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MICROBIAL DIVERSITY AND PHYSICO-CHEMICAL ATTRIBUTES OF TWO HOT WATER SPRINGS IN THE GARHWAL HIMALAYA, INDIA

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ABSTRACT

The water samples were collected from two different hot water springs (Ringigad and Saldhar) of the Garhwal Himalaya for a period of two years. During the study period, the water sample was collected two times (June and August) each year for the analysis of physico-chemical attributes and isolated microorganisms including bacteria, archaea and fungi. A total of fifteen physico-chemical parameters were studied during the study period that includes the water temperature, dissolved oxygen, pH, conductivity, free carbondioxide, salinity, total dissolved solids, chlorides, total alkalinity, nitrates, sulphates, phosphates, total hardness, calcium and magnesium. Seven bacterial strains along with four archaeal and three fungal strains were isolated and identified in Ringigad thermal spring with α-diversity of 14. However, the α-diversity in Saldhar thermal spring was recorded to be 12 with six bacterial, three archaeal and three fungal strains. The temperature of water ranged between 82^{0}C and 89^{0}C for Ringigad and between 87^{0}C and 92^{0}C for Saldhar.

Keywords: Microbial diversity; Physico-chemical properties; Ringigad; Saldhar; Garhwal Himalaya, India

INTRODUCTION

Microorganisms populate in almost every possible region on the earth from those providing favorable circumstances for their survival and reproduction to those offering the severe or extreme conditions for their growth. The microorganisms that can survive in such extreme conditions are widely known as extremophiles. Among the extremophiles, thermophiles have received a huge attention in recent years because of their capability to survive at a very high temperature; even, they are active at an elevated temperature (**Beg *et al.*, 2000**; **Bharadwaj *et al.*, 2010**; **Akmar et al., 2011**; **Huang *et al.*, 2011**).

Thermophilic microbes are found in various biotopes such as thermal springs, geothermal sediments and marine solfataras (**Rothschild and Manicineli, 2001**; **Pathak and Rathod, 2014**). Thermal springs are having very high water temperature or in other words we can say that the temperature is much more than the surroundings (**Sen *et al.*, 2010**).

Thermal springs are the proofs of geological activity that indicate very high temperature and widely available in the Himalayan region (**Kumar *et al.*, 2004**). Thermal springs are the affluent source of thermophilic microorganisms which can be tapped for various applications in different fields. Thermophilic microbes are containing enzymes that are stable at a very high temperature, which make them useful for the pharmaceutical, biotechnological, food processing and chemical industries (**Tekere *et al.*, 2015**). Physico-chemical properties are also playing an important role in the density and diversity of microbes in the thermal springs.

Though a lot of work has been done on various aspects of thermal springs that includes the works of **Kumar *et al.*, (2004)** on soil microbial diversity from two different thermal springs of Uttarakhand, **Sharma *et al.*, (2008)** on characterization and identification of various strains of *Geobacillus spp*. enumerated from Saldhar thermal spring; **Akmar *et al.*, (2011)** on isolation of new thermophilic bacteria from hot spring; **Bhusare and Wakte, (2011)** on hot water spring of Unkeshwar; **Ghati *et al.*, (2013)** on Esterolytic thermophilic bacteria from an Indian hot spring; **Pathak and Rathod, (2014)** on culturable diversity of bacteria in the thermal spring of Unkeshwar, India; **Rawat, (2015)** on bacterial diversity of a sulphur spring in Uttarakhand and **Tekere *et al.*, (2015)** on bacterial diversity in some African thermal springs. But no work has been done so far on the microbial diversity and physico-chemical attributes in the water of Ringigad and Saldhar thermal springs. Therefore, a maiden attempt has been made to provide basic data on microbial diversity and physico-chemical characteristics of Ringigad and Saldhar hot water spring for further studies.

