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Microbial Communities Associated with the Oxidation of Iron and Technetium in Bioreduced Sediments

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Anoxic Tc(IV)-containing sediments representative of the UK Sellafield reprocessing facility were exposed to either air or NO_3^- to investigate redox cycling of technetium and iron. With air, oxidation of Fe(II) in the reduced sediments was accompanied by ~75% mobilization of Tc to solution, as soluble Tc(VII). Nitrate additions resulted in the bio-oxidation of Fe(II), coupled to microbially mediated NO_3^- reduction but was accompanied by only very limited (<5%) mobilization of the reduced, sediment-bound Tc, which remained as Tc(IV). PCR-based 16S rRNA and *narG* gene analyzes were used to investigate changes in the microbial community during sediment oxidation by air and nitrate. Contrasting microbial communities developed in the different treatments and were dominated by *Betaproteobacteria* (including *Herbaspirillum* and *Janthinobacterium* spp.) in the presence of high NO_3^- concentrations. This suggests that the *Betaproteobacteria* are involved in the redox cycling of Fe and N in these systems, but are unable to mediate NO_3^--dependent Tc(IV) oxidation. These microorganisms may play a previously unrecognized yet pivotal role in influencing contaminant fate and transport in these environments which can have implications to the long-term stewardship of radionuclide-contaminated sediments.

INTRODUCTION

Technetium 99 is a radioactive fission product formed in nuclear reactors and is a significant radiological contaminant at nuclear facilities (Kincaid et al. 2000; Lloyd and Renshaw 2005). It is of particular concern due to its long half-life ($\tau^1/_2$ 2.1 × 10^5 years), bioavailability, and potential environmental mobility. In oxic environments, Tc exists as the highly soluble pertechnetate ion ($Tc(VII)O^-_{4\,(aq)}$) which is weakly sorbed to sediments (e.g., Bondietti and Francis 1979). In contrast, during the development of anoxia, Tc(VII) is reduced to its lower valence forms, with Tc(IV) dominating speciation as poorly soluble Tc(IV) hydrous oxide-like phases (Burke et al. 2005; Zachara et al. 2007; Morris et al. 2008). In the majority of environmental studies, Tc(VII) reduction is likely to be dominated by abiotic reduction mediated by, for example, Fe(II)-bearing mineral phases (Lloyd et al. 2000; Fredrickson et al. 2004; Burke et al. 2005), although in Fe-deficient sediments, enzymatic reduction may be important (Wildung et al. 2004). Reflecting this chemical behavior, Tc(VII) immobilization via biostimulation (the addition of electron donors or nutrients to stimulate indigenous microbial communities) has the potential to remove Tc(VII) to sediment most likely by abiotic reduction with biogenic Fe(II) to form Tc(IV). This has been demonstrated in field (Istok et al. 2004) and microcosm studies (e.g. Burke et al. 2005; McBeth et al. 2007; Law et al. 2010) and potentially provides a viable remediation technique for radionuclide contaminated land.

Whilst the mechanisms facilitating the bioreduction of Tc(VII) are now relatively well understood, little is known about the long-term fate of Tc(IV) in biostimulation systems. Of particular concern is the reoxidation potential of Tc(IV) bound to sediments. Several recent studies have suggested that Tc(IV) may be recalcitrant towards oxidation; linkages between the Tc- and Fe-redox cycles were apparent, but the extent of Tc(IV) oxidation and remobilization appeared to be dependent upon the nature of the oxidant (Burke et al. 2006; McBeth et al. 2007). For example, Fe(II) was preferentially oxidized over Tc(IV) under nitrate-reducing conditions, and Tc(IV)-containing phases

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persisted following reoxidation of sediments by nitrate (Burke et al. 2006; McBeth et al. 2007). However, in the presence of air, appreciable Tc(IV) was oxidized in some examples alongside the Fe(II) (Burke et al. 2006; McBeth et al. 2007). The mechanisms of these processes and the microorganisms that catalyse them have been poorly characterised, despite their critical importance in controlling the long-term fate of radionuclides such as technetium in environmental systems.

Aerobic Fe(II) oxidation under acidic conditions is well described and a wide range of microorganisms catalysing this process have been identified (e.g., Johnson et al. 1993; Osorio et al. 2008). It was discovered recently that nitrate-reducing microorganisms are also capable of enzymatic Fe(II) oxidation under anoxic conditions at neutral pH (Straub et al. 1996; Straub and Buchholz-Cleven, 1998; Straub et al. 2004). Furthermore, microorganisms that can couple the reduction of nitrate to the oxidation of Fe(II) have been observed in freshwater sediments (Straub et al. 1996; Straub et al. 2004; Blöthe and Roden, 2009) and several microorganisms that are capable of anaerobic Fe(II) oxidation have been isolated (Straub et al. 1996; Finneran et al. 2002; Lack et al. 2002).

However, laboratory studies have shown that only a few of these organisms can conserve energy for growth through these processes (Weber et al. 2009). Nitrate-dependent Fe(II)-oxidizing Archaea and Bacteria have been identified (Weber et al. 2006b), although among the isolates currently available for study, only a hyperthermophilic archaean (Hafenbradl et al. 1996) and the betaproteobacterium *Pseudogulbenbiania* sp. 2002 (Weber et al. 2006a, 2009) have been demonstrated to oxidize Fe(II) as the sole electron donor and couple this metabolism to autotrophic growth.

For all other isolates described to date, either growth was not demonstrated or a co-substrate (acetate or H_2) was required as an additional electron donor and/or carbon source under Fe(II)-oxidizing conditions (Straub et al. 1996; Straub and Buchholz-Cleven, 1998; Finneran et al. 2002; Lack et al. 2002; Straub et al. 2004; Weber et al. 2009). Finally, an indirect mechanism for microbial Fe(II) oxidation, mediated abiotically by nitrite that accumulates during nitrate reduction, has also been recognized recently (Senko et al. 2005; Li and Krumholz, 2008).

In previous experiments, microcosms containing natural, poorly buffered consolidated alluvial uniformly graded sandy loam sediments representative of the Sellafield nuclear facility were "spiked" with low and high concentrations (1.6 and 325 μM final concentration respectively) of ^{99}Tc (as TcO_4^-) and the electron donor acetate (Law et al. 2010). Experiments were conducted in a carbonate buffered system at circumneutral pH, and a contrasting lower pH, unbuffered "high nitrate" system. All were incubated for 250 days (Law et al. 2010). Reductive removal of Tc(VII) as a hydrous Tc(IV)O_2-like phase occurred alongside Fe(III) reduction in these sediments (Law et al. 2010). In this study, parallel bioreducedTc(IV)-containing sediments from Law et al. (2010) were oxidized with air or 25 mM nitrate to study reoxidation of the reduced technetium and iron.

