Supporting Information

Pilot-scale in situ bioremedation of uranium in a highly contaminated aquifer II: reduction of U(VI) and geochemical control of U(VI) bioavailability

WEI-MIN WU[1], JACK CARLEY[2], TERRY GENTRY[2], MATTHEW A GINDER-VOGEL[3], MICHAEL FIENEN[1], TONIA MEHLHORN[2], HUI YAN[2], SUE CAROLL[2], MOLLY N PACE[2], JENNIFER NYMAN[1], JIAN LUO[1], MARGARET E. GENTILE[1], MATTHEW W. FIELDS[4], ROBERT F. HICKEY[5], BAOHUA GU[2], DAVID WATSON[2], OLAF A. CIRPKA[6], JIZHONG ZHOU[2], SCOTT FENDORF[3], PETER K. KITANIDIS[1], PHILIP M. JARDINE[2], AND CRAIG S. CRIDDLE[1]*

[1] Department of Civil and Environmental Engineering , Stanford University, Stanford, CA 94305.

[2] Environmental Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831.

[3] Department of Geological and Environmental Sciences, Stanford University, Stanford, CA 94305.

[4] Department of Microbiology, Miami University, Oxford, OH 45056.

[5] Ecovation Inc., Victor, NY 14564

[6] Swiss Federal Institute of Aquatical Science & Technology (EAWAG), P.O. Box 611, Ueberlandstrasse 133, CH-8600 Duebendorf, Switzerland

MATERIALS AND METHODS

Choice of electron donor. Lactate, acetate and ethanol were evaluated for U(VI) reduction in the presence of contaminated sediments. Assays were performed in 27-ml anaerobic pressure tubes with He/CO_2 (80:20). Sediment samples (~80 g) from FW103 (depth 11.3 m) were suspended in 120 ml of a 5 mM $CaCl_2$ solution then centrifuged at 9,000 rpm to remove nitrate and aluminum. The solid phase was then re-suspended with supernatant from a denitrifying reactor (*1, 2*) and centrifuged twice more to remove nitrate. The solid phase (24% water content) was distributed into pressure tubes (4 g per tube) then supplemented with reactor supernatant (15 ml) and uranyl nitrate. Denitrifying U(VI)-reducing biomass taken from a pilot-scale FBR (*1, 2*) was added to each tube at 4.3 mg dry weight/g soil. The tubes were sealed with butyl rubber stoppers with aluminum caps, flushed with helium, and pressurized with 3 Pa of CO_2 to give an initial solution pH of 7.4. Sodium acetate, sodium lactate or ethanol stock solutions (1M) were added to give 2 g COD/l. All tubes also received yeast extract (10 mg/L) and 0.2 mM sodium trimetaphosphate. The initial concentrations of nitrate and sulfate were ca. 0.3 mg as NO_3-N/l and 300 mg as SO_4-S/l. Tubes were then incubated at ambient temperature. Samples were periodically withdrawn from each tube and centrifuged at 10,000 rpm for 5 min with the solution phase analyzed for U(VI).

U(VI) sorption as a function of pH. Uranium sorption pH edge experiments were conducted in 30 cm^3 polyethylene centrifuge tubes using 0.1 g of FRC area 3 sediment and 15 ml of solution containing 50 mg/L U(VI) in 0.1 M $NaNO_3$ in a glove box with a 1% CO_2(g) atmosphere. The increased level of CO_2(g) ensured that uranium remained soluble over the pH ranged studied. The solution pH was controlled using a $NaHCO_3$/

CO_2(g) buffer system. The amount of $NaHCO_3$ added to each solution varied depending on the desired pH. The solution was modeled with Geochemist Workbench initially to determine approximately how much $NaHCO_3$ to add to each solution for the desired pH. Test solutions were then prepared to ensure the correct pH. Sediment (0.1 g) was added to each centrifuge tube, placed in the glove box, and 15 ml of solution added. The centrifuge tubes were placed on a rotary shaker in the glove box for 72 h. The tubes were then centrifuged for 15 min at ~2300 RPM, and supernatant was decanted into two separate vials - an acidified scintillation vial for U(VI) analysis and a glass tube for pH measurement. The initial concentration of U(VI) for each pH solution was measured from pre-acidified solution samples previously prepared. U(VI) analysis for all solutions was performed using a kinetic phosphorescence analyzer (KPA).

U(VI) desorption as a function of pH. The effects of pH on the desorption of U(VI) from contaminated soil were evaluated using 27-ml pressure glass tubes at ambient temperature. Each tube contained approximately 13.5 g of contaminated soil core material from well FW104 (11.6-13.1 m depth). The uranium content of the sample was ~700 mg/kg. To simulate the flushing operations planned for field operations and to remove nitrate and aluminum, the solid phase was suspended in 15 ml of acidified (pH 3.6) distilled water creating a slurry suspension. Solids were separated by centrifugation at 7,000 rpm for 10 min, and supernatant collected for U(VI) analysis. This process was then repeated. After the second wash, the sediment was re-suspended to create a slurry, and its pH adjusted to a final value of 3.8 to 11.8 with a mixture of 1 M K_2CO_3 and 1 M KOH (for pH>9.0). Tubes were sealed with butyl rubber stoppers, placed in a shaker for

24 hours at 100 strokes per min, allowed to settle, and the supernatant pH and U(VI) measured. Supernatant samples for each pH level were centrifuged at 16,000 rpm for 5 min then analyzed for U(VI).

