JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises (Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/44/1/MRN-5018

The bacterial population inhabiting a top-ranking High Background Radiation Area: A first-time report

P. Sowptika and G. Anilkumar*

School of Biosciences and Technology, Vellore Institute of Technology, Vellore-632 014, India

***Corresponding Author Email :** ganilkumar@vit.ac.in ***ORCiD:** https://orcid.org/0000-0001-9486-9118

Received: 19.02.2022 *Revised*: 24.05.2022 *Accepted*: 02.09.2022

Abstract

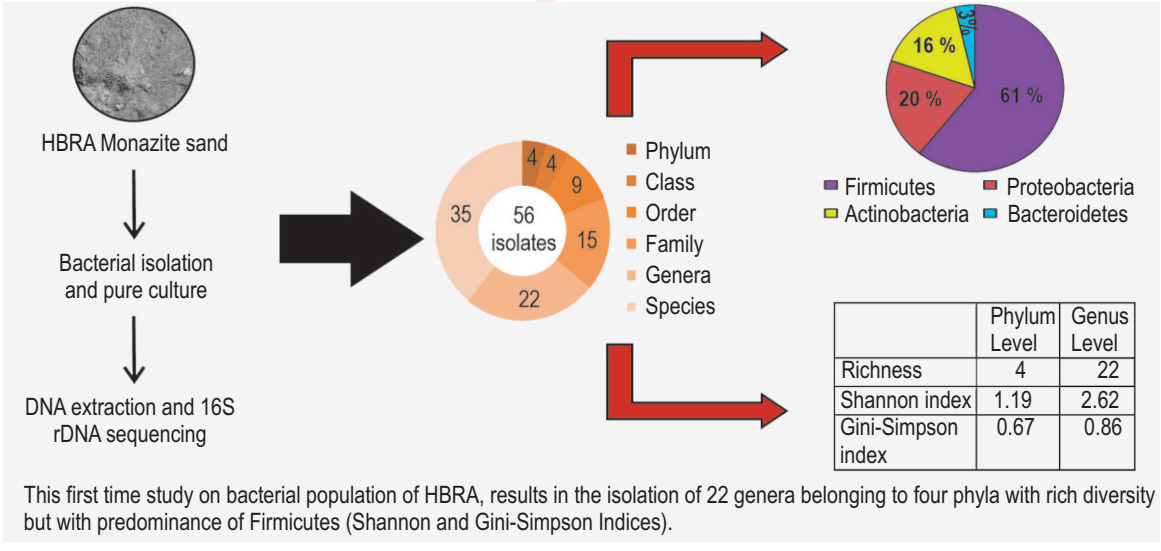
Aim: To study the soil bacterial population inhabiting the High Background Radiation Area (HBRA) of Chavara-Neendakara placer deposits, Kerala, India.

Methodology: The study was performed by culture-dependant methods involving isolation and characterization of microbes through plate culturing and sequencing the 16S rDNA primed PCR amplicons.

Results: Histochemical observations and molecular taxonomic studies through BLAST search, multiple sequence alignment of the sequences and SeaView phylogenetic analyses, resulted in identification of 35 bacterial species belonging to 22 genera. Shannon-Simpson diversity-predominance indices, revealed an over-riding presence of Firmicutes (~60%) in the HBRA soil.

Interpretation: The results reveal for the first time, the composition, structure and dynamics of the bacterial population inhabiting an HBRA.

Key words: Chavara-Neendakara placer deposit, Firmicutes, Gini-Simpson Index, High background radiation area, Shannon index



	Phylum Level	Genus Level
Richness	4	22
Shannon index	1.19	2.62
Gini-Simpson index	0.67	0.86

This first time study on bacterial population of HBRA, results in the isolation of 22 genera belonging to four phyla with rich diversity but with predominance of Firmicutes (Shannon and Gini-Simpson Indices).

How to cite : Sowptika, P. and G. Anilkumar: The bacterial population inhabiting a top-ranking High Background Radiation Area: A first-time report. *J. Environ. Biol.*, **44**, 45-56 (2023).

Introduction

Areas with abnormally high background radiation, caused essentially due to the occurrence of primordial radionuclides (Uranium [^{238}U], Thorium [^{232}Th] and Potassium [^{40}K]), have been reported from several countries. Among these, the coastal regions of Espirito Santo and the Morro Do Forro in Brazil (Paschoa, 2000), Yangjiang in China (Wei *et al.,* 2000), Chavara-Neendakara along the Southwest coast of India (Sunta *et al.,* 1982), Ramsar and Mahallat in Iran (Ghiassi-Nejad *et al.,* 2002), and Rosetta Beach in Egypt (Mubarak *et al.,* 2017), have been identified as High Background Radiation Areas (HBRAs) due to their unusually high levels of radioactivity.While Monazite sands were found to be the source of such high background radiation levels in Brazil, China, Egypt and India, Ramsar has high amounts of ^{226}Ra and its decay products were brought to the surface by hot springs (Aliyu and Ramli, 2015; Chandran and Shreedharan, 2016; Mubarak *et al.,* 2017). Spectrometric studies from our laboratory using HPGe scintillometer revealed that this placer deposit is ranked second in the entire globe, next to Brazil, due to its richness in ^{238}U (~6000 Bq kg^{-1}), ^{232}Th (~41000 Bq kg^{-1}) and ^{40}K (~ 2500 Bq kg^{-1}) and in terms of its high radiation absorbed dose, with annual effective dose of ~9 mSv yr^{-1}, much higher than the world average of 0.07 mSv yr^{-1} (Derin *et al.,* 2012; Vaiserman *et al.,* 2018); the tolerance limit for the general public has been streamlined as 1 mSv y^{-1} (Vaiserman *et al.,* 2018).

To our knowledge, most of the microbial surveys conducted thus far, have been from aquatic (marine/ freshwater/ estuarine) (Tamaki *et al.,* 2005; Sheng *et al.,* 2016; Salazar and Sunagawa, 2017; Wang et al., 2020) and other habitats, including soils from forests (Axelrood *et al.,* 2002; Lladó *et al.,* 2018), grasslands (Nan *et al.,* 2020), farmland (Bevivino *et al.,* 2014), deserts (Lester *et al.,* 2007; Yu *et al.,* 2015; Majid *et al.,* 2016) and marshy areas (including the mangroves) (Michelato Ghizelini *et al.,* 2012; Nathan *et al.,* 2020). Further, microflora from extreme environmental conditions such as high altitude areas (Kumar *et al.,* 2019), hot and cold springs (Selim *et al.,* 2017; Najar *et al.,* 2018; Guðmundsdóttir *et al.,* 2019), abyssal and benthic habitats (Zhang *et al.,* 2015; Li *et al.,* 2019), polar regions (Steven *et al.,* 2007) and even at 20,000 m high in the atmosphere were studied from the stand points of adaptive values (Griffin *et al.,* 2008), but nothing from HBRAs. These studies reveal that the microbiota differs in its structure entrained with different soil horizons. Not only that the soil quality would have an impact on the microbial community structure, but it is as well being increasingly evident that understanding of soil microbiota will have a predictive value on the soil physico-chemical variables and possible applications (Hermans *et al.,* 2020; Camacho *et al.,* 2022).

