A Metagenome Diversity Profile of Springs Microbial for Oligotroph Springs Assessment in UB Forest

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Received: February 15, 2024 **Accepted: October 27, 2024**

Published: June 30, 2025

Abstract: *Ecosystem quality is an emergent property of a complex system that interacts between biotic and abiotic factors. The study aims to determine the bacterial community profile of several springs with different surrounding ecosystems. Metagenomic analysis using next-generation sequencing (NGS) is performed to determine the microbial profile, taxa richness, and relative abundance of spring water from Buk Bejat (BB), Sumber Dampul (SD) 1, 2, and 3, respectively. The community profile of spring water bacteria at the phylum level shows the same pattern in all study areas. Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidota are the dominant phyla in all sites. At the family level, Comamonadaceae is the most plentiful family in all study sites, along with Lactobacillales P5D1-392, which is found to have a low frequency in SD2 and SD3 compared to SD1 and BB. On the other hand, Muribaculaceae, Morganellaceae, Bacteroidaceae, Oscillospiraceae, Bifidobacteriaceae, Saccharimonadaceae, and Peptostreptococcaceae are found with higher frequency in SD2 and SD3 compared to BB and SD1. The hierarchical clustering at the family level shows two closely related clusters composed of the ecosystem, BB-SD1, and SD2-SD3, but this cluster is not followed by bacterial beta diversity. The alpha diversity in BB and SD1 is higher than in SD2 and SD3 based on ACE, Chao1, Margalef, and Simpson indexes.*

Keywords: *Diversity, Microbial profile, Spring water.*

Introduction

The main mountains' raw water source, especially Mount Arjuno in Indonesia, is from springs [30]. Based on the interview with the local people near UB Forest (UBF) – part of Mount Arjuno, it is almost impossible for them to reach the water from the Municipal Waterworks because of the higher location and the higher bill. For several years, the quality and quantity of water springs in UBF have changed. Another study found that the number of springs in Mount Arjuno in 2014 decreased and dried throughout the year [28]. The spring water comes through several layers of materials like sand, clay, and gravel from the ground to reach the surface. The water may contain minerals, but there is a risk of bacterial or viral contamination [27, 33].

Accessing safe water is a fundamental human right, which is the safety level influenced by the water microbes. Certain species of microorganisms, including bacteria, inhabit spring water and can degrade organic and inorganic contaminants. Meanwhile, other types of microorganisms in spring water threaten human health. The terrestrial condition immensely influences the community of water microorganisms in the upstream area, and the shaping of the

microorganism community is dynamic. Surrounding ecosystem conditions, organic fertilizer application, land-use changes, hydrological seasonality, and vegetation may affect dissolved organic matter runoff into aquatic ecosystems, and it may alter the aquatic microbial community. Dissolved organic carbon (DOC) concentration in water is correlated with *Proteobacteria* and *Actinobacteria*. *Proteobacteria* and *Actinobacteria* become more prevalent as DOC levels increase. The bacterial community in aquatic environments is dynamic and adapts to a variety of factors, such as terrestrial activity. DOC levels can be influenced by this terrestrial influence, which in turn affects the abundance of these bacterial phyla [12, 22, 34, 35].

Springs are the most sensitive indicators of global climate change and the window into the status of our groundwater in all aspects, including bacterial diversity. Microorganisms support all trophic life forms, and the community profile depends on the terrestrial ecosystem quality [11]. Using a multidisciplinary approach to examine spring water microbial populations as indicators of groundwater health and terrestrial ecosystem quality is an innovative biomonitoring tool. It also opens the door to novel conservation strategies for aquatic and terrestrial environments, improving water security and ecosystem management. This preliminary study is needed to investigate the metagenome community profile of spring water bacteria. Metagenomic high-throughput sequence analysis can identify detailed and susceptible microbial communities from several spring waters with different terrestrial conditions. The relative abundance of bacteria was determined to provide an initial metagenomic survey of the total microbial content.

Materials and methods

Study area

The study sites are in the UB Forest, part of Mount Arjuno, Indonesia, where the Donowarih and Tawangargo villages are placed (Fig. 1). There are four springs in UBF that are observed and they are used by the local people: 1) Buk Bejat (BB; 1247.55 masl); 2) Sumber Dampul 1 (SD1; 1205.48 masl); 3) Sumber Dampul 2 (SD2; 1171.65 masl); and 4) Sumber Dampul 3 (SD3; 1163.12 masl). SD2 and SD3 are easier to access than SD1 and BB since both springs are correct in the main footpath. Based on the previous study, there are two clusters: BB-SD1 (conserved cluster) and SD2-SD3 (non-conserved cluster), because of their vegetation and phytoplankton community profile. The highest vegetation abundance is found in SD1, while the lowest is in SD2, which is dominated by *Moraceae* family plants. The landslide that happens in SD2 causes low vegetation abundance [37].

