Profiling of Seawater Bacterial Diversity in Tanjung Mas Port Using 16S rRNA eDNA Metabarcoding and Next-Generation Sequencing (NGS)

Naufal Sebastian Anggoro[1], Hermin Pancasakti Kusumaningrum[2]*, Anto Budiharjo[2]
Muhammad Zainuri[3], Wijanarka[2], Tony Hadibarata[4]

[1]*Magister Biology Study Program, Department of Biology Faculty of Science and Mathematics, Universitas Diponegoro*
[2]*Biotechnology Study Program, Department of Biology Faculty of Science and Mathematics, Universitas Diponegoro*
[3]*Oceanography Study Program, Faculty of Fisheries and Marine Science, Universitas Diponegoro*
Jl. Prof. Jacub Rais Tembalang Semarang, Jawa Tengah 50265 Indonesia
[4]*Faculty of Engineering and Science, Curtin University*
Lutong, 98000 Miri, Sarawak, Malaysia
Email: herminpk@live.undip.ac.id

Abstract

Tanjung Emas Port is the entry and exit point for trade commodities, both regional and international filled with many ships. This condition makes biofouling a very massive process in that place by various types of marine bacteria. The initial formation of a biofilm is relevant to bacterial diversity, colonization and adhesion. The objective of the study was assessing bacterial diversity in relation to with biofouling within Tanjung Mas Port Semarang, by using 16S rRNA eDNA metabarcoding Next-Generation Sequencing (NGS). Seawater samples from aquatic sites of Tanjung Mas harbor was used for DNA extraction and amplification of the 16S rRNA V-3-V4 hypervariable region, followed by sequencing and library construction of eDNA Metabarcoding. Sequence processing and analysis was performed in QIIME 2 and RStudio using DADA2 for advanced sequencing processing and Phyloseq. The results of this research showed that bacteria is the predominant taxon constituting 100% of the community. The taxon consists of Proteobacteria (49.38%), Bacteroidota (8.67%), and Firmicutes (8.88%). Alphaproteobacteria (20.92%) and Gammaproteobacteria (12.39%) dominate at the Class level, emphasizing their versatility and ecological influence. At the Order and Family levels reveals the prevalence of Rhodobacterales (10.04%), Chitinophagales (2.53%), Rhizobiales (3.61%), Rhodobacteraceae (5.67%), Saprospiraceae (3.63%), and Rhizobiaceae (2.15%). It was found that the unculturable taxa dominance in Tanjung Mas Port was 44.66%. These taxonomic entities contribute significantly to the taxonomic and functional diversity of the microbial community, influencing nutrient cycling, organic matter degradation, ecosystem stability and biofilm formation.

Keywords: *bacteria, biofouling, Tanjung Mas Port, metabarcoding, NGS*

Introduction

Tanjung Mas Port, located in Semarang, Indonesia, is not merely a geographical location but it also a dynamic convergence point for regional and international trade, serving as a vital gateway for commerce and transportation. This bustling port, characterized by its strategic importance, is intrinsically tied to the intricate dynamics of microbial communities within the surrounding seawater. These microbial ecosystems play a multifaceted role in shaping the port's ecological processes, contributing significantly to nutrient cycling, energy flow, and the overall health of the marine environment (Plait *et al*., 2020).

Microbial communities within the ecosystem of Tanjung Mas Port had face unique challenges posed by the intensity of shipping activities and associated anthropogenic inputs (El *et al*., 2022). Anthropogenic influences, including the discharge of ballast water containing diverse microbial species, have the potential to reshape the composition and structure of local bacterial communities. The port, functioning as a gateway for global trade, becomes a focal point for understanding the intricate dynamics between microbial life and human activities. Despite their critical role, comprehensive studies on bacterial diversity in Tanjung Mas Port especially relating to the biofouling remain limited, highlighting a critical gap in our understanding of microbial responses to anthropogenic stressors in such dynamic maritime environments.

Metabarcoding of environmental DNA (eDNA) targeting the 16S rRNA gene, coupled with Next-Generation Sequencing (NGS), has emerged as a transformative approach in the study of microbial diversity, offering unparalleled insights into complex ecosystems (Valentini *et al*., 2016). This molecular

*) Corresponding author

https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.29.1.61-70
Received : 28-11-2023
Accepted : 20-01-2024

toolkit, characterized by its high-throughput capabilities, provides a detailed exploration of microbial communities, allowing for a comprehensive understanding of their composition and dynamics. Harnessing the power of eDNA metabarcoding and NGS, our study is poised to unlock the intricacies of bacterial diversity within the unique context of Tanjung Mas Port.