Microbial enumeration. Denitrifying bacteria, FeRB and SRB were enumerated using the MPN technique with five tubes for each dilution. Anaerobic pressure tubes (27 ml) containing 10 ml basal medium were sealed with butyl rubber stoppers with aluminum caps. The basal media contained the following components (per liter): NH_4Cl, 0.5g; NaCl, 0.4g; $NaHCO_3$, 0.55g; and mineral solution, 100 ml. The mineral solution contained (per liter): $MnCl_2$, 0.4 g; $MgSO_4$, 1.5 g; $CaCl_2$, 0.5 g; and yeast extract, 0.02g. The medium was prepared under a N_2-CO_2 (99:1, vol/vol) atmosphere and distributed to each pressure tube (10 ml per tube). After autoclaving, a sterile trace element solution (0.4 ml) and a sodium trimetaphosphate solution (50 mM, 0.025 ml) was added into each tube to obtain pH 7.0. The trace element solution contained (per liter): HCl (12N), 6.4 ml; $FeCl_2 \cdot 4H_2O$, 0.3g; $ZnSO_4 \cdot H_2O$, 0.1g; $MnSO_4$, 0.085g; HB, 0.06g; $CoCl_2 \cdot 6H_2O$, 0.02g; $CuSO_4$, 0.004g; $NiSO_4 \cdot 6H_2O$, 0.028 g; and $NaMoO_3 \cdot 2H_2O$, 0.04g. The electron acceptor solution was supplemented to the tubes to obtain 5 mM Fe-citrate for FeRB, 1.0 g/l of sodium thioglycollate and 5 g/l of $FeSO_4$ for SRB, and 1.0 g/l of $NaNO_3$ for denitrifiers, respectively. Ethanol solution (1 M) was added to each tube to give a final concentration of 10mM. Groundwater was pumped from the wells into anaerobic pressure tubes pre-filled with nitrogen gas. The sample was then serially diluted in MPN tubes. The inoculated tubes were incubated at ambient temperature for two months. Tubes were compared to controls for scoring as positive or negative with for production of gas in denitrifying tubes, color change in FeRB tubes and production of black Fe(II) precipitates in SRB tubes. FeRB and SRB tubes were also analyzed and scored for methane production.

Tracer study during the biostimulation period. A tracer study was initiated on day 432 (October 28, 2004). For this study, the recirculation rate in the inner loop between FW104 and FW026 was 0.5 L/min; the extraction flow rate from outer loop FW103 was

0.5 L/min and the injection rate to FW024 was 1.35 L/min. A solution of KBr was injected with ethanol into the recirculation line of the inner loop, resulting in an injected bromide concentration of 320 mg/L and ~5.0 mM of ethanol in well FW104. Injection of KBr and ethanol continued for 6 hours. Samples were withdrawn from all wells every 30 min for about 15 hours then at decreasing frequency for one week.

RESULTS AND DISCUSSION

Choice of electron donor. In batch experiments, ethanol supported faster U(VI) reduction than acetate or lactate (FIGURE S1). Ethanol was therefore selected as the sole electron donor for field-scale experiments. Use of ethanol for bioreduction of U(VI) was previously reported by Abdelouas et al. (*3*). Ethanol supports growth of denitrifying bacteria, sulfate-reducing bacteria (SRB) including *Desulfovibrio* sp. (*4*) and iron-reducing bacteria (FeRB) including *Geobacter* sp. (*5*). Hydrogen, a requirement for U(VI) reduction by several bacteria, is produced during the anaerobic degradation of ethanol either via sulfate reduction or syntrophic acetogenesis (*6*). Acetate, another intermediate in the degradation of ethanol, supports growth of *Geobacter* sp. (*5*) and other FeRB such as *Shewanella* sp. (*7*). Column experiments confirmed that ethanol addition would support microbial U(VI) reduction in FRC soils (*8*).

Adsorption and desorption of U(VI) Uranium (VI) adsorption on soil from the test zone is highly dependent on pH (FIGURE S2, top). Peak sorption occurs at pH 6-6.5, decreasing above and below that range, consistent with prior studies for similar soils (*9*). Desorption of U(VI) was likewise pH dependent (FIGURE S2, bottom). As pH increased from 4 to 5, aqueous U(VI) concentrations decreased from 12.6 μM to 0.42; between pH 4 and 6, most of the U(VI) remained sorbed, and aqueous U(VI) concentrations were low; from pH 6 to 9, aqueous U(VI) increased to high levels.

