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GRAPHICAL ABSTRACT

- Redox gradient shaped community structures within the native aquifer zones.
- Acid injection favors acidophilic chemolithoautotrophic Bacteria and Archaea.
- This acidophilic community shared strong similarities with AMD-related communities.
- Up- and down-stream affected zones showed signs of resilience to ISR fluids.
- Assessing community structures is necessary for the setup of remediation strategies.

ABSTRACT

Received 13 November 2017
Received in revised form 26 January 2018
Accepted 30 January 2018
Available online xxxx

Editor: F.M. Tack

Keywords:

Uranium
In-situ recovery
Kazakhstan
Acid mine drainage
Acidophilic bacteria
Natural attenuation
Next-generation sequencing
Multivariate statistics

A large fraction (47%) of the world's uranium is mined by a technique called "In Situ Recovery" (ISR). This mining technique involves the injection of a leaching fluid (acidic or alkaline) into a uranium-bearing aquifer and the pumping of the resulting solution through cation exchange columns for the recovery of dissolved uranium. The present study reports the in-depth alterations brought to autochthonous microbial communities during acidic ISR activities. Water samples were collected from a uranium roll-front deposit that is part of an ISR mine in operation (Tortkuduk, Kazakhstan). Water samples were obtained at a depth of ca 500 m below ground level from several zones of the Uyuk aquifer following the natural redox zonation inherited from the roll front deposit, including the native mineralized orebody and both upstream and downstream adjacent locations. Samples were collected equally from both the entrance and the exit of the uranium concentration plant. Next-generation sequencing data showed that the redox gradient shaped the community structures, within the anaerobic, reduced, and oligotrophic habitats of the native aquifer zones. Acid injection induced drastic changes in the structures of these communities, with a large decrease in both cell numbers and diversity. Communities present in the acidified (pH values < 2) mining areas exhibited similarities to those present in acid mine drainage, with the dominance of *Sulfobacillus* sp., *Leptospirillum* sp. and *Acidithiobacillus* sp., as well as the archaean *Ferroplasma* sp. Communities located up- and downstream of the mineralized zone under ISR and affected by acidic fluids were blended with additional facultative anaerobic and acidophilic microorganisms. These mixed biomes may

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be suitable communities for the natural attenuation of ISR mining-affected subsurface through the reduction of metals and sulfate. Assessing the effect of acidification on the microbial community is critical to evaluating the potential for natural attenuation or active bioremediation strategies.

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1. Introduction

In Situ Recovery (ISR) is a mining strategy that accounts for almost half of the world's uranium production worldwide (World Nuclear Association, 2016). This alternative to conventional mining utilizes a series of injection and extraction wells to pump a leachate into a mineralized aquifer, resulting in ore dissolution, and to pump back the uranium bearing solution to the surface for further processing (Benes et al., 2001). The dissolved compound of interest (here uranium) is typically removed from solution using ion exchange columns. Further refining purifies the material into a commercial product (Morrell, 2013). ISR is considered advantageous over traditional mining techniques involving mechanical crushing and grinding because it requires lower operational costs and can be applied to relatively low-grade ores (Benes et al., 2001; Rawlings, 2002). ISR is deployed over a limited surface area and mill tailing or waste rock deposit are absent. Mining for uranium with ISR occurs in locations where the ore is deposited within a sedimentary rock or sediment layer with a relatively high hydraulic conductivity (e.g., sandstone) that lies between two relatively impermeable layers, and preferably below the water table (Benes et al., 2001). These deposits are formed when oxidized and mobile uranium precipitates within organic carbon-rich host unit, meeting reducing conditions. Alkaline ISR is used when the composition of the deposit contains typically more than 2% calcite, which corresponds to the vast majority of the cases. Else acid, a relatively dilute solution containing sulfuric acid, is used. Injection of sulfuric acid into the subsurface drastically alters the groundwater geochemistry (Saunders et al., 2016). The leachate, commonly at pH values < 2, dissolves minerals and mobilizes metals, thereby increasing the concentrations of dissolved solids in groundwater. The mobile metals have the potential to travel long distances and contaminate adjacent aquifer sections (Taylor et al., 2004; Belitz et al., 2015; Alrakabi et al., 2012). In addition to being radioactive and forming decay products, such as ²²⁶radium, uranium itself exhibits toxicity. Recent examples of aquifer contamination demonstrate the urgent need to develop technologies for the removal of uranium released by mining activities (Watson et al., 2013; Williams et al., 2013; Romero-González et al., 2016).

The origin and the nature of the microbial communities associated with extremely acidic environments have been described in detail a few years ago (Johnson, 2012). These environments typically develop through the abiotic and microbial oxidation of metal sulfides in the presence of oxygen, producing sulfuric acid (Baker and Banfield, 2003; Kimura et al., 2011). Acid-mine drainage (AMD) is the highly acidic, metal-laden water produced from uncontrolled former mines (Johnson, 2012). Prokaryotes that are metabolically active in AMD have been reviewed in detail elsewhere (Johnson and Hallberg, 2003; Kimura et al., 2011; Dopson and Johnson, 2012; Volant et al., 2014). The main phyla present in these acidic habitats are *Proteobacteria*, *Nitrospira*, *Actinobacteria*, *Firmicutes*, and *Acidobacteria*, with the occasional presence of representatives of the phyla *Bacteroidetes* and TM7 (candidate phylum). Recent work on the ecology of prokaryotes involved in the oxidation of reduced sulfur compounds (such as *Sulfobacillus* sp. and *Acidithiobacillus* sp.) and metals (*Leptospirillum* sp., *Thiobacillus* sp., *Ferroplasma* sp.) in acidic environments has benefited from next-generation sequencing efforts and has shed light on community structure and biogeochemical cycling in these environments (Chen et al., 2016; Méndez-García et al., 2015; Kuang et al., 2013). Further research highlighted the ecological functioning of AMD-related communities, including aspects related to carbon cycle and molecular nitrogen fixation (Huang et al., 2016).

