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# Bacterial Diversity and Composition of an Alkaline Uranium Mine Tailings-Water Interface

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**The microbial diversity and biogeochemical potential associated with a northern Saskatchewan uranium mine water-tailings interface was examined using culture-dependent and -independent techniques. Morphologically-distinct colonies from uranium mine water-tailings and a reference lake (MC) obtained using selective and non-selective media were selected for 16S rRNA gene sequencing and identification, revealing that culturable organisms from the uranium tailings interface were dominated by *Firmicutes* and *Betaproteobacteria*; whereas, MC organisms mainly consisted of *Bacteroidetes* and *Gammaproteobacteria*. Ion Torrent (IT) 16S rRNA metagenomic analysis carried out on extracted DNA from tailings and MC interfaces demonstrated the dominance of *Firmicutes* in both of the systems. Overall, the tailings-water interface environment harbored a distinct bacterial community relative to the MC, reflective of the ambient conditions (i.e., total dissolved solids, pH, salinity, conductivity, heavy metals) dominating the uranium tailings system. Significant correlations among the physicochemical data and the major bacterial groups present in the tailings and MC were also observed. Presence of sulfate reducing bacteria demonstrated by culture-dependent analyses and the dominance of *Desulfosporosinus* spp. indicated by Ion Torrent analyses within the tailings-water interface suggests the existence of anaerobic microenvironments along with the potential for reductive metabolic processes.**

***Keywords***: bacterial diversity, uranium mine tailing, sediment water interface, sulphate reducing bacteria, Ion Torrent analyses

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## Introduction

The Athabasca Basin in northern Saskatchewan hosts the largest high-grade uranium mines and deposits in the world, and represents about one third of global annual uranium production. In this region, Cameco Corporation operates two mines as well as a uranium mill at Key Lake (Natural Resources Canada, 2006; Saskatchewan Mining Association, 2010). The tailings from this mill are deposited within an in-pit tailings management facility (TMF). The potential migration of elements associated with uranium mine tailings, including As, Ni, U, and $^{226}$Ra, present an environmental concern (Pyle *et al*., 2002; Akob *et al*., 2007). Microbial processes can influence the redox status of heavy metal elements and radionuclides, and consequently their mobility (Selenska-Pobell, 2002). It is expected that upon decommissioning of these in-pit TMFs, the hydraulic gradients between the tailings and the regional ground waters would be small enough that the migration of contaminants would be controlled by diffusion, and not advection, with a minimum flux of heavy metals from the tailings into the regional groundwater system. Geotechnical, hydrogeological, and geochemical considerations have been key factors in the design of the TMFs in the Athabasca Basin; however, the potential for microbial metabolic activity to affect these processes upon decommissioning remains poorly understood. The broad habitat range of microorganisms known to participate in a variety of geochemical transformations suggests that they may play a role in the mobilization and subsequent transport of metal elements from tailing bodies into the surrounding environment (Miller *et al*., 1987).

The sediment-water interface overlying tailings deposits is a dynamic and complex aquatic environment characterized by steep gradients of oxygen, nutrients, organic matter, and high bacterial abundance, diversity and activity (Herlihy and Mills, 1985; Mills *et al*., 1989; Shelobolina *et al*., 2003). There have been limited studies carried out on the pore-water and sediment of some acidic mine tailings, but examination of the microbes inhabiting the sediment-water interface of an alkaline uranium mine tailings has not been documented. Furthermore, microbiological studies on acid mine effluent, streams or unsaturated uranium waste piles have focused on U(VI) reduction to the less soluble U(IV) form as a bioremediation strategy (Landa *et al*., 1991; Lovley and Phillips, 1992; Lovley *et al*., 1993), as well as the effect of microbial activity on the partitioning of metals between mobile and immobile phases (Miller *et al*., 1987). These conditions are in sharp contrast to those dominating TMFs in northern Saskatchewan, which are characterized by a high pH (≥9.0) and saturated conditions, and thus the potential

effect of microbial activity on the geochemical behaviour of contaminants associated with TMFs is poorly understood. Furthermore, studies from uranium tailings environments in other regions of the world are of limited use in understanding the geochemical controls in Saskatchewan TMFs, since the ore mined and the milling technology used in the contributing mills are unique. The mined ore at the Deilmann Tailings Management Facility (DTMF) frequently contains relatively high amounts of nickel (2%) and arsenic (1.2%), and tailings are deposited in a very alkaline state as a means of precipitating and immobilizing metals dissolved during processing. The potential effects of microbial diversity and activities on the environmental behavior of immobilized metals such as Cu, Fe, Mo, Ni, and Zn, and oxyanions such as As and Se present in porewaters, at the interface between uranium mine tailings and overlying pond water is similarly not well understood.

It has previously been shown that microbial communities with diverse metabolic profiles exist at the Rabbit Lake TMF, a high pH environment, where the elapsed time since deposition of the tailings (>10 years for the deepest samples collected) did not appear to have a measurable impact on microbial numbers or metabolic profiles (Wolfaardt *et al.*, 2008). No information is presently available on the distribution, relative abundance and activity of microorganisms in the DTMF, or any other high-pH TMF from northern regions. Characterization of the organisms present at the tailings-water interface of the DTMF was therefore undertaken, using both culture-dependent and -independent approaches, to obtain benchmark diversity measurements relative to a nearby reference lake.

## Materials and Methods

### Sampling sites

The Key Lake uranium processing mill is located 570 km north of Saskatoon, Saskatchewan, Canada, on the southern rim of the uranium-rich Athabasca Basin (coordinates at 57°12′24″N and 105°39′33″W). The Key Lake site was an active mining operation since 1984; however, the last ore was mined from the Deilmann Open Pit in 1997, after which mining operations ceased. Uranium processing of ore from regional mining operations has subsequently resulting in tailings that have been deposited in the excavated Dielmann pit (now known as the Dielmann Tailings Management Facility, or DTMF) since 2000. The DTMF is 1,000 m by 600 m, having approximately 60 m of tailings material emplaced under approximately 45 m of water (Bondici *et al.*, 2013). Samples were collected from 3 distinct sites of the DTMF, designated as sites D2, D3, and E2. Site D2 was closest to the shoreline; whereas, E2 was located at the center of the DTMF. Site D3 was located in between D2 and E2 (Fig. 1). The radial distance between the 3 sampling sites was approximately 200 m. McDonald Lake is located approximately 5 km from the DTMF, but is unaffected by mining operations and therefore was selected as a reference lake (hereafter referred to as MC) for comparative microbial analyses (Fig. 1). The MC is shallow (average depth 3.0 m) and surrounded by trees and shrubs. At the time of sampling (August, 2010), an abundance of plant and leaf litter was visible in the littoral zones of the MC.

### Physicochemical properties of the water body and tailings-water interface of the DTMF

A panel of physiochemical parameters was measured at 1 m depth intervals throughout the DTMF water column (from surface to the tailings-water interface) using a Hydrolab (Hydrolab DS5X, Hach, USA). Measured parameters included temperature, pH, conductivity, total dissolved solids (TDS), salinity, dissolved oxygen (DO), and oxidation-reduction potential (ORP). In the MC, only pH, conductivity, TDS and salinity data were available.

