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Infiltration Experiment

DNA Diversity of Microorganisms as a Tracer of Groundwater in the Olkiluoto Infiltration Experiment

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ABSTRACT

Three drillholes and one pumped drillhole including to the infiltration test were investigated for microbial diversity over a one-year period. The infiltration monitoring holes comprised two groundwater observation tubes, OL-PVP21 and OL-PVP22, and one shallow bedrock drillhole, OL-PP69; the pumped deep drillhole was OL-KR14. Biomass was analysed using microscopic counting and quantitative PCR. Cloning and microarray analyses were used to identify DNA signatures in the groundwater samples and to reveal the variation in these signatures over time during the infiltration experiment. The results indicated that DNA signatures could be used to classify groundwater, because differences in DNA signatures were found when comparing the drillhole results. PhyloChip results were verified by cloning, but higher sensitivity meant that a broder diversity of DNA signatures was found using PhyloChip analysis than using cloning. Cloning was therefore used only to analyse samples collected on the first sampling occasion. Groundwaters from four drillholes were compared, revealing that DNA signatures can be successfully used to classify groundwater from different drillholes. In all groundwater samples, qPCR and TNC revealed a relatively constant number of cells over time. DNA signatures suggested a possible migration of groundwaters over time from OL-PVP21 to OL-PP69 and from OL-PP69 to OL-KR14 when pumping the deep drillhole OL-KR14. Chemistry results from the Posiva Oy database POTTI correlated with the PhyloChip results; this was very encouraging, supporting further use of PhyloChip analysis to classify groundwater signatures over time. However, the one-year period of this study was too limited, so more samples should be analyzed over longer periods to follow long-term changes.

Keywords: Infiltration experiment, microbiological diversity, cloning, microarray analysis, Phylochip analysis.

Suotautumiskoe: Mikro-organismien DNA -jakauman käyttö pohjavesityyppien tunnistamisessa

TIIVISTELMÄ

Kolmen suotautumiskokeen monitorointireiän ja kokeessa pumpattavan reiän pohjavedestä tutkittiin mikrobiologista diversiteettiä vuoden ajan. Tutkimuksen kohteena olivat kaksi pohjavesiputkea OL-PVP21 ja OL-PVP22, matala kallioreikä OL-PP69 ja pumppauksen kohteena oleva syvä kairareikä OL-KR14. Biomassa analysoitiin käyttämällä mikroskooppista laskentaa ja kvantitatiivistä PCR:ää. Kloonausta ja DNA-mikrosirua käytettiin mikrobien DNA:n tunnistamiseen ja DNA:n vaihtelun selvittämiseen vuoden aikana. Tutkimuksen perusteella havaittiin, että DNA:ta voidaan käyttää pohjavesien luokitteluun, koska DNA:ssa havaittiin eroja eri kairareikien välillä. PyloChip 16S rRNA-mikrosiru tulokset verifioitiin kloonaamalla. Mikrosiru menetelmän parempi herkkyys aiheutti kuitenkin sen, että sillä saatiin selville laajempi DNA diversiteetti kuin kloonaamalla. Tämän vuoksi kloonausta käytettiin vain ensimmäisille näytteille. Kaikkien neljä tutkimusreiän pohjavesien DNA jakaumaa verrattiin toisiinsa ja havaittiin, että DNA:ta voidaan käyttää pohjavesien alkuperän tunnistamiseen. qPCR- ja TNC-menetelmät antoivat melko tasaisia solumääriä kaikille seurantajaksolla otetuille pohjavesinäytteille. Pohjavesien DNA:n perusteella näytti sille, että OL-PVP21:n pohjavesi on virtaamassa reikään OL-PP69 ja reiän OL-PP69 pohjavesi taas reiän OL-KR14 pumppausväliin. Pohjaveden kemian tulokset tukevat tätä päätelmää. Edellä esitetyn perusteella PyloChip 16S rRNA-mikrosiru -menetelmän käyttö pohjavesien tunnistamiseen on jatkossakin perusteltua. Vuoden tutkimusjakso on kuitenkin melko lyhyt menetelmän testaamiseen, joten lisänäytteitä pidemmältä monitorointijaksolta tarvitaan vielä.

Avainsanat: Suotautumiskoe, mikrobiologinen diversiteetti, kloonaus, DNA-mikrosiru, Pylochip-analysointi.

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1 INTRODUCTION

Three infiltration drillholes and one pumped drillhole were investigated for microbial diversity over time. The infiltration drillholes were two shallow overburden drillholes, OL-PVP21 and OL-PVP22, and one bedrock infiltration drillhole, OL-PP69; the pumped drillhole was denoted OL-KR14. The microbial investigation of the infiltration experiment was performed to track and compare groundwater from the infiltration drillholes versus the pumped drillhole over time, mimicking the effect of water intrusion into the ONKALO tunnel on the basis of DNA signatures. These drillholes were sampled and analysed on four dates. Biomass was analysed using total number of cells (TNC) and quantitative PCR (qPCR). Cloning and microarray analyses were used to identify DNA signatures in the various groundwater samples and to analyse the variation in these signatures during the infiltration experiment.

2 MATERIALS AND METHODS

2.1 Total number of cells

The total number of cells (TNC) mL^{-1} in samples was determined using the acridine orange direct count (AODC) method as devised by Hobbie et al. (1977) and modified by Pedersen and Ekendahl (1990). Acridine orange dye binds to nucleic acids and is fluorescent in blue light, and the method is described in detail in several papers (e.g. Hallbeck and Pedersen 2008). Briefly described, samples were suction filtered (–20 kPa) onto 0.22-µm-pore-size Sudan black-stained polycarbonate filters, 13 mm in diameter, mounted in stainless steel analytical filter holders. The filtered cells were stained for 5–7 min with 200 µL of a 10 mg L^{-1} acridine orange solution, dried, and mounted between microscope slides and cover slips using fluorescence-free immersion oil. The number of cells was counted under blue light (390–490 nm), using a band-pass filter for orange light (530 nm), in an epifluorescence microscope at 1000× magnification. At least 600 cells and 15–30 microscopic fields (1 field = 0.01 mm^2) were counted on each filter. The expected distribution of cells on the filters should follow a normal distribution. Usually, three subsamples filtered on three filters were counted, and the average of these three results was reported together with the standard deviation of the mean. Finally, the personnel conducting the microscopic counting must be inter-calibrated; otherwise, interpretations of what should be counted may differ.

The method is highly reproducible between samples. Typically, three subsamples of a groundwater taken in series reproduce a mean with 15–30 % standard deviation. The detection limit depends on the filtered volume. Typically, 1–10 mL of sample can be filtered without filter clogging problems. At least 20 cells should be counted per counted microscopic field for good statistics and precision, which sets 10^4 cells mL^{-1} as the detection limit. This detection limit can be forced towards 10^3 cells mL^{-1} if the accepted number of cells per field is lowered to two, in which case up to 300 microscopic fields must be counted to reach the 600 cells needed for good reproducibility. Samples with numbers of cells above approximately 10^6 cells mL^{-1} should be diluted; the upper range will then easily exceed typical groundwater TNC numbers.

2.2 qPCR

The gene copy numbers, which reflect the microbial cell number, were determined based on known genetic information using qPCR analysis (Ginzinger 2002). Cells were collected on filters and disintegrated using the DNeasy Blood & Tissue Kit (no. 69504; QIAGEN, Solna, Sweden) according to the manufacturer's protocol for Gram-positive bacteria, and the genetic material, DNA, was extracted. The DNA was amplified using pairs of short DNA sequences (i.e., primers) specific to *Bacteria*, *Archaea*, and *Eukarya* (Table 2-1). Known amounts of DNA from the analyzed genes were included as standards and analyzed concomitantly with the samples. The results were obtained within a few days and the sensitivity of the DNA detection was high, but was related to the available sample volume. All types of microorganisms can be detected provided proper primers are available. As the selected standard gene sequences may differ from those of the sample microorganisms, universal primers or primers designed to detect a specific group of microorganisms may be used to detect as many individual species as

possible of the specific group of organisms. The number of gene copies per cell varies between different microorganism species and strains, which may give rise to some discrepancy between TNC and qPCR results. In addition, DNA-based methods are influenced by sample quality (e.g., traces of metals and organic material might disturb the amplification process), and the extraction procedure may encounter some poor yield issues as well (Lloyd et al. 2010). Details of this methodology have been outlined and discussed elsewhere (Pedersen et al. 2011).

