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Microbial ecology of two hot springs of Sikkim: Predominate population and geochemistry

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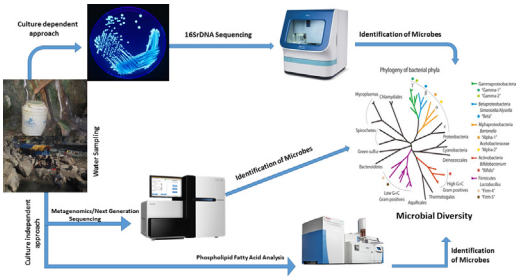
HIGHLIGHTS

- This study describes the geochemistry and microbial ecology of the hot springs of Sikkim, India.
- This study revealed the dominance of proteobacteria and bacteroidetes in the two hot spring.
- The microbial ecology of the two hot springs are depended on the geochemistry of the springs.
- The culture dependent technique was correlative with PLFA studies showing the dominance of gram positve bacteria over gram negative.

GRAPHICAL ABSTRACT



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ABSTRACT

Northeastern regions of India are known for their floral and faunal biodiversity. Especially the state of Sikkim lies in the eastern Himalayan ecological hotspot region. The state harbors many sulfur rich hot springs which have therapeutic and spiritual values. However, these hot springs are yet to be explored for their microbial ecology. The development of neo generation techniques such as metagenomics has provided an opportunity for inclusive study of microbial community of different environment. The present study describes the microbial diversity in two hot springs of Sikkim that is Polok and Borong with the assist of culture dependent and culture independent approaches. The culture independent techniques used in this study were next generation sequencing (NGS) and Phospholipid Fatty Acid Analysis (PLFA). Having relatively distinct geochemistry both the hot springs are thermophilic environments with the temperature range of 50–77 °C and pH range of 5–8. Metagenomic data revealed the dominance of bacteria over archaea. The most abundant phyla were *Proteobacteria* and *Bacteroidetes* although other phyla were also present such as *Acidobacteria*, *Nitrospirae*, *Firmicutes*, *Proteobacteria*, *Parcubacteria* and *Spirochaetes*. The PLFA studies have shown the abundance of Gram Positive bacteria followed by Gram negative bacteria. The culture dependent technique was correlative with PLFA studies. Most abundant bacteria as isolated and identified were Gram-positive genus *Geobacillus* and *Anoxybacillus*. The genus *Geobacillus* has been reported for the first time in North-Eastern states of India. The *Geobacillus* species obtained from the concerned hot springs were *Geobacillus toebii*, *Geobacillus lituanicus*, *Geobacillus Kaustophillus* and the *Anoxybacillus* species includes *Anoxybacillus gonensis* and *Anoxybacillus Caldiproteolyticus*. The distribution of major genera and their statistical correlation analyses with the geochemistry of the springs predicted that the temperature, pH, alkalinity, Ca^{2+}, Mg^{2+}, Cl^{2+}, and sulfur were main environmental variables influencing the microbial community composition and diversity. Also the piper diagram suggested that the water of both the hot springs are Ca-HCO^{3-} type and can be predicted as shallow fresh ground waters. This study has provided an insight into the ecological interaction of the diverse microbial communities and associated physicochemical parameters, which will help in determining the future studies on different biogeochemical pathways in these hot springs.

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1. Introduction

Thermal springs are natural geological phenomena that occur on all the continents. Every thermal spring has its distinctive features which depend on the several promising amalgamations of heat sources, water sources, subsurface rock types, flow paths and chemical reactions (Heasler et al., 2009). Various dissolved minerals such as megnecium, calcium, sodium, chloride, sulfates or silica are generally present in the geothermal water of an area as compared to non-geothermal groundwater (Zangana, 2015). Higher mineralogical compositions support different micro and macro floral community in and on the surrounding environment. Since XIX century, the exploration of hot springs has been in to progress and the physicochemical properties and geological features were the primary areas of study for researchers. However, the isolation and investigation of their thermophilic microbial community did not start until the 1950's (Marsh and Larsen, 1953). In recent scenario hot springs are the hotspots of research in the field of microbial ecology. Microbial community profiling of hot springs, focusing mainly on bacteria and archaea showing thermophilic and hyperthermophilic nature has attributed to different industrial enzymes and proteins of the modern era (López-López et al., 2015).

The thermophilic bacteria are recognized by their metabolic thermostability which are buoyed by their thermophilic protein. The thermostability of the thermophilic enzymes has been established as valuable biocatalysts for various biotechnological and industrial purposes. A prototype is *Taq*-polymerase from *Thermus aquaticus* that led to the advancement of the polymerase chain reaction (PCR) technique (Chien et al., 1976). To understand the geochemistry, geomicrobiology, bioenergetics and biotechnological potential of geothermal systems, various studies have been performed worldwide (Amin et al., 2017) (Liu et al., 2016). However, the conventional classical culture-dependent approach was the primary and sole technique to determine the geomicrobiology of the hot springs before the development of neo molecular techniques. Inability to culture the vast majority of microorganisms with the culture-dependent method has questioned the technique for inclusive profiling of different environment (Amann et al., 1995). To overcome this limitation, different culture-independent techniques including DGGE, PLFA, and Metagenomic studies have revealed a subsequent increase in microbial molecular ecology studies. The first approach to understand the true diversity of distinct environments was provided by a combined approach of PCR amplification of the 16S rRNA genes and their pattern analysis on denaturing gradient gel electrophoresis (DGGE). However, the products spawned during PCR-DGGE of the mixed communities often encumber the application of this technique in quantitative community profiling (Neilson et al., 2013). More recent development of Metagenomic approach has considerably increased the information related to microbial diversity, functional genomics, and transcriptomics (López-López et al., 2015) (DeCastro et al., 2016). This method is precise for gaging the structure of an environmental microbial community since it does not cover any selection and reduces technical biases, particularly the ones presented by amplification of the 16S rRNA gene (Lewin et al., 2013). Besides the abovementioned techniques, an interesting non-culturable technique, i.e., Phospholipid Fatty Acid Analysis or PLFA is available and used since two decades to characterize microbial communities (Willers et al., 2015). The PLFA was first used to assess the microbial biomass from marine and estuarine sediments in 1979 (White et al., 1979). PLFA can be used to measure the viable microbial biomass and to identify the biomarkers for taxonomic evaluation from an environment (Jenkinson and Ladd, 1981).

Aiming the geomicrobiological features, microbial community structure of different geothermal springs has determined worldwide such as Tengchong thermal springs of China (Hou et al., 2013), Nakabusa hot springs of Japan (Kubo et al., 2011), Siloam hot water springs of South Africa (Memory Tekere, 2012), Andean Mountain hot water springs of Colombia (Bohorquez et al., 2012), Solfataric Fields of Iceland (Kvist et al., 2007), Great Basin hot springs (Costa et al., 2009), and Yellowstone National Park (USA) (Spear et al., 2005). In India geological survey has identified about 400 hot springs located in seven geothermal provinces distributed across India (Chandrasekharam, 2005). Of the 400 hot springs, only 28 springs have been explored microbiologically and 12 hot springs have been curtained with cutting-edge metagenomic approaches (Poddar and Das, 2017). Metagenomic studies of hot springs have conferred the microbial diversity and their functional and metabolic framework.

Nestling in the Himalayan mountains, the state of Sikkim is characterized by mountainous topography. Sikkim lies in ecological hotspot zone of the lower eastern Himalayan region. The state hosts several hot springs which are known for their medicinal and therapeutic values. The springs also reportedly have high sulfur content and few known to emit hydrogen (Choudhury, 2006). However, these hot springs are poorly studied for their microbial community structure. Microbial ecology studies could enhance the understanding of different metabolic framework in sulfurous hot springs of the state. The present study was aimed to investigate the unexplored microbial diversity of two hot springs of Sikkim with both the culture-dependent and culture-independent approaches and thus might provide novel insights into the ecological interactions among taxa in these communities, which in turn will also help in defining future study courses in these sites.

2. Materials and methods

2.1. Sampling

2.1.1. Description of a sampling site

The Polok and Borong hot springs selected for the current study were located in South district of Sikkim. Both the hot springs are on the banks of river Rangit which is a tributary of the Teesta River. These two springs are treated as a sacred place with medicinal properties which opened a door to tourism and people from different regions visit the place. The geographical position of coordinates and elevation range of Polok and Borong Tatopani were measured with the help of GPSMAP 78S (Garmin, India). The map of the hot spring site was prepared with the help of Google Earth software (Fig. 1).

2.1.2. Sampling and physiochemical analysis

The water samples were collected aseptically in 1 L sterile thermal flasks in triplicates from both the sampling sites. The samples were divided into three groups based on the experiments to be carried out, such as one group was kept for culture dependent bacterial isolation, the second group for chemical analysis through ICPMS (Inductive Coupled Plasma Mass Spectroscopy) and the third group for PLFA (Phospholipid Fatty Acid Analysis) studies and metagenomic studies. The samples were then immediately transferred to the laboratory and kept at 4 °C. Preliminary physicochemical parameters including temperature, pH, dO_2, TDS, electroconductivity were measured at the sampling site using portable water quality checker (Horiba, Japan; U-50 Series). Elemental analysis was done using ICP-MS (Perkin Elmer, USA).

2.2. Culture-dependent analysis

2.2.1. Isolation of bacterial strains

For culture dependent microbial diversity studies, the samples were enriched immediately after the collection. The bacteria were isolated using ten different media such as Nutrient Agar (NA), Thermus Agar (TA), Luria-Bertani Agar (LBA), Modified Luria-Bertani (mLB), YTP-2 medium, TR medium, R2A, BP medium, GYT and Actinomycetes agar. The composition of the various media used is given in a Supplementary material Table S1. The isolation was done by the standard spread and streak plate methods. The culture plates were incubated at 60 ± 2 °C for 24–72 h. After the incubation, different colonies were selected on the basis of their morphological characteristics and pure culture was obtained by subsequent sub-culturing. Isolated and purified bacterial strains were stored in 50% Glycerol stock at −80 °C till further use.

