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Changes in bacterial community structure correlate with initial operating conditions of a field-scale denitrifying fluidized bed reactor

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Abstract High levels of nitrate are present in groundwater migrating from the former waste disposal ponds at the Y-12 National Security Complex in Oak Ridge, TN. A field-scale denitrifying fluidized bed reactor (FBR) was designed, constructed, and operated with ethanol as an electron donor for the removal of nitrate. After inoculation, biofilms developed on the granular activated carbon particles. Changes in the bacterial community of the FBR were evaluated with clone libraries (n=500 partial sequences) of the small-subunit rRNA gene for samples taken over a 4-month start-up period. Early phases of start-up operation were characterized by a period of selection, followed by low diversity and predominance by *Azoarcus*-like sequences. Possible explanations were high pH and nutrient limitations. After amelioration of these conditions, diversification increased rapidly, with the appearance of *Dechloromonas*, *Pseudomonas*, and *Hydrogenophaga* sequences. Changes in NO_3, SO_4, and pH also likely contributed to shifts in community composition. The detection of sulfate-reducing-bacteria-like sequences closely related to *Desulfovibrio* and *Desulfuromonas* in the FBR have important implications for downstream applications at the field site.

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Introduction

Uranium is a major groundwater contaminant at the US Department of Energy (DOE) NABIR Field Research Center (FRC) on the DOE Oak Ridge Reservation in eastern Tennessee. The sites are also characterized by acidic conditions (pH 3.5), high concentrations of nitrate (up to 160–200 mM), various heavy metals, and other contaminants. A possible strategy for remediation is pH adjustment followed by subsurface delivery of an electron donor to stimulate biological reduction and immobilization of U(VI). A serious obstacle to the in situ reduction of U (VI) is the high nitrate levels at the site. Denitrification intermediates inhibit U(VI) reduction (Senko et al. 2002) and can also cause copious production of biomass and nitrogen gas in the subsurface that would decrease hydraulic control if denitrification occurred in situ. A two-phased approach is currently being used at the FRC to deal with these conditions. The first phase includes neutralization of the groundwater pH and aboveground removal of nitrate, chlorinated solvents, Ca, and Al. The second phase involves recirculation of groundwater supplemented with an electron donor to stimulate microbial growth, including denitrification of residual nitrate and uranium reduction in situ (Luo et al. 2005; Wu et al. 2005).

To remove bulk nitrate, a field-scale denitrifying fluidized bed reactor (FBR) was designed and constructed with granular activated carbon (GAC) as a carrier. In operation, contaminated groundwater is amended with ethanol and pumped through the FBR, and the GAC served as a substratum for microbial biofilms. The treated water can be reinjected into the subsurface and supplemented with an electron donor to further reduce residual subsurface nitrate and to facilitate in situ U(VI) reduction. In this configuration, the FBR functions as a possible source of microorganisms for subsurface denitrification and U(VI) reduction. Recent research demonstrated that the biomass from a pilot-scale denitrifying FBR had U(VI) reduction capability (Wu et al. 2005). Although FBRs are widely used for a range of applications, including denitrification

(US EPA 1993), the microbial population dynamics are not well understood (particularly during initiation).

The objectives of the study were to investigate the relationships between operating conditions and the microbial community structure during start-up and operation to a stable steady state. Bacterial biofilms grown on GAC carrier particles were characterized via small-subunit (SSU) rRNA gene sequences at multiple time points over a 4-month period. The results suggested that a minor subset of the inoculum was initially established, but diversity increased over time during operation. The in-depth analysis provides insight into the conditions needed to achieve a stable community at the FRC.

Materials and methods

Anaerobic denitrifying FBR system

A schematic diagram of the FBR and related treatment system is shown in Fig. 1. Groundwater from extraction wells was pumped to a groundwater storage tank (26.5 m^3), to a vacuum stripper to remove volatile substances, and then to a two-step precipitation system to remove Al^{3+} and Ca^{2+}. The treated water was recarbonated to pH 6.5. Ethanol feed solution (10%, w/w) and nutrient solutions were pumped from two separate tanks (190 l) into the FBR. The nutrient solution contained (g l^{-1} tap water) KH_2PO_4, 4.0; $FeCl_3{\cdot}4H_2O$, 0.3; $ZnSO_4{\cdot}7H_2O$, 0.1; $MnSO_4{\cdot}H_2O$, 0.085; HBO_3, 0.06; $CoCl_2{\cdot}6H_2O$, 0.02; $CuSO_4$, 0.004; $NiSO_4{\cdot}6H_2O$, 0.028; and $Na_2MoO_4{\cdot}2H_2O$, 0.04. The solution was adjusted to a pH less than 3.0 by adding 0.3 ml of HCl (20%) per liter of tap water. The pump rate of the nutrient solution was proportional to the flow rate of groundwater at 1.5 ml l^{-1} of groundwater. Industrial-grade ethanol (88.12% ethanol and 4.65% methanol; Aaper Alcohol & Chemical Co., Shelbyville, KY) was diluted to give a 10% (vol/vol) solution in tap water, resulting in a chemical oxygen demand (COD) concentration of 163 g l^{-1}. The feed rate of ethanol was adjusted daily based on the groundwater feed rate and measured nitrate concentration to maintain a ratio of 4.2:1 as g COD g^{-1} NO_3–N. The FBR was constructed from fiberglass with the following dimensions: height, 3.05 m; diameter, 0.76 m; approximate fluidization volume, 0.5 m^3; and total system volume, 1.6 m^3. GAC (Calgon Type MRX, Calgon Carbon Corp, Pittsburgh, PA) was used as carriers for microbial attachment and growth. Fluidization flow rate was 31.7 m^3 m^{2-1} h^{-1} (14.4 m^3 h^{-1}), which ensured 100% fluidization of the GAC bed. The reactor was operated at ambient temperature conditions (15–30°C) throughout the test period. As more biofilm grew on GAC, the GAC carrier became lighter and entered the upper regions of the reactor. A biofilm growth control device installed at the top of the reactor mechanically sheared biofilm from the particles. Sheared biofilm and GAC particles were separated in a GAC carrier separator, and GAC was recycled back to the FBR. The suspended biomass in the effluent of the FBR was largely removed in a filtration system consisting of two-stage filters. Particle-free treated water was then pumped into the treated water storage tank for subsequent reinjection into the aquifer.

Tap water was used to fill the FBR for hydraulic tests prior to start-up. During start-up, groundwater was pumped to the system to gradually replace the make-up water (approximately 31 m^3). The tap water had a pH of 8.0–8.5 and contained (mg l^{-1}): chloride, 100–120; nitrate, 2.5–3.0; sulfate, 23–25; Ca, 27–30; and Al, <0.2. On day 1, $NaNO_3$ and the industrial-grade ethanol solution were added directly to the reactor to obtain an initial concentration of 6 mM nitrate and 340 mg COD l^{-1}. The reactor was then inoculated with 60 l of denitrifying sludge (0.5 g l^{-1}) from the Y-12 wastewater treatment plant (300 m from the site) and 700 ml of GAC carriers with biofilm from a 34-l FBR at Stanford University that had been fed with synthetic