### Sampling procedures

Samples for microbiological analysis were collected from the tailings-water interface zone (the first 1 m of sediment) of the DTMF and MC. In terms of consistency, the DTMF interface samples were a yellowish gelatinous semisolid; whereas, the MC interface material was clay-like and brown-colored. All interface samples from the DTMF and MC were collected in August, 2010 using a Van Dorn (SKU 1960-G65) horizontal water sampler (Wildco, Wildlife Supply Company, USA). Tailings-water interface and MC interface samples were collected in duplicate glass bottles which were filled completely with sediment and water to eliminate air headspace. In between sampling events, the sampler and associated equipment were rinsed with sterile deionized water. Counting sulfate reducing bacteria (SRB) via dilution and inoculation of pre-prepared media was initiated on-site to minimize external effects. Samples were processed immediately (described below) for enumeration of total culturable cell counts (within 3–4 h after collection) in the laboratory on-site at Key Lake. Samples for remaining analyses were maintained at 4°C before processing. All the analyses were carried out within 15 days of collection.

### Enumeration of total culturable bacteria

Interface samples were serially diluted using sterile physiological saline (0.85% NaCl) and plated on to two types of non-selective agar plates (R2A and TSB; Difco media, USA). Plates were incubated under aerobic and anaerobic conditions (using an anaerobic glovebox with an $O_2$-free atmosphere consisting of 10% $CO_2$, 80% $N_2$, and 10% $H_2$) at room temperature for 15 days, after which time visible colonies

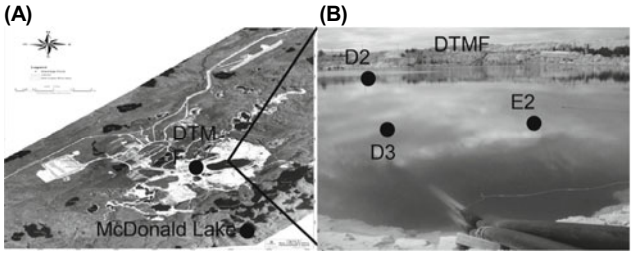


**Fig. 1. Map and location of the sampling sites at the Key Lake uranium mine site.** (A) Map of the DTMF and MC site. (B) D2, D3, and E2 indicate the sampling locations in the DTMF.

were enumerated. All plating was done in triplicate.

### Enumeration of SRB

Sulfate reducing bacteria were enumerated using the three-tube, most probable number (MPN) method. Accordingly, a 1 ml sample of homogenized composite interface material was drawn into a sterile 1 ml syringe and transferred to 10 ml vials containing 9 ml of pre-sterilized and pre-reduced Postgate's B semisolid SRB medium (Jain, 1995), and then serially-diluted to provide up to $10^{-3}$ dilution for the SRB-MPN determination. The MPN tubes were incubated at room temperature for 28 days and observed twice a week for a change in color of the medium (formation of black precipitate) indicative of the presence of SRB. All determinations were performed in triplicate. In parallel, SRB-like organisms were enumerated, in triplicate, using the Sani-Check™ SRB kit (Biosan Laboratories, Inc., USA). The inoculated SRB tubes were inspected daily for 5 days, at which time the results were recorded and interpreted following the procedures of the manufacturer. The tubes showing positive counts after 5 days were confirmed to contain only SRB-like organisms in the samples.

### Isolation of culturable bacteria

After incubation of the interface samples on agar plates at room temperature (~22±2°C) under both aerobic and anaerobic conditions for more than two weeks, unique colonies were picked (based on colony morphology, color, surface texture, and pigmentation) and sub-cultured (2x) onto R2A agar plates to isolate individual colonies. A total of 128 colonies were picked, isolated and purified by consecutive subculturing.

### Identification of culturable bacterial isolates

Isolated bacterial colonies were subjected to DNA extraction using the Qiagen DNA extraction kit (Qiagen, USA). Partial 16S rRNA gene polymerase chain reaction (PCR) amplification was performed using the 8F (5′-AGAGTTTGATCC TGGCTCAG-3′) and 531R (5′-ACGCTTGCACCCTCCG TATT-3′) (Hirkala and Germida, 2004) universal primer sets, yielding a ~500 bp PCR product. The final concentration of reagents used for a 50 µl PCR reaction mixture were as follows: 1.0 µM each of the forward and reverse primers, 1.0 U *Taq* polymerase (Invitrogen, Canada), 1.0 µM $MgCl_2$, 1.0× PCR buffer, and 4.0 µM of deoxyribonucleotide triphosphates (dNTPs). The PCR assay reaction mixture was adjusted with sterile distilled water to a final volume of 50 µl. The thermal cycling profile was set according to Hirkala and Germida (2004). For sequencing, the PCR amplicons were purified using the Qiagen purification kit according to the manufacturer's instructions (Qiagen). Taxonomic identification and clustering of 16S rRNA gene sequences were assigned based on similarities to available strains within the NCBI 16S rRNA sequence database using the BLAST search tool. Sequences of 16S rRNA were verified using the Chromas software program, version 2.32 (Technelysium Pty. Ltd., Australia). The sequence alignments were also edited manually using Chromas. Identification of distinct isolates was based on percent identity where 100% identical sequences were affiliated with the same taxon.

### Comparative analyses of diversity of culturable bacteria from the DTMF and MC interface region

The identified bacterial isolates were group-defined using the Ribosomal Database Project (RDP release 10). Thirty four unique isolates were subjected to further biocomputational analyses and clustered into the major bacterial groups. Diversity and abundance of the cultural bacterial community were determined by enumerating the number of isolated colonies from the selective and non-selective agar media incubated under aerobic and anaerobic conditions and by calculating their percentage and distribution among various bacterial groups. Genetic diversity among the bacterial isolates was determined by phylogenetic analysis of the 16S rRNA sequences.

### Phylogenetic analysis

Phylogenetic trees of the aligned unique sequences and their closest 16S rRNA gene matches were constructed by a neighbor joining method (Saitou and Nei, 1987) using MEGA v5 with 1,000 bootstrap replicates (Tamura *et al*., 2011). Closely-related bacterial sequence data were selected from the NCBI database using BLAST.

### Nucleotide sequence accession numbers

The 16S rRNA sequences for the isolated bacteria were deposited in the EMBL Nucleotide Sequence Database under the accession numbers HE649225 to HE649270.