Table 2-1. *qPCR primers used for the infiltration experiment.*

qPCR gene	Target organism	Primer	Primer name	Primer sequence
16S Bacteria	Universal for *Bacteria*	Forward primer	Q-926f	5′-AAA CT(CT) AAA (GT)GA ATT GAC -3′
		Reverse primer	Q-1100r	5′-GGG TTG CGC TCG TTG-3′
16S Archaea	Universal for *Archaea*	Forward primer	Arch349f	5′-G(CT)G CA (GC) CAG (GT)CG (AC)GA A(AT)-3′
		Reverse primer	Arch806r	5′-GGA CTA C(AGC)(GC) GGG TAT CTA AT-3′
18S Eukarya	Universal for *Eukarya*	Forward primer	18S F	5′-GGC CCT GTA ATT GGA ATG AGTC-3′
		Reverse primer	18S R	5′-CCA AGA TCC AAC TAC GAG CTT-3′

2.3 Cloning and sequencing

Cloning and sequencing were used in determining the microbial diversity and species. Cells were collected on filters and disintegrated using the DNeasy Blood & Tissue Kit (no. 69504; QIAGEN, Solna, Sweden) according to the manufacturer's protocol for Gram-positive bacteria, and the genetic material, DNA, was extracted. The DNA was PCR amplified using pairs of short DNA sequences specific to various microorganisms (Table 2-1). Amplified DNA was cloned into a plasmid of *Escherichia coli* and the target sequences were sequenced after extraction and preparation. The obtained DNA sequences were compared against international databases for identity to deposited genes, and the most closely related sequence in the database revealed species-related information about the analysed microorganisms. The method revealed the diversity of sample microorganisms within a week, including organisms difficult or impossible to cultivate. The cloning method tends to be biased in favour of dominant microorganisms in a sample. Many more than 100 clones may need to be sequenced for a full diversity assessment of one sample, which is expensive and time consuming. Details of this method have been outlined and discussed elsewhere (Pedersen et al. 2011).

2.4 Microarrays

Microarrays, so-called PhyloChips, were run according to a several-step protocol as outlined elsewhere (DeSantis et al. 2007). First, total DNA was extracted from the bacterial communities; for this, cells were collected on filters and disintegrated using the DNeasy Blood & Tissue Kit (no. 69504; QIAGEN, Solna, Sweden) according to the manufacturer's protocol for Gram-positive bacteria. This step was critical, since a low-yielding extraction could reveal less than the actual diversity (Lloyd et al. 2010). The 16S rRNA gene was then amplified using two different universal primer pairs designed on conserved 16S rRNA gene sequences that should pick up all present *Bacteria* and *Archaea*, respectively. The amplicon pool was then fragmented, biotin labelled, and hybridized to a PhyloChip. The fluorescently labelled 16S rRNA fragments were hybridized to complementary probes on the array surface. The microarrays were stained, washed, and scanned. Fluorescence data were then analyzed and microbes identified using a specific statistical computational program (MxProv4.1; AH Diagnostics, Stockholm, Sweden). Thereafter, similarity heat plots were constructed, resulting in a Phylogenetic tree comparing the diversity and abundance of various *Bacteria* and *Archaea* in the samples. The PhyloChip results were interpreted in several ways. The results were obtained as operational taxonomic units (OTUs), which were used for the probe design (each OTU is a group of sequences that are 97 % identical to each other). The strains listed for each OTU should be interpreted with caution since they are just examples of what the OTU may comprise. Generally, many other strains were found in each OTU as well, so the diversity was based primarily on the OTU results and not on specific strains.

2.5 Sampling occasions

Samples were obtained on four occasions:

1. 2–3 December 2008 – OL-PP69, OL-PVP21, and OL-PVP22
2. 12–13 January 2009 – OL-PP69, OL-PVP21, and OL-PVP22
3. 22–23 September 2009 – OL-PP69, OL-PVP21, OL-PVP22, and OLKR14
4. 18–19 November 2009 – OL-PP69, OL-PVP21, OL-PVP22, and OLKR14

3 CLASSIFICATION OF INFILTRATION GROUNDWATER

3.1 Chemistry

The amounts of iron, sulphur, sulphate, and chloride were compared between sampling occasions 2, 3, and 4 (Table 3-1). Samples taken on occasion 1 were not analyzed for chemistry. The general pattern was that the groundwater chemistry of samples taken on occasion 2 differed from the groundwater chemistry of samples taken on occasions 3 and 4 from drillholes OL-PVP21 and OL-PP69. The OL-PVP22 and OL-KR14 groundwaters did not change much in their chemical composition. The total dissolved solids increased somewhat in OL-KR14 during pumping, unlike what would be expected if the more dilute shallow groundwater types had been drawn down. However, if both more saline and diluted groundwater were reaching OL-KR14 at the same time, a shallow intrusion may have been masked by a concomitant up-coning of deeper, saline groundwater. DNA signatures may allow the detailed investigation of the groundwater types actually reaching OL-KR14, now and in the future years of the experiment.

Table 3-1*. Chemistry results (mg L^{-1}) for four groundwater samples.*

Sample	Sampling occasion	Total iron	Ferrous iron	Total Sulphur	Sulphide	Sulphate	Chloride
OL-PVP21	2	0.537		11		33.9	12.8
OL-PVP21	3	2.29		11		34.8	25.5
OL-PVP21	4	2.88		12		37	25.7
OL-PVP22	2	4.62		11		30.7	5.6
OL-PVP22	3	4.30		12		36.7	6.4
OL-PVP22	4	3.44		14		43.1	7.5
OL-PP69	2	8.96		11		35.3	6.8
OL-PP69	3	3.99		14		40.5	20.5
OL-PP69	4	3.02		18		53	23.7
OL-KR14	2	2.26	2.13	16	0.05	51.3	41.9
OL-KR14	3	2.34	2.09	19	0.06	57	51.7
OL-KR14	4	2.34	1.88	22	0.08	66.1	61.1

3.2 Classification by biomass

The total number of cells (TNC) remained constant at approximately 5×10^4 cells mL^{-1} over time and between drillholes (Table 3-3). Some fluctuations were observed, but they were small in the overburden groundwater from drillholes OL-PVP21 and OL-PVP22 and did not vary more than tenfold in groundwater collected from OL-PP69 and OL-KR14. The greatest biomass determined as TNC was found in drillholes OL-PP69 and OL-KR14 groundwater samples. Quantitative PCR of the 16S rRNA *Bacteria* gene quantifying biomass for *Bacteria* produced similar results, but the number of gene copies in most samples was about ten times lower than indicated by TNC. This may be due to general problems in the sample storage solution or to substances inhibiting the

qPCR. *Eukarya* DNA signatures were most frequent in OL-PVP21 followed by OL-PP69 groundwater. The quantity of DNA signatures in OL-PP69 groundwater decreased over time, while the quantity of OL-KR14 DNA signatures increased somewhat over time (Table 3-3). TNC and qPCR of the 16S rRNA gene for *Bacteria* were only used for biomass analyses, so PhyloChip analysis was needed to classify the groundwater samples further.

3.3 Classification of groundwater by cloning

Cloning was used to classify DNA signatures in groundwater collected from drillholes OL-PP69, OL-PVP21, and OL-PVP22 on the first sampling occasion, i.e., 2–3 December 2008. All three groundwater samples possessed a broad diversity of microorganisms, though differences were observed between the drillholes. The microbial community composition appeared to be fairly similar in drillholes OL-PVP21 and OL-PVP22, as deltaproteobacterial DNA signatures dominated the groundwater samples from the overburden drillholes OL-PVP21 (13/29 clones) and OL-PVP22 (14/31 clones) but not the bedrock groundwater from drillhole OL-PP69 (Tables 3-2 and 3-4).

Another discrepancy was in the **gammaproteobacterial** signatures dominating OL-PP69 (5/30 clones), which were lacking in the groundwater of the overburden drillholes. OL-PVP21 and OL-PP-69 groundwaters were similar in the number of DNA signatures belonging to beta- and epsilonproteobacteria.

Epsilonproteobacterial DNA signatures were found in 4/29 clones in OL-PVP21 groundwater, in 1/31 clones in OL-PVP22 and 4/27 clones in OL-PP69 groundwater.

Betaproteobacterial (5/30 clones) and acidobacterial DNA signatures were found as well in OL-PP69 groundwater by means of cloning. One uncultured *Verrucomicrobia* signature was found in OL-PVP22 groundwater and one *Verrucomicrobia* was found by cloning in OL-PP69.

