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Research Article

Diversity of Indigenous Bacteria from Mangrove Sediments in the Waters of Ambon Bay, Maluku

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ABSTRACT

This study aimed to analyze the diversity of indigenous bacteria by comparing culture and non-culture methods and to analyze the physicochemical effects on bacterial diversity in polluted and natural mangrove sediments. The environmental parameter values of mangrove sediments for bacterial growth can change owing to differences in adaptation and tolerance to fluctuations in physicochemical conditions. The number of colonies in natural and polluted areas using the culture method was 6.2 × 10^4 CFU/g and 5.5 × 10^4 CFU/g, respectively. A total of 33 isolates were identified, with 17 and 16 isolates from the natural and polluted areas, respectively. The most common isolates found in both areas were *Acinetobacter haemolyticus* strain FBC636 and *Exiguobacterium acetylicum* strain IAE17. Using the nanopore sequencing method, the total number of colonies in the natural and polluted areas was 69,761 and 58,412 colonies, respectively. A total of 12,954 bacterial species were identified, with 6,837 species in the natural area and 6,117 in the polluted area. The most common isolate found was *Sulfurovum aggregans*. Physicochemical conditions influenced the differences in bacterial diversity between the natural and polluted areas in the mangrove areas of Ambon Bay.

1. Introduction

The mangrove areas in Ambon Bay are spread across several regions, including Waiheru and Poka villages. The diversity of mangrove species in Waiheru varies and still follows the general natural zonation of mangroves (Damayanti *et al.* 2020). On the other hand, the condition of the mangroves in Poka Village is concerning, as many mangroves are polluted and dying due to anthropogenic waste. Based on research by Sairmorsa *et al.* (2024), there has been an increase in heavy metals and indiscriminate waste disposal in this area, leading to cell tissue death in mangroves. This has attracted the attention of governments, researchers, and the public.

The condition of mangrove sediment is related to its microbiological characteristics. Mangrove sediment plays a major role as a source of organic carbon and provides an excellent habitat for microorganisms such as bacteria. The ability of bacteria to act as decomposers allows them to break down organic materials into simpler components (Tyas *et al.* 2018; Fadhila *et al.* 2023). Indigenous bacteria are native bacteria found in their original habitat. Environmental factors influence the diversity of bacteria, and their presence can be used as an indicator of ecosystem stability (Mahrus *et al.* 2020; Palit *et al.* 2022).

To date, bacterial diversity and abundance have been studied using conventional culturing methods. Bacteria were cultivated on artificial growth media for colony counting and morphological observations under a microscope. However, in reality, only approximately 0.1-1% of bacteria in nature are capable of growing on growth media, while the remaining 99% are in an anabiotic

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state, allowing them to survive but not be cultured. This method is still very basic for comprehensively describing the bacterial biodiversity in the environment. Therefore, to analyze the diversity of bacterial communities that cannot be cultured, metagenomic analysis was conducted, as it is considered capable of detecting all bacteria, including those that are difficult to culture, rarely appear or may be overlooked by conventional methods. Additionally, it provides more in-depth information on bacterial diversity in the environment. The application of non-culture-based methods has significantly increased in recent years. Next-generation sequencing (NGS) is a DNA (Deoxyribonucleic Acid) sequencing method capable of sequencing millions of DNA in a single run with accuracy and efficiency (Afianti and Darmayanti 2017; Prayogo *et al.* 2020; Azizi *et al.* 2021).

Exploration of the diversity of indigenous bacteria in mangrove sediments in the waters of Ambon Bay has not been conducted until now. Based on this gap, this research aims to determine the diversity of indigenous bacteria by comparing culturing methods (conventional methods) and non-culture-based methods, namely Next Generation Sequencing (NGS), as well as to analyze the physicochemical influences on the diversity of bacteria from polluted and natural mangrove sediments in the waters of Ambon Bay, Maluku.

2. Materials and Methods

2.1. Sampling and Measurement of Environmental Parameters

The sediment samples were collected from two different locations: one in Waiheru Village as a natural area and the other in Poka Village as a polluted area, with coordinates of 128°13' 37.34" "3°37' 51.61" and 128°11' 35.55 "3 °38' 42.66" ", respectively (Figure 1). Sampling was conducted at a depth of 10-15 cm using a purposive sampling technique (Tyas *et al.* 2018). The measured physical parameters included temperature and salinity. The measured chemical parameters were dissolved oxygen (DO), biological oxygen demand (BOD), pH, nitrate, nitrite, sulfate, and organic matter. The physicochemical data were analyzed using a t-test with a 95% confidence level ($\alpha = 0.05$) using the Statistical Package for the Social Sciences (SPSS) software.