This in turn obviously warrants more studies on soil microbes inhabiting various habitats. It is at this juncture, we report the results of a culture-dependant study on the bacterial population of monazite sand from the top-ranking HBRA, Chavara-Neendakara placer deposits, performed through plate culturing, microscopic observations of the staining reactions and 16S rDNA gene sequencing. As a prelude to have a clearer understanding on the dynamics of microbial communities of this unique habitat, the diversity and prevalence indices were also estimated and compared with those of other ecosystems.

Materials and Methods

Sample collection: A total of 19 sites were identified for sample collection in the 22 km stretch of the HBRA (Chavara-Neendakara placer deposits) by previous investigators from our laboratory (Derin *et al.,* 2012). Before sample collection, these sites were visited by us during 15–20 December 2018, and the sand samples were tested for radioactivity, using a portable Scintillometer (Polimaster PM1405). Among all the (19) sites, the site at Puthenthura of Quilon District, Kerala (Latitude 8° 57'46.46"N; Longitude 76° 31' 47.93"E) (referred to as CH-16, in Derin *et al.,* 2012) was selected for sample collection for the present study, as this area showed the maximum reading of 21000 nGy hr^{-1}. Sand samples were collected from 3 spots (Spots 1-3) chosen at random, situated ~5 m apart in CH-16. The sample from each spot was collected in triplicate from a depth of ~15 cm using a soil borer, and kept in sterile 50 ml polystyrene tubes, in cooler box maintained at 4°C, and transported to our laboratory at the Vellore Institute of Technology, Vellore, Tamil Nadu. Upon reaching the laboratory, the samples were stored at 4°C in a dedicated refrigerator until further processing.

Before processing for bacterial isolation, the sand samples collected in triplicate from each spot were mixed so as to make it three independent samples (Spots 1, 2 and 3), *i.e.,* the samples in triplicate drawn from Spot 1 were mixed together, and named as HB1. Similarly, the triplicates from Spots 2 and 3 were also mixed together and named as HB2 and HB3, respectively.

Isolation and characterization: Bacterial isolates were prepared by serially diluting the sand sample (5 g each), properly mixed with physiological saline (up to 10^{-5}), and spread – plated on Zobell Marine Agar 2216 (HiMedia) (Godson *et al.,* 2014; Pimpliskar and Jadhav, 2015). Colonies were pure-cultured on separate agar plates and incubated at 35°C for 24-48 hr before subjecting to identification.Each pure culture was characterized microscopically with Gram's staining and 3% KOH String test. Malachite Green staining was performed to check for endospore formers on >72 hour grown cultures. Lactophenol cotton blue stain was used for identifying actinomycete-like colonies.

Molecular characterization: Pure cultures, inoculated in 50 ml Zobell Marine 2216 broth, were incubated for 24 hr (35°C) in an orbital shaker (120 rpm). Aliquot of each sample was used to perform DNA extraction by modified version of Phenol Chloroform method (Moore *et al.,* 2008). The concentration and purity of the DNA was assessed using Nanodrop spectrophotometer (Thermo Fisher Scientific), and through Agarose gel electrophoresis. Universal primers, specific for bacterial 16S rRNA (Table 1), were used to PCR (Veriti Thermal cycler, ABI) - amplify the gene in question in a 30 µl reaction for 40 cycles (Table 2). The PCR

products were gel electrophoresed to monitor the integrity of amplicons, and were subsequently column–purified and sequenced in a DNA sequencer (ABI 3130 Genetic Analyser).

Phylogenetic tree: Sequences of PCR amplicons were BLAST-aligned [EzTaxon database] (Kim *et al.,* 2012) for species identification, and were deposited in NCBI GenBank and accession numbers were obtained. SeaView (Version 5.0) software (Galtier *et al.,* 1996) was used to construct the phylogenetic tree with maximum parsimony (1000 bootstrap replicates). Multiple sequence alignment (MSA) was performed using MUSCLE, integrated within SeaView. Sequences of HBRA samples were aligned with their respective type strains retrieved from the EzTaxon database.

Statistical analysis: The extent of diversity of the bacterial community of HBRA was assessed through Shannon index (H') (Shannon, 1948). Dominance of the community was estimated using the Gini-Simpson's (1-D) (Jost, 2006).

HBRA data obtained were superimposed with those of other ecological sites with a view to gauge the extent of identity of the HBRA bacterial population with those of other habitats. Chi squared test was employed to compare the proportionate frequency distribution of the species within the taxa and between the taxa.

Results and Discussion

Repeated examination of samples selected at random revealed similar morphotypes, suggesting autochthonous nature of the population.

Culture-dependent isolation and characterization of samples led to identification of fairly rich microbial population ($4.8\text{-}6.7\times10^5$ cfu ml^{-1}) (Table 3). Several isolates were coloured yellow, orange or red, apparently due to pigmentation including carotenoids, which, bacteria are known to synthesize (Venil *et al.,* 2020). Carotenoid-producing bacteria have been found to be associated with radioactive sites and are known to afford protection against oxidative damage induced by ionizing radiation (Asker *et al.,* 2007; Ruiz-González *et al.,* 2016). Out of 56 isolates, 37 were Gram positive, and 11 were Gram negative, while the remaining colonies were "Gram variable"; the precise identity of "Gram variable" colonies was confirmed with 3% KOH String test (Table 4; Fig. 1). The isolates were mostly rod-shaped, seen in continuous, chain-like formations, clusters and/or pairs, while a few were cocci. With Malachite green staining, endospores were observed either on the terminal end or located centrally, indicating their Bacillales characteristics. Three of the isolates had circular leathery colony with powdery surface resembling actinomycetes colony; upon staining with Lactophenol cotton blue, it displayed characteristic hyphal structure of actinomycetes.

Through sequencing of 16S rDNA, and subsequent BLAST and EzTaxon (a type-strain database revealing similarity to the tune of 98-100%) analysis, 35 different bacterial species belonging to 22 genera were identified, out of which, 10 belonged to Firmicutes, 5 to Proteobacteria, 6 to Actinobacteria and 1 to Bacteroidetes (Table 5; Fig. 2). All the identified species from the HBRA (Fig. 3) showed 99 - 100% branch bootstrap value, signifying their high levels of proximity to the reference sample. Radiation-resistant organisms, used as reference, were, in general, seen to be phylogenetically distant from the HBRA microbes. However, two strains (VITHBRA002 and VITHBRA022) showed strong cladistic similarity to *Bacillus pumilus* (SAFR-032), that survived successfully at the international space station (ISS) (Stepanov *et al.,* 2016). Further, the HBRA sample VITHBRA049 (*Kocuria* sp.) was seen to have common ancestry with *Keinococcus radiotolerance* (84% bootstrap value), a known radio-resistant species. The present study reveals the predominance (~60%) of Firmicutes in HBRA

