INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
23–0273/2023/30–6–381–387
DOI: 10.17957/IJAB/15.2099
http://www.fspublishers.org

Full Length Article

Diversity of Microbial Communities in Three Indonesian Salt Ponds: An Exploration of Extreme Biodiversity

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Received 22 July 2023; Accepted 19 September 2023; Published 30 November 2023

Abstract

Throughout the process of salt crystallization, microbial populations play a crucial role in determining the overall quality of salt. This study aimed to investigate the microbial diversity of traditional salt ponds in Indonesia. Regarding the diversity of Operational Taxonomic Units (OTUs), Tuban exhibited the highest diversity in the high salinity category, Brebes showed the highest diversity in the medium salinity category, and Sampang displayed the highest community diversity in the low salinity category. In all analyzed samples, the dominant bacterial communities belonged to the phyla Proteobacteria (14.64–71.39%), Bacteroidetes (6.26–17.00%) and Actinobacteria (1.89–12.54%), while the archaeal community was predominantly composed of the phylum Euryarchaeota (1.05–72.92%). Despite being in the same Java Sea region, all three salt ponds exhibited variations in their bacterial community profiles. This information provides an effective system for innovative biological solutions aimed at improving the ecological health of traditional salt ponds.

Keywords: Salt pond; Bacteria; Archaea; Salinity

Introduction

Salt ponds represent highly saline ecosystems situated along the coastlines of tropical and subtropical regions (Kalwasińska *et al*. 2018). Besides their significant role in salt production, these ponds exhibit unique environmental and ecosystem characteristics, making them subjects of substantial scientific interest (Rocha *et al*. 2012). Numerous organisms can be found in hypersaline environments such as various taxonomic domains, including bacteria, archaea, viruses and eukaryotes (Oren 2014). Halophilic and archaeal bacteria predominate among these communities in hypersaline environments such as salt ponds, saline lakes and salt crystals (Prashad and Ram 2020).

In these habitats, salinity is a significant factor affecting the variety and quantity of microorganisms (Kalwasińska *et al*. 2018). The distribution and composition of halophilic organisms are closely related to exceptional characteristics influenced by hypersaline environments (Han *et al*. 2017). Notably, in hypersaline environments boasting salinities exceeding 25%, archaea and halophilic bacteria dominate the microbial community (Simachew *et al*. 2016). Several studies have found that archaeal organisms represent approximately 95% of the total organisms identified in Russian salt lakes at salinity levels of 190–290 g/L (Bryanskaya *et al*. 2016). The genera *Halorubellus*, *Halorubrum*, *Halapricum*, *Halonotius*, and *Natronomonas* demonstrated ecological dominance within Chinese salt lakes (Han *et al*. 2017). Similarly, in the salt ponds of Tunisia, the prevalence of *Haloquadratum walsbyi* was found by Ghai *et al*. (2011).

In traditional salt ponds, the process of salt production in a biological system is well known to involve the entire biological community. The implementation of biological systems in salt pond operations had the potential to increase evaporation, leading to improved salt production both in terms of quantity as well as quality. Halophilic bacteria and archaea assume a crucial role in the intricate process of salt crystallization. As studied by Chasanah *et al*. (2020), these microorganisms have the potential to significantly enhance the quality and overall conventional salt production. The study by Chasanah *et al*. (2021) also showed that the distribution of these microorganism in salt water is often overlooked, as well as there is limited information available on the occurrence of halophiles, especially in Indonesia (Chasanah *et al*. 2020) However, researchers have conducted several studies on the use of halophilic bacteria to improve traditional salt ponds. For instance, Marihati *et al*. (2014) employed cultured

To cite this paper: Susilowati R, DG Bengen, M Krisanti, HI Januar, I Rusmana (2023). Diversity of microbial communities in three Indonesian salt ponds: an exploration of extreme biodiversity. *Intl J Agric Biol* 30:381–387

halophilic bacteria and *Artemia* sp. in Madura, Indonesia, resulting in a remarkable 64.7% increase in salt production along with a 2.63% improvement in salt purity.

Brebes, Tuban and Sampang regencies in Indonesia have been renowned for generations as three traditional salt production centers. Brebes and Tuban regencies are in central and eastern Java Island, respectively, while Sampang regency is located on Madura Island which is located east of Java Island and separated by the Madura Strait. The Madura Islands are well-known for producing high-quality salt. This research examined the diversity of halophilic bacteria in three different salt ponds. Furthermore, these data serve as a foundation for innovating biological solutions aimed at enhancing the ecological health of conventional salt ponds. Additionally, Indonesia's conventional salt ponds will have better quality as a result.