2.2. Culturable Method

2.2.1. Bacterial Isolation from Sediment Samples

One gram of sediment sample was dissolved in 9 ml of NaCl for serial dilution from 10^{-1} to 10^{-6} ml was taken from each dilution, and 0.1 ml was spread on Petri dishes containing TSA media using the spread plate technique. The Petri dish was then incubated for 24 to 48 hours. Bacterial cell density was determined using the Total Plate Count (TPC) method, selected isolates with different macroscopic and microscopic characteristics were purified using the quadrant streak method, and the Important Value Index (IVI) was calculated. The importance Value Index (IVI) of bacteria in mangrove areas can be measured through three main parameters: relative frequency (FR), relative density (KR), and relative dominance (DR) (Fadhila 2023). The relationship between bacterial density and physicochemical data was analyzed using Canonical Correlation Analysis (CCA) using the Paleontological Statistics Software Package (PAST) (Chrisnawati *et al.* 2023; Zakaria *et al.* 2024).

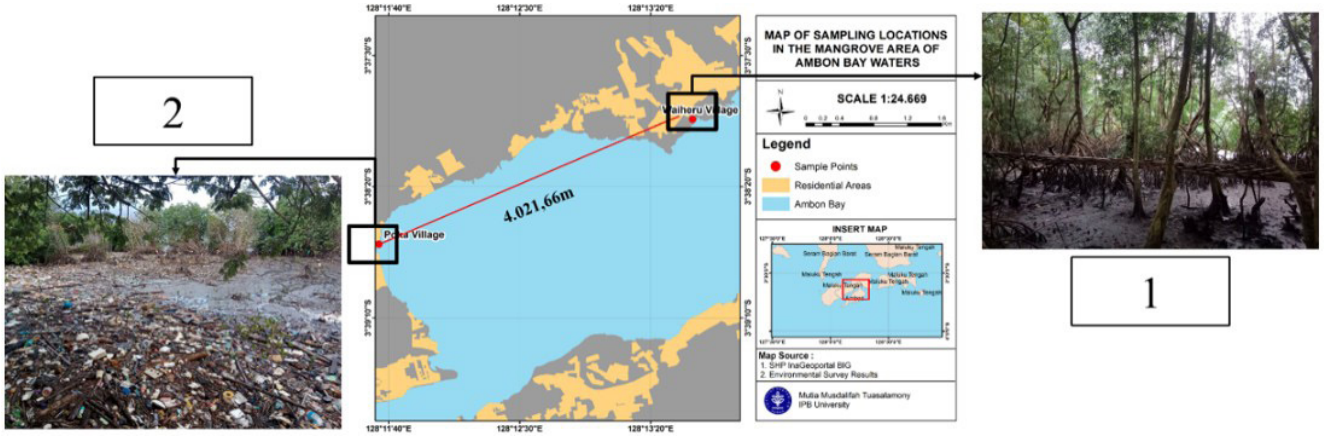

Figure 1. Sampling locations (1: natural area/Waiheru Village; 2: polluted area/Poka Village)

2.2.2. Molecular Identification of Selected Isolates Based on 16S rRNA Gene

The selected isolates with the highest IVI from both natural and polluted areas were extracted using the Quick-DNA Magbead Plus Kit, following the manufacturer's protocol. 16S rRNA gene amplification was performed using Polymerase Chain Reaction (PCR) with the specific universal prokaryotic primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR Master Mix reaction components consist of 9.5 µL ddH_2O, 12.5 µL My Taq HS Red Mix (2X), 1 µL 10 µM 27F Primer, 1 µL 10 µM 1492F Primer, and 1 µL DNA template. The thermal cycles used for the amplification process were pre-denaturation (95°C, 3 minutes), denaturation (95°C, 15 seconds), annealing (52°C, 30 seconds), extension (72°C, 45 seconds), and final extension PCR (72°C, 2 minutes) with a total of 35 cycles. The PCR products were sent for sequencing to the PT. Genetika Science.

2.2.3. Bioinformatics Analysis of 16S rRNA Gene Sequences

Nucleotide sequence similarity analysis was conducted by comparing them with reference bacteria in the GenBank database using the Basic Local Alignment Search Tool Nucleotide (BLASTN) program available on the National Center for Biotechnology Information (NCBI) website. Sequence alignment and phylogenetic tree construction were performed using Molecular Evolutionary Genetics Analysis (MEGA) 11 software with the neighbor-joining method (Tamura-Nei model with 1000× bootstrap) (Tamura *et al.* 2021; Syah 2022).

2.3. Non-culturable Method

2.3.1. DNA Extraction from Sediment Samples

Mangrove sediment was extracted using the ZymoBiomics DNA Miniprep Kit, following the manufacturer's protocol provided by the company. PCR amplification was performed using a combination of primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-AAGGAGGTGATCCAGCCGCA-3') targeting the V1-V9 region (Winand *et al.* 2019; Chavan *et al.* 2022). The amplified products were sequenced using PT. Genetika Science.