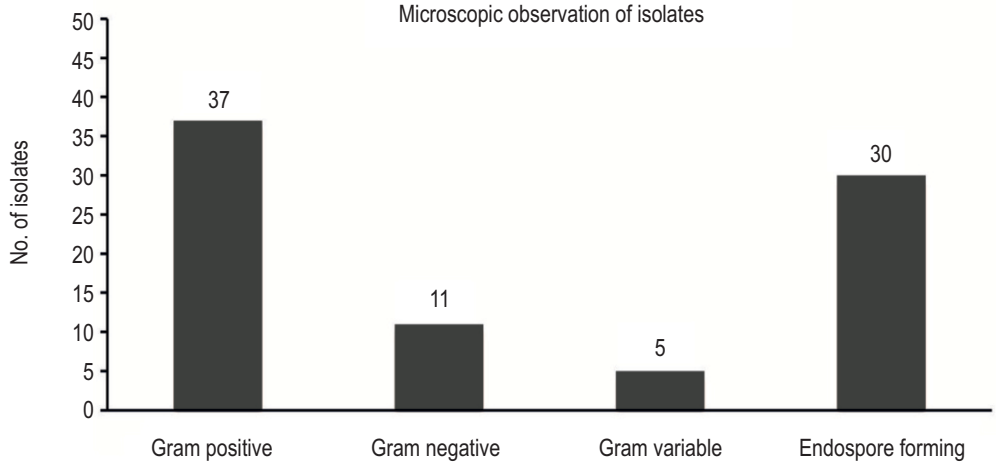


Fig. 1: Graph representing the staining results of isolates as observed under light microscope.

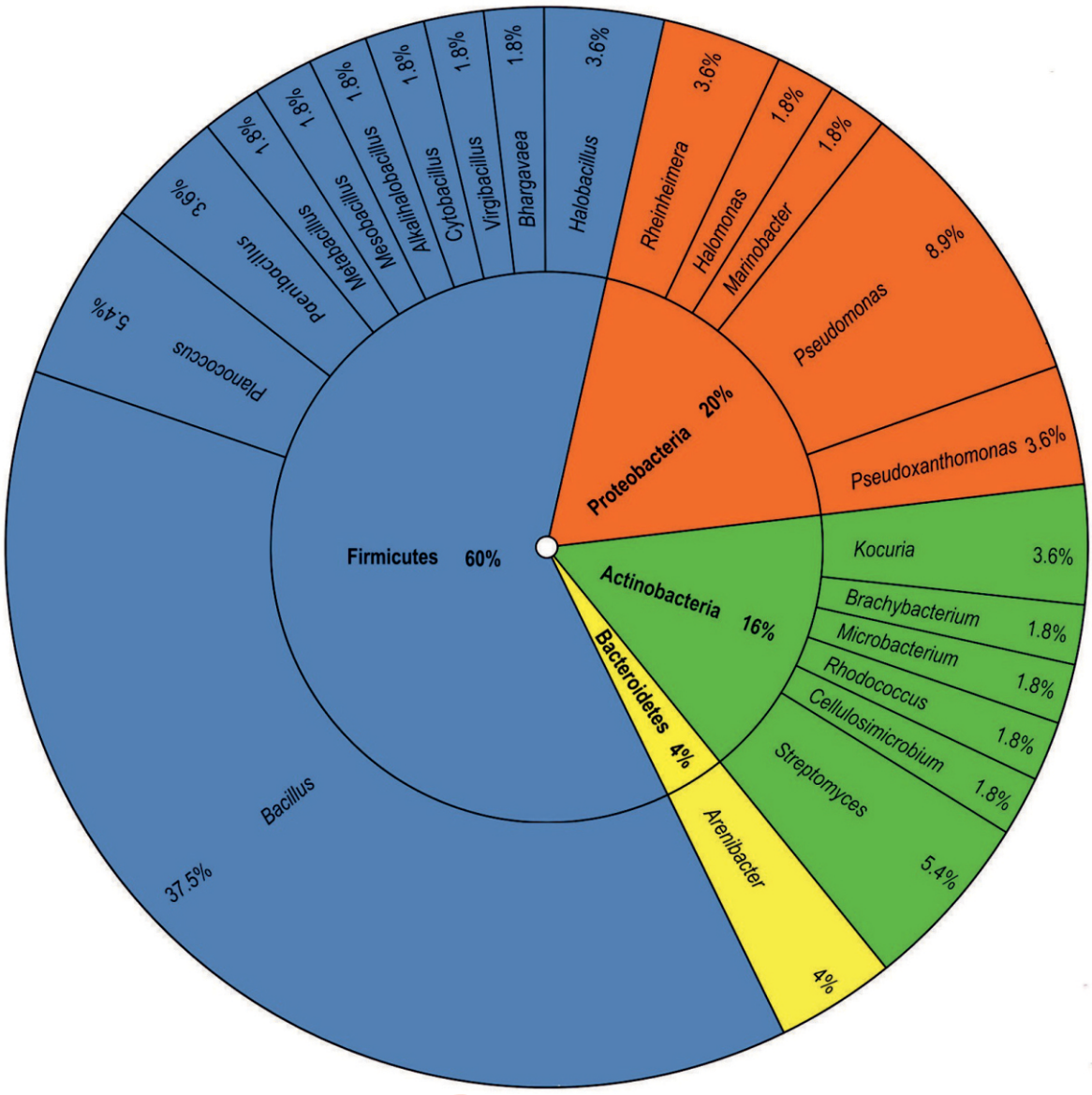


Fig. 2: A hierarchical chart demonstrating the distribution of microbes cultured from HBRA. The inner circle represents the Phylum and the outer ring projects the Genus of the identified HBRA population.

soil, followed by Proteobacteria (~19%), Actinobacteria (~6.5%) and Bacteroidetes (~3.2%) (X2 =0.034, p>0.998) (Table 7). At the genus level, the bacterial population was seen to be fairly diverse, evidenced by Shannon Index (2.62); the diversity index, however, showed signs of decline at the Phylum level, evidently due to Firmicutes' prevalence (Table 6). Further, Gini-Simpson index declined from its value at the genus level (0.86) to that at phylum level (0.67, Table 6), suggesting increased dominance (of Firmicutes) and reduced evenness. While addressing the question of feature(s) that could help Firmicutes to survive better, we are tempted to hypothesize that it could be related to their adaptive capability, such as endospore formation, chemotactic detection of food scarcity and other metabolic adaptations (Fillipidou *et al.,* 2016), which in turn could afford them a selective advantage, an apparent major contributory factor in moulding the bacterial community of HBRA. This hypothesis has further been substantiated through comparative studies of the bacterial populations from various habitats, including those inhabiting the radiation-polluted areas (please also see the section below).

In its proportionate phylogenetic distribution, the HBRA bacterial population showed similarity to those of other habitats

Fig. 3: Phylogenetic tree built using 16S rDNA sequences. 35 different bacterial species of HBRA along with their type strain and reference radiation resistant microbes are used to construct a maximum parsimony tree. Bootstrap value below 40% is not shown in the figure.