Table 3-2. *Cloning results for groundwater samples from the three infiltration drillholes OL-PVP21, OL-PVP22, and OL-PP69 taken on 2–3 December 2008.*

Phylum	**Drillhole**			Total number of clones
	OL-PP69	**OL-PVP21**	**OL-PVP22**	
Acidobacteria	1	1		**2**
Alphaproteobacteria	2		1	**3**
Bacteroidetes	1	1	7	**9**
Betaproteobacteria	5	6	2	**13**
Clostridia	2	2	2	**6**
Deltaproteobacteria		13	14	**27**
Epsilonproteobacteria	4	4	1	**9**
Gammaproteobacteria	5			**5**
Unclassified	9	2	3	**14**
Verrucomicrobia	1		1	**2**
Sum of clones	**30**	**29**	**31**	**90**

Table 3-3. *TNC and qPCR results for* 16S rRNA Bacteria*,* 16S rRNA Archaea, *and* 18S rRNA Eukarya *genes detected in groundwater from the four drillholes on four sampling occasions.*

Sampling occasion	TNC (cells cm^{-2})	Standard deviation	16S rRNA gene Bacteria qPCR		18S rRNA gene Eukarya qPCR		16S rRNA gene Archaea qPCR		qPCR total gene copy number	
			Gene copy number (copies mL^{-1})	Standard deviation	Gene copy number (copies mL^{-1})	Standard deviation	Gene copy number (copies mL^{-1})	Standard deviation	Gene copy number (copies mL^{-1})	Standard deviation
OL-PVP21-1	2.6×10^4	2.6×10^3	1.07×10^3	37	*	-	<100	-	1.07×10^3	*
OL-PVP21-2	3.6×10^4	4.4×10^3	4.11×10^3	0	<100	-	*	-	4.11×10^3	*
OL-PVP21-3	2.0×10^4	7.4×10^2	4.14×10^3	1.23×10^3	3.37×10^3	1.38×10^3	*	-	7.51×10^3	544
OL-PVP21-4	5.1×10^4	9.4×10^3	125	38	1.32×10^4	95	*	-	1.33×10^4	9.25×10^3
OL-PVP22-1	7.5×10^4	9.2×10^3	6.29×10^4	8.81×10^2	*	-	1.28×10^3	3.18×10^2	6.42×10^4	4.36×10^4
OL-PVP22-2	2.8×10^4	3.6×10^3	1.74×10^3	2.41×10^3	<100	-	*	-	1.74×10^3	*
OL-PVP22-3	3.5×10^4	6.4×10^3	2.15×10^3	0	206	38	*	-	2.36×10^3	1.37×10^3
OL-PVP22-4	7.2×10^4	4.5×10^2	157	25	695	195	*	-	852	380
OL-PP69-1	1.4×10^5	2.1×10^4	4.09×10^4	3.97×10^2	*	-	1.02×10^3	1.73×10^2	4.19×10^4	2.82×10^4
OL-PP69-2	2.8×10^4	4.5×10^3	465	526	<100	-	*	-	465	*
OL-PP69-3	7.3×10^4	2.4×10^4	82	49	3.24×10^3	1.36×10^3	*	-	3.32×10^3	2.23×10^3
OL-PP69-4	1.3×10^5	1.2×10^4	1.19×10^3	86	651	128	*	-	1.84×10^3	381
OL-KR14-2	2.1×10^4	6.7×10^3	*	-	*	-	*	-	0	*
OL-KR14-3	1.8×10^5	3.3×10^4	1.11×10^4	5.83×10^3	<10	-	*	-	1.69×10^4	3.73×10^3
OL-KR14-4	1.8×10^5	1.5×10^4	<100	0.03	256	114	*	-	256	*

* Not analysed

Table 3-4. *Cloning analysis of OL-PP69, OL-PVP21, and OL-PVP22 groundwaters sampled on 2–3 December 2008; table shows phylum, species, identity to closest match found in GenBank, accession number submitted to GenBank, and number of clones found in the three drill holes. The number of clones for each drill hole is listed at the bottom of the table.*

Phylum	Species	Identity to closest match in GenBank (%)	GenBank accession number	OL-PP69	OL-PVP21	OL-PVP22
Acidobacteria	*Geothrix fermentans* (GFU41563)	98	FJ823163, FJ823188	1	1	
Alphaproteobacteria	*Roseococcus suduntuyensis* (EU012448)	97	FJ823156	1		
Alphaproteobacteria	Uncultured bacterium clone 3C002620 (EU801354)	98	FJ823172	1		
Alphaproteobacteria	*Sphingomonas suberifaciens* (D13737)	97	FJ823207			1
Bacteroidetes	Uncultured Bacteroidetes bacterium (AB478675)	96	FJ823171	1		
Bacteroidetes	*Flavobacterium xinjiangense* (AF433173)	99	FJ823186		1	
Bacteroidetes	Uncultured Prolixibacter sp. (EU809709)	93	FJ823217			1
Bacteroidetes	Uncultured bacterium (DQ404822)	97	FJ823219			4
Bacteroidetes	Uncultured Bacteroidetes sp. (EF562567)	99	FJ823226			1
Bacteroidetes	Uncultured Bacteroidetes sp. (EU809783)	97	FJ823206			1
Betaproteobacteria	*Variovorax boronicumulans* (AB300597)	99	FJ823165	1		
Betaproteobacteria	Uncultured bacterium (EU937989)	98	FJ823173	1		
Betaproteobacteria	*Gallionella ferruginea subsp. capsiferriformans* (DQ386262)	93	FJ823160	2		
Betaproteobacteria	*Dechloromonas denitrificans* (AJ318917)	97	FJ823161	1		
Betaproteobacteria	*Herbaspirillum lusitanum* (AF543312)	95	FJ823183		1	
Betaproteobacteria	*Gallionella ferruginea* (L07897)	98	FJ823194		1	
Betaproteobacteria	*Oxalobacteraceae bacterium* (FJ165500)	99	FJ823197		1	
Betaproteobacteria	Uncultured bacterium (EF590026)	99	FJ823198		1	
Betaproteobacteria	Uncultured bacterium (EU360492)	97	FJ823199		1	
Betaproteobacteria	*Rhodoferax ferrireduens* (CP000267)	99	FJ823200		1	
Betaproteobacteria	Uncultured bacterium (EF492934)	93	FJ823214			1
Betaproteobacteria	Uncultured bacterium (EU030494)	99	FJ823222			1
Clostridia	Uncultured bacterium (EU266857)	92	FJ823166	1		
Clostridia	Uncultured bacterium (EF400772)	90	FJ823179	1		
Clostridia	*Desulfosporosinus lacus* (AJ582757)	91	FJ823192		2	
Clostridia	Uncultured *Desulfosporosinus sp*. (EF565948)	98	FJ823224			1
Clostridia; Thermoanaerobacter	Uncultured bacterium (AJ583208)	94	FJ823223			1
Deltaproteobacteria	Uncultured *Geobacter sp*. (AY752756)	98	FJ823196		1	
Deltaproteobacteria	Uncultured *Desulfobulbaceae bacterium* (EU016446)	92	FJ823184		6	
Deltaproteobacteria	Uncultured *Desulfobulbaceae bacterium* (EU016417)	99	FJ823202		1	
Deltaproteobacteria	Uncultured bacterium (EU234232)	93	FJ823203		1	
Deltaproteobacteria	Uncultured bacterium (EF667585)	97	FJ823185		1	
Deltaproteobacteria	Uncultured *Desulfobulbaceae bacterium* (EU016449)	98	FJ823190		3	
Deltaproteobacteria	Uncultured bacterium (AM991274)	85	FJ823204			1

Deltaproteobacteria	Uncultured bacterium (EF562080)	96	FJ823213			1
Deltaproteobacteria	Uncultured *Desulfobulbaceae bacterium* (EU016430	93	FJ823215			1
Deltaproteobacteria	Uncultured proteobacterium (AF420340)	87	FJ823209			1
Deltaproteobacteria; Desulfobulbaceae	Uncultured bacterium (DQ833475)	96	FJ823211			1
Deltaproteobacteria; Desulfobulbaceae	Uncultured *Desulfobulbaceae bacterium* (EU016430)	99	FJ823212			4
Deltaproteobacteria; Desulfocapsa	Uncultured bacterium (FJ592590)	88	FJ823218			2
Deltaproteobacteria; Desulfuromonas	Uncultured bacterium (AB294284)	98	FJ823221			1
Deltaproteobacteria; Syntrophus	Uncultured bacterium (FJ437801)	97	FJ823220			1
Deltaproteobacteria; Syntrophus	Uncultured bacterium (EU644230)	98	FJ823210			1
Epsilonproteobacteria	Uncultured bacterium (DQ295666)	99	FJ823182, FJ823195	1	1	
Epsilonproteobacteria	Uncultured bacterium (DQ295660)	82	FJ823158	1		
Epsilonproteobacteria	Uncultured prokaryote (AM268745)	93	FJ823159	1		
Epsilonproteobacteria	Uncultured bacterium (EF667743)	99	FJ823201		1	
Epsilonproteobacteria	*Sulfuricurvum kujiense* (AB080643)	98	FJ823162, FJ823187	1	1	
Epsilonproteobacteria	Uncultured bacterium (DQ787677)	99	FJ823189		1	
Epsilonproteobacteria	Uncultured bacterium (AY667264)	93	FJ823216			1
Gammaproteobacteria	*Methylobacter psychrophilus* (AF152597)	99	FJ823170	3		
Gammaproteobacteria	*Methylobacter tundripaludum* (AJ414655)	95	FJ823174	1		
Gammaproteobacteria	*Methylomonas methanica* (AF150806)	96	FJ823164	1		
Unclassified	Uncultured bacterium (DQ404803)	98	FJ823167	1		
Unclassified	Uncultured bacterium (AY212569)	96	FJ823168	1		
Unclassified	Uncultured bacterium (AY326636)	92	FJ823169	1		
Unclassified	Uncultured bacterium (AB250585)	88	FJ823157	1		
Unclassified	Uncultured bacterium (AM991203)	90	FJ823175	1		
Unclassified	Uncultured bacterium (EU937953)	99	FJ823176	1		
Unclassified	Uncultured bacterium (AJ488070)	99	FJ823177	1		
Unclassified	Uncultured bacterium (AY667262)	93	FJ823178	1		
Unclassified	Uncultured bacterium (AF 507682)	93	FJ823181	1		
Unclassified	Uncultured bacterium (EU801645)	90	FJ823193		1	
Unclassified	Uncultured bacterium (EF667628)	99	FJ823191		1	
Unclassified	Uncultured bacterium (DQ378270)	99	FJ823205			3
Verrucomicrobia	Uncultured bacterium (FJ437883)	98	FJ823180	1		
Verrucomicrobia	Uncultured bacterium (AF407207)	92	FJ823208			1
Total number of clones				30	29	31