2.3.2. Metagenomic Analysis and Bioinformatics of Nanopore Sequencing

Nanopore sequencing was performed using MinKNOW software. The base calling process was performed using Guppy with a high-accuracy model. The sequence reads were classified using a centrifuge classifier. A bacterial index was created using the NCBI 16S RefSeq database. Further analysis and visualization were conducted using the Pavian and Krona tools and RStudio.

3. Results

3.1. Physicochemical Conditions of Mangrove Sediments

Physicochemical measurements were conducted in the mangrove sediment areas of Ambon Bay, covering both the natural and polluted areas (Table 1).

3.2. Bacterial Diversity Using Culturable Method

Bacteria isolated from mangrove sediments in both natural and polluted areas were counted using the TPC method, and the calculation data are presented in Table 2. A total of 33 isolates were successfully identified, consisting of 17 isolates (KAT1-KAT17) from the natural area and 16 isolates (KTT18-KTT33) from the polluted area (Figure 2A). Isolates KAT2 and KTT23 had the highest IVI in the natural and polluted areas,

Table 1. Physicochemical conditions of sediments from natural and polluted mangrove areas

Environmental parameters	Natural area	Polluted area	GR number 22 of 2021
Temperature (°C)	30.4±1.28[a]	29.03±0.16[a]	28-32
pH	8.23±0.09[a]	8.43±0.12[a]	7-8.5
Salinity (‰)	15.3±0.47[a]	14.6±0.47[b]	33-34
DO (mg/L)	3.63±0.06[a]	3.2±0.33[a]	>5
BOD (mg/L)	15.67±5.56[a]	24.3±8.49[a]	20
Nitrate (mg/L)	0.02±0.008[a]	0.01±0.004[a]	0,06
Nitrite (mg/L)	0.05±0.008[a]	0.04±0.02[a]	-
Sulfate (mg/L)	227.03±4.77[a]	215.3±2.49[a]	-
Organic matter (mg/L)	2.36±0.2[a]	2.56±0.2[a]	-

Numbers followed by the same letter in the same parameter are not significantly different based on the t-test ($p<0.05$) and GR (Government Regulations)

Table 2. Total bacterial counts in sediments from natural and polluted mangrove areas

Mangrove sediment sample	Colony count (CFU/g)	Mangrove sediment sample	Colony count (CFU/g)
KA1	6.5×10^4	KT1	5.2×10^4
KA2	6.1×10^4	KT2	5.3×10^4
KA3	6.0×10^4	KT3	4.9×10^4

KA (K: area, A: natural); KT (K: area, T: polluted); (1: first point)

with values of 31.98% and 21.24%, respectively (Figure 2B).

3.3. Correlation of Physicochemical Parameters with Bacterial Isolates

Isolates with the highest IVI values were KAT2 and KTT23. Isolate KAT2 was correlated with DO, nitrate, nitrite, salinity, pH, and BOD (Figure 3A). Isolate KTT23 was correlated with DO and salinity (Figure 3B).

3.4. Molecular Identification Using the Culturable Method

The isolates with the highest IVI values from the natural and polluted areas were KAT2 and KTT23, respectively. The analysis results revealed that isolate KAT2 showed 100% similarity to *Acinetobacter haemolyticus* strain FBC636, whereas KTT23 had 100% similarity to the *Exiguobacterium acetylicum* strain IAE172 (Table 3), which was visualized through the construction of a phylogenetic tree (Figure 4).

3.5. Bacterial Diversity Using the Unculturable Method

The bacterial diversity in the waters of Ambon Bay was identified using Nanopore Sequencing. In the natural area, 43 phyla, 105 classes, 230 orders, 585 families, 2,596 genera, and 6,837 species were detected. The polluted area contained 42 phyla, 105 classes, 231 orders, 580 families,