Table 1: Forward and reverse primers used to PCR amplify the microbial 16S rRNA gene

Primers	Sequence	Reference
Forward	5' AGAGTTTGATCCTGGCTCAG 3'	Weisburg *et al.,* 1991
Reverse	5' ACGGCTACCTTGTTACGACTT 3'	Weisburg *et al.,* 1991

Table 2: 16S rDNA PCR amplification: temperature cycles

	Steps	Temperature	Time
Stage 1	Initial denaturation	95 °C	5 min
	Denaturation	95 °C	1 min
Stage 2	Annealing	58 °C	45 sec
	Extension	72 °C	45 sec
Stage 3	Final extension	72 °C	10 min
	Termination and storage	4 °C	∞

Table 3: Colony characteristics of isolates

Isolates	Colony characteristics						
	Size	Shape	Colour	Margin	Elevation	Consistency	Opacity
VITHBRA001	2 mm	Circular	Pale brown	Even	Flat	Glossy	Opaque
VITHBRA002	1 mm	Circular	White	Uneven	Flat	Glossy	Opaque
VITHBRA003	1.5 mm	Circular	Off white	Even	Low convex	Glossy, inconsistent	Translucent
VITHBRA004	3 mm	Circular	White	Uneven	Flat	Mate	Opaque
VITHBRA005	3.5 mm	Irregular	Periphery-off white, center reddish brown	Uneven	Flat	Mate	Opaque
VITHBRA006	1.5 mm	Circular	White	Even	Flat	Glossy	Opaque
VITHBRA007	1 mm	Circular	Creamish white	Uneven	Low convex	Glossy	Opaque
VITHBRA008	2.5 mm	Irregular	White periphery, center pinkish	Uneven	Flat	Dry, wrinkled surface	Translucent
VITHBRA009	3.5 mm	Circular	White	Even	Flat	Mate	Opaque
VITHBRA010	2 mm	Circular	Cherry red	Even	Convex	Mate	Opaque
VITHBRA011	1 mm	Circular	Creamish white	Uneven	Low convex	Glossy	Opaque
VITHBRA012	3 mm	Circular	Pale orangish	Uneven	Low convex	Glossy	Translucent
VITHBRA013	3.5 mm	Circular	White	Even	Flat	Mate	Opaque
VITHBRA014	2 mm	Circular	Creamish White	Even	Low convex	Wrinkled surface	Opaque
VITHBRA015	Pinpoint	Circular	Pale Orange	Even	Low Convex	Butyrous	Translucent
VITHBRA016	~2 mm	Circular	Orange	Even	Flat	Butyrous	Opaque
VITHBRA017	1 mm	Circular	Brownish	Even	Low Convex	Dew-Drop Like	Transparent
VITHBRA018	Pinpoint	Circular	Pale orange	Even	Flat	Butyrous	Translucent
VITHBRA019	1 mm	Circular	Pale yellow	Even	Low convex	Butyrous	Translucent
VITHBRA020	1 mm	Circular	Pale yellow	Even	Low Convex	Butyrous	Translucent
VITHBRA021	~2 mm	Circular	Creamish	Even	Low Convex	Butyrous	Translucent
VITHBRA022	~1 mm	Circular	White	Even	Low Convex	Dry	Opaque
VITHBRA023	1 mm	Circular	Creamish	Even	Flat	Butyrous	Opaque
VITHBRA024	~2 mm	Circular	Creamish periphery, center brick red	Irregular	Uneven	Slimy, butyrous	Opaque
VITHBRA025	1mm	Circular	Brownish	Even	Low Convex	Slimy	Translucent
VITHBRA026	~1 mm	Circular	Creamish	Diffused	Flat	Butyrous	Opaque
VITHBRA027	3 mm	Irregular	White	Uneven	Low convex	Glossy	Opaque
VITHBRA028	4 mm	Circular	Brown centre, white periphery	Uneven	Low convex	Dry, Butyrous	Opaque
VITHBRA029	2 mm	Circular	Yellow centre,	Even	Convex	Dry	Opaque

Table continued

Isolates	Colony characteristics						
	Size	Shape	Colour	Margin	Elevation	Consistency	Opacity
VITHBRA031	2 mm	Irregular	white periphery Orange centre, white periphery	Uneven	Flat	Dry	Opaque
VITHBRA032	2 mm	Circular	Creamish	Uneven	Flat	Glossy, Butyrous	Opaque
VITHBRA033	1 mm	Circular	Yellowish-white	Uneven	Low convex	Glossy	Translucent
VITHBRA034	3 mm	Circular	Creamish	Uneven	Low convex	Glossy, Butyrous	Opaque
VITHBRA035	<1 mm	Circular	Bright yellow	Even	Flat	Glossy	Translucent
VITHBRA036	1.5 mm	Circular	Light yellow	Even	Flat	Glossy	Opaque
VITHBRA037	2.5 mm	Circular	Creamish	Uneven	Low convex	Glossy, Butyrous	Opaque
VITHBRA038	1 mm	Circular	Yellow	Even	Convex	Glossy	Translucent
VITHBRA039	2 mm	Circular	Whitish-yellow	Even	Convex	Glossy	Opaque
VITHBRA040	1 mm	Circular	Yellowish-white	Uneven	Flat	Glossy, Dew-drop	Transparent
VITHBRA041	4 mm	Circular	Yellow centre, white periphery	Uneven	Flat	Glossy, Butyrous	Translucent
VITHBRA042	2 mm	Circular	Creamish	Uneven	Low convex	Glossy	Translucent
VITHBRA044	3 mm	Circular	White	Uneven	Convex	Glossy, Butyrous	Opaque
VITHBRA045	Pinpoint	Circular	White	Even	Low convex	Glossy	Opaque
VITHBRA046	6 mm	Circular	Light orange centre, white periphery	Uneven	Low convex	Glossy, Butyrous	Opaque
VITHBRA047	1 mm	Circular	Bright orange	Even	Low convex	Glossy	Opaque
VITHBRA048	3 mm	Circular	Creamish	Uneven	Flat	Dry, Butyrous	Translucent
VITHBRA049	1 mm	Circular	Bright orange	Even	Low convex	Glossy	Opaque
VITHBRA050	2 mm	Circular	Yellow	Even	Convex	Glossy	Translucent
VITHBRA051	4.5 mm	Circular	Orange centre, white periphery	Uneven	Low convex	Glossy, Butyrous	Opaque
VITHBRA052	3.5 mm	Irregular	Orange centre, white periphery	Uneven	Flat	Glossy, Butyrous	Translucent
VITHBRA053	4 mm	Irregular	Yellow centre, white periphery	Uneven	Flat	Dry, Butyrous	Translucent
VITHBRA054	<1mm	Circular	Orange	Even	Low convex	Glossy	Opaque
VITHBRA055	3.5 mm	Circular	Brown centre, white periphery	Uneven	Flat	Dry, Butyrous	Opaque
VITHBRA056	2 mm	Circular	Whitish-yellow	Even	Convex	Glossy	Opaque
VITHBRA057	4.5 mm	Circular	Creamish	Uneven	Flat	Glossy, Butyrous	Transparent
VITHBRA058	3.5 mm	Circular	Creamish	Uneven	Flat	Glossy, Butyrous	Transparent