Air reoxidation will be important on disturbance of anoxic sediments, with a fluctuating water table and on exposure of reduced sediments to oxygenated waters. Nitrate as nitric acid is a particular concern with respect to reoxidation and radionuclide behavior as it is a very common co-contaminant at nuclear sites (Lloyd and Renshaw, 2005; Singleton et al. 2005). The microbial communities associated with sediments undergoing nitrate-dependent Fe(II) oxidation were studied in detail to help constrain the organisms and mechanisms involved. This is important, as although it has been over a decade since nitrate-dependent Fe(II) oxidation was first identified as a microbial process, there is still relatively little known about the organisms responsible. A better understanding of the redox cycling of Fe in relevant subsurface sediments alongside the direct and indirect linkages to radionuclide speciation and the identification of the microorganisms and mechanisms responsible are important steps in understanding the long-term stewardship of legacy radioactive wastes.

MATERIALS AND METHODS

Safety

^{99}Tc is a radioactive beta emitter (E_{max} = 294 keV) and should be handled in a properly equipped laboratory. The possession and use of radioactive materials is subject to statutory controls.

Sample Collection

Unsaturated sediments representative of the Quaternary unconsolidated alluvial flood plain deposits that underlie the Sellafield site were collected from the Calder Valley, Cumbria, during December 2006 and are best described as a uniformly graded sandy loam with an approximate particle composition of 53% sand, 42% silt, and 5% clay (Law et al. 2010). The sampling area was located approximately 2 km from the Sellafield site (Lat 54°26′30 N, Long 03°28′09 W). Sediments were transferred directly into a sterile HDPE sample container, sealed, and stored at 5°C in darkness.

Oxidation of Pre-Reduced Sediments

To prepare reduced sediments containing Tc(IV) for oxidation, initial bioreduction microcosm experiments were prepared in sterile 120 ml serum bottles containing 10 ± 1 g of sediment and 100 ± 1 ml of synthetic groundwater, with 10 mM sodium acetate added as an electron donor. The preparation of the microcosms is described fully by Law et al. (2010). Briefly, microcosm bottles were prepared and flushed with filtered N_2/CO_2 (80/20) and sealed with butyl rubber stoppers. Bioreduction groundwater compositions were: (A) carbonate buffered groundwaters at pH 7 and (B) unbuffered high nitrate (10 mM) groundwaters at pH 5. Technetium 99 was added at a final concentration of 1.6 μM TcO_4^- (as pertechnate; LEA-CERCA, France).

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All microcosms were incubated for 250 days at 21°C in the dark, and in both systems Tc(VII) reduction to Tc(IV) was commensurate with the development of Fe(III)-reducing conditions (Law et al. 2010). Nitrate was fully utilised during bioreduction, and the pH of the un-buffered high nitrate system increased from ~5.5 to ~7 due to the production of alkalinity during denitrification (Law et al. 2010).

After bioreduction, parallel, bioreduced and Tc(IV)-labelled microcosms were used for the oxidation experiments described in this manuscript. After anaerobic incubation, oxidation samples (in triplicate) were exposed to air (O_2 oxidation) or injected with an anaerobic NO_3^- solution to a final concentration of 25 mM. In addition, sterile control experiments were prepared by autoclaving (20 min, × 3 times) bioreduced microcosms and then exposing them to air or nitrate. Sampling of sediment slurries was done using sterile Ar flushed syringes. Porewater and sediment samples were collected from the slurry via centrifugation (13,000*g*, 10 min). Total Tc, NO_3^-, NO_2^-, Fe, pH, and Eh were determined in porewaters. Additionally, sediment samples were analyzed for 0.5 N HCl extractable Fe(II) and total Fe and for each time point 0.5 g of untreated sediment was stored under sterile conditions at −80°C for analysis of changes in the microbial community.

Geochemical Analyzes

Technetium concentrations were determined by liquid scintillation counting (LSC) on a Packard Tri-carb 2100TR (detection limit ~0.4 Bq ml^{-1}). In addition, the speciation of aqueous Tc was determined at selected time points using extraction with tetraphenylarsoniumchloride (TPAC; Wildung et al. 2000). Total dissolved Fe and NO_2^- were measured with standard UV-Vis spectroscopy methods on a Cecil CE 3021 spectrophotometer (Viollier et al. 2000; Harris and Mortimer, 2002). Aqueous NO_3^- and SO_4^{2-} were measured by ion chromatography (Dionex ICS-90). Total bioavailable Fe(III) and the proportion of extractable Fe(II) in the sediment was estimated by digestion of 0.1 g of sediment in 5 ml of 0.5 N HCl for 60 min followed by a colorimetric assay using ferrozine (Lovley and Phillips, 1987). The pH and Eh were measured with an Orion 420A digital meter and calibrated electrodes. Standards were used routinely to check the reliability of all methods and calibration regressions had $R^2 \geq$ 0.99.

XAS Sample Preparation

The TcO_4^- spike (325 μM) was injected into pre-reduced sediment microcosms that contained >70% 0.5 N HCl extractable Fe as Fe(II) and experiments were equilibrated for two weeks prior to sampling. One microcosm from each of the reduced systems was retained for XAS analysis of Tc speciation under bioreducing conditions. Results for these microcosms are presented by Law et al. (2010), who described the presence of hydrous Tc(IV)O_2-like phases in these reduced sediments. The remaining microcosms were then oxidized by uncapping and exposure to air with gentle manual agitation, or injection with NO_3^- to 25 mM. After 30 days of incubation at 21°C each microcosm was centrifuged (12 min, 1000 *g*) and approximately 0.5 g of the resulting sediment (water content ~40–50%) was then mounted for XAS analysis on airtight XAS Perspex sample cells sealed with Kapton windows. Porewaters were also collected and analyzed as described here. The XAS sample cells were triple contained in heat sealed plastic bags and stored at −80°C under Ar until analysis. All sample manipulations were conducted in an O_2-free atmosphere.

XAS Analysis

Eight scans were collected for each sample and averaged to improve the signal to noise ratio. The EXAFS spectra were background subtracted using EXPLINE and analyzed with EXCURV98 using full curved wave theory (Gurman et al. 1984). Phase shifts were determined from *ab initio* calculations using Hedin-Lundqvist potentials and von Barth ground states (Binsted 1998). The data were fitted in k^3 space by defining a theoretical model which was informed by the relevant literature (Lukens et al. 2000; Wharton et al. 2000; Maes et al. 2004; Morris et al. 2008) and used whole integer values for shell of backscatters around the central atom. Shells of backscatter atoms were added around a central ^{99}Tc atom and by refining an energy correction E*f* (the fermi energy), the absorber scatter distance, and the number of atoms in each shell, the least square residual (the R factor; (Binsted et al. 1992) was minimized (Morris et al. 2008). Shells were only included in the model fit if the overall R-factor was improved by ≥5%.

