Electron flow in acidic subsurface sediments co-contaminated with nitrate and uranium

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Abstract

The combination of low pH and high concentrations of nitrate and radionuclides in the subsurface is representative of many sites within the U.S. nuclear weapons complex managed by the Department of Energy (DOE), including the DOE's Environmental Remediation Sciences Program Field Research Center (ORFRC), in Oak Ridge, Tennessee. In order to provide a further understanding of the coupled microbiological and geochemical processes limiting radionuclide bioremediation, we determined the rates and pathways of terminal-electron accepting processes (TEAPs) in microcosm experiments using close to *in situ* conditions with ORFRC subsurface materials. At the *in situ* pH range of 4–5, carbon substrate utilization and TEAP rates were diminished, such that nitrate was not depleted and metal reduction was prevented. Upon biostimulation by pH neutralization and carbon substrate addition, TEAPs were stimulated to rates that rival those measured in organic-rich surficial sediments of aquatic environments, and extremely high nitrate concentrations (0.4–0.5 M) were not found to be toxic to microbial metabolism. Metal reduction under neutral pH conditions started once nitrate was depleted to low levels in response to biostimulation. Acidity controlled not only the rates but also the pathways of microbial activity. Denitrification predominated in sediments originating from neutral pH zones, while dissimilatory nitrate reduction to ammonium occurred in neutralized acidic microcosms amended with glucose. Electron donors were determined to stimulate microbial metabolism leading to metal reduction in the following order: glucose > ethanol > lactate > hydrogen. In microcosms of neutralized acidic sediments, 80–90% of C equivalents were recovered as fermentation products, mainly as acetate. Due to the stress imposed by low pH on microbial metabolism, our results indicate that the TEAPs of acidic subsurface sediment are inherently different from those of neutral pH environments and neutralization will be necessary to achieve sufficient metabolic rates for radionuclide remediation.
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1. Introduction

Extraction and processing of uranium ore during the cold-war era have left many sites around the world contaminated with uranium. Because of the use of nitric acid during the processing of uranium and uranium-bearing waste, nitrate is often a co-contaminant with uranium and contaminated environments are mostly acidic near the source zone (Riley and Zachara, 1992; Brooks, 2001; Moon et al., 2006). Leaching of uranium into the groundwater is of major concern because oxidized uranium, U(VI), is toxic, soluble, and therefore mobile in subsurface environments where the majority of contamination resides.

Uranium [U(VI)] can be microbiologically or abiotically immobilized from water by its reduction to insoluble U(IV) oxide (Mohagheghi et al., 1985; Lovley et al., 1991; Liger et al., 1999; Wielinga et al., 2001; Fredrickson et al., 2002; Gu and Chien, 2003; Lovley et al., 2004). The half-cell potential for this reaction is "intermediate" and at circumneutral pH, various chemical reductants (such as complexed iron, Fe^{2+}) or microorganisms will mediate rapid U(VI) reduction to U(IV). One of the more promising

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strategies for the *in situ* remediation of uranium waste involves the "biostimulation" of uranium immobilization. Biostimulation is defined as the addition of "nutrients" (electron donors/acceptors, inorganic nutrients) which serve to increase the number or activity of indigenous microorganisms available for bioremediation activity (NABIR Bioremediation Primer, 2003). Biostimulation leads to anoxia and enhanced metal reduction, thereby creating a permeable treatment zone in contaminated aquifers that removes uranium from the aqueous phase before they enter sensitive water supplies (Anderson et al., 2003; Istok et al., 2004).

Iron-reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB) have been shown to rapidly reduce U(VI), and some of these organisms are capable of growth using U(VI) as the sole electron acceptor (Lovley et al., 1991, 2004; Truex et al., 1996; Tebo and Obraztsova, 1998; Lovley and Anderson, 2000; Suzuki et al., 2005; DiChristina et al., 2005). In the terrestrial subsurface, FeRB are likely to outcompete SRB since Fe(III) is usually a much more abundant electron acceptor than sulfate in subsurface sediments. However, geochemical evidence from subsurface aquifers indicates that Fe(III) and sulfate reduction zones often overlap (Jakobsen and Postma, 1999), and physiological studies corroborate the geochemical evidence suggesting that the processes may be catalyzed by overlapping populations. Overlap of these processes could be due to variations in the bioavailability and/or redox potential of Fe(III) forms, along with the relative concentrations of alternate electron acceptors present. Iron(III)-reducing consortia enriched from acidic, radionuclide contaminated sediments utilize Fe(III) minerals as a sole electron acceptor (Kostka et al., 2002) and rapidly reduce U(VI) (Petrie et al., 2003). Members of the *Deltaproteobacteria* (*Geobacter* spp., *Anaeromyxobacter* spp.) might be important metal-reducers in acidic co-contaminated subsurface sediments upon pH neutralization (Petrie et al., 2003). Sequences related to metal reducing *Deltaproteobacteria* increased in subsurface sediments treated *in situ* by pH neutralization and carbon substrate addition (North et al., 2004).

Nitrate serves as a competing and energetically more favorable electron acceptor for metal-reducing bacteria in nitric acid contaminated sediments (Finneran et al., 2002a; Istok et al., 2004). Little or no net U(VI) reduction seems to occur in co-contaminated sediments in the presence of nitrate (Senko et al., 2002; Finneran et al., 2002a; Elias et al., 2003; Shelobolina et al., 2003). After nitrate depletion, U(VI) and Fe(III) are reduced concurrently (Finneran et al., 2002a). Many FeRB and SRB, such as members of the *Geobacteraceae* and *Shewanellae* families, possess the ability to reduce nitrate (DiChristina, 1992; Lovley et al., 2004; DiChristina et al., 2005), but none are capable of complete denitrification. *In situ* bioremediation of uranium waste seems feasible, since coincident U(VI) and Fe(III) reduction was stimulated after the addition of electron donors in field bioremediation experiments (Anderson et al., 2003; Istok et al., 2004). However, reduced U may be remobilized via biotically or abiotically catalyzed reactions with nitrate and denitrification intermediates (Finneran et al., 2002b; Senko et al., 2002, 2005). In particular, U(IV) may be oxidized chemically by nitrite or Fe(III), and both of these oxidants may be regenerated by microbially mediated processes under nitrate-reducing conditions (Senko et al., 2005). Thus, optimization of U(VI) bioremediation strategies requires an extensive knowledge of the microbial communities catalyzing metal- and nitrate reduction along with the environmental parameters controlling their activity.