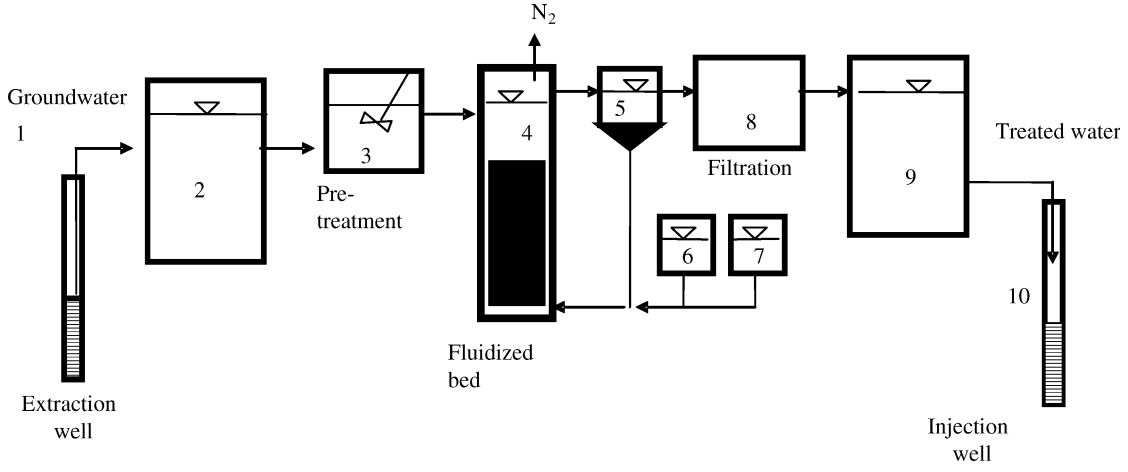

Fig. 1 Schematic diagram of fluidized bed reactor system for removal of nitrate from contaminated groundwater. *1* indicates extraction well; *2*, groundwater storage tank; *3*, pretreatment units (VOC removal, pH adjustment, and dissolved Al and Ca removal); *4*, fluidized bed reactor; *5*, GAC carrier separator; *6*, ethanol solution tank; *7*, nutrient solution tank; *8*, filtration treatment; *9*, treated water storage tank; and *10*, injection well

wastewater containing ethanol and lactate (1:1 as COD) and operated at pH 7.35–7.5 (Wu et al. 2005).

The inoculation contained approximately 30 g of biomass from the two samples. For 48 h, the reactor was maintained in recirculation mode without added influent to facilitate microbial attachment to the carrier. The reactor then received make-up water supplemented with a $NaNO_3$ stock solution (59 g l^{-1} as NO_3–N), trimetaphosphate (0.29 g l^{-1} as P), and ethanol at a COD to NO_3–N ratio of 4.20:1. Make-up water was added continuously for 28 days. Measurements of nitrate in the reactor influent indicated that groundwater eventually displaced the tap water and arrived at the last storage tank of the pretreatment system on day 31. Feeding of $NaNO_3$ stock solution was then stopped. Ethanol was fed to the FBR continuously, and the feed rate was adjusted based on the incoming nitrate concentration to maintain a COD/NO_3–N ratio sufficient for efficient nitrate removal [effluent nitrate (<1.0 mM) and low effluent COD (<30 mg l^{-1})]. The clean treated water was reintroduced to the subsurface. Samples from the FBR and pretreatment system were analyzed daily for nitrate, COD, and pH.

Samples of biofilm-coated GAC were withdrawn from the upper portion of the fluidized GAC bed (about 0.8–1.0 m above the reactor bottom). Previous characterization of the bench-scale FBR indicated that planktonic and biofilm communities were similar; therefore, composite samples consisted of both the liquid fraction and the biofilm. The recirculation rate required for fluidization was sufficient to ensure well-mixed conditions throughout the reactor. Thus, the collected samples were representative of other locations within the reactor.

DNA extraction and purification

GAC particle/biofilm samples were collected and stored at −80°C. DNA was extracted from the initial, pilot-scale FBR inoculum and samples from operational days 12, 34, 48, 69, and 118. Samples were suspended in a lysis buffer, and the cells were disrupted by two cycles of freeze–thaw and grinding with sterile sand (Zhou et al. 1996). DNA was extracted and purified with a PowerSoil DNA Isolation Sample Kit (MO BIO, Carlsbad, CA) following the manufacturer's protocol.

Polymerase chain reaction amplification and cloning

The SSU rRNA genes were amplified in a PTC-200 Peltier Thermal Cycler (MJ Research, Bio-Rad, Weltham, MA) with the primer pair FD1 F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1540R (5′ AAG GAG GTG ATC CAG CC 3′). The polymerase chain reaction (PCR) (25 μl) contained 12.5 μl BioMix (Bioline, Randolph, MA), 1 μl each primer, 9.5 μl sterilized Milli-Q water, and 1 μl purified DNA (5–10 ng). To minimize PCR-induced artifacts, the optimal number of cycles was determined, and five PCR reactions were combined prior to cloning as described previously (Qiu et al. 2001). The PCR parameters were as follows: 80°C for 1.5 min; 94°C for 2 min; 94°C for 30 s; 58°C for 1 min; 72°C for 1 min; 20 cycles; 72°C for 7 min. An aliquot of 5 μl of PCR product was run in a 0.8% agarose Tris-acetate EDTA (TAE) gel stained with ethidium bromide to evaluate the quality of the amplified fragment.

PCR products of the SSU rRNA genes were purified using a Promega kit (Promega, Madison, WI). The purified fragments were cloned using the vector PCR 2.1-TOPO and *Escherichia coli* DH5α competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). From each of the six libraries, 48–144 white colonies were randomly selected, and the cloned inserts were amplified with vector-specific primers M13 forward and M13 reverse (30 cycles of the PCR parameters described above, but with an annealing temperature of 60°C and a final extension time of 10 min). The resulting amplification products were analyzed as described above.

Sequence and phylogenetic analysis

Polymerase chain reaction products (20 μl) amplified with vector-specific primers were purified with a Montage PCR_{u96} plate according to the manufacturer's instructions (Millipore, Bedford, MA). DNA sequences were determined with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions at 1:4 dilutions with an internal primer (529r, *E. coli* designation) as previously described (Fields et al. 2005). Sequence reaction products were analyzed on the ABI model 3100 and 3730 DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequences were assembled and edited using the Sequencher program (v. 4.0, Gene Codes Corporation, Ann Arbor, MI).

The SSU rDNA sequence identification was performed with the nucleotide–nucleotide basic local align search tool (BLASTn) server (http://www.ncbi.nlm.nih.gov/BLAST) and the Ribosomal Database Project (RDP)-II http://rdp.cme.msu.edu/index.jsp). Unique SSU rDNA clones from each library were identified and designated as OTUs (operational taxonomic units) at a 97% sequence identity level. The sequences were aligned with ClustalW (Thompson et al. 1994). Phylogenetic trees were constructed with the unweighted pair group method with arithmetic mean (UPGMA) and a Kimura 2-parameter model, and bootstrap analysis was performed with programs within MEGA version 2.1 (Kumar et al. 2001). Rarefaction analyses indicated that a majority of the sampled diversity was obtained within approximately 50 clones for each time point (data not shown).

Data analyses

Species diversity was calculated from the distribution of unique OTUs in the clone libraries using indices for species richness (Shannon–Wiener and reciprocal of Simpson's

index) and evenness (equitability) computed as described previously by Krebs (Brown and Bowman 2001).