DNA Extraction and Amplification

Microbial community DNA was extracted from sediment samples (0.2 g) using the PowerSoilDNA Isolation Kit (PowerSoil DNA Isolation Kit, MO BIO Laboratories INC, USA). The 16S-23S intergenic spacer region from the bacterial RNA operon was amplified from community DNA by PCR with the primers S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18 (Ranjard et al. 2000). Amplification was performed in a BioRadiCycler (BioRad, UK) as described by (Ranjard et al. 2000) but with 35 cycles. The amplified products were separated by electrophoresis in a 3% tris-acetate-EDTA (TAE) gel. DNA was stained with ethidium bromide and viewed under short-wave UV light using a BioRadGeldoc 2000 system (BioRad, UK).

The universal bacterial primers 8F (5′-AGAGTTT GATCCTGGCTCAG-3prime;) (Edwards et al. 1989), and 1492R (5′- TACGGYTACCTTGTTACGACTT-3′) (Lane 1991) were used to amplify 16S rRNA gene fragments from the extracted and purified chromosomal DNA. PCR was performed with a BioRadiCycler using 0.25 μM of each primer, 0.2 mMdNTPs, 1 × PCR buffer, 2.5 mM $MgCl_2$ and 1.25 units of JumpStart^{TM} *Taq*DNA Polymerase (Sigma, UK), which were made up to a final volume of 50 μl with sterile water. The PCR amplification protocol was as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles each consisting of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, elongation

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at 72°C for 1 min and followed by a final extension step at 72°C for 10 min.

*Nar*G gene fragments were amplified by PCR as described previously (Geissler 2007) using the primers from (Goregues et al. 2005). The PCR amplifications were performed in a BioRadiCycler (BioRad, UK) with an initial denaturation at 94°C for 7 min, followed by 35 cycles consisting of a 30 s denaturing step at 94°C, an annealing step at 50°C for 30 s, and a 40 s elongation step at 72°C. The amplification was completed with an extension period of 5 min at 72°C. PCR products were separated and visualized by agarose (1%) gel electrophoresis. DNA was stained with ethidium bromide and viewed under shortwave UV light using a BioRadGeldoc 2000 system (BioRad, UK).

Construction of Clone Libraries, Restriction Fragment Length Polymorphism (RFLP) and Phylogenetic Analysis

PCR products with the correct size were purified using a QIAquick PCR purification kit (Qiagen, UK). The PCR products were then cloned with a Strata Clone^{TM} PCR cloning Kit (Stratagene, USA) according to the manufacturer's instructions. Plasmid inserts of approximately 55 clones were directly amplified from the transformed cells by PCR with primers that were complementary to the flanking regions of the PCR insertion site of the cloning vector. The PCR amplification protocol was: initial denaturation at 94°C for 4 min, melting at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min; 35 cycles, followed by a final extension step at 72°C for 5 min. RFLP patterns of cloned 16S rRNA gene fragments were obtained by digestion of the PCR products with the restriction enzymes *Sau*3A and *Msp*I in parallel. The *narG* gene containing inserts were analyzed by restriction fragment length polymorphism (RFLP) with the four-base-specific restriction endonuclease *Hae*III and *Alu*I in parallel as previously described (Geissler 2007). The digested fragments were then separated by agarose (3%) gel electrophoresis. RFLP patterns were grouped visually and representative clones were chosen for sequencing.

Nucleotide sequences were determined by the dideoxynucleotide method and the primer 1492r was used for sequencing of the 16S rRNA gene sequences and plasmid specific primers M13rev (5′-CAGGAAACAGCTATGACC-′3) and M13uni (5′-TGTAAAACGACGGCCAGT-′3) for the *narG*gene sequences. Sequences were obtained using an ABI Prism BigDye Terminator Cycle Sequencing Kit in combination with an ABI Prism 877 Integrated Thermal Cycler and ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems, UK). Sequences were compared with those available in the GenBank by using BLAST analysis (Basic Local Alignment Search Tool(Zhang et al. 2000)). The *nar*G gene sequences were first translated into amino acid sequences (www.expasy.ch).

The possibility of chimera formation by 16S rRNA gene sequences was checked by submitting sequences and their closest phylogenetic relative to the pintail program (www.cardiff.ac.uk/biosi/research/biosoft). Possible chimeras were excluded from the phylogenetic analyses. Phylogenetic affiliation of approximately 800 bases per sequence was estimated by using BLAST and the CLASSIFIER function in RDP (Maidak et al. 2000). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura et al. 2007). The sequences were manually aligned to related sequences obtained from GenBank by using CLUSTALW and phylogenetic tree was generated using the neighbour-joining algorithm and bootstrapped (500 trial replicates). The 16S rRNA gene sequences reported here were deposited to the GenBank under accession numbers GU233536 to GU233634 and the *narG* gene sequences were deposited to the EMBL nucleotide sequence database under accession numbers FN658505 to FN658536.

Enrichment Culture

In nitrate reoxidation experiments, after one week of oxidation with nitrate and under optimal conditions when the majority of Fe(II) was oxidized and nitrate was reduced, 5 ml of the sediment slurry was added anoxically to 100 ml of bicarbonate buffered freshwater medium containing 20 mM $FeSO_4$ and 10 mM $NaNO_3$ (Straub and Buchholz-Cleven 1998) to establish a nitrate-reducing Fe(II)-oxidizing consortium. The colour of the medium changed from blue/green to red/brown within 3weeks, which was indicative of Fe(II) oxidation. After 11 sub-cultures (∼30 weeks), DNA was extracted from the enrichment culture as described here, and a 16S rRNA gene clone library was constructed to characterize the bacterial community.