For ISR, acidification of the subsurface with sulfuric acid is hypothesized to contribute to the establishment of a microbial community with similarities to AMD communities. For either natural attenuation or active bioremediation to be successful post-ISR, it is critical to understand the effect of acidification on the microbial communities within the impacted groundwater. Remediation of uranium contamination typically relies on the establishment of reducing conditions in the subsurface and natural or engineered processes resulting in a reduced zone would allow the aquifer section affected by ISR to return to its original state. For instance, stimulation of anaerobic subsurface bacteria with organic matter has the potential to neutralize the acid and either create mineral species capable of uranium reduction or to directly (enzymatically) precipitate uranium species (Wall and Krumholz, 2006; Bernier-Latmani et al., 2010; Williams et al., 2013; Newsome et al., 2015). Furthermore, metals could be immobilized as sulfides precipitates (Watson et al., 2013). The goals of this study are i) to assess comprehensively the impact of ISR fluids on the autochthonous microbial communities present within an aquifer undergoing mining activities and ii) to compare these communities to those found commonly in AMD habitats.

2. Material and methods

2.1. Groundwater sampling

More than 50% of the uranium production in Kazakhstan comes from the Chu-Sarysu artesian basin (Dahlkamp, 2009). This region is a geological depression located between the Karataou and Chu-Illi mountains (Yazhikov, 1996). Samples (Table 1) were obtained from various redox compartments of the Uyk aquifer within the Tortkuduk deposit (Eastern Chu-Sarysu), in agreement with the roll front deposit geometry.

With the exception of the "IN" and "OUT" samples, groundwater samples were taken from a depth of ca. 500 m below ground level (bgl) using a submerged pump. Sampling was carried out after stabilization of the on-line parameters measured in-situ (temperature, pH, redox, conductivity, dissolved oxygen) and the pumping out of a minimum of three well volumes. Samples were collected in 10 l plastic vessels treated beforehand with 5% hydrochloric acid for 24 h. Samples were kept in a cooled ice-box protected from light and processed within the next 4 h.

Table 1

List of samples collected from the Uyk aquifer and the uranium collection factory. Both "upstream" and "downstream" are relative to the roll front area in which ISR is conducted.

Sample names	Sample origins	Details
UP	Uyk aquifer	Upstream zone, native aquifer, pristine
MIN	Uyk aquifer	Mineralized, roll-front uranium-rich, native aquifer, pristine
DWN	Uyk aquifer	Downstream zone, native aquifer, pristine
UP-acid	Uyk aquifer	Upstream zone, affected by ISR fluids
DWN1-acid	Uyk aquifer	Downstream, affected by ISR fluids
DWN2-acid	Uyk aquifer	Downstream, affected by ISR fluids
IN	Concentration factory	Inlet, before uranium concentration
OUT	Concentration factory	Outlet, after uranium concentration and before re-injection into the aquifer

2.2. Chemical analyses

Water samples for chemical analysis was filtered through 0.22 µm filters (Millipore, USA) in 1-l HDPE plastic containers. Samples for cations analysis were acidified with 0.05% ultra-pure nitric acid. Cations and anions were analyzed with IC (Ion Chromatography System ICS-3000, Dionex). Metals were analyzed with ICP-OES (Multitype ICP Emission Spectrometer, ICPE-9000, Shimadzu). TOC and TIC were analyzed on a TOC-V Series (Shimadzu).

2.3. DNA extraction

Microbial cells were collected onto 0.2 µm, 142 mm diameter, sterile PES filters using a stainless steel filter holder (all Millipore, USA). Each filter collected cells from 2 to 3 l of aquifer sample and was then rinsed with 1 l of sterile deionized water followed by 1 l of sterile TE buffer. Filters were folded and inserted into sterile plastic bags (Whirl-pack, Nasco, USA) and frozen at -20°C until DNA extraction. DNA extraction was carried out as mentioned in Tarnawski et al. (2016).

2.4. Next-generation library preparation

Library preparation was carried out as shown in Diaby et al. (2015). Briefly, PCR amplification of the 16S rRNA hypervariable regions V1–V3 was carried out using bacterial HPLC-purified bacterial primers 28f and 519rm (5'-GAGTTTGATCCTGGCTCAG-3' and 5'-GTNTTACNGCGGCKGCTG-3' respectively) and archaeal primers 109f and 915r (5'-ACKGCTCAGTAACAGT-3' and 5'-GTGTCCTCCCGCCAATTC respectively), with 0.1 ng/µl of template DNA (final concentration). Amplicons ca. 520 bp were generated in 50 µl reaction volumes containing 5 µl of 10× PCR Buffer, 5 µl of 25 mM MgCl_2 , 9 µl of Enhancer P, 3.6 µl of dNTPs (2.5 µM each), 1 µl of each primer (10 µM) and 0.5 µl of PfuGold DNA polymerase (all PfuLab, Germany). PCR amplification conditions were as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s, and a final elongation step of 72°C for 5 min. Amplicons purification was carried out with magnetic beads (AxyPrep Mag PCR Clean-Up, Axygen, USA). Amplicons were reduced in size using the enzymes provided in the Ion Xpress Plus Fragment Library Kit and were fused with both adaptors A and the barcoded Pi (Ion Xpress Barcode Adapters) according to the manufacturer's instructions (Life Technologies). Size selection (max 490 bp) was carried out on agarose gels (E-Gel System, Life Technologies). Quantification of the fragments was carried out using a BioAnalyser 2100 and the High Sensitivity DNA chips (Agilent technologies).