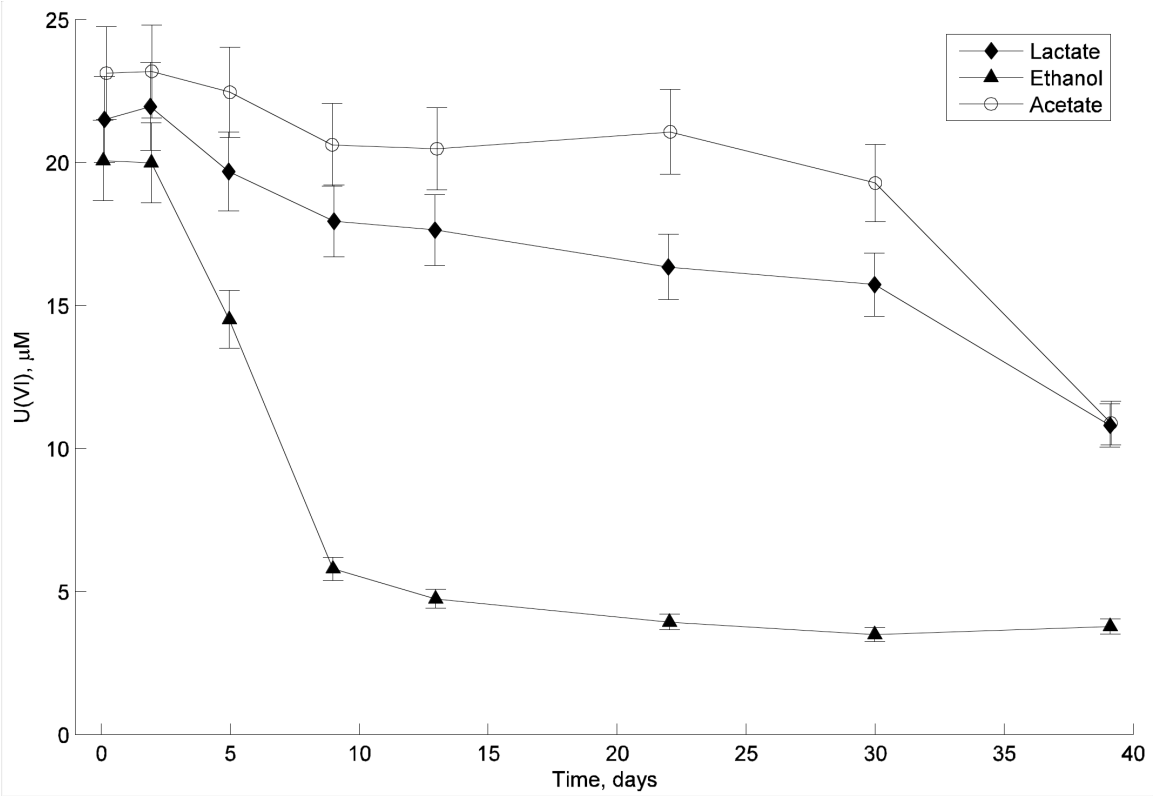

FIGURE S1. Effects of different electron donors on U(VI) reduction of contaminated sediments (data are averages of duplicates, with the range indicated by error bars).

Changes in water table level due to well clogging. FIGURE S3 illustrates changes in water level in the inner loop wells for days 140-480. An increase in the water level in the injection well and drop in the water level within extraction well FW026 indicated clogging. After cleanup, water levels returned to their baseline levels. The injection well FW104 clogged more frequently than the extraction well FW026. Clogging was not observed in the outer loop injection well FW024 or the extraction well FW103, though seasonal water level fluctuations were observed (FIGURE S3B).