### Ion Torrent analyses

Next-generation sequence analyses were conducted using the Ion Torrent Personal Genome Machine (PGM; Life Technologies, USA) (Yergeau *et al*., 2012; Bondici *et al*., 2013). A set of primers specific to the V5 region of the 16S rRNA gene was used for the Ion Torrent sequencing. To these primers, Ion Torrent adapter A (forward) and adapter P1 (reverse) fragments were attached. In addition to the adapters, the target-specific forward primer also included a key tag and a multiplex identifier (MID). The sequence of the primers were F5′-CCA TCT CAT CCC TGC GTG TCT CCGACTCAGMIDGATTAGATACCCTGGTAG and R5′-CTCTCTATGGGCAGTCGGTGATCCGTCAATTCCTT TRAGTTT, respectively. The sequencing of 5 base pair MIDs used for each of the samples was as follows: ATCAG (D2), CGTGT (D3), CTCGC (E2), and TGATA (MC). The PCR reactions were carried out in 50 µl volumes containing 2 µl of template DNA, 1.0 µM each primer, 1.0 U *Taq* polymerase (Invitrogen, USA), 1.0 µM $MgCl_2$, 1.0× PCR buffer (both provided with the *Taq* polymerase), and 4.0 µM of dNTPs. Cycling conditions involved an initial 5 min denaturing step at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 57°C, and 45 sec at 72°C, and a final elongation step of 10 min at 72°C. The PCR amplicons were purified using the Qiagen purification kit according to the manufacturer's instructions and quantified using a NanoDrop spectrophotometer. Pooled, key-tagged gene amplicons were sequenced at the Biotechnology Research Institute (Montreal, Quebec,

**Table 1.** Physicochemical properties of the water body and tailings–water interface of DTMF

| Sites | Location/depth | Temp. (°C) | TDS[a] (g/L) | Conduct. (µS/cm) | pH | Salinity (ppt) | ORP[b] (mV) | DO[c] (%Sat.) | DO (mg/L) |
|---|---|---|---|---|---|---|---|---|---|
| E2 | 1 m | 14.2 | 1.0 | 1531 | 5.9 | 0.78 | 255 | 107.5 | 10.31 |
| D2 | | 14.2 | 0.9 | 1472 | 6.9 | 0.81 | 204 | 109.4 | 10.49 |
| D3 | | 14.3 | 1.0 | 1528 | 6.9 | 0.81 | 228 | 108.4 | 10.37 |
| E2 | 20 m | 8.2 | 1.2 | 1815 | 6.1 | 0.97 | 224 | 20.4 | 2.24 |
| D2 | | 8.1 | 1.2 | 1863 | 6.5 | 0.99 | 193 | 22.1 | 2.43 |
| D3 | | 8.2 | 1.2 | 1905 | 6.5 | 1.02 | 190 | 20.6 | 2.27 |
| E2 | Interface (43 m) | 12.2 | 1.6 | 2467 | 10.2 | 1.33 | 118 | 3.2 | 0.32 |
| D2 | | 10.9 | 1.5 | 2300 | 9.4 | 1.23 | 126 | 1.7 | 0.18 |
| D3 | | 10.8 | 1.6 | 2571 | 9.2 | 1.38 | 118 | 1.8 | 0.18 |
| McDonald Lake | Interface | ND | 0.18 | 21 | 7.0 | 0.00 | ND | ND | ND |

[a] TDS, total dissolved solids
[b] ORP, oxidation-reduction potential
[c] DO, dissolved oxygen

Canada) using the Ion Torrent PGM and a 314 chip. The sequences retrieved from Ion Torrent analyses were processed using RDP pyrosequencing pipeline tools. Low-quality sequences with lower scores and shorter than 100 bp were removed using the pipeline initial process tool. Hierarchical clustering of sequences was obtained by the RDP classifier with bootstrap cut-off value of 50%.

### Statistical analyses

Analyses of variance (ANOVA) was used to demonstrate differences among the sample means at $P<0.05$ using SPSS v17 (Statistical Package for the Social Sciences). The number of culturable bacteria and culture-independent sequence frequencies were used for principal components analyses (PCA) using PRIMER v6 software (PrimerE Ltd., UK). Similarity and dissimilarity analyses were carried out using the paired sample t-test and a $P<0.05$ significance level using SPSS. Correlation among the class-wise sequence data and physicochemical data (TDS, conductivity, salinity, and pH) from the DTMF and MC were done using means and standard deviation by bivariate analyses at the <0.01 and <0.05 significance levels.

## Results and Discussion

### Physicochemical properties of the water and tailings-water interface of the DTMF

Physicochemical parameters were measured for the DTMF water body at 1 m intervals from the air-water interface to the tailings-water interface for each of the three sampling locations (D2, D3, and E2). Representative physicochemical data is presented for the 1, 20, and 43 m (interface) sampling depths (Table 1). TDS, conductivity, pH and salinity increased with depth, ranging from 1.0 to 1.6 g/L, 1510 to 2446 µS/cm, 6.6 to 9.6 and 0.8 to 1.4 ppt, respectively. In contrast, redox potential, DO (% saturation) and DO (mg/L) gradually decreased with depth, ranging from 229 to 121 mV, 108 to 2.2% and 10.39 to 0.23 mg/L, respectively. An exception to this trend existed for temperature, which was highest at the air-water interface (14°C), rapidly decreased to ~8°C at 20 m, and then increased again with depth to 10–12°C at the water-sediment interface (Table 1). It is assumed that the variation in temperature was due to solar heating of the upper layers of the DTMF. Physicochemical parameters for DTMF sites D2 and D3 were similar; whereas, E2 was slightly different from D2 and D3 with regard to temperature, pH and DO profiles (Table 1). The DTMF tailings body was highly-alkaline, with an average pH of ~10. A limited physicochemical data set was available for the MC, where TDS, pH, salinity and conductivity were determined to be 0.18 g/L, 7.0, 21 µS/cm and 0 ppt, respectively. The average temperature of the DTMF tailings (11.3°C) was considerably higher than the average temperature of Rabbit Lake uranium mine tailings (~0°C) also located in northern Saskatchewan; however, the pH of Rabbit Lake tailings (9.9) determined by Moldovan and Hendry (2005) and Wolfaardt *et al.* (2008) were very similar to the DTMF. The concentration of metal elements within the DTMF pore water and MC water has been described by Shaw *et al.* (2011) and also compiled from internal company reports (Harm Maathuis, Cameco Corporation), respectively. Accordingly, the average concentrations of arsenic, cadmium, iron, manganese, molybdenum, nickel and selenium in DTMF porewater were 2.9100, 0.0026, 0.0086, 0.1396, 13.1484, 0.04495, and 0.0384 mg/L, respectively, and 0.0002, <0.0001, 0.0310, 0.0180, <0.0001, 0.01 and <0.0001 mg/L, respectively, in the MC.

Natural alkaline environments are relatively rare. The best-studied alkaline environments are temperate and subtropical alkaline soda lakes with pH values ranging from 8 to more than 12, containing large amounts of sodium carbonates in combination with low concentrations of $Mg^{2+}$ and $Ca^{2+}$ (Duckworth *et al.*, 1996; Jones *et al.*, 1998). These extreme conditions are reflected by the adaptation of the microorganisms found in the lakes, many species of which are both

**Table 2.** Total culturable bacteria counts from the DTMF and MC interfaces

| Sites | Counts $Log_{10}$ CFU/ml (SD)[a] |
|---|---|
| DTMF–D2 | 6.89 (±0.21) |
| DTMF–D3 | 6.59 (±0.09) |
| DTMF–E2 | 6.33 (±0.26) |
| MC | 6.14 (±0.0) |
| Rabbit Lake[b] | 5.17 (±0.0) |

[a] SD, standard deviation
[b] Data from Wolfaardt *et al.* (2008)

