitrogen, phosphorus, and methanol; nitrate and methanol; nitrate; nitrogen, phosphorus, methanol, and sodium molybdate; nitrogen, phosphorus, and nitrite; and nitrite and methanol. Potassium nitrate (KNO_3; reagent grade; Sigma) was added to corresponding treatments at a molar BEX- and GRO-C to nitrate-N ratio of 10.0 as an enhancement for denitrifying bacteria. Methanol (MeOH; reagent grade; Sigma) was added in a molar amount equal to the carbon substrate present in corresponding treatments to enhance BEX solubility and, possibly, BEX degradation. Ammonium chloride (NH_4Cl; reagent grade; Fisher Scientific International, Hampton, NH, USA) and dibasic potassium phosphate (K_2HPO_4; reagent grade; Fisher Scientific) were used as nitrogen and phosphorus (nutrient) sources, respectively, and these were added to corresponding treatments in a molar C to N to P ratio of 100:10:2. Sodium molybdate (Na_2MoO_4; reagent grade; Sigma) was added at a concentration of 20 mM molybdate (MoO_4^{2-}) to corresponding treatments as a specific inhibitor to SRB [30]; and potassium nitrite (KNO_2; reagent grade, Sigma) was added at a concentration of 3 mM nitrite (NO_2) to corresponding treatments as a competitive inhibitor of SRB [31–34].

Microcosms were sealed and incubated at room temperature (20°C) and then stored in the dark for 127 d. Samples were drawn from each microcosm on days 0 (after addition of sediment, amendments, and groundwater), 18, 39, 80, and 127 and analyzed for BEX; samples drawn on days 0, 18, 64, and 127 also were analyzed for sulfate and sulfide. On day 70, all microcosms were spiked with 700, 200, and 300 μg/L of ben-

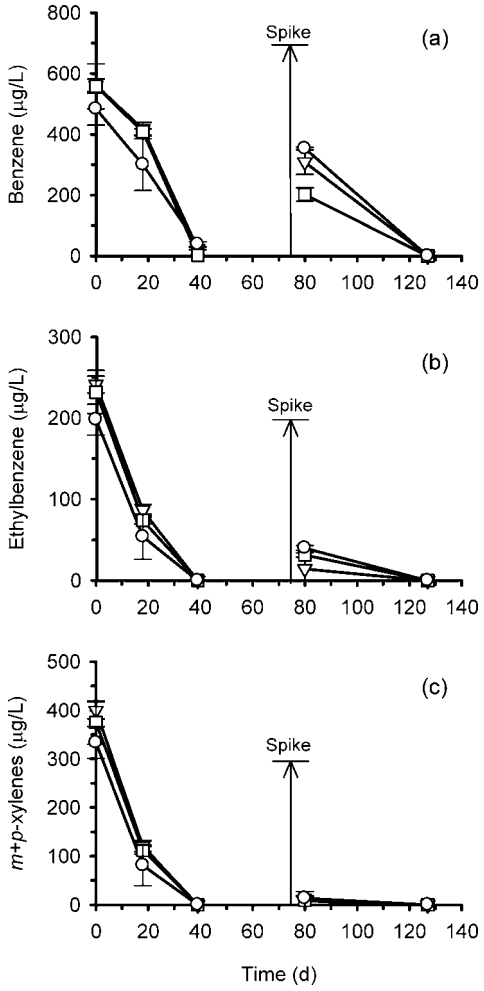

Fig. 1. Biodegradation of benzene (**a**), ethylbenzene (**b**), and *m*+*p*-xylenes (**c**) in microcosms in the laboratory containing groundwater and sediment from a monitoring well near South Lovedale (OK, USA). All microcosms were treated with $FeCl_2$ and the following amendments: No additional amendments (▽); nitrogen and phosphorus (□); or nitrogen, phosphorus, and methanol (○). Background concentrations of benzene, ethylbenzene, and *m*+*p*-xylenes in the groundwater were 700, 220, and 310 µg/L, respectively, approximately 2 d before addition to the microcosms. Error bars represent the standard error of the mean (n = 3). The arrows at day 70 indicate the concentration of each chemical spike that was injected into the microcosms on that day.

zene (reagent grade; Sigma), ethylbenzene (reagent grade; Sigma), and *p*-xylene (reagent grade; Sigma), respectively, because nearly all the BEX in the microcosms had been degraded by day 39. We used *p*-xylene to spike the microcosms instead of *m*+*p*-xylenes, because our analytical method did not distinguish between the two.