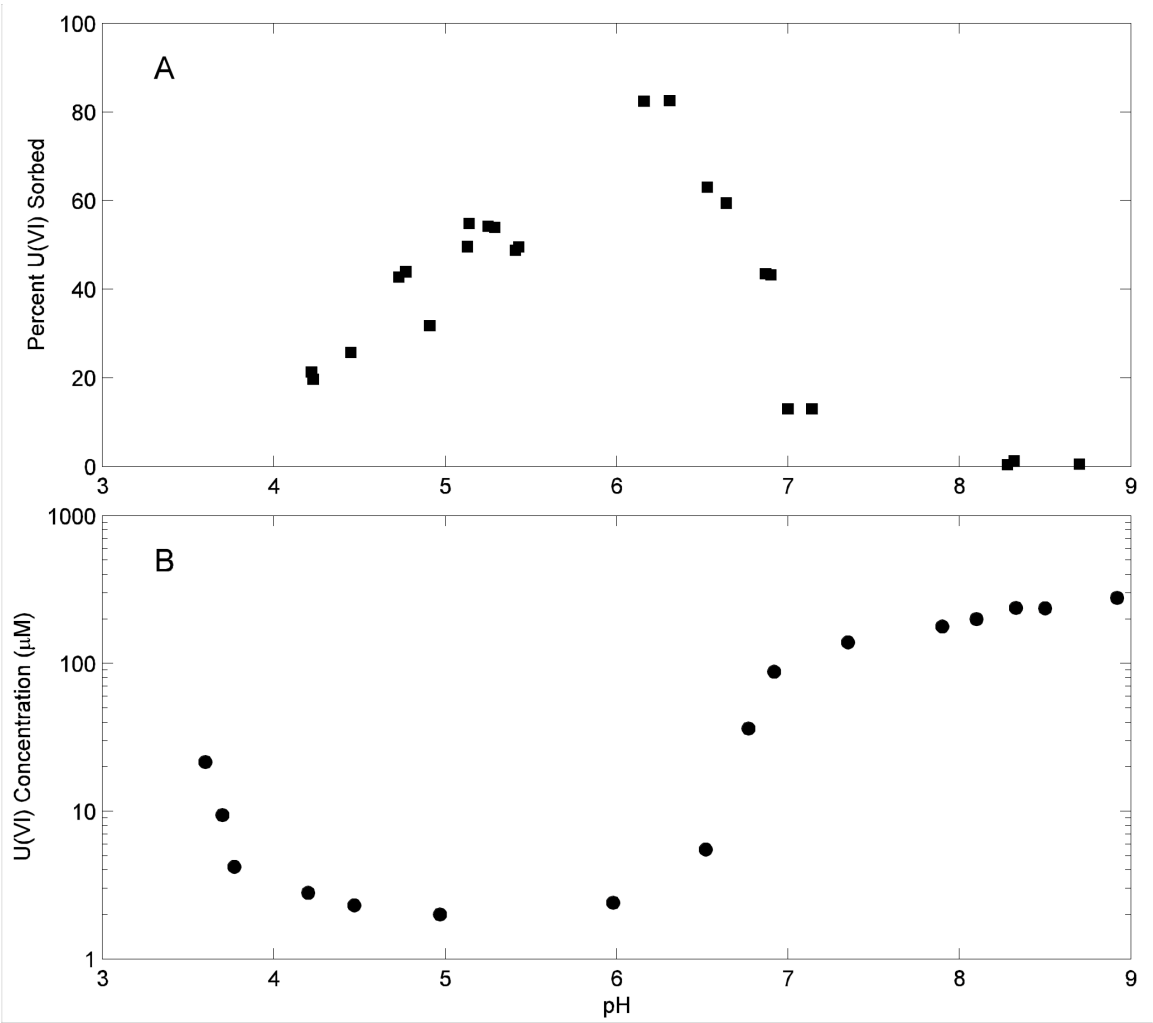

FIGURE S2. A. Adsorption of U(VI) by non-contaminated sediments of Area 3 at FRC site as a function of pH; B. Desorption of U(VI) from contaminated sediments as a function of pH.

Tracer study during biostimulation. On day 432, a tracer study was initiated to determine whether breakthrough profiles had changed compared to the profiles obtained in a tracer study conducted before conditioning and biostimulation (*10*). Bromide was added to the inner loop along with ethanol. Wells that exhibited a significant response in the earlier study were targeted for frequent sampling. As shown in FIGURE S4, the results were similar, but not identical to those obtained in the previous tracer study. Bromide breakthrough profiles were rapid and more pronounced in the MLS wells at depths between 12.2 and 13.7 m. Unlike the earlier study, neither bromide nor ethanol was detected at FW100 - probably due to the shorter duration of this test. Injected ethanol

concentration in FW104 was 5 mM, about 4 times higher than that used during the routine biostimulation experiments (1.2-1.5 mM). Consequently, ethanol (2-2.5 mM) accumulated at FW101-2 and FW102-3. During transport, the ratio of ethanol to bromide decreased, confirming biodegradation of ethanol, and allowing estimates of its rate of consumption.