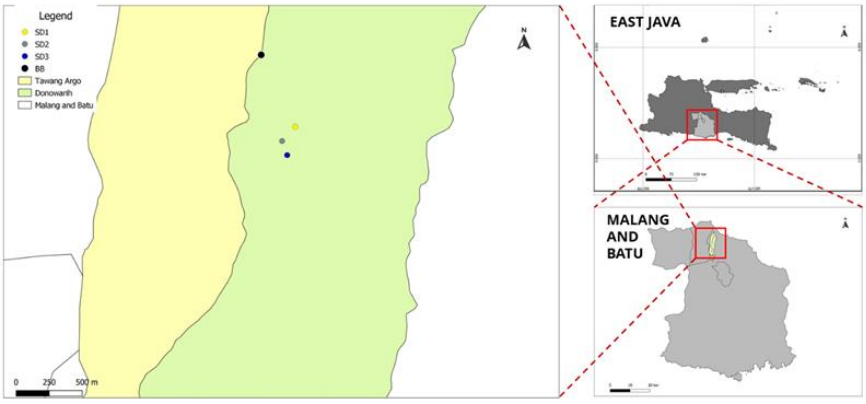


Fig. 1 The springs' location in UBF area. BB spring is located in the border between Tawang Argo region of India and Donowarih region of Indonesia, and SD1, SD2, SD3 are in the Donowarih region of Indonesia.

Sample collection and preparation

Water samples from springs are from three different points at each study site. The spring water collected for DNA analysis in this study is a composite from three replications for every site. It is placed in a sterile bottle and stored in the icebox. As much as a liter of spring water is filtered by vacuum filtration at room temperature (23-25 ℃). Spring waters are passed through a 0.45 μm pore size sterile Whatman, which traps microorganisms. However, the volume of water filtered from every site is similar to a liter. The filtrate on the membrane from all sites is stored at -78°C until DNA preparation.

DNA extraction and sequencing

Total DNA is isolated from the microbial biomass collected on filter membranes for each spring water sample using the ZymoBIOMICSTM DNA Miniprep Kit. The isolated DNA is evaluated and confirmed using a nanodrop and electrophoresis before sequencing. Sequencing is performed using the Illumina NovaSeq 6000 platform provided by Novogene (China). The sequencing target is the bacterial 16S rRNA gene region V3-V4 with primary sequences 341F – CCTAYGGGRBGCASCAG (forward) and 806R – GGACTACNNGGGTATCTAAT (reverse).

Importing data, identifying taxa, and diversity analysis

Sequences in the fastq format are analyzed using the pipeline from QIIME2 2021.4 [8]. The 16S region V3-V4 sequences are imported into the program then de-multiplexing is performed to determine the quality of the sequences. Furthermore, the sequences are trimmed to remove non-biological sequences using the cut-adapt plugin [25]. The sequences are filtered and combined (forward and reverse) using the DADA2 plugin [10]. Sequences below 20 have poor quality, so those sequences are filtered and not included in the next analysis.

Sequence taxonomy identification is carried out using the SILVA version 138 weighted classifier database for bacteria [41] using the q2-feature-classifier plugin [7] with the option of classifying sklearn [29]. The alpha diversity of each sample is determined based on several parameters, such as Shannon, Simpson, Margalef, Chao1, and the ACE diversity index. Beta diversity is evaluated based on Bray-Curtis, weighted UniFrac, and unweighted UniFrac Distance Index. Rarefaction curves are also constructed to determine the effect of sampling depth on the diversity value of each sample. The relative abundance is analyzed based on the relative number of taxa in each sample, and it is visualized in the taxa barplot and heatmap [16].