Statistical analysis

We conducted statistical comparisons with analysis of variance (α = 0.05), followed by the Tukey honestly significant difference post hoc pairwise comparisons using Minitab™ (Ver 13.31; Minitab, State College, PA, USA) for data at each sampling period, but we did not compare data through time. Because of possible correlations in these time series data, we made a Bonferroni adjustment to our α-level from 0.05 to 0.01 for the BEX data (i.e., α = 0.05/5 sampling periods) and from 0.05 to 0.013 for the sulfate data (i.e., α = 0.05/4 sampling periods) for detecting significant differences between treatments that received different amendments.

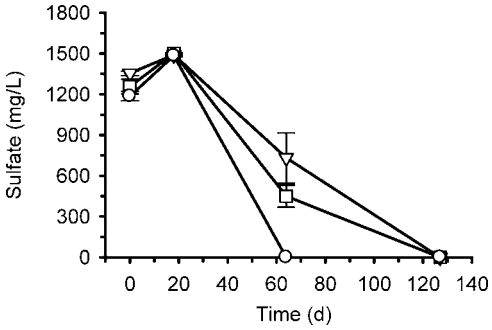

Fig. 2. Removal of sulfate through microbial sulfate reduction in laboratory microcosms containing groundwater and sediment from a monitoring well near South Lovedale (OK, USA). All microcosms were treated with $FeCl_2$ and the following amendments: No additional amendments (▽); nitrogen and phosphorus (□); or nitrogen, phosphorus, and methanol (○). The background concentration of sulfate in the groundwater was 1,730 mg/L at 2 d before addition to the microcosms. Error bars represent the standard error of the mean (n = 3).

RESULTS

Toluene concentrations were less than the MDL in the groundwater used to establish the microcosms in this experiment (Table 1); therefore, we only present the results concerning BEX degradation. The pH of groundwater samples and the background concentrations of BEX, GRO, sulfate, sulfide, and nitrogen are listed in Table 1. During the experiment, BEX and sulfate concentrations decreased in all treatments, but the most rapid and complete degradation occurred in the nonamended, nutrient-amended (nitrogen and phosphorus), and methanol–nutrient treatments. Therefore, for simplicity, we discuss only the results from these three treatments.

Mean benzene concentrations in the nonamended, nutrient-amended, and methanol–nutrient treatments decreased from an initial concentration of 700 µg/L (background concentration) (Table 1) to between 483 and 561 µg/L on day 0 after the addition of amendments and sediment to the microcosms. Concentrations continued to decrease to less than 38 µg/L during the first 39 d of the experiment. Ten days after the initial spiking of these microcosms with benzene, the benzene concentrations ranged from 202 to 353 µg/L, and complete benzene degradation (less than the MDL of 20 µg/L) occurred within 57 d of this additional benzene addition (Fig. 1a). Similarly, complete ethylbenzene and *m*+*p*-xylene degradation occurred in these microcosms during the first 39 d. Furthermore, after adding spikes of 200 and 300 µg/L of ethylbenzene and *p*-xylene on day 70, respectively, concentrations quickly decreased to less than 40 and 14 µg/L, respectively, within 10 d. Complete degradation of these two constituents occurred within 57 d of these additional spikes (Fig. 1b and 1c). No significant differences were found in the benzene, ethylbenzene, or *m*+*p*-xylenes concentrations between any of these three treatments at any one sampling period.

Sulfate concentrations in the nonamended, nutrient-amended, and methanol–nutrient treatments initially increased, likely because of desorption from the sediment mixture in each microcosm, but then steadily decreased throughout this 127-d experiment to concentrations less than the MDL (8 mg/L) (Fig. 2). Sulfate concentrations in the methanol–nutrient treatments were much lower than those in the nonamended and nutrient-

amended treatments on day 64. Whether this difference was significant or not is debatable, because the p value was 0.013 and our α-level for this comparison also was 0.013. This, however, only illustrates that the sulfate concentration in the methanol–nutrient treatment decreased considerably faster than that in the other two treatments. As expected, sulfide concentrations in all microcosms remained very low and did not exceed 1.3 mg/L during the 127-d experiment.

DISCUSSION

The present study demonstrated BEX biodegradation under sulfate-reducing conditions using water and sediments from the actual contaminated field site without adding artificial enrichment media other than $FeCl_2$. Although some studies have demonstrated BTEX biodegradation by SRB using the water and sediments from a contaminated site [8,12,13], these studies have not addressed the production of sulfide and its toxic/inhibitory effects on SRB.