like heavy metal contaminated soil (sandy site in Scotland) (Ellis *et al.,* 2003), T22 Trench of Chernobyl with (anthropogenic) background radiation (Chapon *et al.,* 2012; Ruiz-González *et al.,* 2016) and the Uranium ore deposit of Domiasiat, North-east India (Kumar *et al.,* 2013). Further, metagenomic studies on airborne microbes of Beijing revealed a temporal fluctuation in the bacterial strains; Firmicutes were shown to be significantly prominent ($p<0.05$) during unfavourable situation of smog in January (Qin *et al.,* 2020). These evidences strengthen our hypothesis that the predominance of a taxon (Firmicutes in the present study) could be related to "extreme" nature of the habitats (heavy metal contamination, exposure to radiation etc.).

The recent understanding on the expression of biosynthesis gene cluster (BGC) among microbes including Firmicutes (*Bacillus subtilis*, showing resistance to ionizing radiations) (Li *et al.,* 2015; Sayed *et al.,* 2020), would help us to explain their adaptive strategies in extreme environments. Contrarily, Firmicutes' predominance was not noticeable in British Columbia forest soil and that of agricultural land of Sardinia (Italy) (Table 7). The high fertility with dense foliage, consisting of rich sources of organic components, decomposers, nitrogen fixers and carbon cyclers in the forest and farm lands, could support a rich diversity of bacterial species, with hardly any influence of a limiting factor to promote the predominance of a particular taxon (Lladó and Baldrian, 2017). We tried to correlate the association (if any) between environmental conditions of these study areas and the population structure of the inhabitant bacterial communities, but were unable to find any significant correlation due to paucity of data. Admittedly, however, the inherent limitations of the present culture-dependent study, would not allow us to contend affirmatively that Firmicutes predominance related to "extreme" environment would be ubiquitous. More research is required to make any generalization in this regard.

Table 4: Cellular morphology under microscope

Isolates	KOH string Test[a]	Gram staining	Cellular morphology	Endospore
VITHBRA001	-	+	Long rods	+ (central and swollen)
VITHBRA002	-	+	Rods with round ends, occasionally 2 in pairs	+
VITHBRA003	-	+	Short rods	+ (central and swollen)
VITHBRA004	-	+	Long rods	+ (terminal)
VITHBRA005	-	+	Short rods in long chains	+ (central)
VITHBRA006	-	+	Long rods with tapering end-forming short chains	+
VITHBRA007	-	+	Long rods with tapering end, forming short chains	+
VITHBRA008	-	+	Long Rods in cluster	+
VITHBRA009	-	+	Long thick rods with long chains and flat ends	+
VITHBRA010	-	+	Long slender rods	-
VITHBRA011	-	+	Long rods with tapering end,forming short chains	+
VITHBRA012	-	+	Very short rods in cluster	-
VITHBRA013	-	+	Long thick rods with long chains and flat ends	+
VITHBRA014	-	+	Short rods	+
VITHBRA015	-	+	Cocci in cluster	-
VITHBRA016	-	+	Long rods	+
VITHBRA017	+	+/ -	Rods	-
VITHBRA018	-	+	Cocci in cluster	-
VITHBRA019	+	-	Short rods	-
VITHBRA020	+	-	Short rods	-
VITHBRA021	+	+/-	Rods	-
VITHBRA022	-	+	Short rods paired	+
VITHBRA023	-	+	Rods	+
VITHBRA024	-	+	Long rods	+ (Terminal Swollen)
VITHBRA025	+	-	Rods	-
VITHBRA026	-	+	Long rods	+
VITHBRA027	-	+	Long slender rods	+
VITHBRA028	-	+	Long Rods, forming long chain	+
VITHBRA031	-	+	Short Rods, short chains	+
VITHBRA032	-	+	Short Rods in cluster	+
VITHBRA033	+	-	Very Short Rods, short chains	-
VITHBRA034	-	+	Coccoid rods in cluster	-
VITHBRA035	-	+	Very Short Rods in cluster	-
VITHBRA036	-	+	Very Short Rods in cluster	-
VITHBRA037	-	+	Long Rods in cluster	+ (Terminal)
VITHBRA038	+	-	Short Rods with round ends in cluster	+
VITHBRA040	+	-	Long Rods in cluster of two mostly	-
VITHBRA041	-	+	Long Rods with long chains	+
VITHBRA042	+	-	Very Short Rods in cluster	-
VITHBRA044	-	+/-	Short Rods with round ends forming chains	+
VITHBRA045	-	+	Coccoid in cluster	-
VITHBRA046	-	+/-	Short Rods with short chains	+
VITHBRA047	-	+	Cocci in cluster	-
VITHBRA048	-	+	Long Rods with short chains	+
VITHBRA049	-	+	Cocci in cluster	-
VITHBRA050	-	+/-	Short Rods with short chains	+
VITHBRA051	-	+	Long Rods with short chains	+
VITHBRA052	+	-	Very Short Rods in cluster	-
VITHBRA053	+	-	Very Short Rods in cluster	-
VITHBRA054	-	+	Cocci in cluster	-
VITHBRA055	-	+	Short Rods in cluster	+
VITHBRA057	+	-	Very Short Rods in cluster	-
VITHBRA058	+	-	Very Short Rods in cluster	-

[a] indicated as '+' for string formation and '-' with no string indicated; *Cotton blue staining data of three Actinomycetes isolated not provided.