The environmental characteristics of Tanjung Mas Port, shaped by a dynamic interplay of natural and anthropogenic factors, underscore the significance of investigating microbial communities in this setting (Tjahjono *et al*., 2018). Previous research has highlighted the vulnerability of port environments to the establishment of non-indigenous species, potentially impacting ecosystem functions and local biodiversity (El *et al*., 2022). In our study, the focus on bacterial diversity within Tanjung Mas Port positions it as a pivotal investigation at the intersection of molecular ecology, port management, and global environmental health. By employing eDNA metabarcoding and NGS, our research transcends the limitations of traditional methods, offering a more comprehensive and nuanced view of microbial communities in Tanjung Mas Port. The choice of the 16S rRNA V34 region as the target for metabarcoding ensures a focused exploration of bacterial taxa, allowing us to discern fine-scale patterns in microbial diversity. To understand the dynamic community structure, composition, and functional prediction of the bacterial community from biofouling at Tanjung Mas Port, Semarang, the use of the eDNA metabarcoding method is very important.

Materials and Methods

Sample collection

A seawater sample was meticulously collected from 6°57'16.83"S and 110°25'17.44"E coordinate location in Tanjung Mas Port in Semarang, Indonesia, ensuring an encompassing snapshot of the dynamic microbial community within this maritime environment. The sample was collected using modified protocol (Monaghan *et al*., 2020), comprising 2 L of seawater, and carefully extracted from a depth of 1 m using a sterile bottle to minimize contamination.

Filtration and sample preservation

The solitary seawater sample underwent immediate on-site filtration to concentrate microbial biomass. A Pall paper filter with a pore size of 0.22 micrometers was employed to effectively capture microbial cells. Filtration was conducted using aseptic techniques to prevent external contamination. Following filtration, the filter, containing the entire microbial biomass, was cautiously removed and stored in a sterile 2 mL tube, maintaining the integrity of the collected microbial community. To preserve the genetic material within the microbial biomass, the filter paper was immersed in 1 mL of DNA/RNA Shield solution. DNA/RNA Shield served as an efficient stabilizing medium, ensuring the protection of nucleic acids from degradation during transport and storage. The use of DNA/RNA Shield facilitated subsequent extraction of high-quality environmental DNA (eDNA), crucial for accurate metabarcoding analysis (Thijs *et al*., 2017).

DNA extraction and eDNA metabarcoding

DNA extraction from the singular filter paper was performed using the Zymo Quick-DNA/RNA Miniprep Kit. The extraction protocol included bead beating for efficient disruption of microbial cells and subsequent release of genetic material. The extracted DNA was eluted in a final volume of 50 µL, providing a concentrated template for subsequent analysis.

Library construction

Library construction for Illumina sequencing involved a two-step process: amplification and indexing. The 16S rRNA V3-V4 hypervariable region was targeted using primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') for amplification (Thijs *et al*., 2017). PCR amplification of the V3-V4 region was carried out using the KAPA HiFi HotStart ReadyMix. The thermal cycling conditions included an initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with a final extension at 72°C for 5 min. Indexing of the amplicons was performed in a second PCR using unique dual-index barcodes. The thermal cycling conditions were similar to the first PCR, with the number of cycles adjusted to avoid over-amplification.

Sequencing

The constructed libraries underwent sequencing on the Illumina NovaSeq 6000 platform, employing paired-end sequencing with a 2x250 bp configuration for enhanced resolution (Teeling *et al*., 2016). The resulting high-throughput sequence data provided a comprehensive dataset for subsequent analysis, enabling a detailed exploration of microbial diversity within Tanjung Mas Port. The Illumina NovaSeq 6000 sequencing platform generated raw sequence reads in the form of forward and reverse reads. The output data, typically in FastQ format, consisted of paired-end reads representing both ends of the DNA fragments. These paired reads were then

merged to reconstruct the full-length 16S rRNA V3-V4 region sequence for each microbial taxon (Abellen *et al.*, 2021).

Data quality control

The raw sequencing data underwent initial quality control steps, including the removal of low-quality reads, adapter trimming, and filtering out sequences below a specified length threshold. Subsequently, the high-quality, pre-processed sequences were utilized for downstream bioinformatics analyses. The joined sequences were subjected to operational taxonomic unit (OTU) clustering based on sequence similarity, defining distinct microbial entities within the dataset. Taxonomic assignment of OTUs was performed using reference SILVA databases.