Much of our knowledge of terminal-electron-accepting processes (TEAPs) in the subsurface, including metal reduction, is based on mechanistic data collected at neutral pH. The acidic subsurface likely contains different functional groups of microorganisms and/or different metabolic controls (Petrie et al., 2003). Thus, this study focused on the function or activity of microbial communities with a high bioremediation potential in acidic subsurface sediments contaminated with U(VI). To optimize *in situ* bioremediation strategies, terminal-electron accepting processes and carbon substrate utilization were examined by manipulating a variety of relevant environmental parameters in sediment microcosms. In particular, high nitrate concentrations, low and neutralized pH conditions, and a variety of electron donors were assessed for effectiveness in targeting specific communities capable of nitrate reduction and iron reduction (and therefore the potential for uranium reduction).

2. Methods

2.1. Site description and sampling procedures

This study focused on contaminated subsurface sediments collected from the Oak Ridge Field Research Center (ORFRC) designated by the Environmental Remediation Sciences Program (ERSP) at the Department of Energy. The ORFRC is located at the Y-12 complex within the Oak Ridge National Laboratory (ORNL) reservation at Oak Ridge, Tennessee. The contaminated plot lies adjacent to a parking lot, which caps four former waste ponds (S-3 ponds) containing uranium and nitric acid waste generated during weapons production (see Petrie et al., 2003, for further details). Contaminated subsurface sediments were sampled from the saturated zone of shallow residuum overlying Nolichucky Shale, where elevated concentrations of uranium and nitrate have been observed (Brooks, 2001; Jardine et al., 2003; Moon et al., 2006).

Sediment cores were sampled on 9 July, 2003, using a Geoprobe equipped with polyurethane sleeves lining the corer. Sediment core depths ranged from 3 to 6 m below the surface. Samples were sealed under argon, and shipped to Florida State University on artificial ice packets via FedEx priority overnight. Groundwater samples of approximately one-liter volume were withdrawn from injection

wells approximately 7–8 m deep and 3.2 cm in diameter into airtight bottles for shipment as described above.

2.2. Solid phase geochemistry in sediment cores

The sediment pH was determined by diluting 2 ml of sediment with 2 ml of deionized water. The 1:1 dilution was shaken for 1 h, centrifuged, and then the pH of the supernatant was measured using a calibrated digital pH meter (McLean, 1982). Nitrate was extracted for 1 h with a 1:1 dilution of deionized water followed by centrifugation and measured by chemiluminesence detection after vanadium reduction (Braman and Hendrix, 1989; Petrie et al., 2003; North et al., 2004). Poorly crystalline Fe oxide minerals were quantified using a 4 h oxalate extraction followed by colorimetric determination of total Fe, and Fe(II) was similarly extracted from fresh frozen sediment in anoxic oxalate (Phillips and Lovely, 1987; Thamdrup and Canfield, 1996). Oxalate extractable Fe(III) was defined as the difference between these two measures. Calibration experiments with pure Fe phases have confirmed the selectivity of this extraction towards poorly crystalline Fe phases (Canfield, 1989; Kostka and Luther, 1994). Sediment sulfate concentrations in microcosm porewaters were measured using ion chromatography.

2.3. Microcosm experiments I-1 to I-5

This microcosm experiment was conducted to separate the impacts of low pH and high nitrate on terminal-electron-accepting processes (TEAPs) under close to *in situ* conditions using groundwater and sediments from the ORFRC site (Table 1). 20 g (wet wt) of sediment was mixed with 30 ml anoxic groundwater inside a Coy anaerobic chamber (95% N_2; 5% H_2) until a uniform consistency was achieved. The sediment suspensions were transferred into 80 ml glass bottles and closed with screw caps. Sediment was paired with groundwater sampled from the closest well to the collection site of the relevant sediment core (Table 1). The final pH of microcosms I-1 and I-2 were neutral, while microcosms I-3, I-4, and I-5 were acidic sediment and groundwater microcosms that were neutralized with sterile anoxic 1 M $NaHCO_3$ prior to incubation. Each set of microcosms contained two replicates of the following treatments: no amendment, ethanol, or glucose. The final concentration of carbon substrate added from sterile stock solutions approximated 10 mM for each substrate. Sediment microcosms were incubated statically in the chamber at 28 °C in the dark and sampled at regular intervals over a 1 year period.

2.4. Microcosm experiments II-1 to II-6

The second microcosm experiment sought to quantify the rates and pathways of TEAPs across a range of geochemical parameters in acidic subsurface sediments. To reduce nitrate and potential toxicant concentrations, deionized water was used in place of groundwater and paired with sediment from the relevant core (Table 1). Thirty grams (wet wt) of thoroughly mixed anoxic sediment were added to 60 ml of anoxic sterilized double deionized water inside a Mecaplex anaerobic chamber (100% N_2 gas phase). Sediment suspensions were transferred into sterile 150 ml infusion bottles (Merck ABS, Dieton, Switzerland) and sealed with sterile rubber stoppers and screw-cap seals. Bottles were placed onto a rotary shaker for 30 min, then purged with sterile argon for 30 min ending with an overpressure of 300–400 mbar argon at room temperature. Treatments were performed in triplicate (Table 1). The final concentrations of lactate, glucose, or ethanol approximated 20 mM. H_2 was added as sterile gas so that it comprised approximately 20% of headspace vol-

Table 1
Summary of microcosm treatments

Microcosm	Core/groundwater samples	Neutralized, nitrate added, and/or washed	Amendments[a]	Nitrate ($\mu mol\,g^{-1}$)[b]	Microcosm pH[b]
I-1	61-01-00/FW20	No	Gluc, EtOH	22.5–35.8	7.0
I-2	61-01-24/FW20	No	Gluc, EtOH	22.3–25.8	6.8
I-3	61-03-00/FW21	Neut	Gluc, EtOH	372.4–452.1	6.8
I-4	61-04-00/FW21	Neut	Gluc, EtOH	404.2–451.4	6.8
I-5	61-05-24/FW21	Neut	Gluc, EtOH	406.9–415.0	6.8
II-1	61-01-24	No	Gluc, EtOH, H_2, Lact	0	5.5–6
II-2	61-01-00	+Nitrate	Gluc, EtOH, H_2, Lact	57.1–64.5	5.5–6
II-3	61-03-25	No	Gluc, EtOH	19.7–25.6	4.3
II-4	61-03-25	Neut.	Gluc, EtOH	20–25.5	7.0
II-5	61-03-25	Washed	Gluc, EtOH	2.1–2.4	6.6
II-6	61-03-25	Washed, Neut.	Gluc, EtOH	3.1–4.1	7.1

Experiments I-1 to I-5 used sediments/groundwater collected from the ORFRC site and had two replicates per treatment. Experiments II-1 to II-6 were conducted with sediments/double deionized water and had three replicates per treatment.