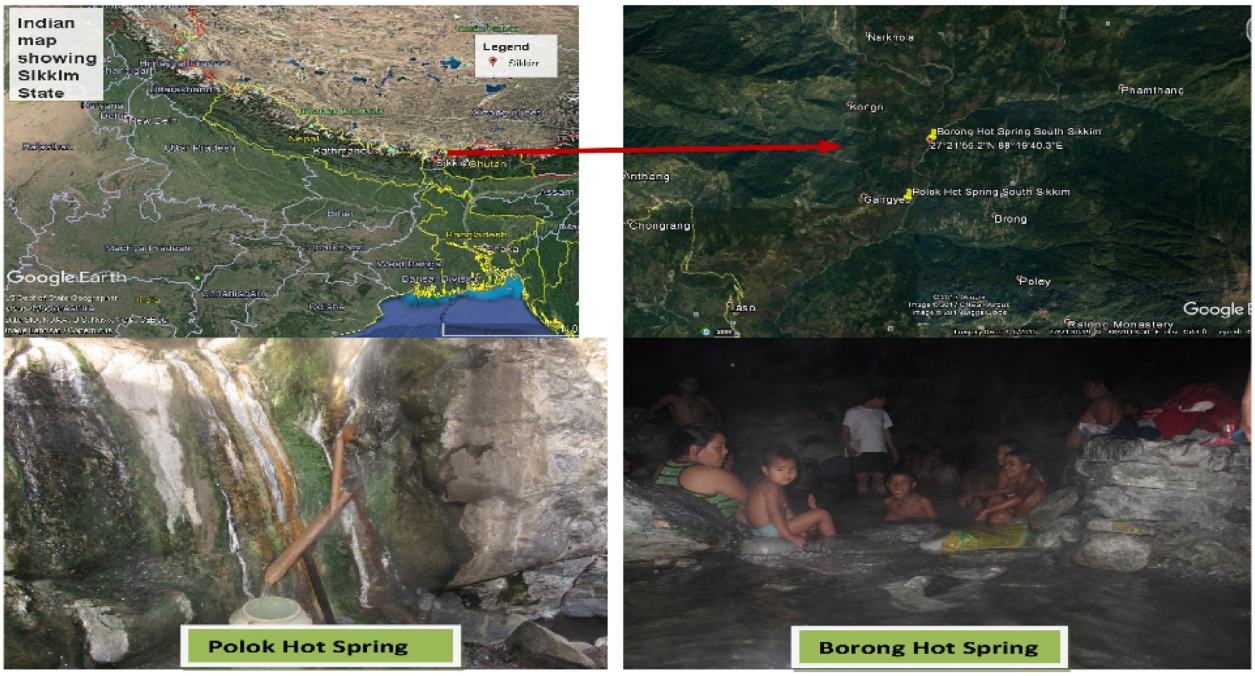

Fig. 1. Google based map of two hot springs Borong and Polok of South Sikkim India. The geographical position of coordinates and elevation range of Polok and Borong hot spring were measured with the help of GPSMAP 78S (Garmin, India).

2.2.2. Morphological, physiological and biochemical characterizations of the isolates

Colony morphology (color, form, margins, elevation, and density) was checked. The general cell morphology was observed under light microscope. The shape (short rods, long rods, filaments, commas, spirals), and arrangement (simple, pair, chains, clusters) were recorded systemically. Gram-staining and spore staining was done by standard methods (Arayan et al., 2008; Arya et al., 2015). The physiological characteristics including the effect of temperature, pH, and NaCl concentrations on growth were measured. The optimal physiological temperature for growth was determined by incubating the isolates in a gradient of temperature from 20 to 80 °C in Thermus Agar medium. The pH tolerance and effect of NaCl concentration was tested in a range of 4.0–10.0 and 0.5–5% in TA medium respectively (Arya et al., 2015). The biochemical characterization of the isolates was done by qualitative analysis of various enzymes such as catalase, amylase, protease, lipase, urease, oxidase, and nitrate reductase. The carbohydrate fermentation test was performed using dextrose, maltose, fructose, mannitol, mannose, raffinose, ribose, sucrose, xylose, and arabinose (Arayan et al., 2008; Arya et al., 2015)

2.2.3. Molecular identification and phylogeny

For culture-dependent techniques, the DNA was extracted using Qiagen QIAamp DNA Mini Kit (Qiagen, USA) as per the manufacturer's instructions. The extracted DNA was stored at −80 °C for further analysis. The 16S rRNA gene amplifications were done by using two universal primers 27F(5′-AGAGTTTGATCMTGGCTCAG-3′) and 1406R(5′-GACGGGCGGTGTGTRCA-3′). The polymerase chain reaction was performed in 25 μL volume using 12.5 μL GoTaq Green Master Mix 2× (Promega), 20 pM of forward and reverse primers and 2 μL of the template. The PCR cycle was designed as, 5 min at 94 °C; 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C; and a final extension step of 10 min at 72 °C. The PCR products were analyzed by electrophoresis at 100 V for 1 h in a 0.8% Agarose gel (Sigma Aldrich, USA). The PCR product was purified using a QIAquick PCR purification kit (Qiagen, USA) for cycle sequencing. The purified 16S rDNA was then sequenced using a BigDye™Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) as manufacturer's instructions using Automated DNA Sequencer (ABS/Genetic 3500 Analyzer). The sequence obtained was assembled with Codon Code Aligner (ver. 5.2). Assembled sequences were compared with nr/nt database of NCBI using BLAST sequence homology search for taxonomic identification. A phylogenetic tree was constructed to identify the evolutionary lineage of the isolates. The tree was constructed using neighbor-joining method (Saitou and Nei, 1987) with jukes-cantor evolutionary distance measurement (Erickson, 2010) in MEGA 7.0 software.

2.3. Culture independent techniques

2.3.1. Phospholipid Fatty Acid Analysis (PLFA)

For PLFA analysis, the phospholipids were extracted according to the standard protocol (Fan et al., 2017) (Quideau et al., 2016) and were analyzed using Sherlock-MIDI identification system. The calibrated standards were used by the microbial identification system (MIDI) for annotation of generated phospholipid peaks. The Equivalent carbon length (ECL) values in comparison to the expected ECL value in the PLFA peak are mentioned in Supplementary Table S2. The minimum limit of detection (LOD) for the MIDI-PLFA method per 2uL injection, is 1 ng of fatty acid.

2.3.2. Metagenomic DNA extraction

Environmental DNA was extracted using DNeasy PowerWater Kit (MO BIO Laboratories, Carlsbad, CA, USA) in accordance with the manufacturer's instruction. Quality of the DNA was checked on 0.8% agarose gel and DNA was quantified using Qubit Fluorometer (Thermofisher Scientific, USA), with a detection limit of 10–100 ng/μL.

Table 1
Physical Parameters of Polok and Borong hot springs.

Hot spring	Temperature (in °C)	pH	Conductivity mS/cm	D.O. (mg/L)	D.O. (%)	TDS (g/L)
Polok	76.3	7.52	0.756	5.56	92	0.483
Borong	52.3	5.32	0.205	6.56	98.3	0.133

Table 2
Elemental analysis of Polok and Borong hot springs.

SNO.	1	2	3	4	5	6	7
Test parameters	Color	Aluminium	Ammonia	Boron	Calcium	Chloride	Copper
Unit	Hazen	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	<1	<0.03	<0.5	<0.5	14	70	<0.05
Borong	<1	<0.03	<0.5	<0.5	12	17	<0.05

SNO.	9	10	11	12	13	14	15
Test parameters	Free residual chlorine	Iron	Magnesium	Manganese	Nitrate	Phenolic compounds	Selenium
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	0.22	0.08	7	<0.1	10	0.07	<0.01
Borong	0.2	0.06	4	<0.1	8	0.18	<0.01

SNO.	17	18	19	20	21	22	23
Test parameters	Sulfide	Total alkalinity	Zinc	Cadmium	Cyanide	Lead	Mercury
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	0.4	327	<0.05	<0.003	<0.05	<0.01	<0.001
Borong	0.4	75	<0.05	<0.003	<0.05	<0.01	<0.001

SNO.	25	26	27	28	29	30	31
Test parameters	Nickel	Total arsenic	Total chromium	COD	BOD	Colloidal sulfur	Total phosphate
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	<0.02	<0.01	<0.05	30	11	8.7	<0.05
Borong	<0.02	<0.01	<0.05	20	8.4	10.2	<0.05

2.3.3. 16S metagenomic sequencing library preparation and sequencing

Amplifications of the V3 and V4 regions of bacterial 16S rRNA gene were done using two primers (16SV3F = 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16SV3R = 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3′) (Klindworth et al., 2013). The amplicon libraries were prepared using Nextera XT Index Kit (Illumina inc.), accordance with 16S metagenomic sequencing library preparation protocol (Faircloth et al, 2014). The amplicon library was purified with AMPure XP beads. The amplified library was checked by Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chips and concentration was quantified by Qubit fluorometer. Based on the data obtained from the Qubit fluorometer and the bioanalyzer, 500uL of the 10pM library was loaded into MiSeq cartridge for cluster generation and sequencing. Paired-end sequencing method was used. After the sequencing, high-quality metagenome reads were trimmed to remove the barcode and adaptor sequences.

2.3.4. Metagenomic data analysis

The adapter trimmed sequence were subjected to pre-processing for De-replication, Singleton removal, OTU Clustering, Chimera filtering with SolexaQA. Sequences with Phred score lower than 20 and

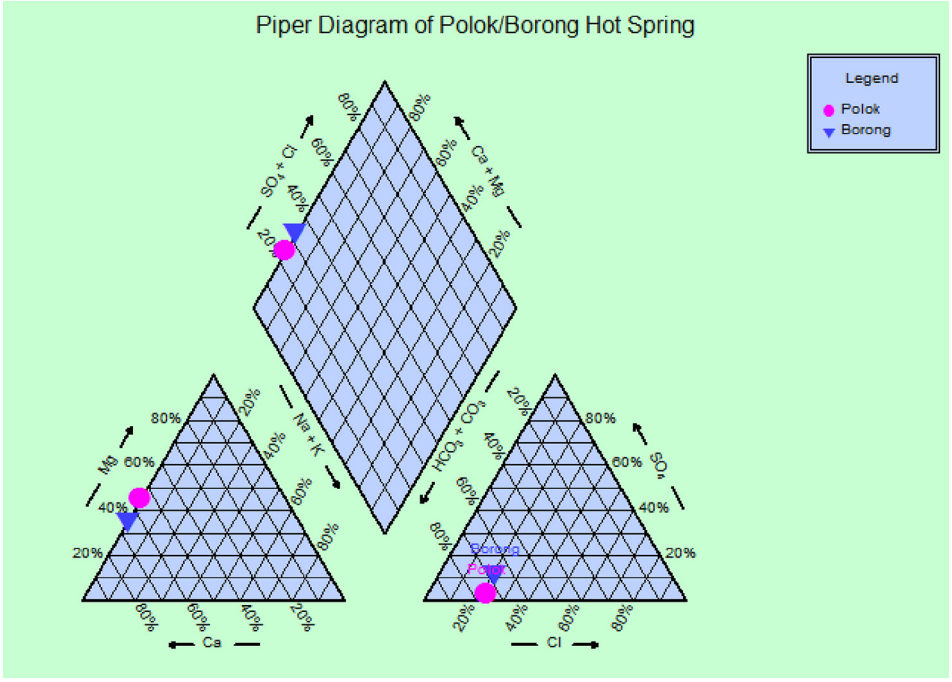

Fig. 2. Piper diagram of Polok and Borong hot spring.