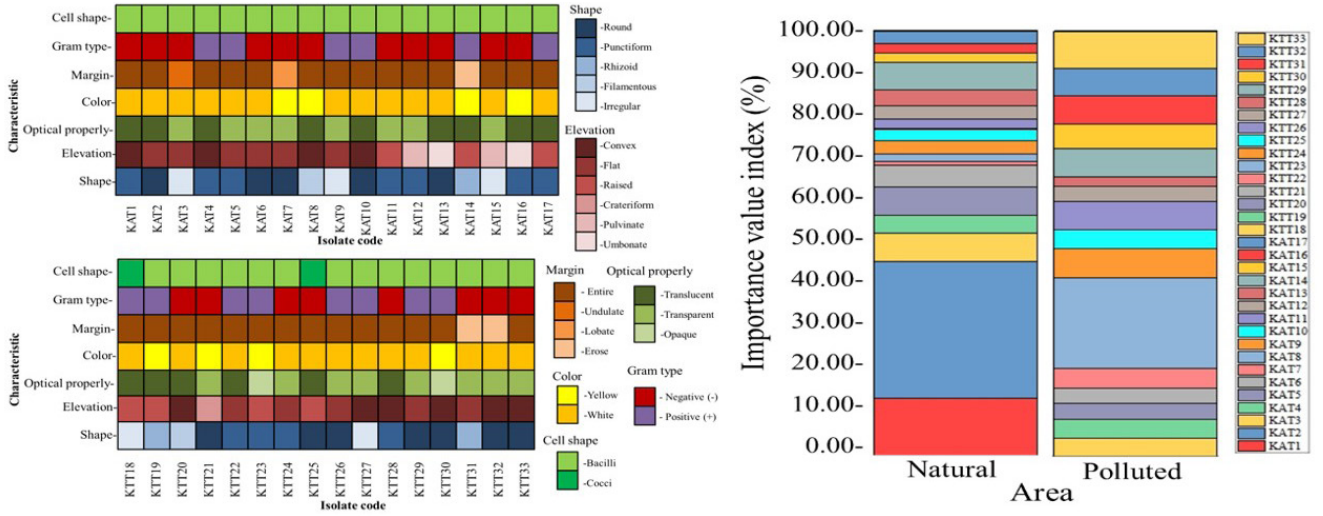

Figure 2. Macroscopic (shape, elevation, optical properly, color, and margin) and microscopic (Gram type and cell shape) characteristics of bacterial isolates (A) Importance value index of bacterial isolates (B) in natural and polluted areas

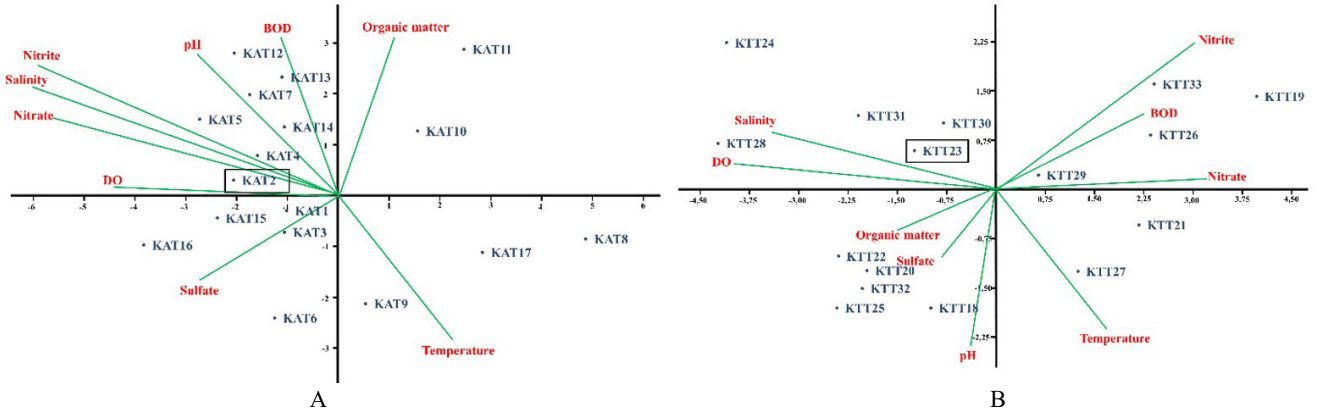

Figure 3. Correlation between physicochemical properties and natural (A) and polluted (B) areas

Table 3. Sequence similarity analysis of bacterial isolates' 16S rRNA gene using the BLAST-N program

Sample code	Description	Query cover (%)	E-value	Percent identify (%)	Acces number
KAT2	*Acinetobacter haemolyticus* strain FBC636	100	0	100	OP615118.1
KTT23	*Exiguobacterium acetylicum* strain IAE172	100	0	100	MK414870