[a] In addition to controls.
[b] Initial concentration or pH.

ume. The final concentration of nitrate approximated 20 mM by adding 3.0 ml of 0.5 M filter sterilized $NaNO_3$. Sediment pH neutralization was achieved by adding sterile 1 M $NaHCO_3$ that had been bubbled with sterile argon gas and autoclaved. Washing of sediment was done three times in a 1:10 dilution using sterile anoxic double deionized water, with the supernatant being discarded each time after centrifugation. Following microcosm amendments, all bottles were placed onto the shaker for an additional 30 min of equilibration, prior to initial sampling. Sediment microcosms were incubated statically at 30 °C in the dark and sampled over regular intervals over a 2–3 month period.

2.5. Analysis of microcosm constituents

Headspace gases CO_2, N_2, H_2, CH_4, and N_2O were quantified with Hewlett-Packard C. (Palo Alto, CA) 5980 series II flame ionization gas chromatographs (Küsel and Drake, 1995; Karsten and Drake, 1997). Gas values were estimated by Henry's law and included the total amounts in both gas and liquid phases. Sediment suspensions were sampled using sterile Argon flushed syringes with 19 gauge needles. 0.2 ml of slurry sample was used immediately for Fe(II) measurements. The suspension was centrifuged, and the supernatant was filtered through a 0.2 μm nylon syringe filter. Nitrate and nitrite determinations were performed within 3 days. Nitrate concentrations were determined spectrophotometrically using an initial amidosulfuric acid (10% w/v) extraction followed by a salicylic acid in sulfuric acid (5% w/v) extraction. The reaction was terminated by an addition of 2 M NaOH before reading absorbance at a wavelength 410 nm on a spectrophotometer (Cataldo et al., 1975). Nitrite concentrations were determined spectrophotometrically (Harrigan and McCance, 1966). Remaining sample material was stored at −20 °C. Soluble ammonium concentrations were determined from previously frozen filtered porewater using flow injection with conductivity detection (Hall and Aller, 1992). At random time intervals, pH was measured in the supernatant. Short chain aliphatic acids, alcohols and sugars were determined using Hewlett Packard 1090 series II high performance liquid chromatographs (Küsel and Drake, 1995).

3. Results

3.1. Geochemistry of sediment cores

Sediment pH ranged from moderately acidic (3.5) to near neutral (6.7) values (Table 2). Extractable nitrate varied in the range from 0.1 to 40.1 μmol g^{-1} among contaminated cores (Table 2). The general trend indicated that sediment pH decreased and nitrate concentrations increased with sediment depth, with a high spatial heterogeneity. Solid phase oxalate extractable iron concentrations ranged from 7.4 to 196.0 μmol g^{-1} (Table 2), with the majority of the Fe being oxidized (solid FeII ranged from

Table 2
Subsurface sediment characteristics of the contaminated sites at the ORFRC

Sample (taken from top of core)	Core segment depth (inches)[b]	pH	Nitrate (μmol g^{-1})[c]	Fe-oxalate extract (μmol g^{-1})[c]
61-01-00	93–111	6.7	0.6	31.5
61-01-24	111–120	6.1	0.1	17.0
61-02-00	120–131	3.9	25.4	30.4
61-02-24	135–150	3.9	15.9	21.6
61-03-00	150–163	3.9	17.8	17.3
61-03-24	168–180	3.7	40.1	18.6
61-04-00	180–200	3.7	29.8	9.6
61-04-22	200–210	3.5	73.1	196.0
61-05-00	210–226	3.7	36.0	25.3
61-05-24	232–240	4.1	35.2	12.1
61-05-24[a]	232–240	4.4	23.3	7.4

Sediment cores were collected 8–18 feet below the surface.

[a] Sample taken from the bottom of the core.
[b] Calculated from core depth, recovery, and quantity of sediment.
[c] Average of sample duplicates from sediment cores.

0.07 to 0.75 μmol g^{-1}). Sediment sulfate concentrations were below 0.2 μmol g^{-1} in deionized water extracts.

3.2. Microcosms I-1 to I-5

In microcosms with an initial neutral pH (I-1 and I-2), all sediments (control, glucose and ethanol) showed a depletion of initial 25 μmol g^{-1} nitrate within the first 3 to 4 weeks (Fig. 1A and Table 3), followed by an accumu-
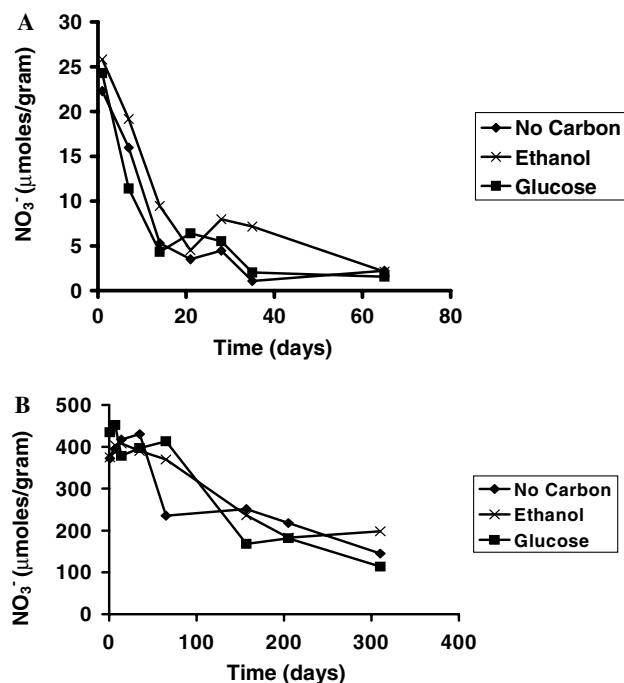

Fig. 1. Determination of nitrate depletion in microcosms from experiment I for near neutral pH sediments containing moderate nitrate levels (A; I-1 and I-2), and neutralized acidic sediments containing higher levels of nitrate (B; I-3 to I-5). Note the different time scales in (A) and (B).

Table 3
Summary of electron acceptor utilization in microcosm experiment I

Microcosm	Amendments	Nitrate reduction rates (μmol g^{-1}d^{-1})[b]	Fe(II) production rates (μmol g^{-1}d^{-1})[b]
I-1	Control	1.15	.09
I-1	Ethanol	1.10	.40
I-1	Glucose	1.80	.50
I-2	Control	0.92	.31
I-2	Ethanol	1.10	.12
I-2	Glucose	1.00	.12
I-3	Control	0.87	ND[a]
I-3	Ethanol	1.00	ND
I-3	Glucose	1.15	ND
I-4	Control	1.25	ND
I-4	Ethanol	1.12	ND
I-4	Glucose	1.22	ND
I-5	Control	0.95	ND
I-5	Ethanol	1.17	ND
I-5	Glucose	1.62	ND

Rates are averages from duplicate microcosm incubations.

