Microbial colonisation in diverse surface soil types in Surtsey and diversity analysis of its subsurface microbiota

V. Marteinsson[1, 3], A. Klonowski[1], E. Reynisson[1], P. Vannier[1], B.D. Sigurdsson[3] and M. Ólafsson[2,]

[1] Matís, Food safety, Environment & Genetics, Vínlandsleið 12, IS-113 Reykjavík, Iceland

[2] ÍSOR, Gensásvegi 9, 108 Reykjavík, Iceland

[3] Agricultural University of Iceland, Hvanneyri, IS-311 Borgarnes, Iceland

Correspondence to: V. Marteinsson (viggo@matis.is)

Abstract

Colonisation of life on Surtsey has been observed systematically since the formation of the island 50 years ago. Although the first colonisers were prokaryotes, such as bacteria and blue-green algae, most studies have been focused on settlement of plants and animals but less on microbial succession. To explore microbial colonization in diverse soils and the influence of associate vegetation and birds on numbers of environmental bacteria, we collected 45 samples from different soils types on the surface of the island. Total viable bacterial counts were performed with plate count at 22°, 30° and 37°C for all soils samples and the amount of organic matter and nitrogen (N) was measured. Selected samples were also tested for coliforms, faecal coliforms aerobic and anaerobic bacteria. The subsurface biosphere was investigated by collecting liquid subsurface samples from a 182 meters borehole with a special sampler. Diversity analysis of uncultivated biota in samples was performed by 16S rRNA gene sequences analysis and cultivation. Correlation was observed between nutrient deficits and the number of microorganisms in surface soils samples. The lowest number of bacteria ($1x10^4$-$1x10^5$/g) was detected in almost pure pumice but the count was significant higher ($1x10^6$-$1x10^9$/g) in vegetated soil or pumice with bird droppings. The number of faecal bacteria correlated also to the total number of bacteria and type of soil. Bacteria belonging to *Enterobacteriaceae* were only detected in vegetated and samples containing bird droppings. The human pathogens *Salmonella*, *Campylobacter* and *Listeria* were not in any sample. Both thermophilic bacteria and archaea 16S rDNA sequences were found in the subsurface samples

collected at 145 m and 172 m depth at 80°C and 54°C, respectively, but no growth was observed in enrichments. The microbiota sequences generally showed low affiliation to any known 16S rRNA gene sequences.

1 Introduction

Microorganisms are typically in a great abundance and high diversity in common soil and their integrated activity drives nutrient cycling on the ecosystem scale. Organic matter (OM) inputs from plant production support microbial heterotrophic soil microbial communities that drive also processes that make nutrients available in the system. This, in turn, supports plant primary productivity and basic food webs on the ground and in the subsurface (Fenchel et al., 2012; Roesch et al., 2007; Schlesinger, 1997; Whitman et al., 1998). Moreover, as soil develops, soil geochemistry and OM availability changes (Vitousek and Farrington, 1997) due to mineral-OM interactions and geochemical constraints on biological activity (Kleber et al., 2007; Sinsabaugh et al., 2008). Nutrient limitations can constrain plant and food web development, thus shaping the rate of succession of plant and animal life within the ecosystem (Odum, 1969; Walker and del Moral, 2003).

Subsequent of a volcanic eruption, lava flow and ash deposition, new surfaces are created where both organismal growth and weathering processes are effectively reset. Microbial cells colonizing new volcanic deposits must be successful in either growing autotrophically, by fixing C and N using light or inorganic energy sources for growth, e.g. *Cyanobacteria* and sulfate-reducing bacteria (Edwards et al., 2003; Ernst, 1908; Konhauser et al., 2002) or using carbon monoxide as a C and energy source (Dunfield and King, 2004; King and Weber, 2008) or by growing heterotrophically using trace amounts of organic carbon (Cockell et al., 2009; Wu et al., 2007). Studies on the microbiota of volcanic terrains have only emerged within the past few years, revealing that such habitats are capable of harbouring significant microbial diversity, despite their extreme nature (Gomez-Alvarez et al., 2007; Kelly et al., 2010). However, completely isolated volcanic terrains, such as islands, are extremely rare. One of few such places is Surtsey, a neo-volcanic island, created by a series of volcanic eruption that started in 1963 and ended in 1967 (Þórarinsson, 1967, 1968; Þórarinsson, 1965). The island of Surtsey provides a unique laboratory for the investigation of biological establishment and succession on relatively newly deposited volcanic substrata, on the surface and in the subsurface. The first reports of living forms in Surtsey were from 1964 - 1966 (Brock, 1966; Friðriksson, 1965), when the first cyanobacteria were observed, even before the end of the

eruption. Phototrophs were further investigated in 1968 (Schwabe, 1970) and with subsequent investigations in following years (Brock, 1973; Schwabe and Behre, 1972). However, despite such remarkable habitat, very little research on the microbiology has been performed since the first years of the island formation despite frequent research expeditions and the most recent report on microbes in Surtsey is only from the end of last century (Frederiksen et al., 2000). Besides, no reports or data exist on heterotrophic growth or distribution of such bacteria in the surface soils of the island and nothing is known about distribution of faecal bacteria or pathogens possibly brought by bird inputs of organic matter, such as faeces. Additionally, even less is known about the island subsurface life, but such life is well known in subseafloor sediments and within the deep biosphere where high number of microbes are present and active (Kallmeyer et al., 2012).

The overall aim of this study was to explore microbial colonization in different surface soil types and in the subsurface below 160 m depth in a drill hole in Surtsey. That was done by obtaining viable count and distribution of heterotrophic microbes on the island surface and by obtaining the correlation of nutrients and other environmental measurements to different soil types and determine how that affects microbial communities in Surtsey. By investigating presence, survival and possible dissemination routes of pathogenic bacteria into such remote environments and by investigating the existence and diversity of subsurface microbial biosphere and their possible dissemination routes.

2 Material and methods

2.1 Surface sampling and study sites

Samples were collected during a sample expedition at Surtsey in July 2009. About 1.0 cm thick layer was retrieved inside a frame of 20 x 20 cm ($0.04 m^2$) with spoon that was washed with 70% ethanol between samples. Samples were collected into sterile plastic box and stored outside in the shade and then at 4°C when arrival to the laboratory in Reykjavík until they were processed. Samples were retrieved all around the island and the GPS location of each sample taken. The samples can be divided into three types of samples, SS samples (sand or pumice with bird droppings), SJ samples (pure sand or pumice) and SR samples (vegetated, partly vegetated and none vegetated area) that were taken at permanent study plots in Surtsey (Magnusson et al., 2014).

2.1.1 Media and cultural conditions

The basic methodology used at the Laboratory for media and culturing were NMKL methods (Nordisk Metodikkomité for Næringsmidler) and methods from the Compendium of Methods for the Microbiological Examination of Foods published by the American Public Health Association (APHA-2001). About 25g of each surface samples was weighed and 225ml Peptone water was mixed in before using a stomacher for blending the soil for 1 min. The supernatant of each sample was used and analysed with different methods.

2.1.2 Total viable count of environmental bacteria

The conventional "pour-plate" method was used on Plate Count Agar. Briefly, 1 ml of homogenate sample was used with 20 ml melted and cooled culture medium. Incubation temperatures was at 22°C for 72 hours and 30°C for xx days in aerobic an anaerobic conditions (FDA, 2001, chapter 3 (pour plate), NMKL 86, 4th ed., 2006, NMKL 74, 3rd ed., 2000). Total viable count was also estimated by filtering 0.1, 1, 10 and 100 ml samples through a sterile 0.22 μm cellulose membrane filter (Millipore Corporation, MA, USA) to capture microbial cells and placed onto Reasoner's 2A (R2A) agar (Difco, Kansas, USA) and incubated in 22°C for 4-5 days for evaluation of total viable count. For better results, 20 ml of sterile phosphate buffer (FB) was used with the 0.1 and 1 ml samples to increase the volume filtered, allowing better dispersion of cells to be grown on the filter paper. All 44 surface samples were tested with these methods and the results expressed as cfu/g.

2.1.3 Total coliforms, faecal coliforms and *Escherichia coli*.

A reference method based on most probable number (MPN) from NMKL (NMKL 96, 4th ed., 2009, Compendium 4th ed., 2001, chapter 8 (8.71, 8.72, 8.81) was used to estimate total coliforms, faecal coliforms and *Escherichia coli*. Pre-enrichment was in LST broth (37°C for 48 hours) and confirmation tests were done in BGLB broth for total coliforms (37°C for 48 hours) and in EC broth for faecal coliforms (44°C for 24 hours). *Escherichia coli* was confirmed by the testing of indol production. The expression of results are in cfu/g.

2.1.4 Total viable count of *Enterobacteriaceae.*

A reference method from NMKL (Reference: NMKL 144, 3rd ed., 2005.) was used to estimate total *Enterobacteriaceae* in all 44 surface samples. The medium Violet Red Bile

Glucose Agar (VRBGA) was used (pour-plate method with overlay). Plates were incubated for 24 hours at 37°C and typical colonies counted. Oxidase test was used for confirmation. The expression of results is cfu/g.