Table 3.a
Morphological and biochemical characterization of various isolates; (+) indicates positive (−) indicates negative results.

Isolates	Gram stain	Spore	Form	Biochemical tests				Carbohydrate fermentation								
				Catalase	Amylase	oxidase	Nitrate reductase	Maltose	Mannitol	Dextrose	Mannose	Ribose	Sucrose	Xylose	Fructose	Cellobiose
TP3	+	+	Rods	+	−	−	−	+	+	+	−	+	−	−	+	−
TP2	+	+	Rods	+	−	−	−	+	+	+	−	−	−	−	+	−
BPP2	+	+	Rods	+	−	−	+	+	−	−	+	−	−	−	+	+
TP5,10PHP1	+	+	Rods	+	−	−	+	+	+	+	−	+	−	−	+	−
10PHP2	+	+	Rods	+	−	+	+	+	−	+	−	−	−	−	+	−
TP9	+	−	Rods	+	+	−	−	+	+	+	−	−	+	+	+	−
TP11	+	+	Rods	+	−	−	+	+	+	+	+	+	−	+	+	−
BPP1	+	+	Rods	+	−	−	−	+	−	−	+	−	−	−	−	−
TB10	+	−	Rods	+	−	−	−	+	+	+	+	+	−	+	+	−
TB7	+	−	Rods	+	−	−	−	+	+	+	+	+	−	−	+	−
TB3	+	+	Rods	+	−	−	+	+	+	+	−	+	−	−	+	−
BPB1	+	+	Rods	−	−	−	+	+	−	−	−	−	−	−	−	+
10PHB1	+	+	Rods	+	−	−	+	+	+	+	−	−	−	−	+	−
YTPB1	+	+	Rods	+	−	+	+	+	−	+	−	+	−	−	−	−
TRB1	+	+	Rods	+	−	−	+	+	−	+	−	+	−	+	+	−
TB9	+	+	Rods	+	−	−	−	+	+	+	+	+	−	−	+	−
TB1	+	−	Rods	+	−	−	+	+	+	+	+	+	−	−	+	−

ambiguous bases having primer mismatch and low read length <100 bp were removed. Annotation and normalization of operational taxonomic unit (OUT) was done using UPARSE OUT clustering and QIIME at 97% similarity (Edgar, 2013). For normalization, inbuilt script as well as METAGEN assist was used. The resulting representative OUT was aligned and given taxonomic classing using Greengenes database (http://greengenes.lbl.gov/). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. Sequences without homologous pair were classified as unknown.

2.3.5. Statistical analysis

The principal component analysis (PCA) was used to correlate between bacterial diversity and physicochemical parameters of the samples using PAST. The Analysis of variance was used to check the significance of various data with the help of Graph Pad Prism and XLSTAT. The piper diagram was made with the help of Rock Ware AQUA version 1.5. The Shannon diversity indices, chao1 were calculated with the help of EstimateS and PAST software (Chao et al., 2006). The heat map was used to analyze the comparative bacterial diversity among each of the two hot springs and between previously reported microbial diversity of different hot springs from Central India, Northeast India, and Tibet with the help of R software using Bray Curtis Dissimilarity matrix (package: ggplot, function: heatmap.2).

2.4. Data availability and accession number

Raw metagenomics reads were submitted to Sequence Read Archive (SRA), NCBI under accession numbers SAMN08038921, SRA: SRS2697425 for Polok Hot Spring with sample name as POLV4 and SAMN08038632, SRA: SRS2697438 for Borong Hot Spring with sample name as BORV4. The BioSample records will be accessible with the following links for Polok and Borong hot springs respectively http://www.ncbi.nlm.nih.gov/biosample/8038921 and http://www.ncbi.nlm.nih.gov/biosample/8038632.

3. Results

3.1. Site description

The geographical location of Polok and Borong hot spring was determined with the help of GPSMAP 78S (Garmin, USA) as per the guidelines are given by the manufacturer. The coordinates of the sites of

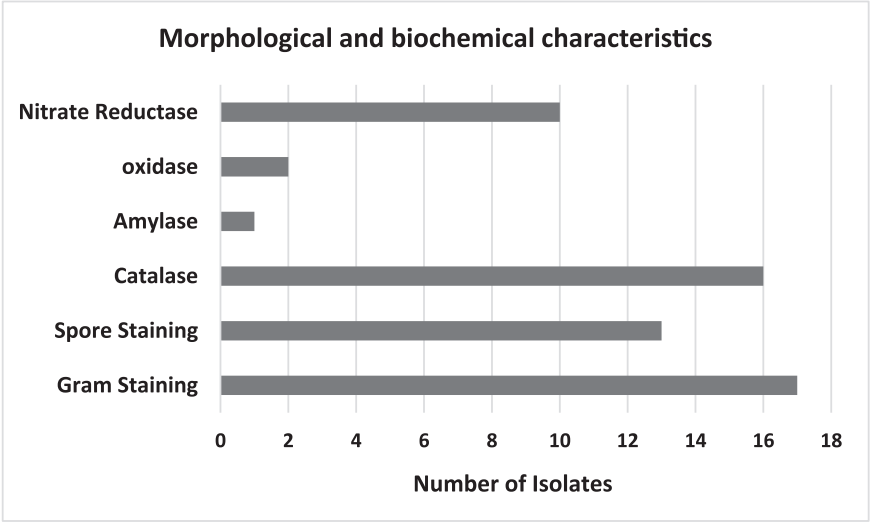

Fig. 3. Morphological and biochemical characteristics of isolates isolated from both Polok and Borong hot springs.

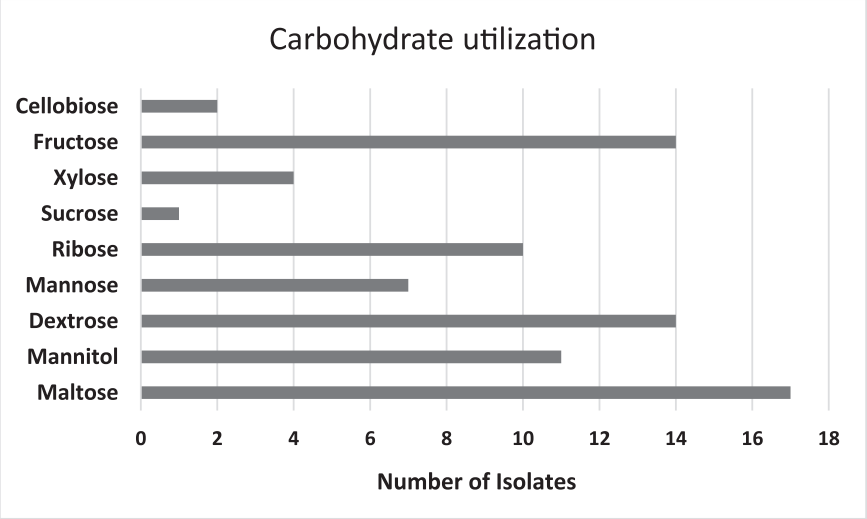

Fig. 4. Carbohydrate utilization of isolates isolated from both Polok and Borong hot springs.

Polokhot spring is 27°21′00.29″N longitude and 88°19′21.99″E latitude. The Borong is located at 27°21′93.57″N longitude and 88°19′67.01″E latitude. The elevation range of the locations was 3108 m and 3404 m for Polok and Borong hot springs respectively.

3.2. Physicochemical analysis of two hot springs

The temperature and pH of the sites were found considerably different. The Polok hot spring was little warmer than the Borong with a temperature of 75–77 °C, Borong was recorded with 50–52 °C. The pH of Polok hot spring was in between 7.5 and 8.5 defining the normal to the alkaline condition of the water while the water of Borong was acidic in nature with a pH of 5.1–5.6. The conductivity, dissolved oxygen and TDS were measured as 0.75 mS/cm, 4.5–5.5 mg/L, and 0.4 g/L respectively for Polok hot spring while the Borong hot spring had 0.20 mS/cm, 5.5–6.2 mg/L, and 0.13 g/L respectively (Table 1). The elemental analysis with ICP-MS showed more or less similar pattern of elemental composition in both the springs (Table 2). However, the Polok hot spring was found to be rich in chloride, calcium, magnesium along with COD and BOD (Table 2). Ionic concentration of elements in the hot springs was plotted as piper diagram (Fig. 2) for classification on the basis of chemical composition (Piper, 1944). Piper diagram is a combination of triangle plots representing anionic and cationic element on a common baseline. The apexes of the cation plot were magnesium, calcium, sodium, and potassium cations, while the apexes of the anion plot were chloride, sulfate, carbonate and hydrogen carbonate anions. The two ternary plots are then anticipated onto a diamond which can be used to describe different water types. Piper divided water into four basic types conferring to their location near the four corners of the diamond. Water that plots at the top of the diamond is high in $Ca^{2+} + Mg^{2+}$ and $Cl^- + SO4^{2-}$, which results in an area of permanent hardness. The water that plots near the left corner is rich in $Ca^{2+} + Mg^{2+}$ and HCO^{3-} and is in an area of temporary hardness. Water plotted at the lower corner of the diamond is mainly composed of alkali carbonates ($Na^+ + K^+$ and $HCO^{3-} + CO3^{2-}$). Water present near the right-hand side of the diamond may be reflected as saline ($Na^+ + K^+$ and $Cl^- + SO4^{2-}$). The piper diagram suggested that the water of both the hot springs are Ca-HCO^{3-} type and can be predicted as shallow fresh ground waters (Fig. 2).

3.3. Bacterial isolation and physicochemical characterization

A total of 100 thermophilic bacteria were isolated from two springs. On the basis of morphological and biochemical differences, 25 isolates from each hot spring were selected for further analysis. The molecular identification based on 16S rRNA further identified 17 distinct bacteria, which were taken as a representative of autochthonous thermophiles from the springs. The cell morphology of the bacteria suggested that most of the isolates were Gram-positive rods, however, some of the isolates showed Gram variable reaction. Most of the isolates were spore-forming except few such as TP9, TB10, TB7, and TB1 (Table 3.a). Of the 17 isolates, 16 were catalase positive, whereas amylase, oxidase, and nitrate reductase activity were less common among the isolates (Fig. 3). Carbohydrate fermentation test showed that most of the isolates were

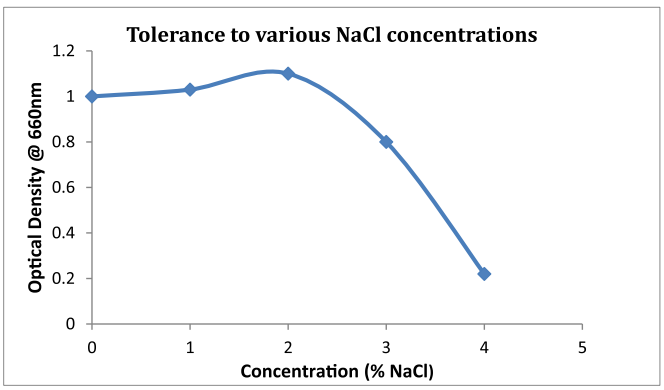

Fig. 5. Tolerance to different NaCl concentrations.