[a] ND, none detected.

[b] Rates determined by the linear regression of the change in concentration with time (production or depletion), unless otherwise indicated in text.

lation of Fe(II) (Table 3). Incubations of neutralized acidic sediments (I-3, I-4, and I-5), which contained 400–500 μmol g^{-1} initial nitrate, showed more extensive nitrate consumption over a longer time period (Fig. 1B). However, nitrate consumption rates ranged from 0.87 to 1.80 μmol g^{-1}d^{-1} in all microcosms of experiment I with little difference between control and amended incubations (Table 3). Further, nitrate was not completely consumed

and no Fe(II) production was detected after nearly one year of incubation for neutralized acidic sediments (I-3, I-4, and I-5; Table 3 and Fig. 1B).

3.3. Microcosms II-1 to II-6

In microcosm II-1 (which contained little to no nitrate), microbial activity was minimal, as indicated by small amounts of CO_2 produced and substrates utilized. Fe(II) accumulation rates in controls averaged 0.25 μmol g^{-1}d^{-1}, whereas decreased rates of 0.15 μmol g^{-1}d^{-1} were observed in ethanol and H_2 treatments (Table 4). Minimal Fe(III) reduction occurred in lactate and glucose treatments.

Microcosms of neutral pH sediments containing moderate nitrate concentrations (II-2; $\sim$60 μmol g^{-1} nitrate) showed high activities in glucose and ethanol treatments as well as one lactate replicate; the remaining treatments including the control exhibited minimal activities (Figs. 2–4 and Table 4). Glucose and ethanol treatments showed the highest CO_2 production rates of 2.50 μmol g^{-1}d^{-1} and 1.35 μmol g^{-1}d^{-1}, respectively (Table 4; in glucose treatments, the peak of carbon dioxide production occurred in the first 32 days giving a rate of 5.3 μmol g^{-1}d^{-1}). One lactate replicate bottle showed a similar CO_2 production rate equivalent to glucose and ethanol amended treatments, resulting in an overall average rate of 0.58 μmol g^{-1}d^1. Nearly complete nitrate depletion was observed in glucose and ethanol treatments (and in one lactate replicate) during the first 21 days at rates of 2.84 μmol g^{-1}d^{-1} and

Table 4
Summary of electron acceptor and donor utilization in microcosm experiment II

Microcosm	Amendments	Nitrate reduction rates (μmol g^{-1}d^{-1})[b]	Fe(II) production rates (μmol g^{-1}d^{-1})[b]	CO_2 production rates (μmol g^{-1}d^{-1})[b]	Substrate utilization (μmol g^{-1}d^{-1})[b]
II-1	Control	NA[a]	0.25	0.10	NA
II-1	Lactate	NA	0.01	0.05	0.10
II-1	Ethanol	NA	0.15	0.07	0.25
II-1	Glucose	NA	0.02	0.04	0.07
II-1	Hydrogen	NA	0.15	0.05	0.13
II-2	Control	0.26	0.00	0.10	NA
II-2	Lactate	0.57	0.56	0.59	0.40
II-2	Ethanol	2.70	1.36	1.35	1.11
II-2	Glucose	2.84	1.44	2.50	1.10
II-2	Hydrogen	0.27	0.00	0.08	0.06
II-3	Control	0.13	0.00	0.00	NA
II-3	Ethanol	0.28	0.00	0.00	0.11
II-3	Glucose	0.26	0.00	0.00	0.12
II-4	Control	0.18	0.00	2.40	NA
II-4	Ethanol	2.82	0.04	0.41	1.30
II-4	Glucose	1.68	0.28	1.11	1.28
II-5	Control	0.02	0.00	0.00	NA
II-5	Ethanol	0.25	0.01	0.05	0.00
II-5	Glucose	0.01	0.05	0.01	0.00
II-6	Control	0.07	0.00	1.66	NA
II-6	Ethanol	0.32	0.00	1.34	0.01
II-6	Glucose	0.22	0.82	2.28	1.09

[a] NA, not applicable.

[b] Rates determined by the linear regression of the change in concentration with time (production or depletion), unless otherwise indicated in text.
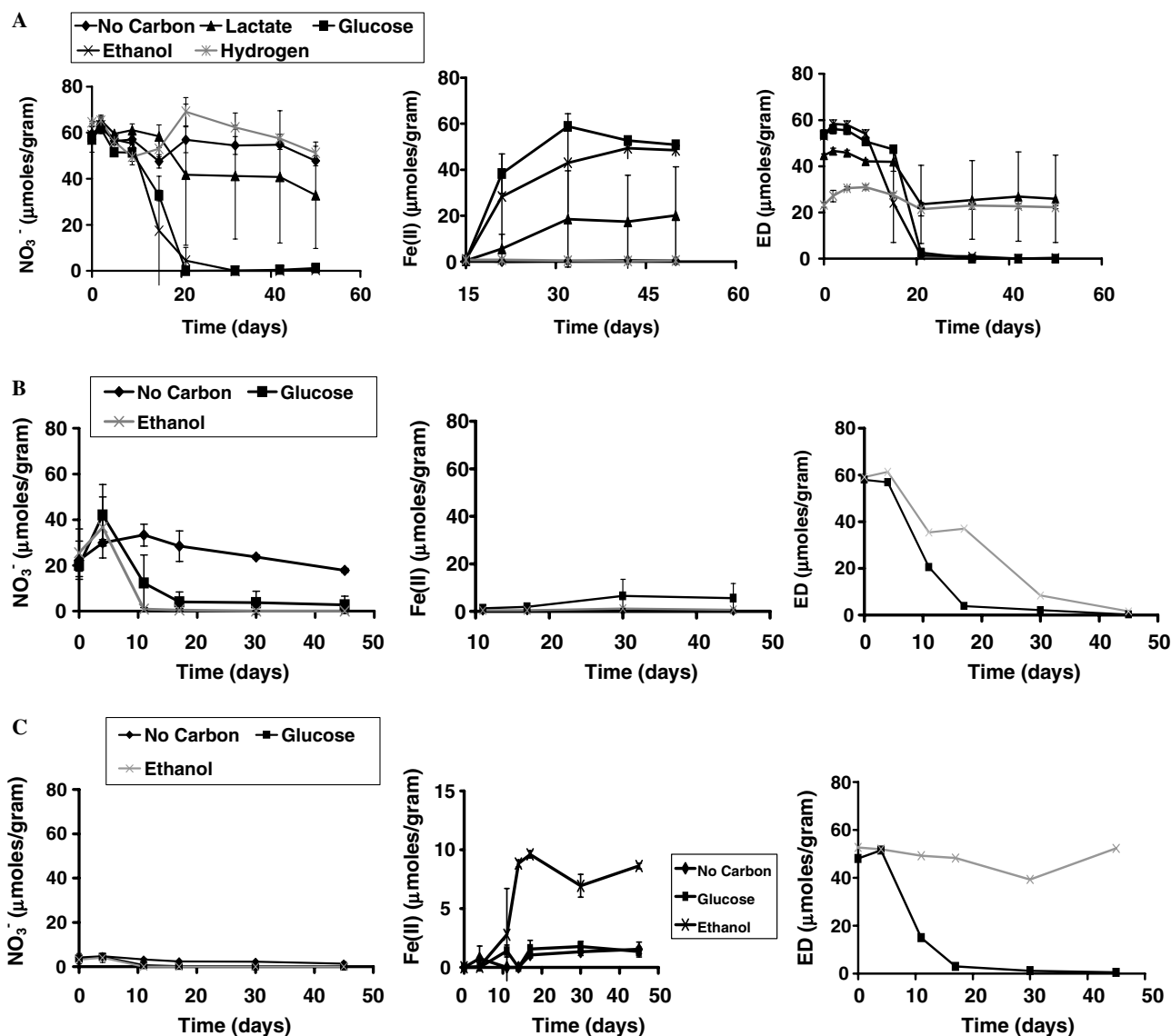
Fig. 2. Determination of nitrate depletion (left graph), and Fe(II) production (middle graph) as well as electron donor utilization (right graph) in microcosms of near neutral pH sediments containing moderate nitrate levels (II-2) (A), neutralized acidic sediments containing moderate nitrate levels (II-4) (B), and neutralized washed acidic sediments containing low levels of nitrate (II-6) (C).