2.1.5 Detection of pathogens

Reference methods from NMKL was used to estimate total number of pathogens or for *Salmonella*, *Campylobacter* and *Listeria*. The following NMKL method (Reference: NMKL 71, 5th ed., 1999, ISO 6579:2002, Wellcolex- serogroup identification) was used for Salmonella detection. Briefly, pre-enrichment was in BPW broth (37°C for 24 hours), 25 g into 225 of enrichment broth. Second enrichment was in RV broth (41.5°C for 24 hours) and tetrathionate broth (41.5°C for 24 hours). Broths from these enrichments were streaked onto two solid media: XLD and BG (37°C for 24 hours). Typical colonies (2-4 or as needed) were inoculated into TSI- and LI-agarslants (37°C for 24 hours). Confirmation was done by testing for flagellar (H) and somatic (O) antigens. The expression of results was pos/neg in 25 g and 17 selected surface samples were tested.

The following NMKL method (NMKL 119, 3rd ed., 2007) was used for *Campylobacter jejuni/coli* detection. Briefly, enrichment was in Bolton broth, 11 g of sample into 100 ml of enrichment broth. (41.5°C for 48 hours). Broths from these enrichments were streaked onto mCCDA agar and incubated at 41.5°C for 48 hours in an anaerobic jar with microaerobic atmosphere. The expression of results is pos/neg in 11 g and 17 selected surface samples were tested (NMKL 119, 3rd ed., 2007).

The following NMKL method (Reference: NMKL 136, 5th ed., 2010) was used for *Listeria monocytogenes* detection Briefly, pre-enrichment in *Listeria* broth, 25 g into 225 ml of enrichment broth (30°C for 24 hours). Then further inoculation was in Fraser broth (37°C for up to 48 hours). Both primary and secondary enrichment cultures were streaked onto Oxford and OCLA agar (37°C for 24 and 48 hours). Confirmation tests were done on 5 colonies from each plate and include Gram-staining, catalase and motility. Species identification includes haemolysis on Blood agar and testing on API Listeria (System for the identification of *Listeria*, bioMérieux SA/France). The expression of results was pos/neg in 25 g and 17 selected surface samples were tested.

2.1.6 Soil geochemistry, chemical and soil moisture analysis

The total amount of nitrogen (totN%) was measured on a nitrogen analyzer (2400 Kjeltec Analyzer unit Foss Tecator). About 3 g of soil was analyzed at 420°C for 2.5 hours according to the method ISO 5983-2:2005. The total amount of carbon (totC%) was calculated from loss on ignition after heating at 550°C for 4 hours, assuming the organic matter contained 50% carbon according to the method ISO 5984-2002 (E). Soil gravimetric water content (GWC) was measured as the mass lost from soil after drying 5 g soil for 24 hours at 103°C

2.1.7 CO_2 flux measurements

The measurement of Net Ecosystem Exchange (NEE, μmol CO_2 m^{-2} s^{-1}) and ecosystem Respiration (Re, μmol CO_2 m^{-2} s^{-1}) were performed as described by Sigurdsson and Magnusson (2010) on top of microbial samples market as SR-samples that were collected from permanent vegetation survey plots, which are 10×10 m in area (Magnusson et al., 2014; Sigurdsson and Magnusson, 2010). Briefly, an EGM-4 infrared gas analyzer and a CPY-2 transparent respiration chamber (PP Systems, UK) were first used to measure NEE in light, which was measured as Photosynthetically Active Radiation (PAR) inside the chamber. Then Re was subsequently measured in dark by covering the chamber. The total gross ecosystem CO_2 uptake rate (GPP), was then calculated by the difference between Re and NEE. Vegetation surface cover (Cov., %) was also recorded under the flux chamber at each measurement point and soil temperature was recorded at 5 and 10 cm depth (Ts05 and Ts10, °C) with a temperature probe placed adjacent to the respiration chamber.

2.1.8 Multivariate analysis of measured parameters

Multivariate analysis was performed on the environmental parameters collected in order to visualise environmental similarities between sample sites. The parameters were: temperature, total carbon, total nitrogen, water content, total microbial count on PCA and counts of *Enterobacteriaceae*. Samples containing missing values were excluded in the analysis except in in six occasions were total nitrogen values were not available. In these cases the values were estimated based on other similar samples in the dataset. The other option would have been to exclude those samples from the analysis. Data were normalised with ln (x+0.1) or ln (x+1), latter for bacterial counts and standardised with (x-mean)/stdev. Non-metric multidimensional scaling (NMDS) using Euclidean similarity measures were performed (Ramette, 2007) using the environmental statistical analysis program PAST.

2.2 Subsurface sampling

2.2.1 Sampling and temperature data

The subsurface was sampled through continuously cored drill hole SE-1 (Moore, 1982; Ólafsson and Jakobsson, 2009). The temperature was measured along the drill hole at 1 m interval from the surface down to the bottom at 180 m with a borehole temperature meter. A temperature logger (DST milli-PU logger from StarOddi, Reykjavík, Iceland, was placed for approximately 21 hours at 168 m depth in the borehole and the temperature was recorded every 15 minutes with SeaStar software. Samples were collected in a "homemade" downhole water sampler made of stainless steel. The total capacity of the sampler is about 1.3 L that was kept open (flow through) to the sampling depth and closed with a messenger. Contamination of samples were avoided washing the sampler with several equivalent volumes of 70% ethanol before operation. Samples SB1, 2, 4, 5 and SB6 were retrieved from 57, 58, 145, 168 and 170 m respectively. Samples SB4, 5 and 6 were sampled below the see level (58 m). Samples were reduced by Na_2S solution (0.05% w/v final concentration) and kept under anaerobic conditions at low temperature during the field trip and at 4°C in the laboratory.

2.2.2 Enrichment cultures of subsurface samples

Media for enrichments of chemolithotrophic and chemoorganotrophic organisms were prepared by using 0.5 ml sample and 4.5 ml 0.2 μm-filtered water from the subsurface sample. Cultures were incubated under aerobic (ambient headspace) and anaerobic conditions at 40°C, 60°C and 80°C. Each enrichment was prepared in Hungate culture tubes with 0.01% yeast extract, vitamins solution, Balch element solution (Balch et al., 1979), S^0 and resazurin and incubated under pure N_2 and 0.025% final wt/v $Na_2S \bullet 9H_2O$, same but aerobically with ambient headspace and incubation with 80%/20% H_2/CO_2 and 0.025% final wt/v $Na_2S \bullet 9H_2O$ Additional enrichments used R_2A medium and 162 *Thermus* medium (Degryse et al., 1978), both aerobically with ambient headspace; and *Thermotoga* ("Toga") medium (Marteinsson et al., 1997) and YPS medium (Marteinsson et al., 2001a) under pure N_2 headspace. Growth in enrichments was examined with phase-contrast microscopy (Olympus BX51).

2.2.3 DNA Extraction and PCR reactions subsurface samples

To capture microbial cells for DNA extraction and analysis, 250 ml of sample was filtered through a 47 mm, 0.22 μm-pore size cellulose membrane filter (Millipore Corporation, Bedford, MA, USA) in our laboratory in Reykjavik. Isolation of chromosomal DNA

extraction from and obtained biomass from filter was performed as described by Marteinsson et al., 2001a.

2.2.4 Clone library construction and sequencing

PCR amplification was performed according to the protocol in Skírnisdóttir *et al.* (2001) with primers 9F (`5-GAGTTTGATCCTGGCTCAG-3`) and 805R (`5-GACTACCAGGGTATCTAATCC-3`) (Skírnisdóttir et al., 2001). PCR product was cloned by the TA method using a TOPO TA cloning kit (Invitrogen). Plasmid DNA from single colonies was isolated and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI sequencer (PE Applied Biosystems). Clones were sequenced using the reverse primer 805R. Cloned sequences were analysed and edited by using the program Sequencer 4.8 from ABI. A total of 41 clones sequences were grouped into operational taxonomic units (OTUs) at a threshold of 98% sequence identity and then aligned by using ClustalW within the MEGA package, version 5.1 (Thompson et al., 1994). In order to check for species identification, sequences were searched against those deposited in GenBank, through the NCBI BLAST (Altschul et al., 1990). Neighbor-joining phylogenetic trees were constructed with MEGA 5.1 (Tamura et al., 2011) using a representative sequence from each OTU and related GenBank sequences.