RESULTS

Oxidation by Air

In the carbonate buffered and unbuffered high nitrate systems, >80% of extractable sediment Fe(II) was oxidized after 14 days of air exposure. Slower rates of oxidation were then observed until 45 days when >95 % of 0.5 N HCl extractable Fe was present as Fe(III). Commensurate remobilization of Tc was observed, with ∼75% of sediment-bound Tc returned to solution after 45 days (Figure 1). Interestingly, ∼25% of the Tc associated with the sediment in both the carbonate buffered and unbuffered high nitrate systems appeared to be recalcitrant towards remobilization. This is similar to observations by other workers (e.g.,Burke et al. 2006; McBeth et al. 2007; Begg et al. 2008; Fredrickson et al. 2009) and may be due to formation of Tc(IV)/Fe containing molecular species that are postulated to be recalcitrant to oxidation (Fredrickson et al. 2009). Finally, parallel air oxidation experiments, conducted with the higher concentrations of Tc necessary for XAS studies, permitted evaluation of the predominant Tc oxidation state and coordination environment in the oxidized systems. Here (Figure 2; Table 1), the k^3 weighted EXAFS and associated Fourier transforms indicated that hydrous Tc(IV)O_2-like phases were present in the sediment, with diagnostic features for TcO_2 found at 2.00 Å. Additional third shell fits were attempted with Tc and Fe, and fitting with Tc at 2.52 Å gave a marginally better fit than Fe at 2.68 Å,

TABLE 1
Summary of Tc EXAFS modelling results for nitrate and air oxidized sediments

Sample /Treatment	Shell (n)	O-T	r(Å)	$2\sigma^2$ (Å^2)	R
Carbonate-buffered Air	1	6-O	1.998	0.015	39
	1	6-O	2.004	0.016	27
	2	1-Tc	2.534	0.013	
	1	6-O	2.000	0.016	33
	2	1-Fe	2.703	0.011	
Carbonate-buffered Nitrate	1	6-O	1.994	0.016	44
	1	6-O	2.001	0.017	28
	2	1-Tc	2.527	0.011	
	1	6-O	1.996	0.016	36
	2	1-Fe	2.690	0.009	
Non-buffered Air	1	6-O	1.990	0.016	49
	1	6-O	2.009	0.017	36
	2	1-Tc	2.527	0.018	
	1	6-O	1.998	0.016	42
	2	1-Fe	2.700	0.012	
Non-buffered Nitrate	1	6-O	1.998	0.016	51
	1	6-O	1.995	0.016	41
	2	1-Tc	2.528	0.011	
	1	6-O	1.993	0.016	46
	2	1-Fe	2.701	0.01	

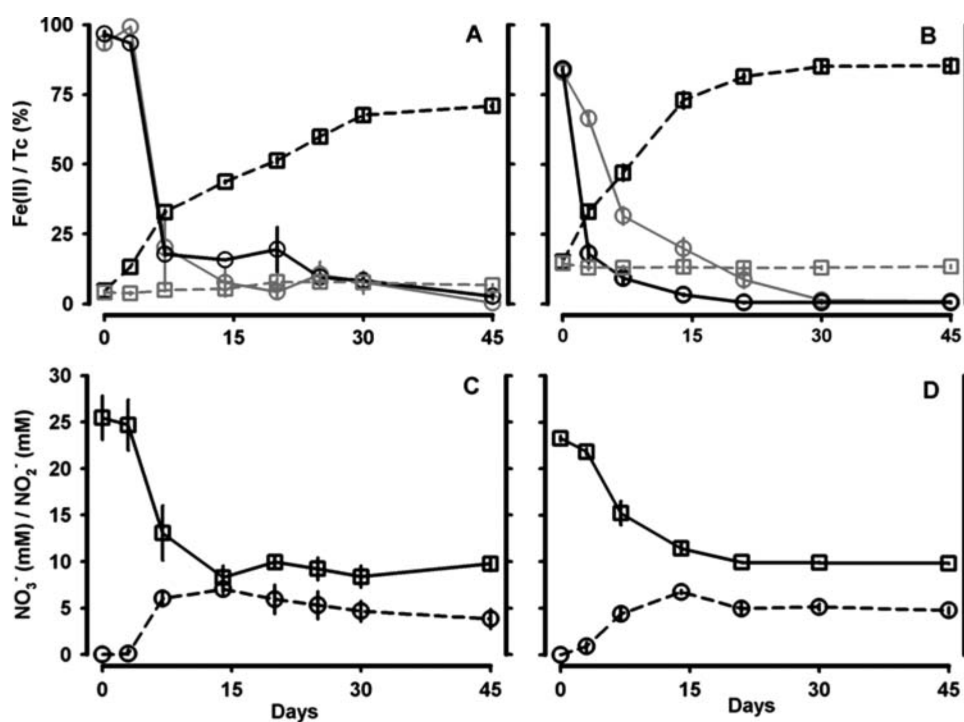

FIG. 1. Microcosm incubation time-series data (day 0–45). Panels A and C carbonate buffered microcosms, panels B and D unbuffered high nitrate microcosms. Symbols: Panels A and B—light grey symbols = NO_3^- oxidation, black symbols = air oxidation (○ = Fe(II), □ = Tc); panels C and D—open □ = NO_3^-, open ○ = NO_2^-. Error bars represent 1 σ experimental uncertainty from triplicate microcosm experiments (where not visible, error bars are within the symbol size).

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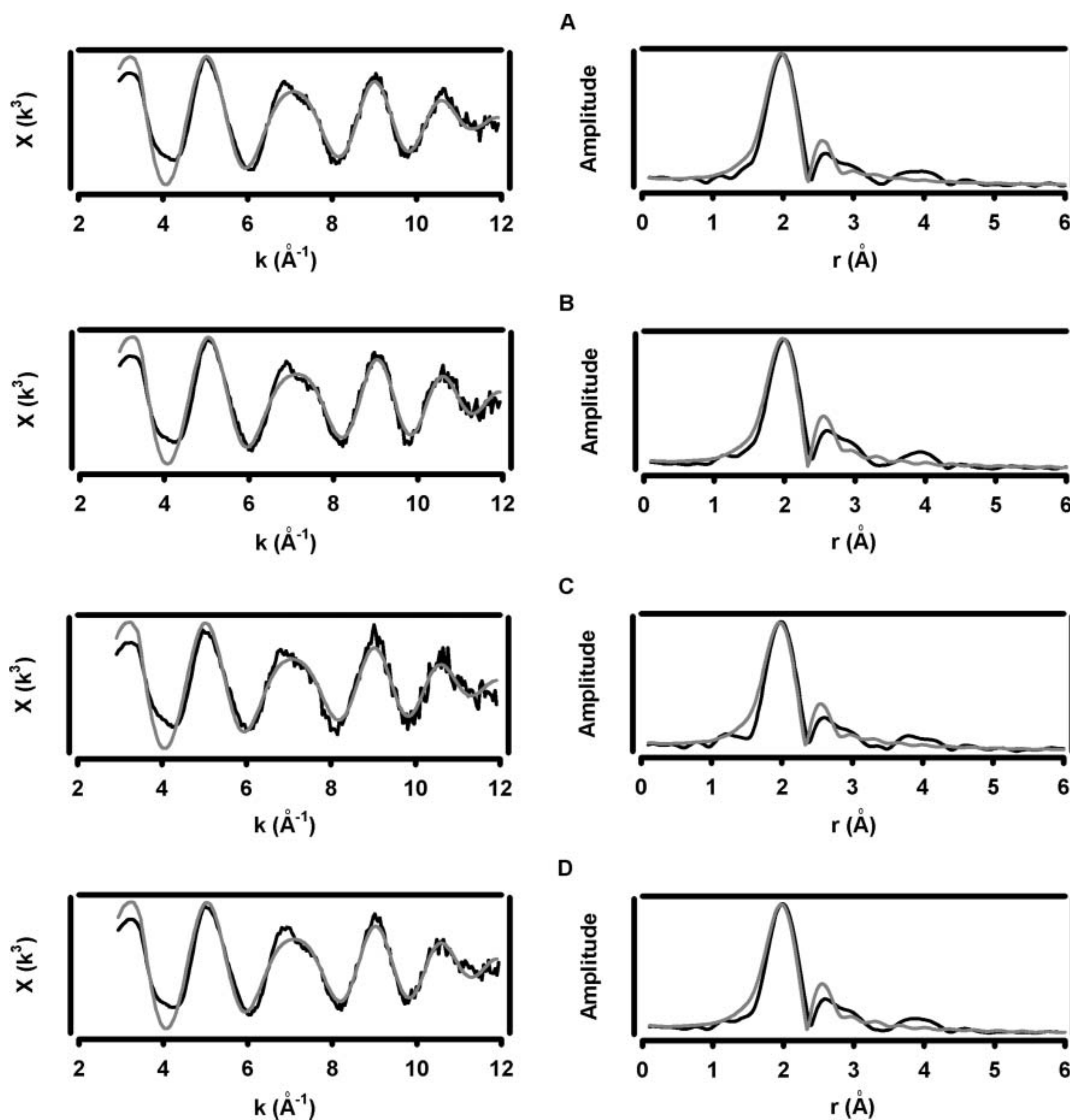