4 ACCESSION NUMBERS

The sequence data from this study have been submitted to the GenBank database and were given the accession numbers FJ823156-FJ823226 and FJ895109.

4.1 Classification of groundwater using PhyloChip analysis

PhyloChips were used to detect microbial diversity in groundwater collected from drillholes OL-PP69, OL-PVP21, and OL-PVP22 on four sampling occasions, i.e., 1) 2–3 December 2008, 2) 12–13 January 2009, 3) 22–23 September 2009, and 4) 18–19 November 2009, and in OL-KR14 groundwater on two sampling occasions, i.e., 22 September 2009 and 19 November 2009. PhyloChip analysis revealed a broader diversity than did cloning, making it a more sensitive diversity analysis method. Because cloning is more time consuming and expensive than is PhyloChip analysis and because cloning and PhyloChip data from the first sampling occasion agreed, PhyloChips were used for analysis of samples from the remaining three sampling occasions. Small differences were observed between the data for the four sampling occasions over time, but clear differences were observed between the drillholes (Tables 3-2 and 3-4). OL-PP69 samples belong together in the same group, which also included the OL-KR14 sample taken on the fourth occasion, possibly reflecting the intrusion of water from drillhole OL-PP69 to OL-KR14. The OL-KR14 groundwater sampled on 22 September 2009 constitutes a group of its own and may indicate that the microbial community of this drillhole groundwater differs significantly from those of the other drillholes. The second groundwater samples from OL-PVP21 and OL-PVP22 taken on 22–23 January 2009 belong together in a single group differing from all other samples, indicating that seasonal variation in the overburden groundwater can be significant. Such seasonal variation may be greater than the variation between the sampling depths of the overburden, so the intrusion of groundwater through rock fractures may be difficult to detect given the methods and low sampling interval used here. Turning to the Archaea, the OL-KR14 and OL-PP69 samples belong together in the same group.

Operational taxonomic units (OTUs) indicate the microbial water signatures found using PhyloChip analysis. OTUs are recognized by their identification numbers, which can be found at www.greengenes.gov. The DNA signatures detected in the various analysed groundwater samples were divided into the groups presented below. The OTU groups were divided according to the microorganisms found into the following signatures:

Acidobacterial DNA signatures were rare in OL-KR14, OL-PVP21, and OL-PVP22 groundwaters (Appendix Table 1), but were found in the second and third, but not first or fourth, groundwater samples from drillhole OL-PVP21. They were only found in the last two samples in OL-PVP22. These signatures were found in the second groundwater sample from OL-PP69, but were rare. Actinobacterial signatures were found as one OTU group in the first sample but not at all in the second sample from OL-KR14.

Alphaproteobacterial DNA signatures were common in the first groundwater sample from drillhole OL-PVP22, but decreased in number over time. They were also found in OL-PVP21 groundwater but only on the two last sampling occasions. They were common, remaining constant in number over time in all four groundwater samples from

OL-PP69. Few alphaproteobacterial DNA signatures were found in OL-KR14 groundwater, but they increased somewhat in number over time.

Bacteroidetes DNA signatures from five OTU groups were common in OL-PVP21 and OL-PVP22 groundwaters and relatively constantly found over time. They were common in OL-PP69 groundwater samples taken on all sampling occasions, but most common in the last sample; they belonged to five OTUs. These DNA signatures were also common in samples of OL-KR14 groundwater taken on both sampling occasions, containing five and four OTU groups, respectively.

Betaproteobacterial DNA signatures belonging to 30 OTUs were relatively common in all OL-PP69 groundwater samples. DNA signatures from 30 OTUs were very rare but increased in number over time in OL-PVP21 groundwater. They were found as 29 OTUs in OL-PVP22 groundwater. Betaproteobacterial DNA signatures were rare in OL-KR14 groundwater from both sampling occasions.

Other common OTUs belonged to 13 OTUs clustering into **deltaproteobacterial** DNA signatures in groundwater from the overburden drillholes OL-PVP21 and OL-PVP22. These increased in number on the two last sampling occasions. Sulphate-reducing bacteria (SRB) belonging to deltaproteobacterial signatures remained constant in number between the sampling occasions in several OTU groups in OL-KR14 groundwater. These DNA signatures were absent in OL-PP69 groundwater according to the PhyloChip analyses.

Epsilonproteobacterial DNA signatures were found in 15 OTUs in the first and fourth samples of OL-PP69 groundwater according to PhyloChip results. Epsilonproteobacterial signatures were found in one OTU in the first sample but not at all in the second groundwater sample from OL-KR14. Epsilonproteobacterial DNA signatures belonging to 15 OTUs were found, but were rare, in the first groundwater sample from OL-PVP21. One OTU group was found in the last groundwater sample from OL-PVP21.

Firmicutes were common (17 OTUs), increasing in number over time in OL-PVP21 groundwaters. However, the first sample displayed very low concentrations of these DNA signatures. The same trend was seen in OL-PVP22 (18 OTUs) groundwater. Two OTUs were common in OL-PP69 groundwater. Firmicutes dominated groundwater from the first sampling occasion in OL-KR14 groundwater (11 OTUs), but decreased in number in the second sample (four OTU).

Gammaproteobacterial DNA signatures were common, being constantly found over time in all OL-PP69 groundwater samples. They increased over time from one to four OTUs in OL-KR14 groundwater. DNA signatures from two OTUs were found in OL-PVP21 and OL-PVP22 groundwater. A slight decrease in number was found over time.

OP9 and **OP11** DNA signatures were found in the first and last samples of OL-PP69 groundwater.

OP8, OP9, OP11, Planctomycetes, and **Nitrospiral** DNA signatures were found in the two last samples of OL-PVP22 groundwater but were absent in OL-PP69 groundwater.

Bacteria belonging to **OP9** and **OP11** DNA signatures were found, but were rare, in the second sample of OL-KR14 groundwater.

Nitrospira, OP8, and **Planctomycetes** signatures were found by means of PhyloChip analysis in the last samples of overburden groundwaters from drillholes OL-PVP21 and OL-PVP22. Nitrospira and OP8 signatures were absent in OL-KR14 groundwater while Planctomycetes were rare.

Verrucomicrobial DNA signatures were present in the second samples of OL-PVP21 and OL-PVP22 groundwaters and in the first and last samples of OL-PP69 (6 OTU) groundwater. Verrucomicrobial signatures were rare but present in the last sample of OL-KR14 groundwater.