MATERIALS AND METHODS

Study area

Ringigad is a thermal spring located at 17 Km from Joshimath towards Suraithota (Figure 1) between latitude 30^{0}33'14" N and longitude 79^{0}40'0.06" E at an altitude of 1,850 m above mean sea level in the Chamoli district of Uttarakhand. The approximate area covered by this hot water spring is 45 m^2. The maximum *in-situ* temperature of Ringigad hot water spring is 89^{0}C.

However, Saldhar is also a thermal spring located at 19 Km from Joshimath towards Suraithota (Figure 2) just near to the road on the right side. It is situated between latitude 39^{0}29'25" N and 79^{0}39'29" E at an altitude of 1,900 m above mean sea level in the Chamoli district of Uttarakhand. The approximate area that is covered by this hot water spring was 70 m^2. The maximum *in-situ* temperature of Saldhar is 92^{0}C.

Figure 1 Study area (Ringigad, a thermal spring near Joshimath)

Figure 2 Study area (Saldhar, a thermal spring near Joshimath)

Water sampling

Water of both the thermal springs was sampled during the year 2014 and 2015 in two sampling operations each year. In both the years, the water sample was collected in the month of June and August to observe any possibility of change among the physico-chemical parameters during the dry and rainy months. The main objective of current study is to explore the microbial diversity in two hot water springs. Water samples were collected from the origin place of both the hot water springs in autoclaved thermoflask. However, water samples collected for the isolation and identification of
culturable microbial diversity was placed in an ice box filled with freezed ice packs and analyzed within 24 hours. Few of the water quality parameters like pH, water temperature, dissolved oxygen and free carbon dioxide were measured at the sampling site whereas, for the remaining physico-chemical characteristics, the water samples were transferred to the Laboratory of Environmental Microbiology and Biotechnology, Department of Environmental Sciences, H.N.B. Garhwal University, Srinagar-Garhwal, Uttarakhand, India at its earliest possible. All the physico-chemical characteristics were analyzed by following the standard protocols available in **APHA, (2005)** and microbial diversity by following the standard methods outlined in **Harley and Prescott, (2002)**; **Morello *et al*., (2003)**.

Physico-chemical characteristics

Water samples were analyzed for a predefined set of physical and chemical characteristics. Water temperature was measured by dipping the digital thermometer 10 cm in the hot water of the spring carefully and noted down the readings. The temperature range of digital thermometer was (-50 ^{0}C to +300 ^{0}C)
pH was measured both at the site by using portable pH meter of Electronics India (Model No. 7011) and in the laboratory by using the Toshcon bench top multiparameter analyzer (Model No. TPC-17). Dissolved oxygen was measured by using the modified Winkler method at the sampling site. Conductivity, salinity and total dissolved solids (TDS) were measured by using the Toshcon
Bench Top Multiparameter analyzer (Model No. TPC-17). Free CO_2, total alkalinity, chlorides, total hardness, Calcium and Magnesium were measured by following the protocols outlined in **APHA, (2005)**. Nitrates, Sulphates and Phosphates were measured by Spectrophotometric method by using Systronic UV-VIS Spectrophotometer (model No. 117).

Microbal isolation and enumeration

Nutrient Agar media (HiMEDIA) was used for the estimation of colony forming units (CFUs) of bacteria. Media pH for microbial isolation was adjusted according to the pH of sampling sites. SDA media was used for isolation of fungal species. It was supplemented with 50 mg/l of each (Streptomycin and Ampicillin) to prevent the bacterial contamination. AIA media was used for the isolation of actinomycetes. After the isolation of microbial colonies, each unique colony was streaked on separate media plate to get the pure culture of each microbial colony (**Clesceri *et al.*, 1998**).

Morphological studies

To study the morphological characteristics, the purified selected microbial isolates were observed by naked eyes and under the Phase Contrast Microscope (Nikon Eclipse TS100). Morphology of selected bacterial isolates that was observed in the laboratory is given in Table 1.