FIG. 2. Background subtracted, normalized, and k^3 weighted ^{99}Tc K edge EXAFS spectra (left) and corresponding Fourier transforms (right) for (A) carbonate buffered air oxidized sediment, (B) carbonate buffered NO_3^- oxidized sediment, (C) unbuffered high nitrate air oxidized sediment, and (D) unbuffered high nitrate NO_3^- oxidized sediment. Experimental data (black) best modelled fit (Table 1) (grey).

suggesting short range order in the Tc(IV) precipitate. Interestingly, evidence for mixed Tc(VII)/Tc(IV) phases, which have been described previously in some unwashed Tc "reoxidation" treatments (Burke et al. 2006; McBeth et al. 2007; Fredrickson et al. 2009) was absent. Chemical extraction (TPAC) of the $Tc_{(aq)}$ that was mobilized by oxidation revealed that it was present as Tc(VII).

Oxidation by Nitrate

The addition of 25 mM NO_3^- to the pre-reduced microbially-active carbonate-buffered and unbuffered high nitrate systems resulted in the oxidation of Fe(II). In the carbonate buffered system the rate of NO_3^--mediated Fe(II) oxidation was similar to that observed during treatment with air (Figure 1). However, in the sediments that were initially unbuffered and contained high nitrate during the pre-reduction phase, the addition of 25 mM NO_3^- resulted in a slower initial rate of Fe(II)-oxidation than was observed during air exposure (Figure 1). Regardless, in both the carbonate-buffered and unbuffered high nitrate systems, when 25 mM NO_3^- was added, 15 mM NO_3^- was consumed within 14 days, leaving ∼10 mM NO_3^- unreacted. This remained until the end of the experiment (45 days).

Within the first 10–14 days after NO_3^- addition up to 7 mM NO_2^- was produced and the NO_2^- concentration had only decreased to ∼5 mM by the end of the experiment. By contrast in the sterile controls, no NO_3^- reduction, NO_2^- production

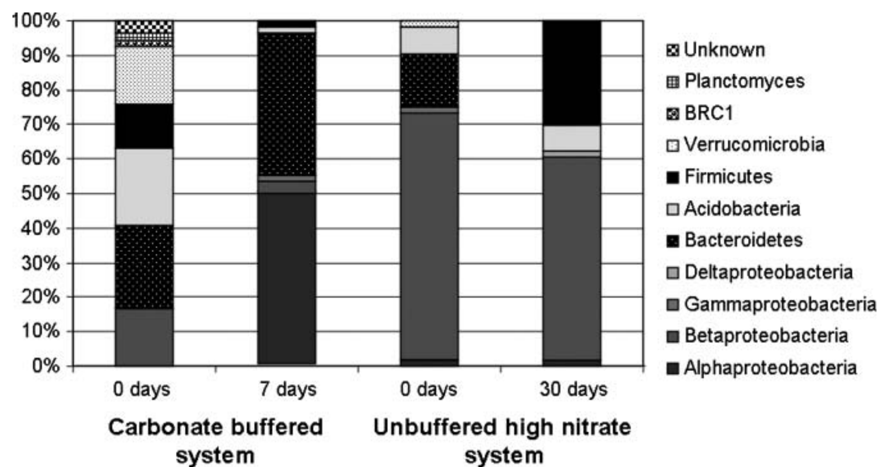

FIG. 3. Distribution (% of the total) of clones from the carbonate buffered system and the unbuffered high nitrate system before and after treatment with air according to all observed bacterial divisions.

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or Fe(II) oxidation was observed, which confirms that these processes are microbially-mediated. In contrast to the air treatment, less than 5% of sediment-associated Tc was remobilized to solution during treatment with NO_3^- in the carbonate buffered and unbuffered high nitrate systems (Figure 1). Again, XAS analysis of analogue NO_3^- oxidation experiments (Figure 2; Table 1) conducted with higher Tc concentrations, indicated the presence of hydrous Tc(IV)O_2-like phase(s) in the sediments and TPAC analysis of porewater indicated that remobilized Tc was present as Tc(VII).

Changes in Microbial Community Structure

RISA analysis from time courses of the pre-reduced carbonate buffered and unbuffered high nitrate systems showed broad alterations in the microbial community structure during subsequent oxidation with air and nitrate (data not shown). Samples taken from the carbonate buffered system at 0 days, and during Fe(II)-oxidation with air and nitrate (7 days) were used to analyze further the extant bacterial communities by construction of 16S rRNA gene clone libraries. In parallel analyzes on the unbuffered high nitrate "pre-reduced" system (pH 5 + 10 mM NO_3^-) samples were taken at 0 days, during oxidation with nitrate (7 days), and during oxidation with air or nitrate at 30 days.