2.5. Semiconductor sequencing

Next-generation sequencing was conducted on an Ion Torrent Personal Genome Machine as shown in Diaby et al., 2015. Emulsion PCR was carried out applying the Ion Xpress Plus Template Kit (Life Technologies) as described by the manufacturer. Sequencing of the amplicons was carried out using the Ion Sequencing 400 kit (Life Technologies) on a 316 chip following the corresponding protocol. Pooled barcoded samples were loaded on the same chip.

2.6. Sequence analysis

Data were processed on Mothur (Schloss et al., 2009), including denoising with the Single Linkage Preclustering (SLP) method (Huse et al., 2010) and testing for absence of chimera using the UCHIME algorithm (Edgar et al., 2011) and the SILVA database (Quast et al., 2013). Hierarchical clustering was carried out using Esprit-Tree (Cai and Sun, 2011). The freeware R (R Development Core Team, 2009) was used subsequently for numerical ecology analysis and inference statistics, for the computation of Fishers's α index (a semi-parametric index independent from the size of the sampling) and Pielou's evenness. Multifactorial

analysis (MFA) and correlation analysis were conducted on chemical and microbial data sets. MFA is a symmetrical analysis, in which both data sets play the same role, and exposes correlative structures without any reference to causal relationship (Borcard et al., 2011).

2.7. Quantitative PCR analysis

Amplification of the 16S rRNA gene was carried out using the bacteria primers 338f (5'-ACTCCTACGGGAGGAGCAG-3') and 520r (5'-ATTACCGGGGCTGCTGG-3'), as well as the archaeal primers 931f (5'-AGGAATTGGCGGGGAGCA-3') and 1100r (5'-BGGGTCTCGCTCGTTRCC-3'), respectively. Amplification was carried out in triplicate in a Rotor-Gene 3000 cyclor (Corbett, Australia) as follows: 10 µl reactions containing 2.5 µl template DNA, 2.1 µl water, 0.2 µl each primer (100 mM stock) and 5 µl of 2× KAPA SYBR Fast Universal qPCR kit (KAPA Biosystems, USA) were cycled (40 cycles) at 95°C for 10 s, followed by an extension at 62°C for 30 s and an acquisition at 72°C for 20 s. The final melting step was carried out from 72°C to 95°C , at a rate of 0.1°C/s . Analysis of the results was carried out using the built-in analytical software (micPCR, Bio Molecular Systems, ver. 2.2).

2.8. Data availability

Raw sequences were deposited in the European Nucleotide Archive (<http://www.ebi.ac.uk/ena>) and are available under the accession number PRJEB22952.

3. Results and discussions

3.1. Chemical analysis

Sediment samples associated with the Uyük aquifer have been extensively described as permeable and mostly composed of sandy silicates (Dahlkamp, 2013), making the aquifer adequate for ISR processes. However, the high hydraulic conductivity of the aquifer may also allow groundwater to move past the roll front orebody, up- and downstream of the zone under ISR exploitation. Table 2 presents the water chemistry measured for the samples involved in this study (a comprehensive list of data is presented in Table SI-A-1).

Groundwater present within the upstream compartment of the aquifer (sample "UP") showed an oxidizing signature (250.2 mV/SHE) before it comes into contact with a more reduced zone (sample "MIN"), with a negative ORP value (-281.1 mV/SHE), allowing for uranium precipitation from solution, thereby forming the roll-front orebody structure (Saunders et al., 2016). Sample "DWN" was taken from the reduced zone of the aquifer, located downstream of the mineralized zone (-14.1 mV/SHE). With the exception of ORP and Fe, both "UP" and "DWN" samples showed a similar chemical signature and close pH values (Table SI-A-1). Samples "IN" and "OUT" were taken at the entrance and the exit (respectively) of the uranium concentration factory. As such, these samples represent a mixture of acidic leachates pumped from various wells in operation. The very low pH and high ORP values measured in these two samples reflect the conditions induced by ISR mining, and underscore the presence of significant concentrations of metals and metalloids, including Al, as a result of the dissolution of clay particles (up to 832 mg/l in the 'DWN-1' sample, see Table SI-A-1) (Robin et al., 2015a; Robin et al., 2016). Sulfate and iron showed the highest proportional increase in concentration, with maximal concentrations reaching respectively 22'810 and 1'210 mg/l (Table 2). The presence of oxygen is equally a characteristic of these samples, with up to 3.89 mg/l in the "IN" sample. Samples "IN" and "OUT" were comparable, at least for iron and pH values, to samples taken by Coupland and Johnson (2004) from an abandoned copper mine in the North of Wales. However, sulfate concentrations were substantially higher in the present study, as a result of the injection of sulfuric acid in the aquifer. Two cases of AMD, reported by Johnson and