Library shuffling (LIBSHUFF) analysis computes the homologous and heterologous coverage within and between clone libraries (Singleton et al. 2001). The analysis estimates the similarity between clone libraries from two different samples based upon evolutionary distances of all sequences. Thus, the sampled diversity of a community can be directly compared to another community. The predicted coverage of a sampled library is denoted by the homologous coverage, and the heterologous coverage is the observance of a similar sequence in a separate library. The values are reported over a sequence similarity range or evolutionary distance (*D*) based upon a distance matrix. Analyses were performed according to specified directions given at the LIBSHUFF website http://www.arches.uga.edu/~whitman/libshuff.html). Principal component analysis (PCA) used the programs in the SYSTAT statistical computing package (v. 9.0, SPSS, Inc., Chicago, IL).

Chemicals

Chemicals used for the FBR were industrial grade. $NaNO_3$ granules and solutions of K_2CO_3 (50%, w/w) and HCl (20%, w/w) were from The Dycho Company, Niota, TN. Industrial ethanol (88.12% ethanol and 4.65% methanol, w/w) was obtained from Aaper. All chemicals used for laboratory tests were analytical grade.

Analytical methods

U(VI) concentration in all groundwater samples was determined by kinetic phosphorescence analysis using a KPA-11 analyzer (ChemChek Instruments, Richland, WA) (Brooks et al. 2003). Chemical oxygen demand concentration and sulfide were determined using Hach method 430 and method 690, respectively, with a Hach DR 2000 spectrophotometer (Hach Chemical, Loveland, CO). Anions (including NO_3^-, Cl^-, SO_4^{2-}, and PO_4^{3-}) were analyzed with an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex DX-120, Sunnyvale, CA). Cations (Al, Ca, Fe, Mn, Mg, K, etc.) were determined using an inductively coupled plasma (ICP) atomic emission spectrophotometer (Thermo Jarrell Ash PolyScan Iris Spectrometer, Genesis Laboratory Systems, Grand Junction, CO; Gu et al. 2003).

Results

FBR start-up and operation

Operational results for days 1–150 are summarized in Fig. 2. Between days 3 and 31, the reactor received nitrate-amended tap water; between days 32 and 150, the reactor received FRC groundwater. Treated water was stored in the treated-water storage tank and then injected into the subsurface, and the influent nitrate decreased from 200 mM to

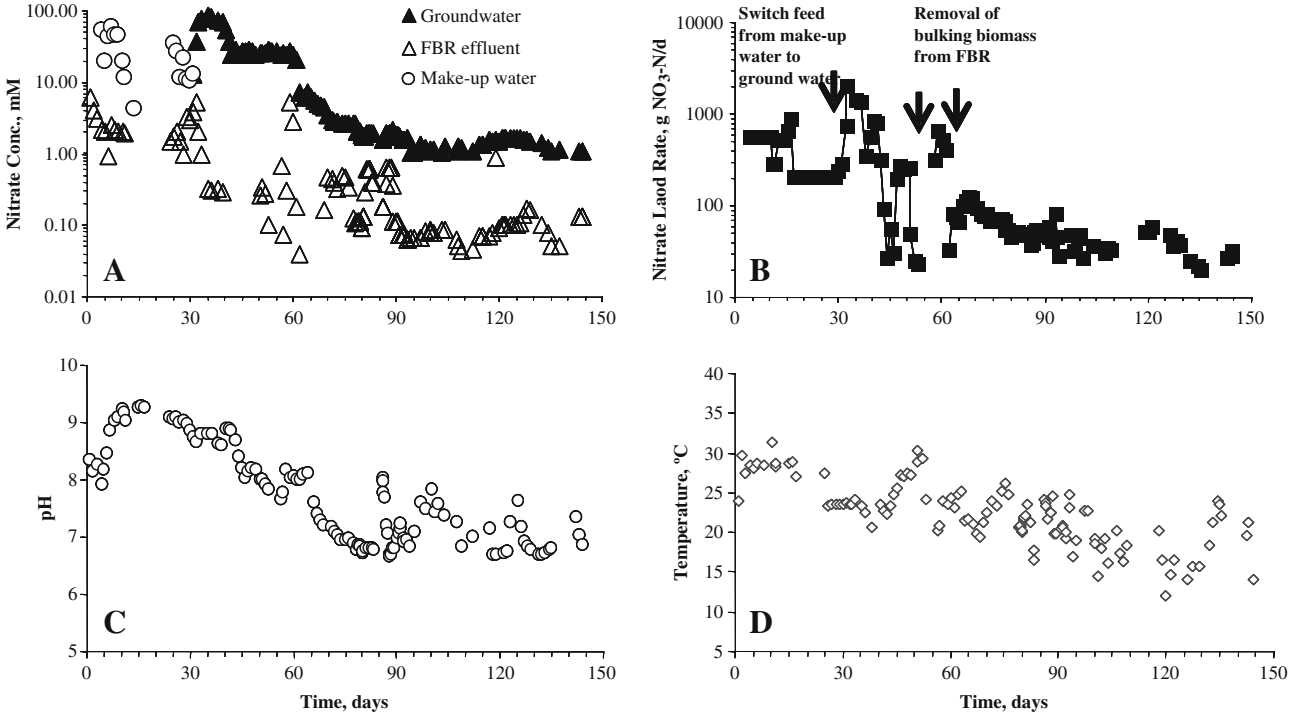

Fig. 2 Operational performance of FBR. **a** Influent and effluent nitrate concentrations. **b** Nitrate loading rates. **c** Change of pH during operational period. **d** Change of temperature during operational period

below 1.0 mM over the 150-day period (data not shown). Low-effluent nitrate was needed to create the low nitrate environment necessary for U(VI) reduction (Wu et al. 2005; Gu et al. 2005; Luo et al. 2005). During the first 60 days of start-up, the reactor received influent with relatively high nitrate concentrations, and biofilm attachment and growth was observed on the GAC carrier. The average hydraulic retention time was approximately 48 h. After the biofilm developed, stable performance was observed from days 61 to 150. The influent nitrate was approximately 2–6 mM, and the effluent nitrate was less than 0.1 mM. After day 150, the treatment system was operated intermittently.

Changes in nitrate concentration in the FBR influent are indicated in Fig. 2a. On day 1, the FBR contained make-up water supplemented with sodium nitrate at 6.0 mM and ethanol (340 mg l^{-1} as COD). The reactor was then inoculated with denitrifying cultures and maintained in recirculation mode without additional electron donor or nitrate for 48 h. Gas bubbles appeared at the surface of the reactor 8 h after inoculation and suggested active denitrification as previously described (Gentile et al., 2005). Operation in a recirculation-only mode continued until nitrate concentration in the FBR decreased from 6.0 mM to less than 2.0 mM. On day 3, the FBR received make-up water supplemented with $NaNO_3$. At the same time, groundwater (up to 200 mM nitrate) was pumped continuously to the storage tank and gradually entered the pretreatment system. Movement of the groundwater through the pretreatment system was monitored by tracking nitrate concentrations that varied between 10 and 60 mM. By day 31, make-up water had flushed out of the pretreatment system, and the concentration of nitrate entering the FBR increased from close to 0 to 12 mM. Subsequently, influent nitrate concentration increased to a peak of 80 mM on day 35 (Fig. 2a). Thereafter, it gradually decreased from 80 to 24 mM on day 44 and remained at this level until day 60. Finally, nitrate concentrations declined to the 2.5-mM level on day 75 and declined further to approximately 1.0 mM on day 150. The decrease in influent nitrate concentration was the result of belowground recirculation and dilution. Eventually, subsurface nitrate was diluted by the injection of treated clean water, so the influent nitrate progressively declined. During the initial 30 days (when make-up water was provided), the nitrate concentration in the FBR effluent was stable at 1.0–5.0 mM, and thereafter remained at 1.0 mM or less to meet the needs of subsurface operations except days 31–33 and days 59–60 (described below).