Bioinformatics analysis

The bioinformatics analysis commenced with the processing of raw Illumina NovaSeq data in QIIME 2. Utilizing the DADA2 plugin, a stringent quality control procedure was implemented, involving filtering out low-quality reads, trimming adapters, and merging paired-end reads for subsequent analyses. The taxonomic assignment was performed using reference databases, and alpha and beta diversity metrics were calculated to evaluate the microbial community in the singular seawater sample. The processed data were further analyzed in RStudio using DADA2 for advanced sequence processing and Phyloseq, ggplot2 for visualization. DADA2 in RStudio facilitated denoising and the inference of amplicon sequence variants (ASVs), while Phyloseq ggplot2 generated insightful visualizations, including taxonomic composition and diversity plots. Statistical assessments were performed within RStudio to identify significant differences in microbial community composition. The resulting outputs comprised taxonomic profiles, diversity metrics, and visually informative plots, providing a comprehensive overview of the microbial community within the seawater sample from Tanjung Mas Port.

Result and Discussion

Environmental DNA (eDNA) refers on DNA that can be extracted from environmental samples such as water, soil or feces without isolating the target organism (Taberlet *et al.*, 2018). Environmental DNA (eDNA) is a powerful metabarcoding for monitoring biodiversity. Our analysis revealed an observed richness of 1080 operational taxonomic units (OTUs) as exhibited on Table 1., indicative of the initial bacterial diversity present in the sample. Estimates of species richness using the Chao1 and ACE indices further supported the notion that the true richness in the community may be comparable to the observed richness, with Chao1 and ACE values both at 1080.

The investigation of bacterial community structure revealed a rich and diverse ecosystem, as evidenced by a comprehensive set of diversity metrics. The observed count of 1080 operational taxonomic units (OTUs) hinted at the apparent species richness within the community. Utilizing the Chao1 estimator (Chao, 1984), which accounts for unobserved species, confirmed this richness with an estimate mirroring the observed count. Remarkably, the low standard error associated with the Chao1 estimate suggested a high level of confidence in this determination. The Abundance-based Coverage Estimator (ACE) further underscored the community's diversity, yielding an estimate congruent with both the observed count and Chao1 estimate (Martiny *et al.*, 2001). The slight standard error associated with ACE, measured at 16.42987, introduced an element of uncertainty, highlighting the variability in estimating the total species pool. The Shannon diversity index, reflecting both richness and evenness, delivered a value of 5.260632, affirming a complex and diverse microbial community. Similarly, the Simpson and Inverse Simpson indices provided insights into species probability and overall biodiversity, with values of 0.9763994 and 42.37181, respectively (Morris *et al.*, 2014). The Fisher alpha diversity index concluded the analysis with a robust value of 185.442, consolidating the evidence for a highly diverse bacterial community (Willis, 2019).

Phylum and class-level composition

Proteobacteria was found to dominate the phylum and class-level composition constituting 49.38% of the community. Other significant contributors included Bacteroidota (17.25%), Firmicutes (8.92%), Cyanobacteria, and Unidentified phyla. Figure 1 showcases the top 10 phyla, shedding light on the nuanced distribution within Seawater Tanjung Mas. At the Phylum level, the landscape reveals the dominance of Proteobacteria, substantiated by a robust 2568 occurrences, constituting approximately 49.38% of the total sequences. This phylum's prevalence underscores its remarkable adaptability to diverse ecological niches, playing an integral role in nutrient cycling and ecosystem stability. Simultaneously, the contributions of Bacteroidota, Firmicutes, and Cyanobacteria, collectively constituting 24.59% of the community, unveil the taxonomic tapestry that contributes to the intricacies of Tanjung Mas's microbial ecosystem. Bacteroidota, with 897 occurrences, manifests its importance in organic matter degradation and nutrient cycling, adding layers to the microbial community's functional diversity (Wong *et al.*, 2021). Alphaproteobacteria

emerged as the leading class, comprising 25.32% of the microbiome at the class level (Figure 2.),. The top 10 classes included Gammaproteobacteria (15.83%), Bacteroidia (13.83%), and others. The class-level distribution highlighted the richness and differential abundance of microbial classes.