2.2.5 Pyrosequencing and analysis

Two sets of reactions targeting the v4-v6 regions of the archeal 16S rRNA gene were performed using the VAMPS primers (Sogin et al., 2006). First, pyrosequencing of short reads, 70-100 nt of the archeal v6 variable region (primers 958F and 1048R; ´5-AATTGGANTCAACGCCGG-3` and ´5-CGRCGGCCATGCACCWC-3`) in the 16S ribosomal gene was performed with a 454 GS-FLX (Roche) on sample SB4. Cycling conditions included an initial denaturation at 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 57-60°C for 45 seconds, and 72°C for 1 minute; and a final extension at 72°C for 2 minutes. Tags shorter than 60 nt were discarded when trimmed by the GS FLX software. Second, a 454 GS-FLX with Titanium chemistry on samples SB-4, SB-5 and SB-6 for longer reads using v4-v6 Vamps primers (5´YCTACGGRNGGCWGCAG-3´and 5´-CGACRRCCATGCANCACCT-3´). Titanium adaptors A and B were attached to the forward and reverse primers respectively along with multiplex identifier (MID) adaptors recommended by Roche to be used in the FLX pyrosequencing. The PCR was performed in a 25 uL reaction volume using FastStart High Fidelity polymerase system (Roche, Madison,

WI). The PCR program was as follows: 94°C 10 min, 35 cycles of 94°C for 40 s, 52°C for 40 s and 72°C for 60 s and a final extension step at 72°C for 7 min. After the recovery of a PCR product from the DNA the rest of the workflow prior to sequencing was done according to manufacturer instructions for FLX amplicon sequencing using the GS Titanium SV emPCR Lib-A kit (Roche, Madison, WI). With both short and long amplicons, the raw sequences were filtered, trimmed and processed through the Qiime pipeline using the Greenegene database (version 12.1). The first steps included various quality processing including filtering sequences which were under 200 bp and over 1000 bp, contained incorrect primer sequences (>1 mismatch) and removal of chimera using Decipher (Wright et al., 2011). Sequences were assigned to samples through the MID sequences and clustered into Operational Taxonomic Units based on 97% similarity in the 16S rRNA sequences using Uclust and then assigned phylogenetic taxonomy through RDP classifier. OTU sequences where then aligned with PyNast.

3 Results

3.1 Surface sampling and study sites

At total 44 surface samples were collected around the island. An overview of the sampling site is shown in Figure 1. Most of the samples were collected on the South site of the island were the soil was highly variable ranging from sand to completely vegetated environment with significant interactive effects of bird association including nesting seabirds.

3.1.1 Viable count of total environmental bacteria and *Enterobacteriaceae.*

A good visual correlation was found between total bacterial counts with plate count agar method and growth on R2A media from all samples incubated at 22°C (Figure 3). Positive relationship was also observed between the reduced vegetation or nutrient deficits soils and the number of microorganisms in the samples. The lowest number of bacteria ($1x10^4$-$1x10^5$/g) was detected in almost pure sand or pumice but the count was significantly higher ($1x10^6$-$1x10^9$/g) in vegetated soil, sand or pumice with bird drop (Figure 3). The number or detection of *Enterobacteriaceae* in the soil samples showed similar correlation to the viable count and no growth was observed in samples with low numbers of bacteria or < $1x10^6$cfu/g except in one sample (SS10).

3.1.2 Counts of total coliforms, faecal coliforms *Escherichia coli*, aerobic and anaerobic bacteria growing at 30°C and pathogen detection.

A total of 12 soil samples that showed significantly high numbers of environmental bacteria or > $1x10^6$cfu/g were selected for further testing of viable count of: total coliforms, faecal coliforms, *Escherichia coli*, aerobic and anaerobic bacteria growing at 30°C and detection of pathogens. Additionally samples containing various soil types and with low viable count of total environmental bacteria < $1x10^6$ cfu/g were also tested as controls. The results are summarized in Table 1. *Listeria*, *Campylobacter* or *Salmonella* were not detected in any of the selected samples.

3.1.3 Soil environment and biogeochemical variables

Soil nitrogen, carbon and moisture measurements were performed for all samples with sufficient soil quantity for analysis. Measurements of total nitrogen, carbon and water content was performed in 37 samples except in 6 samples that lacks totN% measurements. Seven samples could not be measured (Table 1). Average totN% measurements were similar in SJ and SR samples, 0.01 and 0.02 respectively but SS samples containing bird droppings was at least 60 times higher or 0.68. Average totC% was also highest in SS samples or 4.68 and SJ and SR samples were 1.17 and 2.74 respectively. Average water content in SJ, SR and SS samples were 0.34, 0.91 and 0.61 respectively.

3.1.4 Multivariate analysis of environmental parameters

In order to capture the niche similarities between sampling sites multivariate NMDS analysis was performed based on measurements environmental parameters. The analysis showed that the SS samples are separated from other samples while the SR and SJ samples overlap. Samples SR15-17 are well separated from all other samples which is due to it higher load of *Enterobacteriaceae*, total viable counts and higher water content compared to other sampling sites (Figure 4). For selected samples, more environmental data was recorded (NEE, Re., GPP, PAR, Ts05, Ts10, Cov.) and was used as a base for another sub-NMDS analysis which confirmed previous analysis and clustered the most vegetated samples together (data not shown). Table 2 shows the CO" flux measurements. Other SR samples were gathered together except for SR-3 which is also in a great distance geographically from the other SR samples. Subsurface sampling

3.1.5 Sampling and temperature data

The temperature was measured along the drill hole at 1 m interval from the surface down to the bottom at 178 m with a borehole temperature meter. The temperature measurements are showed in figure 5a in correlation to the depth in the drill hole. The maximum temperature was 130°C at 95 m depth and the bottom temperature was 40°C at 178 m depth. The temperature was 54.8±0.1°C at 168 m depth and remained stable over 21 (h) measurements (Figure 5b). About 250 ml were sampled at every depth, 57 m (SB-1) and 58 m depth (SB-2), both samples at 100°C, at 145 m depth (SB-4) at 80°C, at 168 m (SB-5) and 170 m depth (SB-6) both samples at 54-55°C. The pH was little above 8.0 in the samples and the salinity was above sea salinity or around 3.7%.

3.1.6 Enrichment cultures of subsurface samples

No growth could be observed after about 6 weeks of incubation in any of the enrichments incubated at 40°C, 60°C and 80°C.

3.1.7 DNA extraction, PCR reactions and clone library construction

Very small pellets of unsure biomass was obtained from all SB samples and DNA concentration was extremely low. PCR amplification products were achieved from SB-4, SB-5 and SB-6 with both universal bacterial and archaeal primers. Libraries construction was successful with clones containing bacterial 16S rRNA genes that were amplified in samples SB-5 and SB-6 and with archaeal genes in sample SB-6.

3.1.8 Subsurface diversity analysis, clonal and next generation sequencing

Three approaches were used assess the bacterial and archeal taxa composition in the samples; partial sequencing of cloned 16S rRNA fragments, pyrosequencing of short fragment of the v6 region and pyrosequencing of a longer fragment of v4-v6 region. Clone libraries of the 40 archaeal 16S rRNA genes (500 bp) in sample SB-6 showed high homology (99%) to uncultured subsurface archaeon related sequences (Genbank accession DQ354739.1) from subsurface water of the Kalahari Shield, South Africa by BLAST method. All the clones were dominated by this one sequence except two clones which showed high homology to uncultured subsurface archaeon related sequences (DQ988142) (AB301979.1), from methane cycling in subsurface marine sediments and from in hydrothermal sediments at the Yonaguni Knoll IV hydrothermal field in the Southern Okinawa trough, respectively. Clone libraries of the bacterial 16S rRNA genes in sample SB-5 and SB-6 and their closest known relatives are

presented in a neighbor-joining trees of sequences which is summarized in figure 6. The SB-4 v6 library consists mostly or 94.5% of a single taxon affiliated with genus *Archeoglobus* from the Phylum *Euryarchaeota* (18.08724.000 short sequences), 0.1% was affiliated to *Methanomicrobia*, 3.5% to unassignable *Euryarchaeota*, 0.1% to *Crenarchaeota* and 1.8% to undefined Archaea. The longer reads of the v4-v6 regions with Titanium chemistry on samples SB-5 and SB-6 showed the vast majority of pyrosequencing reads taxonomically affiliated with one taxa *Methanobacteriales*, SB-6 76.5% (5121 sequences) and SB-5 84.2% (8307 sequences).The results are summarized in figure 7.