Table 1 Morphological and Biochemical characterization of bacterial isolates identified from hot springs of the Garhwal Himalaya

Characteristics	**Bacterial Isolates**					
	Brevibacillus parabrevis	***Bacillus cerus***	***Geobacillus stearothermophilus***	***Streptococcus pyogenes***	***Bacillus cibi***	***Bacillus subtilis***
Shape	Round	Round	Circular	Spherical	Circular	Round
Size	2 mm	2-5 mm	2-3 mm	1 mm	3-4 mm	2-3 mm
Margin	Smooth	Smooth	Entire	Smooth	Irregular	Lobate
Elevation	Flat	Undulate	Convex	Convex	Raised	Flat
Color	Yellowish grey	Grayish yellow	Cream	Translucent	Orange yellow	White
Cell shape	Rod	Rod	Rod	Cocci	Rod	Rod
Spore formation	+	+	+	-	+	-
Motility	Motile	Motile	Motile	Non-motile	Motile	Motile
Grams staining	v	+	+	+	V	+
Flagella	Peritrichous	Peritrichous	Monotrichous	Atrichous	Peritrichous	Peritrichous
Catalase	+	+	-	-	+	+
Citrate	v	+	v	+	+	+
Urease	-	+	-	-	-	-
Methyl Red (MR)	-	-	-	-	-	-
Voges Proskauer (VP)	-	+	-	-	-	+
Fructose	-	+	-	+	+	+
Indole Test	-	-	-	-	-	-
Raffinose	-	-	-	-	+	+
Ribose	+	+	+	-	-	+
Sorbitol	-	-	-	-	-	+
Sucrose	v	v	-	+	+	+
Xylose	-	-	+	-	-	+
Trehalose	+	+	-	+	+	+
Mannose	-	-	-	+	+	+
Mannitol	+	-	+	-	-	+
Lactose	-	-	-	+	-	v
Maltose	+	+	-	+	+	+

Abbreviations: +: positive; -: negative; v: variable

Biochemical tests

Moreover, detailed biochemical characterizations were carried out to identify the bacterial and archeal isolates from the hot water spring, up to possible genus or species level. The result of biochemical tests for selected bacterial isolates recorded in the laboratory is given in Table 1. Identification of all the fungal isolates were made by microscopic analysis by using the taxonomic keys and standard procedures. To confirm the identification of the microbes done in the laboratory, the pure culture of each isolate was collected and then sent for further identification and confirmation to National Centre for Microbial Resources (NCMR), National Centre for Cell Sciences (NCCS), Pune by using the MALDI-TOF MS.

Statistical analysis

Statistical treatment (minimum; maximum; mean; standard deviation) of the physico-chemical parameters of water was conducted.

RESULTS AND DISCUSSION

Physico-chemical characteristics

Data of all the fifteen physico-chemical characteristics obtained under two sampling operations each year during a period of two years (2014-2015) from the Ringigad and Saldhar hot water springs of the Garhwal Himalaya. The data for physico-chemical parameters of Ringigad and Saldhar hot water springs has been presented in Table 2 & 3.

The minimum water temperature of Ringigad thermal spring was recorded 82^{0}C as minimum and 89^{0}C as maximum at the site. The pH of water of Ringigad thermal spring varied from 6.7 to 6.9, indicated the slightly acidic nature of water. Similar range of pH was also recorded by **Kumar *et al.* (2013)** for hot springs of Kullu district in Himachal Pradesh; **Singh *et al.* (2015)** for hot springs of Jharkhand and West Bengal region; **Ghilamicael *et al.* (2017)** for hot springs in Eritrea.

The value of dissolved oxygen (DO) varied from 0.8 mg/l to 1.2 mg/l. Dissolved oxygen concentration is inversely proportional to the temperature. As the temperature of water goes up the concentration of dissolved oxygen in the water goes down (**Rana *et al.* 2018**). **Fazlzadeh *et al.* (2017)** recorded the DO concentration within a range of 3.25 mg/l to 3.57 mg/l for thermal springs in Iran. The electrical conductivity (EC) was ranged from 4.48 mS/cm to 4.68 mS/cm in the water of hot spring. **Haki and Gezmu (2012)** recorded the similar range of electrical conductivity for the hyperthermal springs of Ethiopia. Salinity of the samples ranged from 2.1^{SAL} to 2.3^{SAL} throughout the sampling period. Similar range of salinity was also recorded by **Hamzah *et al.* (2013)** for thermal springs of Malaysia.