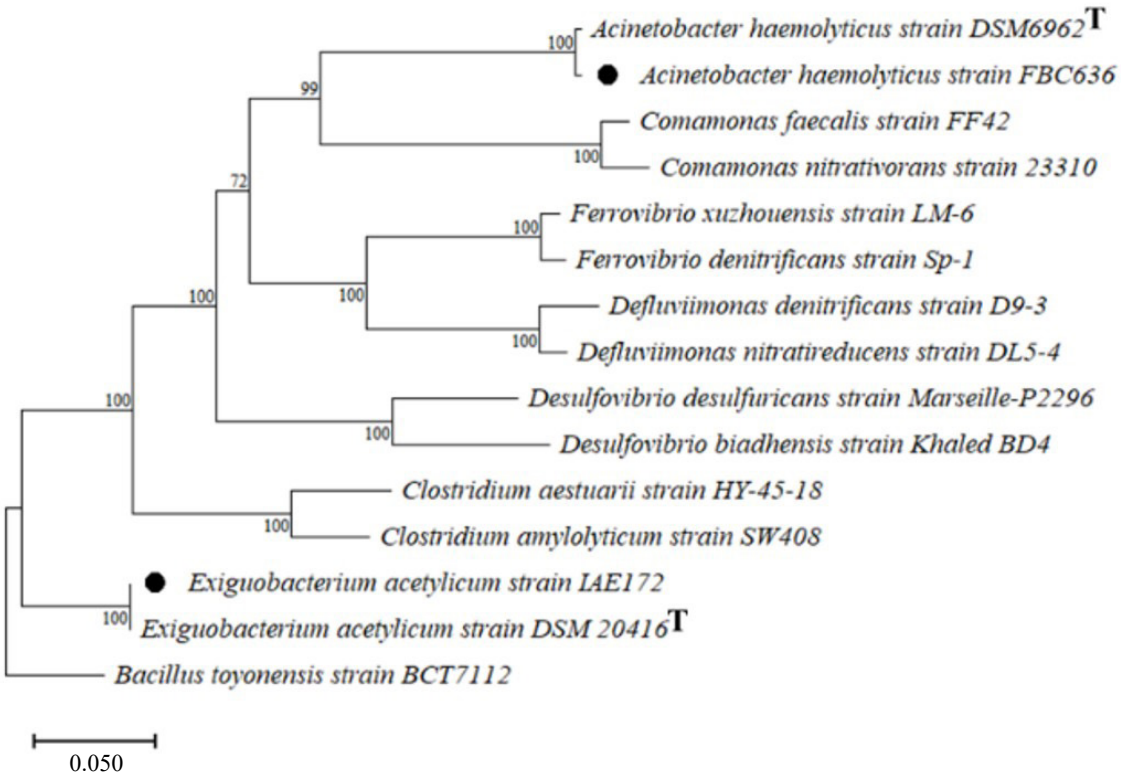

Figure 4. Phylogenetic tree construction of 16S rRNA gene sequences of isolates *Acinetobacter haemolyticus* strain FBC636 and *Exiguobacterium acetylicum* strain IAE172

2,421 genera, and 6,117 species. The most abundant phylum found in both the natural and polluted areas was *Pseudomonadota*, with abundances of 35.05% and 41.81%, respectively (Figure 5A). The highest classes in both areas were *Gammaproteobacteria* and *Alphaproteobacteria*, with abundances of 18.10% and 23.21%, respectively (Figure 5B). The highest orders in both areas were *Desulfobacterales* and *Rhodobacterales*, with abundances of 8.85% and 12.67%, respectively (Figure 5C). The most abundant families in both areas were *Sulfurovaceae* and *Roseobacteraceae*, with abundances of 6.62% and 6.89%, respectively (Figure 5D). *Sulfurovum* was the most abundant genus, with abundances of 6.83% and 6.41%, respectively (Figure 5E). *Sulfurovum aggregans* was the most abundant species, with abundances of 3.91% and 4.55% (Figure 5F).

3.6. Alpha Diversity

Bacterial diversity was demonstrated by two samples that were isolated and identified using alpha diversity. This diversity was demonstrated using the Chao1, ACE, Shannon, and Simpson indices (Figure 6A). The overall alpha diversity boxplot analysis for each index showed high bacterial community diversity in the natural area, with 12,954 species observed in both areas (Figure 6B).

The number of abundant colonies in the natural area was 69,761, whereas that in the polluted area was 58,412 (Figure 6A). The natural and polluted areas had 28.23% (2,406) and 19.78% (1,686) of unique and distinct bacterial species, respectively, which were not found in each other (Figure 6B). Additionally, 51.99% (4,431) of the bacterial species overlapped between the two areas (Figure 7).

4. Discussion

The physicochemical conditions of mangrove sediments in the natural area (Waiheru Village) and polluted area (Poka Village) met the quality standards based on Government Regulation Number 22 of 2021 (for marine biota), except for the BOD value in the polluted area. Physicochemical measurements aim to evaluate the sediment quality in relation to bacterial growth (Adesuyi *et al.* 2016). According to Zakaria *et al.* (2024), mangrove areas in the waters of Teluk Ambon have a semi-enclosed structure, indicating that the water circulation pattern and seasonal influence affect the physicochemical data in these areas. This resulted in some physicochemical data showing no significant difference. A comparison of environmental parameters between the natural and polluted areas showed no significant differences in