The nitrate loading rate is shown in Fig. 2b. From days 3 to 31, the FBR received tap water supplemented with a $NaNO_3$ solution, and the nitrate loading rate was 500–800 g NO_3–N per day or higher. It was maintained at 200 g d^{-1} between days 17 and 30. On day 31, groundwater reached the FBR, and the $NaNO_3$ supplementation was stopped. The nitrate loading rate increased to 700–1,500 g day^{-1} as groundwater that contained high nitrate concentrations (up to 80 mM) entered the system, and this resulted in a relatively high effluent nitrate concentration. Accordingly, for days 34–59, the groundwater feed rate was reduced to decrease the nitrate loading rate to 50–200 g day^{-1}. During this period, the biofilm grown on GAC carrier was unstable, and biomass floated to the top (as described below). Operation of the FBR was stopped twice—on days 50–51 and days 53–56—to remove floating biomass. The reactor was restarted on day 57 at an initial loading rate of approximately 100 g d^{-1}. The groundwater flow rate was constant thereafter, and because water recirculating through the subsurface contained progressively less nitrate, the loading rate declined with time.

After start-up, pH increased from 8.0 to greater than 9.0 (Fig. 2c) and remained at that level for more than 40 days. The pH change could be explained by the following stoichiometry:

$$5CH_3CH_2OH + 12NaNO_3 \rightarrow 6N_2 + 8HCO_3 + 2CO_3^{2-} + 11H_2O + 12Na^+$$

The above stoichiometry indicated that 1 mol of alkalinity was generated for every mole of nitrate converted into N_2. If the reaction alone controlled solution pH, a pH exceeding 10 would be expected. Denitrifiers are not especially pH-sensitive, but values outside the optimal range of 7–8 can lead to a detrimental accumulation of intermediates (Rittimann and McCarty 2001), and high values may interfere with the availability of essential nutrients, especially phosphorus. Although pilot-scale laboratory studies indicated stable long-term operation at pH 9, provisions were made to prevent detrimental increases in pH, and the FBR was designed so that the pH of make-up water was first decreased from 8.0 to 6.5 by carbonation with CO_2. The amount of CO_2 required for this step is significant because prior to entering the FBR, the pH of the added groundwater was adjusted to 8.5 with K_2CO_3 to precipitate calcium. The water was then carbonated with CO_2 to pH 6.5. During start-up, however, the FBR received make-up water of lower-than-expected buffer capacity. This meant that adjustment of influent pH to 6.5 occurred with less CO_2 addition (around 1.5–2 mM) than expected for normal operation treating groundwater.

After groundwater entered the FBR, effluent pH decreased to less than 9.0. The decrease in pH became even more pronounced after the influent concentration of nitrate decreased below 20 mM on day 62. Under these operating conditions, less alkalinity was produced, and the influent pH remained at 6.5. Changes in temperature of the FBR are shown in Fig. 2d. Temperature varied between 20 and 30°C during the initial 60-day start-up period and gradually decreased to 12 to 27°C in the winter months. The effluent of FBR usually contained residual COD from 10 to 30 mg l^{-1} due to excess supply of ethanol for denitrification. At extreme cases, the effluent COD reached 50 mg l^{-1} in 1–2 days. The residual COD was further degraded in flowing holding tanks to 10 mg l^{-1} or lower before injection to subsurface.

Analytical problems initially prevented monitoring of nitrite, but after day 60, nitrite concentrations were below

the detection limits (<0.01 mM). Uranium(VI) levels showed almost no change during passage through the reactor and indicated that little U(VI) reduction or removal occurred in the FBR, although FBR biomass has been recently shown to have some capacity for U(VI) reduction (Wu et al. 2005). One possible explanation for the lack of removal might be the presence of residual nitrate (about 0.1 mM) in the FBR and operation at a pH and carbonate concentration that prevented appreciable adsorption of U (VI) to the GAC.

FBR biofilm

Thin biofilms developed on the GAC carrier 2 weeks after start-up. Rapid growth of a gray biofilm was observed on days 25–30. As growth continued and particle density decreased, the top level of the fluidized bed expanded until it reached a level where further expansion was prevented by the biofilm growth control device. On days 50 and 53, a large amount of GAC floated to the top of the reactor. The biofilm was white with trapped gas bubbles. To remove floating biomass, the FBR was turned off on day 50 for 12 h and again on day 53 for 72 h. Floating biomass of this description had not been previously reported for denitrifying FBRs, but based on past experience with bulking sludge in activated sludge systems, we hypothesized that a phosphorus deficiency might have caused an overproduction of extracellular polymers (Rittimann and McCarty 2001). Accordingly, on day 56, we added additional KH_2PO_4 directly to the FBR at a concentration of 5 mg l^{-1} as P, and we reduced nitrate loading from more than 250 g day^{-1} to less than 100 g day^{-1} (Fig. 2b). The floating biomass was eliminated, and efficient removal of nitrate was observed thereafter.

The color of the biofilm became dark gray. As discussed below, the data suggested that changes in the bacterial community structure may have corresponded to changes in pH, nitrate overloading, and nutrient levels. The hydraulic retention time of groundwater in FBR varied from 15 to 48 h, with an average of approximately 20 h (overflow rate of 0.078 m^3 h^{-1}) during the operation. Based on the recycle flow rate of 14.4 m^3 h^{-1}, the ratio of overflow/recycle was 0.0054, and these results suggested that microorganisms from the input stream could grow in the reactor and attach onto GAC carriers.

Community structure based on partial SSU rRNA gene sequences

Approximately 500 clones were screened via comparison of partial sequences (450 nt), and rarefaction analysis indicated that the majority of recovered diversity was sampled for each time point (data not shown). For each sample, between 43 and 124 clones were screened (Table 1). The inoculum was predominated by *Alcaligenes*-like sequences (Acli-group 14), β-*Proteobacterium* sequences (B-NOS3-group 10), *Diaphorobacter*-like sequences (DNP-group 12), and *Acidovorax*-like sequences (AVPD-group 11 and AVG8-group 9). The majority of the OTUs from the inoculum were unique, and only four OTUs from the inoculum appeared to be closely related to OTUs at other time points (Fig. 3). Ecological indices indicated that the inoculum displayed the highest diversity and evenness values of all the time points tested. Twenty-one OTUs were estimated based on 97% similarity of the SSU rRNA gene sequences (Table 1).

Azoarcus-like sequences (AZ-group18) predominated the day 12 sample and comprised a majority of the library (Fig. 3). The day 12 sample was also the point of lowest diversity and evenness (Table 1). *Azoarcus*-like sequences predominated on day 34 and constituted 94% of the sampled diversity (Fig. 3). The minor OTUs at day 34 were similar to OTUs detected at subsequent time points (days 48 and 69), but OTU AqA2-34d-12 was the only minor OTU that was observed past day 69. At day 48, the *Azoarcus*-like OTU declined, and the diversity increased compared to days 12 and 34 (Table 1). Several distinct OTUs were observed. AqA4Rd-group 2, AqA4-group 3, and AqA2-group 4, which were closely related to *Rhodobacter* sp., comprised 9.3, 7, and 11.6% of the day 48 clonal library, respectively. *Hydrogenophaga*-like sequences (HaHd-group 13) represented 9.3% of the day 48 clonal library and were also observed on day 69 (6.25%), with an increased proportion on day 118 (28%) (Fig. 3).