The concentration of total dissolved solids (TDS) was ranged between 2.47 mg/l to 2.53 mg/l in the water of Ringigad thermal spring. High range of total dissolved solids was recorded by **Hamzah *et al.* (2013)** for thermal springs of Malaysia. The concentration of free CO_2 was ranged between 35.2 mg/l and 48.4 mg/l during the study period.

The concentration of total hardness varied from 228 mg/l to 234 mg/l. High range of alkalinity (196 mg/l) was recorded by **Kumar *et al.* (2013)** for thermal springs of Kullu district in Himachal Pradesh. The concentration of Calcium varied from 63.2 mg/l to 69.6 mg/l. Magnesium concentration varied from 14.20 mg/l to 17.13 mg/l. A similar range (12.60 mg/l to 15.62 mg/l) of magnesium was recorded by **Singh *et al.* (2015)** for thermal springs of Jharkhand and West Bengal. The concentration of chlorides varied from 11.36 mg/l to 14.2 mg/l concentration observed in the sample. Extreme high range of alkalinity (197.38 mg/l) was recorded by **Kumar *et al.* (2013)** for thermal springs of Kullu district in Himachal Pradesh.

Alkalinity of the water ranged from 270.0 mg/l to 300 mg/l during the study period. However, a very high range (165.2 mg/l) of alkalinity was recorded by Kumar et al. (2013) for thermal springs of Kullu district in Himachal Pradesh. The concentration of nitrates present in the water sample was ranged from 0.239mg/l to 0.256 mg/l. Sulphates concentration were also found within a range of 0.276 mg/l to 0.289 mg/l. The concentration of phosphates in the water sample was ranged from 0.026 mg/l to 0.028 mg/l. A very high range of sulphates and nitrates were recorded by **Sherpa *et al.* (2013)** for thermal springs of Sikkim in India.

The minimum water temperature of Saldhar thermal spring was recorded 87^{0}C as minimum and 92^{0}C as maximum at the site. The pH of water of Saldhar thermal spring varied from 9.1 to 9.3, indicating that the water is alkaline in nature. This high pH may be due to the presence of cyanobacteria present at the site in the form of algal mat. The cyanobacteria use the carbonates and bicarbonates that increase the pH of the water at a high level. Similar range of pH (6.9-9.5) was recorded by **Singh *et al.* (2015)** for the water samples of thermal springs in Jharkhand and West Bengal of India; **Guzman *et al.* (2004)** also recorded the similar range of pH (7.76 to 9.98) for thermal springs in the pacific coast of Guerrero, Mexico.

The values of dissolved oxygen (DO) varied from 0.4 mg/l to 0.6 mg/l. **Fazlzadeh *et al.* (2017)** recorded the DO concentration within a range of 3.25 mg/l to 3.57 mg/l for thermal springs in Iran and **Kumar *et al.* (2013)** recorded 2.52 mg/l of dissolved oxygen for hot springs of Kullu district in Himachal Pradesh. The electrical conductivity (EC) was ranged from 6.04 mS/cm to 6.08 mS/cm in the water of hot spring. Homma and Tsukahara (2008) recorded the similar range of conductivity for the Northernmost area of the Itoigawa Shizuoka Tectonic Line. Salinity of the samples ranged from 3.0^{SAL} to 3.28^{SAL} throughout the sampling period.

The concentration of total dissolved solids (TDS) was ranged between 3.10 mg/l to 3.34 mg/l in the water of Saldhar thermal spring. The concentration of free CO_2 was recorded between 30.8 mg/l and 35.2 mg/l during the study period. High range of total dissolved solids was recorded by **Hamzah *et al.* (2013)** for thermal springs of Malaysia. The concentration of free CO_2 was ranged between 35.2 mg/l and 48.4 mg/l during the study period.