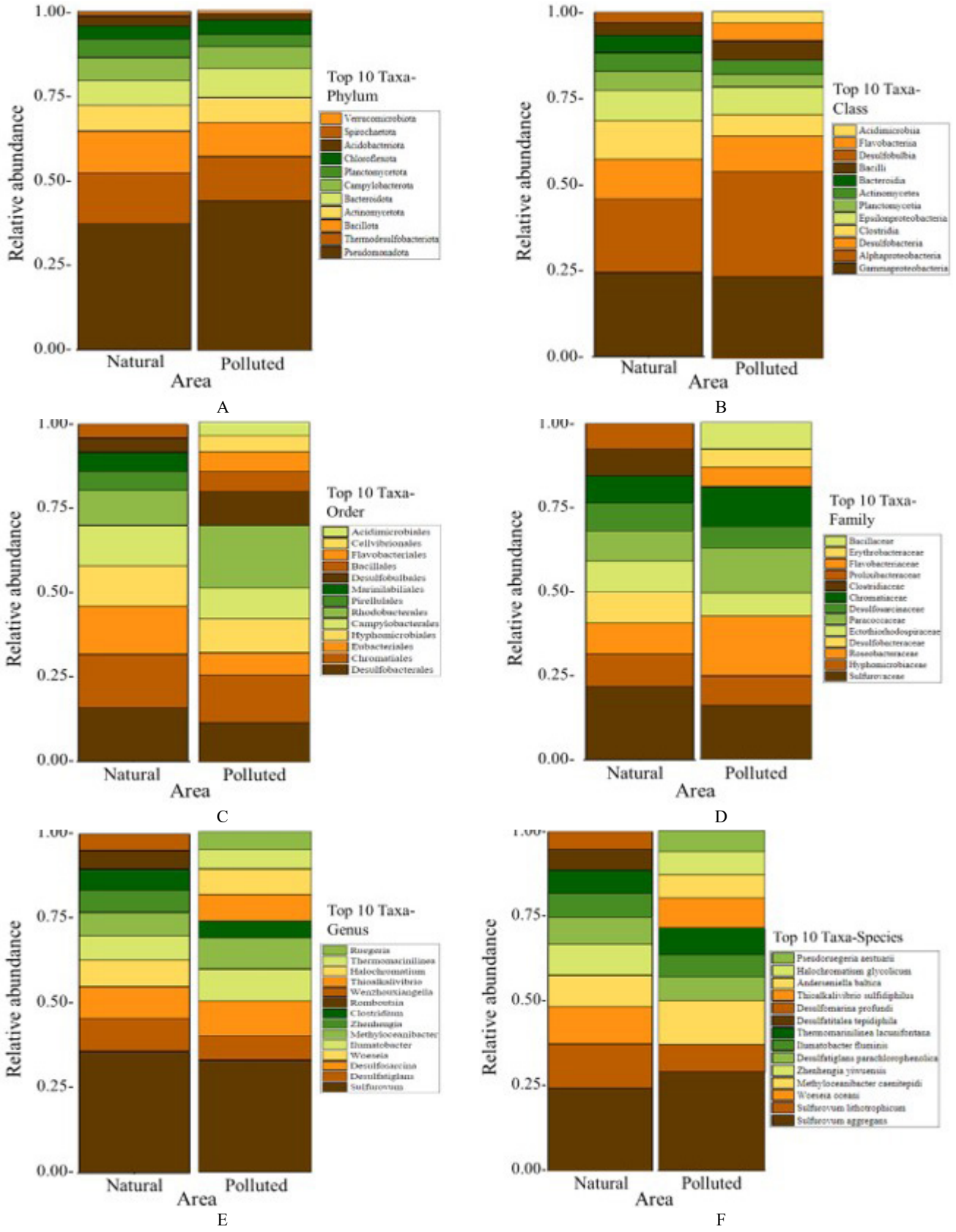

Figure 5. Relative abundance of the top ten phyla (A), classes (B), orders (C), families (D), genera (E), and species (F) in natural and polluted areas based on metagenome data