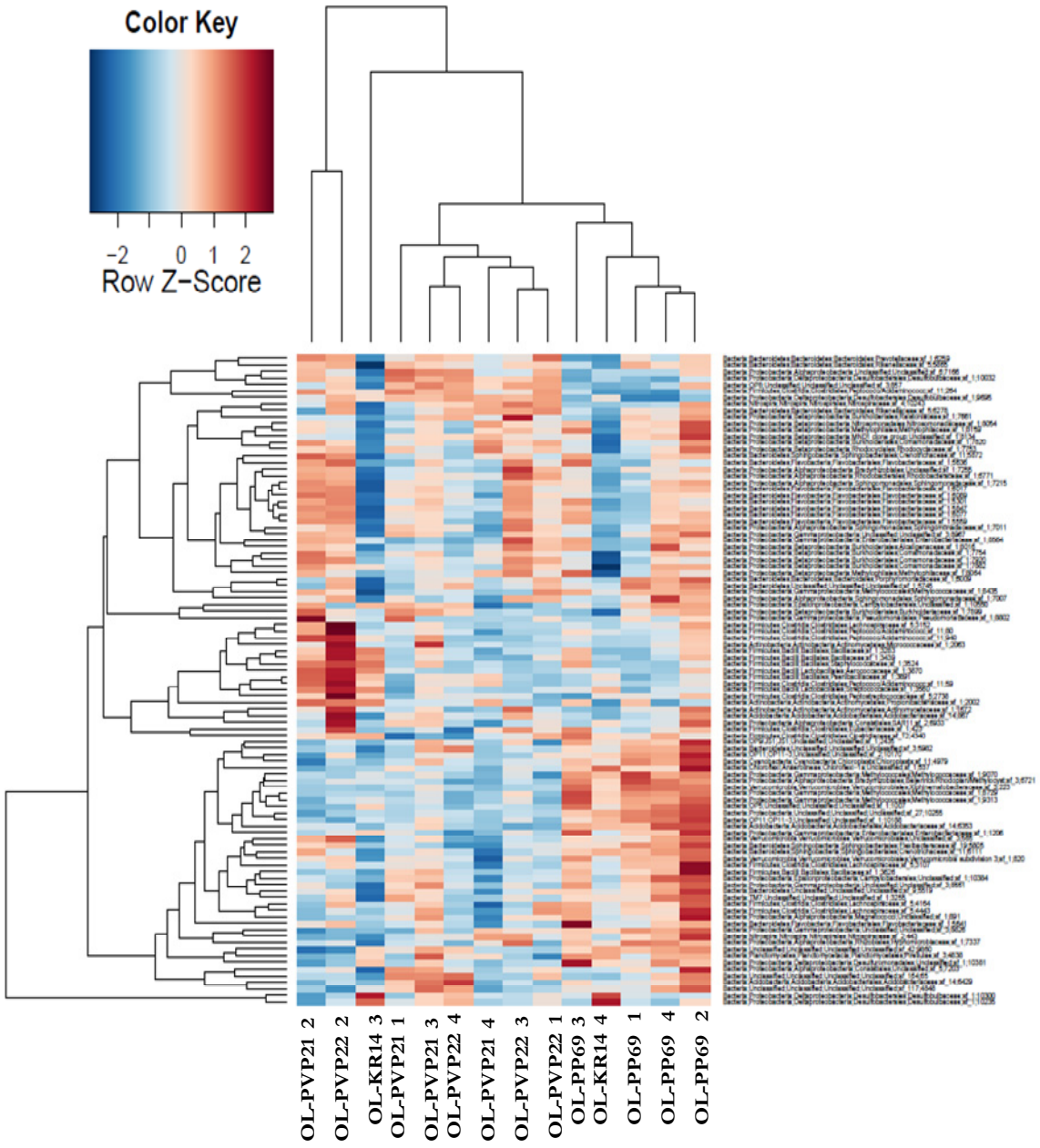

Figure 4-1. *A similarity heat plot shows DNA signatures from OL-KR14, OL-PP69, OL-PVP21, and OL-PVP22 groundwaters sampled on four occasions, i.e., 1) 2–3 December 2008, 2) 12–13 January 2009, 3) 22–23 September 2009, and 4) 18–19 November 2009, and analyzed for OTU numbers using 16S rRNA gene PCR for the domain* Bacteria*. The darker red the signal, the more intense it is, and the more organisms of the species in question. Changes in abundance over time are indicated by the red and blue clusters. The diversity table to the right in the figure is summarized in the text and displayed in full scale in Appendix Table 2.*

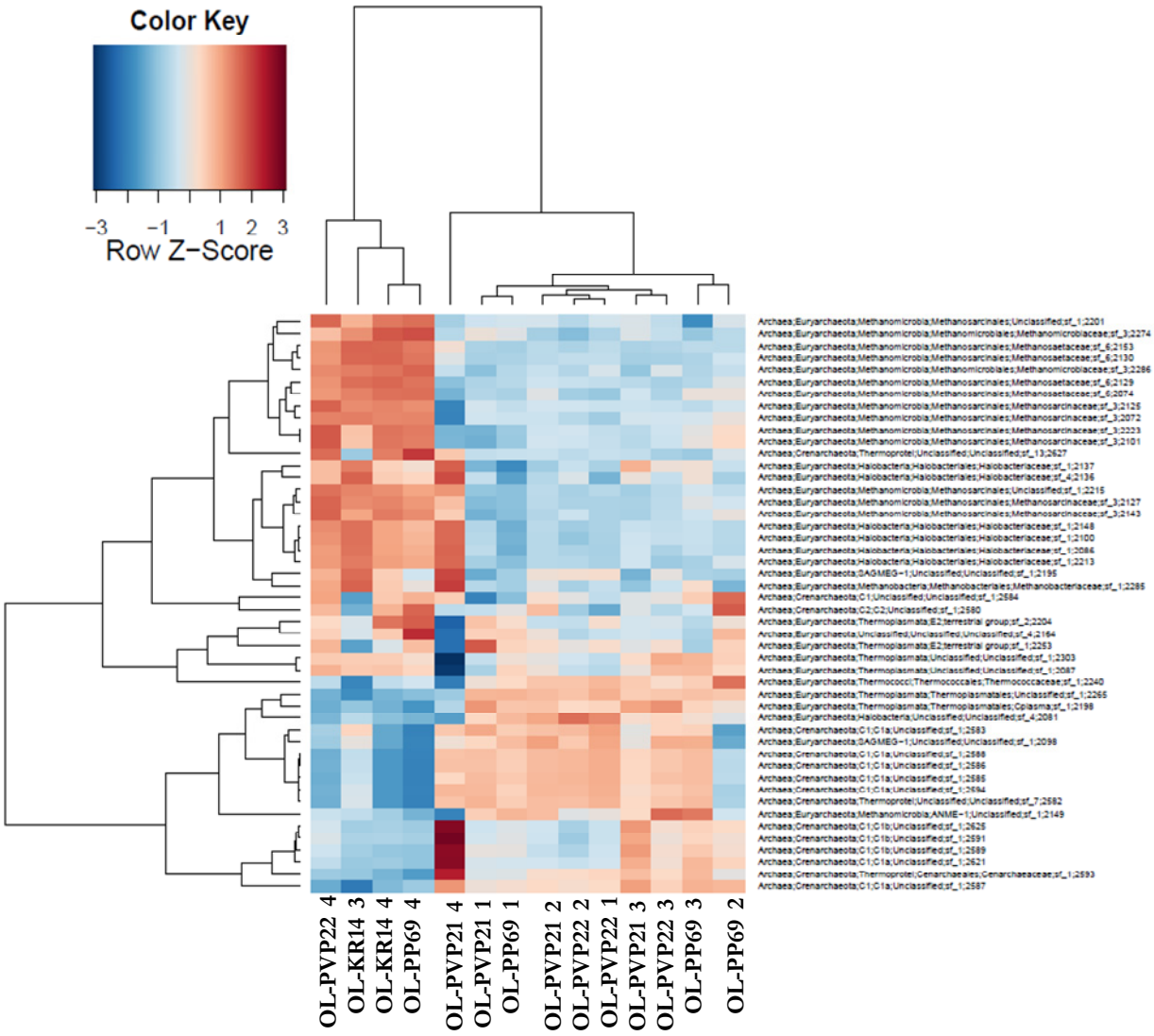

Figure 4-2. *A similarity heat plot shows groundwater signatures from OL-KR14, OL-PP69, OL-PVP21, and OL-PVP22 sampled on four occasions, i.e., 1) 2–3 December 2008, 2) 12–13 January 2009, 3), 22–23 September 2009, and 4) 18–19 November 2009, and analyzed for OTU numbers using 16S rRNA gene PCR for the domain* Archaea*. Changes in abundance over time are indicated by the red and blue clusters. The diversity table to the right in the figure is summarized in the text and displayed in full scale in Appendix Table 3.*

5 EVALUATION OF CLASSIFICATION DATA

PhyloChip results were verified by cloning results, although the higher-sensitivity PhyloChip analysis found a broader diversity of DNA signatures than did cloning. Cloning was therefore applied only to samples collected on the first occasion. PhyloChip analysis was used with 16S rRNA gene qPCR for *Bacteria*, with 18S rRNA gene qPCR for *Eukarya*, and with TNC to analyse biomass from the groundwater samples. In samples taken on four occasions over one year, PhyloChip data revealed a resemblance between groundwater samples from the same drillhole over this period. The groundwaters from four drillholes were compared, indicating that DNA signatures can be used successfully to classify groundwater from different drillholes. Analysis by means of qPCR and TNC revealed a relatively constant number of cells over time in all groundwater samples. DNA signatures suggested a possible migration of groundwaters over time from drillholes OL-PVP21 to OL-PP69 and from OL-PP69 to OL-KR14 when pumping the deep drillhole OL-KR14. Chemistry results from the Posiva Oy database POTTI correlated with the PhyloChip results. This finding was very encouraging, supporting further use of PhyloChip analysis to classify groundwater signatures over time. However, the one-year period of this study was too limited, so more samples should be analyzed over longer periods to follow long-term changes.