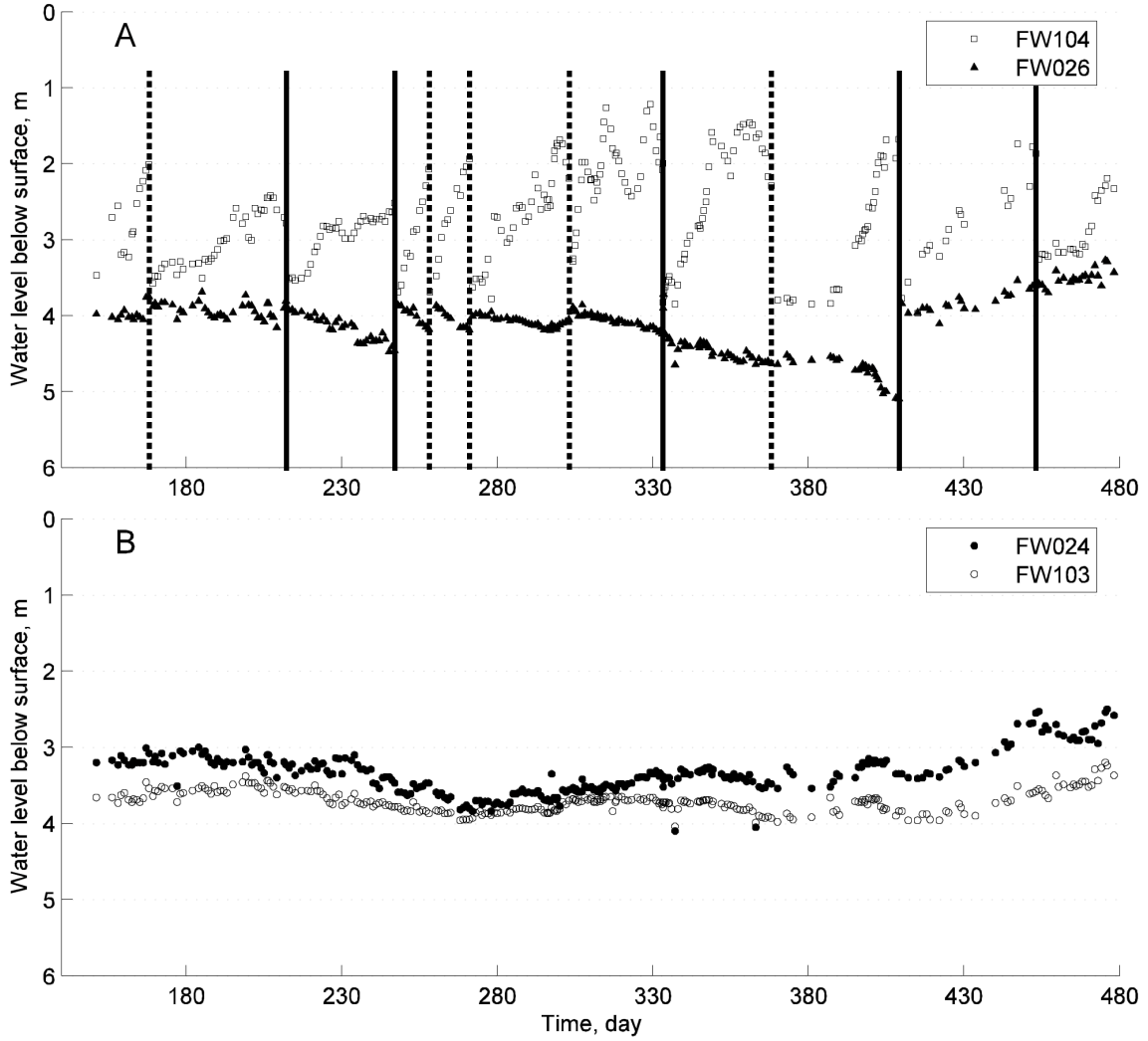

FIGURE S3. Water table changes during in-situ biostimulation. A. Inner loop injection well (FW104) and extraction well (FW026). Solid lines indicate clean-up of both FW026 and FW104. Dashed vertical lines indicate clean-up of FW104 only. B. Outer loop injection well FW024 and extraction well FW103.

U(VI) reduction in Winter (days 185-535). FIGURE S5 illustrates a sequence of base and ethanol addition obtained during winter operation on days 469-476 (December 4-11, 2004, groundwater temperature of 12-14^{o}C). The results indicated that nitrate, sulfate, and U(VI) were all reduced at lower temperatures. Prior to ethanol injection, a solution of K_2CO_3 was injected at FW104 for 38 hours. By day 471, pH levels at both wells 101-2 and 102-3 had increased, with the most dramatic increase at 101-2 where the pH increased from 6.2 to 6.5 (Panel C), and U(VI) concentrations increased from 0.7 to close to 2 μM (Panel E). Ethanol injection (200 mg/L as COD) was then initiated. Over a three-day period, COD increased to ~120 mg/L in FW 101-2 and to ~60 mg/L in FW 102-3. More COD penetrated to injection well FW 102-3 than in summer months (FIGURE S5, panel A) probably because of the slower rates of ethanol consumption at cooler temperatures.

In this experiment, the sequence of base and ethanol addition was modified to assess the effects of ethanol addition in the absence of added carbonate. Accordingly, on day 472, addition of K_2CO_3 was stopped, while ethanol addition continued. When K_2CO_3 injection stopped, the pH in the injection well decreased rapidly from 7.2 to 6, but there was little change at the multilevel wells, possibly because bicarbonate buffer continued due to both denitrification and sulfate-reduction. The patterns of nitrate removal, sulfate removal and sulfide production were similar to those observed in summer months (FIGURE 4B, D and F), but removal occurred over ~3 days versus 2 days during the summer.

The response at FW 102-3 differed for summer and winter months. During the summer, insufficient ethanol was available for removal of the aqueous U(VI) (FIGURE 4, panel E). In winter, however, ethanol addition stimulated removal of aqueous U(VI), decreasing its concentration from 2.4 μM to 1.6 μM in one day (FIGURE S5, panel E). When carbonate addition stopped, both sorption and reduction contributed to removal of aqueous U(VI), and U(VI) concentrations fell to 1.4 μM. When ethanol addition then stopped, microbial reduction stopped, and U(VI) levels increased to 1.8 μM (FIGURE

S5, Panel E). Similar patterns were observed at the injection and extraction wells (FW 104 and FW 026) and at FW 101-2.