Table 2

Chemistry of the water samples. All elements are expressed in mg/L. ORP and conductivity (Cond) are respectively expressed in mV/SHE and mS/cm. TIC: total inorganic carbon. TOC: total organic carbon.

	pH	ORP	Cond	Na	Ca	Mg	K	SO ₄	Cl	TIC	TOC	Si	Fe	Al	U
UP	7.6	250.2	1000	106	57	27	4.7	183.3	125	35	1.9	11	0	0	0
MIN	7.5	−281	1120	116	53	26	4	182.2	138	42	2.8	12	0.1	0	0
DWN	8.1	−14.1	1048	115	48	25	3.9	268.8	129	34	1.3	8.9	0.3	0.1	0
UP-acid	5	−223	2647	114	127	87	14	1544.7	125	5.3	1	13	43.2	1.1	0
DWN2-acid	3.5	120.2	7757	188	300	499	60	10,182	126	5.7	1	25	479	252	51
DWN1-acid	1.7	468.8	22,810	209	563	762	131	20,666	129	2.5	1.3	136	1210	832	23
OUT	1.4	244.8	14,310	195	523	701	111	19,724	133	2.8	1.1	105	1140	556	0
IN	1.3	245.8	14,600	190	519	652	100	19,724	168	6.7	1.6	106	1070	446	99

Hallberg (2003), showed a chemistry that was similar to the measured acid solution present in the “IN” and “OUT” samples in terms of sulfate and metals in general, including a sample of the Rio Tinto (Spain) (Amils, 2016). Uranium reached 98.5 mg/l in “IN” and was absent in “OUT”, demonstrating the effectiveness of the retention system based on ion-exchange resins. Uranium concentration measured in “IN” was also similar to those published in other studies on contaminated aquifers and sediments (Wu et al., 2006; Cardenas et al., 2008).

ISR fluids affected wells adjacent to the mineralized section of the aquifer under mining activities. The typical chemical signature present in the “IN” sample was found in wells located in the upstream (“UP-acid”) and downstream (“DWN1-acid” and “DWN2-acid”) sections of the aquifer. ‘UP-acid’ was affected moderately, showing negative ORP, pH > 5, limited amounts of dissolved elements resulting from the dissolution of the geological matrix and no U. Both samples taken downstream of the mining activity zone (‘DWN1-acid’, ‘DWN2-acid’) were affected to a higher extend by the intrusion of ISR fluid, as shown in Table 2.

3.2. Microbial quantitative analyses

16S rRNA gene copy numbers and inference statistics for Bacteria and Archaea are presented in Tables 3 and SI-A-4 respectively. Bacterial cell numbers and diversity indices varied according to the sampling location. The highest copy number was observed in the mineralized area of the aquifer (sample “MIN”) with 6.02×10^6 copies of the 16S rRNA gene per liter. The sample showing the lowest amount was “DWN”, a water sample taken from a pristine area located downstream of the mining area, with 3.41×10^4 copies of the 16S rRNA gene per liter. This latter showed the highest cell diversity, with a Fisher's alpha parameter of 80.72. The impact of the ISR process was manifest in the mineralized area, with a significant decrease in the total number of cells. Sample “IN” contained less than 1.70×10^5 copies of the 16S rRNA genes, corresponding to a 50-fold reduction relative to the mineralized (pristine) “MIN” sample. However, the apparent diversity of this sample was large, with a Fisher's alpha parameter of 53.72. The mixing of leach fluids pumped from all wells under mining activity may induce this relatively high diversity value. Furthermore, the uranium processing plant apparently acts as an enrichment system, for more than 6.59×10^5 copies of the 16S rRNA gene were quantified in the “OUT” sample, which is a 5-fold enrichment relative to the “IN” sample. Conversely, this sample

exhibited a sharp decline in the apparent diversity as measured with Fisher's alpha parameter (a value of 20). Few AMD studies have characterized cell abundance and diversity in the same manner as was done here. Kimura et al. (2011) showed that significant bacterial cell numbers were present within water samples from an abandoned pyrite mine in Wales. Under acidic conditions (pH values varying from 1.85 to 2.30), these authors counted from 1×10^7 to 7×10^8 cells per ml by fluorescence. Edwards et al. (1999) measured very similar numbers in effluents of the Richmond mine (California) using fluorescent probes. In our acidic samples going into and out of the uranium plant (“IN” and “OUT”), the number of 16S rRNA copies were more than 3 and 4 orders of magnitude lower for Bacteria (Table 3) and Archaea (Table SI-A-4), respectively.

The impact of ISR on bacterial communities inhabiting areas adjacent to the production area occurs to varying degrees and is a function of the natural redox zonation inherited from the roll front deposit. This impact is also possibly a function of the time of exposure to the ISR leaching fluids as well as their concentration. Upstream of the mineralized section of the aquifer (sample “UP-acid”), the impact of the intrusion of acid was moderate, with a lowering of the pH value from 7.64 to 5.01. For the bacterial community, the impact was significant with a large decrease in cell numbers, as well as in apparent diversity (Table 3). Downstream, data showed a moderate decline in cell numbers for sample ‘DWN1-acid’ and the relative stability of its apparent diversity relative to the mineralized zone (sample “MIN”), despite the significant decrease in the pH value and a chemical composition very close to the one measured for the “IN” and “OUT” samples. Paradoxically, sample “DWN2-acid”, apparently less affected in terms of water chemistry, showed a more significant reduction in cell numbers as well as in the apparent diversity, with one of the lowest Fisher's alpha parameter (30.34).