5.1 Classification of groundwater samples over time

DNA signatures found using PhyloChip analyses are discussed below, as cloning was performed on only one occasion and not over time. The chemistry analyses were considered as well, as they complemented PhyloChip analyses, and the combined methods allowed minor changes between the samples to be observed over time. However, the groundwater could be distinguished between the drillholes. Small changes were observed in the bacterial signatures of OL-KR14 groundwater that may indicate mixing with OL-PP69 groundwater. In the first year, these groundwaters did not mix, but then the DNA signatures obtained using PhyloChip analysis indicated that the groundwater started to mix as the pumping continued. Variations in DNA signatures were found between the various drillhole groundwater samples, indicating that DNA signatures may be used to classify groundwater from different drillholes. However, only small variations were found in the groundwater sample series over time. Additional analyses may reveal whether this remains the case over a longer period of time than the present one-year observation period. Though “increase” and “decrease” are used in the text below, it should be borne in mind that only small variations were found.

Alphaproteobacterial DNA signatures are found under aerobic conditions, and bacteria with these DNA signatures carry out photosynthesis (Table 5-1). Such bacteria found in the two overburden groundwaters from drillholes OL-PVP21 and OL-PVP22 differed from those in the bedrock groundwater from OL-PP69 and in groundwater from the deep drillhole OL-KR14. Several alphaproteobacterial DNA signatures were commonly found in OL-PVP21 and OL-PVP22 groundwaters, but decreased in number over time. In OL-PP69 and OL-KR14 groundwaters, this DNA signature was less common, remaining constant over time in the former and increasing in number in the latter (Figure 4-1 and Table 5-2).

Actinobacterial signatures are found mostly in aerobic environments (Table 5-1) and were unusual but found in OL-KR14 and OL-PP69 groundwaters.

Betaproteobacterial DNA signatures include several nitrogen-fixing bacteria (Table 5-1). Nitrogen fixation is the process by which atmospheric nitrogen gas is converted into ammonia, which is subsequently available for many important biological molecules, such as amino acids, proteins, vitamins, and nucleic acids. According to betaproteobacterial DNA signatures, the overburden groundwater samples seem to cluster together, bedrock groundwater differs, and deep drillhole water seems to differ the most from the rest. Nitrogen fixation thus seems to be more abundant in the overburden groundwaters from drillholes OL-PVP21 and OL-PVP22 than in the bedrock groundwater from OL-PP69 or the deep groundwater from OL-KR14. Betaproteobacterial DNA signatures were rare in the first OL-PVP21 and OL-PVP22 groundwater samples, increasing somewhat in number over time in OL-PVP21 groundwater and decreasing in OL-PVP22 groundwater. Groundwater from OL-PP69 was similar in composition to OL-PVP21 groundwater. Very few betaproteobacterial signatures were found in OL-KR14 groundwater, and they increased somewhat in number over time.

Deltaproteobacterial DNA signatures commonly represent SRB, which use sulphate as electron acceptor to produce hydrogen sulphide under anaerobic conditions (Table 5-1). In the groundwater in which these signatures were found, sulphate reduction can be an ongoing process and the groundwater may be enriched in sulphide. Chemistry analyses indicated that total sulphur and sulphate concentrations were low and remained relatively constant, increasing somewhat over time in the overburden groundwaters. Sulphide concentrations were measured only in OL-KR14 groundwater. Chloride concentrations were relatively high and increased over time (Table 3-1). Groundwater from the overburden drillholes OL-PVP21 and especially OL-PVP22 contained several DNA signatures clustering into deltaproteobacterial DNA signatures. These DNA signatures were extremely rare in OL-PP69 groundwater, but were common in OL-KR14 groundwater in which they decreased in number over time. This may be another dilution effect if other groundwaters mix with the OL-KR14 groundwater when the drillhole is pumped. The sulphide concentration decreased over time in OL-KR14 groundwater, further supporting the decreased number of deltaproteobacterial signatures observed using PhyloChip analysis.

Epsilonproteobacterial DNA signatures indicate the presence of strictly or facultatively anaerobic bacteria (Table 5-1). Total sulphur increased over time in OL-PVP22 groundwater, supporting the increase in the population of sulphur-oxidizing bacteria belonging to epsilonproteobacteria (Table 3-1). These epsilonproteobacterial DNA signatures were found in all four groundwaters, but were most common in OL-PVP21 and OL-PP69 groundwaters, increasing in number over time in OL-PVP22 and decreasing over time in OL-PVP21 and OL-KR14 groundwaters. High concentrations of total sulphur may explain the numerous epsilonproteobacterial DNA signatures in OL-PP69 groundwater, as these bacteria can use elemental sulphur as a source of energy. Total sulphur was highest in OL-KR14 groundwater, supporting the presence of epsilonproteobacterial DNA signatures.

Firmicute (Gram-positive bacteria) DNA signatures indicate various anaerobic or aerobic environments (Table 5-1) and were common in OL-PVP21, OL-PVP22, and OL-PP69 groundwaters. These DNA signatures increased in number over time in OL-PVP21 groundwater, but decreased somewhat over time in OL-PVP22 and OL-PP69 groundwaters. Firmicute DNA signatures were common in the first sample of OL-KR14 groundwater, but had decreased in the last sample, possibly due to a change in water type due to pumping.

Gammaproteobacterial DNA signatures were rare in the overburden groundwaters (OL-PVP21 and OL-PVP22) but dominated the bedrock groundwater (OL-PP69). All four OL-PP69 groundwater samples were dominated by gammaproteobacterial DNA signatures. The first sample of OL-KR14 groundwater contained few gammaproteobacterial DNA signatures, but these increased in number over time, somewhat supporting the idea of groundwater mixing. These results indicated a shift in water composition as OL-KR14 was pumped, possibly due to OL-PP69 groundwater mixing with OL-KR14 groundwater.

Verrucomicrobial DNA signatures (Table 5-1) were most abundant in the first OL-PP69 groundwater sample but were also present in the first OL-PVP22 sample. Their numbers remained constant in the OL-PP69, OL-PVP21, and OL-PVP22 groundwaters but increased in OL-KR14 groundwater.

Thermoplasmata of the genus Euryarchaeota and **Thermoprotei 1** of the genus *Crenarchaeota* remained constant in numbers over time in the OL-PVP21, OL-PVP22, and OL-PP69 groundwaters.

Methanomicrobial DNA signatures, which indicate methane-enriched groundwaters (Table 5-1), belong to the Euryarchaeota branch. They increased in number over time in OL-PVP21 and even more in OL-PVP22 and OL-PP69 groundwaters. However, most were found in OL-KR14 groundwater, in numbers that remained constant over time. These variations may be seasonal or simply represent a bias in the last groundwater sample. More samples are needed to be able to form any conclusions (Figure 4-2 and Table 5-3).

Halobacterial DNA signatures indicate aerobic or anaerobic groundwaters that are saturated or nearly saturated with salt (Table 5-1). They were rare but increased somewhat in number over time in OL-PVP21, OL-PVP22, and OL-PP69 groundwater. However, most halobacterial DNA signatures were found in OL-KR14 groundwater, in which they remained constantly relatively high in number over time.

Crenarchaeota also increased in number over time in OL-PVP21, OL-PVP22, and OL-PP69 groundwaters, but occurred in very low numbers on all sampling occasions in OL-KR14 groundwater.

Table 5-1. *DNA signatures found in the four infiltration groundwater samples by means of cloning and PhyloChip analysis. Their modes of action are listed together with a reference.*

DNA signature	**Mode of action**	**Reference**
Acidobacteria	Favour or demand acidic environments, so waters containing these DNA signatures are likely acidic.	Barns et al. (2007)
Actinobacteria	Mostly aerobic but can live in anaerobic environments.	Ventura et al. (2007)
Alphaproteobacteria	Aerobic environments. Most OTU groups found in these groundwaters photosynthesize to acquire energy. They use the energy from sunlight to convert carbon dioxide and H_2S into organic materials to be utilized in cellular functions such as biosynthesis and respiration.	Madigan et al. (2009)
Bacteroidetes		Madigan et al. (2009)
Betaproteobacteria	Aerobic or facultatively aerobic. Fix nitrogen in various types of plants, oxidizing ammonium to produce nitrite. May use photosynthesis.	Madigan et al. (2009)
Chloroflexi	Facultatively aerobic. Bacteria belonging to these DNA signatures produce energy from light, and hence are phototrophic.	Madigan et al. (2009)
Cyanobacteria	Facultatively aerobic. Bacteria with this DNA signature obtain their energy through photosynthesis. May reduce elemental sulphur.	Madigan et al. (2009)
Gammaproteobacteria	Several bacteria are found in this DNA signature group. Among them are bacteria able to oxidize methane and hydrogen sulphide to produce sulphur.	Madigan et al. (2009)
Deltaproteobacteria	Anaerobic bacteria belong to these DNA signatures. Many members of this group are sulphate-reducing bacteria (SRB). Sulphide is produced that may cause copper corrosion.	Madigan et al. (2009)