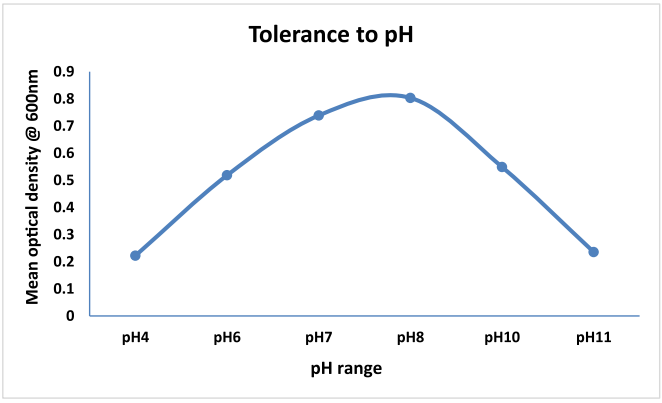

Fig. 6. Tolerance of isolates to different pH.

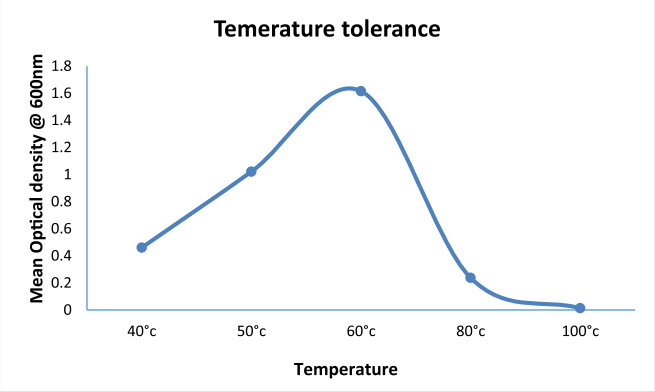

Fig. 7. Tolerance of isolates to various temperatures.

active utilizers of simple sugars like dextrose, maltose, ribose, fructose, mannitol, while they were unable to utilize complex sugars like sucrose, arabinose, raffinose, cellobiose, and dulcitol (Fig. 4). Physiological analysis showed the isolates could tolerate a wide range of temperature, pH and NaCl concentration as mentioned in Figs. 5–7. Growth was observed up to 5% of NaCl concentration (Fig. 5) while interestingly the isolates were able to grow both at acidic and alkali condition of pH ranging from 4 to 11. However, optimum pH for most of the isolate was pH 8.0 though few isolates showed growth up to pH 11.0 (10PHP1, 10PHP2, and 10PHB1) (Fig. 6). The isolates could actively grow in the temperature gradient of 30 °C −80 °C. However, most of the isolates showed the optimum temperature of 60 °C (Fig. 7).

3.4. Molecular identification and phylogeny

Molecular identification showed the singular dominance of phylum *Firmicutes*. Major genus found in the study is *Geobacillus* with a few representatives of genus *Anoxybacillus*. Identified isolates of *Geobacillus* were as *G. lituanicus* TP11, *G. toebii* TP3/TP5, *Parageobacillus toebii* 10PHP2, *G. Kaustophillus* YTPB1, *G.* sp., BPP2, and *G sp*., TB7. The representative isolates of genus *Anoxybacillus* were *Anoxybacillus gonensis* TP9 and *Anoxybacillus Caldiproteolyticus* TRB1. The alignment and similarity search of 16S rRNA sequence with nr/nt database of NCBI have shown that many of the isolates have a distinct percentage of identity from <95%. These results suggested the novelty of these isolated bacteria. The identified species, the percentage of identity and their NCBI Accession numbers are given in Table 3.b. The phylogenetic tree was made by Neighbor-joining method using the jukes-cantor model as shown in Fig. 8.

Table 3.b
Identification of bacteria isolates based on 16SrRNA, there percentage identity and accession numbers.

Isolates	Identification based on 16SrRNA	% identity	Accession No.
TP3	*Geobacillus toebii* TP3	94	MG603308
TP2	*Geobacillus* sp. TP2	91	MG603309
BPP2	*Geobacillus* sp. BPP2	91	MG603313
TP5,10PHP1	*Geobacillus toebii* TP5	90/88	MG603315
10PHP2	*Parageobacillus toebii* 10PHP2	95	MG731573
TP9	*Anoxybacillus gonensis* TP9	100	KX894322
TP11	*Geobacillus lituanicus* TP11	99	MG603317
BPP1	Geobacillus *toebii* BPP1	95	MG731574
TB10	*Geobacillus* sp. TB10	91	MG603310
TB7	*Geobacillus* sp.TB7	91	MG603311
TB3	*Geobacillus toebii* TB3	90	MG603312
BPB1	*Geobacillus* sp.BPB1	90	MG603314
10PHB1	*Geobacillus toebii* 10PHB1	90	MG603316
YTPB1	*Geobacillus kaustophillus* YTPB1	99	MG603318
TRB1	*Anoxybacillus caldiproteolyticus* TRB1	98	MG603319
TB9	*Geobacillus* sp.TB9	93	MG731576
TB1	*Geobacillus* sp.TB1	95	MG731575

3.5. PLFA studies

The major fatty acids significantly varied among the Polok and Borong hot springs. It was found that the straight chain fatty acids were abundant in case of Borong (11.28) as compared to Polok (3.34), whereas branched-chain fatty acids were similar in both the springs (B – 26.79; P – 27.07). However, the polyunsaturated fatty acids (PUFA) were relatively higher in case of Polok as shown in Table 4. The PLFA results showed that the two hot springs, i.e., Polok and Borong were considerably different with respect to their biomass content. The biomass content of Borong was higher (1044.939 nmoles/g) than that of Polok hot spring (838.859 nmoles/g) (Table 5). Fatty acid marker analysis with Sherlock PLFA tool defined the community structure of two springs with the abundance of Gram-positive bacteria, Gram-negative bacteria, anaerobic bacteria, fungi, and eukaryotes. The results showed that Gram-positive bacteria were relatively higher in Borong hot spring (30.03%) than the Polok (27.94%), while Gram-negative bacteria were higher in Polok (7.17%) than the Borong (4.85%). The percentage of Fungi and Eukaryotes were similar in both the hot Springs as shown in Table 5 and Fig. 9. The abundance of various fatty acid types was also investigated and it was found that there was no much distinction between the fatty acids present in both the hot springs (Supplementary S2). The fatty acid found in the springs were 15:3 ω3c, 15:0 anteiso, 16:00, 17:1 iso ω9c, 17:0 anteiso, 18:2 ω6c, 18:1 ω9c, 19:3 ω3c and 21:3 ω3c. However, the fatty acid 12:0 was abundant in Borong hot spring which was not recorded in Polok. The abundance of various fatty acids was represented by plot matrix as shown in Fig. 10. The principle component analysis (PCA) of the fatty acids showed 15:3 ω3c, 15:0 anteiso, 17:0 anteiso, 19:3 ω3c and 21:3 ω3c are positively correlated between the two springs while 12:0 fatty acid was correlated to the Borong (Fig. 11).

3.6. Metagenomic analysis

3.6.1. Diversity index and rarefaction curve

The diversity indices such as Shannon H, Fisher Alpha, and Chao1 were estimated using PAST and EstimateS software packages. The results have shown that the Polok is more diverse than Borong hot spring. The Shannon index was 3.54 and 2.78 for Polok and Borong respectively (Fig. 12). The Chao1 index was also higher in case of Polok hot spring (Table 6).

Rarefaction allows the calculation of species richness in a sample. The curve is a plot of a total number of species annotated as a function of the number of sequences sample (Das et al., 2017). The steep slope at the beginning on the left side signifies most common species have been identified and the plateau at the right side signifies further intensified sampling could lead to the identification of few rarest species (Fig. 13)

3.6.2. Metagenomic studies

The 16 s rRNA gene library of Borong was comprised of 3, 72,480 reads with average sequence length of 301 bp. The average GC content was 53%. While, 3, 98,782 reads were obtained from 16 s rRNA gene library of Polok hot spring with an average sequence length of 301 bp. The mean GC content was estimated to be 54%. A total of 409 OTUs were clustered using UClust. Approximately, 104 OTUs were obtained from Borong hot spring and 360 OTUs were obtained from Polok hot spring. Annotation with reference library showed, both the hot spring possesses lesser amount of archaeal communities (<1% but they are significantly distinct). Borong hot spring consisted of *Crenarchaeota* (0.96%), whereas Polok had *Euryarchaeota* (0.56%). The order and genus under *Crenarchaeota* were identified as *Desulfurococcales* and *Desulfurococcus* respectively and in case of *Euryarchaeota*, the order and genus were identified as *Methanomicrobiales* and *Methanospirillum*. The bacterial community showed significant variation between the springs. The phylum wise diversity showed the dominance of *Proteobacteria* (62.50%), *Bacteroidetes* (15.38%), *Acidobacteria* (3.85%),