Archaeal 16S rRNA gene copies were found in very low numbers in all sections of the aquifer. Copies were highest in the “MIN” sample, with 4.90×10^4 copies per liter. This sample also displayed the highest relative bacteria diversity with a Fisher's alpha parameter of 4.64. In the “IN” and “OUT” samples, Archaeal cells were almost absent, reaching barely 787 copies per liter in sample “OUT”. In the affected sections of the aquifer, copies varied from 269 to 1/189 copies per liter (“UP-acid” and “DWN2-acid”, respectively) (Table SI-A-4). Kock and Schippers (2008) showed that Archaea were sometimes absent from AMD, for being highly sensitive to extreme conditions of temperature and pH.

Table 3

Quantitative PCR on the 16S rDNA genes and inference statistics at the genus level (3% similarity).

Samples	pH	Genus richness	OTUs	ACE	CHA01	Pielou's evenness	Fisher's alpha	16S rRNA copies/l
UP	7.64	391	9833	35,080	31,088	0.67	60.14	1,639,900
MIN	7.49	247	4399	13,394	12,031	0.49	35.09	6,017,900
DWN	8.09	501	9743	27,489	24,780	0.59	80.72	34,100
UP-acid	5.01	276	3080	9009	8155	0.51	39.94	68,400
DWN1-acid	1.65	360	6597	24,372	20,757	0.56	54.56	1,547,900
DWN2-acid	3.54	218	1860	5039	4590	0.34	30.34	971,000
IN	1.25	355	2961	8463	7250	0.36	53.72	169,700
OUT	1.35	152	1070	3236	2955	0.31	20.00	659,700

With the exception of the members of the Euryarchaeota, other archaeal phyla classically composed a minor fraction of the microbial communities present in AMDs (Chen et al., 2016).

3.3. Next generation sequencing of the microbial communities

A simplified table displaying the relative contributions of major phyla is presented (Table 4) and a table summarizing all the phyla is provided in the Appendix (Table SI-A-2). A comprehensive data set measured in this study for the Domains Bacteria and Archaea is presented as an Excel spreadsheet in a separate Excel file (Document SI-B).

Ten major bacterial phyla were sufficient to account for all phyla present, contributing from 68.4% (sample “UP”) to 97.8% (sample “DWN2-acid”) for all sections of the aquifer. The complete list included 54 Phyla, with rare ones, such as AC1, *Deferribacteres*, and *Synergistetes*, which were detected in proportions below 1% (Document SI-B). For the Domain Archaea, three phyla only contributed from 80.4% (sample “UP”) to 99.9% (sample “OUT”) to the communities.

A high proportion of the sequences are affiliated with the Proteobacteria, present in all compartments of the Uyuq aquifer (Tableau 4). This Phylum dominated the autochthonous communities present within the upstream and downstream pristine sections of the aquifer (26.02% and 16.35% in “UP” and “DWN” samples, respectively). These pristine sections were populated by a rich diversity of families classically associated with anoxic habitats and involved in fermentation and anaerobic respiration (Fig. 1), such as Desulfobacteraceae, Desulfobulbaceae, Pelobacteraceae, and Syntrophaceae (δ -Proteobacteria), Comamonadaceae and Rhodocyclaceae (β -Proteobacteria) (Florentino et al., 2016). Classes α -, ε - and γ -Proteobacteria were present in low proportions only (Document SI-B). The family Rhodocyclaceae was abundant in the “UP” sample with 3.15% of all sequences. In contrast, the sample from the mineralized zone (“MIN”) contained only 8.55% of sequences affiliated with this phylum, with 7.27% of δ -Proteobacteria belonging to the above-mentioned families as well as the Syntrophobacteraceae (2.22%), which appeared only in this section of the aquifer. In the zones affected by ISR fluids, the predominance of Proteobacteria was even greater, with a maximum of 68.84% of all sequences for sample “DWN2-acid”. Sample “UP-acid” was characterized by the presence of the highest proportion of β -Proteobacteria (13.21%) with Gallionellaceae (3.73%) and Hydrogenophilaceae (*Thiobacillus* sp., 3.45%) (Document SI-B). This sample displayed equally significant proportions of organisms such as *Acidithiobacillus* sp., a γ -Proteobacteria present in 2.27%, which is commonly identified within acidophilic sulfur-oxidizing biota (Méndez-García et al., 2015). In contrast, low contributions (<1%) of sequences affiliated with neutrophilic δ -Proteobacteria were observed. The apparent mixing of organisms typically associated with distinct environments

may have resulted from the mixing of fluid from the adjacent ISR production cell, and the reduced and neutrophilic water from the upstream section of the aquifer. This mixing resulted in the formation of an ecotone, confirmed by the presence of *Gallionella* sp., which typically grows in microaerophilic water laden with ferrous iron (Hallbeck and Pedersen, 2005). In downstream sections of the aquifer affected by the ISR process, the predominance in Proteobacteria was evidenced by large contributions from γ -Proteobacteria, reaching 35.99% and 66.49% of all sequences in “DWN1-acid” and “DWN2-acid”, respectively. The phylogeny of the vast majority of these sequences was unknown (23.94% and 57.78%, respectively), the rest being shared between sequences affiliated to Alteromonadales and Pseudomonadales (Fig. 1).