Results and discussion

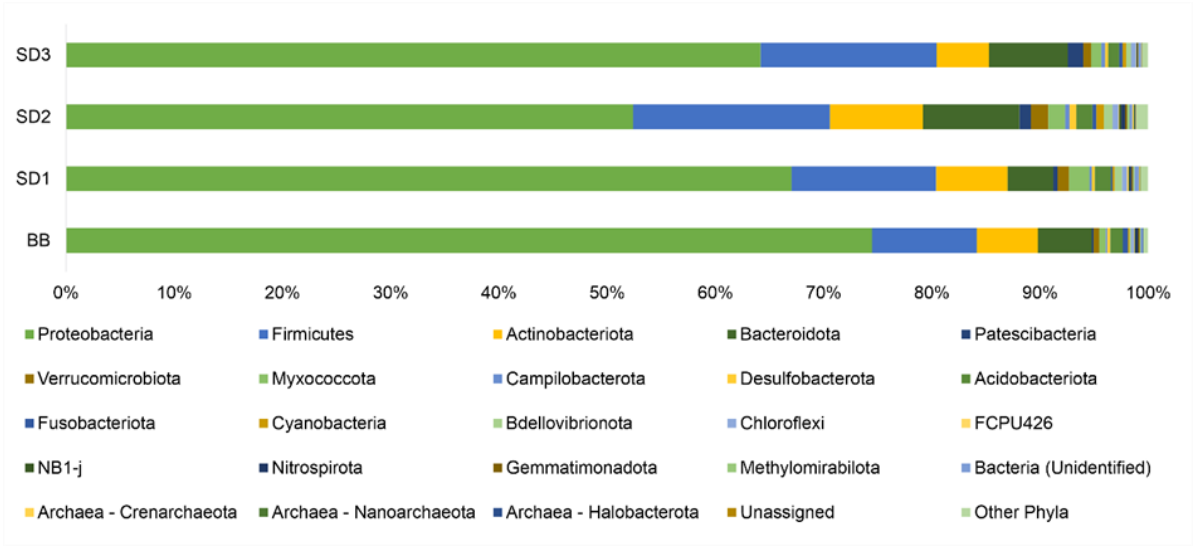
Variation of bacterial community among sites

The bacterial community among the springs of UBF varies based on phylum level, and the pattern is similar. This study identifies 31 bacterial phyla found in four sites dominated by several phyla, including *Proteobacteria*, *Firmicutes*, *Actinobacteriota*, and *Bacteroidota*. Although the predominant phylum in each location is the same, the relative abundance of each phylum in each location is relatively different. *Proteobacteria* are the most abundant phylum in each location, with a total relative abundance exceeding 50%. BB had the most generous number of *Proteobacteria* (more than 70%), followed by SD1 (almost 70%), and both locations are more pristine. In other aquatic ecosystems, including rivers and glacial lakes, *Proteobacteria* are found to be more dominant than other phyla [9, 44]. Based on these data, the relative abundance of *Proteobacteria* at the conserved location is higher than in the unconserved area. *Firmicutes* are the second most abundant phylum, with more SD2 (20%) and SD3 (18%) than SD1 and BB. *Bacteriodota* phylum also had a similar pattern with *Firmicutes*,

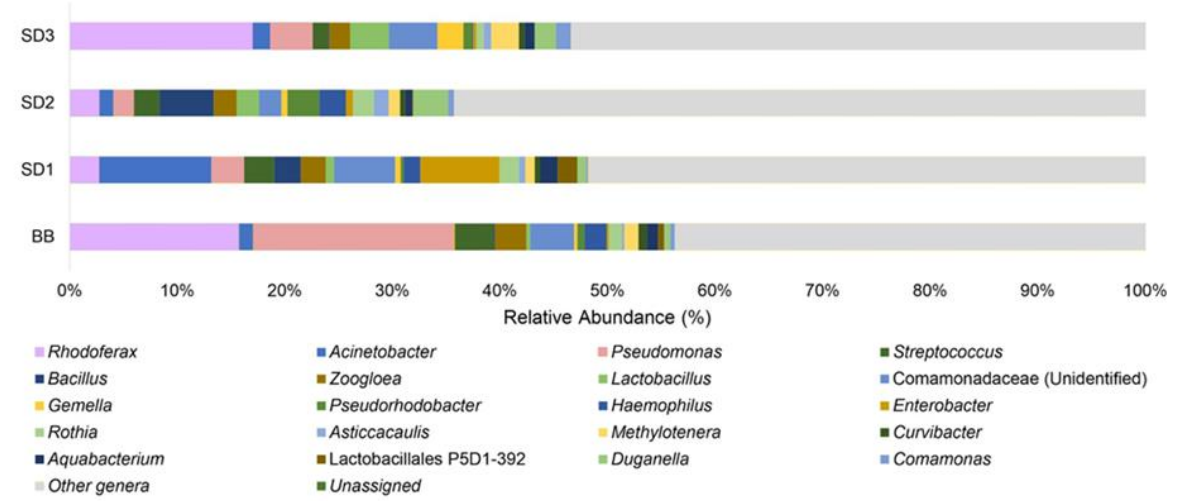
which is more abundant in SD2 and SD3 (Fig. 2A). Meanwhile, [26] reported that *Proteobacteria* is the dominant phylum in the river with low anthropogenic activities, and it is followed by *Bacteroidota*, *Patescibacteria*, *Firmicutes*, and *Actinobacteria*.