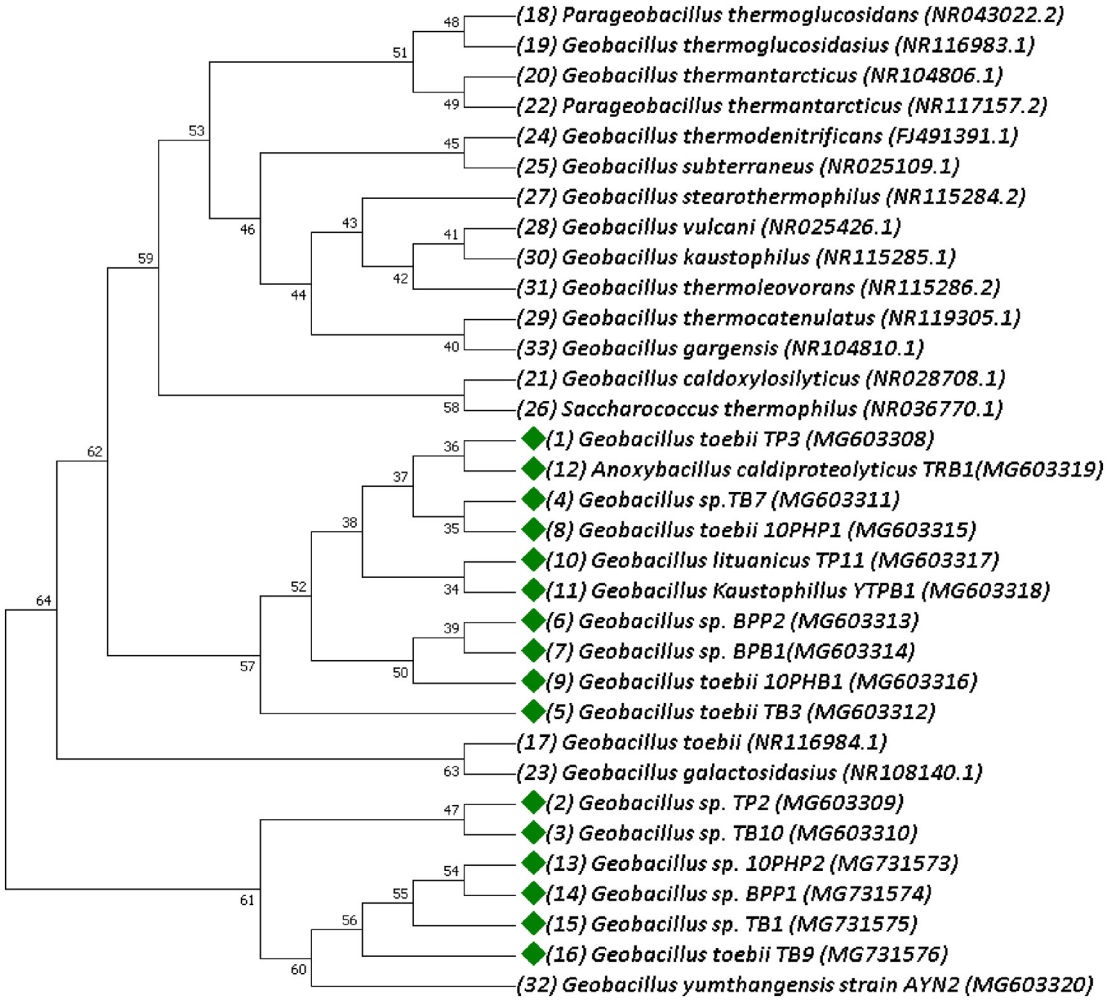

Fig. 8. Phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.39 is shown. The evolutionary distances were computed using the Jukes-Cantor method [2] and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There. Evolutionary analyses were conducted in MEGA7.

Nitrospirae (3.85%) and *Firmicutes* (2.88%) in Borong whereas Polok was dominated by *Proteobacteria* (47.22%), *Bacteroidetes* (3.61%), *Firmicutes* (3.06%), *Parcubacteria*(3.06%) and Spirochaetes (2.50%) (Figs. 14, 16). The major genus present in Borong hot spring was *Acinetobacter* (7.69%), *Flavobacterium* (3.85%), *Vogesella* (3.85%), *Ignavibacterium* (2.88%), *Sediminibacterium* (2.88%), *Thermodesulfovibrio* (2.88%) and *Acidovorax* (1.92%). While the major genera in Polok was *Flavobacterium* (3.33%), *Parcubacteria genera Incertae sedis* (3.06%), *Sediminibacterium* (2.78%), *Pseudomonas* (1.67%), *Treponema* (1.68%) and *Opitutus* (1.39%) Figs. 15, 17.

3.6.3. Correlation of bacterial diversity and geochemistry of Polok and Borong hot spring

Principle component analysis was accomplished to analyze the correlation between bacterial diversity at phylum level and geochemistry of the two hot springs. The top five phyla from both the hot springs and geochemical parameters such as temperature, pH, alkalinity, Ca^{+2}, Mg^{+2}, Cl^{+2}, and sulfur were studied as shown in Fig. 18. The PCA revealed that the community composition was differentially correlated to various concerned geochemical parameters. The *Proteobacteria* were highly correlated to temperature, whereas, *Bacteroidetes* showed a high correlation to chlorine and alkalinity. Contrary, Ca^{+2}, Mg^{+2}, sulfur, and pH were positively correlated with *Parcubacteria*, *Firmicutes*, and *Spirochaetes*. However, *Parcubacteria*, *Firmicutes*, and *Spirochaetes* were negatively correlated with temperature, chloride and alkalinity.

3.6.4. Comparison of microbial diversity

Microbial community structure of hot springs reported earlier from different regions of northeast India (Panda et al., 2015) were compared with our studies using heatmap plot with Bray Curtis dissimilarity method. Phylum level diversity comparison showed that the community structure of Borong and Polok were correlative with the Yumthang hot spring of Sikkim (Panda et al., 2017) as earlier reported. Whereas, Jarkem hot spring of Meghalaya showed comparatively different microbial community structure. The major phylum of hot springs Polok and Borong are *Proteobacteria* and *Bacteroidetes* while major phylum of Jarkem is *Firmicute* sand *Chloroflexi*. This difference indicates possible role of geographical location in shaping the microbial community (Fig. 19.)

Table 4
Major Fatty acids types present in both the hot springs.

Hot spring sample info	Straight 001: straight	Branched 002: branched	Cyclo 004: cyclo	MUFA 005: MUFA	PUFA	DMA	18:1 w9c	18:2 w6, 9c	10-methyl	Hydroxy
Borong	11.28	26.79	–	3.04	55.32	0.54	1.25	1.79	–	–
Polok	3.34	27.07	–	4.75	60.46	0.94	1.44	1.98	–	–

Table 5
PLFA analysis of two hot springs. Fatty acid types converted to microbial groups by Sherlock software.

Hot spring	Gram positive	Gram negative	Anaerobe	Actinomycetes	AM fungi	Fungi	Methanobacter	Eukaryote	Total biomass (nmoles/g)
Borong	30.03	4.85	0.61	–	–	2.03	–	62.41	1044.9
Polok	27.94	7.17	0.43	–	–	2.05	–	62.41	838.8

4. Discussion

The systems with a continuous circulation of heat and fluid, where fluid enters the reservoir from the recharge zones and leaves through discharge constitute geothermal fields. The unique spots within these fields are mainly hot springs, which are present all over the world. Although the hot springs are not merely the ponds where hot water oozes out, these thermal springs have been in use for religious and/or medicinal tenacities before 2000 BCE in India and for hundreds of years in China, Egypt, Japan, Turkey and in many European and Middle-Eastern countries as shown by archaeological marks (Olivier and Jonker, 2013). Many thermal springs urbanized into thriving centers of religion, culture, and health, such as those at Bath in England, Vichy in France and Baden-Baden in Germany (Olivier et al., 2011).

The main importance of these hot springs is the microbiota present in them. The microorganisms present in such hot springs can thrive under extreme temperatures. These microorganisms are known as thermophiles, which can grow into the temperatures above 45 °C (Stetter, 1999). The ideal growth temperature of the thermophiles may vary from 45 °C to above 100 °C (Andrade Jr et al., 1999). Some extremophiles have been known for >40 years, but the detection and isolation of new thermophilic microorganisms have increased for the last decades or ever since thermophilic bacteria were first discovered in the 1960's (Rampelotto, 2013). Many bacteria and archaea have been isolated from hot springs. *Aquificales* are the dominant group of thermophilic bacteria. Also, *Thermotoga*, *Thermus* (*T. thermophilus*), *Bacillus*, *Clostridium*, *Synechococcus*, *Chloroflexus* etc., have also been identified from many hot springs. In environments with a temperature above 90 °C, archaeal communities are dominating (Reysenbach and Shock, 2002). Archaea such as *Methanopyrus*, *Pyrodictium*, *Thermoproteus*, *Methanothermus*, *Archaeoglobus*, *Thermofilum*, *Thermococcus*, *Sulfolobus* etc. have been isolated from many such springs (Rampelotto, 2013; Stetter, 1999). It is important to study the thermophiles of hot springs as their chemical constituents and various metabolites are stable especially at high temperature. Thermophilic enzymes of these microorganisms got a special attention from the scientists from all over the world. These enzymes not only withstand high temperature but are also stable at different pH and resistant to different kinds of chemical reagent. These thermophilic enzymes have vast applications in industries and also in genetic engineering. Thus it is of great importance to investigate the microflora of these hot springs by culture-dependent and culture-independent techniques to find new promising enzymes or protein compounds.

The biodiversity of several hot environments such as hot springs, oil reservoirs, composter were studied using shotgun metagenomics sequencing.

(Kotlar et al., 2011; Martins et al., 2013; Mehetre et al., 2016). These new molecular methodologies have enhanced the microbial ecology studies by aiding in the analysis of comprehensive microbial community structure of an environment. Geothermal craters in earth crust are naturally diverse in microbial community structure. However, due to the uncultivated status of the major taxa in hot springs, culture-dependent analysis led to the identification of few distinct genera from the Polok and Borong hot springs. The major or the singular dominant phyla identified was *Firmicutes* and the major genus identified was *Geobacillus*. Homology with reference strains from nucleotide database of NCBI showed identity percentage as low as 93%, which predict the possibilities of finding novel species from the springs. The various microorganisms identified were *G. lituanicus* TP11, *G. toebii* TP3/TP5, *Parageobacillus toebii*10PHP2, *G. Kaustophillus* YTPB1, *G.* sp., BPP2, *G* sp., TB7, *Anoxybacillus gonensis* TP9 and *Anoxybacillus Caldiproteolyticus* TRB1. The genus *Geobacillus* is earlier reported from the Himalayan geothermal provinces of Himachal Pradesh (Mehta et al., 2012; Sharma et al., 2013) and Uttarakhand regions (Dheeran et al., 2010). But, to the best of our knowledge, it is the first report of finding the *Geobacillus* species from the hot springs of the northeastern region of India. Apart from Himachal, there was no such known report of *Geobacillus* from other geothermal provinces in India such as Sohana geothermal province (Sareen and Mishra, 2008), west coastal geothermal province (Mehetre et al., 2016), Mahanadi geothermal province (Badhai et al., 2015). The biochemical characterization indicated the isolated bacteria could use a wide range of carbon and nitrogen sources for energy. Most of the strains are able to utilize simple sugars like dextrose, maltose, fructose, mannose etc. The physiological characterization showed the thermophilic nature of the isolates. The isolates could grow in wide range of temperature (30–80 °C) with optimum of 60 °C. The pH-dependent growth analysis showed acidic to extreme alkali nature of the isolates which could actively survive and grow in pH 3–12. Correlative results could be found from the study of Pandey et al. (2015). Isolated bacteria from Soldhar and Ringigad hot springs of Uttarakhand, India exhibited tolerance to a wide range of temperature (20–80 °C), from mesophilic(+20° to +45 °C) to thermophilic (+46° to +75 °C); and few in hyperthermophilic range (+76 °C). The isolates also had a wide range of pH (4–14) and moderate salt tolerance (Pandey et al., 2015). The possible reasons could be hypothesized that this wide range of temperature and pH tolerance shown by these bacteria may be due to their presence in fluctuating surrounding environments at different geothermal heights. The hot waters present in reservoirs deep down to earth flows out through high pressures. The geochemistry of water as in the deep-down geothermal point's get changes in the surface with changing physicochemical parameters. The bacteria present may come in contact with varying water contents with varying physicochemical composition at varying heights. Thus these fluctuating environments with respect to distinct physicochemical contents help these bacteria to become tolerant and adaptive to the changing environment.