Samples “IN” and “OUT” exhibit a distinct microbial structure relative to the phylum Proteobacteria. Specifically, sequences affiliated with the family Acidithiobacillaceae and the genus *Acidithiobacillus* representing 61.81% and 22.59% of the sequences, respectively. This genus is composed of strictly aerobic and acidophilic organisms that use reduced sulfur to support autotrophic growth (Nuñez et al., 2017). Some strains have been shown to use ferrous iron as well as U(IV) as a source of electrons and energy (Kelly and Wood, 2015). The majority of these sequences could not be assigned at the species level, and only a minor fraction was affiliated to *At. caldus* and *At. albertensis*. *Acidithiobacillus* sp. was also present in smaller proportion in the upstream and downstream affected sections of the aquifer, confirming the intrusion of ISR fluids. In samples “IN” and “OUT”, respectively 3.29% and 7.10% of all sequences were affiliated with mitochondrial sequences (Document SI-B). The presence of sequences affiliated with eukaryotic cells (unclassified) in the samples strongly affected by acid confirmed the results obtained earlier by Kock and Schippers (2008) in the analysis of AMD samples.

Sequences affiliated with the Phylum Firmicutes (Fig. 2) showed the clear predominance of the Sulfobacillaceae (genus *Sulfobacillus* sp.) in samples “IN” (6.25%) and “OUT” (17.41%), as well as in the DWN1-acid sample (19.04%) (Document SI-B). When the impact of the acidic fluid was moderate, with pH values >3, the Fe(II)-oxidizing *Alicyclobacillus* sp. was most abundant, as in the “UP-acid” and “DWN2-acid” samples. This organism is a facultative anaerobe and mixotroph, providing a strong ecological advantage in these sections of the aquifer (Yahya et al., 2008; Chen et al., 2016).

The distribution of individual families within the Phylum Firmicutes was highly dependent on the spatial location and the redox zonation relative to the mineralized zone. In the “UP” community, the Firmicutes are represented by Peptococcaceae (7.65%), with representatives of the genus *Desulfurispora* sp., which are endospore-forming, sulfate reducers. In contrast, the “DWN” sample was heterogeneous with approximately equal contributions from a number of families, Peptococcaceae, Streptococcaceae and Erysipelotrichaceae. Sample “MIN” showed a

Table 4
List of major phyla and their relative contributions (in %) to the Bacterial and Archaeal communities.

	UP	MIN	DWN	UP-acid	DWN1-acid	DWN2-acid	IN	OUT
Actinobacteria	1.17	0.46	5.41	9.90	8.75	10.75	5.61	2.06
Bacteroidetes	7.26	9.03	7.06	22.09	10.35	0.82	3.75	0.21
Chlorobi	6.28	0.49	2.63	6.41	0.38	0.37	0.06	0.01
Chloroflexi	6.85	0.22	11.02	0.37	0.66	0.27	0.48	0.02
Firmicutes	12.04	3.04	8.00	4.22	24.50	10.67	9.20	17.95
Lentisphaerae	0.03	28.92	0.01	0	0.04	0.02	0.03	0
Nitrospirae	1.37	0.38	11.64	0.09	0	0.15	0.13	46.63
OD1	5.15	8.62	0.36	0.61	0.02	0.75	0.03	0
OP11	2.28	9.81	0.21	0.66	0.01	5.18	0.02	0
Proteobacteria	26.02	8.55	16.35	25.14	44.89	68.84	76.78	30.92
Unclass. Bacteria	18.75	19.92	32.29	27.83	4.97	1.76	1.35	1.92
Crenarchaeota	10.83	1.96	19.72	13.55	6.66	5.58	4.91	4.16
Euryarchaeota	44.56	19.54	70.41	85.60	92.92	92.93	94.52	95.57
[Parvarchaeota]	25.03	70.47	3.66	0.00	0.18	1.17	0.40	0.13
Unclass. Archaea	19.58	8.02	6.21	0.84	0.24	0.32	0.17	0.14

Fig. 1. Left: copies of the Bacteria 16S rDNA genes per liter (white bars) and relative contributions of sequences belonging to the Phylum *Proteobacteria* to the communities (in %, gray bars). Black squares: pH values. Right: relative contributions (in %) of Families present within the Phylum.

different pattern, with the large dominance of members affiliated with the Ruminococcaceae (1.69%). This sample also displayed the highest proportion of the phylum Lentisphaerae (28.92%), which was almost absent in all other samples (Fig. SI-A-7).

3.4. Comparative analysis with AMD-related communities

Comparison with data collected worldwide showed that the immediate area receiving sulfuric acid was chemically similar to acid mining drainage (AMD). However, the ISR samples are comparatively different with respect to the high nitrogen and phosphorus contents (Table SI-A-1), which is not typical of AMD systems (Baker and Banfield, 2003), due to the precipitation of ferric phosphate (Falagán et al., 2014). These limitations can be mitigated in AMD by the fixation of nitrogen, which was found in the genus *Leptospirillum* and *Acidithiobacillus*, providing these genera with an ecological advantage and resulting in their abundance (Huang et al., 2016). The analysis carried out in the present study showed that phosphorus and nitrogen compounds were absent in the water samples taken from the pristine sections of the aquifer, despite low amounts present within minerals and organic compounds within

the aquifer matrix (data not shown). Intrusion of high concentrations of both compounds with the incoming of ISR fluids in the affected sections of the aquifer is likely to explain the reason for the presence of high 16S rRNA copy numbers as well as the persistence of highly diversified microbial communities.