Proteobacteria is a major group (phylum) of bacteria that consists of a wide variety of pathogens; others are free-living and nitrogen fixers that typically live in several habitats, including soil, freshwater, and wastewater. This finding agrees with the other results, which examined the bacterial community structures in wastewater treatment bioreactors via high-throughput sequencing [4], urban surface waters [18], and an oligotrophic cave environment [14]. The spring's location in the forest may cause an abundance level of *Proteobacteria* due to the plentiful availability of organic matter. Nitrite-oxidizing bacteria are mainly from the *Proteobacteria* phyla, which converts ammonia into its the most oxidized form [15]. Another study shows that the number of nitrogen-fixing bacteria increases in undisturbed forest areas more than in agriculture [42]. *Firmicutes* are the second largest group in UBF springs. This phylum is plentiful in the environment, especially in waters exposed to anthropogenic fecal contamination [18] also dominated by oligotrophic aquatic ecosystems [5]. Apart from humans and mammals, *Firmicutes* are also found in the digestive system of freshwater and marine fish [43]. *Actinobacteria* is the third largest group in all of the sites. Since the springs are in the forest and agricultural land, they can be washed into aquatic habitats. *Actinobacteria* have significant contributions to the decomposition of organic materials, such as cellulose and chitin [2, 32, 39]. A genus of *Actinobacteria* that inhabits freshwater is *Micromonospora*, and they can turn over cellulose, chitin, and lignin [2]. *Bacteroidetes*, the fourth largest abundance, are also microbiota in the gut tract, and they constitute about 70-90% of relative abundance in the lotic ecosystem [1, 19, 45]. *Muribaculaceae*, *Morganellaceae*, *Bacteroidoceae*, *Bifidobacteriaceae*, *Saccharimonadaceae*, *Oscillospiraceae*, and *Peptostreptococcaceae* are several families that are from *Firmicutes* and *Bacteriodota*/*Bacteroidetes* as the gut microbiome. With the abundance of *Firmicutes* in the fourth sites, especially in SD2, *Firmicutes* contamination of spring water may originate from mammals or soil that are washed by water flow or rain. Furthermore, interference from animals or human activities influences spring water quality.

Activities and ecosystem quality near water sources influence the ecosystem and water quality of water sources, including springs [21, 31, 38]. More than 400 bacterial genera are identified in this study. Moreover, the spring water bacteria community structure shows variations. Genera *Rhodoferax*, *Pseudomonas*, *Bacillus*, *Comamonas*, and family *Comamonadaceae* play a role in the sulfur, iron, and nitrogen cycle [6, 17, 23] and exist in all of the springs (relative abundance total SD2 < SD1 < BB < SD3). Even though SD2 has the highest total nitrate concentration [37], the number of bacteria in the nitrogen cycle is the lowest among other locations. Bacteria lead the biogeochemical cycles of carbon, nitrogen, and other nutrients in an ecosystem. Indigenous microbial communities and environmental conditions determine the rate of organic matter degradation in an ecosystem [13]. On the other hand, several pathogenic bacteria are commonly found in humans and animals, including *Acinetobacter*, *Gemella*, *Haemphillus*, and *Enterobacter* (Fig. 2B). SD1 is where pathogenic bacteria are located more abundantly than the others. Based on a previous study, SD1 is the conserved area based on phytoplankton and vegetation profile, and it has the highest water debit among other locations. It may cause more to humans and animals to visit SD1 than other locations. Changes in land use or ecosystem around the springs and poor management may result in fluctuation of the microbial community, with consequences to water quality.