The conventional culture-dependent methods fail to give comprehensive microbial community structure of an environment due to the

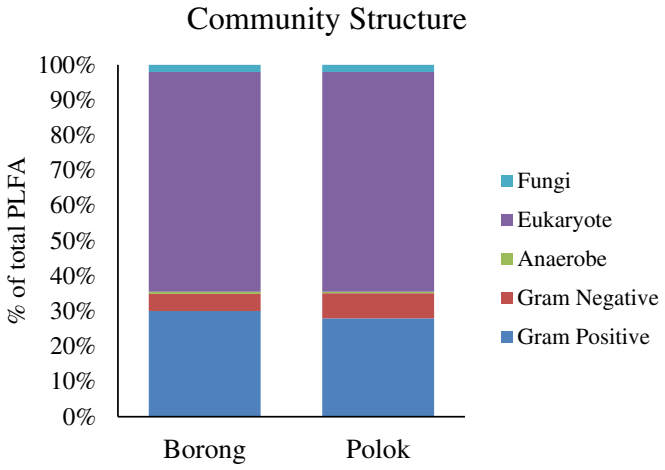

Fig. 9. Community Structure of two hot springs based on PLFA analysis.

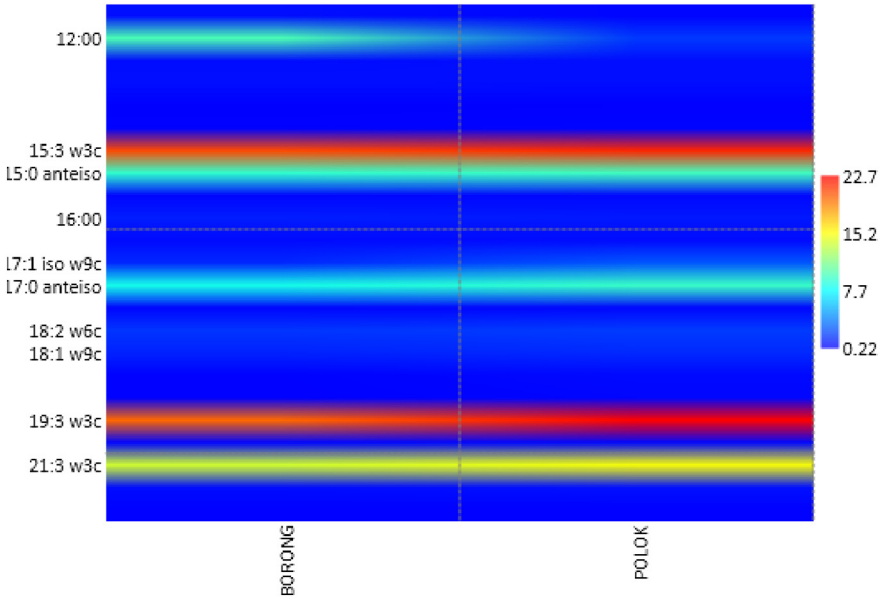

Fig. 10. Abundance of various fatty acids in Borong and Polok hot springs.

large unculturable status of the microbial world. The development of wide range of culture-independent methods has widened the scientific knowledge about previously unknown microorganisms and their involvement in different biological processes. Culture-independent techniques such as PLFA analysis which was a pioneer in the field and first used in 1979 to estimate the microbial biomass from marine sediments (White et al., 1979). This technique is important to determine viable microbial biomass, microbial community composition and metabolic activity in an environment (Rzonca and Schulze-Makuch, 2003). Phospholipid fatty acids (PLFA) are an essential structural component of microbial cell membrane, which makes it an important chemotaxonomic marker. PLFA analysis is widely used to estimate the total biomass and to observe the changes in community composition of the microbiota in soil and aqueous environment. The PLFA results have shown the abundance of Gram-positive bacteria in both the hot springs. Borong had 30% of Gram-positive bacterial community while Polok had 27%. Gram-negative bacteria were 4% and 7% respectively in Borong and Polok. The Anaerobes, Fungi, and Eukaryotes were equally abundant in both the hot springs. The various fatty acids abundant in both the hot springs were 15:3 ω3c, 15:0 anteiso, 16:00, 17:1 iso ω9c, 17:0 anteiso, 18:2 ω 6c, 18:1 ω 9c, 19:3 ω 3c and 21:3 ω 3c. The abundant fatty acids showed the dominance of biomarkers for Gram-positive bacteria such as 15:0 anteiso, 16:00, 17:0 anteiso, 18:0 (Willers et al., 2015). The total biomass of Borong hot spring (1044.9 nmoles/g) was higher

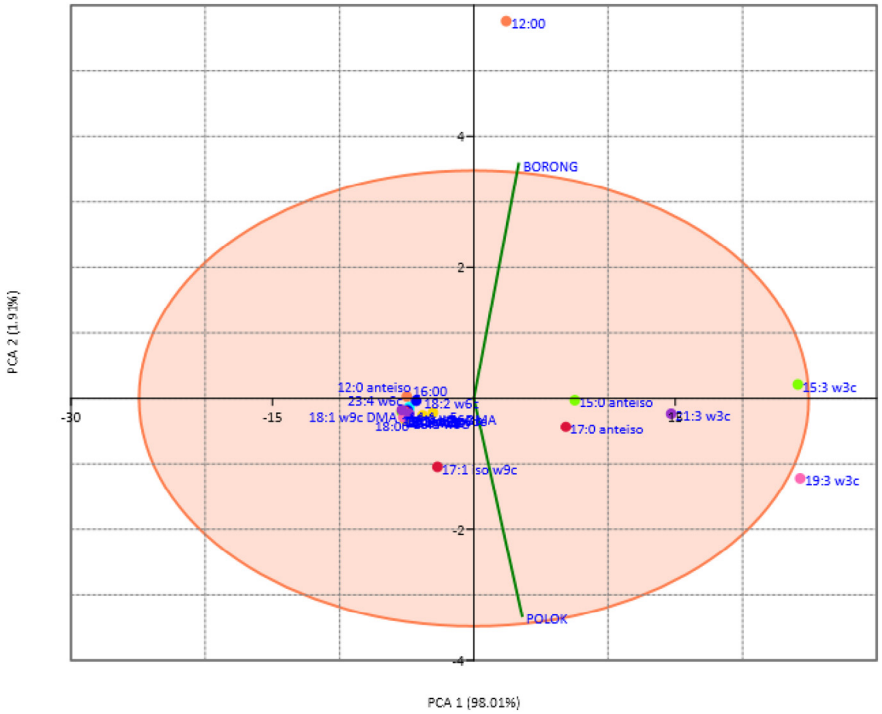

Fig. 11. Principle component analysis of various fatty acids with respect to Polok and Borong hot springs.

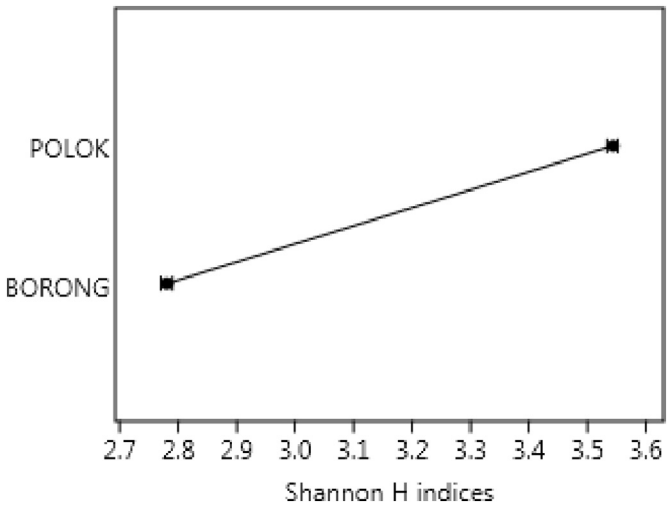

Fig. 12. Representation of the abundance of bacterial diversity in both the hot springs with Polok having higher Shannon H index than Borong hot spring.

than that of Polok hot spring (838.8 nmoles/g). The similar studies have been performed by (Rzonca and Schulze-Makuch, 2003), however, they have found an abundance of Gram-negative bacteria than that of Gram-positive bacteria.

The metagenomic studies revealed the major dominance of *Proteobacteria* and *Bacteroidetes* in both the hot springs. The *Proteobacteria* constituted 62.50% and 47.22% of the total community in Borong and Polok hot springs respectively. The second abundant phylum in both the hot springs was *Bacteroidetes* which constituted 22.78% and 15.38% of the metagenome library of Polok and Borong hot springs respectively. In many geothermal environments, the phylum *Proteobacteria* and *Bacteroidetes* have been found as the dominant phylum (Amin et al., 2017; Lau et al., 2009; López-López et al., 2015). An earlier report on the microbial diversity of the Yumthang hot spring of north Sikkim similarly recorded the abundance of *Proteobacteria* (83.68%) and *Bacteroidetes* (10.93%) as the dominant phyla (Panda et al., 2016).

As the main characteristic feature of any hot spring is temperature, thus, the abundance of a particular phylum in the hot springs has been correlated and interpreted by many researchers as a function of temperature. Subudhi et al. (2017) have shown the predominant shifting of thermophilic cyanobacteria as a function of temperature and also, have shown the abundant growth of different strains at different temperatures (Subudhi et al., 2017). Similarly, Sahoo et al., 2017, have correlated and linked the predominant nature of Proteobacteria in the hot springs of Odisa, India, as a function of temperature (Sahoo et al., 2017). In our study, the phylum level diversity of two hot springs is similar to a great extent, but the relative abundance is considerably different. Borong hot spring is having higher abundance of Proteobacteria than that of Polok hot spring. These hot springs are located in the same area, only few miles apart and at a similar altitude and similar chemical constituents. Thus having similar geographical and geological features, this migt be the reason for having similar bacterial diversity in the two hot springs. However there is a considerable difference in their temperature. The phylum Proteobacteria are known to dominate in moderate temperatures (Wang et al., 2013). Since, Borong possess the lower temperature than Polok hot spring and therefore, the function temperature may be the reason for proteobacterial predominance in Borong hot spring. The other characteristic feature of phylum Proteobacteria is that they are known to tolerate higher concentration

Table 6
Diversity indices of hot spring microbial communities.