Azoarcus-like sequences also dominated on day 69 and constituted 67.5% of the sampled diversity. The day 69 sample had 16 estimated OTUs (second highest after the inoculum); however, a majority of the OTUs comprised less than 3% of the sampled diversity. Uncultured clone-like (UBC-I12-group 17) sequences and *Hydrogenophaga*-like (HaHd-group 13) sequences were relatively abundant, and both comprised 6.25% of the library (Fig. 3).

Day 118 was also predominated by *Azoarcus*-like sequences, although at a decreased proportion compared to the other samples, and it also had high diversity. UBC-I12-group 17 and HaHd-group 13 sequences observed at day 69 were also observed at day 118 and at higher occur-

Table 1 Characteristics and diversity estimates for SSU rRNA gene clones from FBR time point samples

Time points	Number of clones[a]	OTU[b]	H′[c]	1/*D*[d]	Evenness[e]
Inoculum	124	21	3.60	9.58	0.82
Day 12	86	3	0.25	1.07	0.16
Day 34	82	6	0.47	1.13	0.18
Day 48	43	10	2.48	3.83	0.75
Day 69	80	16	2.06	2.17	0.52
Day 118	86	10	2.41	4.22	0.72

[a]Number of clones sequenced from each library
[b]Operational taxonomic units based on distinct, partial (450 nt) SSU rRNA gene sequences (≤97%)
[c]Shannon–Weiner index, higher number represents more diversity
[d]Reciprocal of Simpson's index, higher number represents more diversity
[e]As evenness approaches 1, the population is more evenly distributed

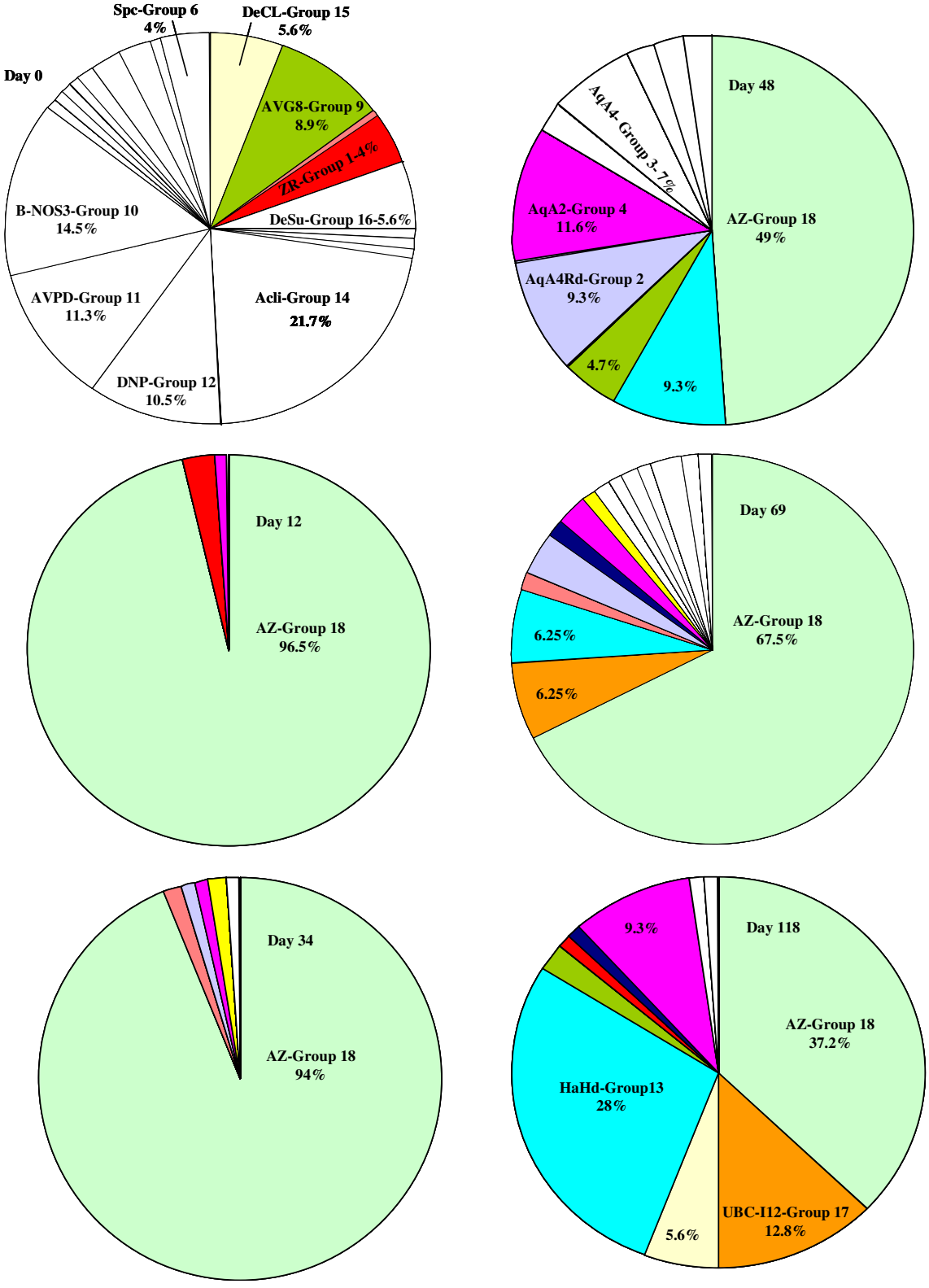

Fig. 3 The clone distribution and overlap among time points for the FBR SSU rRNA gene sequences. Each color is a unique sequence the sample color shared between sites in the same sequence. *Pie segments* depicted as *white* represent unique sequences observed only at the respective time point. The sequence names are denoted for predominant clones and the respective percentage for that time point

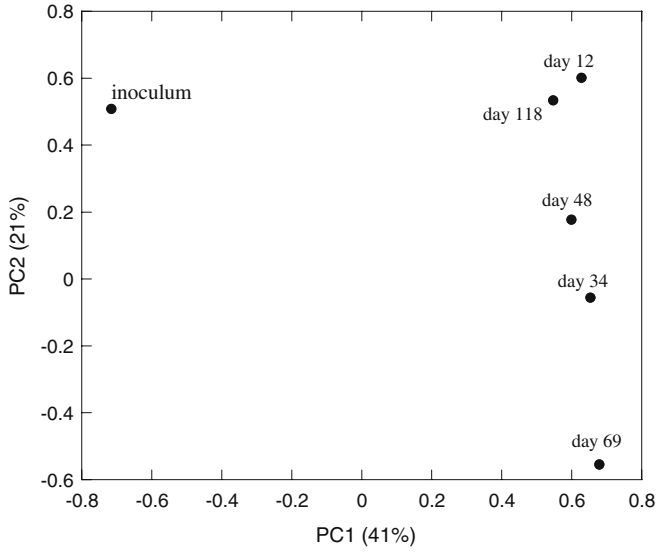

Fig. 4 Principal component analysis (PCA) of the six FBR time points based on physical and chemical parameters (pH and nitrate concentration)

rences (12.8 and 28% of the respective libraries). AqA2-group 4 was also relatively abundant and comprised 9% of the clone library (Fig. 3). It should be noted that the reported sequences are biomarkers for present populations, and the identification of different gene sequences underscores the need for further isolation, identification, and characterization of the microorganisms.