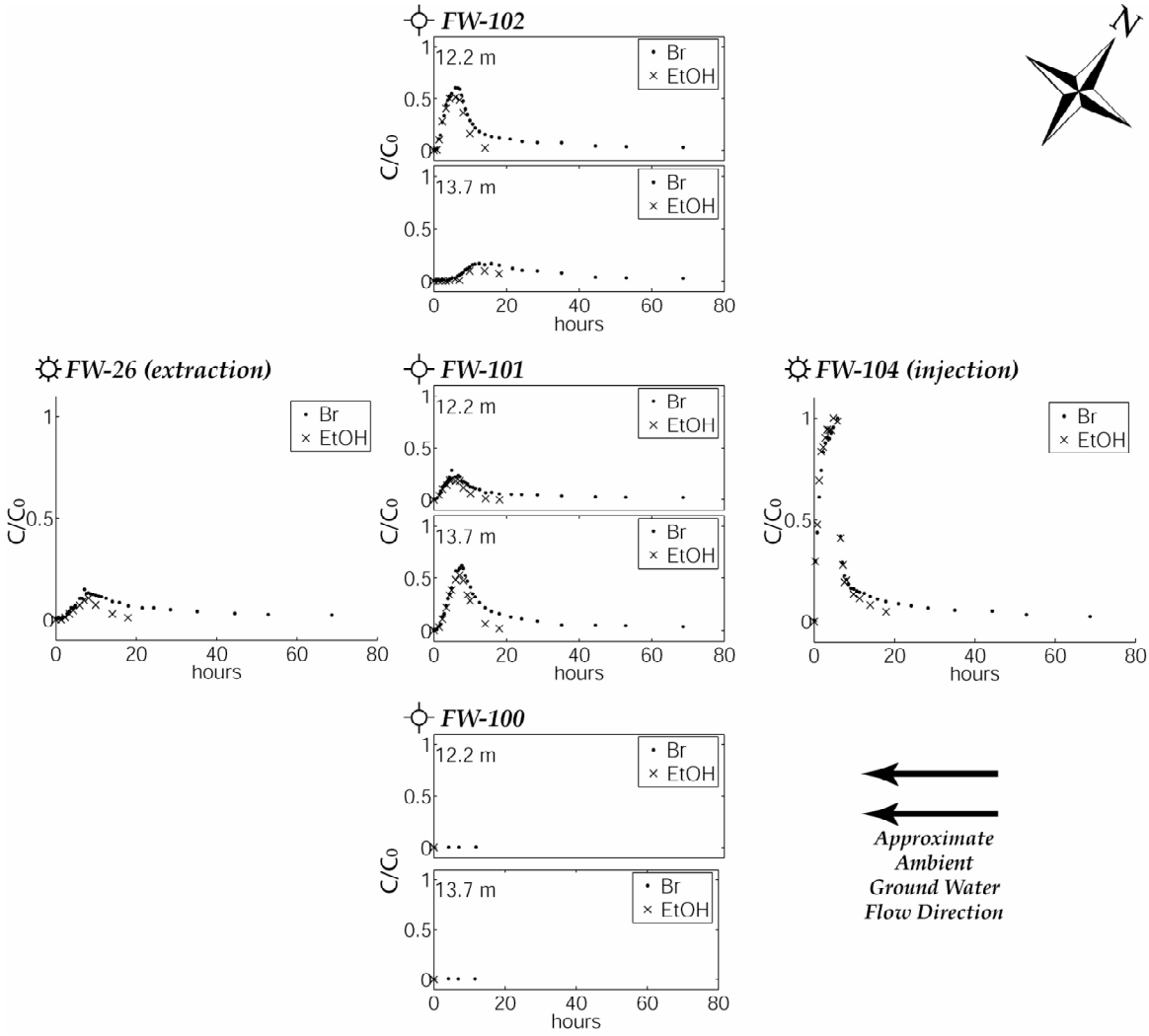

FIGURE S4. Schematic plan view showing wells at approximate locations and concentration breakthrough curves of bromide and ethanol in the tracer test on day 432 (October 28-29, 2004). The sequence of MLS wells (top to bottom): FW102-2, FW102-3, FW101-2, FW101-3, FW100-2, and FW100-3.