Hot springs	Total number of reads	G + C content	Average sequence length	Total number of OTUs	Shannon H index	Fisher alpha	Chao1
Borong	372,480	53	301	104	2.78	11.46	104
Polok	398,782	54	301	360	3.54	44.5	360

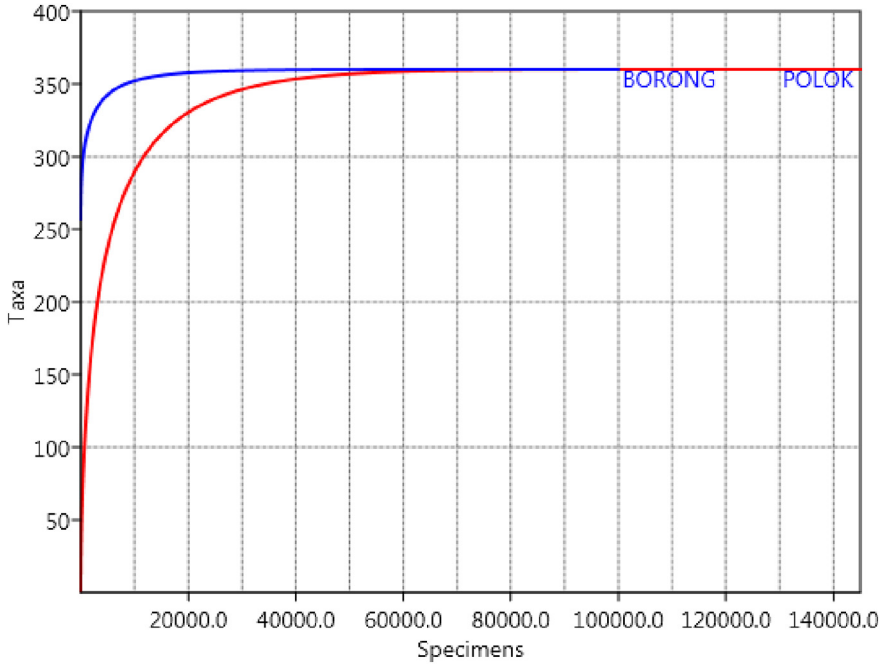

Fig. 13. Rarefaction curve, red curve shows species richness in Polok Hot Spring whereas blue line represents Borong hot spring.

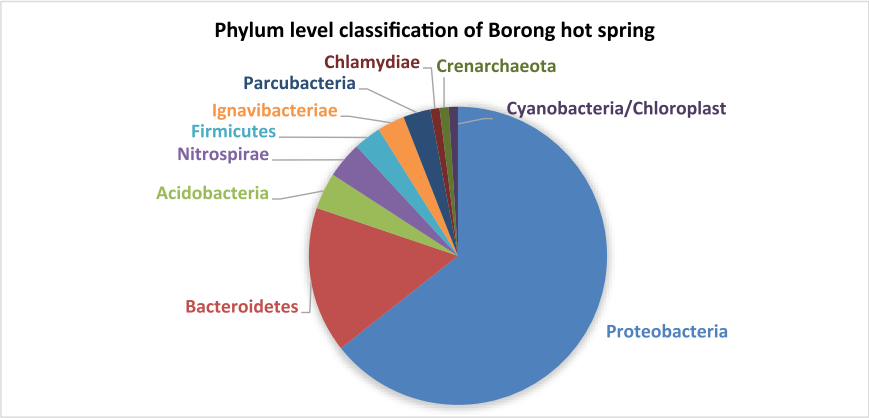

Fig. 14. Phylum level classification of Borong hot spring.

of sulfur and utilize sulfur as an electron donor during their physiological process (Bolhuis et al., 2014). Therefore, it is not surprising that Borong hot spring has higher abundance of proteobacteria, as Borong hot spring possess high sulfur content then Polok hot spring which was confirmed by ICPMS results. This correlation of temperature and sulfur concentration to phylum Proteobacteria has been supported by our PCA results which is in accordance with Sahoo et al., 2017 (Sahoo et al., 2017).

Subdivision of *Proteobacteria* to class level hierarchy showed the presence of *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* and *Deltaproteobacteria* in both the hot springs with the dominance of *Gammaproteobacteria*. The *Epsilonproteobacteria* was least while zetaproteobacteria was not recorded. Following the *Proteobacteria* and *Bacteroidetes*, the other dominant phyla were quite distinct with respect to each spring. *Verrucomicrobia* (3.61%) and *Firmicutes* (3.06%) are the third and fourth dominant bacterial phyla in Polok followed by *Parcubacteria* (3.06%) and *Spirochaetes* (2.50%). However, Borong hot spring was dominated by *Acidobacteria* (3.85%) and *Nitrospirae* (3.85%).

The major genus present in Borong hot spring was *Acinetobacter* (7.69%), *Flavobacterium* (3.85%), *Vogesella* (3.85%), *Ignavibacterium* (2.88%), *Sediminibacterium* (2.88%), *Thermodesulfovibrio* (2.88%) and *Acidovorax* (1.92%). While the major genera in Polok was *Flavobacterium* (3.33%), *Parcubacteria genera Incertaesedis* (3.06%), *Sediminibacterium* (2.78%), *Pseudomonas* (1.67%), *Treponema* (1.68%) and *Opitutus* (1.39%). Most of the genus represented was Gram-negative in nature. The genus *Acinetobacter* possess Gram-negative, non-fermentative bacteria which are ubiquitous organisms in soil, water, and sewage. These are known for the biopolymers and biosurfactant production (Towner, 2006). The genus *Flavobacterium* contains Gram-negative aerobic rods that are motile by gliding and are widely distributed in soil and freshwater habitats (Bernardet et al., 1996). The genus *vogesella* contains singular or paired short chained bacteria. The colonies are a deep royal blue with a metallic copper-colored sheen, due to the production of indigoidine (Krieg, 2015). The genus *Ignavibacterium* possess Gram-negative bacteria which are strictly anaerobic, moderately thermophilic, neutrophilic and obligately heterotrophic in nature (Iino et al., 2010). The genus *Sediminibacterium* belongs to phylum *Bacteroidetes* which includes Gram-negative, strictly aerobic, rods, motile by gliding and having isoC15: 1 G and isoC15: 0 as the major cellular fatty acids (Kang et al., 2014). The genus *Thermo desulfovibrio* was described as a group of obligately anaerobic, curved rod-shaped, thermophilic bacteria that reduce sulfate and other sulfur compounds (Maki, 2015). At species level, the diversity of bacteria is quite distinct in both the hot springs. However, the uncultured bacteria dominated both the hot springs. The top five species of Polok hot spring were *Sediminibacterium goheungense*, *Opitutusterrae*, *Treponema caldarium*, and *Ignavibacterium album*. Top five species of Borong hot spring were *Ignavibacterium album*, *Thermodesulfovibrio yellowstonii*, *Flavobacterium cheonhonense*, *Rheinheimera aquatic*, and *Sediminibacterium goheungense*.

Comparison with the reported microbial diversity of hot springs from different provinces of India showed a significant pattern of differentiation and correlation along the community structure. This difference in microfloral diversity may be due to the geographical and geochemical distinction among the hot springs. The heatmap constructed with Bray Curtis Dissimilarity distance showed two major clades or groups with two sub-clades each and one out-group. Four of the Tibetan hot springs (Rongma, Gulu, Jiwa1 and Jiwa2) (Huang et al., 2011) and two hot springs from Barkeshwar, West Bengal, India (Bengal et al., 2017), Junagarh, Gujrat, India (TulsiShyam) (Ghelani et al., 2015), one from Shillong, Meghalaya, India (Jakrem) (Panda et al., 2015) and one from Odisha (Atri) (Badhai et al., 2015), formed a single group, where the diversity of Atri and Jakrem was similar with the dominance of *Chloroflexi*. The other hot springs formed the second sub group

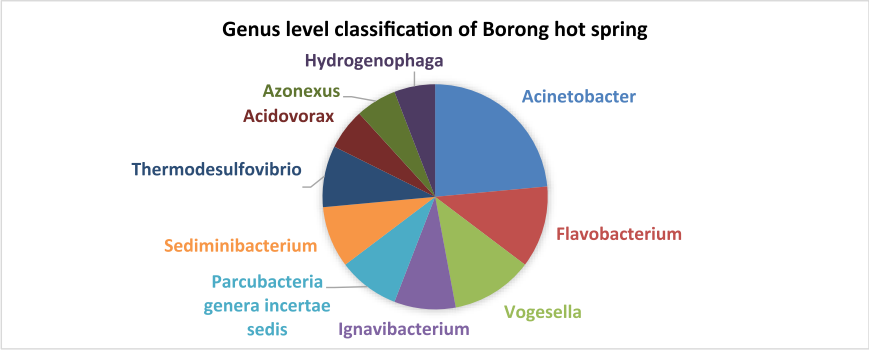

Fig. 15. Genus level classification of Borong hot spring.

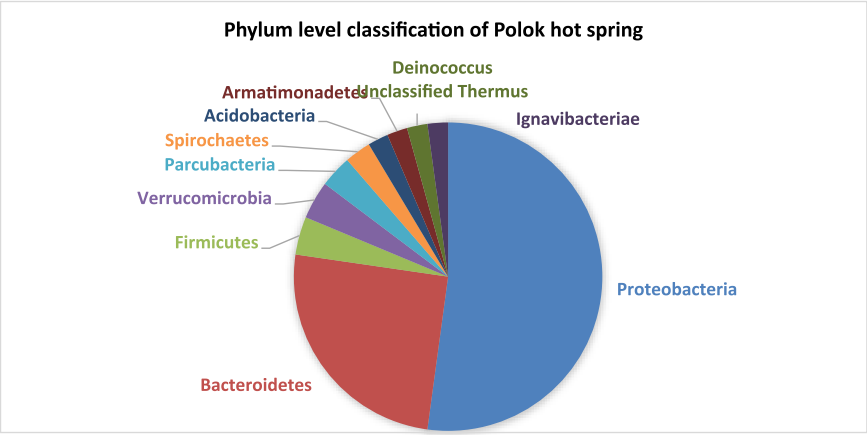

Fig. 16. Phylum level classification of Polok hot spring.

where the dominant group is *Firmicutes*. The Rongma of Tibet formed a single branch in the second subgroup of first clade as it the only spring in first group with dominance of *Acidobactera* and *Bacteroidetes*. The second clade was made by seven hot springs including three hot springs of Sikkim (Polok, Borong and Yumthang) and one from Shimla, Himachal (Tattapani) (Mohanrao et al., 2016), two hot springs from Odisha, India (Athamallik and Tarabalo) (Badhai et al., 2015), and one from Tibet (Gulu 2) (Huang et al., 2011) respectively. The hot springs from the second clade were dominated by *Proteobacteria*. Polok and Borong in the second clade showed correlative diversity with the dominance of *Proteobacteria* and *Bacteroidetes*. Anhoni hot spring from Madhya Pradesh, India (Saxena et al., 2017) formed the out-group, where the relative abundance of the phyla was least in comparison to the other springs.