A)



B)

Fig. 2 Relative abundance of water bacteria in A) phyla, and B) top twenty genera level in each of the fourth locations

The performed hierarchical clustering informs the relatedness of the studied ecosystems in terms of their microbial community features. Hierarchical clustering at the family level shows two closely related clusters composed of the ecosystem, BB-SD1, and SD2-SD3. This finding is similar to the previous study, which mentions two groups (BB-SD1 and SD2-SD3) based on vegetation and the phytoplankton community [37]. *Comamonadaceae* is the family with the highest abundance in all samples, along with *Lactobacillales* P5D1-392, which is found with a low frequency in SD2-SD3 compared to SD1-BB. On the other hand, *Muribaculaceae*, *Morganellaceae*, *Bacteroidaceae*, *Oscillospiraceae*, *Bifidobacteriaceae*, *Saccharimonadaceae*, and *Peptostreptococcaceae* were found with higher frequency in SD2 and SD3 compared to BB and SD1 (Fig. 3). Those families are the microflora of the mammalian digestive tract [45].

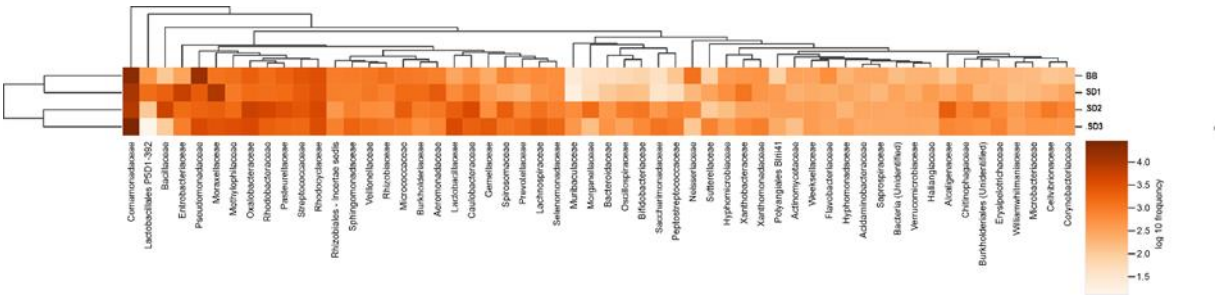


Fig. 3 Heat map of bacteria family level, generated in cluster and visualized using tree-view. The red color indicates a higher frequency, and the white color indicates no frequency.

Bacterial diversity

The alpha diversity describes the taxa richness for every site, and several diversity indexes appropriately show it. Table 1 shows that the taxa richness of every site is diverse. Based on Operational Taxonomic Units (OTUs), the most varied taxa belong to SD1 (2563 OTUs). Then it is followed by SD2 (2332 OTUs), SD3 (2262 OTUs), and BB (2188 OTUs). Based on ACE, Chao1, Margalef, and Simpson, the alpha diversity of SD1 is the highest, but the Shannon index says that the diversity of SD1 and SD2 is precisely the same. The variety of microbes at every site is relatively wide since the Shannon index value is more than eight. The evenness level of every location is similar since the Simpson index value is relatively equal. The community profile of bacteria in a freshwater ecosystem is influenced by several natural and anthropogenic factors, including land use, vegetation, leaching from the soil, weathering of rocks, depositions due to wind, runoff, etc. [13].

Table 1. Several non-phylogenetic diversity indexes of the springs

Location	Operational taxonomic units, (OTUs)	Diversity indexes				
		ACE	Chao1	Margalef	Shannon	Simpson
BB	2188	2229.37	2211.94	193.98	8.24	0.98
SD1	2563	2607.46	2587.20	227.03	8.86	0.99
SD2	2332	2354.08	2345.87	201.91	9.38	0.99
SD3	2262	2283.96	2275.00	195.21	8.95	0.98

A total of 329 features were found in all samples analyzed, with 1432, 1793, 1369, and 1349 features found only specific to samples BB, SD1, SD2, and SD3, respectively (Fig. 4). This means that as many as 329 taxa might have a wide tolerance range to environmental changes that occur in the four study sites. Along with some studies, the existence of a core number of bacteria shows that bacteria have adapted to survive and thrive in a variety of ecosystem conditions [20, 40]. It also proves the resilience of ecosystems to environmental changes and disturbances, as well as biogeochemical cycling [24]. The specific OTUs inform the number of unique bacteria with the highest alpha diversity, which belong to pristine locations (BB and SD1). Unique bacteria in different aquatic ecosystems refer to the distinct bacterial groups that are specifically adapted to thrive in specific aquatic environments and have evolved to survive and thrive in their respective environments [36]. For example, *Polaromonas* sp. is a unique species of bacteria involved in decomposition in glacial areas, and *Sideroxydans lithotrophicus* in subglacial locations [3].