The groundwater and sediments at our field site contained high concentrations of BEX, which were being biodegraded through SRB activity that was facilitated by high sulfate concentrations at the site. Eventually, however, biodegradation of these contaminants was inhibited by high sulfide concentrations that accumulated because of the low ionic strength of the groundwater.

In these experiments, the initial decrease in BEX concentrations on day 0 in the microcosms likely resulted from adsorption to sediment and glass surfaces in the serum bottles. Following this initial decrease, BEX was completely degraded under anaerobic conditions within 39 d (Fig. 1), and the increased SRB activity, illustrated by the steadily decreasing sulfate concentrations (Fig. 2), suggests that SRB were using BEX as a substrate. Furthermore, BEX degradation occurred even faster following the BEX spike added to each microcosm on day 70 (Fig. 1). Interestingly, the treatment with no nutrient or substrate amendments (other than $FeCl_2$) performed as well as the treatments amended with typical nutrients, such as phosphorus and nitrogen (Fig. 1). Therefore, it appears that the only limiting factor to BEX degradation in the groundwater at the field site was inhibition of the SRB by high sulfide concentrations.

The sulfate concentration decreased more rapidly in the methanol–nutrient treatment than in the other two treatments (Fig. 2), which suggests that SRB activity was highest in this treatment and, thus, that BEX degradation might have occurred more quickly in the presence of methanol. No significant differences, however, were found between the BEX concentrations in each treatment at the different sampling times; therefore, the addition of methanol did not enhance BEX degradation over the course of this 127-d experiment.

The SRB consortia in the groundwater from our field site were capable of rapidly ($<$39 d) biodegrading environmentally relevant BEX concentrations to less than the MDL under anaerobic conditions and without additional nutrient or substrate amendments (other than $FeCl_2$). Furthermore, because we used water and sediment samples from the field, the results of this experiment were successfully extrapolated to pilot-scale remediation of the field site (data not shown).

Acknowledgement—This research was conducted and supported by Western Research Institute (WRI) and Duke Energy. Financial support was provided by the U.S. Department of Energy (DoE) through Duke Energy and WRI's Cooperative Agreement DE-FC26-98FT40322 with the DoE. We also thank Trisha Elizondo and Greg Nelson (Arcadis G&M) for their involvement in this project.

REFERENCES

1. U.S. Environmental Protection Agency. 2003. National Primary Drinking Water Standards. EPA 816-F-03-016. Washington, DC.
2. Al-Bashir B, Cseh T, Leduc R, Samson R. 1990. Effect of soil/contaminant interactions on the biodegradation of naphthalene in flooded soil under denitrifying conditions. *Appl Microbiol Biotechnol* 34:414–419.
3. Hutchins SR, Sewell GW, Kovacs DA, Smith GA. 1991. Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. *Environ Sci Technol* 25:68–76.
4. Rabus R, Widdel F. 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch Microbiol* 163:96–103.
5. Aeckersberg F, Bak F, Widdel F. 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. *Arch Microbiol* 156:5–14.
6. Beller HR, Grbic-Galic D, Reinhard M. 1992. Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. *Appl Environ Microbiol* 58:786–793.
7. Beller HR, Spormann AM. 1997. Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. *Appl Environ Microbiol* 63:3729–3731.
8. Coates JD, Anderson RT, Lovley DR. 1996. Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing conditions and artificially iron-reducing conditions. *Environ Sci Technol* 30:2784–2789.
9. Coates JD, Woodward J, Allen J, Philp P, Lovley DR. 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Appl Environ Microbiol* 63:3589–3593.
10. Edwards EA, Willis LE, Reinhard M, Grbic-Galic D. 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate reducing conditions. *Appl Environ Microbiol* 58:794–800.
11. Elshahed MS, McInerney MJ. 2001. Is interspecies hydrogen transfer needed for toluene degradation under sulfate-reducing conditions? *FEMS Microbiol Ecol* 35:163–169.
12. Kleikemper J, Schroth MH, Sigler WV, Schmucki M, Bernasconi SM, Zeyer J. 2002. Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. *Appl Environ Microbiol* 68:1516–1523.
13. Lovley DR, Coates JD, Woodward JC, Phillips EJP. 1995. Benzene oxidation coupled with sulfate reduction. *Appl Environ Microbiol* 61:953–958.
14. Noh SL, Choi JM, An YJ, Park SS, Cho KS. 2003. Anaerobic biodegradation of toluene coupled to sulfate reduction in oil-contaminated soils: Optimum environmental conditions for field applications. *J Environ Sci Health Part A Toxic-Hazard Subst Environ Eng* A38:1087–1097.
15. Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F. 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulfate-reducing bacteria. *Nature* 372: 455–458.
16. Schmitt R, Langguth HR, Puttmann W, Rohns HP, Eckert P, Schubert J. 1996. Biodegradation of aromatic hydrocarbons under anoxic conditions in a shallow sand and gravel aquifer of the Lower Rhine Valley, Germany. *Organic Geochemistry* 25:41–50.
17. Koster IW, Rinzema A, de Vegt AL, Lettinga G. 1986. Sulfide inhibition of the methanogenic activity of granular sludge at different pH levels. *Water Res* 20:1561–1567.
18. McCartney DM, Oleskiewicz JA. 1991. Sulfide inhibition of anaerobic degradation of lactate and acetate. *Water Res* 25:203–209.
19. O'Flaherty V, Mahoney T, O'Kennedy R, Colleran E. 1998. Effect of pH on growth kinetics and sulfide toxicity thresholds of a range of methanogenic, syntrophic, and sulfate-reducing bacteria. *Process Biochem* 33:555–569.
20. Okabe S, Nielson PH, Characklis WG. 1992. Factors affecting microbial sulfate reduction by *Desulfovibrio desulfuricans* in continuous culture: Limiting nutrients and sulfide concentrations. *Biotechnol Bioeng* 40:725–734.
21. Oleskiewicz JA, Marstaller T, McCartney DM. 1989. Effects of