The nitrate-reducing community was also analyzed at 0 and 7 days in both systems during oxidation with nitrate by *narG* gene analysis to target the gene for the alpha-subunit of the membrane-bound nitrate reductase. Initially, at 0 days when the oxidation of the sediments was initiated (which corresponded to bioreduced sediment with 250 days anaerobic incubation, see (Law et al. 2010)), the microbial community in the carbonate buffered system was diverse, with representatives of the phyla *Betaproteobacteria, Bacteroidetes, Acidobacteria, Firmicutes*and *Verrucomicrobia* dominating (Figure 3). In contrast, in the sediments originally bioreduced in the presence of high

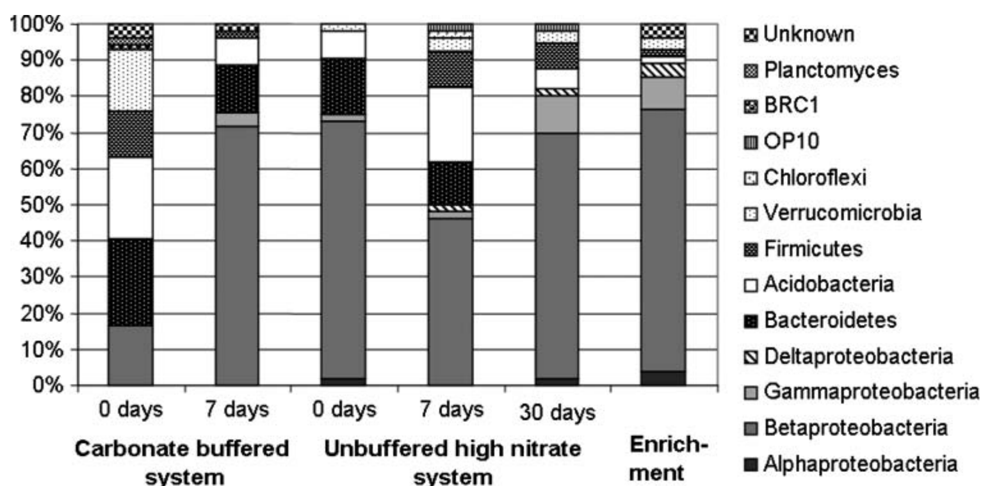

FIG. 4. Distribution (% of the total) of clones from the carbonate buffered system and the unbuffered high nitrate system before and after treatment with 25 mM nitrate according to all observed bacterial divisions.

nitrate (10 mM), representatives of the phylum *Betaproteobacteria* dominated (71%) the microbial community (Figure 3).

Oxidation by Air

In the carbonate buffered pre-reduced system, after 7 days of exposure to air, an enrichment of representatives of *Alphaproteobacteria* occurred (none detected at 0 days and 50% at day 7), although *Bacteroidetes* still represented a significant component (41.1%) of the sequences detected in the community (Figure 3). In the unbuffered high nitrate system after 30 days of air oxidation, *Betaproteobacteria* still dominated (71.1% of the clone libraries at 0 days versus 57.4% at 30 days) but sequences affiliated with *Firmicutes* also became important (29.6% of the library) (Figure 3).

Oxidation by Nitrate

Analyzes of the clone libraries showed a change in the community structure during nitrate-dependent Fe(II) oxidation in the carbonate buffered system (Figure 4). The most significant change was an increase in the number of clones associated with *Betaproteobacteria*, from 16% to 71% during nitrate-dependent Fe(II) oxidation. *Janthinobacterium* sp. was the most frequently identified phylogenetic group (66% of all sequences) among the *Betaproteobacteria* clones.

The clone libraries from the anaerobic sediments originally prepared with high nitrate additions revealed only small shifts in community structure (Figure 4). At 7 days the frequency of *Betaproteobacteria* sequences in the clone libraries decreased to approximately 46 %, but increased at 30 days to 67%. Sequences affiliated with *Herbaspirillum* sp. dominated the *Betaproteobacteria* clones in the high nitrate system (40% at 7 days and 46% at 30 days).

NarG gene analysis was used to further analyze the molecular ecology of the denitrifying communities in these experiments. Most of the *narG* gene sequences in the bicarbonate system at day 0 were related to the *narG*gene of environmental sequences retrieved from uncultured bacteria (Figure 5). However, at 7 days a strong shift in the *narG* gene pool in the carbonate buffered system was observed, with more than 90 % of the *narG* gene sequences most closely related to a nitrate reductase gene sequence from the betaproteobacterium *Herminimonasarsenicoxidans* (Figure 5). Additionally, three of the other *narG* gene sequences detected were closely affiliated (91.8%) with the nitrate reductase of *Rhodoferax ferrireducens*.

Initially, most of the *narG* gene sequences from the unbuffered high nitrate system were related to *narG* genes retrieved from uncultured bacteria (Figure 5). During reoxidation, mediated by the addition of 25 mM NO_3^-, the *narG* gene pool evolved. After 7 days of nitrate-mediated oxidation >60% of the *narG* gene sequences were most closely related to the *narG* gene of the betaproteobacterium *Polaromonas naphthalenivorans* CJ2 (Figure 5). At the end of the experiment the *narG* gene pool was more diverse (Figure 5).

Enriched Fe(II)-Oxidizing Consortium

To identify further the organisms responsible for nitrate-dependent Fe(II) oxidation, an enrichment culture was established using a medium containing Fe(II) as the sole electron donor and nitrate as the sole electron acceptor. The 16S rRNA gene clone library of the consortium enriched in this medium was dominated (73% of the library) by clones that were affiliated with *Betaproteobacteria*, with almost all closely related (99%) to *Herbaspirillum* sp. G8A1 (Figure 4). The remaining 27% of the clone library was comprised of *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Firmicutes*, and uncultured bacteria (Figure 4).

DISCUSSION

Tc(IV) Oxidation

Under the conditions studied, Tc(IV) removed to pre-reduced sediments was reoxidized significantly by air but was resistant to reoxidation by nitrate, consistent with previous studies (Burke et al. 2006; McBeth et al. 2007; Fredrickson et al. 2009) and formed a recalcitrant Tc(IV) phase. These results have positive implications for the predicted remobilization behavior of technetium in contaminated environments, especially where bioreduction has been stimulated to minimize transport of the radionuclide and suggests that Tc(IV) may be stable in the presence of nitrate contaminated groundwaters even when bio-oxidation of Fe(II) is occurring. Indeed, even though appreciable quantities of Fe(III) formed during bio-oxidation with NO_3^-, the resultant NO_2^- and Fe(III) species were unable to oxidize and mobilize the Tc(IV) from the sediments. Thus the oxidation of Fe(II) and Tc(IV) are not coupled directly in the sediment type that we have studied.

Fe(II) Oxidation

In both oxidation treatments (air and nitrate), Fe(II) was oxidized efficiently. In the bio-oxidized system, the depletion of added nitrate was commensurate with Fe(II) oxidation, and the absence of these processes in analogue sterile controls indicates that as expected these processes are microbially-mediated. The mechanism(s) for this are unclear as Fe(III) may be produced during microbial nitrate reduction by oxidation of Fe(II) with nitrite (Senko et al. 2005; Li and Krumholz, 2008), or by enzymatic Fe(II) oxidation coupled directly to nitrate reduction (Straub et al. 1996; Weber et al. 2001; Weber et al. 2009). The chemical oxidation of Fe(II) by nitrite can occur when nitrate reduction is microbially catalysed in the presence of an organic electron donor (Senko et al. 2005; Li and Krumholz, 2008).