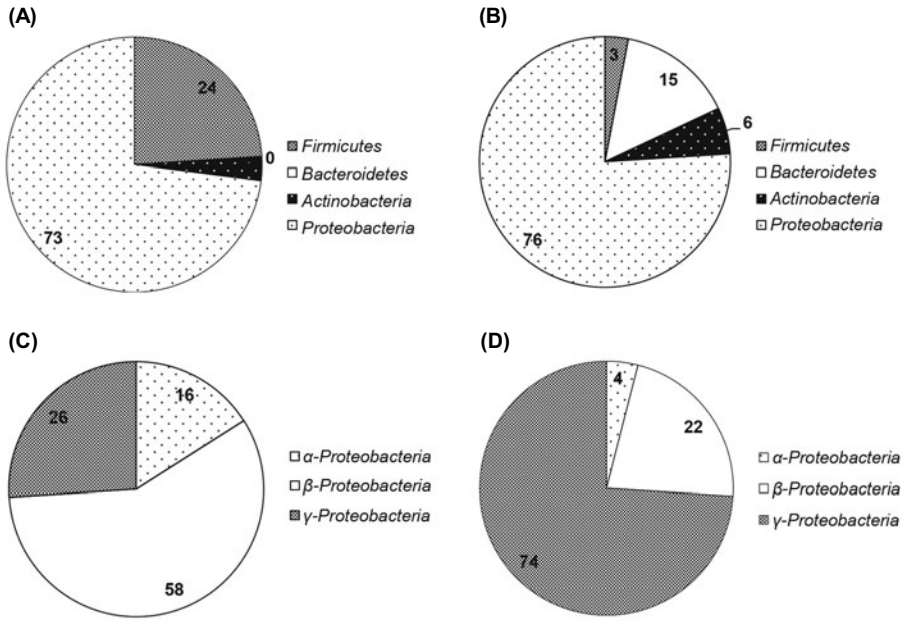


**Fig. 2. Diversity and distribution of culturable bacteria from the DTMF and MC interfaces.** (A) distribution of major culturable bacterial groups in DTMF; (B) distribution of major culturable bacterial groups in MC; (C) distribution of culturable *Proteobacteria* in DTMF and (D) distribution of culturable *Proteobacteria* in MC. Percentages of bacterial groups were calculated based on the number of total unique bacterial isolates by culture- based methods.

alkaliphilic and halophilic, or extremely halotolerant (Sorokin *et al*., 2003; Ma *et al*., 2004). The DTMF, with a pH around 10, along with high conductivity, salinity, TDS and low oxygen and ORP, may be considered a somewhat extreme environment and thus may also contain adapted and metabolically-distinct microorganisms.

### Total culturable bacterial counts

Total culturable bacterial counts from the DTMF sites (ranging from 6.33 to 6.89 $Log_{10}$ CFU/ml) were approximately 0.5 $Log_{10}$ higher than for the MC (6.14 $Log_{10}$ CFU/ml) (Table 2). The total bacterial counts from the interface of the DTMF were 1 to 2 $Log_{10}$ higher than previously reported counts on 10% trypticase soy agar (TSA) for the TMF at Rabbit Lake, also a high-pH system (Wolfaardt *et al*., 2008), indicating that the DTMF interface region is relatively more microbiologically-active, based on culturable cell counts. The total bacterial counts from the MC near the DTMF were also 2 $Log_{10}$ higher than the upstream surrounding lakes in the Rabbit Lake area (Wolfaardt *et al*., 2008). The elevated numbers of bacteria in the DTMF in relation to Rabbit Lake could be linked with greater carbon availability (on average; the DOC available in DTMF was 20 µg/ml vs <0.8 µg/g in the Rabbit Lake region (Wolfaardt *et al*., 2008) occurring either as a consequence of $UO_2$ processing (requiring chemicals such as kerosene, isodecanol, amines, etc.) or arising naturally (airborne sediment, surface runoff, algal primary production, etc.). Differences in the temperature profiles in the two systems could also provide an entirely-reasonable explanation for the elevated numbers in the DTMF, as the temperature was almost 10°C higher than in the Rabbit Lake TMF (Wolfaardt *et al*., 2008).

### Culturable bacterial isolates from DTMF and MC

Following plating of samples from the DTMF and MC, a total of 128 bacterial isolates were recovered, 74 of which were from the DTMF and 54 from the MC. Gene sequence (16S rRNA) analysis was used to determine that of these 128 isolates, 34 were unique (18 were from the DTMF and 16 were from the MC) and that from the four sampling sites (three from DTMF and 1 from MC), each yielded 21 to 32% of the total unique isolates. Pooling the bacterial isolates from both the DTMF and MC revealed the following proportional groupings; *Proteobacteria* (75%), *Firmicutes* (14%), *Bacteroidetes* (7%), and *Actinobacteria* (4%). The DTMF microflora was dominated by *Proteobacteria* isolates (73%), whereas no *Bacteroidetes* were detected. Similarly, *Proteobacteria* was the proportionally-dominant culturable phyla in the DTMF tailings waste and *Bacteroidetes* was the least frequent (Bondici *et al*., 2013). Twenty-four percent of the DTMF bacterial community consisted of *Firmicutes* and only 3% were *Actinobacteria* (Fig. 2A). In the MC, the microflora was again dominated by *Proteobacteria* (76%) followed by *Bacteroidetes* (15%). Six and 3% of the total culturable bacterial community from the MC were *Actinobacteria* and *Firmicutes*, respectively (Fig. 2B). Despite the fact that 75% of the isolates from the DTMF and MC were *Proteobacteria*, there were distinct differences in *Proteobacteria* composition between the two bacterial communities. For example, the DTMF consisted of mainly *Betaproteobacteria* (58%), often thought to be more dominant in nutrient/carbon rich environments (Ley *et al*., 2006), followed by *Gammaproteobacteria* (26%) and *Alphaproteobacteria* (16%) (Fig. 2C). In contrast, the MC was dominated by *Gammaproteobacteria* (72%), followed by *Betaproteobacteria* (22%), and with *Alphaproteobacteria* making up only (4%) of the culturable isolates (Fig. 2D). Neither of the study sites yielded any culturable *Deltaproteobacteria*. In general, it is noteworthy that the proportionally-higher percentage of culturable *Proteobacteria* may reflect the fact that they tend to be much easier to cultivate on agar media than *Firmicutes* (Wagner, 2008), leading to a relatively-higher apparent abundance.