Microbial communities populating AMDs show strong disparities in their phylogenetic make-up (Huang et al., 2016). The biomes found worldwide harbor abundant acidophilic chemolithotrophic organisms belonging to the Phyla *Proteobacteria*, *Nitrospira*, *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Aquificae* and the candidate Phylum TM7 (Chen et al., 2016). However, the relative proportion of each phylum as well as the relative contributions of each species within the phylum strongly depended on local environmental factors (Kuang et al., 2013). These discrepancies were found at a high phylogenetic level (phylum), but also at low level, with the presence of specific and local phylotypes, as it was the case for *Acidithiobacillus* sp. and *Sulfobacillus* sp. (Justice et al., 2014; Nuñez et al., 2017). In the present study, *Sulfobacillus* sp. was present in samples “IN” and “OUT”, as well as in all samples affected by ISR fluids. Some *Sulfobacillus* species are mixotrophs, which could provide them with the ability to live in a wide range of environmental

Fig. 2. Left: copies of the Bacteria 16S rDNA genes per liter (white bars) and relative contributions of sequences belonging to the Phylum *Firmicutes* to the communities (in %, gray bars). Black squares: pH values. Right: relative contributions (in %) of Families present within the Phylum.

conditions. Others are reported to be able to use alternative electron acceptors, such as nitrate, and could potentially contribute to the acidification of their habitat in absence of oxygen (Justice et al., 2014). *Leptospirillum* sp. contributed up to 46.52% of all sequences found in the “OUT” samples, making this genus the highest contributor within this sample (Fig. 4). This genus however contributed marginally to the communities present within the affected sections of the aquifer, with 0.13% and 0.04% in samples “IN” and “UP-acid”, respectively (Document SI-B). 16S rRNA gene copy numbers increased almost four fold when passing through the uranium processing plant, from 1.69E5 to 6.59E5 copies per liter, suggesting that the plant offers a highly favorable environment for *Leptospirillum* (Table 3). This may be because of the corrosion of steel pipes within the U concentration plant, which releases Fe (II), the substrate for aerobic iron oxidation by *Leptospirillum*. As for *Sulfobacillus* sp., data obtained here cannot however prove with certainty that *Leptospirillum* sp. contributed to the oxidation of the native Fe(II) within the aquifer, and therefore contributed to the mobilization of U(VI).

Alicyclobacillus sp. (and Acidomicrobiaceae in general, with a maximum of 7.25% in ‘DWN2-acid’) were present preferentially in the affected sections of the aquifer (Fig. 2). Members of this genus were documented as heterotrophs or chemolithoautotrophs in mine environments or AMD treatment plants (Chen et al., 2016). In the present study, results showed that these organisms were less abundant in the strongly oxidative and acidic conditions of ISR. Dissimilatory sulfate reducers belonging to the Phylum *Nitrospirae* were found at very low contributions

in sections of the aquifer affected by the ISR fluids, and reached up to 11.16% of all sequences in ‘DWN1-acid’. Members of the genus *Thermodesulfovibrio* can use thiosulfate and/or sulfite and nitrate as alternative electron acceptors. All isolates are also capable of a chemolithoheterotrophic lifestyle using H₂ as an electron donor and acetate as a carbon source (Daims, 2014).

Most of the Archaea identified in the present study showed low abundances. *Ferroplasma* sp. (Family Ferroplasmaceae) was the only purely acidophilic genus belonging to the domain Archaea, and was found solely in samples “IN” and “OUT” (Fig. SI-A-9). As it was the case for *Sulfobacillus* sp. and *Leptospirillum* sp., *Ferroplasma* sp. was enriched during the passage of the ISR fluid in the pipes of the uranium concentration plant. The relative proportion of 16S rRNA sequences was increased, going from 0.45% in “IN” to more than 31.15% in “OUT”. Samples taken from “IN” and “OUT”, as well as from the affected sections of the aquifer were composed of ca. 85% to 95% of sequences that were affiliated with the phylum *Euryarchaeota*. These methanogens were affiliated with organisms belonging to the families Methanobacteriaceae, Methanospirillaceae and especially Methanosaetaceae. Among the latter, genus *Methanosaeta* sp. was particularly abundant in acid samples, contributing 51.04% of all Archaea sequences present in the sample “IN” (Fig. SI-A-9). Sections of the native aquifer were populated of *Euryarchaeota* (between 19 and 69%) in addition to the relative proportions of sequences that are affiliated to the phylum *Parvarchaeota* (Fig. SI-A-10), as it was the case for sample “MIN” with unknown sequences

Fig. 3. Left: copies of the Bacteria 16S rDNA genes per liter (white bars) and relative contributions of sequences belonging to the Phylum *Nitrospirae* to the communities (in %, gray bars). Black squares: pH values. Right: relative contributions (in %) of Families present within the Phylum.

affiliated to the Orders WCHD3-30 and YLA114 (66.03% of the sequences).