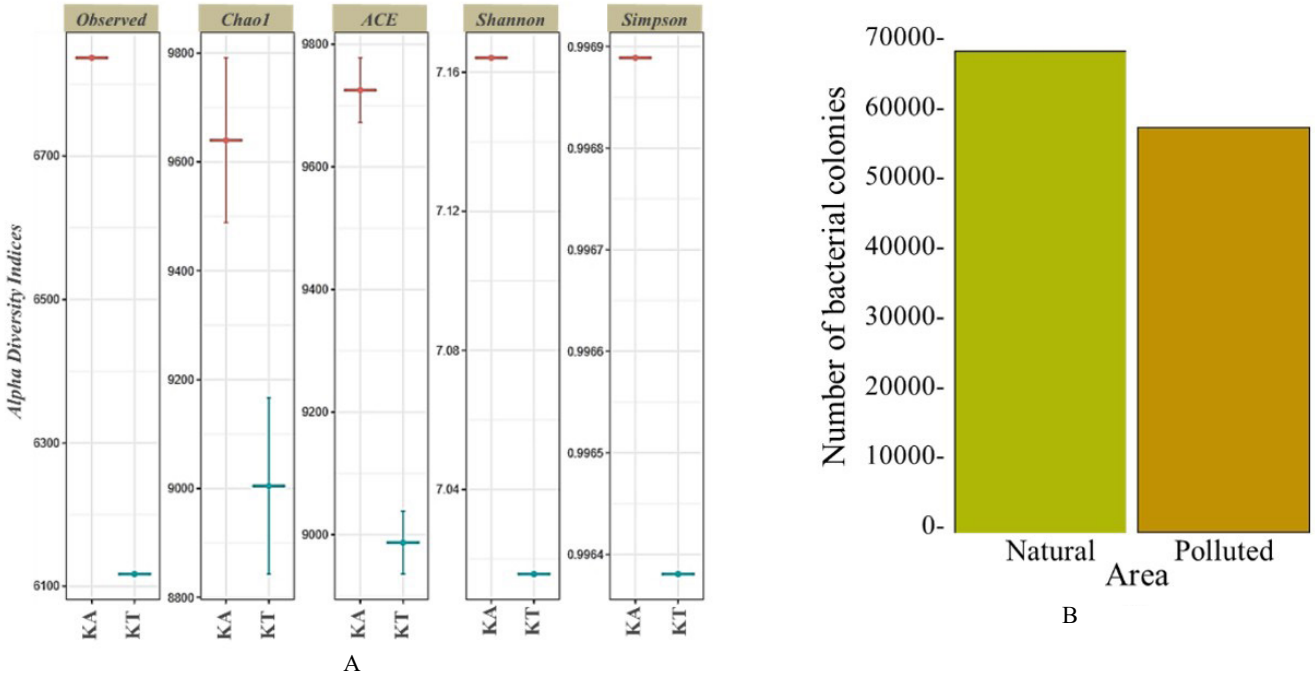

Figure 6. (A) Alpha diversity index, (B) colony count, (KA) in natural areas, (KT) polluted areas (KT)

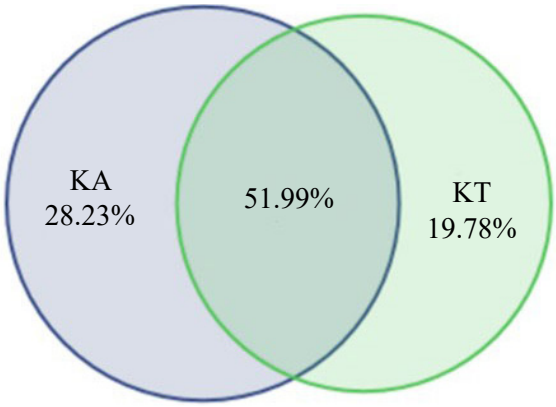

Figure 7. Venn diagram of natural and polluted areas (KA: natural area; KT: polluted area)

temperature, pH, sulfate, DO, BOD, nitrate, nitrite, and organic matter. However, salinity differed significantly between the two areas. The proximity of the polluted area to the river mouth and anthropogenic waste may dilute the salt content, thus lowering salinity (Matatula *et al.* 2019; Tuahatu *et al.* 2022; Mariwy *et al.* 2024). The natural area has a mangrove density of 1,433±565 trees/ha, which is considered to be dense. The mangrove density in the polluted area was 233±103 trees/ha, categorized as very low, in line with the criteria for degraded mangroves as stipulated in Ministerial Decree No. 201 of 2004, which defines degraded mangroves as having a tree density <1000 trees/ha (Damayanti *et al.* 2020; Matitaputy *et al.* 2024). Visual observations of the polluted area revealed various types of waste around the mangroves. This finding is consistent with the research of Sairmorsa *et al.* (2024), who stated that domestic waste and oil contamination may have caused the damage and death of mangroves.

Bacterial growth is generally optimal at temperatures between 20°C and 40°C and at pH levels ranging from 6.5 to 8 (Talaro and Chess 2018). The salinity of mangrove sediments affects bacterial growth (Mahrus *et al.* 2020). Mangroves have muddy sediments with low DO content, which is inversely proportional to the temperature and salinity of the water (Putri *et al.* 2021). Sulfate levels in mangroves are influenced by runoff from waste that contains sulfur compounds. The BOD content in mangrove areas is associated with high bacterial activity in the decomposition of easily degradable organic matter (Supriyantini *et al.* 2017). Bacterial activity in mangrove environments influences nitrate and nitrite levels (Zhang *et al.* 2023). Sulfate concentrations depend on the activity of sulfate-reducing bacteria in sediments (Abdi *et al.* 2018). The concentration of organic matter is directly proportional to the total number of bacteria in the water and affects bacterial growth (Mahrus *et al.* 2020; Qian *et al.* 2023). The values of environmental parameters in mangrove sediments in relation to bacterial growth can change owing to bacterial adaptation and tolerance to fluctuations in physicochemical conditions (Lee *et al.* 2023).