2.70 $\mu mol\,g^{-1}d^{-1}$, respectively (Fig. 2A and Table 4). In addition, these treatments produced 1.2 $\mu mol\,g^{-1}d^{-1}$ of dinitrogen gas (Fig. 3A), indicative that 74–83% of the nitrate was denitrified, considering a 2:1 stoichiometry $(NO_3^- : N_2)$. Nitrite was produced as an intermediate during nitrate reduction, and glucose microcosms showed a transient nitrite accumulation of 3.2 $\mu mol\,g^{-1}$ (data not shown). Fe(III) reduction only occurred following nitrate depletion, and glucose treatments produced the highest Fe(II) accumulation rates (1.44 $\mu mol\,g^{-1}d^{-1}$; Fig. 2A and Table 4). Supplemental glucose and ethanol were completely consumed and yielded primarily acetate, butyrate, and propionate or acetate, respectively, as end products (Fig. 4A). The majority of H_2 and lactate was not consumed (Fig. 2A).

The carbon recovery in glucose amended treatments averaged 82% (96% if using peak CO_2 produced) at the end of

incubation, 44% of which were recovered in fermentation products. Approximately 22% of C equivalents were utilized for complete denitrification based on a stoichiometry of $5C : 4NO_3^-$, and 5% of C equivalents were utilized for Fe(III) reduction based on a stoichiometry of 1C:4Fe(II). Ethanol-amended treatments had an average recovery of 78% (85% if using peak CO_2 produced), and 81% of carbon added was utilized by respiration processes (68%—denitrification; 12%—Fe(III) reduction). Of the total carbon added, 14% was detected in acetate at the end of the incubation.

In acidic microcosms (II-3; $\sim$19.7–25.6 $\mu mol\,g^{-1}$ nitrate), minimal CO_2- or Fe(II)-producing activity was observed in control or amended sediments. Minimal nitrate was reduced, and no utilization of carbon substrates was evident (Table 4). However upon neutralization of these acidic sediments (II-4), complete utilization of both glucose and ethanol was observed (Figs. 2–4 and Table 4). Glucose
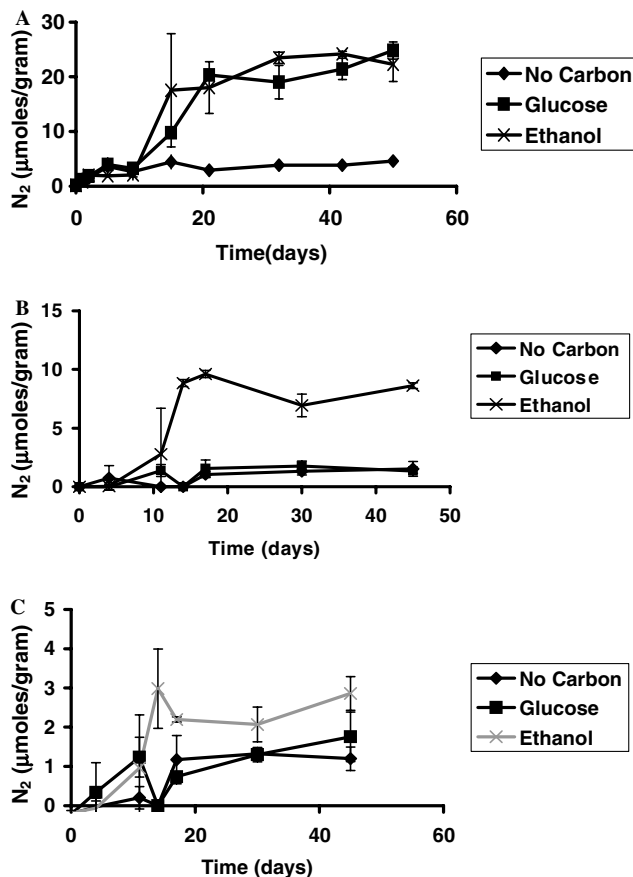
Fig. 3. Accumulation of N_2 from denitrification in microcosms of near neutral pH (II-2; A), neutralized acidic sediments (II-4; B), and washed neutralized acidic sediments (II-6; C). Values include N_2 measured in the headspace and calculated in aqueous phase.

treatments yielded 15–20 μmol H_2 production (data not shown). The highest CO_2 production rates were found in glucose treatments within the first 17 days ($11.5\,\mu mol\,g^{-1}\,d^{-1}$), while lower rates relative to control treatments were observed with ethanol addition (control $= 0.6\,\mu mol\,g^{-1}\,d^{-1}$ and ethanol $= 4.9\,\mu mol\,g^{-1}\,d^{-1}$). Nitrate was almost completely reduced in the ethanol treatments at a rate of $2.82\,\mu mol\,g^{-1}\,d^{-1}$ (Fig. 2B), and dinitrogen gas was produced in stoichiometric amounts from nitrate reduced (1:2 ratio; Fig. 3B). In glucose-amended treatments, nitrate was rapidly reduced ($1.68\,\mu mol\,g^{-1}\,d^{-1}$; Fig. 2B), but little dinitrogen gas was produced (Fig. 3B). Ammonium accumulated in the porewater of glucose-amended treatments up to 1 mM during nitrate reduction (data not shown). Increased Fe(II) production was observed in glucose treatments ($0.28\,\mu mol\,g^{-1}\,d^{-1}$) in comparison to control and ethanol treatments (0–$0.04\,\mu mol\,g^{-1}\,d^{-1}$; Fig. 2B and Table 4).