4 Discussion

4.1 Surface soil samples

As shown with an overview of the sampling sites on the Surtsey Island, most of the samples were collected on the South site of the island, at the same area as seagull (*Larus* spp.) colony has been established but also outside that area that contained less vegetation. A significant correlation was observed between the nutrient levels in soils and the number of heterotrophic environmental microorganisms grown on two different media at 22°C. The lowest number of bacteria ($1x10^4$-$1x10^5$/g) was measured in more or less pure pumice but the count was significant higher ($1x10^6$-$1x10^9$/g) in vegetated soil or pumice with bird drop (Figure 3). Moreover, the number of *Enterobacteriaceae* bacteria in all the soils samples showed strong correlation to the counts of total environmental bacteria in samples and therefore with nutrient content and also to samples encompassing bird droppings. Such samples (SS samples) enclosed also *Enterobacteriaceae* but in low numbers (10^1/g). Correlation of microbes to organic matter can be anticipated as organic matter inputs from plant production support growth of microbial heterotrophic soil microbial communities (Fenchel et al., 2012; Roesch et al., 2007; Schlesinger, 1997; Whitman et al., 1998). It has also been demonstrated that high number of viable counts of environmental bacteria and organic matter could designate other and more diverse groups of bacteria such as coliforms, faecal coliforms, *Escherichia coli*, aerobic and anaerobic bacteria growing at 30°C and even pathogenic bacteria (Girdwood et al., 1985; Pommepuy et al., 1992). Consequently, samples that showed significantly high number of bacteria and few other samples with low bacterial counts were selected for further investigation by these previously mentioned accredited methods. Furthermore, these samples were also tested for presence of pathogenic microbes such as *Listeria, Campylobacter* or *Salmonella*. Interestingly, the results showed relatively little variance among soils types, the

controls or pure pumice samples showed little growth by any of these tests as expected but soils with some vegetation and bird droppings revealed also low cell counts apart from aerobic bacteria growing at 30°C. Noteworthy, despite high vegetation and seagull activity, we measured low number of faecal coliforms except in one sample (SR-17) and few other samples with bird droppings but none of the samples contained any of the pathogens that were tested for. This confirm that the coliforms and faecal coliforms cannot survive long in the soil (Avery et al., 2004; Sun et al., 2006).

In the early stage of primary succession the plant nutrients are retained within the soil system and within microorganisms. The content of organic matter such as carbon and nitrogen is extremely low in the soil of Surtsey where there is no vegetation but can be extremely high in vegetated soils with bird droppings. Before sampling, we classified the surface samples into three sorts of samples according to their visual appearance in the field; pumice soil with bird droppings (10 SS samples), pure pumice soil (15 SJ samples) and square samples (19 SR samples). The SR samples were soil that was totally, partly or not vegetated and collected inside of defined squares used for activity measurements of the soil and vascular plants (Magnusson et al., 2014; Sigurdsson and Magnusson, 2010). Ecosystem respiration (Re) was measured inside these squares in order to investigate soil properties and surface cover of vascular plants. The plots were distributed among the juvenile communities of the island, inside and outside a seagull colony established on the island (Sigurdsson, 2009). Our classification by using multivariate NMDS analysis based on our results confirm our previous visual classification to certain extend. The analysis showed that the all SS samples were clearly separated from the other samples while the other two types of soil samples, SR and SJ were gathered into one big group, that could be divided into two smaller sub groups and one small group completely apart. This unique group (SR samples) contained samples that were vastly vegetated. The vegetated samples i.e. SR-14 to SR-19 were particular and different from all other samples due to higher load of *Enterobacteriaceae,* total viable counts, higher % of carbon, nitrogen and water content compared to other sampling sites (Figure 4, Table 1). Moreover, by taking into account data only from samples (all SR samples) collected for ecosystem respiration (Re), they could be divided mainly into two groups reflecting the soil properties or vegetation, inside and outside the seagull colony. SR16, 15, and 19 were clustered inside the main seagull colony on the Southern part of the island were SR7, 11, 5 and 9 are clustered just beside the main seagull colony or South-east part of the island, while

the two most dissimilar samples SR-3 and SR-1 were collected far away from the seagull colony or on the Northern part of the island.

4.2 Subsurface samples

An access to the deep biosphere in a remote neo volcanic island is extremely unique. We were able for the first time to collect hot subsurface samples deep in the centre of a volcanic island, created by a series of volcanic eruption only 42 years after the eruption break. Equally, as reported for geothermal boreholes in Reykjavík, the surface of the drill hole in Surtsey can be regarded as a window to the deep subsurface biosphere of the island (Marteinsson et al., 2001a). This window has been open for 30 years before our sampling in 2009 as the borehole was finished in August 1979 (Jakobsson and Moore, 1982). The purpose of the drill hole was to obtain a core for studying the structure of the island and the hydrothermal alteration of the tephra formed during the Surtsey eruption (Ólafsson and Jakobsson, 2009). The drill site is located on the edge of the Surtur tephra crater at 58 m above sea level with a total depth of 181 m. Several temperature measurements have been taken along the depth of the drill hole since the drilling and it appears that the hole has cooled since 1980 (Ólafsson and Jakobsson, 2009). Our temperature measurements along the drill hole at 1 m interval from the surface down to the bottom at 180 m showed drastic temperature changes compared to previously measurements. Our highest temperature measurement was 126.5°C which is about 14°C lower than maximal heat reported in 1980 and 3.5 °C lower than in 2004 (Ólafsson and Jakobsson, 2009). In our study we were able to record with 15 min interval the temperature with a temperature logger for 21 hours at 168 m depth in the borehole and the temperature showed to be remarkable stable at this depth at 54.8±0.1°C. This could indicate very little cooling effect of the cold seawater into the system. To our knowledge, this is the first long term temperature measurements in the borehole of Surtsey. Such deep environment with temperature below 100°C and high temperature barrier (130°C) atop, are ideal conditions for the growth of extreme microorganisms. The high temperature and the casing of the borehole down to 165 m isolates the bottom environment from the upper layers or surface microorganisms (Ólafsson and Jakobsson, 2009). The high sterilizing temperature atop of the borehole suggest indigenous subterrestrial microbiota that has probably disseminated from the below faults and cracks of the seafloor in a similar manner as has been reported in other various subterrestrial environments, geothermal boreholes in Reykjavik (Marteinsson et al., 2001a), fresh water hydrothermal vent cones in Eyjafjörður (Marteinsson et al., 2001b) and in

subglacial lakes on Vatnajökull (Marteinsson et al., 2013). Furthermore, our results on the microbial diversity supports such deep indigenous subterrestrial microbiota speculations as our 16S rRNA gene sequence showed only similarity to uncultivated taxon originated from the deep biosphere. Our archeal cone libraries of the 40 archaeal 16S rRNA genes in sample SB-6 at 172 m depth showed high homology (99%) to uncultured subsurface archaeon related sequences from subsurface water of the Kalahari Shield, South Africa by BLAST method (Genbank accession DQ354739.1). All the clones were dominated by this one sequence except two clones which showed high homology to uncultured subsurface archaeon related sequences from methane cycling in subsurface marine sediments and from in hydrothermal sediments at the Yonaguni Knoll IV hydrothermal field in the Southern Okinawa trough, (DQ988142) and (AB301979.1) respectively. The bacterial clone libraries obtained from samples SB-5 and SB-6 showed high diversity as is presented in a neighbor-joining tree of sequences in Figure 6. All these clones could not be affiliated with high homology to any cultivated bacteria and their closest relatives were uncultivated bacterium clones from various subsurfaces or sediments. Interestingly, few clones (12 clones) showed homology to clone SUBT-5 from geothermal boreholes in Reykjavik (Marteinsson et al., 2001a). Similar, with our deep pyrosequencing results, the SB-4 v6 library consists mostly or 94.5% of a single taxon affiliated with genus *Archeoglobus* from the phylum *Euryarchaeota*, 0.1% was affiliated to *Methanomicrobia*, 3,5% to unassignable *Euryarchaeota*, 0,1% to *Crenarchaeota* and 1,8% to Archaea (Figure 7). It is noteworthy that *Archeoglobus* species has been isolated from various marine environments and has optimum growth temperature at 80°C or at the same temperature measured at 145 m depth of the borehole (SB-4) (Huber et al., 1995; Stetter et al., 1993; Stetter et al., 1987). The longer reads of the v4-v6 regions with Titanium chemistry on samples collected at 172 m depth at 55°C (SB-5 and SB-6) showed the vast majority of pyrosequencing reads taxonomically affiliated with one taxa *Methanobacteriales*, 84.2% and 76.5% respectively (Figure 7). Interestingly, many methonogens grow at similar temperatures as found in these sample depths. Finally, despite various enrichment conditions and media, we were not able to enrich for any microbes with our culture techniques. This may suggest that we have not been able to create the right physical growth conditions and/or to use the right media composition for developing growth.

Conclusion:

We have explored for the first time microbial colonization in diverse soils and the influence of associate vegetation and birds on viable counts of environmental bacteria at the surface of the Surtsey. The number of faecal bacteria correlated to the total number of environmental bacteria and type of soil but no pathogenic microbes were detected in any sample tested. We were able for the first time to collect hot subsurface samples deep in the centre of this volcanic island and record the temperature for 21 hours at 168 m depth. Both uncultivated bacteria and archaea were found in the subsurface samples collected below 145 m. The microbial community at 54°C and 172m depth was dominating with diverse bacteria and a homogeny archaeal community of *Methanobacteriales* while the archaeal community at 145 m depth and 80°C was dominated by *Archaeoglobus* like sequences. The subsurface microbial community in Surtsey may be regarded as indigenous subterrestrial microbiota as both bacteria and archaea showed low affiliation to any known microbiota and there is a high temperature barrier (130°C) atop

Acknowledgements

The authors thank Árni R. Rúnarsson and Sveinn M. Magnússun for their technical contribution and the scientific party during the Surtsey expedition in 2009, especially Borgþór Magnússon and Erpur Snær Hansen. We would also like to thank Sveinn Jakobsson for helpful discussions and Náttúrufræðistofnun Íslands for the topographic map of Surstey. Special thanks are to Sigmar Guðbjörnsson for providing a temperature logger from Star Oddi. Many thanks are also due to the Matis staff working in the division of analysis and consulting. This work was partly financed by preliminary grant from The Icelandic centre for research (RANNIS) and the European Union program MaCuMBA (grant agreement 311945).