Epsilonproteobacteria	Strictly or facultatively anaerobic bacteria that are hydrogen or sulphur oxidizing, sulphur or nitrate reducing belong to these DNA signatures.	Takai et al. (2005)
Firmicutes	Obligate or facultatively aerobic bacteria belong to these DNA signatures.	Madigan et al. (2009)
Halobacterial	Aerobic or anaerobic environments. Found in water saturated or nearly saturated with salt. They are also called halophiles. They are common in most environments where large amounts of salt, moisture, and organic material are available. Large blooms of Halobacteria appear reddish from the pigment bacteriorhodopsin.	DasSarma et al. (2006)
Methanobacterial	Anaerobic environments. Carbon dioxide-reducing methanogens use hydrogen or formate as the reducing agent to produce methane.	Madigan et al. (2009)
Nitrospira	Nitrite-oxidizing bacteria belong to these DNA signatures.	Madigan et al. (2009)
OP8	Nitrite-oxidizing bacteria belong to these DNA signatures.	Madigan et al. (2009)
OP9	Facultatively aerobic. Energy is obtained through photosynthesis by *Bacteria* found with this DNA signature. They may reduce elemental sulphur.	Madigan et al. (2009)
OP11	OP11 DNA signatures are generally found in reduced environments with a conspicuous presence of sulphur compounds, such as sulphate and sulphide. This may indicate that the detected organisms engage in sulphur cycling in the environment.	Harris et al. (2004)
Planctomycetes	Facultatively aerobic bacteria, growing by respiration of sugars.	Madigan et al. (2009)
Thermoplasmata	-	-
Verrucomicrobia	Aerobic or facultatively aerobic bacteria fermenting various sugars.	Madigan et al. (2009)

Table 5-2. *Comparison of various bacterial DNA signatures found in groundwaters from the four drillholes chosen for the infiltration experiment. The numbers stand for the relative amounts of the various OTU groups (DNA signatures) listed on the left-hand side, and the boldface numbers 1–4 stand for the sampling occasions.*

Phylum	Drillhole													
	OL-KR14		OL-PP69				OL-PVP21				OL-PVP22			
	3	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Acidobacteria		+	+		+									+
Actinobacteria	+			+					+	+		2.5	+	
Alphaproteobacteria	+	2.5	2	3	2	2.5	4.5	3	2.5	+	5	3	4	3
Bacteroidetes	2.5	3	4	4	3.5	5.5	6	6	4	7.5	5	6	5	4
Betaproteobacteria	+		2	3	2.5	4	2	4.5	3.5	5.5	4.5	3	5	+
Chloroflexi		+												
Cyanobacteria		+												
Gammaproteobacteria	+	3	4	3	4	3	2.5	2.5	1.5	+	3	1.5	3	
Deltaproteobacteria	3.5	2.5					+		2	2	2	+	+	3
Epsilonproteobacteria	+		+			+	+							+
Firmicutes	10	3	4	4	4.5	2	2	6	5	4	2	6.5	1.5	3
Nitrospira										2			+	+
OP8										+				+
OP9		+												+
OP11		1.5	+			+								+
Planctomycetes	+	+								+				+
Unclassified	2	3	2.5	2	2	2	2		+		+	+		2.5
Verrucomicrobia		+	+			+		+				+		

Note: All data below 3 % were marked as they were considered to be below detection. Only red OTU fields from the heat plot were considered, and every "+" indicates approximately 4 % of the total number of OTUs in the drillhole of interest on a specific sampling occasion.

Table 5-3. *Comparison of various archaeal DNA signatures between groundwaters collected from drillholes OL-PVP21, OL-PVP22, OL-PP9, and OL-KR14 on four sampling occasions. The numbers in the table stand for the relative amounts of the various OTU groups (DNA signatures) listed on the left-hand side, and the boldface numbers 1–4 stand for the sampling occasions.*

Phylum	Drillhole													
	OL-KR14		OL-PP69				OL-PVP21				OL-PVP22			
	3	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Euryarchaeota; Methanomicrobia	17	18	1		1	15	1	1	1	5			1	14
Euryarchaeota; Halobacteria	6	6	1		2	6	1	1	2	5	1	1	2	6
Euryarchaeota; Thermoplasmata	3	3	6	4	5	4	4	4	4		4	2	5	4
Euryarchaeota; SAGMEG	1	1	1		1			2		1	2	2	1	1
Euryarchaeota; Thermococci			1	1	1		1	1	1		1	1	1	
Euryarchaeota; Unclassified		1	1	1	1	1	1							1
Crenarchaeota; Thermoprote1		1	1		2	1	2	2	2	3	2	2	2	1
Crenarchaeota; C1		1	6	6	11	1	5	7	9	11	6	6	9	1
Crenarchaeota; C2				1		1		1						1

Note: Only red OTU fields from the heat plot were considered and every number indicates one OTU group found using PhyloChip analysis.

6 CONCLUSIONS

- DNA signatures could successfully be used to classify groundwater. Clear differences were observed in DNA signatures between the drillholes, but the individual groundwaters remained relatively stable over time.

- TNC remained fairly constant over time in OL-PVP21 and OL-PVP22 groundwaters, while TNC in OL-PP69 groundwater decreased tenfold and in OL-KR14 increased tenfold from the first to the second sampling occasions. These results were supported by 16S rRNA gene qPCR. Bacterial DNA signatures indicated small changes over time in the groundwaters from the four infiltration drillholes, supporting the idea of waters mixing to some extent while overall remaining relatively constant. Archaeal DNA signatures remained constant in OL-KR14 groundwater, but *Methanomicrobia* signatures increased in number in the last samples from drillholes OL-PP69, OL-PVP21, and OL-PVP22.

- The pumped groundwater (OL-KR14) differed significantly from the bedrock (OL-PP69) and overburden (OL-PVP21 and OL-PVP22) groundwaters in terms of archaeal DNA signatures, as it contained the most methanomicrobial and halobacterial signatures, indicating the presence of methane and salt. Low concentrations of deltaproteobacterial, epsilonproteobacterial, and gamma-proteobacterial signatures were found as well.

- Bedrock groundwater from OL-PP69 was dominated by gammaproteobacteria.

- Overburden groundwater differed from the bedrock groundwater from drillhole OL-PP69 and from the deep groundwater from OL-KR14, as it was dominated by deltaproteobacterial DNA signatures indicating high concentrations of sulphide. Betaproteobacteria were found in the overburden groundwaters, indicating that these groundwaters may be rich in nitrogen and/or ammonia. Alphaproteobacteria were commonly found in drillholes OL-PVP21, OL-PVP22, OL-PP69, and OL-KR14. The presence of alphaproteobacteria indicates aerobic conditions, as they include photosynthesizing bacteria.

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APPENDIX

Table 1. *Phyla obtained using 16S rRNA gene* Bacteria *qPCR and PhyloChip analysis in four drillholes, on two sampling occasions for OL-KR14 and on four sampling occasions for OL-PP69, OL-PVP21, and OL-PVP22.*

Phylum	Drillhole				Total number of observations
	OL-KR14	OL-PP69	OL-PVP21	OL-PVP22	
Acidobacteria	1	8	3	1	13
Actinobacteria	1	6	7	6	20
Alphaproteobacteria	4	26	21	27	78
Bacteroidetes	9	45	41	38	133
Betaproteobacteria	1	31	29	29	90
Chloroflexi	1	4	1	1	7
Cyanobacteria	1	4	1	0	6
Gammaproteobacteria	5	36	14	15	70
Deltaproteobacteria	7	5	10	12	34
Epsilonproteobacteria	1	7	5	1	14
Firmicutes	15	35	34	27	111
Nitrospira	0	5	3	5	13
OP8	0	1	3	4	8
OP9	1	4	1	2	8
OP11	2	8	2	4	16
Planctomycetes	2	3	2	1	8
Unclassified	6	21	7	8	42
Verrucomicrobia	1	7	5	3	16
Sum	57	249	184	181	687

Table 2. List of diversity in Figure 4-1.

Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;sf_1;10235
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;sf_1;10300
Bacteria;Unclassified;Unclassified;Unclassified;Unclassified;sf_117;4848
Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;sf_14;6429
Bacteria;Unclassified;Unclassified;Unclassified;Unclassified;sf_164;65
Bacteria;Proteobacteria;Alphaproteobacteria;Consistiales;Unclassified;sf_5;7203
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Unclassified;sf_1;10381
Bacteria;Planctomycetes;Planctomycetacia;Planctomycetales;Pirellulae;sf_3;4838
Bacteria;Unclassified;Unclassified;Unclassified;Unclassified;sf_42;9860
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae;sf_1;7337
Bacteria;Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;sf_2;443
Bacteria;Proteobacteria;Gammaproteobacteria;Unclassified;Unclassified;sf_3;8826
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;5641
Bacteria;Proteobacteria;Alphaproteobacteria;Magnetococci;Unclassified;sf_1;891
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;sf_5;4443
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;sf_5;4164
Bacteria;TM7;Unclassified;Unclassified;Unclassified;sf_1;3255
Bacteria;Bacteroidetes;Unclassified;Unclassified;Unclassified;sf_9;5519
Bacteria;Proteobacteria;Gammaproteobacteria;Unclassified;Unclassified;sf_3;8551
Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Unclassified;sf_1;10384
Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;sf_1;3626
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;sf_5;3107
Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobia subdivision 3;sf_1;820
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Crenotrichaceae;sf_11;6111
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Flexibacteraceae;sf_19;5805
Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Unclassified;sf_3;565
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;sf_1;1206
Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;sf_14;6353
Bacteria;OP11;OP11−3;Unclassified;Unclassified;sf_1;10188
Bacteria;Proteobacteria;Unclassified;Unclassified;Unclassified;sf_27;10255
Bacteria;OP5;Unclassified;Unclassified;Unclassified;sf_1;1007
Bacteria;Proteobacteria;Gammaproteobacteria;Methylococcales;Methylococcaceae;sf_1;9313
Bacteria;Proteobacteria;Gammaproteobacteria;Methylococcales;Methylococcaceae;sf_1;8729
Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Xiphinematobacteraceae;sf_3;223
Bacteria;Proteobacteria;Alphaproteobacteria;Bradyrhizobiales;Beijerinck/Rhodoplan/Methylocyst;sf_3;6721
Bacteria;Proteobacteria;Gammaproteobacteria;Methylococcales;Methylococcaceae;sf_1;9070
Bacteria;Chloroflexi;Anaerolineae;Chloroflexi−1a;Unclassified;sf_1;537
Bacteria;Cyanobacteria;Cyanobacteria;Chloroplasts;Chloroplasts;sf_11;4979
Bacteria;OP11;OP11−3;Unclassified;Unclassified;sf_2;10170
Bacteria;Bacteroidetes;Unclassified;Unclassified;Unclassified;sf_3;5982
Bacteria;OP9/JS1;JS1;Unclassified;Unclassified;sf_1;2435
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;sf_12;4340
Bacteria;Firmicutes;Clostridia;Clostridiales;Eubacteriaceae;sf_1;423
Bacteria;Proteobacteria;Alphaproteobacteria;Consistiales;SAR11;sf_2;6933
Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;sf_14;867
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Actinomycetaceae;sf_1;1672
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;sf_1;2002
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;sf_5;2738
Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;sf_1;3560
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococc/Acidaminococc;sf_11;59
Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae;sf_1;3891
Bacteria;Firmicutes;Bacilli;Lactobacillales;Aerococcaceae;sf_1;3870
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;sf_1;3524
Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;sf_1;3439

Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;sf_1;3283
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Micrococcaceae;sf_1;2063
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococc/Acidaminococc;sf_11;940
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococc/Acidaminococc;sf_11;80
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;sf_5;3152
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;sf_1;8802
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae;sf_1;7899
Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Unclassified;sf_1;10550
Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;sf_1;7007
Bacteria;Proteobacteria;Gammaproteobacteria;Methylococcales;Methylococcaceae;sf_1;8435
Bacteria;Bacteroidetes;Unclassified;Unclassified;Unclassified;sf_1;5745
Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Porphyromonadaceae;sf_1;6009
Bacteria;Proteobacteria;Betaproteobacteria;Methylophilales;Methylophilaceae;sf_1;8064
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;sf_1;7882
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;sf_1;7920
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;sf_1;7754
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;sf_1;8016
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;sf_1;8564
Bacteria;Proteobacteria;Gammaproteobacteria;Unclassified;Unclassified;sf_3;8967
Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;sf_1;7011
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;5559
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;6077
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;5847
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;5301
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;6089
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;6017
Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;sf_1;7215
Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;sf_1;6771
Bacteria;Proteobacteria;Alphaproteobacteria;Bradyrhizobiales;Unclassified;sf_1;7255
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;sf_1;5636
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Crenotrichaceae;sf_11;5872
Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;sf_1;7753
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;sf_1;7820
Bacteria;Proteobacteria;Betaproteobacteria;MND1 clone group;Unclassified;sf_1;8134
Bacteria;Proteobacteria;Betaproteobacteria;Methylophilales;Methylophilaceae;sf_1;8159
Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;sf_1;8054
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Ralstoniaceae;sf_1;7661
Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Rikenellaceae;sf_5;6278
Bacteria;Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;sf_4;10243
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;sf_1;9695
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococc/Acidaminococc;sf_11;264
Bacteria;OP8;Unclassified;Unclassified;Unclassified;sf_3;857
Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;sf_1;10032
Bacteria;Proteobacteria;Alphaproteobacteria;Unclassified;Unclassified;sf_6;7166
Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Rikenellaceae;sf_5;5865
Bacteria;

Table 3. *List of diversity in Figure 4-2.*

Archaea;Crenarchaeota;C1;C1a;Unclassified;sf_1;2587
Archaea;Crenarchaeota;Thermoprotei;Cenarchaeales;Cenarchaeaceae;sf_1;2593
Archaea;Crenarchaeota;C1;C1a;Unclassified;sf_1;2621
Archaea;Crenarchaeota;C1;C1b;Unclassified;sf_1;2589
Archaea;Crenarchaeota;C1;C1b;Unclassified;sf_1;2591
Archaea;Crenarchaeota;C1;C1b;Unclassified;sf_1;2625
Archaea;Euryarchaeota;Methanomicrobia;ANME−1;Unclassified;sf_1;2149
Archaea;Crenarchaeota;Thermoprotei;Unclassified;Unclassified;sf_7;2582
Archaea;Crenarchaeota;C1;C1a;Unclassified;sf_1;2594
Archaea;Crenarchaeota;C1;C1a;Unclassified;sf_1;2585
Archaea;Crenarchaeota;C1;C1a;Unclassified;sf_1;2586
Archaea;Crenarchaeota;C1;C1a;Unclassified;sf_1;2588
Archaea;Euryarchaeota;SAGMEG−1;Unclassified;Unclassified;sf_1;2098
Archaea;Crenarchaeota;C1;C1a;Unclassified;sf_1;2583
Archaea;Euryarchaeota;Halobacteria;Unclassified;Unclassified;sf_4;2081
Archaea;Euryarchaeota;Thermoplasmata;Thermoplasmatales;Cplasma;sf_1;2198
Archaea;Euryarchaeota;Thermoplasmata;Thermoplasmatales;Unclassified;sf_1;2265
Archaea;Euryarchaeota;Thermococci;Thermococcales;Thermococcaceae;sf_1;2240
Archaea;Euryarchaeota;Thermoplasmata;Unclassified;Unclassified;sf_1;2087
Archaea;Euryarchaeota;Thermoplasmata;Unclassified;Unclassified;sf_1;2303
Archaea;Euryarchaeota;Thermoplasmata;E2;terrestrial group;sf_1;2253
Archaea;Euryarchaeota;Unclassified;Unclassified;Unclassified;sf_4;2164
Archaea;Euryarchaeota;Thermoplasmata;E2;terrestrial group;sf_2;2204
Archaea;Crenarchaeota;C2;C2;Unclassified;sf_1;2580
Archaea;Crenarchaeota;C1;Unclassified;Unclassified;sf_1;2584
Archaea;Euryarchaeota;Methanobacteria;Methanobacteriales;Methanobacteriaceae;sf_1;2285
Archaea;Euryarchaeota;SAGMEG−1;Unclassified;Unclassified;sf_1;2195
Archaea;Euryarchaeota;Halobacteria;Halobacteriales;Halobacteriaceae;sf_1;2213
Archaea;Euryarchaeota;Halobacteria;Halobacteriales;Halobacteriaceae;sf_1;2086
Archaea;Euryarchaeota;Halobacteria;Halobacteriales;Halobacteriaceae;sf_1;2100
Archaea;Euryarchaeota;Halobacteria;Halobacteriales;Halobacteriaceae;sf_1;2148
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;sf_3;2143
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;sf_3;2127
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Unclassified;sf_1;2215
Archaea;Euryarchaeota;Halobacteria;Halobacteriales;Halobacteriaceae;sf_4;2136
Archaea;Euryarchaeota;Halobacteria;Halobacteriales;Halobacteriaceae;sf_1;2137
Archaea;Crenarchaeota;Thermoprotei;Unclassified;Unclassified;sf_13;2627
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;sf_3;2101
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;sf_3;2223
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;sf_3;2072
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcinaceae;sf_3;2125
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;sf_6;2074
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;sf_6;2129
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;sf_3;2286
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;sf_6;2130
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;sf_6;2153
Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;sf_3;2274
Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Unclassified;sf_1;2201