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Full Length Research Paper

Metagenomic analysis of bacterial diversity of Siloam hot water spring, Limpopo, South Africa

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The bacterial diversity of Siloam hot water spring was determined using 454 pyrosequencing of two 16S rRNA variable regions V1-3 and V4-7. Analysis of the community DNA revealed that the phyla Proteobacteria, Cyanobacteria, Bacteriodetes, Planctomycetes, Firmicutes, Chloroflexi and Verrucomicrobia were the most abundant. The bacterial diversity detectable and classifiable was greater when the V4-7 variable region was used compared to the V1-3 region. The most abundant bacteria genera detected with region V1-3 were; *Stenotrophomonas* (23.3%), *Aquaspirillum* (5.11%), *Zavarzinella* (2.73%), *Haliscomenobacteria* (1.25%), *Rheinheimera* (1.14%) and *Tepidomonas* (1.14%). All the other detectable genera were below 0.6%. Genera detected with region V4-7 from most abundant were; *Stenotrophomonas* (17.96%), *Zavarzinella* (5.81%), *Aquaspirillum* (4.75%), *Rheinheimera* (3.52%), *GPI* (1.41%), *Gemmata* (1.41%) and *Syntrophobacter* (1.06%). All the other genera detected were below 0.7%. Siloam is one of the hottest thermal springs in South Africa (63°C), the water has a pH of 9.5 and is relatively high in fluoride and bromide; it is possible that the physicochemical properties could have some influence on the diversity of bacteria. This article reports on the first phylogenetic analysis of a South African thermal spring bacterial community.

Key words: Thermophilic, hot springs, biodiversity, 454 sequencing, South Africa.

INTRODUCTION

Natural environments harbour a large diversity of micro-organisms, most of which have not yet been identified/or characterised. Despite numerous studies of bacterial communities in different natural habitats, it is an acceptable norm that exploitable microbial diversity is inexhaustive and microorganisms represent the largest reservoir of undescribed biodiversity (Nichols, 2002). Extreme environments form a major source of potentially significant untapped microbial diversity for potential use as resources for biotechnological processes and products. Thermal hot water springs harbour a large diversity of microorganisms (Satyanarayana et al., 2005) and microbial community diversity in hot spring habitats not yet studied, remain largely unknown. Thermal hot springs in South Africa have not yet been studied with respect to microbial diversity. Study of microbial diversity in these areas is necessary for it is known that differrent extreme environments due to different physical and chemical conditions, biogeography and geological history, have different microbial phenotypes (Lau et al., 2009; Narayan, 2008).

Metagenomic studies, a culture independent approach to investigate the composition of mixed microbial communities from environmental niches, allows for the identification of not only culturable but also nonculturable undescribed bacteria (Petrosino et al., 2009; Krause et al., 2006; Chen and Patcher, 2005). Direct access to the genomic DNA of co-existing microbial species can give a

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better understanding of evolution, lifestyle and diversity of the microorganisms and expose more of the hidden world of microbes (Krause et al., 2008). The use of metagenomics combined with post-Sanger sequencing offers the ability to rapidly identify, characterise and understand microbial communities in detail (Liu, 2008; Roesch et al., 2007; Krause et al., 2006; Schloss and Handelsman, 2005). The recently launched GS-FLX-titanium sequencer based on pyrosequencing allows for quick and inexpensive analysis of microbial diversity in different samples in a single run and without the need for cloning (Liu et al., 2008). Literature suggests that post-Sanger sequencing can detect rare minority organisms in a community when compared to approaches such as Sanger sequencing with low depth of coverage that misses these microbes entirely (Krause et al., 2008; Petrosino et al., 2009).