Materials and Methods

Study site

Brebes (B.10, B.15 and B.20), Tuban (T.10, T.15 and T.20), and Sampang (S.10, S.15 and S.20) traditional salt ponds were investigated. The pond was divided into three sections: the low-salinity ponds, each having a water depth of 50–60 cm and a salinity level below 100 practical salinity unit (PSU) (B.10, T.10, S.10); the medium-salinity section (B.15, T.15, S.15) with a range of 100 to 150 psu and water depths between 15–20 cm; and finally, the high-salinity section with a range from 150 to greater than > 200 psu, and water depth varying from 5 to 10 cm (B.20, T.20, S.20). Traditional salt ponds occupied an area ranging from 1 to 1.5 Ha, with each individual pond spanning 100 to 150 m^2 in size. These salt ponds were situated at specific coordinates 113°08'21.67"E-7°12'33.76"S, while those in Brebes Regency were discovered at 109°01'07.0"E-6°48'06.0"S, and Tuban Regency at 112°09'44.8"E-6°54'24.3"S. The samples were taken in October 2021.

Microbial sampling and DNA extraction

One liter of water was randomly selected from ponds that had different salinity gradients. The water underwent filtration using a Corning vacuum pump fitted with micropore membrane filters, which included a 0.45 and 0.22 µm membrane filter (Silva *et al*. 2023). DNA extraction was carried out using the ZymoBIOMICS DNA Miniprep Kit isolation kit. The purity of the extracted DNA was confirmed by subjecting it to 1% agarose gel electrophoresis and visualizing it with ethidium bromide.

PCR amplification, library preparation, and illumina MiSeq sequencing

The 515F-806R primer was used to amplify the hypervariable region V4 of the 16S rRNA gene (Walters *et al.* 2016). The Biolab New England Phusion High Fidelity PCR Master Mix was added to the PCR process. Following the manufacturer's instructions, sequencing libraries were created using the NEBNext® UltraTM DNA Library Prep Kit for Illumina from New England Biolabs. The Qubit@ 2.0 Fluorometer from Thermo Fisher Scientific Inc. and the Agilent Bioanalyzer 2100 system from Agilent Technology, Inc. were used to evaluate the library's quality. The library was sequenced on an Illumina HiSeq 2500 platform, generating 250 bp reads.

Analysis of data

The samples' distinctive barcodes were used to assign pair-end reads. Afterward, the samples' barcode and primer sequences were trimmed before being combined using FLASH v1.2.7 (Magoˇc and Salzberg 2011). The obtained raw tags were processed using QIIME v1.7.0 for optimal quality control (Caporaso *et al.* 2010). The UCHIME algorithm was applied to eliminate chimera sequences (Haas *et al*. 2011). The UPARSE tool was used to analyze the collected effective tags (Edgar 2013). Effective tag sequences were provided Operational Taxonomic Units (OTUs) with a similarity level of less than 97%. To annotate representative sequences obtained from OTU clustering using the MOTHUR software and the SILVA database, employing a threshold of 0.8–1 (Schloss *et al*. 2009). Alpha diversity metrics, including observed species, Chao1, ACE, Shannon Index, Simpson Index and Good's coverage, were calculated using QIIME v1.7.0 and visualized using R v2.15.3. QIIME v1.7.0 software was used to compute beta diversity based on unweighted and weighted distances of UniFrac. UPGMA clustering to interpret the distance matrix was conducted using QIIME v1.7.0 software.

Results

Abundance of prokaryotes in salt ponds

Using high-throughput Illumina sequencing, the bacteria in 27 samples from salt ponds were investigated to determine their composition. The results showed that 4,085,031 16S rRNA gene sequences were examined, of which 3,939,964 sequences were of high quality. These sequences were divided into 6,390 operational taxonomic units (OTUs) with a 97% similarity level. The sample with the highest number of detected OTUs (3,370) in the low salinity samples was at Sampang (S.10) (Table 1). Brebes (B.15) had 2,615 OTUs, which was the most in the medium salinity group. Continuing to the high salinity group, the sample from Tuban (T.20) had the most OTUs (2,203). In all samples, the goods coverage for each sample ranged from 0.99 to 1. The rarefaction curves demonstrated a flat or non-decreasing trend. In other words, the rarefaction curve provided information about the species diversity in the samples and confirmed that the generated data was adequate to obtain an accurate representation (Fig. 1).