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Figures

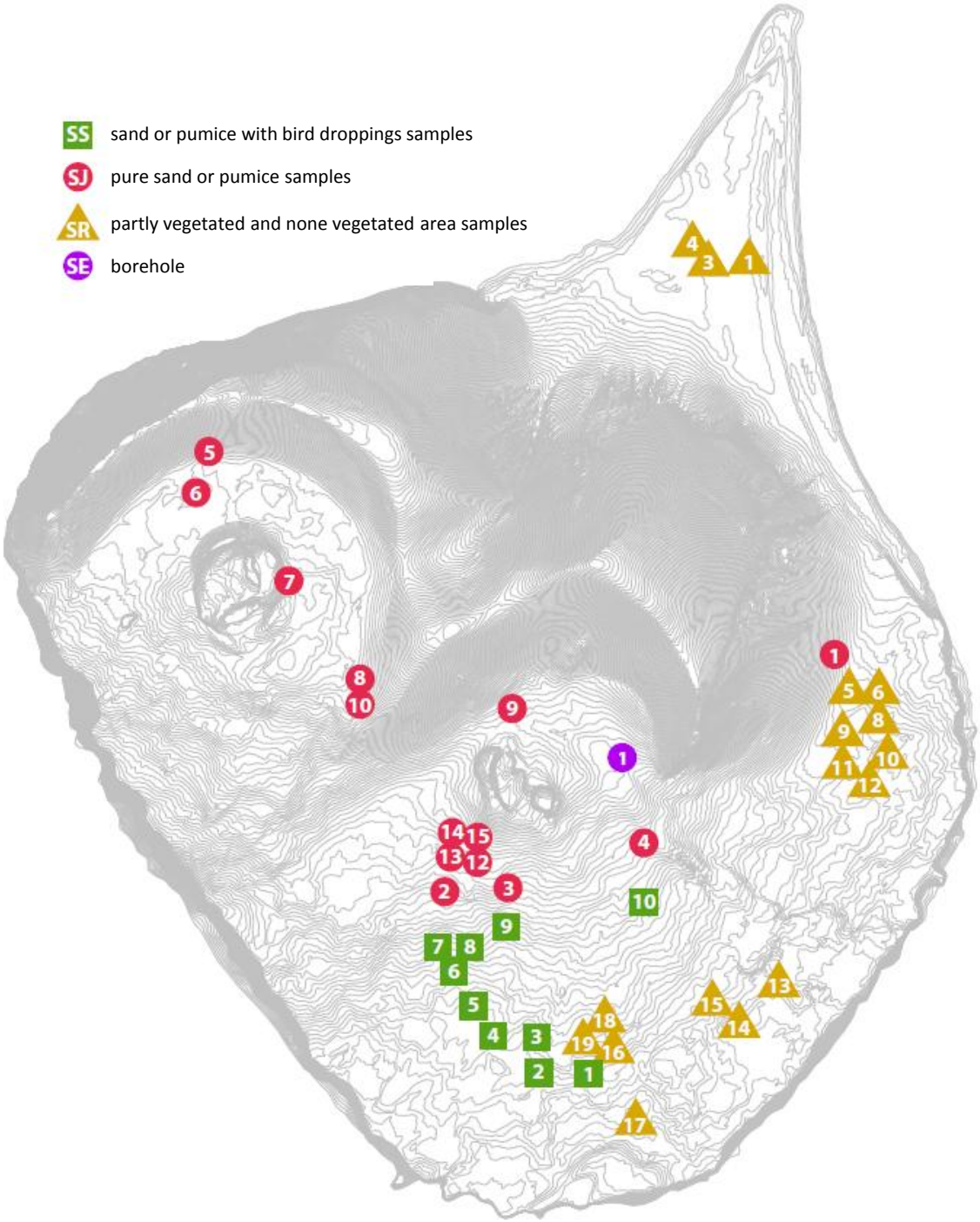

Figure 1. An overview of the sampling site. The sites are marked with green squares for sand or pumice with bird droppings samples (SS), pink circles for pure sand or pumice and vegetated samples (SJ), yellow triangles for partly vegetated and none vegetated area samples (SR) collected inside squares for activity measurements and purple circle for the drill hole site (SE) and are distributed with GPS points.

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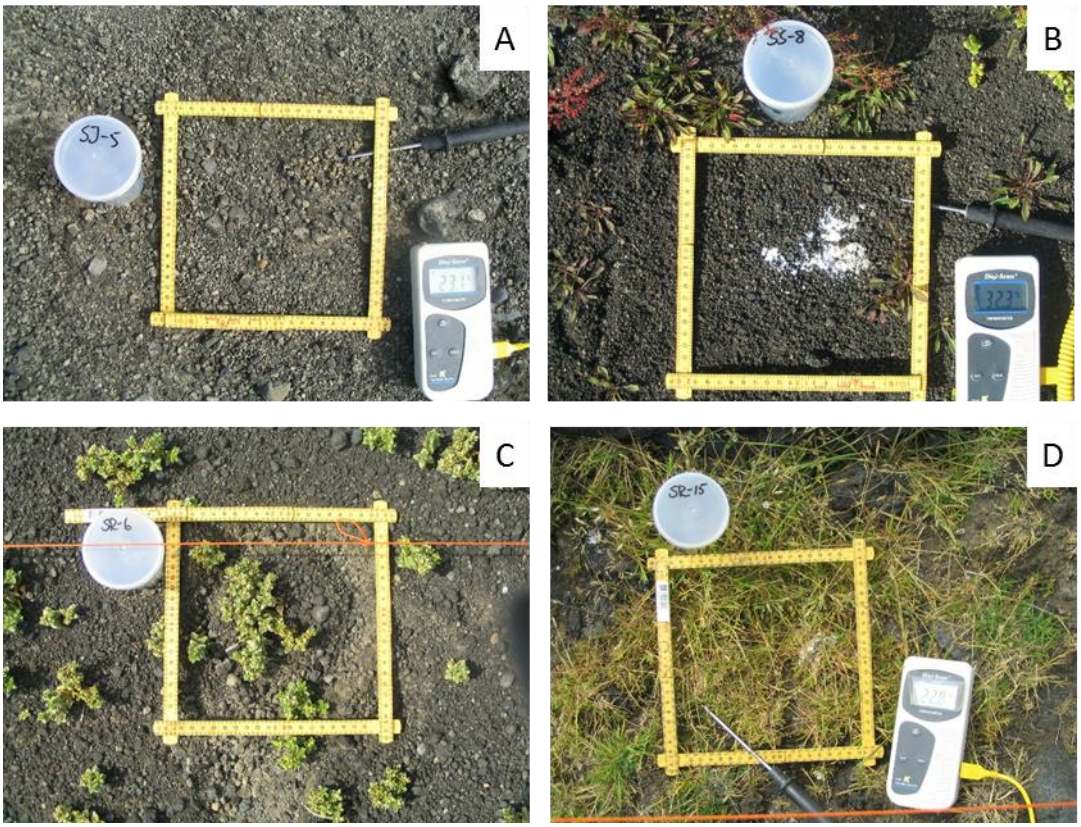


Figure 2. Pictures of the sample types. Samples were divided into three types: a) SS samples (Barren sand or pumice with bird droppings) see SS-8, b) SJ samples (Barren sand or pumice without bird droppings (SJ)) see SJ-5, and c) SR samples were partly vegetated surfaces SR6, d) or totally vegetated surfaces SR-15. Some of samples were part of a serial study area in Surtsey such as SR6 and SR15, the track after respiration measurement can be seen on the photos.