PCA and LIBSHUFF analysis

When the number of unique OTUs was compared to the pH or nitrate loading values of the FBR, the number of unique OTUs decreased as the pH increased or the nitrate loading rate was increased (data not shown). Similar results were observed when pH or nitrate levels were compared to diversity ($1/D$). These results suggested that stable FBR operation required a given number of populations in the system. When the presence or absence of unique OTUs was tabulated, PCA separated the sampling dates (Fig. 4). The inoculum was distinct from the other samples, and 62% of the variability could be explained by two components. Interestingly, component 1 was responsible for the majority of separation between the inoculum and subsequent sampling dates, and component 2 was associated with the majority of separation between samples day 12 through day 118.

Based upon PCA, a sequence that differentiated day 118 was closely related to a clone previously observed from a polychlorinated-dioxin-degrading enrichment. Sample day 69 had one of the lower diversity indices, but PCA suggested that several unique populations may have been factors in the grouping profile. The closest matches for some of the unique OTUs at day 69 included a clone from a reactor used to treat monochlorobenzene-contaminated groundwater (clone GOUTB3), denitrifying *Thauera* sp. 27, *Desulfuromonas* sp., *Fusibacter* SA1 from a South African Au-mine, and *Dechlorosoma*. For sample day 48, unique OTUs included *Hydrogenophaga atypica*, *Rhodobacter* sp., and clone PL5B10 observed at a Canadian oil reservoir. Sample day 34 had one major unique OTU that was most closely related to *Acidovorax* BSB421 that was isolated from activated sludge.

The SSU rDNA clonal libraries were compared between time points via LIBSHUFF analyses. The inoculum had the highest ΔC_{xy} values when compared to other time points and was most dissimilar to the clone library on day 12 (Table 2). At other time points, ΔC_{xy} values decreased as diversity increased, except on day 69, which had the second-highest ΔC_{xy} value (Table 2). Excluding the inoculum, other time points displayed increased dissimilarity (increased ΔC_{xy}) with day 12 when the entire SSU rDNA libraries were compared (Table 2).

Phylogenetic analysis

The unique OTUs were compared to the closest related sequences and were determined for clones with less than 97% similarity to previously identified SSU rRNA gene sequences. All time points were predominated by β-*Proteobacteria*, and sequences classified as δ-*Proteobacteria* and *Sphingobacteria* comprised less than 2% of the entire clonal library. The OTU represented by 00d-GAC-C13 had 99% sequence identity to *Desulfovibrio aerotolerans*, and the OTU represented by 69d-10.31-7 had 98% sequence

Table 2 LIBSHUFF analyses of the FBR communities at different time points based on SSU rRNA clonal libraries

ΔC_{xy}	Inoculum	Day 12	Day 34	Day 48	Day 69	Day 118
Inoculum	–					
Day 12	12.671	–				
Day 34	7.985	0.092	–			
Day 48	5.742	0.105	0.107	–		
Day 69	9.208	0.127	0.081	0.727	–	
Day 118	5.852	0.166	0.130	1.169	0.521	–

ΔC_{xy} represents the difference in coverage of the two sequence libraries (an increased ΔC_{xy} represents greater dissimilarity between the given communities; P value=0.001). The software for the analysis was used according to specified directions (http://www.arches.uga.edu/~whitman/libshuff.html)

identity with *Desulfuromonas acetexigens*. A few γ-*Proteobacteria* sequences were observed and included sequences in clone group 5 that had 99% sequence identity to *Pseudomonas stutzeri* and sequence 118d-12.19-2.6 that had 92% sequence identity to an *Ectothiorhodospira* sp.

The predominant OTU (clone group 18; *n*=377; 75%) for days 12–118 had 99% sequence identity with an uncultivated *Azoarcus* sp. (Fig. 5). Unique OTUs to the inoculum had at least 98% sequence identity with the following: α-*Proteobacteria* NOS3, *Acidovorax* sp. PD-10, *Diaphorobacter nitroreducens*, *Alcaligenes* sp. PCNB-2, and *Dechlorosoma suillum*.

The OTU DeCL-group 15 had 98% sequence identity with *Dechloromonas* sp. CL and was detected in the inoculum and day 118 sample. The OTU AVG8-group 9 had 99% sequence identity to *Acidovorax* sp. G8B1 (Fig. 5) and was observed in the inoculum library and, to a lesser extent, at days 48 and 118.