3.5. Correlations between community structures and chemical data sets

At the family level, Bacteria and Archaea were statistically correlated principally with elements linked with pH, cationic exchange (Robin et al., 2015b) and dissolution mechanisms, such as calcium, iron, magnesium, manganese, titanium and zinc (Table SI-A-3). No significant

correlation were present with redox (ORP), dissolved oxygen, aluminum and total organic carbon (TOC). In comparison, correlation between pH value and community composition was identified in several habitats affected by AMD (Kuang et al., 2013). In a recent review, Huang et al. (2016) presented seven studies for which this statistical correlation was demonstrated. However, these authors also identified two studies showing a statistical correlation between metal concentrations and microbial communities in water samples affected by AMD (García-Moyano et al., 2012; Yang et al., 2014).

Fig. 4. Left: multifactorial analysis (MFA) site scores using both Bacteria and Archaea phylogeny at the Genus level (spe) and the chemical variables (env); open circles: individual scores; full circles: sample score centroid. Right: correlations between chemical data and MFA site scores. Variables and Genus showing statistically significant correlations are displayed only.

Results from the MFA analysis (Fig. 3, left) shows that three clusters of samples are formed, separated mainly on Axis 1 (explaining 51.7% of the variance), which is interpreted as the expression of the pH variable (Fig. 3, right). The first cluster includes all the samples from the native aquifer ('UP', 'DWN', and 'MIN'), as well as the affected sample "UP-acid". Short distances separate the centroids of the pristine sample scores, supporting the statistical similarity between both set of data. Sample 'UP-acid' is located close to these samples. This sample is characterized by the presence of genera typically associated with AMD, as well as those typical of anoxic and reduced areas (the latter were more abundant). Such a mixed community may form with the mixing of relatively low amounts of ISR fluid, rich in oxygen, organic matter, nitrogen and phosphorus within the reduced water present in the native sections of the pristine aquifer. This juxtaposition of microbial genera potentially represents evidence for a certain degree of resilience of the autochthonous community, with which allochthonous acidophilic organisms are blended. The resulting mixed biome is likely to compose an ecotone, which could contribute potentially to the natural attenuation of the aquifer.

The second cluster shown by the MFA analysis consists of samples related to the uranium concentration plant ('IN' and 'OUT'). Both samples showed greater distances between chemical and microbiological components, possibly reflecting the mixing of multiple sources reaching the uranium concentration plant. The third group is composed of the two affected samples located downstream of the mineralized area under ISR activity. Here again, both samples showed large distances between biological and chemical components. These distances may be interpreted as the impact of the mixing of ISR fluids within the reduced section of the aquifer, inducing an imbalance between community and chemical compositions.

The three clusters mentioned above corresponded to three major groups of Families (Fig. 3, right), sharing specific oxidation or reduction activities. The first cluster, representing pristine conditions and neutral pH values, included heterotrophic organisms typically found in reductive habitats, such as the genera *Desulfurispora*, *Desulfovibrio* and *Desulfococcus*. In contrast, the second cluster harbored representatives of the genera *Ferroplasma*, *Acidithiobacillus* and *Leptospirillum*, which are typically present at low pH values and at high concentrations of dissolution products. The mixotrophic genus *Sulfobacillus* sp. was not closely associated with this group, probably due to its ability to oxidize both reduced sulfur and iron compounds. The third cluster of organisms was related to representatives of the affected aquifer section located downstream, such as members of the Family Acidimicrobiaceae and genus *Alicyclobacillus*.

4. Conclusions

In this study, we provide an in-depth analysis of microbial communities affected by ISR mining. As expected, the addition of acid strongly affected the chemistry and the associated microbial communities and this change was observable even at the phylum level. The taxa involved in the acidification process followed a strong niche differentiation, including the implication of deeply diverging bacterial guilds. This result indicated that high bacterial taxa distribution was ecologically coherent with ecosystem functioning. pH was the main driving force for the chemical and microbial community changes observed in the aquifer. Bacterial diversity and numbers of the original neutrophilic communities were decreased in favor of acidophilic species in ISR areas. Sections affected by ISR fluids showed specific characteristics, halfway between the two, resulting in the formation of an ecotone showing microbial assemblages able to cope with limited amounts of mining leach fluid. This result suggests an intrinsic natural attenuation capacity of the aquifer. However, it was not possible to quantify this capacity based on this study. Sulfuric acid injection liberated metals and increased the amount of sulfate within the aquifer, thus creating conditions that were similar to AMD. Consequently, the resulting microbial community harbored

some genera typically associated with AMD. This affords a considerable advantage to future experimental design, as AMD is a well-studied system. The similarity of the microbial community between the acidic samples from Kazakhstan and AMD suggested that the lack of carbon within the subsurface would retard full natural attenuation. Thus, biostimulation of the metal- and sulfate-reducing guilds may be promoted with the addition of an adequate carbon sources (e.g., ethanol and glycerol). Future work should focus on ways to promote specifically the growth of metal- and sulfate-reducers capable of raising pH and precipitating metals and uranium from the acidic solutions, using known techniques related to AMD remediation.

Competing Interest Statement

The authors declare no competing interests.

Acknowledgement

EPFL and Areva Mines (France) funded the research described here. We acknowledge Emmanuelle Rohrbach, Brian C. Reinsch and the CEL team at EPFL for their technical assistance, Thierry Lefebvre and Olivier Regnault and local field technicians at KATCO for their precious contribution to the sampling process, as well as Evelyne Ruchti (Blueprint Design) for the design of the graphical abstract. Mention of trade names and commercial products does not constitute recommendation for use.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.01.321>.

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