Tabel 1: Prokaryotic abundance and diversity across different salinity gradient groups in three salt pond locations (Tuban, Brebes, Sampang)

Samples	Total sequences	High-quality sequences	Number of OTUs	Shannon diversity index (H')	Simpson diversity index (D)	Chao1	ACE	Goods coverage
T.10	148023±6728.48	140148.67±21621.63	3115±255.065	8.30±0.098	0.99±0.002	3066.44±305.179	3183.49±334.161	0.99±0.001
T.15	147769±6192.78	140963.67±12056.06	2537±249.949	7.14±0.300	0.97±0.023	2501.53±258.827	2610.66±223.752	0.99±0.000
T.20	153684±10186.90	145871.00±10820.68	2203±183.665	6.24±0.205	0.94±0.014	2170.26±179.391	2303.15±185.587	1.00±0.001
S.10	149383±4906.61	137090.33±5225.30	3370±124.102	8.56±0.185	0.99±0.001	3303.43±144.022	3407.19±118.033	0.99±0.000
S.15	155493±4108.80	145472.00±4184.71	2205±203.281	7.29±0.168	0.98±0.004	2153.06±219.041	2275.97±246.326	1.00±0.001
S.20	149415±8618.58	136449.67±6096.70	1675±284.256	5.92±1.031	0.92±0.059	1642.02±307.248	1722.64±329.749	1.00±0.001
B.10	152124±10022.55	129415.33±9737.81	1808±162.022	7.06±0.090	0.98±0.001	1895.50±207.033	1959.61±199.861	1.00±0.001
B.15	151325±6788.28	130763.00±7747.05	2615±313.741	7.32±0.678	0.96±0.017	2556.01±343.301	2626.04±306.526	1.00±0.001
B.20	154462±3981.22	145902.33±3692.15	1460±304.974	6.48±0.724	0.96±0.043	1432.51±324.488	1514.08±351.981	1.00±0.001

Mean ± standard deviation

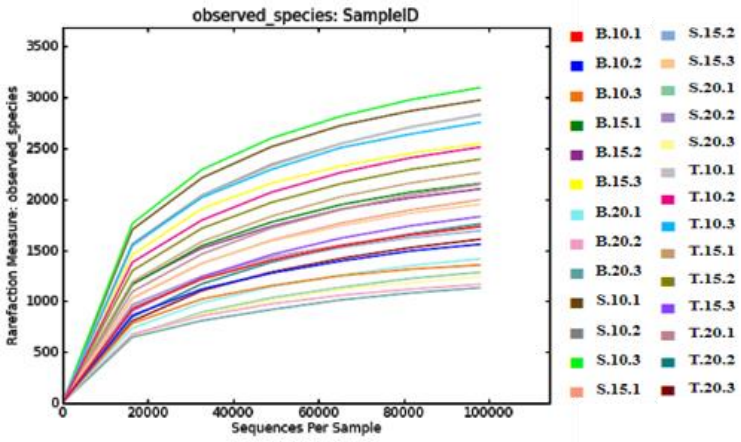

Fig. 1: Rarefaction of each sample

In the overall analyzed samples, bacteria dominated with a percentage of approximately 72.42% of the total 5,660 OTUs, while Archaea accounted for 27.30% of the total 684 OTUs, and other microorganisms constituted 0.266% of the total 46 OTUs. The bacterial community included 46 phyla, 113 classes, 166 orders, 318 families, and 607 genera. On the other hand, in the archaeal community, there were 5 phyla, 7 classes, 5 orders, 3 families, and 39 genera. Based on Fig. 2A, the relative abundance in three locations showed three dominant groups in the bacterial community, which included the phyla *Proteobacteria* (14.64–71.39%), *Bacteroidetes* (6.26–17.00%), and *Actinobacteria* (1.89–12.54%). Meanwhile, the archaeal community was dominated by the phylum *Euryarchaeota* (1.05–72.92%). Furthermore, at the class level, bacteria had a dominant class, namely *Gammaproteobacteria* (9.17–60.18%), while archaea were dominated by the class *Halobacteria* (8.10–72.92%) (Fig. 2B). The order level, there was a dominant order in the bacterial community, including *Alteromonadales* (0.92–26.55%), while archaea were dominated by the order *Halobacteriales* (1.05–72.97%) (Fig. 2C).