In microcosm II-4, 72% of glucose amended carbon and 109% of ethanol amended carbon was recovered as CO_2 or fermentation products (Fig. 4B). In glucose amended microcosms, nitrate reduction comprised 6–12% of carbon utilization (assuming dissimilatory nitrate reduction to ammonium, with stoichiometry of $1C:1NO_3$), and Fe(III)

reduction comprised 2% of carbon utilization (Figs. 2B, 3B, and 4B). When glucose was totally consumed , 58% of carbon was accounted for as fermentation products, mainly as acetate. In ethanol amended treatments, 19–31% of carbon added was utilized for denitrification, with only 0.2% used for Fe(III) reduction, and 94% of carbon was present as acetate or butyrate at the end of the incubations (Figs. 2B, 3B, and 4B).

Washing the sediments in microcosms II-5 and II-6 removed nitrate and presumably toxic metals while increasing the pH to near neutral (Table 1). In microcosm II-5 (washed acidic sediment) minimal activity was observed in control and glucose treatments (Table 4). Nitrate reduction was only observed in ethanol treatments. In those treatments, nitrate (2–4 $\mu mol\,g^{-1}$ sediment; Table 1) was completely reduced at a low rate of $0.25\,\mu mol\,g^{-1}\,d^{-1}$ (Table 4); and 3 $\mu mol\,g^{-1}$ of dinitrogen gas was produced, indicative of complete nitrate reduction. Glucose treatments and controls of II-5 showed a similar lack of activity as seen in II-3. Little carbon substrate utilization, CO_2 production or Fe(III) reduction occurred in any treatments of the washed, acidic sediments in the microcosms of experiment II-5 (Table 4).

Microcosm experiment II-6 (washed, neutralized acidic sediment) showed activity patterns similar to that in II-4, with glucose and ethanol treatments displaying the highest nitrate reduction rates, and the glucose treatments having the highest Fe(III) reduction rates (Table 4). The highest CO_2 production rate from glucose treatments occurred within the first 17 days ($11.1\,\mu mol\,g^{-1}\,d^{-1}$; rate over entire incubation time $= 2.3\,\mu mol\,g^{-1}\,d^{-1}$). Nitrate reduction occurred in glucose and ethanol bottles at 0.22 and $0.32\,\mu mol\,g^{-1}\,d^{-1}$, respectively (Table 4). The majority of nitrate went to complete denitrification in all treatments of experiment II-6 (Fig. 3C). While glucose was totally consumed yielding fermentation products, minimal ethanol was consumed (Figs. 2C and 4C). During glucose consumption, 29.1 $\mu mol\,g^{-1}$ H_2 was formed, and Fe(II) production rates were enhanced in comparison to control and ethanol treatments to $0.82\,\mu mol\,g^{-1}\,d^{-1}$ (Table 4). This rate was the highest observed Fe(II) production from stimulated acidic sediments.

Microcosm II-6 showed a carbon recovery balance of 94% with glucose amended treatments, while only negligible ethanol was utilized in ethanol amended treatments (Fig. 4C). Glucose amended treatments utilized 1% of added carbon equivalents to incompletely reduce nitrate, and 3% of carbon to reduce 37.4 $\mu mol\,g^{-1}$ of Fe(III). 60% of initial carbon was present at the end of the incubation as fermentation products.

4. Discussion

The dominant contaminants in ORFRC sediment include radionuclides (uranium and technicium), toxic metals (nickel, aluminum, barium, chromium, mercury), chelating agents (EDTA), chlorinated hydrocarbons (TCE, perchloroethylene), polychlorinated biphenyls, and fuel hydrocarbons (toluene, benzene) (Brooks, 2001; ERSP website
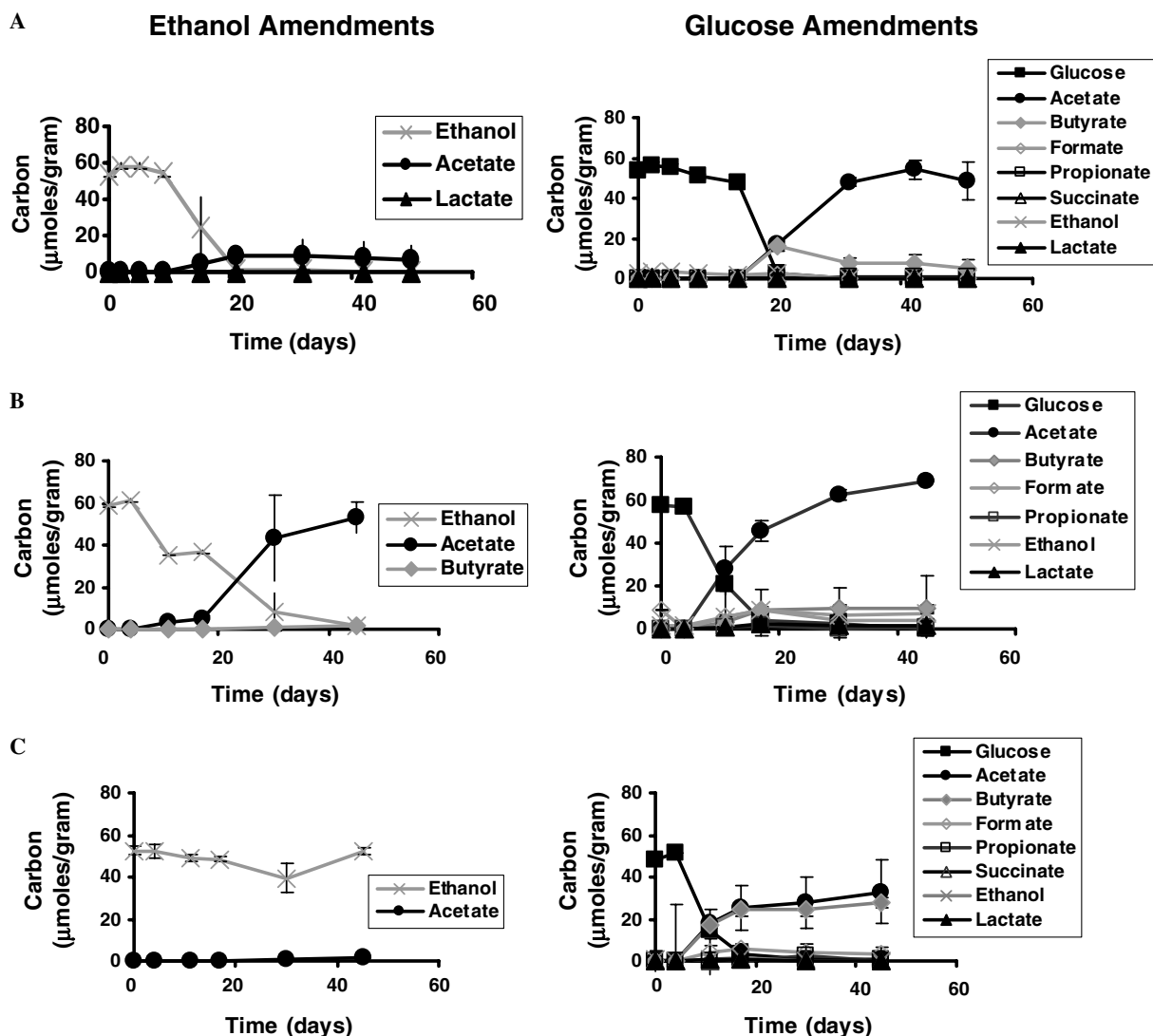
Fig. 4. Utilization of carbon substrates in neutral sediment microcosms (A; II-2) as compared to neutralized acidic sediment microcosms (B; II-4) and washed neutralized microcosms (C; II-6) showing ethanol (first panel) and glucose (second panel) amendments.