The *Betaproteobacteria* includes several groups of aerobic

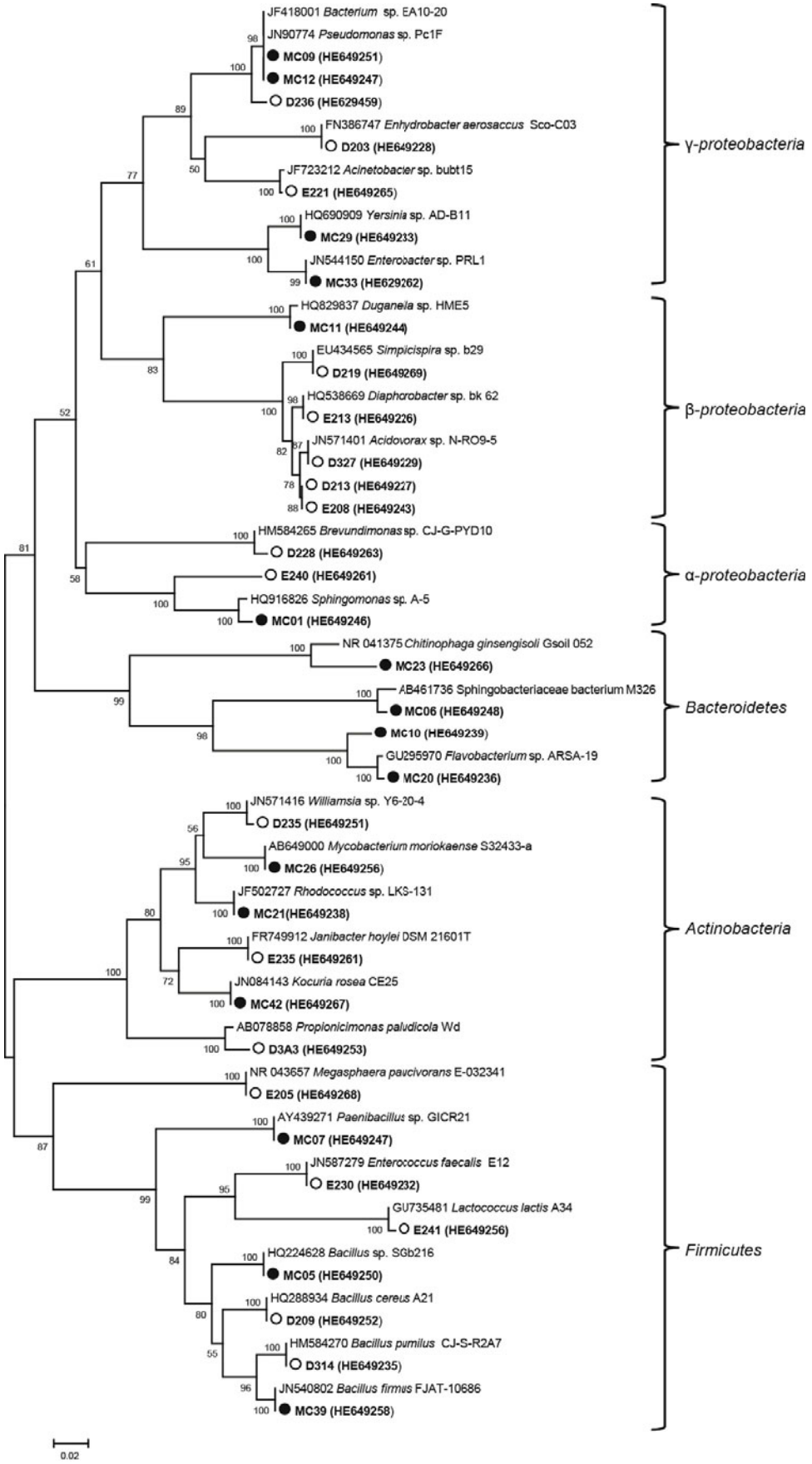


**Fig. 3. Unrooted Neighbor Joining Tree (N-J tree) of the partial 16S rRNA gene sequence data showing the diversity and distribution of the culturable bacteria isolated from the DTMF (open circle) and MC (solid black circle) interface along with the closely-related 16S rRNA sequence data from the NCBI database.** N-J tree was generated with Kimura 2 correction (Kimura, 1980) for distance calculations. Bootstrap percentages retrieved in 1,000 replications are shown at the nodes. Clusters and bootstrap values that were 50% or greater are indicated. The scale bar (0.02) indicates the number of nucleotide substitutions per site.

or facultatively-anaerobic bacteria with highly-versatile degradation capacities, including chemolithotrophic genera and some phototrophs. The observed abundance of *Betaproteobacteria* amongst DTMF isolates is in contrast with previous reports indicating the prevalence of *Gammaproteobacteria* in underground uranium mine gallery of Jaduguda uranium mine, India (Islam and Sar, 2011) and uranium mill tailings in Shiprock, New Mexico, USA (Selenska-Pobell *et al*., 2001; Radeva and Selenska-Pobell, 2005). However, the prevailing conditions in these two sites are significantly different from that of the DTMF at Key Lake in terms of key physicochemical parameters, offering a reasonable explanation for the observed differences. The observed pH and conductivity at the Jaduguda uranium mine site was 6.4 and 200 μS/cm (Islam and Sar, 2011); whereas, the DTMF interface had an average pH and conductivity of 9.9 and 2446 μS/cm, respectively. Similarly, Radeva and Selenska-Pobell (2005) reported the average pH of the Shiprock uranium mill sampling site to be 6.8. Generally, the abundance of alkaline-adapted *Betaproteobacteria* in the DTMF suggests a potential for bioconversion of heavy metals in the DTMF system (Miller *et al*., 1987; Burkhardt *et al*., 2010).

The *Alphaproteobacteria* comprise most of the phototrophic genera, but also includes several genera capable of metabolising C1-compounds (single carbon compounds), symbionts of plants and animals, and a group of pathogens. The low frequency or absence of *Alphaproteobacteria* has also been seen in other studies, for example, in soil samples from uranium mill tailings at Gittersee/Coschütz in Germany where *Alphaproteobacteria* were not predominant (Selenska-Pobell, 2002).

The *Gammaproteobacteria* consist mainly of human and animal pathogens, and are generally present in eutrophic environments; however, their presence in uranium mine tailings has been indicated by several studies (Selenska-Pobell, 2002; Radeva and Selenska-Pobell, 2005; Moreels *et al*., 2008). The presence of 75% of the total *Proteobacteria* being *Gammaproteobacteria* in the DTMF is suggestive of possible exposure of the system to animal faecal material, and reflective of the routine use of the DTMF for disposal of human sewage.

*Firmicutes* are one of the most common bacterial groups isolated from extreme environments (Bowers *et al*., 2009; Dib *et al*., 2009). Many *Firmicutes* produce endospores which are resistant to unfavourable conditions and might overcome the conditions of high pH, low oxygen, high or low temperatures, high NaCl and elevated heavy metal concentrations associated with industrial activities (Bowers *et al*., 2009). The prevalence of *Firmicutes* in the DTMF interface zone is consistent with the physicochemical conditions and selective pressure of the DTMF. *Bacteroidetes*, which were predominant in the MC, are widely-distributed bacteria mainly found in environments enriched with warm blooded animal feces; thus, an impact of animals and waterfowl activity at the MC sites appears likely. As both the DTMF and MC are open environments in close geographic proximity, we expected to see some similarity in the microbial diversities in both systems. The distinct differences observed in the DTMF environment, which has been developed recently in comparison to MC, suggests that the physical and chemical conditions indeed impose a significant selective pressure, likely based on the combined effects of elemental toxicity, pH, and salinity. In such an environment, the existence and proliferation of a distinctive bacterial community is not surprising, and accordingly a number of the isolates found in the DTMF interface were determined to be putatively-novel species based on their having less than 97% sequence homology to known 16S rRNA sequences. In contrast, the MC contained fewer putative novel species.