Table 5: HBRA microbes obtained from culturing by spread plate method

Isolated HBRA strain	Accession no. of isolates on GenBank submission	Closest type strain with accession no.	Similarity percentage
Firmicutes			
VITHBRA001	KY386292	*Metabacillus halosaccharovorans* E33 (HQ433447)	99.5
VITHBRA002	MK850856	*Bacillus safensis sub* sp. *safensis* FO-36b (MK424279.1)	99.79
VITHBRA032	MZ057734		100
VITHBRA037	MZ057737		99.79
VITHBRA003	MK850857	*Mesobacillus subterraneus* DSM 13966 (RSFW01000004)	99.48
VITHBRA004	MK850860	*Bacillus infantis* NRRL B-14911 (CP006643)	99.72
VITHBRA046	MZ057745		99.71
VITHBRA005	KY386291	*Bacillus filamentosus* SGD-14 (NR_134701.1)	99.84
VITHBRA028	MZ057730		99.86
VITHBRA051	MZ057750		100
VITHBRA007	MK966435	*Bacillus xiamenensis* HYC-10 (AMSH01000114)	99.78
VITHBRA011	MK966436		99.93
VITHBRA008	MK966437	*Bacillus subtilis* JCM 1465 (MH145363.1)	99.79
VITHBRA009	MK966438	*Bacillus aryabhattai* B8W22 (EF114313.2)	100
VITHBRA013	MK966439		100
VITHBRA014	MK966442	*Bacillus altitudinis* 41KF2B (NR_042337.1)	100
VITHBRA023	MK966441		99.78
VITHBRA016	MK966444	*Bacillus vietnamensis* NBRC 101237(NR_113995.1)	98.43
VITHBRA022	MK966451	*Bacillus australimaris* NH7I_1 (JX680098)	99.93
VITHBRA024	MK966449	*Bacillus paralicheniformis* KJ-16 (KY694465.1)	99.71
VITHBRA031	MZ057733		99.36
VITHBRA026	MK966452	*Cytobacillus kochii* WCC 4582 (NR_117050.1)	99.79
VITHBRA044	MZ057743	*Bacillus paramycoides* NH24A2 (MAOI01000012)	99.86
VITHBRA055	MZ057754	*Bacillus licheniformis* ATCC 14580 (AE017333)	99.93
VITHBRA041	MZ057741	*Alkalihalobacillus clausii* DSM8716 (CP019985.1)	99.56
VITHBRA006	KY317934	*Paenibacillus lautus* NBRC 15380 (BIMF01000051)	99.37
VITHBRA027	KY386288	*Paenibacillus dendritiformis* CIP 105967 (AY359885)	99.77
VITHBRA015	MK966445	*Planococcus plakorditis* DSM 23997 (CP016539)	99.61
VITHBRA018	MK966446		99.61
VITHBRA054	MZ057753		99.37
VITHBRA034	MZ057735	*Bhargavaea beijingensis* ge10 (EF371374)	99.64
VITHBRA048	MZ057747	*Virgibacillus dokdonensis* DSW-10 (AY822043)	99.93
VITHBRA038	MZ057738	*Halobacillus marinus* KGW1 (KJ563233.1)	99.93
VITHBRA050	MZ057749		100
Proteobacteria			
VITHBRA017	MK966447	*Rheinheimera pleomorphica* PKS7 (KJ563231)	100
VITHBRA025	MK966448		100
VITHBRA021	MK966450	*Halomonas piezotolerans* NBT06E8 (MN435603)	99.86
VITHBRA012	MK966440	*Marinobacter litoralis* SW-45 (NR_028841.1)	99.71
VITHBRA033	MT435126	*Pseudoxanthomonas indica* P15 (NR_116019.1)	99.42
VITHBRA040	MZ057740		99.44
VITHBRA052	MZ057752	*Pseudomonas stutzeri* ATCC 17588 (MT027239.1)	99.93
VITHBRA053	MT435128		99.78
VITHBRA042	MZ057742	*Pseudomonas benzenivorans* DSM 8628 (NR_116904.1)	98.68
VITHBRA057	MZ057756		98.29
VITHBRA058	MT435129		98.3
Actinobacteria			
VITHBRA010	MK850858	*Rhodococcus pyridinivorans* DSM44555 (LRRI01000001)	100
VITHBRA036	MT435127	*Cellulosimicrobium cellulans* LMG 16121 (CAOI01000359)	99.92
VITHBRA029	MZ057731	*Streptomyces rochei* NRRL B-2410 (MUMD01000370)	100
VITHBRA039	MZ057739		100
VITHBRA056	MZ057755		100

Table continued

Isolated HBRA strain	Accession no. of isolates on GenBank submission	Closest type strain with accession no.	Similarity percentage
VITHBRA035	MZ057736	*Microbacterium paraoxydans* NBRC 103076 (BCRH01000180)	99.78
VITHBRA045	MZ057744	*Brachybacterium paraconglomeratum* LMG 19861 (NR_025502.1)	99.78
VITHBRA047	MZ057746	*Kocuria oceani* FXJ8.095 (NR_156033.1)	99.78
VITHBRA049	MZ057748		99.28
Bacteroidetes			
VITHBRA019	MK966453	*Arenibacter latericius* KMM 426 (AF052742)	99.2
VITHBRA020	MK966454		99.13

Table 6: Diversity indices compared at the Phylum and Genus level for HBRA microbial community

Diversity Index	Phylum level	Genus level
Richness	4	22
Shannon index	1.19	2.62
Gini-Simpson index	0.67	0.86

Table 7: Proportionate distribution of various taxa across different habitat

Phylum	Locations				
	HBRA sample	Heavy metal contaminated soil (Ellis *et al.*, 2003)	T22 Trench of Chernobyl (Chapon *et al.*, 2012)	British Columbia Forest Soil (Axelrood *et al.*, 2002)	Agricultural Land (Bevivino *et al.*, 2014)
Firmicutes	34 (60)	275 (57)	197 (65)	296 (22)	30 (15)
Proteobacteria	11 (20)	130 (27)	62 (20)	602 (45)	100 (50)
Actinobacteria	9 (16)	50 (10)	39 (13)	420 (31)	41 (21)
Bacteroidetes	2 (4)	30 (6)	5 (2)	21 (2)	27 (14)
Total	56	485	303	1339	198
Chi-Square value	0.034[a]	3.2379	1.4083	47.1069	47.8304
p-value	0.9983	0.3564	0.703583	0.00001	0.00001

Each column represents number of isolates identified under each phylum and in parentheses represents the expected proportionate values. The Chi-squared value of each habitat is obtained by comparing with HBRA. [a]A goodness of fit is calculated to show the predominance of Firmicutes in the HBRA sample

To conclude, the present study not only brings out first time information on the bacterial communities inhabiting HBRA, but it could also be considered as a prelude to decipher the impact (if any) of natural background radiation on the diversity and distribution of microbes in the ecosystem. In spite of appearing harmless by itself, long-term exposure to low-dose radiation is also shown to have negative impacts (Hwang *et al.,* 2008). That the long-term exposure to (low-dose) radiation has made any impact on the HBRA microbial community, is speculative, but an investigatively challenging problem, inviting further study. Previous investigations have revealed that long-term exposure of microbes to radiation has produced diverse results, sometimes "biphasic", with beneficial effects at low doses (hormetic for instance), but detrimental at high doses (Calabrese, 2016), making the issue more complex. The results of experimentation by Ruiz-González *et al.* (2016), conducted on the barn swallow feather-associated bacterial population of Chernobyl areas, post-nuclear holocaust, deserves mention at this juncture. Here the investigators have identified three areas around Chernobyl, based on background radiation levels: of "high" (2.9 µGy hr^{-1}), "intermediate" (0.45 µGy hr^{-1}) and "low" (0.1 µGy hr^{-1}), and the control (0.03 – 0.05 µGy hr^{-1}) area. Pertinently, the bacteria inhabiting "high" and "intermediate" areas have shown perceptibly better survival rate and resistance against induced radiation, than those of the control and "low-dose" areas. That the radiation dose of Chavara-Neendakara HBRA falls within the span of "high" and "intermediate" areas of Chernobyl, makes the present study more relevant and interesting. Although radioactivity of this HBRA is known for several decades, its ecological relevance has not been explored so far.