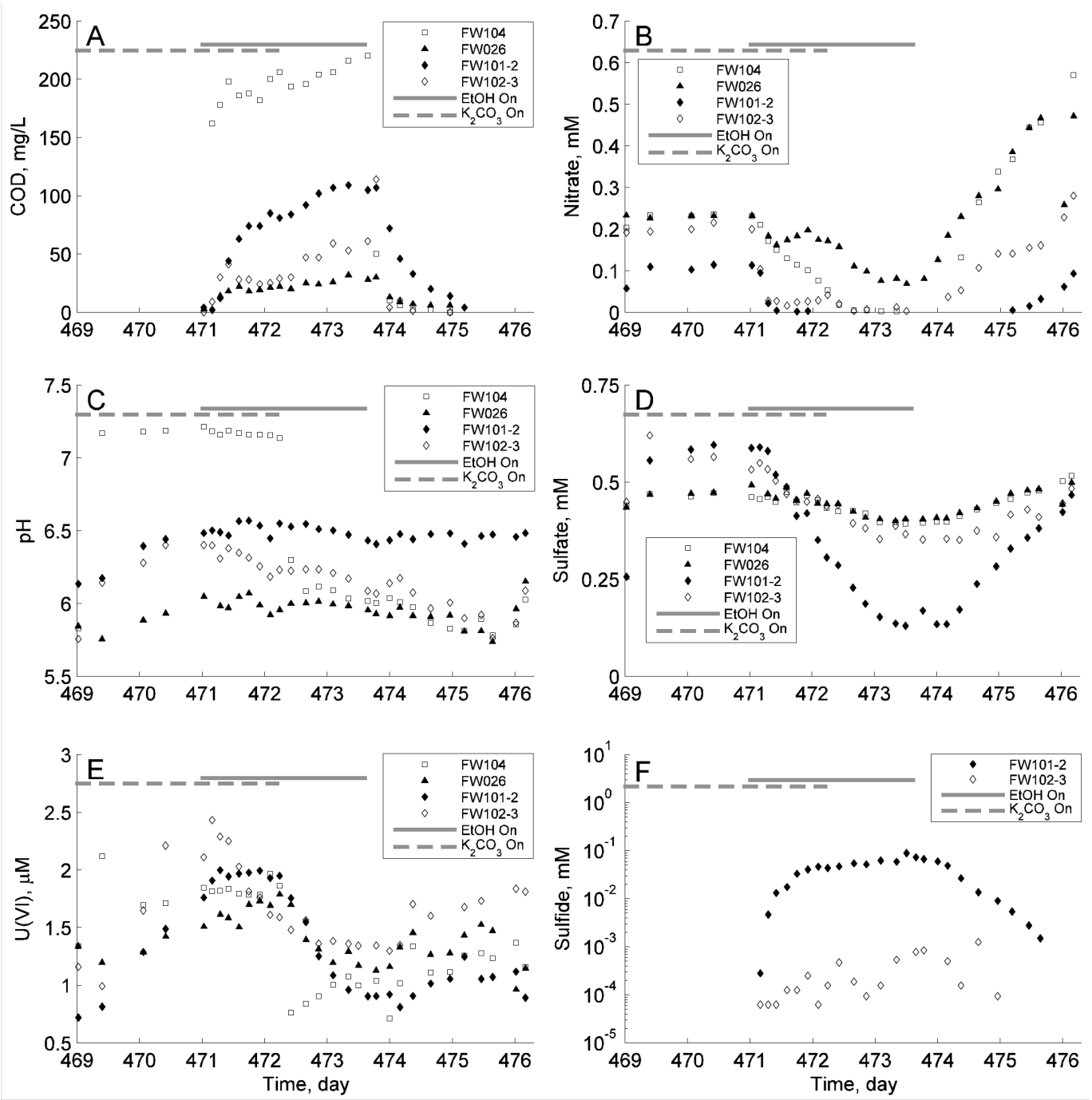

FIGURE S5. Changes in groundwater quality of inner loop wells when carbonate and ethanol were added during the winter (days 469-476). The period of ethanol addition is indicated by the horizontal solid bars; the period of carbonate addition by the horizontal dashed bars. A. COD. B. Nitrate. C. pH. D. Sulfate. E. U(VI). F. Sulfide.

Ethanol metabolism. During the initial denitrification phase, the only organic compound detected was ethanol. With the onset of sulfate- and uranium-reduction, acetate was also

detected. Its presence may be due to the activity of SRB, such as *Desulfovibrio* spp., that incompletely oxidize ethanol to acetate (6) by the reaction:

$2\ CH_3CH_2OH + SO_4^{2-} = 2\ CH_3COO^- + 2\ H_2O + H_2S.$

At FW101-2, acetate was detected along with relatively high levels of sulfide (up to 0.16 mM) (FIGURE S6). Production of acetate by SRB is consistent with the MPN results for FW101-2, showing more SRB (~10^4 cells/ml) than FeRB (~10^3 cells/ml) on day 453 (TABLE 1). FeRB are probable acetate consumers, and this might explain their levels at extraction well FW026 (TABLE 1).

Negligible acetate was detected at FW 102-3 (FIGURE S6). The difference in acetate levels at FW 101-2 and 102-3 could be due to differences in the flow paths connecting these wells to the injection well FW 104. Travel from the injection well to FW 101-2 is short and direct, so there may have been insufficient time for acetate removal and Fe(III) on the solids may be depleted. By contrast, water travel from FW 104 to FW 102-3 proceeds via a lengthier path, allowing for complete removal of acetate. Opportunities for denitrification and iron reduction are also likely greater near the outer boundary of the flow cell where 102-3 is located, and both of these processes would support complete ethanol oxidation and consumption of acetate.