In the present study, the culture-dependent taxonomic profiling and PLFA studies showed a positive correlation. Both the methods showed the dominance of Gram-positive bacteria over Gram-negative in the hot springs. Some of the additional studies with environmental samples from our lab (unpublished data) also supports the similar findings, where PLFA and culture dependent data are correlative. The similar results were found by Pandey et al., 2015. They have also found only Gram positive bacteria while investigating the diversity of two hot springs of Uttrakhand (Pandey et al., 2015). However, the NGS data was relatively contradictory to both the PLFA and culture-dependent approach. The dominant bacterial phylotypes in the metagenome library were Gram-negative (*Proteobacteria*). The earlier findings of dominant Gram-negative bacteria with a metagenomic approach from the *Yumthang* hot springs of Sikkim correlates with our results (Panda et al., 2017) In order to limit the contradictions, various media were used to culture the enriched samples along with different pH and temperature. However, the results were synonymous. Thus a probable reason for these findings can be hypothesized that the ubiquitous nature of phylum *Firmicutes* (the dominant phyla in culture-dependent study) and their ability to adopt a wide range temperature, pH, and salinity makes them stable and culturable. But, the large dominance of unculturable Gram-negative bacteria (*Proteobacteria*) in the environment masked the dominance of Gram-positive phyla (*Firmicutes*) in the metagenomic study. Also the present study including the two different culture independent techniques i.e., PLFA and metagenomics which are based on the analysis of two different molecules such as fatty acids and DNA respectively. It is also evident that the DNA is more stable than phospholipid fatty acids as the later gets degraded immediately after the decease of bacteria. Whereas in case of metagenomics study, the DNA of both the live and dead bacterial cell are present and investigated. Thus this makes it important to study the microbial diversity of any ecological niche with the help of diverse techniques.

Geochemical parameters have often been the driving factor in shaping the microfloral community in such environment holds an important aspect of such studies. Several researchers in order to determine the evolution of thermophilic microbial communities have compared the metagenomic data with geochemistry of hot springs (Rzonca and Schulze-Makuch, 2003). The piper diagram for physicochemical parameters revealed, both the hot springs are Ca-HCO^{3-} type and can be predicted as shallow fresh ground waters. However, in veracity, sampled spring waters almost always represent mixtures from deep thermal fluids diluted by more shallow ground waters, therefore they must be considered as deep geothermal waters derivatives (Goff and Grigsby, 1982). The correlation between various physicochemical parameters

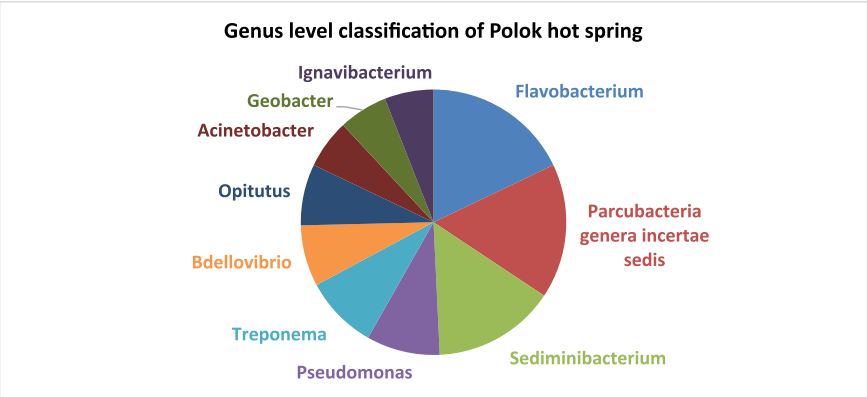

Fig. 17. Genus level classification of Polok hot spring.

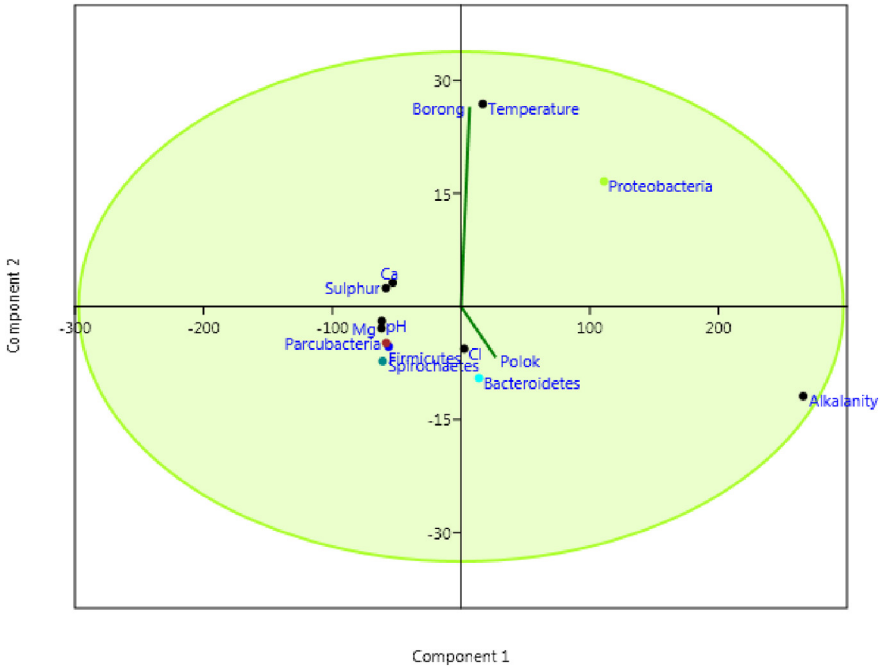

Fig. 18. Principle component analysis (PCA), to analyze the correlation between bacterial diversity at phylum level and geochemistry of the Polok and Borong hot springs.

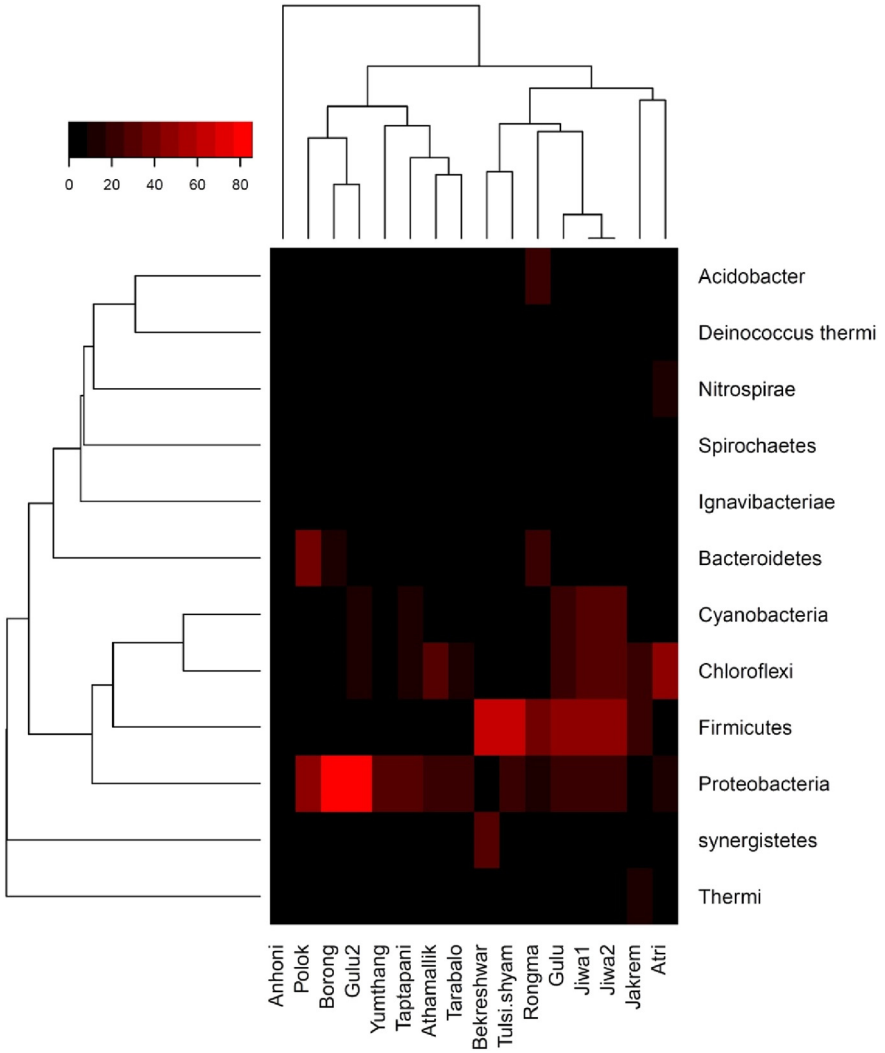

Fig. 19. Heat map (Comparative analysis of top phylums among various hot springs of central India, North-east India and Tibet).

and bacterial diversity from both the hot springs showed chlorine, alkalinity was positively correlated with *Bacteroidetes* and temperature is closely correlated with the abundance of *Proteobacteria*. These results are supported by earlier studies (Rzonca and Schulze-Makuch, 2003; Panda et al., 2016). They have found the significant correlation between community composition and various physicochemical parameters such as temperature, dissolved SiO_2, elemental sulfur, total sulfide, and calcium.

5. Conclusion

Metagenomic analysis revealed a wide and diverse bacterial population in both the Polok and Borong hot springs of Sikkim. These hot springs of Indo-Tibetan plateau are home to many possibly unknown and novel microbes as indicated by the abundance of 16.35% and 15.28% uncultured bacteria in Borong and Polok respectively. However, the most abundant phyla present were *Proteobacteria* and *Bacteroidetes*. The PLFA analysis showed the abundance of Gram-positive bacteria and eukaryotes in both the hot springs. Also the culture dependent 16S rRNA analysis showed the abundance of genus *Geobacillus* followed by *Anoxybacillus*. The correlation of physicochemical characteristics with most dominant phyla suggested that various physicochemical parameters such as temperature, pH, alkalinity, Ca^{+2}, Mg^{+2}, Cl^{+2}, and sulfur content shapes the microbial community composition and diversity. The piper diagram suggested that the water of both the hot springs are Ca-HCO^{3-} type and can be predicted as shallow fresh ground waters. To the best of our knowledge this is the first study which revealed the microbial diversity of Polok and Borong hot springs of Sikkim. The results of this study significantly expand the understanding of the microbial community structure of Polok and Borong hot springs and provided a basis for comparative analysis with other geothermal systems.

Conflict of interest

Authors have no conflict of Interests.

Author's contribution

NT designed the study, INN did the experimental works, MTS and SKD helped in the sample collection and field study, INN and SD did the analysis and prepared the manuscript, NT reviewed and edited the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.05.037.

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