The bacterial diversity as determined by sequecing the hypervariable regions V1-3 and V4-7 of the 16S rRNA belonging to the microbial community and the associated physicochemical conditions of the Siloam hot spring are presented here. Different hyper variable regions have been found to have different efficacies with respect to detecting community members (Sundquist et al., 2007; Petrosino et al., 2009). The two variable regions, V1-3 and V4-7 were amplified and sequenced in order to determine if the two variable regions would give the same phylotypes, and also determine which region gives a more resolved representation of the diversity .The diversity as determined using each variable region and a comparison of the results is given here. The microbial diversity of this hot water spring has not yet been studied and this report presents the first study to describe the bacterial diversity present at Siloam hot spring.

The prevailing bacterial diversity in a habitat can be affected by physical and chemical environmental variables. Temperature is one of the most important factors governing microbial distribution (Abou-Shanab, 2007; Takacs-Vesbach et al., 2008). In this study, physical and chemical parameters at the time of sampling were determined. The study also seeks to relate these to the bacterial members of the community identified.

MATERIALS AND METHODS

Study area and sampling

Siloam hot water spring is located in the Limpopo province of South Africa at 22°54'S; 30°11'E. Water samples for chemical and microbial community studies were collected on the 24th of August 2010. Water quality parameters, namely; temperature, pH, electrical conductivity (EC), total dissolved solid (TDS) and disolved oxygen (DO) were measured *in situ* using the relevant field meters (Mettler Toledo meters, UK). Since a steel pipe has been fitted onto the point where the water comes out of the spring, a pooled water sample was obtained by sampling from the pipe and from the water pool directly below the pipe that receives the continuous outflow of spring hot water . This water was at the same temperature as the water from the pipe outlet. The sample were collected into a sterile 2 L bottles and placed inside a cooler box for transportation to the laboratory for physico-chemical analyses and bacterial diversity studies. Analyses for physical and chemical parameters of the water were conducted by the Institute for Soil, Climate and Water (Agricultural Research Council), Pretoria using standard methods.

DNA extraction

The water samples were concentrated by both filtration for the clear water and centrifugation (7500 rpm for 10 min) for water with the bulk of the biofilm, and subsequently re-suspended in 20 ml of phosphate buffer saline (PBS) (10 mM). Two millilitres of the samples (both filtration and biofilm samples) were centrifuged at 7500 rpm for 10 min to collect the pellet. The pellet was re-suspended in 1 ml PBS (10 mM) as an additional wash step. DNA was extracted with the Genomic DNA Tissue Mini-Prep Kit (Zymo Research), as per protocol; with an additional DNA wash-step. Cell lysis was followed by modern fast-Spin column purification technology. Five microliter of the extracted DNA was run on a 1% agarose gel at 90 V for 30 min to verify the success of the extractions.

PCR amplification

The PCR reaction was performed on the extracted DNA samples using universal degenerate primers 27F.1 and 1492R (De Santis et al., 2007) shown in Table 1. Each PCR reaction contained 5 µl of 10 × Taq buffer, 2 mM $MgCl_2$, 1.5 U Super-Therm DNA Polymerase (Southern Cross), 0.25 mM dNTP's, 0.1 µM of each primer, 1 µl of extracted DNA and Nuclease Free Water (NFW) up to the final reaction volume of 50 µl. The PCR cycle started with an initial denaturation step at 94°C for 10 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min that was then followed by cooling to 4°C. Few microliters of the samples were run on a 1% agarose gel at 90 V for 30 min in order to verify amplification.

The entire PCR reaction was loaded onto a 1% agarose gel and the correct band size (approximately 1500 bp) was excised. The DNA was recovered from the gel slices by using the GeneJET™ gel extraction kit (Fermentas).

The DNA was subsequently re-amplified with two sets of primers in order to amplify two variable regions of the 16S rRNA gene (V1-3 and V4-7). These primers contained the appropriate adaptor and barcode sequences that were necessary for running the samples on the GS-FLX-Titanium (Roche) as set out in Table 1. Each PCR reaction was done as described previously except that the annealing temperature was either 50 or 56°C dependent on the variable region amplified (Table 1).