A neighbour-joining phylogenetic tree showed clustering of the DTMF and MC bacterial isolates (Fig. 3), including very distinct clades of *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. *Proteobacteria* also showed clear subdivisions of *Alpha*, *Beta*, and *Gammaproteobacteria*. In most cases, the DTMF isolates separated into a distinct branch of the tree with a higher bootstrap value, further supporting the notion that the DTMF interface represented a distinct environment. The group *Bacteroidetes* was comprised solely of MC isolates and was similar to the cluster formed by *Gammaproteobacteria*, where again most of the isolates originated from the MC. Out of 7 *Gammaproteobacteria*, only 3 came from the DTMF, 2 of which formed a separate cluster, which were *Acinetobacter* sp. (97% similarity) and *Enhydrobacter* sp. (100% similarity). All other isolates from the MC formed two distinct clades. DTMF isolates D213, D327, D219, E213, and E208 were part of the *Betaproteobacteria* and distantly related to the MC isolates. The DTMF interface was dominated by special groups of culturable bacteria from the *Betaproteobacteria* and *Firmicutes*; whereas, the *Gammaproteobacteria* and *Bacteroidetes* were dominant in the MC (Fig. 3). The contrasts evident in the respective culturable bacterial communities clearly point to distinct physicochemical properties of the DTMF compared with the MC, and provides some insight into the possible metabolic activities that may be occurring in the DTMF interface zone. A detailed phylogenetic analysis was previously carried out on the bacterial communities in uranium ores and surrounding soils from the Banduhurang open cast uranium mine, India, by 16S rRNA sequence analyses (Islam *et al*., 2011). The *Gammaproteobacteria* from this study were represented by 7 ribotypes, covering 56–65% of the communities present in ore samples, while showing relatively lower abundance (30%) in association with soils. All ribotypes under the subphylum *Betaproteobacteria* retrieved from ores were affiliated to the order *Burkholderiales* (Islam *et al*., 2011). In contrast, no *Burkholderia* isolate was recovered in the present study from any of the DTMF interface samples. Among the *Alphaproteobacteria*, *Brevundimonas* spp. (99% similarity)

**Table 3. Total SRB counts in DTMF and MC determined by MPN method and SANICHECK SRB kit**

| Sites | SRB counts/ml (MPN) | SRB like organisms counts/ml (SANICHECK kit) |
|---|---|---|
| D2 | 23 | ≤10[a] |
| D3 | 6 | ≤10 |
| E2 | 6 | ≤10 |
| MC | >700[b] | >10 |

[a] Black precipitate was observed after 5 days of incubation thus the counts were considered as ≤ 10 $ml^{-1}$ in accordance with the manufacturer's instructions.
[b] Samples were positive for all 3 dilution tubes.

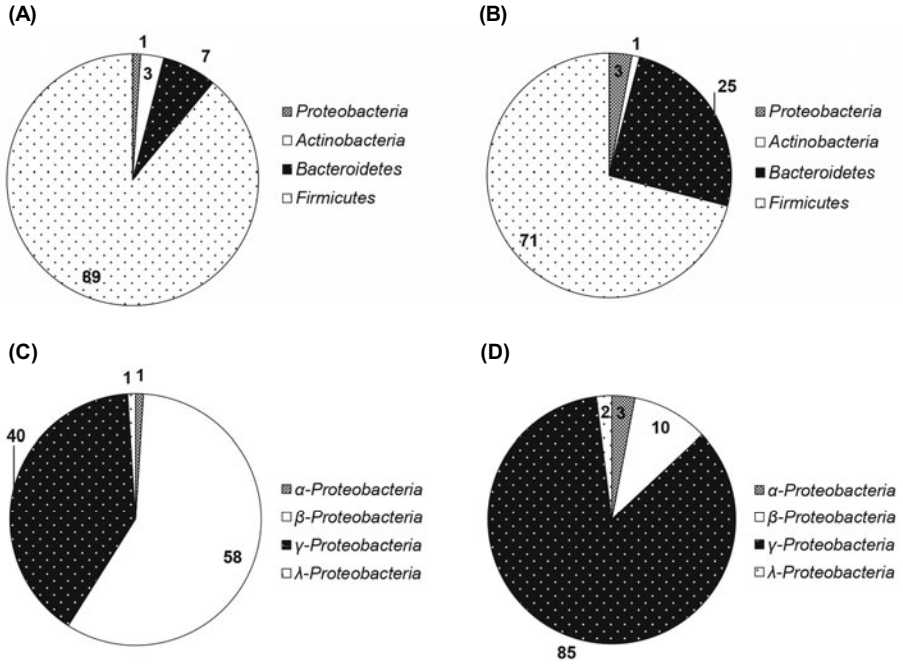


**Fig. 4. Diversity and distribution of bacteria in DTMF and MC interface as revealed by Ion Torrent analyses.** (A) distribution of major bacterial groups in DTMF; (B) distribution of major bacterial groups in MC; (C) distribution of *Proteobacteria* in DTMF and (D) distribution of *Proteobacteria* in MC. Percentage distribution was calculated based on the total number of sequences available in each bacterial group.

was present in our study and was also observed by Islam *et al.* (2011). Among the *Firmicutes*, Bacilli were common and represented at least 5 unique bacterial isolates from the present study; whereas, Islam *et al.* (2011) found only one *Bacillus* sp. in their uranium ore sample. Presence and abundance of these isolates at the DTMF water tailings interface were similar to the isolates in the tailings body (Bondici *et al*., 2013).

## SRB counts

Based on the MPN data (Table 3), the MC interface sample contained ~100 times higher SRB counts than the DTMF samples ( >700 SRB/ml for MC versus 23, 6 and 6 SRB/ml for sampling sites D2, D3, and E2, respectively) (Table 3). The SANICHECK$^{TM}$ kit yielded similar low counts of ≤10 SRB-like organisms per ml in DTMF and >$10^4$ $ml^{-1}$ in MC. The DTMF SRB counts are similar to the low SRB counts determined from a uranium pit mine in Brazil (Benedetto *et al*., 2005), and SRB numbers in metal- and radionuclide-contaminated soils in a former mining area near Ronnenberg, Germany (Sitte *et al*., 2010). These culture-based findings suggest that the highly-oxic chemical state of the ferrihydrite-complexed tailings was not conducive to the recovery and growth of the strictly anaerobic SRB.

## Bacterial diversity in DTMF and MC by Ion Torrent analyses

Using Ion Torrent high-throughput sequence analysis, an estimated 36000 classified sequences were obtained from all four sampling sites. The number of sequences varied from 20 to 28% in E2, MC, D3, and D2 sites, respectively. In the DTMF, 89% of sequences retrieved were *Firmicutes* and 7, 3, and 1% were from *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, respectively (Fig. 4A). In contrast, 71% of the sequences from the MC were *Firmicutes* and 25, 3, and 1% were *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (Fig. 4B), respectively. When the total *Proteobacteria* data were analysed (as they were dominant in culture-based analyses), 58% were found to be *Betaproteobacteria* in the DTMF and 40% were found to be *Gammaproteobacteria* (Fig. 4C). In the MC, *Gammaproteobacteria* constituted 85% of the total *Proteobacteria* and only 10% were *Betaproteobacteria* (Fig. 4D). *Deltaproteobacteria* (2% of total *Proteobacteria*) was present in the MC but not in the DTMF. The low numbers of *Proteobacterial* sequences found using IT sequencing contrasts with the high number of culturable *Proteobacteria* isolates. This observation is thought to be reflective of the respective ease and difficulty of culturing *Proteobacteria* and *Firmicutes*, respectively, and reveals the potential to reach erroneous conclusions when either culture-dependent

**Table 4. Percentage distribution of 5 dominant bacterial genus in DTMF and MC demonstrated by Ion Torrent analyses.** Percentage is calculated based on the total number of sequences.