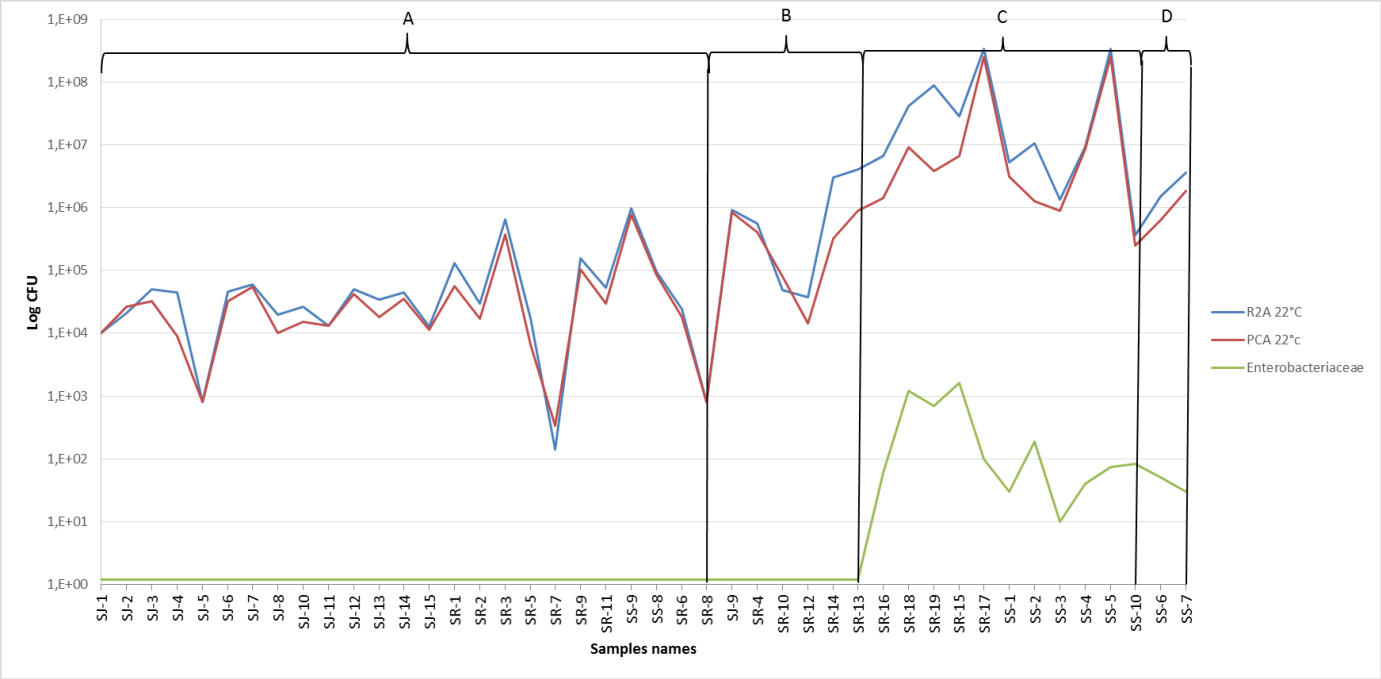


Figure 3. Total bacterial counts with plate count agar method at 22°C. The blue line shows the total environmental bacterial counts with plate count agar method and the red line the numbers grown on R2A media at 22°C. The number of *Enterobacteriaceae* in the soil samples are showed as green line. The nature of the samples are divided as sand or pumice including sample SR8 (A), partly vegetation (B), total vegetation with bird droppings including sample SR13 (C) and sand with bird droppings including sample SS10 (D). SJ-no, SS-no and SR-no stands for sand or pumice with bird droppings, pure sand or pumice and vegetated, partly vegetated and none vegetated area, respectively. See Fig. 1 for locations.

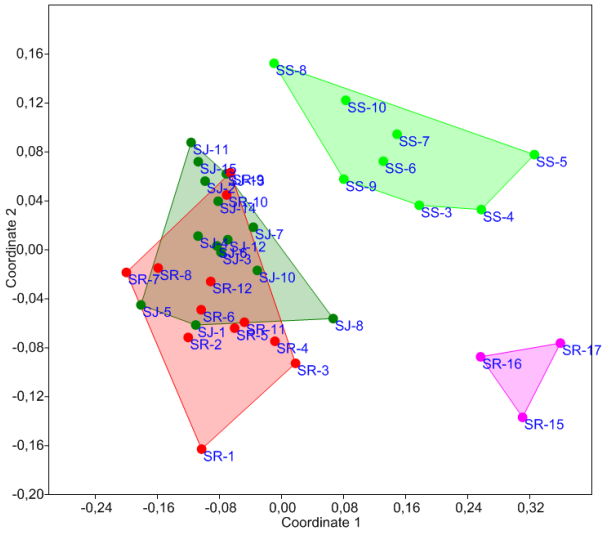


Figure 4. Non-metric multidimensional scaling, Euclidean distances. Environmental parameters included in analysis: temperature, total carbon, total nitrogen, water content, total microbial count on PCA and counts of *Enterobacteriaceae*. SS samples in light green (sand or pumice with bird droppings), SJ samples in dark green (pure sand or pumice) and SR samples in red (partly vegetated and none vegetated area) and SR in purple (vegetated).

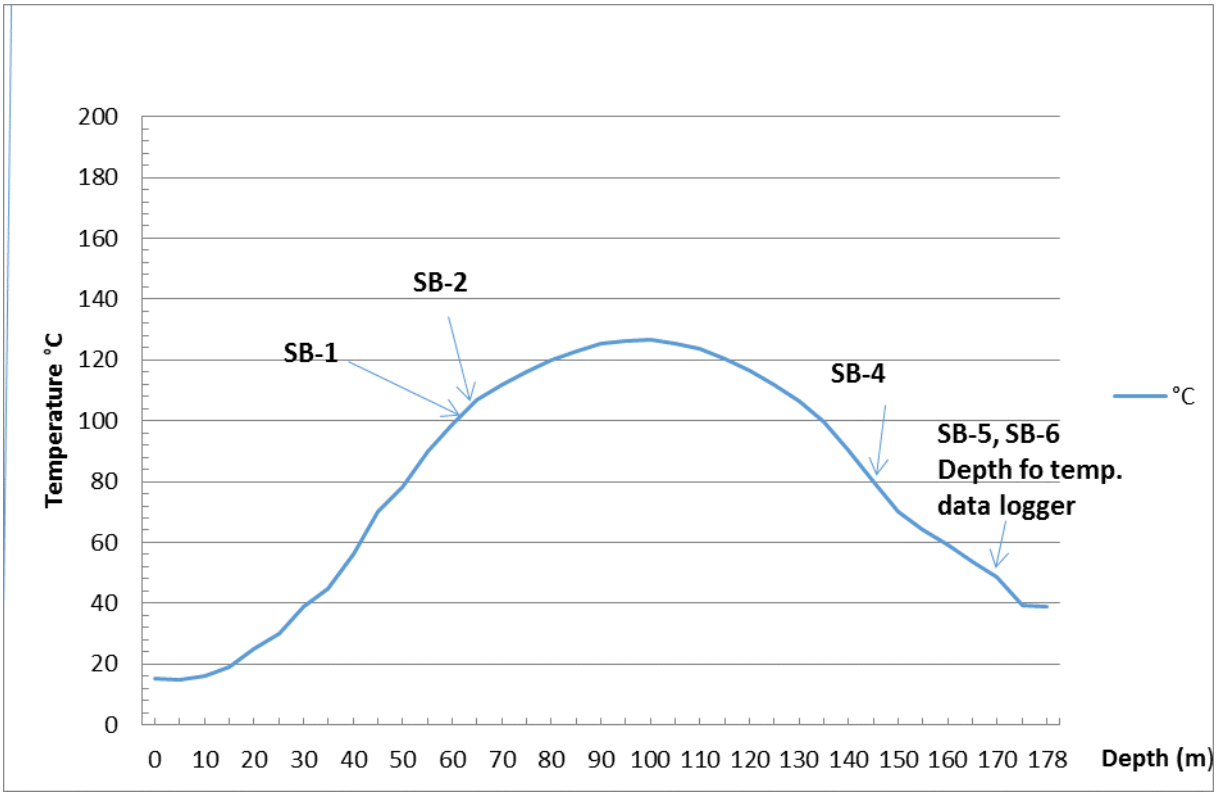


Figure 5. Temperature measurements along the drill hole. Temperatures were measured along the drill hole at 1 m interval from the surface down to the bottom at 178 m with a borehole temperature meter. The figure shows the depth and temperature of a temperature logger that measured the temperature for 21 hours.