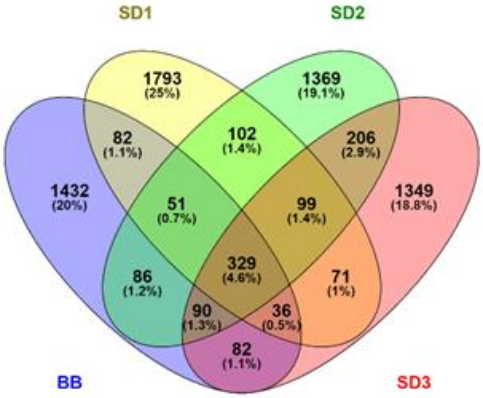


Fig. 4 Venn diagram displays the identified features based on the molecular sequencing data. The numbers within the circles represent the specific bacterial OTUs in the sites while common bacterial OTUs between locations are represented in the overlaps. The prevalent bacterial OTUs present in all locations are represented by the core number.

The beta diversity provides information about the interrelationships of the spring ecosystem based on its microbial community. Beta diversity analysis shows no prominent clustering pattern among the analyzed sites. Based on the Bray-Curtis index, each site is separated from the others without any indication of grouping (Fig. 5A). A similar result is also shown by the plotting results of the UniFrac weighted index. SD1 and SD3 tend to be identical to each other according to the unweighted UniFrac distance index (Fig. 5B). However, the SD2 and SD3 samples tend to be dissimilar, with a different value of more than 50% (Fig. 5C). The beta diversity analysis provides information that the environment around spring may influence the bacterial water community. For example, vertical vegetation structures (aboveground vegetation cover, surface litter layer, and underground roots), plant diversity, vegetation patterns, and scale characteristics are responsible for runoff and soil loss, including organic matter and bacteria [22, 34].

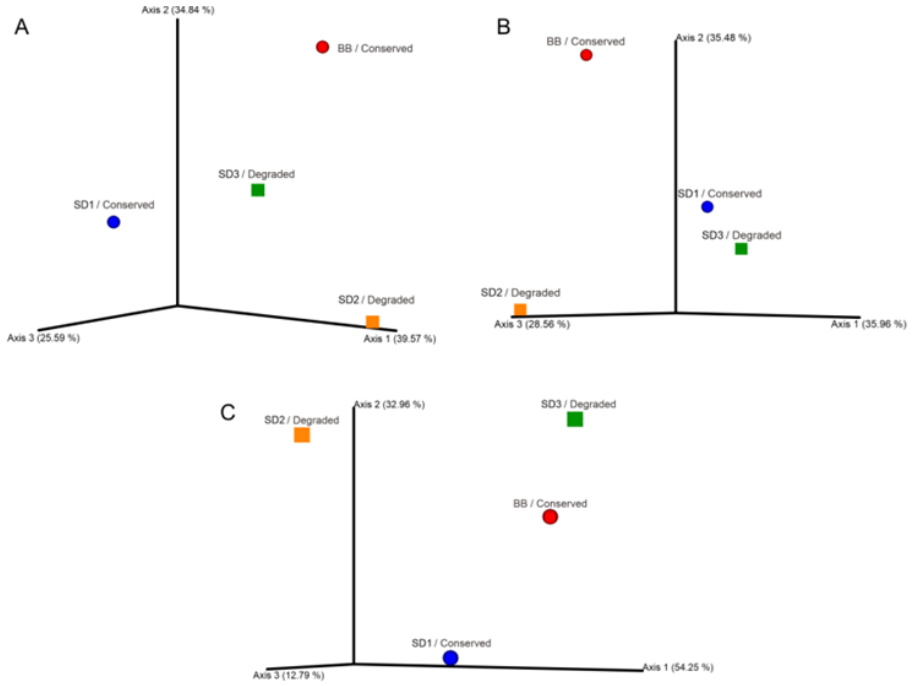


Fig. 5 Beta diversity represented in the emperor plot based on:
A) the Bray-Curtis index, B) unweighted UniFrac, and C) weighted UniFrac

Conclusion

Different springs with different surrounding ecosystems create a variety of microbial water profiles. The condition affects the composition and diversity of bacteria. Several phyla, including *Proteobacteria*, *Firmicutes*, *Actinobacteriota* and *Bacteroidota* are found predominant in each location, but the relative abundance of each phylum in each location is relatively different. This study also finds that the springs in the less disturbed area have a higher alpha diversity than in the disturbed area.

Acknowledgements

The authors would like to thank the Director and the staff members of UB Forest for the research permission, Dr. Muhammad Yusuf, Dr. Purnomo and Nuansa Farah Pratiwi for sampling and data collection.

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