The entire PCR product was loaded onto a 1% agarose gel and the correct band size (500 to 600 bp) was excised from the gel and subsequently purified as before. The DNA concentrations were quantified by using a Nanodrop spectrophotometer. The samples were pooled at equal concentrations of the filtration and biofilm samples, and V1-3 samples were equal to V4-7 samples. The pooled samples were sequenced on the GS-FLX-Titanium series (Roche) by Inqaba Biotechnology, South Africa. The resulting data were classified using the RDP Naive Bayesian rRNA Classifier Version 2.2, March 2010, RDP training set 6, based on nomenclatural taxonomy and Bergey's Manual with an 80% confidence threshold on the RDP-database. Only sequences that were 50 bp or more were included in the analyses.

Table 1. PCR primers and annealing temperatures used for PCR amplification.

Primer name	Reference (primer used)	Forward (F)/ reverse (R)	Primer sequence	Variable region/ annealing temperature
27F.1	DeSantis et al., 2007	F	5'AGRGTTTGATCMTGGCTCAG 3'	Entire 16S/58°C
1492r	DeSantis et al., 2007	R	5'GGTTACCTTGTTACGACTT 3'	
A1.4	DeSantis et al., 2007 (27F.1)	F	5'**CGTATCGCCTCCCTCGCGCCATCAG**tct* ctatgcgAGRGTTTGATCMTGGCTCAG3'*	V1-3/50°C
B1	Coenye et al., 1999 (pD)	R	5'CTATGCGCCTTGCCAGCCCGCTCAGGT ATTACCGCGGCTGCTG3'*	
A2.4	Dowd et al., 2008 (530F)	F	5'**CGTATCGCCTCCCTCGCGCCATCAG**tga *tacgtctGTGCCAGCMGCNGCGG3'*	V4-7/56°C
B2	Sundquist et al., 2007 (1073R)	R	5'CTATGCGCCTTGCCAGCCCGCTCAGAC GAGCTGACGACARCCATG3'*	

The capital letters in bold indicate the adapter sequences of the primer that is required for pyrosequencing. The lowercase letters indicate the barcode sequences. The capital letters that are not bold indicate the primers that are template specific.

RESULTS

Physico-chemical parameters are known to have a great influence on the thermophilic microbial diversity. Tables 2 and 3 show the physical and chemical characteristics of water collected from Siloam thermal spring**.** The hot spring temperature was 63°C and based on the temperature classification of hot springs (Kent, 1949), Siloam is a scalding hot spring and one with the highest temperature amongst the hot springs described previously in South Africa (Olivier et al., 2010). The Siloam hot springs are alkaline with a pH of 9.5. Bromine, boron and strontium were the most abundant trace elements.

The 16S rRNA pyrosequencing used to identify the bac-terial community at Siloam targeted two DNA regions; V1-3 and V4-7. Table 4 gives a summary of the sequence reads obtained. Although, region V4-7 had less sequences in total, compared to region V1-3, the average read length was longer, thus allowing for better classification.

A total of 880 sequences were identified in waters at Siloam (V1-3 region). The bacterial phylotypes detected are given in Table 5. It can be seen that the phyla Proteobacteria, Bacteroidetes and Planctomycetes were the most abundant, with Proteobacteria constituting 48 and 56% of the total bacteria identified in the V4-7 and V1-3 regions, respectively. A significant number of the sequences; 24.77 and 15.49% for V1-3 and V4-7, respectively, could not be linked to the exsisting database and were recorded as unclassified bacteria.

A total of 23 classes were identified of which 21 was detected with region V1-3 and 20 with region V4-7. 18 classes were shared between region V1-3 and V4-7. The most dominant class of the Proteobacteria at this hot water spring were the γ - Proteobacteria followed by the β-Proteobacteria, α- Proteobacteria and δ- Proteobacteria respectively. The γ – Proteobacteria constituted 29.20 and 29.05%, β – Proteobacteria, 17.05 and 13.20%; α-Proteobacteria 2.27 and 1.23% and δ- Proteobacteria 1.36 and 2.82% of the total Proteobacteria class for regions V1-3 and V4-7, respectively. Other major classes detected for regions V1-3 and V4-7 respectively were Planctomycetacia, 8.30 and 10.74%; Sphingobacteria, 3.18 and 5.63% and Cyanobacteria 0.57 and 5.46%. The bacterial classes detected are shown in Figure 1.