Alpha diversity of prokaryotes in salt ponds

The analysis of Shannon Diversity Index (H), Simpson Diversity Index (D), Chao1, and ACE was conducted to determine the diversity of prokaryotic microorganisms in the salt pond environment. According to Table 1, it was observed that prokaryotic abundance and diversity varied across different salinity gradient groups in three salt pond locations.

Beta diversity of prokaryotes in salt ponds

The uniFrac distance heatmap in at low salinity Tuban (T.10) and Sampang (S.10) groups were the smallest values for weighted unifrac distance (WUD) was 0.249 and unweighted uniFrac distance (UUD) was 0.381 (Fig. 3A.). The largest values were between Tuban (T.10) and Brebes (B.10) groups, with WUD (0.350) and UUD (0.528). This means that the bacterial community diversity in Tuban (T.10) sample had the least difference or the most similarity to Sampang (S.10) compared to Brebes (B.10). In the medium salinity group (T.15, B.15, S.15), Tuban (T.15) had the most similarity in bacterial communities with Sampang (S.15), with the smallest values for WUD (0.235) and UUD (0.391). In the high salinity group (T.20, B.20, S.20), the bacterial community similarity can be observed between Tuban (T.20) and Brebes (B.20) samples, with the smallest values for WUD (0.313) and UUD (0.494).

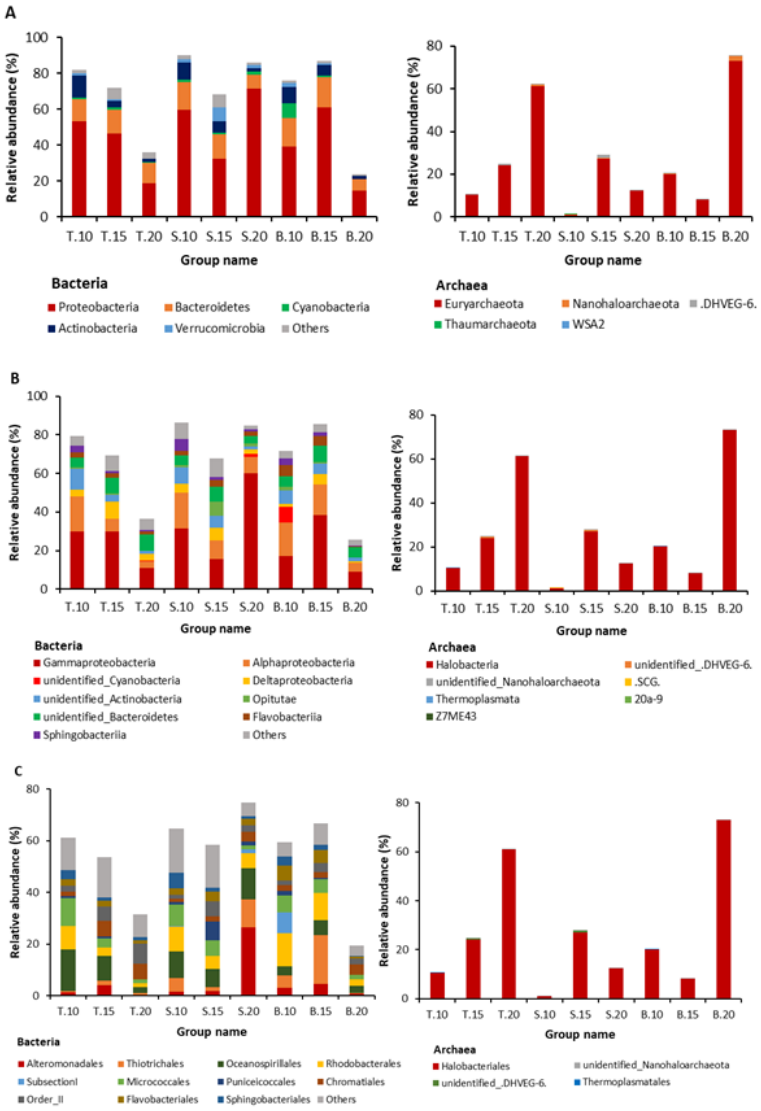

Fig. 2: Relative abundance of procaryota (bacteria and archaea) communities at different taxonomic units in the three salt ponds, Tuban (T.10, T.15, T.20), Sampang (S.10, S.15, S.20), Brebes (B.10, B.15, B.20). (A) phylum, (B) class, (C) orde

The weighted PCoA plot showed closely clustered microbial distances, except for the Sampang (S.20) (Fig. 3B). These results indicated that the microbial communities in the three locations tended to have a similar community structure. In the weighted PCoA plot, the first principal coordinate (PC1), explaining 76.29% of the data variation, showed the level of similarity between the communities of the three pond locations. Based on UPGMA (Fig. 3C), relative diversity at the phylum level showed each dendrogram divided into two main cluster groups. In low salinity samples from Tuban (T.10) and Sampang (S.10), clustered together, as well as medium salinity samples from Tuban (T.15) and Sampang (S.15). However, at high salinity, the Brebes (B.20) sample formed one main cluster with Tuban (T.20), indicating their similarity and close phylogenetic.