The present study implicates that the background radiation prevalent in the HBRA is not ignorable, as it could exert a selection pressure, a matter of ecological and evolutionary

significance. Future longitudinal studies assessing the microbial community structure of HBRA over time should be able to address this question effectively. Being inherently and perpetually radioactive, HBRA could be an ideal "natural platform" for experimentation on long-term exposure to radiation, and the inhabiting microbes, the "natural model" for future investigations.

Acknowledgment

The receipt of VIT SEED GRANT from Vellore Institute of Technology (VIT), Vellore (India), is gratefully acknowledged.

Add-on Information

Authors' contribution: P. Sowptika: Laboratory work; **G. Anilkumar:** Conceptualized, planned and edited.

Funding: This work was funded by Vellore Institute of Technology, Vellore (India) through VIT SEED GRANT.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

References

Aliyu, A.S. and A.T. Ramli: The world's high background natural radiation areas (HBNRAs) revisited: A broad overview of the dosimetric, epidemiological and radiobiological issues. *Radiat. Meas.,* **73**, 51–59 (2015).

Asker, D., T. Beppu and K. Ueda: Unique diversity of carotenoid-producing bacteria isolated from Misasa, a radioactive site in Japan. *Appl. Microbiol. Biotechnol.,* **77**, 383–392 (2007).

Axelrood, P.E., M.L. Chow, C.S. Arnold, K. Lu, J.M. McDermott and J. Davies: Cultivation-dependent characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. *Can. J. Microbiol.,* **48**, 643–654 (2002).

Bevivino, A., P. Paganin, G. Bacci, A. Florio, M.S. Pellicer, M.C. Papaleo, A. Mengoni, L. Ledda, R. Fani, A. Benedetti and C. Dalmastri: Soil bacterial community response to differences in agricultural management along with seasonal changes in a Mediterranean region. *PLoS ONE*, **9**, e105515 (2014).

Calabrese, E.J.: The emergence of the dose-response concept in biology and medicine. I*nt. J. Mol. Sci.,* **17**, 2034 (2016).

Camacho, A., C. Mora, A. Picazo, C. Rochera, A. Camacho-Santamans, D. Morant, L. Roca-Pérez, J.J. Ramos-Miras, J.A. Rodríguez-Martín and R. Boluda: Effects of soil quality on the microbial community structure of poorly evolved Mediterranean soils. *Toxics*, **1**,14 (2022).

Chandran, G.A. and H. Sreedharan: Review on studies in high background radiation areas (HBRAs) of various parts of the world. *Int. J. Adv. Res. Biol. Sci.,* **3**, 163–169 (2016).

Chapon, V., L. Piette, M.H. Vesvres, F. Coppin, C.L. Marrec, R. Christen, N. Theodorakopoulos, L. Février, S. Levchuk, A.M. Garin, C.Berthomieu and C. Sergeant: Microbial diversity in contaminated soils along the T22 trench of the Chernobyl experimental platform. *Appl. Geochem.,* **27**, 1375–1383 (2012).

Derin, M.T., P. Vijayagopal, B. Venkatraman, R. Chaubey and A. Gopinathan: Radionuclides and radiation indices of high background radiation area in Chavara-Neendakara Placer Deposits (Kerala, India). *PLoS ONE*, **7**, e50648 (2012).

Ellis, R.J., P. Morgan, A.J. Weightman and J.C. Fry: Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl. Environ. Microbiol.,* **69**, 3223–3230 (2003).

Filippidou, S., T. Wunderlin, T. Junier, N. Jeanneret, C. Dorador, V. Molina, D. Johnson and P. Junier: A combination of extreme environmental conditions favor the prevalence of endospore-forming firmicutes. *Front. Microbiol.,* **7**, 1707 (2016).

Galtier, N., M. Gouy and C. Gautier: Seaview and phylo_ win: Two graphic tools for sequence alignment and molecular phylogeny. *Comput. Appl. Biosci.,* **12**, 543-548 (1996).

Ghiassi-Nejad, M., S.M.J. Mortazavi, J.R. Cameron, A. Niroomand-rad and P.A. Karam: Very high background radiation areas of Ramsar, Iran: Preliminary biological studies. *Hlth. Phys.,* **82**, 87-93 (2002).

Godson, P.S., N. Chandrasekar, S.K. Kumar and P.V. Vimi: Microbial diversity in coastal sediment during pre and post tsunami periods in the south east coast of India. *Front. Biol.,* **9**, 161-167 (2014).

Griffin, D.W.: Non-spore forming eubacteria isolated at an altitude of 20,000 m in Earth's atmosphere: Extended incubation periods needed for culture-based assays. *Aerobiologia*, **24**, 19–25 (2008).

Guðmundsdóttir, R., A.K. Kreiling, B.K. Kristjánsson, V. Marteinsson and S. Pálsson: Bacterial diversity in Icelandic cold spring sources and in relation to the groundwater amphipod *Crangonyx islandicus*. *PLoS ONE*, **14**, e0222527 (2019).

Hermans, S.M., H.L. Buckley, B.S. Case, F. Curran-Cournane, M. Taylor and G. Lear: Using soil bacterial communities to predict physico-chemical variables and soil quality. *Microbiome*, **8**, 79 (2020).

Hwang, S.L., J.S. Hwang, Y.T. Yang, W.A. Hsieh, T.C. Chang, H.R. Guo, M.H. Tsai, J.L. Tang, I.F. Lin and W.P. Chang: Estimates of relative risks for cancers in a population after prolonged low-dose-rate radiation exposure: A follow-up assessment from 1983 to 2005. *Radiat. Res.,* **170**, 143-148 (2008).

Jost, L.: Entropy and diversity. *Oikos*, **113**, 363–375 (2006).

Kim, O.S., Y.J. Cho and K. Lee: Introducing EzTaxon-e: A prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.,* **62**, 716–721 (2012).

Kumar, R., M. Nongkhlaw, C. Acharya and S.R. Joshi: Uranium (U)-tolerant bacterial diversity from U ore deposit of domiasiat in North-East India and its prospective utilisation in bioremediation. *Microbes Environ.,* **28**, 33–41 (2013).

Kumar, S., D.C. Suyal, A. Yadav, Y. Shouche and R. Goel: Microbial diversity and soil physiochemical characteristic of higher altitude. *PLoS ONE*, **14**, 1–15 (2019).

Lester, E.D., M. Satomi and A. Ponce: Microflora of extreme arid Atacama Desert soils. *Soil. Biol. Biochem.,* **39**, 704-708 (2007).

Li, A.Z., X.B. Han, M.X. Zhang, Y. Zhou, M. Chen, Q. Yao and H. Zhu: Culture-dependent and -independent analyses reveal the diversity, structure, and assembly mechanism of benthic bacterial community in the Ross Sea, Antarctica. *Front. Microbiol.,* **10**, 2523 (2019).

Li, Y., Z. Li, K. Yamanaka, Y. Xu, W. Zhang, H. Vlamakis, R. Kolter, B. Moore and P. Qian: Directed natural product biosynthesis gene

cluster capture and expression in the model bacterium *Bacillus subtilis*. *Sci. Rep.,* **5**, 9383 (2015).