The sediments used in our study most certainly contained bioavailable organics that could support heterotrophic metabolism, as in preparing the bioreduced materials for study, metal reduction was stimulated by the addition of acetate. This acetate would have augmented naturally occurring organics. By the end of the 250 day pre-reduction process and the subsequent

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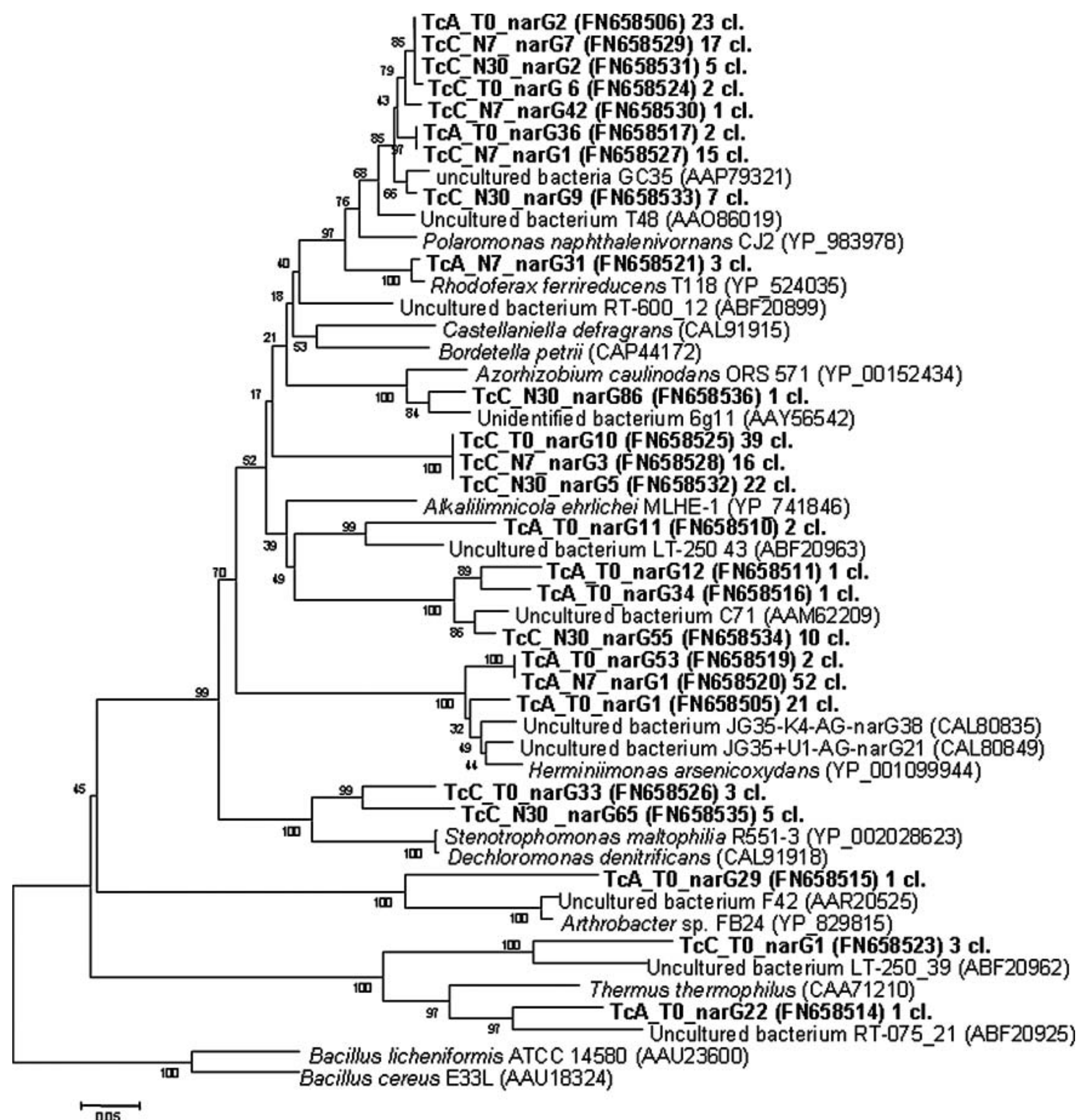

FIG. 5. Phylogenetic dendrogram of the deduced protein sequences from *nar*G gene sequences retrieved from the carbonate buffered system (TcA) and the high nitrate system (TcC) as well as their closest amino acid sequences. N stands for the treatment with nitrate followed by the incubation time. The accession numbers of the NarG sequences are given in brackets followed by the numbers of the clones retrieved. The dendrogram was generated using distance-matrix and neighbour-joining methods.

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oxidation steps described in this manuscript, it is highly likely that this bioavailable organic pool was depleted; however, both NO_2^- and enzymatic Fe(II) routes to bio-oxidation are possible in the sediment systems. Interestingly, in the enrichment cultures, no organic electron donor was added to the Fe(II) media, and by ∼6 subcultures, these systems will have been essentially completely autrophic.

Therefore, it is conceivable that a proportion of nitrate-dependent Fe(II) oxidation was catalysed by lithoautotrophic organisms in the sediment experiments; certainly chemolithoautotrophic organisms were able to catalyse nitrate-dependent Fe(II) oxidation in the sub-cultures from these experiments. To date, only a very restricted number of organisms (an archaean and one bacterium) have been shown conclusively to use Fe(II) as the sole electron donor for nitrate reduction, and to couple this metabolism to autotrophic growth (Weber et al. 2009). For all other isolates to date either no growth was observed or a co-substrate was required (Weber et al. 2009).