The order Xanthomonadales belonging to the class γ-Proteobacteria of the phylum Proteobacteria was the most abundant followed by the *Planctomycetales, Sphingobacteriales, Neisseriales, Chromatiales, Burkholderiales* and *Rhodocyclales*, all detectable above 1% for both regions. Legionellales, Clostridiales, Myxo-coccales and Syntrophotobacteria were all detectable at above 1% for the V4-7 region. Figure 2 shows the different orders detected.

From the 37 orders, a total of 67 different genera were identified; 43 from V1-3 and 47 from V4-7 the region. Only twenty two genera were detected in both V1-3 and V4-7 regions. These genera are shown in Figure 3. The most abundant bacteria genera detected with region V1-3 were; *Stenotrophomonas,* 23.3%; *Aquaspirillum*, 5.11%, *Zavarzinella,* 2.73%; *Haliscomenobacteria,* 1.25%; *Rheinheimera*, 1.14%; and *Tepidomonas*, 1.14%; all the other detectable genera were below 0.6%. Genera

Table 2. Physical and chemical characteristics of the water at Siloam hot water spring.

Physical/ chemical parameter	Concentration (mg/L)
temperature (°C)	63
pH	9.5
TDS	197.32
Conductivity(mS/m)	39.00
DO (%)	40.0
Cations (mg/L)	
Sodium (Na)	66.24
Potassium (K)	2.82
Calcium (Ca)	1.38
Magnesium (Mg)	13.33
Anions	
Fluoride (F)	6.11
Chloride (Cl)	44.35
Nitrate (NO_3)	0.00
Sulphate (SO_4)	10.44
Phosphate (PO_4)	0.00
Carbonate (CO_3)	14.40
Bicarbonate (HCO_3)	107.36

Table 3. Trace elements composition at Siloam Hot spring.

Element	Concentration (µg/L)	Element	Concentration (µg/L)	Element	Concentration µg/L
Antimony	0.0	Cobalt	0.1	Strontium	20.4
Arsenic	0.3	Copper	0.0	Tellurium	0.0
Barium	4.2	Iodine	1.9	Thallium	0.0
Beryllium	0.0	Lead	0.1	Titanium	6.1
Bismuth	0.0	Manganese	0.8	Tungsten	0.5
Boron	57.9	Mercury	0.5	Uranium	0.0
Bromine	93.1	Molybdenum	2.2	Vanadium	2.3
Cadmium	0.0	Nickel	0.0	Zinc	3.5
Chromium	1.0	Selenium	0.7		

Table 4. Summary of pyrosequencing data from Siloam hot springs.

Sequence	V1-3	V4-7
Number of sequences	880	568
Total length of sequences (bp)	263 930	208 353
Average length of sequences (bp)	300	367

detected with region V4-7 from most abundant were; *Stenotrophomonas* (17.96%), *Zavarzinella* (5.81%), *Aquaspirillum* (4.75%), *Rheinheimera* (3.52%), *GPI* (1.41%), *Gemmata*- (1.41%) and *Syntrophobacter* (1.06%). The other genera detected were all below 0.7%.

DISCUSSION

The total of 43 and 47 genera were detected in the Siloam hot spring using the V1-3 and V4-7 regions, respectively using pyrosequencing. High water

Table 5. Relative abundance and phylogenetic diversity of bacteria in Siloam hot spring.