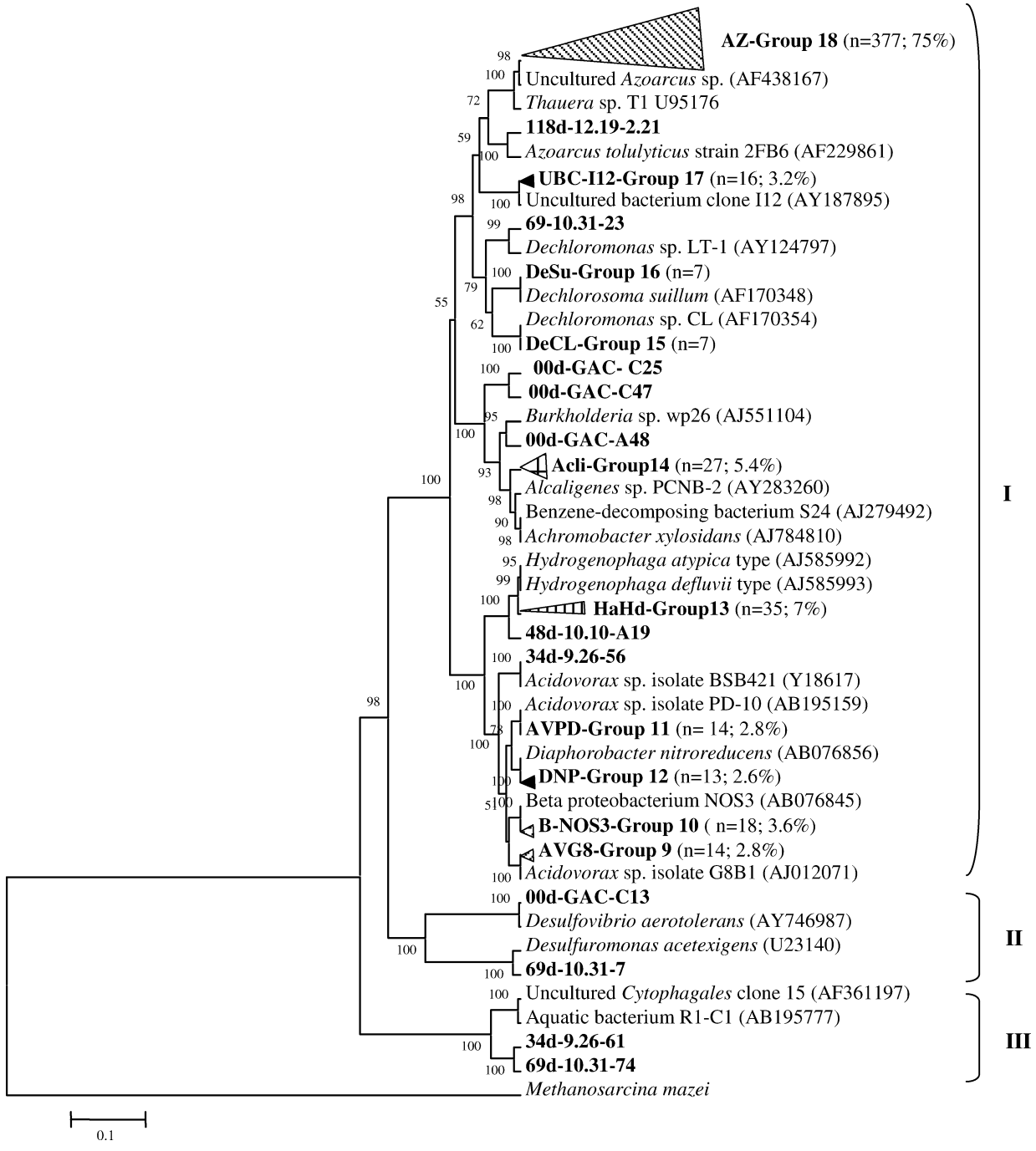

Fig. 5 Phylogenetic relationships of unique cloned SSU rRNA gene sequences from the FBR and reference sequences from GenBank classified in the β-*Proteobacteria* (*I*), δ-*Proteobacteria* (*II*), and *Sphingobacteria* (*III*), and *Methanosarcina mazei* was the outgroup. The percentage (%) represents the abundance of predominant clones from the sampled diversity. Sequences in *bold* are FBR clones or clone groups. The *accession numbers* for reference sequence are listed in the *tree* [e.g., *Thauera* sp. T1 (U95176)]

The UBC-I12-group 17 had 99% sequence identity to an uncultivated bacterium (I12) and was detected at days 69 and 118. The HaHd-group 13 had 98% sequence identity to *Hydrogenophaga* sp. and was observed in samples from days 48, 69, and 118. Other OTUs represented by sequences with 96% or less sequence identity were related to *H. atypica* (48d-10.10-A19), *Burkholderia* sp. Wp26 (00d-CA-A48), *Dechloromonas* sp. LT-1 (69d-10.31-23), *Azoarcus tolulyticus* (118d-12.19-2.21), and a benzene-decomposing bacterium, S24 (00d-CA-C25 and 00d-CA-C47) (Fig. 5).

The obtained α-*Proteobacteria* sequences were most closely related to *Zoogloea*, uncultivated clones, and *Rhodobacter* sp. The FBR clones had between 93 and 99% sequence identity with the closest relatives in the database (Fig. 6). The OTU represented by the sequences 69d-10.31-95 and 118d-12.19-1.3 had 95 and 91% sequence identity, respectively, to an uncultured bacterium clone that had 98% sequence identity to *Ochrobactrum anthropi*. Bacterial isolates related to *O. anthropi* have been isolated from groundwater at the FRC (M.W. Fields, unpublished data), and *Ochrobactrum* can be floc-forming bacteria that are typically present in activated sludge

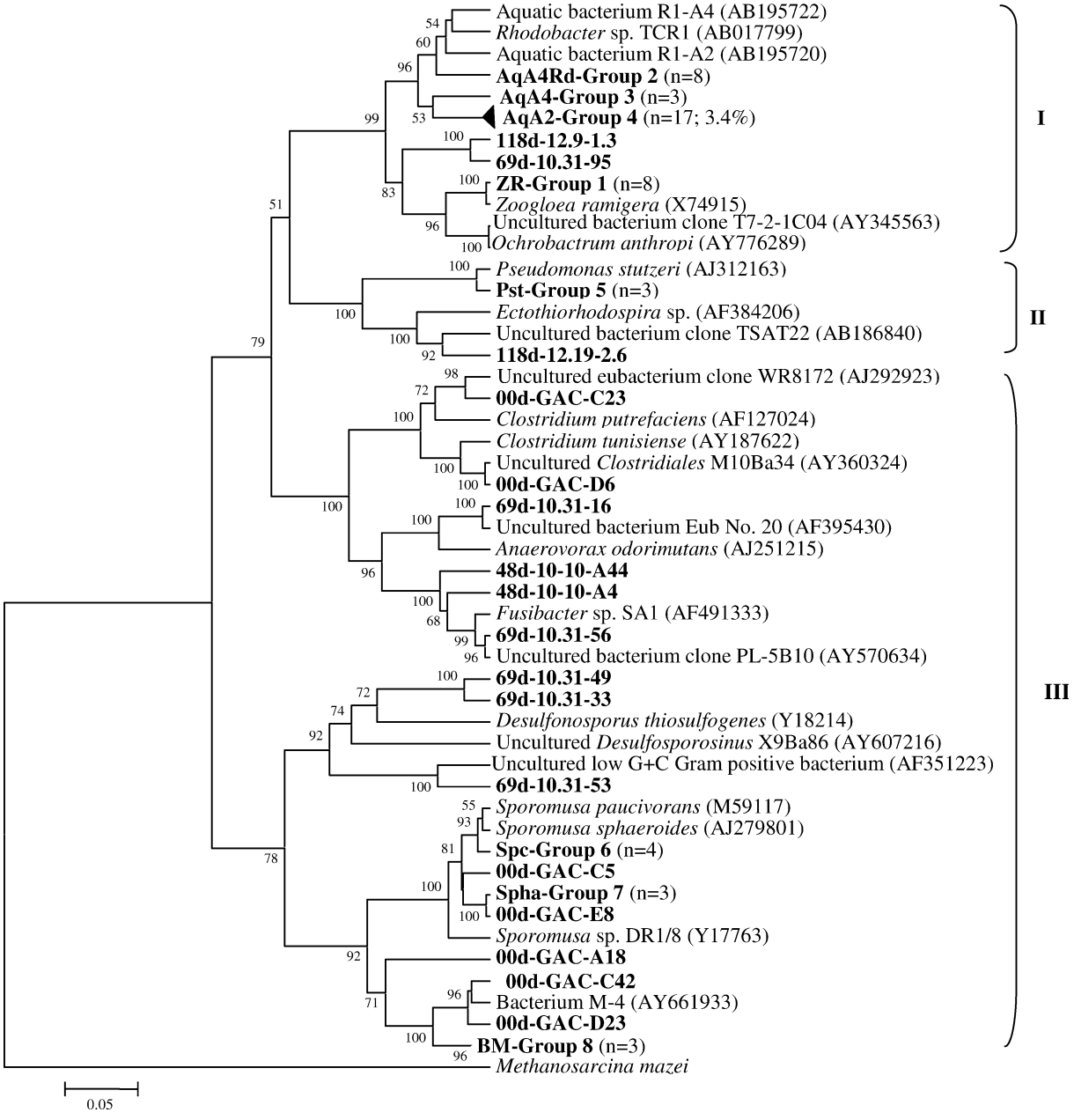

Fig. 6 Phylogenetic relationships of unique cloned SSU rRNA gene sequences from the FBR and reference sequences from GenBank classified in the α-*Proteobacteria* (*I*), γ-*Proteobacteria* (*II*), and *Clostridia* (*III*), and *Methanosarcina mazei* was the outgroup. The percentage (%) represents the abundance of predominant clones from the sampled diversity. Sequences in *bold* are FBR clones or clone groups. The *accession numbers* for reference sequence are listed in the *tree*

(Ozdemir et al. 2003). The OTU represented by AqA2-group 4 was detected at all time points and had 94% sequence identity to the aquatic bacterium clone R1-A2 and *Rhodobacter* sp.