Discussion

The rarefaction curves (Fig. 1) showed a flat or non-decreasing line, indicating that the amount of sequencing data available was sufficient to provide a good picture of the species diversity in the sample. These sequences were divided into 6390 operational taxonomic units (OTUs) with a 97% similarity level. These research samples had less OTUs compared Taiwanese salt ponds, which had approximately 8085 OTUs (Tran *et al.* 2019), while salt ponds in Bolivia had 149 OTUs (Haferburg *et al.* 2017).

The phylum *Proteobacteria* was found to dominate the bacterial community at all locations. Bacterial abundance >5% at high salinity was represented by the genus *Pseudoalteromonas* (16.57%), which dominated in the Sampang. In previous studies, it was also found that

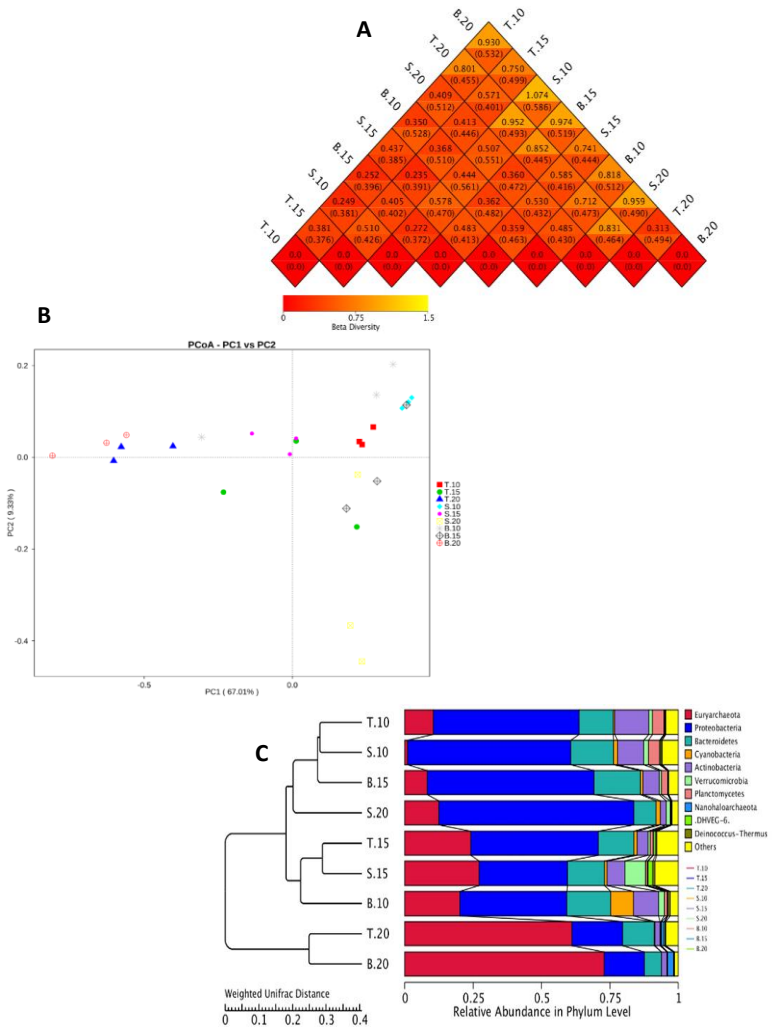

Fig. 3: (A). Heatmap of Weighted Unifrac and Unweighted Unifrac distances. (B) Biplot ordination of UniFrac-weighted principal coordinate analysis (PCoA), (C) UPGMA/hierarchical clustering analysis based on weighted UniFrac distances displaying the relative abundance of the most abundant micobial phylum