Phylum detected with V1-3	Abundance as percentage of sequence detected with V1-3	Phylum detected with V4-7	Abundance as percentage of sequence detected with V4-7
OP10	0.23	OP10	0.35
Spirochaetes	0.23	Spirochaetes	0.18
Actinobacteria	0.23	Actinobacteria	0.35
Cyanobacteria	0.57	Cyanobacteria	5.46
Synergistetes	0.11	Synergistetes	0.53
Verrucomicrobia	1.14	Verrucomicrobia	2.46
Deinococcus-Thermus	0.45	Deinococcus-Thermus	0.70
Acidobacteria	0.23	Acidobacteria	0.00
Bacteroidetes	6.93	Bacteroidetes	13.03
Planctomycetes	8.30	Planctomycetes	10.74
Fusobacteria	0.11	Fusobacteria	0.00
Chloroflexi	0.91	Chloroflexi	0.88
Firmicutes	1.25	Firmicutes	1.58
Proteobacteria	54.43	Proteobacteria	48.06
Unclassified Bacteria	24.77	Unclassified Bacteria	15.49
Unclassified	0.11	Unclassified	0.18

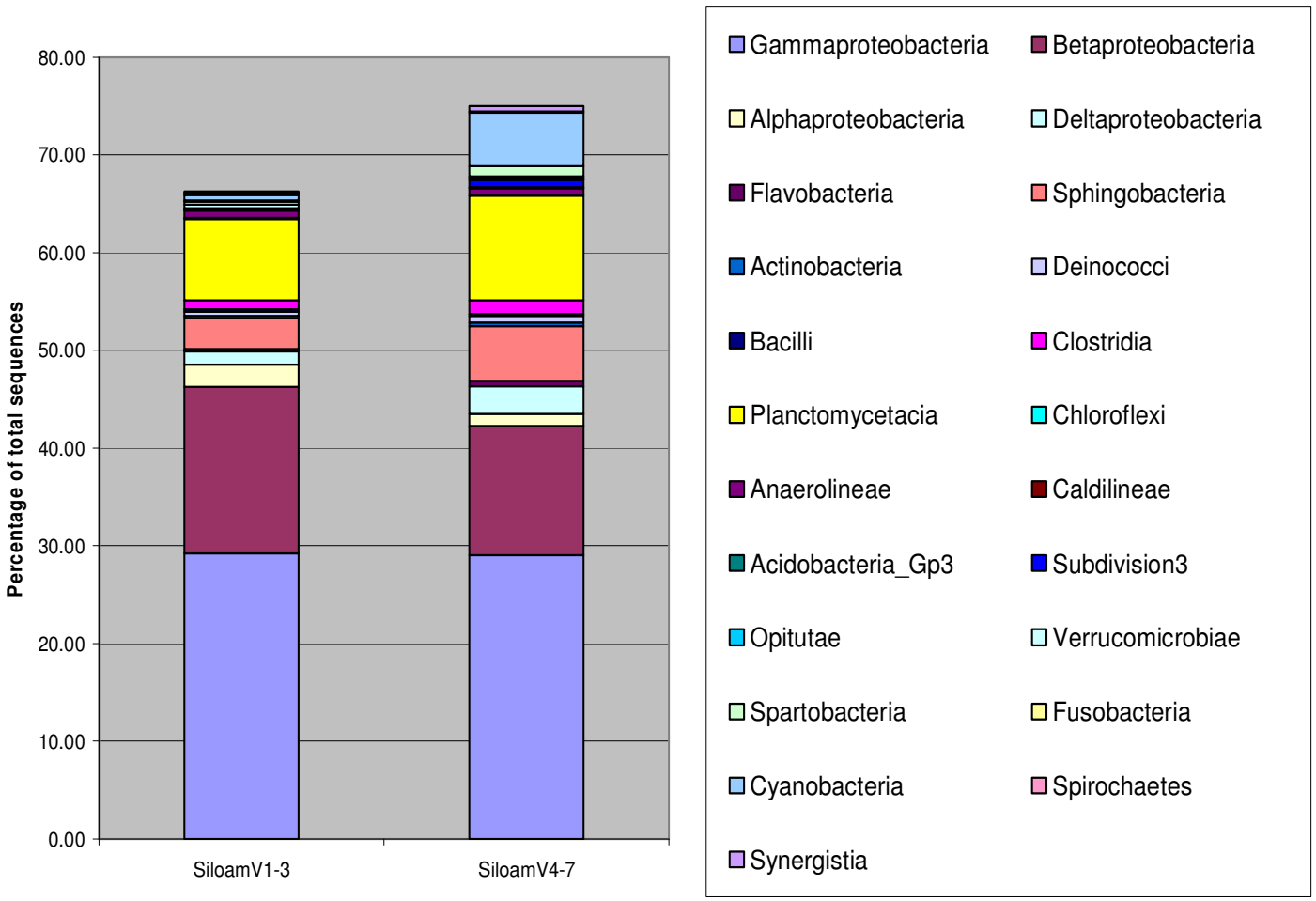

Figure 1. Microbial classes' composition detected in Siloam hot spring sequencing of the variable regions V1-3 and V4-7 of the 16S rRNA genes.