http://www.lbl.gov/NABIR/). The combination of a low pH and high concentrations of nitrate and radionuclides in a subsurface environment is representative of many sites within the U.S. nuclear weapons complex as well as radionuclide-contaminated sites in eastern Europe (Riley and Zachara, 1992; NABIR Bioremediation Primer, 2003; Moon et al., 2006). Thus, our results are applicable to more than only bioremediation research at the ORFRC. Through quantification of the rates and pathways of TEAPs in acidic subsurface sediments, we provide improved inputs for reaction-based biogeochemical models that will aid in the development of radionuclide remediation strategies.

4.1. Terminal-Electron-Accepting Processes (TEAPs) in the acidic subsurface

We hypothesized that regardless of the electron acceptor and donor availability, microbial metabolism would be limited by low pH in the contaminated ORFRC subsur-

face. Our hypothesis was confirmed as microbial activity was minimal at pH 5 or below and could not be stimulated by adding electron donors (Table 4). Biostimulated activity was observed upon raising the pH to neutral conditions (Figs. 2–4). As expected based on thermodynamic and geochemical considerations (Chapelle, 2000), nitrate and Fe(III) reduction were the predominant TEAPs observed in ORFRC sediments under conditions relevant to bioremediation, and in concurrence with previous work, metal reduction in the acidic subsurface did not occur until nitrate was depleted to low levels (Fig. 2; Finneran et al., 2002a,b; Senko et al., 2002). In microcosms of ORFRC sediments at near neutral pH or those neutralized by bicarbonate addition, nitrate or Fe(III) reduction rates were among the most rapid reported for neutral subsurface environments (Snoeyenbos-West et al., 2000; Elias et al., 2003; Istok et al., 2004) or aquatic sediments (Roden and Wetzel, 1996; Thamdrup, 2000). Although we cannot rule out the importance of other TEAPs, little to no Mn(II) or methane

accumulated in microcosm porewaters, and sulfate concentrations were below 0.2 µmol g^{-1} in microcosm porewaters (data not shown).

A primary concern in the remediation of metal contaminants in the acidic subsurface is the extremely high nitrate concentrations (0.3–0.5 M at ORFRC; ERSP website http://www.lbl.gov/NABIR/) that often accompany low pH in uranium-contaminated areas (Riley and Zachara, 1992; Brooks, 2001; NABIR Bioremediation Primer, 2003; Moon et al., 2006) and may inhibit microbial activity. In field bioremediation tests and sediment incubations with materials from the ORFRC, relatively slow rates of nitrate reduction were observed under moderately acidic conditions (pH 3–5; Shelobolina et al., 2003; Istok et al., 2004). Our results indicate that microbial communities were inhibited by low pH rather than by high nitrate levels. In other words, the extremely high nitrate concentrations present in the ORFRC subsurface do not appear to be toxic to microorganisms. Upon neutralization of acidic sediments or in pH neutral sediments from other areas of the ORFRC, rapid nitrate reduction rates were observed despite high initial nitrate concentrations ranging from 20 to nearly 500 µmol g^{-1} when ethanol or glucose were added as electron donor (Figs. 1 and 2). Thus, increased rates of nitrate reduction can be achieved in acidic subsurface sediments through neutralization in conjunction with added electron donor, as supported by recent field results (Istok et al., 2004; North et al., 2004).

In addition to metabolic rates, the electron-accepting pathways of subsurface sediments appear to be controlled by pH. In microcosms of sediments with initial neutral pH, complete denitrification occurred according to the stochiometric production of dinitrogen gas (Fig. 3). Microcosms of neutralized acidic sediments revealed that the majority of nitrate was reduced by denitrification only in ethanol treatments, while dissimilatory nitrate reduction to ammonium (DNRA) predominated in glucose-amended microcosms. The two processes of nitrate reduction are executed by different bacterial groups. Competition for carbon substrates can determine which of the processes is favored. Since fermentative bacteria are responsible for the DNRA proceeding through nitrite, the addition of glucose to neutralized acidic sediments might have favored the activity of fermentative nitrate reducers. In general, high soil pH favors both nitrite accumulation and DNRA (Stevens et al., 1998).

The majority of cultivated metal-reducing bacteria are incapable of denitrification and preferentially reduce nitrate to ammonium (DiChristina, 1992; Lovley et al., 2004). Thus, the shift to DNRA suggests an overlap between nitrate- and Fe(III)-reducers which might be advantageous for bioremediation, since abundant nitrate could support the growth of a larger population of Fe(III)-reducers. Indeed, an increase in the sequences of members of the *Deltaproteobacteria*, mainly *Anaeromyxobacter* and *Geobacter* groups was observed after biostimulation by glucose or ethanol addition (Peacock et al., 2004; Istok et al., 2004;

North et al., 2004), both of which are known Fe(III)-reducing organisms capable of dissimilatory nitrate reduction to ammonium (Lovley et al., 2004).