Table 2 Physico-chemical parameters for water of Ringigad thermal spring in the Garhwal Himalaya

Physico-chemical Parameters	2014		2015		Min.	Max.	Mean ± SD
	June	August	June	August			
Water Temperature (^{0}C)	82.0	89.0	83.0	87.0	82.0	89.0	85.25±3.30
Dissolved oxygen (mg/l)	1.2	0.8	1.2	0.8	0.8	1.2	1.0±0.03
pH	6.7	6.8	6.9	6.8	6.7	6.9	6.8±0.08
Conductivity (mS/cm)	4.48	4.62	4.54	4.68	4.48	4.68	4.58±0.09
Free CO_2 (mg/l)	35.2	44.0	35.2	48.4	35.2	48.4	40.7±6.6
Salinity (^{SAL})	2.3	2.3	2.1	2.3	2.1	2.3	2.25±0.1
TDS (mg/l)	2.47	2.51	2.49	2.53	2.47	2.53	2.5±0.03
Chlorides (mg/l)	11.36	14.20	14.20	14.20	11.36	14.2	13.49±1.42
Total Alkalinity (mg/l)	270.0	300.0	275.0	290.0	270.0	300.0	283.75±13.77
Nitrates (mg/l)	0.239	0.250	0.247	0.256	0.239	0.256	0.248±0.01
Sulphates (mg/l)	0.276	0.283	0.281	0.289	0.276	0.289	0.282±0.01
Phosphates (mg/l)	0.026	0.028	0.028	0.027	0.026	0.028	0.027±0.00
Total Hardness (mg/l)	228.0	232.0	228.0	234.0	228.0	234.0	230.5±3.0
Calcium (mg/l)	63.2	66.6	68.0	69.6	63.2	69.6	66.85±2.72
Magnesium (mg/l)	17.13	16.5	14.20	14.70	14.2	17.13	15.63±1.40

Table 3 Physico-chemical parameters for water of Saldhar thermal spring in the Garhwal Himalaya

Physico-chemical Parameters	2014		2015		Min	Max	Mean ± SD
	June	August	June	August			
Water Temperature (^{0}C)	89.0	92.0	87.0	90.0	87.0	92.0	89.5±2.08
Dissolved oxygen (mg/l)	0.6	0.4	0.6	0.4	0.4	0.6	0.5±0.12
pH	9.1	9.3	9.1	9.1	9.1	9.3	9.15±0.10
Conductivity (mS/cm)	6.08	6.08	6.05	6.04	6.04	6.08	6.06±0.02
Free CO_2 (mg/l)	30.8	35.2	30.8	30.8	30.8	35.2	31.9±2.20
Salinity (^{SAL})	3.14	3.00	3.28	3.16	3.0	3.28	3.15±0.11
TDS (mg/l)	3.10	3.18	3.34	3.34	3.1	3.34	3.24±0.12
Chlorides (mg/l)	9.94	14.2	9.94	12.78	9.94	14.2	11.72±2.13
Total Alkalinity (mg/l)	295.0	320.0	290.0	310.0	290.0	320.0	303.75±13.77
Nitrates (mg/l)	0.089	0.091	0.091	0.096	0.089	0.096	0.092±0.00
Sulphates (mg/l)	0.287	0.311	0.296	0.310	0.287	0.311	0.301±0.01
Phosphates (mg/l)	0.023	0.023	0.021	0.024	0.021	0.024	0.023±0.00
Total Hardness (mg/l)	248.0	256.0	252.0	256.0	248.0	256.0	253.0±3.83
Calcium (mg/l)	52.0	56.11	54.4	55.2	52.0	56.11	54.43±1.76
Magnesium (mg/l)	28.83	28.28	28.34	28.83	28.28	28.83	28.57±0.30

The concentration of total hardness varied from 228 mg/l to 234 mg/l. High range of alkalinity (196 mg/l) was recorded by **Kumar *et al.* (2013)** for thermal springs of Kullu district in Himachal Pradesh. The concentration of Calcium varied from 63.2 mg/l to 69.6 mg/l. Magnesium concentration varied from 14.20 mg/l to 17.13 mg/l. A similar range (12.60 mg/l to 15.62 mg/l) of magnesium was recorded by **Singh *et al.* (2015)** for thermal springs of Jharkhand and West Bengal.