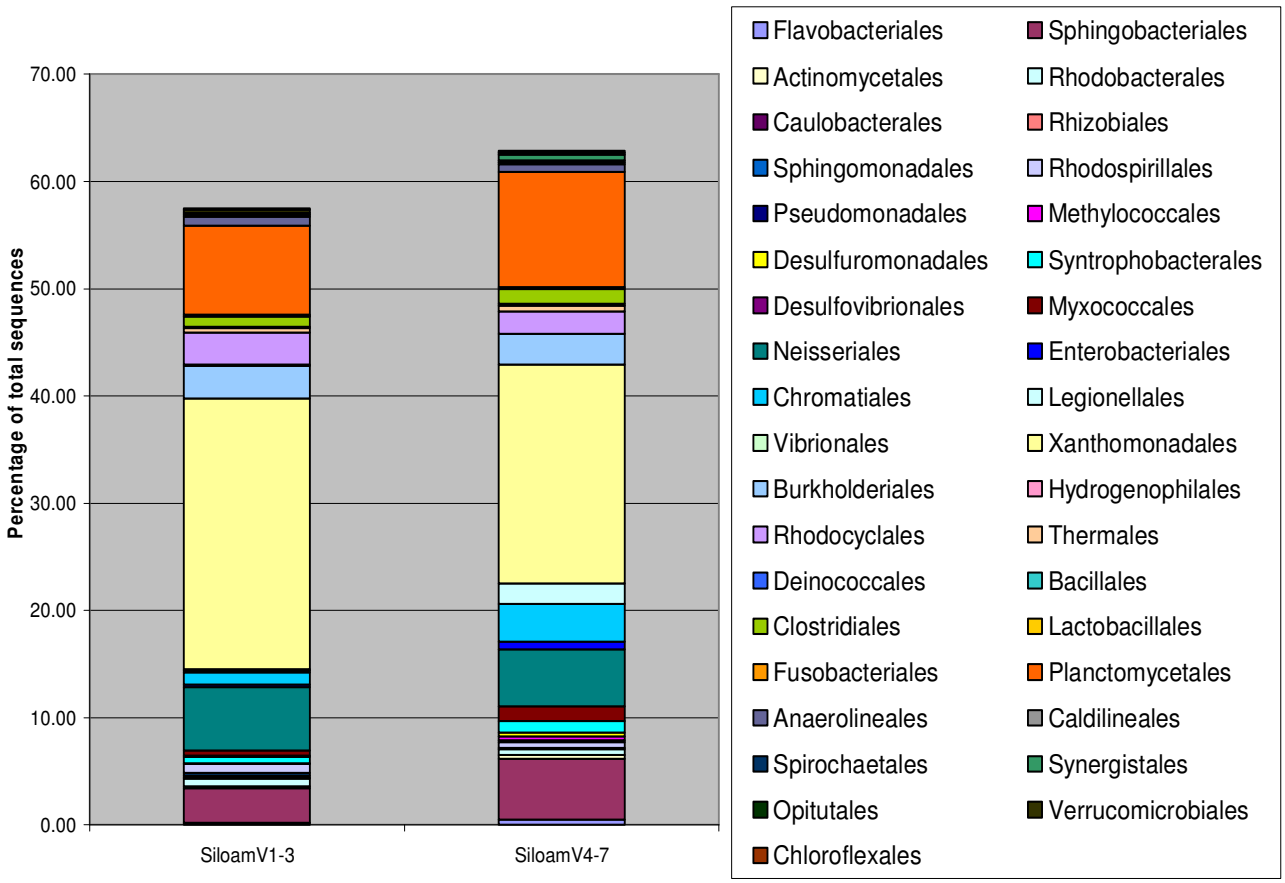

Figure 2. Microbial orders composition detected in Siloam hot spring with sequencing of the variable regions V1-3 and V4-7 of the 16S rRNA genes.

temperature exerts stress on microbial species that only specific species can tolerate and survive (Abou-Shanab, 2007). The most dominant phylotypes were found to be Proteobacteria, Planctomycetes, Bacteriodetes and Cyanobacteria which are widely distributed in both terrrestial and aquatic environments. This indicates that the hot water spring could harbour microorganisms of terrestrial as well as aquatic origin and bacteria known to be either mesophilic or thermophilic inhabitants. However, no attempt was made in this study to characterise the bacteria with respect to their thermophilic status. Groups important to a particular environment are generally known to be enriched and also be in correlation to the hydro-geochemistry of the area (Edwards et al., 2006; Meyer-Dombard et al., 2005; Whitakker et al., 2003). The chemical analysis of the thermal spring water indicates geochemical anomalies with respect to fluoride and bromide concentrations.

The most abundant genera, *Stenotrophomonas, Zavarzinella*, and A*quaspirillum*, are not generally classified as thermophiles. However, specific species from genera such as *Thiobacillus* and *Meiothermus* have been found in thermal springs elsewhere (Pires et al., 2005; Chen et al., 2002). Although, the dominant groups are more emphasised, it is worth noting that the micro-organisms found in low abundance in rare or extreme environment are important individuals of the community that could constitute an unexplored reservoir of genomic variation and novelty (Sogin et al., 2006).

The challenge usually encountered with analysis post-Sanger sequencing results includes accurate assignment of each read to be able to describe the composition of microbes in a community. Accurate taxonomic assign-ment is dependent on the region of the 16S rRNA gene that is targeted during sequencing (Liu et al., 2008). Different hyper variable regions have been found to have different efficacies with respect to identification of the bacterium (Sundquist et al., 2007; Petrosino et al., 2009). The primers used in this study (Table 1) amplified two variable regions (V1-3 and V4-7) and were used in order to determine if the two variable regions would give the same phylotypes or resolved classification. In this study, the V4-7 and the V1-3 hypervariable regions had similar and overlapping efficacies; the V4-7 region was more effective in detecting genera which could not be detected and classified by the V1-3 region. A significant number of