The calculation results show that the total bacterial count in the natural area is higher than in the polluted area, with average values of 6.2 × 10^4 CFU/g and 5.5 × 10^4 CFU/g, respectively. A total of 33 isolates were successfully obtained, with 17 and 16 isolates from natural and polluted areas, respectively. This finding is supported by a previous study by Fadhila *et al.* (2023), who reported that the total bacterial count in mangrove sediments at Tirang Beach ranged from 10^3 to 10^4 CFU/g, with a total of 32 bacterial isolates. Physicochemical conditions and the availability of nutrients in the sediment can influence differences in total bacteria and the number of isolates. The adaptability and competitive abilities of bacteria contribute to variability in IVI. Based on the macroscopic characteristics (shape, elevation, optical features, color, and colony edges) and microscopic characteristics (Gram staining and cell morphology), each isolate exhibited varying characteristics. However, most were dominated by a round shape, convex elevation, translucent optical features, white color, smooth edges, gram-negative classification, and rod-shaped cells.

The correlation between bacterial abundance and environmental variables is shown in the CCA plot, comparing bacterial isolates in natural and polluted mangrove sediment areas. Each isolate exhibited a significant response and was influenced by the physicochemical conditions of the sediment. Isolates that correlated with DO were aerobic or facultative anaerobic. Isolates that were correlated with organic matter were heterotrophic. Isolates that were positively correlated with nitrate, nitrite, and sulfate levels were involved in denitrification, nitrification, and sulfate reduction.

Phylogenetic analysis based on 16S rRNA gene sequences was performed to determine the relationships between the species. *Acinetobacter haemolyticus* strain FBC636 (KAT2) is a gram-negative aerobic bacterium that plays a role in denitrification and heavy metal bioremediation (Alhashimi *et al.* 2023). *Exiguobacterium acetylicum* strain IAE172 (KTT23) is a gram-positive, facultative anaerobic bacterium that is tolerant to polluted environments and capable of degrading heavy metals and organic pollutants (Xiao *et al.* 2024). These findings align with the macroscopic and microscopic characteristics and CCA analysis.

Sulfurovum aggregans was the most frequently found species in both natural and polluted areas, accounting for 3.91% and 4.56%, respectively. Mangrove sediments have low oxygen levels and are rich in sulfur compounds. Sulfurovum aggregans is a facultative anaerobe, gram-negative bacterium that utilizes sulfur compounds as an energy source and plays a role in the sulfur cycle in mangrove sediments (Mino *et al.* 2014).

Alpha diversity analyses, such as Observed, Chao1, ACE, Shannon, and Simpson, were used to describe the bacterial community diversity (Figure 6A). The Observed index counts the number of observed species. Chao1 estimates the total number of species, including those that may not have been observed, and corrects for bias toward rare species. The Abundance-based Coverage Estimator (ACE) focuses on the abundance of rare or uncommon species. The Shannon index reflects species diversity and ecosystem stability, with values of H" >3 indicating high species diversity in an environment. The Simpson index ranges from 0 to 1, reflecting species dominance. Each index shows that the natural area has the highest alpha diversity index compared to the polluted area. This suggests that more than half of bacterial species can survive in both natural and polluted environments, indicating the presence of adaptive or persistent bacterial species under different environmental conditions. Physicochemical parameters influence bacterial diversity and abundance (Mahrus *et al.* 2020; Xia & Sun 2023).

This study found that the colony counts using the culturable method in the natural and polluted areas were 6.2 × 10^4 CFU/g and 5.5 × 10^4 CFU/g, respectively, with a total of 33 isolates, consisting of 17 isolates from the natural area and 16 isolates from the polluted area. *Acinetobacter haemolyticus* and *Exiguobacterium acetylicum* were the dominant isolates from each area. The unculturable method identified more colonies, with 69,761 in the natural area and 58,412 in the polluted area, totaling 12,954 species, with 6,837 species from the natural area and 6,117 species from the polluted area, including *Sulfurovum aggregans* as the dominant species. The non-culture-based method proved to be superior in revealing the bacterial diversity. The observed physicochemical parameter data generally showed no significant differences between the natural and polluted areas; however, they still played an important role in the distribution and composition of bacteria.

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