pH on sulfide toxicity to anaerobic processes. *Environ Technol Lett* 10:815–822.
22. Reis MAM, Almeida JS, Lemos PC, Carrondo MJT. 1992. Effect of hydrogen sulfide on the growth of sulfate reducing bacteria. *Biotechnol Bioeng* 40:593–600.
23. Roychoudury AN, van Cappellen P, Kostka JE, Viollier E. 2003. Kinetics of microbially mediated reactions: Dissimilatory sulfate reduction in saltmarsh sediments (Sapelo Island, Georgia, USA). *Estuar Coast Shelf Sci* 56:1001–1010.
24. Canty M. 1998. Overview of the sulfate-reducing bacteria demonstration project under the Mine Waste Technology Program. *Mineral Processing and Extractive Metallurgy Review* 19:61–80.
25. Dvorak DH, Hedin RS, Edenborn HM, McIntire PE. 1992. Treatment of metal-contaminated water using bacterial sulfate reduction: Results from pilot scale reactors. *Biotechnol Bioeng* 40:609–616.
26. Tabak HH, Scharp R, Burckle J, Kawahara FK, Govind R. 2003. Advances in biotreatment of acid mine drainage and biorecovery of metals: 1. Metal precipitation for recovery and recycle. *Biodegradation* 14:423–436.
27. Keeney DR, Nelson DW. 1982. Nitrogen—Inorganic forms. In Page AL, Miller RH, Keeney DR, eds, *Methods of Soil Analysis,* Part 2—Chemical and Microbiological Properties, 2nd ed. Agronomy Monograph 9. American Society of Agronomy–Soil Science Society of America, Madison, WI, USA, pp 643–698.
28. U.S. Environmental Protection Agency. 2003. Sulfide–methylene Blue. EPA method 0376.2. Washington, DC.
29. U.S. Environmental Protection Agency. 1996. Nonhalogenated Organics Using GC/FID. Method 8015B. Washington, DC.
30. Oremland RS, Capone DG. 1988. Use of ''specific'' inhibitors in biogeochemistry and microbial ecology. *Adv Microb Ecol* 10: 285–383.
31. Greene EA, Hubert C, Nemati M, Jenneman GE, Voordouw G. 2003. Nitrite reductase activity of sulfate-reducing bacteria prevents their inhibition by nitrate-reducing, sulfide-oxidizing bacteria. *Environ Microbiol* 5:607–617.
32. Haveman SA, Brunelle V, Voordouw JK, Voordouw G, Heidelberg JF, Rabus R. 2003. Gene expression analysis of energy metabolism mutants of *Desulfovibrio vulgaris* Hildenburough indicates an important role for alcohol dehydrogenase. *J Bacteriol* 185:4345–4353.
33. Haveman SA, Greene EA, Stilwell CP, Voordouw JK, Voordouw G. 2004. Physiological and gene expression analysis of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrite. *J Bacteriol* 186:7944–7950.
34. Londry KL, Suflita JM. 1999. Use of nitrate to control sulfide generation by sulfate-reducing bacteria associated with oily waste. *J Ind Microbiol Biotechnol* 22:582–589.