| Genus | Class | Percentage in total sequences | |
|---|---|---|---|
| | | DTMF (average) | MC |
| *Anaerovorax* | *Clostridia* | 0 | 6.6 |
| *Buttiauxella* | *Gammaproteobacteria* | 0 | 13.2 |
| *Desulfosporosinus* | *Clostridia* | 55.2 | 20.6 |
| *Dysgonomonas* | *Bacteroidia* | 4.1 | 13.2 |
| *Proteiniclasticum* | *Clostridia* | 16.3 | 0.1 |

**Table 5. Correlation coefficient analyses (two-tailed) among bacterial classes (number of isolates and sequence frequencies) and physicochemical parameters in DTMF and MC.** APB, *Alphaproteobacteria*; BPB, *Betaproteobacteria*, GPB, *Gammaproteobacteria*; DPB, *Deltaproteobacteria*; SPB, *Sphingobacteria*; BAC, *Bacteroidia*; BCL, *Bacilli*; CLD, *Clostridia*, ACB, *Actinobacteria*; MLC, *Mollicutes* and MMB, *Methanomicrobia*.

| Factors | Bacterial classes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | APB | BPB | GPB | DPB | SPB | BCD | BCL | CLD | ACB | MCL | MMB |
| TDS | -0.930 | 0.679 | -0.785 | -0.998[a] | -0.998[a] | -0.274 | 0.358 | 0.340 | 0.268 | 0.484 | -0.998[a] |
| Conductivity | -0.936 | 0.612 | -0.763 | -0.996[a] | -0.996[a] | -0.238 | 0.388 | 0.309 | 0.305 | 0.498 | -0.996[a] |
| pH | -0.994[a] | 0.690 | -0.885 | -0.959[b] | -0.922 | -0.469 | 0.130 | 0.536 | 0.051 | 0.730 | -0.959[b] |
| Salinity | -0.937 | 0.611 | -0.763 | -0.996[a] | -0.992[a] | -0.239 | 0.387 | 0.310 | 0.304 | 0.501 | -0.996[a] |

[a] Correlation is significant at the 0.01 level (2-tailed).
[b] Correlation is significant at the 0.05 level (2-tailed).

or culture-independent community characterization techniques are used in isolation. A total of 73 genera were identified in both the DTMF and MC by IT analyses, of which, 43 were present in DTMF, 37 were present in MC, and 14 were common to both. Five most dominant bacterial genera were selected to observe their abundance in DTMF and MC by calculating their percentage in the total number of sequences (Table 4). It was observed that *Desulfosporosinus*, *Dysgonomonas*, and *Proteiniclasticum* were the most predominant genera in the DTMF of which *Desulfosporosinus* comprised 55.1% of the total sequences. On the other hand, MC also showed 20.6% abundance of sequences of *Desulfosporosinus* (Table 4). The genus *Desulfosporosinus* from the *Firmicutes* group was the most abundant bacterial genus in both systems.

Ion Torrent data indicated that the most abundant bacteria in the DTMF and the MC was in fact the SRB, *Desulfosporosinus*, despite the fact that MPN and SANICHECK data suggested that SRB were relatively rare. No other study besides the tailings of this site has yet demonstrated the presence of *Desulfosporosinus* in an alkaline uranium mine impacted site. *Desulfosporosinus* spp. are obligate anaerobes, autotrophs and spore-formers (Stackebrandt *et al.*, 2003) capable of consuming short-chain fatty acids as members of

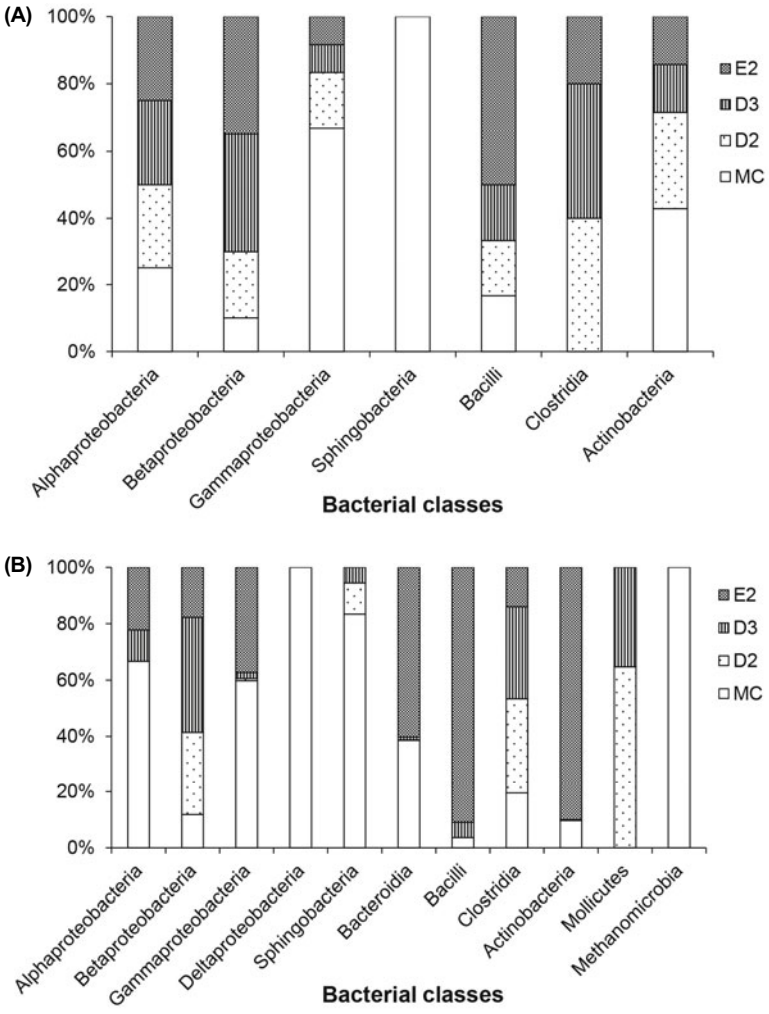


**Fig. 5. Comparative site-wise community composition and diversity of bacteria for DTMF and MC interface samples.** D2, D3, and E2 are the DTMF sampling locations; whereas, MC is the MC sampling location. (A) diversity of bacteria based on culturable data and (B) diversity of bacteria based on culture-independent data by Ion Torrent analyses. Percentage was calculated based on available unique bacterial isolates (A) and number of sequences observed in the DNA sample (B) in each sampling site.

consortia that collectively degrade petroleum hydrocarbons (Suzuki *et al*., 2002). The present work is the first report of *Desulfosporosinus* in an engineered, highly alkaline environmental setting. Members of the genus mainly inhabit industrially-impacted soil and sediments, coal mining-impacted lakes and radionuclide-contaminated sediments (Singleton, 1993; Vatsurina *et al*., 2008). *Desulfosporosinus*-like strains have repeatedly been seen to dominate subsurface bacterial communities associated with radionuclide-contaminated sediment that include the Midnight Mine, Washington (Suzuki *et al*., 2002), the DOE Field Research Center in Oak Ridge, Tennessee (Shelobolina *et al*., 2003), and uranium mine tailings in Shiprock, New Mexico (Shelobolina *et al*., 2003). Since sulfate is present in the DTMF at concentrations ranging from 2 to 3 orders of magnitude greater than that of uranium, sulfate might play a dominant role in *Desulfosporosinus* metabolism. However, it may also be the case that since the DTMF is an "open" system, significant numbers of *Desulfosporosinus* may have entered the system, but then encountered poor conditions for growth and proliferation and thus were in the process of dying off (yet still detectable using DNA-based detection techniques). Additional work is therefore needed to elucidate the potential for SRB like those found in the DTMF to survive, and potentially proliferate, under the metal-rich, oxic conditions prevalent in the tailings.