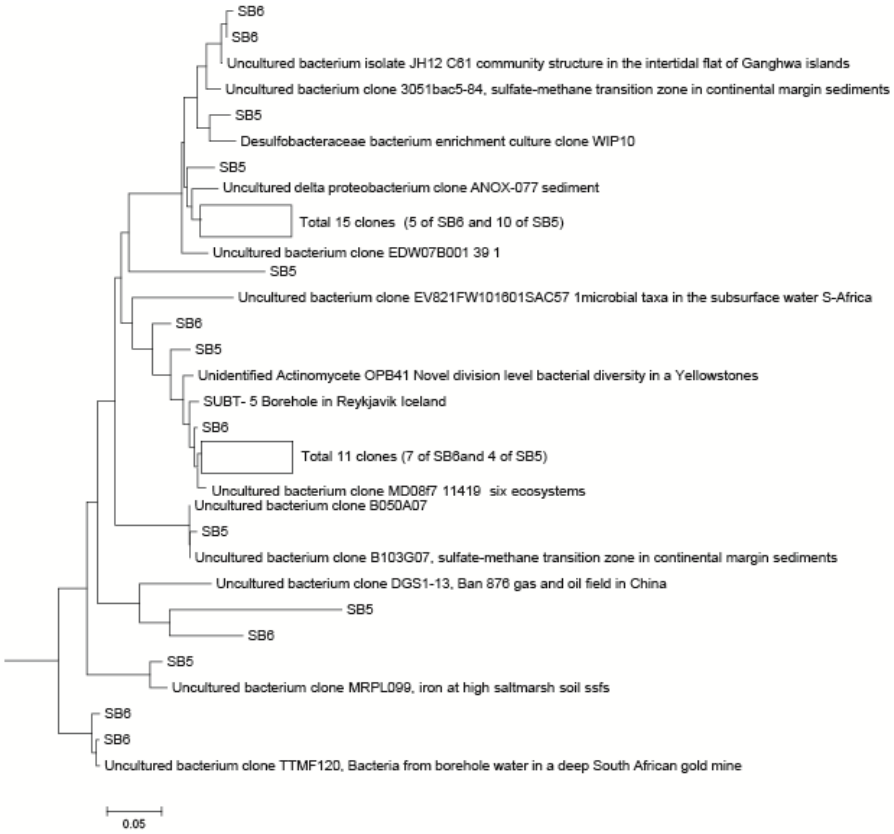
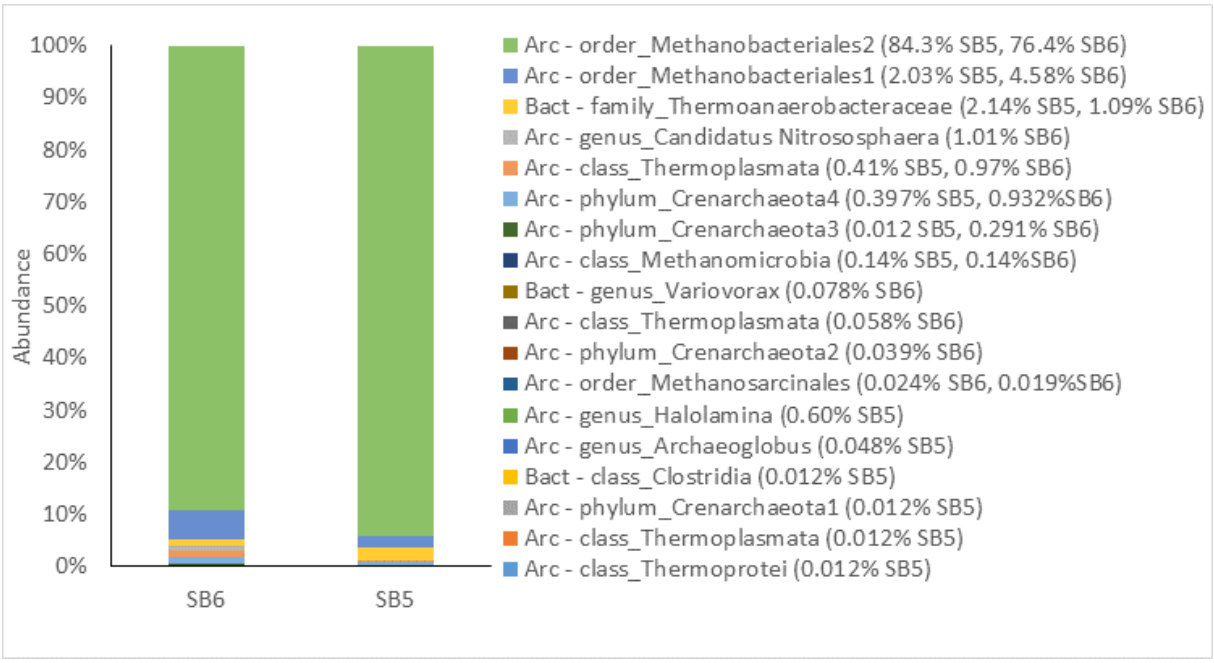


Figure 6. Neighbor-joining trees of sequences from the 16S rRNA clone libraries, databases showing phylogenetic relationships. The scale bar represents the expected % of substitutions per nucleotide position and a marine *Crenarchaeon* was used as outgroup. The cluster in uncultured delta *proteobacterium* clone ANOX-077 represents 11 clones with 99% sequence similarity (5 SB-6 and 10 SB-5).The cluster in uncultured bacterium clone MD08f7 11 clones with 99% sequence similarity (7 SB-6 and 5 SB-5).

a



b

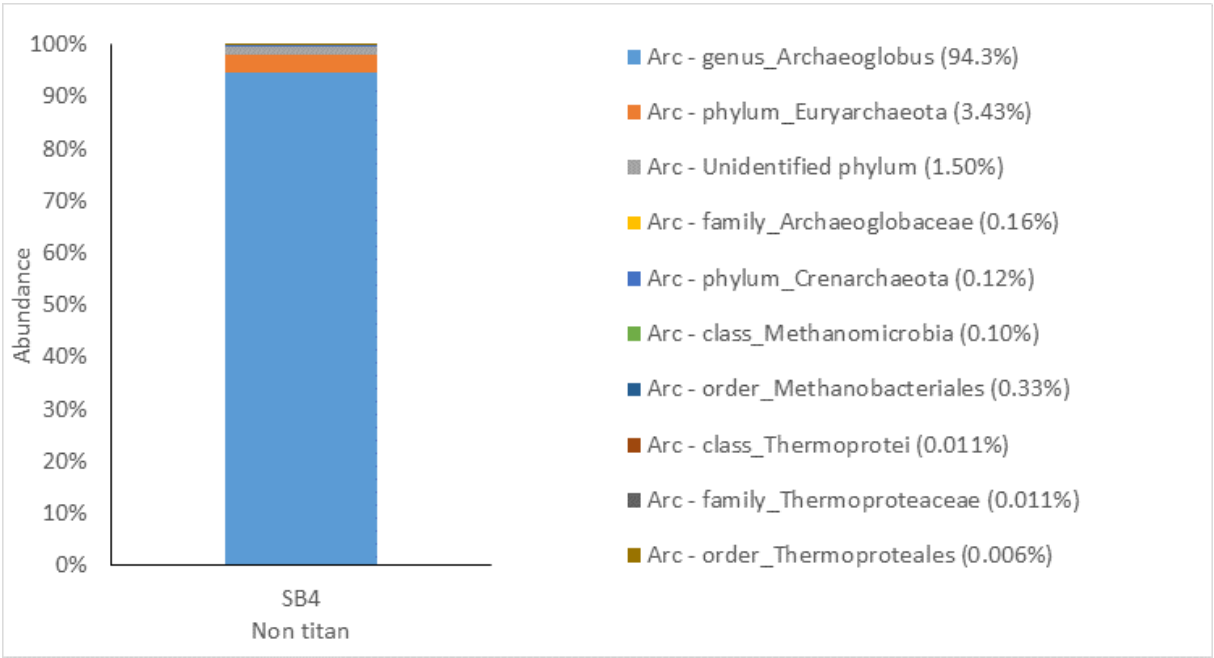


Figure 7. Sequencing results of the 16S rRNA gene with next generation sequencing method. a) the longer reads of the v4-v6 regions with Titanium pyrosequencing on samples SB-5 and SB-6; b) pyrosequencing of short fragment of the v6 region of the SB-4 v6 library. The columns and the colours show the % of each taxon (see text and % of each taxon on the right side).

Table

Table 1 : Viable count of total coliforms, faecal coliforms, *Escherichia coli*, aerobic and anaerobic bacteria growing at 30°C and detection of pathogens on different media (PCA, R2A, VRBGA).