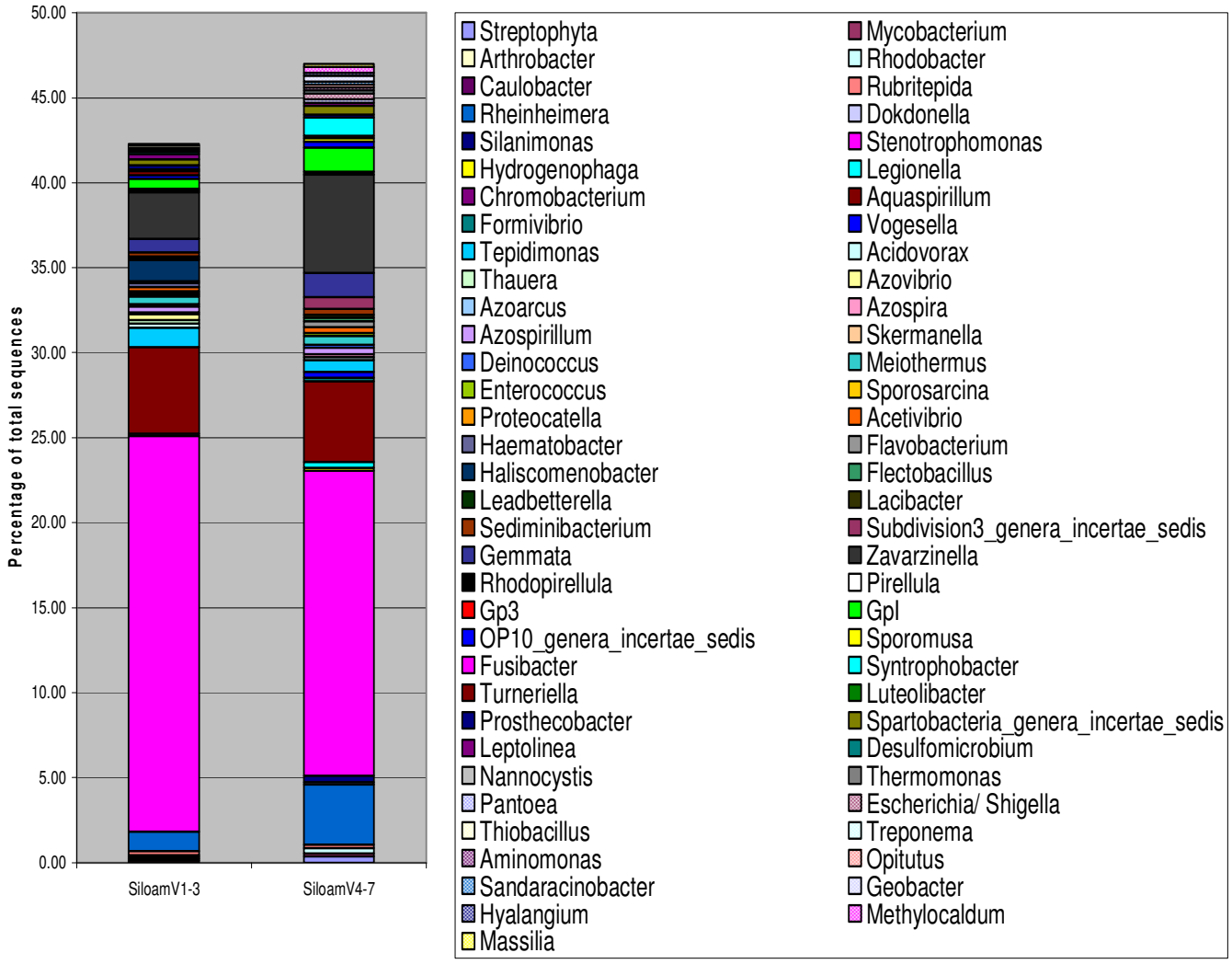

Figure 3. Compostion of the genera detected in Siloam hot spring with sequencing of the variable regions V1-3 and V4-7 of the 16S rRNA genes.

the sequences could not be assigned to any phyla, giving 24.77 % and 15.49 % for V1-3 and V4-7 respectively and were grouped as unclassified bacteria. This group might represent bacteria that have not yet been classified or detected before. Contamination of the hot water spring by normal surface water, soil, and spores cannot be excluded, but it can, however, be concluded that the bacterial phylotypes detected can possibly all proliferate in this thermophilic environment. This study shows that a considerable diversity of microbial groups can be revealed by metagenomics using post-Sanger sequencing and that insight into the microbial genetic diversity, community composition, distribution and abundance increases steadily with each new survey. Here, we described the bacterial community diversity at Siloam hot water spring in South Africa. The results report in this article constitute the first regarding the bacterial diversity of thermal springs in South Africa.

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