### Comparative analyses of bacterial diversity in the DTMF and MC as demonstrated by culture-based and Ion Torrent techniques

The major bacterial communities in the DTMF and MC showed clear differences; seven culturable bacterial classes represented the DTMF and MC (Fig. 5A), whereas 11 bacterial classes were present in both systems (Fig. 5B) based on Ion Torrent analyses. *Deltaproteobacteria*, *Bacteroidia*, *Mollicutes*, and *Methanomicrobia* were not identified by culture methods (Fig. 5A) but were identified by the culture-independent approach (Figs. 5A and 5B). In comparison to culture-based findings, Ion Torrent results were dominated by *Deltaproteobacteria* sequences, possibly due to their slow-growing nature on agar plates (Mitsui *et al*., 1997) and/or specific physiological/nutritional requirements that were not met during repetitive subculture in the present study. This kind of contrast amongst culture-dependent and culture-independent analyses is not an uncommon phenomenon in microbial diversity studies (Ellis *et al*., 2003). Among the eleven classes demonstrated by culture-independent analyses, *Deltaproteobacteria* and *Methanomicrobia* were present only in the MC. In contrast, *Mollicutes* were present only in the DTMF (Fig. 5B). In the DTMF, *Betaproteobacteria*, *Bacilli* and *Clostridia* represented the majority of sequences in comparison to MC where *Bacteroidia* and *Clostridia* were dominant (Fig. 5B). Principle component analysis (PCA) of the class-wise culturable bacterial isolates (Fig. 6A) and culture-independent bacterial sequences frequencies (Fig. 6B) in the DTMF (D2, D3, and E2) and the MC revealed significant differences ($P$<0.01; R=0.737–0.831). Not surprisingly, no significant differences were observed among the three sites of DTMF (D2, D3, and E2) ($P$>0.05; R=0.405–0.543). However, the PCA analysis of the data from both culture-based and culture-independent analyses confirmed the presence of a distinctive environmental ecosystem harboring a specialized microbial community in the DTMF (Figs. 6A and 6B).

### Role of physicochemical parameters on the culture-independent bacterial diversity

A bivariate correlation analysis was carried out among the

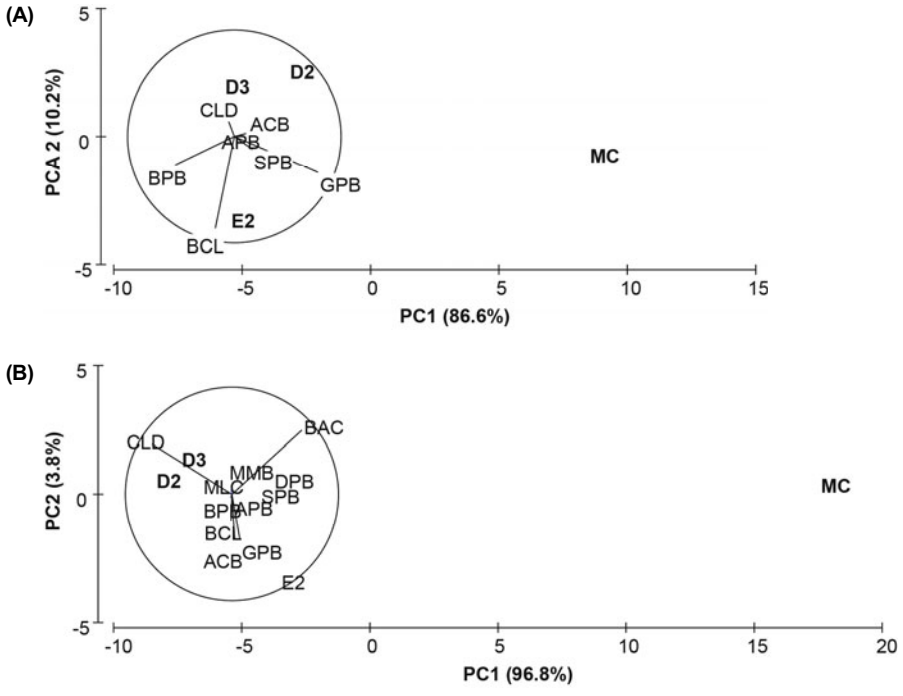


**Fig. 6. Principal component analysis (PCA) of the community structure of bacteria based on the number of available sequences of bacterial classes in each sampling location.** D2, D3, and E2 are the DTMF sampling locations whereas MC is the MC sampling location. (A) PCA of the culturable bacterial isolates present in DTMF and MC samples; and (B) PCA of the culture–independent sequence analyses of the DNA samples isolated from DTMF and MC interface samples. APB, *Alphaproteobacteria*; BPB, *Betaproteobacteria*, GPB, *Gammaproteobacteria*; DPB, *Deltaproteobacteria*; SPB, *Sphingobacteria*; BAC, *Bacteroidia*; BCL, *Bacilli*; CLD, *Clostridia*, ACB, *Actinobacteria*; MLC, *Mollicutes* and MMB, *Methanomicrobia*.

available sequence frequencies of various bacterial classes and physicochemical parameters in the DTMF and MC. TDS, conductivity, salinity and pH were included in these analyses whereas other parameters (e.g., the concentrations of metal elements) were not included as they were not measured in MC. Table 5 showed that the abundance of *Alphaproteobacteria* was negatively-correlated with pH ($P<0.01$). *Deltaproteobacteria* and *Methanomicrobia* were also negatively correlated with pH ($P<0.05$). *Betaproteobacteria*, *Clostridia* and *Mollicutes* were positively-correlated with pH; whereas, *Deltaproteobacteria*, *Sphingobacteria*, and *Methanomicrobia* were negatively correlated ($P<0.01$) with TDS, conductivity and salinity. *Betaproteobacteria*, *Bacilli*, *Clostridia*, and *Mollicutes* all showed a positive correlation with TDS, conductivity, pH and salinity. The four tested parameters (each of which were individually higher in the DTMF than in the MC) all positively-correlated with the dominant bacterial classes in the DTMF (Table 5). In contrast, these four parameters negatively-correlated with different bacterial clusters in the MC (Table 5).

## Conclusions

The results of this study have identified the presence of specialized groups of bacteria inhabiting the water-tailings interface of the DTMF. Overall, it is evident that the distinctive conditions imposed within the DTMF (e.g., pH, TDS, conductivity, salinity, and metal ion concentrations) exert selective pressures on the resident microbial communities. As there are no other reports of the culturable and culture-independent bacterial populations and diversity existing at the interface of alkaline uranium mine tailings, the present study will aid in the understanding of these environments along with potential microbial activities occurring therein. In particular, these findings may help understand the changes in microbiological diversity and metabolic potential that occur in microbial communities that have been incorporated within the tailings mass for extended periods of time. The potential for the use of metal elements present in the DTMF as electron acceptors by SRB, as well as the activity of heterotrophic microorganisms could, in general, influence the chemical stability of the system over long periods, and thus additional study of these effects is warranted.

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