Lladó, S., R. López-Mondéjar and P. Baldrian: Drivers of microbial community structure in forest soils. *Appl. Microbiol. Biotechnol.,* **102**, 4331–4338 (2018).

Lladó, S. and P. Baldrian: Community-level physiological profiling analyses show potential to identify the copiotrophic bacteria present in soil environments. *PLoS ONE*, **12**, e0171638 (2017).

Majid, S.A., M.F. Graw, A.D. Chatziefthimiou, H. Nguyen, R. Richer, M. Louge, A.A. Sultan, P. Schloss and A.G. Hay: Microbial characterization of Qatari Barchans and Dunes. *PLoS ONE*, **11**, 1–22 (2016).

Michelato Ghizelini, A., L. Cristina Santana Mendonça-Hagler and A. Macrae: Microbial diversity in brazilian mangrove sediments - A mini review. *Brazilian J. Microbiol.*, **43**, 1242–1254 (2012).

Moore, E., A. Arnscheidt, A. KrÜger, C. StrÖmpl and M. Mau: Section 1 update: Simplified protocols for the preparation of genomic DNA from bacterial cultures. In: Molecular Microbial Ecology Manual. Springer Netherlands, Dordrecht, pp. 1905–1919 (2008).

Mubarak, F., M. Fayez-Hassan, N.A. Mansour, T.S. Ahmed and A. Ali: Radiological investigation of high background radiation areas. *Sci. Rep.,* **7**, 15223 (2017).

Nan, J., L. Chao, X. Ma, D. Xu, L. Mo, X. Zhang, X. Zhao and Y. Bao: Microbial diversity in the rhizosphere soils of three Stipa species from the eastern Inner Mongolian grasslands. *Glob. Ecol. Conserv.,* **22**, e00992 (2020).

Nathan, V.K., J. Vijayan and P. Ammini: Comparison of bacterial diversity from two mangrove ecosystems from India through metagenomic sequencing: Comparative mangrove bacterial diversity using metagenomics. *Reg. Stud. Mar. Sci.,* **35**, 101184 (2020).

Najar, I.N., M.T. Sherpa, S. Das and N. Thakur: Microbial ecology of two hot springs of Sikkim: Predominate population and geochemistry. *Sci. Total Environ.,* **637–638**, 730–745 (2018).

Paschoa, A.S.: More than forty years of studies of natural radioactivity in Brazil. *Technology*, **7**, 193–212 (2000).

Pimpliskar, M.R. and R.N. Jadhav: Study on bioactive property of marine bacteria isolated from soil sediment along Vasai coast. *J. Pure. Appl. Microbiol.,* 9, 1573-1577 (2015).

Qin, N., P. Liang, C. Wu, G. Wang, Q. Xu, X. Xiong, T. Wang, M. Zolfo, N. Segata, H. Qin, R. Knight, J. Gilbert and T. Zhu: Longitudinal survey of microbiome associated with particulate matter in a megacity. *Genome Biol.,* **21**, 55 (2020).

Ruiz-González, M.X., G.Á. Czirják, P. Genevaux, A. Møller, T. Mousseau and P. Heeb: Resistance of feather-associated bacteria to intermediate levels of Ionizing radiation near Chernobyl. *Sci. Rep.,* **6**, 22969 (2016).

Salazar, G. and S. Sunagawa: Marine microbial diversity. *Curr. Biol.,* **27**, R489–R494 (2017).

Sayed, A.M., M.H.A. Hassan, H.A. Alhadrami, H.M. Hassan, M. Goodfellow and M.E. Rateb: Extreme environments: microbiology leading to specialized metabolites. *J. Appl. Microbiol.,* **128**, 630–657 (2020).

Selim, S., S. Hassan, N. Hagagy, L. Kraková, T. Grivalský and D. Pangallo: Assessment of microbial diversity in Saudi springs by culture-dependent and culture-independent methods. *Geomicrobiol. J.,* **34**, 443–453 (2017).

Shannon, C.E.: A Mathematical Theory of Communication. *Bell Syst. Tech. J.,* **27**, **379–423**, 623–656 (1948).

Sheng, P., Y. Yu, G. Zhang, J. Huang, L. He and J. Ding: Bacterial diversity and distribution in seven different estuarine sediments of Poyang Lake, China. *Environ. Earth. Sci.,* **75**, 1-9 (2016).

Stepanov, V.G., M.R. Tirumalai, S. Montazari, A. Checinska, K. Venkateswaran and G.E. Fox: *Bacillus pumilus* SAFR-032 genome revisited: Sequence update and re-annotation. *PLoS ONE,* **11**, e0157331 (2016).

Steven, B., G. Briggs, C.P. McKay, W.H. Pollard, C.W. Greer and L.G. Whyte: Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. *FEMS Microbiol. Ecol.,* **59**, 513–523 (2007).

Sunta, C.M., M. David, M.C. Abani, A.S. Basu and K.S.V. Nambi: Analysis of dosimetry data of high natural radioactivity areas of southwest coast of India. In: The Natural Radiation Environment (Eds.: K.G. Vohra, U.C. Mishra, K.C. Pillai and S. Sadasivan). Wiley Eastern Limited, India, pp. 35-42 (1982).

Tamaki, H., Y. Sekiguchi, S. Hanada, K. Nakamura, N. Nomura, M. Matsumura and Y. Kamagata: Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. *Appl. Environ. Microbiol.,* **71**, 2162–2169 (2005).

Vaiserman, A., A. Koliada, O. Zabuga and Y. Socol: Health impacts of low-dose ionizing radiation: Current Scientific Debates and Regulatory Issues. *Dose Response*,**16**, 1559325818796331 (2018).

Venil, C.K., L. Dufossé and P. Renuka Devi: Bacterial pigments: sustainable compounds with market potential for pharma and food industry. *Front. Sustain. Food. Syst.,* **4**, 100 (2020).

Wang, M., S. Noor, R. Huan, C. Liu, J. Li, Q. Shi, Y. Zhang, C. Wu and H. He: Comparison of the diversity of cultured and total bacterial communities in marine sediment using culture-dependent and sequencing methods. *Peer J.,* **8**, e10060 (2020).

Wei, L. and T. Sugahara: An introductory overview of the epidemiological study on the population at the high background radiation areas in Yangjiang, China. *J. Radiat. Res.,* **41**, Suppl:1–7 (2000).

Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane: 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.,* **173**, 697–703 (1991).

Yu, L.Z.H., X.S. Luo, M. Liu and Q. Huang: Diversity of ionizing radiation-resistant bacteria obtained from the Taklimakan Desert. *J. Basic. Microbiol.,* **55**, 135–140 (2015).

Zhang, J., Q.L. Sun, Z.G. Zeng, S. Chen and L. Sun: Microbial diversity in the deep-sea sediments of Iheya North and Iheya Ridge, Okinawa Trough. *Microbiol. Res.,* **177**, 43-52 (2015).