The taxonomic exploration at the Class level delves into the nuances of Tanjung Mas seawater microbial communities, unveiling a dynamic interplay among taxonomic groups. Alphaproteobacteria, Gammaproteobacteria, and Bacteroidia collectively seize the spotlight, commanding 48.50% of the sequences and exemplifying the intricate taxonomic tapestry within Proteobacteria and Bacteroidota. Alphaproteobacteria, constituting a substantial portion, exhibits a remarkable ecological versatility, adapting to various marine niches and influencing ecosystem dynamics (Dang *et al*., 2019). Simultaneously, the presence of Clostridia, representing 6.69% of the community, contributes to the broader taxonomic spectrum within the Firmicutes, adding layers to the microbial community's structural diversity (Rea *et al*., 2011). The prominence of Alphaproteobacteria and Gammaproteobacteria at the Class level is particularly intriguing. Alphaproteobacteria, known for their symbiotic associations and key roles in nitrogen cycling, contribute to the complexity of nutrient dynamics in the Tanjung Mas marine environment (Rädecker *et al*., 2015; Kamiri *et al*., 2018). Meanwhile, Gammaproteobacteria, recognized for their diverse metabolic capabilities, including sulfur oxidation and methane production, further amplify the functional diversity within this taxonomic class (Zhou *et al*., 2020). Bacteroidia's presence at the Class level signifies the importance of Bacteroidota in the microbial community structure. Members of Bacteroidia are renowned for their ability to degrade complex organic compounds, playing a crucial role in nutrient cycling and organic matter decomposition (Zoppini *et al*., 2020).

Order and family diversity

Rhodobacterales (10.13%) and Chitinophagales (7.02%) were among the prominent orders at the order-level composition as illustrated on Figure 3. A notable percentage remained unidentified, emphasizing the challenges in taxonomic classification.

The Order level reveals the prevalence of Rhodobacterales, Chitinophagales, and Rhizobiales, collectively constituting 18.13% of the community. Rhodobacterales, a dominant order, stands out with 10.04% presence, playing a pivotal role in nutrient cycling and microbial interactions within the Tanjung Mas marine environment (Li *et al*., 2018). Chitinophagales and Rhizobiales contribute an additional 4.09% to the taxonomic diversity at this level, indicating the specificity of taxonomic groups that shape the microbial community structure. Rhodobacterales, in particular, encompasses a diverse range of bacteria with crucial ecological roles. These bacteria are adept at utilizing sunlight to generate energy, contributing to the overall productivity of the ecosystem (Kieft *et al*., 2018).

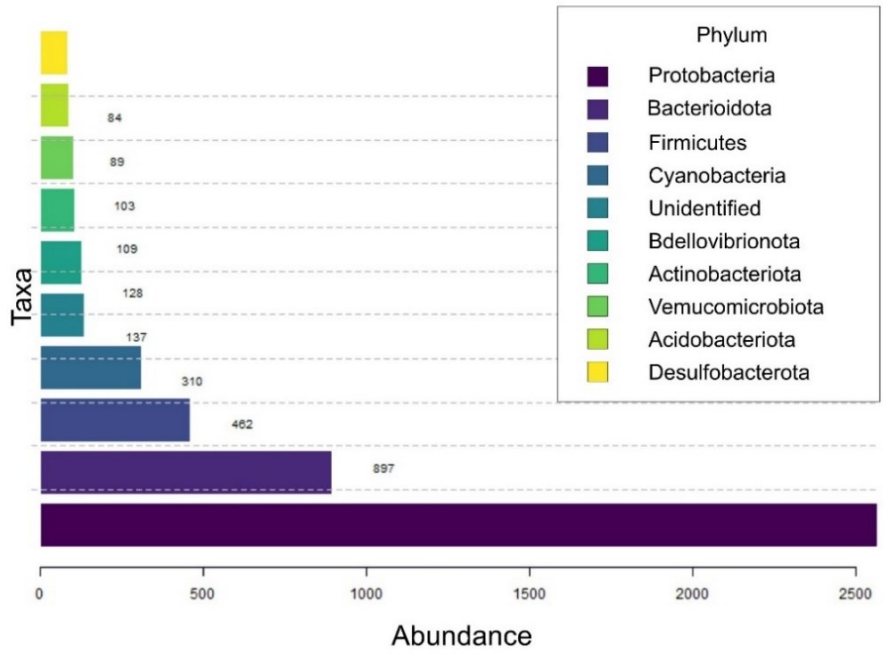

Figure 1. Bar plot illustrating top 10 phylum-level composition in Seawater Tanjung Mas.