Soil #	T°(C)	PCA 22°C (CFU/g)	R2A 22°C (CFU/g)	VRBGA 37°C (CFU/g)	CFU 30°C		Coliforms /MPN	Faecal *coli* /MPN	*E. coli* /MPN	totN (% of dw)	totC (% of dw)	GWC
					Aerobic	Anaerobic						
SJ-1	21.2	$1.0x10^4$	$1.0x10^4$	0	nd	nd	nd	nd	nd	0.0095	0.24	0.20
SJ-2	30.6	$2.6x10^4$	$2.1x10^4$	0	nd	nd	nd	nd	nd	0.0110	0.28	0.18
SJ-3	25.7	$3.2x10^4$	$5.0x10^4$	0	nd	nd	nd	nd	nd	0.0280	0.43	0.15
SJ-4	27.0	$9.0x10^3$	$4.4x10^4$	0	nd	nd	nd	nd	nd	0.0020	0.18	0.14
SJ-5	23.1	$8.0x10^2$	$8.0x10^2$	0	nd	nd	nd	nd	nd	0.0010	0.09	0.09
SJ-6	26.5	$3.2x10^4$	$4.6x10^4$	0	nd	nd	nd	nd	nd	0.0030	0.20	0.30
SJ-7	27.0	$5.4x10^4$	$6.0x10^4$	0	nd	nd	nd	nd	nd	nd	2.49	0.13
SJ-8	26.8	$1.0x10^4$	$2.0x10^4$	0	nd	nd	nd	nd	nd	nd	9.06	1.37
SJ-9	25.9	$8.5x10^5$	$9.3x10^5$	0	nd	nd	nd	nd	nd	nd	nd	nd
SJ-10	26.0	$1.5x10^4$	$2.6x10^4$	0	nd	nd	nd	nd	nd	0.0180	0.99	0.84
SJ-11	33.0	$1.3x10^4$	$1.3x10^4$	0	nd	nd	nd	nd	nd	nd	0.38	0.17
SJ-12	27.0	$4.2x10^4$	$5.0x10^4$	0	$3.1x10^2$	$1.0x10^1$	$3.0x10^0$	$3.0x10^0$	$3.0x10^0$	0.0090	0.36	0.34
SJ-13	31.0	$1.79x10^4$	$3.4x10^4$	0	$8.2x10^2$	$1.0x10^1$	$3.0x10^0$	$3.0x10^0$	$3.0x10^0$	0.0165	1.00	0.30
SJ-14	29.5	$3.5x10^4$	$4.4x10^4$	0	nd	nd	nd	nd	nd	0.0030	0.33	0.28

SJ-15	32.0	$1.14x10^{4}$	$1.26x10^{4}$	0	nd	nd	nd	nd	nd	nd	0.41	0.29
SR-1	15.5	$5.6x10^{4}$	$1.29x10^{5}$	0	nd	nd	nd	nd	nd	0.0060	0.21	0.14
SR-2	21.0	$1.73x10^{4}$	$3.0x10^{4}$	0	nd	nd	nd	nd	nd	0.0060	0.08	0.14
SR-3	21.0	$3.7x10^{5}$	$6.4x10^{5}$	0	nd	nd	nd	nd	nd	0.0030	1.28	0.99
SR-4	21.0	$4.1x10^{5}$	$5.6x10^{5}$	0	$2.2x10^{3}$	$1.5x10^{2}$	$3.0x10^{0}$	$3.0x10^{0}$	$3.0x10^{0}$	0.0120	0.73	0.49
SR-5	22.4	$6.5x10^{3}$	$1.71x10^{4}$	0	nd	nd	nd	nd	nd	0.0210	2.42	0.23
SR-6	22.3	$1.8x10^{4}$	$2.43x10^{4}$	0	nd	nd	nd	nd	nd	0.0030	0.18	0.18
SR-7	24.7	$3.4x10^{2}$	$1.4x10^{2}$	0	nd	nd	nd	nd	nd	0.0340	0.18	0.18
SR-8	25.3	$8.0x10^{2}$	$8.0x10^{2}$	0	nd	nd	nd	nd	nd	0.0015	0.24	0.24
SR-9	30.7	$1.04x10^{5}$	$1.57x10^{5}$	0	$1.2x10^{2}$	$1.0x10^{1}$	$3.0x10^{0}$	$3.0x10^{0}$	$3.0x10^{0}$	nd	0.35	0.22
SR-10	29.7	$7.9x10^{4}$	$4.9x10^{4}$	0	nd	nd	nd	nd	nd	0.0165	0.32	0.30
SR-11	22.0	$3.0x10^{4}$	$5.3x10^{4}$	0	nd	nd	nd	nd	nd	0.0030	0.61	0.59
SR-12	24.0	$1.43x10^{4}$	$3.7x10^{4}$	0	nd	nd	nd	nd	nd	nd	0.25	0.24
SR-13	26.3	$9.0x10^{5}$	$4.1x10^{6}$	$2.2x10^{2}$	nd	nd	nd	nd	nd	nd	nd	nd
SR-14	23.7	$3.2x10^{5}$	$3.0x10^{6}$	0	nd	nd	nd	nd	nd	nd	nd	nd
SR-15	22.7	$6.7x10^{6}$	$2.8x10^{7}$	$1.63x10^{3}$	$5.8x10^{4}$	$3.0x10^{1}$	$1.5x10^{2}$	$9.3x10^{1}$	$9.3x10^{1}$	0.0210	6.14	2.82
SR-16	25.0	$1.42x10^{6}$	$6.7x10^{6}$	$1.2x10^{2}$	$8.8x10^{4}$	$1.9x10^{3}$	$3.0x10^{0}$	$3.0x10^{0}$	$3.0x10^{0}$	0.0690	14.22	3.99
SR-17	23.0	$1.0x10^{8}$	$1.0x10^{8}$	$2.0x10^{2}$	$1.4x10^{4}$	$1.0x10^{1}$	$9.3x10^{2}$	$9.3x10^{2}$	$9.3x10^{2}$	0.0420	13.87	2.85
SR-18	nd	$9.1x10^{6}$	$4.1x10^{7}$	$2.42x10^{3}$	$7.6x10^{4}$	$4.5x10^{2}$	$3.0x10^{0}$	$3.0x10^{0}$	$3.0x10^{0}$	nd	nd	nd
SR-19	27.9	$3.8x10^{6}$	$8.7x10^{7}$	$6.92x10^{2}$	$4.1x10^{3}$	$5.0x10^{1}$	$3.0x10^{0}$	$3.0x10^{0}$	$3.0x10^{0}$	nd	nd	nd
SS-1	27.1	$3.1x10^{6}$	$5.3x10^{6}$	$6.0x10^{1}$	$3.7x10^{4}$	$1.3x10^{2}$	$2.3x10^{1}$	$9.0x10^{0}$	$9.0x10^{0}$	nd	nd	nd

SS-2	26.0	$1.26x10^{6}$	$1.05x10^{7}$	$3.8x10^{2}$	$2.4x10^{4}$	$6.0x10^{1}$	$4.0x10^{0}$	$3.0x10^{0}$	$3.0x10^{0}$	nd	nd	nd
SS-3	26.7	$9.0x10^{5}$	$1.34x10^{6}$	$2.0x10^{1}$	$1.4x10^{3}$	$4.0x10^{1}$	$9.3x10^{2}$	$9.3x10^{2}$	$9.3x10^{2}$	0.6785	7.30	0.98
SS-4	25.7	$8.5x10^{6}$	$9.3x10^{6}$	$4.0x10^{1}$	$1.0x10^{4}$	$5.0x10^{1}$	$4.3x10^{1}$	$4.3x10^{1}$	$4.3x10^{1}$	0.6785	14.90	1.56
SS-5	28.4	$2.52x10^{8}$	$3.37x10^{8}$	$7.5x10^{1}$	$1.4x10^{4}$	$1.0x10^{1}$	$9.3x10^{2}$	$9.3x10^{2}$	$9.3x10^{2}$	0.6785	8.10	1.00
SS-6	26.5	$6.3x10^{5}$	$1.49x10^{6}$	$5.0x10^{1}$	$4.6x10^{3}$	$3.0x10^{2}$	$3.0x10^{0}$	$3.0x10^{0}$	$3.0x10^{0}$	0.6785	1.70	0.45
SS-7	26.5	$1.82x10^{6}$	$3.6x10^{6}$	$3.0x10^{1}$	$3.0x10^{4}$	$2.0x10^{4}$	$9.3x10^{2}$	$9.3x10^{2}$	$9.3x10^{2}$	0.6785	2.30	0.28
SS-8	32.3	$8.5x10^{4}$	$9.6x10^{4}$	0	nd	nd	nd	nd	nd	0.6785	0.60	0.19
SS-9	25.5	$7.6x10^{5}$	$9.6x10^{5}$	0	nd	nd	nd	nd	nd	0.6785	2.10	0.29
SS-10	25.3	$2.47x10^{5}$	$3.6x10^{5}$	$8.4x10^{1}$	$3.3x10^{3}$	$6.2x10^{2}$	$4.6x10^{2}$	$4.6x10^{2}$	$4.6x10^{2}$	0.6785	0.50	0.15

nd : not determinated

CFU : Colony-forming unit

MPN : Most Probable Number

totN (% of dw) : percentage of nitrogen

totC (% of dw) : percentage of carbon

GWC : Soil gravimetric water content

T°(C): Surface temperature

Table 2. CO_2 flux measurements from selected samples.

	NEE (umol CO2 m-2 s-1)	Respiration (umol CO2 m-2 s-1)	GPP (umol CO2 m-2 s-1)	PAR (umol photons m-2 s-1)	Vegetation (%)
SR-1	-1.59	0.26	-1.85	761	90
SR-3	0.06	0.1	-0.04	802	1
SR-5	0.05	0.05	0	1270	0
SR-7	0.15	0.15	0	870	0
SR-9	0.01	0.01	0	1170	0
SR-11	0.02	0.02	0	800	0
SR-15	-0.02	0.02	-0.04	854	60
SR-16	-0.18	0.4	-0.58	687	100
SR-19	-0.07	0.27	-0.34	1209	100