Microbial Communities Associated with Fe-N Redox Cycling

16S rRNA and *narG* gene analyzes suggest that *Betaproteobacteria* are involved in the redox cycling of Fe and

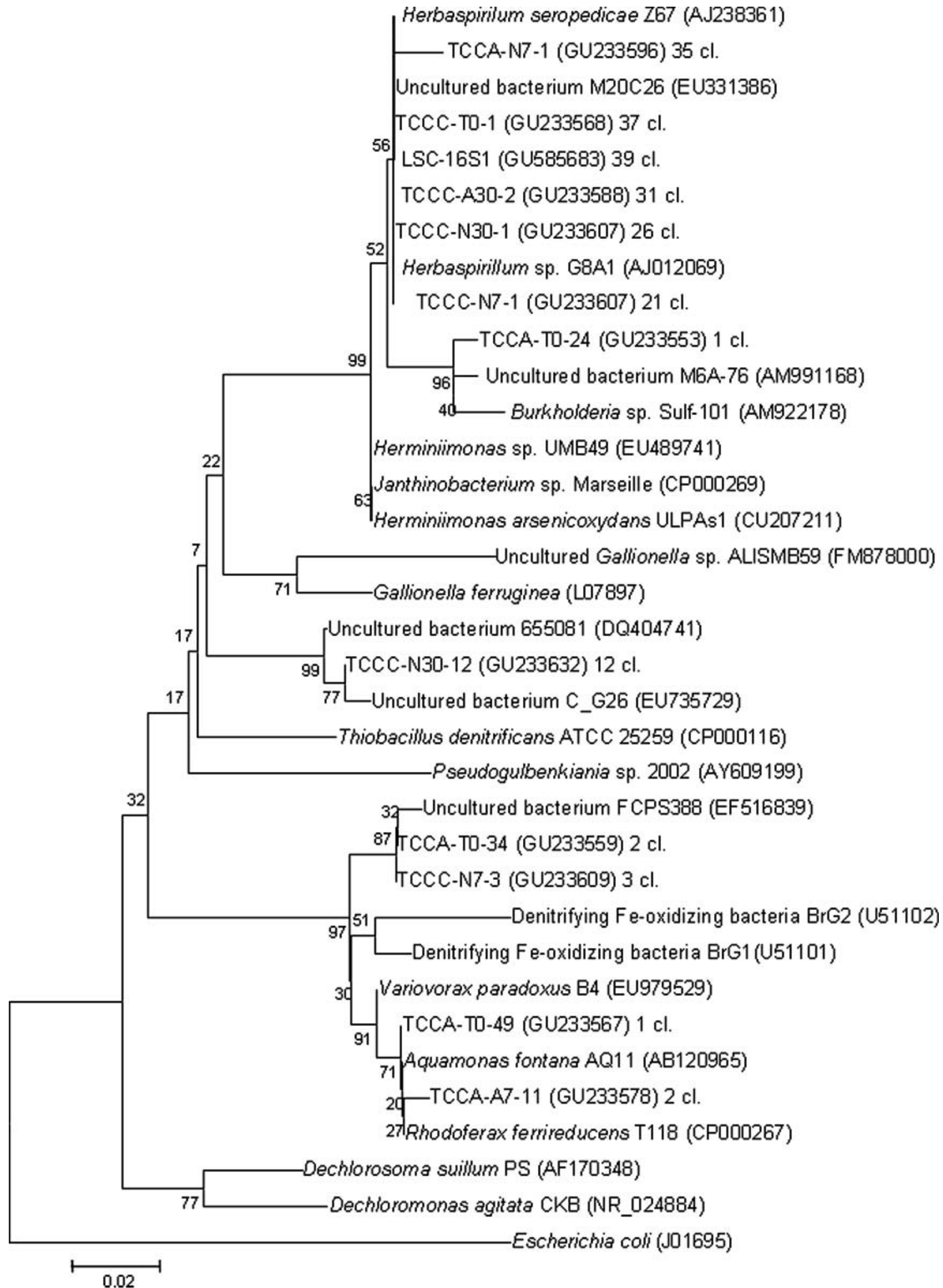

FIG. 6. Phylogenetic dendrogram of betaproteobacterial 16S rRNA gene sequences from the carbonate buffered system (TCCA) and the unbuffered high nitrate system (TCCC) as well as their closest relatives. N and A stand for the treatment with nitrate or air respectively followed by the incubation time. The accession numbers of the sequences are given in brackets and followed by the numbers of the clones retrieved. The dendrogram was generated using distance-matrix and neighbour-joining methods.

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N in the Sellafield sediments studied. *Betaproteobacteria* were also an important part of the bacterial community facilitating organotrophic NO_3^- reduction in microcosms containing these sediments (Law et al. 2010), and it is likely that this group may also mediate NO_3^--dependent Fe(II) oxidation.

Betaproteobacterial 16S rRNA gene sequences that were dominant in clone libraries from the nitrate-dependent Fe(II) oxidation phase in the two systems studied and in the enrichment culture, were phylogenetically distinct from *Betaproteobacteria* known to be involved in anaerobic Fe(II) oxidation, for example *Pseudogulbenbiania* sp. 2002 (Weber et al. 2009) or the isolates BrG1 and BrG2 (Straub et al. 1996, 2004) (Figure 6). They were, however, most closely related to *Herbaspirillum* sp. and *Janthinobacterium* sp. These organisms are known denitrifiers (Probian et al. 2003; Audic et al. 2007), which would suggest that they were involved in the reduction of nitrate to nitrite, with the resultant nitrite oxidizing Fe(II) abiotically via an indirect mechanism. However, the consortium which was obtained by inoculating a medium containing Fe(II) as the sole electron donor and nitrate as the electron acceptor, with sediment from a microcosm oxidized with added nitrate, was also dominated by *Betaproteobacteria* including *Herbaspirillum* sp. The survival of the consortium under these chemolithoautrophic conditions implies that these bacteria may be involved in a direct coupling between nitrate reduction and Fe(II) oxidation.

Biogeochemical Significance

Under the conditions studied, Tc(IV) sorbed to bioreduced sediments was largely oxidized by air but not by nitrate, and thus the NO_2^- and Fe(III) formed under denitrifying conditions did not oxidize the Tc(IV). These observations, which are consistent with other studies (Burke et al. 2006; McBeth et al. 2007; Morris et al. 2008; Fredrickson et al. 2009), impact on our understanding of the biogeochemical cycling of Tc in contaminated environments, especially on the management of land where bioreduction has been stimulated as an active remediation strategy to minimize the transport of the radionuclide. Of particular interest is the long term stability of the reduced Tc(IV) phases formed in sediments oxidized by NO_3^- additions, as they could be potentially protected (e.g., by Fe mineral assemblages) from mobilization by other strong oxidizing agents (Zachara et al. 2007). Also of interest is the role of microorganisms in a mechanistic understanding of metal oxidation in these sediments. Only a restricted number of organisms have been shown to catalyse the anaerobic oxidation of Fe(II) using NO_3^- as the sole electron acceptor, and the mechanisms of this process have not as yet been investigated previously. Our results from enrichment studies certainly suggest that *Betaproteobacteria* may play an important role in mediating this process at a nuclear relevant field site. Overall, our study highlights the fact that these microorganisms may play a previously undefined yet pivotal role in influencing contaminant fate and transport in these environments. Indeed, understanding the full redox cycle of these systems (including reoxidation scenarios) both in model, pure culture experiments and in more complex lab and field simulations is essential to implementation of optimal management at these nuclear legacy sites.

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