Sequences related to *Clostridia* were detected less frequently (inoculum, days 48 and 69), and the OTU represented by 00d-CA-C23 had 100% sequence identity to an uncultivated clone and 93% sequence identity to *Clostridium putrefaciens* (Fig. 6). The OTU represented by 69d-10.31-16 had 99% sequence identity to a previously observed clone and 92% sequence identity to *Anaerovorax odorimutans*, a putrescine-degrading strict anaerobe (Matthies et al. 2000). The OTUs represented by 69d-10.31-33 and 69d-10.31-49 had 93% sequence identity to an uncultivated *Desulfonosporus* clone. Sequences in Spc-group 6 and Spha-group 7 had 97% sequence identity to *Sporomusa paucivorans* or *Sporomusa sphaeroides*, respectively. Sequences in BM-group 8 had between 95 and 97% sequence identity to the bacterial isolate M-4, and isolate M-4 was obtained from groundwater enrichments from the FRC (Fields et al. 2005).

Discussion

Small-subunit rRNA gene analyses of the microbial community indicated the predominance of *Alcaligenes*, *Acidovorax*, *Diaphorobacter*, and *Dechloromonas* (β-*Proteobacteria*) in the pilot-scale FBR inoculum (Wu et al. 2005; Gentile et al., 2005). In the pilot-scale reactor, *Acidovorax*, *Diaphorobacter*, and *Dechloromonas* populations were consistently present for over 1 year despite two biofilm shearing events and an increase in loading rate. Sequences from DNP-group 12 showed a high similarity level (99%) with *D. nitroreducens*, a respiratory denitrifier (Khan and Hiraishi 2002). *Acidovorax* species are commonly observed in wastewater treatment reactors and have been shown to be able to metabolize several different carbon sources (Snaidr et al. 1997; Kniemeyer et al. 1999). In addition, *Acidovorax*-like sequences were observed in FRC groundwater in the same area where the FBR was being utilized (Fields et al. 2005).

Interestingly, some of the clones from the FBR biofilm were most closely related to sequences from acetogenic bacteria. *Sporomusa* species are homoacetogens and have been isolated from the anoxic bulk soil of flooded rice microcosms (Rosencrantz et al. 1999). In addition, *Sporomusa* sequences were observed in the FBR biofilm that was used as an inoculum, and the biofilm was capable of uranium reduction (Wu et al. 2005). Acetogens are generally considered to be obligate anaerobes, but it can colonize habitats that are not completely anoxic. Recently, Boga and Brune (2003) hypothesized that homoacetogens could reestablish anoxic conditions because of tolerance to temporarily low pO_2 and the capacity to reduce O_2 in the termite gut. These results suggested the possible existence of acetogenic microorganisms in the FBR biofilm. The presence of such bacteria could alleviate O_2-related toxicity in the denitrifying biofilm, but further work is needed to address the niche(s) for the organisms represented by these sequences.

Others have noted that the composition of biofilms on FBR carriers can change dramatically from the original inoculum as populations respond to selection pressures during bioreactor initialization and operation (Massol-

Table 3 FBR operating conditions and changes in key features of community structure throughout the start-up period

		Key features of community structure		
		Diversity	Evenness	Dominant clones
0	Inoculation pH 8.3 Nitrate level 6.0 mM	High	High	*Alcaligenes*-like (21.7%) β-*Proteobacterium*-like (14.5%) *Acidovorax*-like (20.1%) *Diaphorobacter*-like (10.5%)
12	pH 9.2 Nitrate level 2.0 mM Efficiency 70–80%	Low	Low	*Azoarcus*-like (96.5%)
34	pH 8.2 Nitrate level 1.0 mM Efficiency >98%	Low	Low	*Azoarcus*-like (94%)
48	pH 8.2 Nitrate level <1.0 mM Efficiency >96%	Medium	High	*Azoarcus*-like (49%) Aquatic bacterium-like (28%) *Hydrogenophaga*-like (14%)
69	Stable operation pH 7.2 Nitrate level <0.3 mM Efficiency >93%	Medium	Medium	*Azoarcus*-like (67.5%) *Hydrogenophaga*-like (6.25%) Aquatic bacterium-like (3.7%)
118	Stable operation pH 7.0 Nitrate level <0.2 mM Efficiency >87%	Medium	High	*Azoarcus*-like (37.2%) *Hydrogenophaga*-like (28%) Aquatic bacterium-like (9.3%)

Deya et al. 1997). In the current study, LIBSHUFF analysis and PCA of the FBR community indicated that the inoculum was markedly different from the community present at other time points. PCA indicated that component 2 was the major group of factors that could explain most of the variation between the samples from days 12 to 118. Factor loadings from PCA suggested that particular populations could be associated with the changes between the communities at different days. Interestingly, day 69 showed the highest number of unique OTUs and suggested that of the days sampled, day 69 could be considered a climax community. It was at this time period that the pH of the reactor declined to circumneutral values, and the nitrate loading rate was lowered.

The community analysis suggested that *Azoarcus* was a major population throughout FBR operation. *Azoarcus* species have been previously shown to degrade toluene and other organics under denitrifying conditions (van Schie and Young 1998; Zhou et al. 1996), and sequences from the AZ-group 18 had 99% sequence identity to an uncultivated *Azoarcus* species from an enrichment obtained from organic wastewater used for generating electricity (Kim et al. 2004). Sequences were also closely related to *Thauera* species, most of which have been previously reported for their capacity for aromatic compound degradation under denitrifying conditions (Song et al. 1998, 2000). It should also be noted that *Azoarcus* sequences were shown to predominate in groundwater samples at three different sites at the FRC (Fields et al. 2005), and the groundwater contained detectable levels of various organics.

Clone groups closely related to *Hydrogenophaga* species and two different groups of aquatic bacteria (93% sequence identity to *Rhodobacter* sp.) appeared to fluctuate simultaneously. A similar phenomenon was observed by Zumstein et al. (2000), and the authors suggested that microorganisms and associations of microorganisms can occupy the same ecological niche successively and can correspond to the ecological unit termed an "ecotype" (Zumstein et al. 2000). *H. atypica* was isolated from an activated sludge facility in Munich, Germany (Kampfer et al. 2005), and a photosynthetic *Rhodobacte*r species was isolated from an anaerobic swine waste lagoon that appeared to outcompete sulfate-reducing bacteria, methanogens, and denitrifiers (Do et al. 2003).

Only two clones in the δ-*Proteobacteria* were observed in all of the clone libraries. One was closely related to *D. aerotolerans*, an oxygen-tolerant sulfate reducer isolated from activated sludge (Mogensen et al. 2005), and the other was closely related to *D. acetexigens*. Members of the genus *Delsufuromonas* are respiratory organisms that can couple the oxidation of simple organic compounds to the reduction of S^0 (Pfennig and Widdel 1982). Sulfate-reducing bacteria (SRB) were detected in the FBR biomass used for the inoculum (Wu et al. 2005) and may persist at a low level within GAC biofilms.

The initial predominance of *Azoarcus*-like sequences corresponded to low nitrate removal efficiencies during this period (Table 3). These results are consistent with previous observations that indicated low diversity within a functional group (i.e., denitrification) correlated with greater likelihood of process failure (Wagner et al. 2002) and is also consistent with lower productivity (i.e. denitrification) at lower community diversity. The inoculum obtained from the pilot-scale FBR operated at Stanford University had been previously acclimated to a lower pH of 7.35–7.5 (Wu et al. 2005). Thus, it appeared that the field-scale FBR passed through a selection event due to high pH and possibly high nitrate levels. The community composition shifted to increased diversity and evenness as the pH of the FBR returned to optimal operating range. The high diversity in the microbial community may have helped to promote the denitrification process at later time points when nitrate concentrations decreased (Table 3). The data indicated that molecular methods can provide insights into shifts in community composition due to process operational changes and suggested a relationship between process stability and community structure dynamics during bioreactor start-up.

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