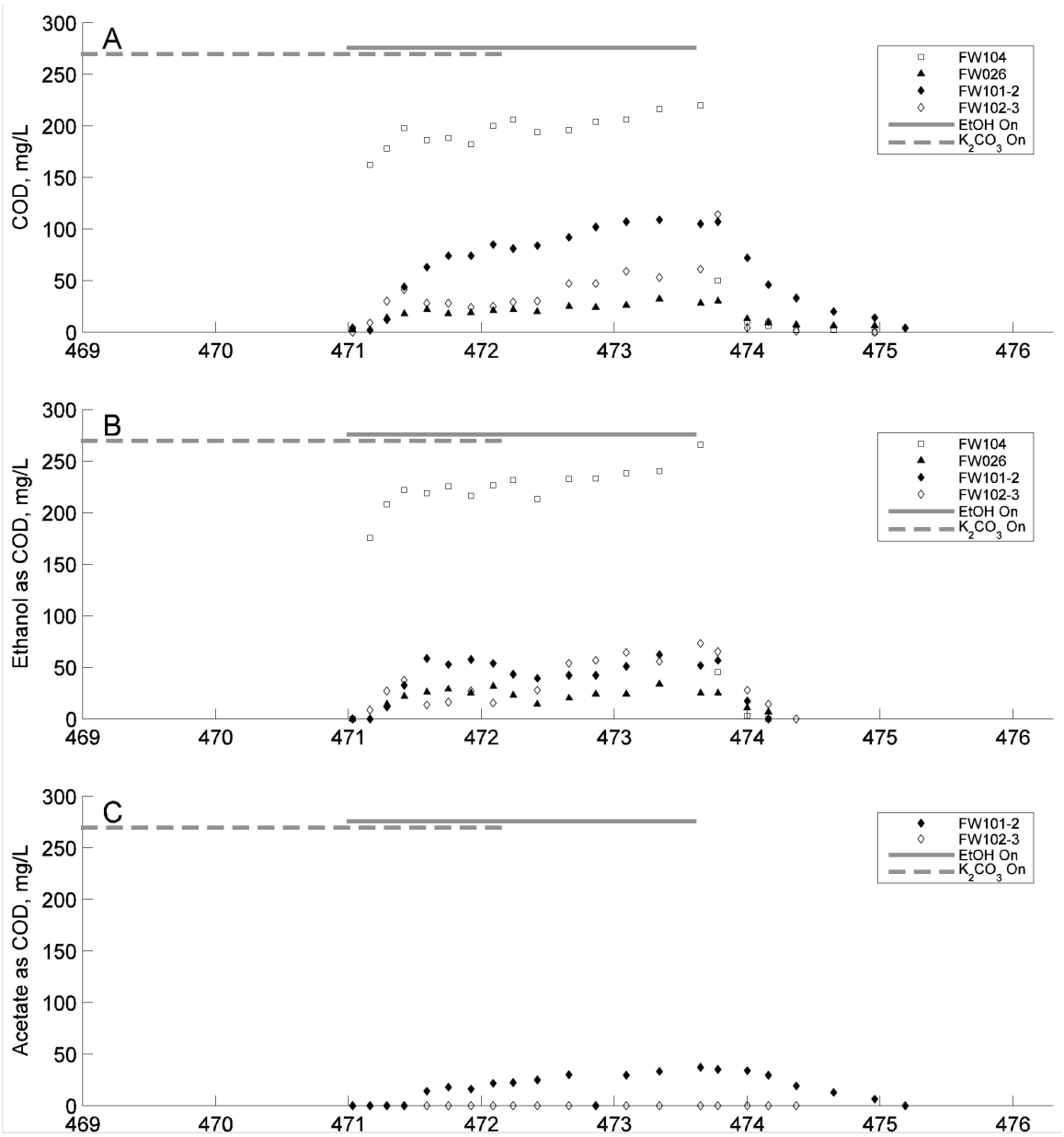

FIGURE S6. Ethanol degradation monitored on days 400-405. Acetate was detected as an intermediate. A. COD concentration. B. Ethanol concentration as COD (1 mg ethanol = 2.435 mg COD). C. Acetate concentration as COD (1 mg acetate = 1.067 mg COD).

REFERENCES

1. Gentile, M., T. Yan, S. M. Tiquia, M.W. Fields, J. Nyman, J. Zhou, and C. S. Criddle. 2005. Stability in a denitrifying fluidized bed reactor. Accepted for publication in Microbial Ecology.

2. Wu, W.-M., B. Gu, M. W. Fields, M. Gentile, Y.-K. Ku, H. Yan, S. Tiquias, T. Yan, J. Nyman, J. Zhou, P.M. Jardine, and C. S. Criddle. 2005. Uranium (VI) reduction by denitrifying biomass. Bioremediation J. 9: 1-13.

3. Abdelouas, A., Y. Lu, W. Lutze, and H. E. Nuttall, 1998. Reduction of U(VI) to U(IV) by indigenous bacteria in contaminated ground water. J. Contaminant Hydrol. 35:217-233.

4. Hansen, T.A. 1994. Metabolism of sulfate-reducing prokaryotes. Antonie Leeuwenhoek. 66:165-185.

5. Coates, J. D., V. K. Bhupathiraju, L. A. Achenbach, M. J. McInerney, and D. R. Lovley. 2001. *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. *Int. J. Sys. Evol. Microbiol.*, *51*, 581-588.

6. Wu, W-M., R. F. Hickey, and J. G. Zeikus, 1991. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: role of sulfate-reducing bacteria. Appl. Environ. Microbiol. 57:3438-3449.

7. Ivanova, E. P., T. Sawabe, N. M. Gorshkova, V. I. Svetashev, V. V. Mikhailov, D. V. Nicolau, and R. Christen. 2001. *Shewanella japonica* sp. nov. Int.J. Syst. Evol. Microbiol. 51, 1027-1033.

8. Gu, B., W. Wu, M. W. Fields, M. A. Ginder-Vogel, H. Yan, S. Fendorf, C. S. Criddle, and P. M. Jardine, 2005. Bioreduction of uranium in a contaminated soil column. Environ. Science and Technology 39:4841-4847.

9. Barnett, M. O.; Jardine, P. M.; Brooks, S. C. 2002. U(VI) adsorption to heterogeneous subsurface media: application of a surface complexation model. Environ. Sci. Technol., 36, 937-942.