the largest bacterial community in Salt Lake in China consisted of phyla *Proteobacteria* (85.08%) (Wang and Bao 2022). *Proteobacteria* was found to be the most prevalent bacterial phylum in lakes with various salinity levels (low, medium, and high), as reported by He *et al*. (2022). In a hypersaline lake in Iran, among the *Proteobacteria* phylum, class *Gammaproteobacteria* and genera (*Pseudoalteromonas*, *Salinibacter*) was found to dominate at salinity levels of 50–300 psu (Naghoni *et al*. 2017), while the genus *Spiribacter* was found at a salinity level of 190 psu in Spain (Leon *et al*. 2013). At medium salinity, the genus *Methylophaga* (18.47%) dominated in the Brebes. *Gammaproteobacterial* groups that are halophilic methylotrophs are known to have the ability to survive in a variety of environments, including conditions that are alkaline, hypersaline, or at low or high temperatures (Kumar *et al*. 2019). At low salinity, the genus *Litoricola* (4.96%) dominated in the Sampang location. Song *et al*. (2022) found *Litoricola* in a solar saltern in China and revealed that it had a favorable association with pH but a significantly negative interaction with salinity.

The phylum *Euryarchaeota* dominated the archaeal community at all three sites. Generally, Archaea dominated at high salinity, and the highest relative abundance of the phylum *Euryarchaeota* was represented by the class *Halobacteria*. This was consistent with findings of other authors who studied the microbial diversity in Taiwanese salt ponds, where the archaeal community represented about 37.6% of the total population, and the family and order *Halobacteriaceae* dominated (Tran *et al*. 2019). The most found genera in all samples of brine water from Poland, according to Kalwasińska *et al*. (2018), were *Halorubrum*, *Halohasta*, *Halonotius*, and *Halolamina*.

Based on Table 1, in the current study Chao1 (1,432.51–3,303.43) and ACE values (1514.08–3407.19) were higher when compared to Chasanah *et al*. (2020), they who reported values ranging from 522 to 1073 for Chao1 and 543 to 1,088 for ACE in the Indramayu salt ponds, which represented one of the locations in West Java, Indonesia. According to Cheng *et al*. (2023). The UPGMA dendrogram diagram provided a general overview of the clustering of bacterial phyla in the three locations (Fig. 3C). We demonstrated that the addition of this method significantly altered the taxonomic and migratory profiles of the genomic DNA samples. In the weighted UPGMA, low salinity samples from Tuban (T10) and Sampang (S10) clustered together, as well as medium salinity samples from Tuban (T15) and Sampang (S15), indicating similar environmental conditions between Tuban and Sampang. Varying physical, chemical, and biological conditions in the salt pond environment affected the composition and diversity of the existing microbes. Cao *et al*. (2008) suggested that environmental factors, nutrient availability and pollution extent of could microbial communities in salt pond environment.

In addition, the differences in bacterial abundance and diversity can also be influenced by the extracellular DNA metabolism by some *Haloarchaea*. Genetic material from various organisms, including some fungi, bacteria, and Archaea, can be released into the extracellular medium as extracellular DNA (eDNA) (Aldecoa *et al*. 2017). The microbial community in the Isla Cristina saltern (Spain) primarily utilizes extracellular DNA as a source of phosphorus (P) and preferentially takes up eDNA in accordance with its metabolism (Hua *et al*. 2021). Consequently, the availability of extracellular DNA or inorganic phosphorus (Pi) affects the community's taxonomic composition (Akpolat *et al*. 2021). This also suggests that in hypersaline environments, the taxonomic composition of microbial communities is influenced by the availability of phosphorus, including eDNA and Pi. Moreover, the preferences and needs of microbes for P sources can significantly impact the composition of microbial communities in such environments. Microorganisms exhibit selectivity in choosing eDNA that is like their own in order to obtain the required P for growth and metabolism, and microbial DNA methylation shows a distinct tendency (Cheng *et al*. 2023).

Conclusion

There were variations in bacterial community profiles among the three salt ponds located in the same region, particularly the Java Sea. Notably microbial communities are dynamic and can change over time due to shifts in environmental conditions and other factors. Therefore, ongoing monitoring and analysis are necessary to understand the specific reasons behind the observed variations in bacterial community profiles among three salt ponds in the Java Sea region.

Acknowledgements

We would like to thank The Ministry of Marine Affairs and Fisheries, Indonesia for funding the research.

Author Contributions

All authors contributed equally to study design, sampling, methodology, interpretation of results, and manuscript writing.

Conflicts of Interest

All authors declare no conflict of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

Funding Source

The Ministry of Marine Affairs and Fisheries, Indonesia funded this study.

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