The highest Fe(III) reduction rates measured in pH neutral or neutralized acidic ORFRC stimulated microcosms (0.01–1.4 µmol g^{-1} d^{-1}) were up to six times higher than the highest rates observed in pH neutral, uranium-contaminated aquifer sediments (0.021–0.226 µmol g^{-1} d^{-1}; Elias et al., 2003) or in sediments from acidic coal mining lakes (0.425 µmol Fe(II) g^{-1} d^{-1}; Peine et al., 2000). In fact, Fe(III) reduction rates from stimulated acidic sediment microcosms were well within the range of those measured in surficial organic-rich aquatic sediments (Thamdrup, 2000). However, few studies have determined Fe(III) reduction rates in subsurface sediments (Snoeyenbos-West et al., 2000; Finneran et al., 2002a,b; Elias et al., 2003; Shelobolina et al., 2003), and no *in situ* rates are available from the acidic subsurface. Nitrate reduction and electron donor utilization rates of this study were in the range of those of field studies conducted at the ORFRC (Istok et al., 2004; North et al., 2004), suggesting that the sediment microcosms represented the *in situ* conditions reasonably well. Reactive transport models relying on Fe(III) reduction rates determined with pure culture studies are currently used to develop bioremediation strategies for metal contaminants at the ORFRC and other sites managed by the DOE. Fe(III) reduction rates obtained from ORFRC sediments with indigenous microbial communities will better mimic *in situ* conditions.

4.2. Electron donor utilization in acidic subsurface sediments

Microbial growth is impacted under acidic conditions as enzymatic activity is lowered (Langworthy, 1978; Baronofsky et al., 1984; Kobayashi et al., 2000). Decreased metabolic activity can lead to an accumulation of carbon intermediates in acidic sediments (Goodwin and Zeikus, 1987; Hines et al., 2001; Horn et al., 2003), and ORFRC groundwaters contain high concentrations of non metabolized short chain organic acids (0.49 mM acetate and 0.11 mM citrate; Shelobolina et al., 2003). Stress imposed by low pH can also cause a decrease in the abundance and diversity of microorganisms in acidic subsurface environments (Petrie et al., 2003; Reardon et al., 2004; Fields et al., 2005). Acidity affected both the rates (Table 4) and pathways (Fig. 3) of microbial metabolism here, as indicated by the differences observed in moderately acidic versus neutral pH ORFRC sediment microcosms. At an *in situ* pH of below 5, little to no glucose fermentation was observed in contaminated ORFRC sediment microcosms (Table 4), similar to acidic mining-impacted sediments (Küsel et al., 1999; Küsel and Dorsch, 2000). Thus, microbial communities were inhibited by acidity which apparently is a master variable in the contaminated ORFRC sediments.

Even relatively low organic acid concentrations can have a significant impact on the pH generating system of cells at

low external pH (Goodwin and Zeikus, 1987). The effect of organic acids on the transmembrane pH gradient has been attributed to their ability to diffuse across the cell membrane in an undissociated form in response to the difference in pH between the two sides acting as uncoupler for proton motive force (Goodwin and Zeikus, 1987). Fermentors like *Clostridium acetobutylicum* concentrate acetate and butyrate within the cell under acidic conditions and lower the intracellular pH. The high intracellular butyrate concentration leads to induction of water retention, thereby circumventing a decrease in the intracellular pH great enough to be deleterious to the cell (Gottschal and Morris, 1981; Huang et al., 1986). However, both acidic and neutral fermentation products have been shown to interfere with membrane related functions such as energy generation which are responsible for generation of the transmembrane pH gradient. Butanol has been shown to increase lipid fluidity and to inhibit some membrane-related functions, such as ATPase activity and nutrient transport systems, in *C. acetobutylicum*. Thus, the failure to maintain a high intracellular pH in acidic subsurface sediments caused by damage to the proton pump or to the shortage of energy might explain the low activities observed in acidic microcosms.

Electron donors were observed to stimulate electron-accepting pathways in the following order: glucose > ethanol > lactate > hydrogen. Ethanol and glucose have been the primary electron donors considered in bioremediation tests at the ORFRC (Istok et al., 2004; North et al., 2004; Peacock et al., 2004). Rates of donor utilization were equally high whether the initial sediment pH was near neutral or acidic sediment was neutralized with bicarbonate (Tables 3 and 4). The majority of sediments containing high levels of uranium-contamination are acidic at the ORFRC and thus conditions in neutralized acidic microcosms would be most relevant for bioremediation treatment of the source zone. In neutralized acidic microcosms, 80–90% of C equivalents theoretically obtained from glucose were recovered as fermentation products, mainly as acetate (Fig. 4). The majority of added ethanol was completely oxidized in neutral pH microcosms, but ethanol was not consumed or only incompletely oxidized to acetate in washed neutralized or neutralized sediment microcosms, respectively. Clearly, biostimulation of acidic subsurface sediments with either ethanol or glucose would lead to mostly incomplete carbon oxidation. Acetate accumulated even in some cases when Fe(III) was available at neutral pH, which is in contrast to metal reduction studies conducted in neutrophilic uranium contaminated aquifers or aquifer materials (Senko et al., 2002; Finneran et al., 2002a,b; Anderson et al., 2003).

During biostimulation, carbon substrates will not be limiting and metal reduction may be catalyzed in parallel by respiratory and fermentative pathways. The abundance of sequences of known fermentative metal-reducing bacteria (*Serratia*, *Clostridium*) in acidic ORFRC sediments doubled in response to pH neutralization and biostimulation (North et al., 2004). Further, 16S rRNA gene sequences affiliated with fermentative organisms (*Paenibacillus*, *Brevibacillus*, *Anaerovibrio*) occur in parallel with sequences of known respiratory FeRB (*Anaeromyxobacter*, *Geobacter*) in cultures of Fe(III)-reducing bacteria enriched from the same ORFRC subsurface (Petrie et al., 2003; North et al., 2004). Thus, fermentative pathways should be considered in addition to respiration pathways when designing bioremediation strategies for metal contaminants in the acidic subsurface.

In microcosms of neutralized acidic sediments, ethanol appeared to be utilized primarily for denitrification, while DNRA was favored by glucose addition. Members of the *Deltaproteobacteria* may be sensitive to high levels of ethanol, such that they were outcompeted by non metal-reducing denitrifiers in the ethanol treatments. Complete denitrification could be less advantageous in remediation efforts as large amounts of nitrogen gas could clog the aquifer by occupying pore spaces. Further, glucose treatments of neutralized acidic sediments showed the highest Fe(III) reduction rates, implying a high potential for metal (including uranium) reduction. Therefore, glucose might be more effective than ethanol as an electron donor for radionuclide remediation in acidic subsurface sediments.

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