The concentration of total hardness varied from 248 mg/l to 256 mg/l. The concentration of Calcium varied from 52.20 mg/l to 56.11 mg/l. Magnesium concentration varied from 28.28 mg/l to 28.83 mg/l. The concentration of chlorides varied from 9.94 mg/l to 14.20 mg/l observed in the water sample.

Alkalinity of the water ranged from 290 mg/l to 320 mg/l during the study period. The concentration of nitrates present in the water sample was ranged from 0.089 mg/l to 0.096 mg/l. Sulphates concentration were also found within a range of 0.287 mg/l to 0.311 mg/l. The concentration of phosphates in the water sample was ranged from 0.021 mg/l to 0.24 mg/l.

Microbial diversity

In Ringigad, an overall, seven species of bacteria (*Brevibacillus borstelensis, Aeromonas veronii, Paenibacillus dendritiformis, Bacillus cerus, Bacillus cibi, Streptococcus pyogenes* and *Strenotrophomonas maltophila*) and four species of actinomycetes (*Streptomyces albus, Streptomyces canescens, Thermoactinomyces candidus* and *Thermoactinomyces thalopophilum*) were recorded. However, three species of fungi (*Sclerotium rolfsii, Fusarium oxysporum* and Sclerotinia sclerotiorum) were also recorded. The α-diversity of microbes in the Ringigad thermal spring was found to be fourteen during the study period (Table 4). In Saldhar, an overall, six species of bacteria (*Bacillus cerus, Streptococcus pyogenes, Bacillus subtilis, Brevibacillus parabrevis, Brevibacillus reuszeri* and *Geobacillus stearothermophilus*) and three species of actinomycetes (*Streptomyces albus, Thermoactinomyces candidus* and *Thermoactinomyces thalopophilum*) were recorded. However, three species of fungi (*Aspergillus tubingensis, Trichoderma harzianum* and
Sclerotinia sclerotiorum) were also recorded. The α-diversity of microbes in Saldhar spring was found to be twelve during the study period (Table 4). The microbes that were found in both the thermal springs were typical to other thermal springs. All the microbial species are hyperthermophiles and can be identified only in the thermal springs having such an extreme temperature.

Table 4 Microbial diversity of Ringigad and Saldhar hot water springs of Garhwal Himalaya (Abbreviations: +: present; -: absent)

Microorganisms	Ringigad	Saldhar
Bacteria		
Brevibacillus borestelensis	+	-
Aeromonas veronii	+	-
Stenotrophomonas maltophilia	+	-
Paenibacillus dendritiformis	+	-
Bacillus cerus	+	+
Bacillus cibi	+	-
Streptococcus pyogenes	+	+
Bacillus subtilis	-	+
Brevibacillus parabrevis	-	+
Brevibacillus reuszeri	-	+
Geobacillus stearothermophilus	-	+
Actinomycetes		
Thermoactinomyces candidus	+	+
Thermoactinomyces thalpophilum	+	+
Streptomyces albus	+	+
Streptomyces canescens	+	-
Fungi		
Sclerotium rolfsii	+	-
Fusarium oxysporum	+	-
Sclerotinia sclerotiorum	+	+
Aspergillus tubingensis	-	+
Trichoderma harzianum	-	+
Total	**14**	**12**

CONCLUSION

Keeping in view, it has been concluded that both the thermal springs (Ringigad and Saldhar) are having a very high temperature. The concentration of dissolved oxygen is very low in which only the thermophilic microbes can survive. The pH of both the springs revealed the alkaline nature of the water of the hot springs. Apart of the high temperature, the α-diversity of microbes in Ringigad is 14 and in Saldhar it is 12.

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