Table 1. Taxonomic Abundance Analysis

Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
1080	1080	0	1080	16.42987	5.260632	0.9763994	42.37181	185.442

The prevalence of Chitinophagales, known for their capacity to degrade chitin, and Rhizobiales, associated with nitrogen fixation, further emphasizes the functional diversity within Tanjung Mas's microbial landsxape(Pohlner *et al*., 2019; Gillan *et al*., 2023).

At Family-level insights revealed the dominance of Rhodobacteraceae (10.06%) and Saprospiraceae (6.42%), providing a more granular view of the microbial community structure (Figure 4). Rhodobacteraceae, Saprospiraceae, and Rhizobiaceae emerge as prominent contributors, representing 13.14% of the communit. Rhodobacteraceae, with its diverse genera, is known for its roles in organic matter degradation and carbon cycling (20). Saprospiraceae, comprising chemoorganotrophic bacteria, adds another layer to the functional diversity of Tanjung Mas microbial communities (Povlovska *et al*., 2021). Meanwhile, Rhizobiaceae, with its association with nitrogen-fixing bacteria, contributes to nutrient cycling dynamics (Igiehon and Babalola, 2018). The Rizobiaceae family found in Tanjung Mas Harbor comes from sea water. Despite being primarily linked to soil and plant hosts, the metabolic capabilities and ecological roles of Rhizobiaceae in marine environments remain elusive due to the limited availability of cultured marine isolates and their sequenced genomes. Some of the family of Rhizobiaceae can potentially be used for bioremediation and biodegradation of heavy metals and toxic compounds (Alves *et al.*, 2014). Aoki *et al.* (2022) found a Rhizobiaceae-associated metagenome-assembled genome (MAG) from a marine manganese-oxidizing enrichment culture, shedding light on its role in Nada coastal area ecosystems. This study enhances our understanding of Rhizobiaceae's ecological significance and metabolic potential in marine environments, providing valuable insights into nutrient cycling dynamics.

Simultaneously, Flavobacteriaceae, Lachnospiraceae, and Cyclobacteriaceae enrich the taxonomic spectrum at the Family level, collectively representing 4.84% of the community. Flavobacteriaceae members are often associated with the degradation of complex organic compounds and contribute to the overall metabolic diversity of the ecosystem (Pollet *et al*., 2018). Lachnospiraceae, known for their roles in carbohydrate metabolism, and Cyclobacteriaceae, contributing to the breakdown of organic matter, further underscore the functional versatility within the Tanjung Mas microbial community (Mahmoud *et al*., 2021; Frates *et al*., 2023).

Genus and species-level richness

Exploring genus-level diversity the "Unidentified" genus dominated (39.51%). Other notable genera included unculturable (44.66%) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (Figure 5). The Genus level unfolds with the dominance of unculturable taxa, representing 44.66% of the sequences. This prevalence underscores the vast undiscovered diversity within the microbial community, a common challenge in marine metagenomic studies (Behzad *et al*., 2015). The unidentified nature of these taxa poses intriguing questions about the functional roles they may play in the ecosystem. The challenges associated with assigning specific names to a significant portion of the sequences highlight the need for advanced genomic techniques and further taxonomic resolution (Behzad *et al*., 2015). In contrast, the isolation of certain *Rhizobium* species from seawater sources, as demonstrated by Liu *et al.* (2015) and Fu *et al.* (2017), provides a parallel perspective on the adaptability of specific Rhizobium strains to marine environments. The presence of specific genera, such as *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* , contributes to 0.95% of the community, offering glimpses into known genera and their potential ecological roles. The dominance of unculturable taxa at the Genus level hints at the vast reservoir of unexplored microbial diversity in Tanjung Mas. This unidentified diversity represents an exciting frontier for further research, as these taxa may hold novel biochemical pathways, metabolic capabilities, and ecological functions that contribute to the resilience and adaptability of the marine ecosystem.

A study of various types of bacteria in Tanjung Emas Harbor has also been carried out by Husna *et al.* (2022) especially pathogenic bacteria. Nevertheless, at the species level, a significant proportion of the observed count pertains to species categorized as "Unidentified" (48.89%). Other top species included Pseudomonas geniculata, unculturable_organism, and more, as exhibited on Figure 6.

At the species level, a substantial 68.46% of the sequences remain unidentified, emphasizing the intricate nature of the microbial community and the

necessity for finer taxonomic resolution. We found *P. geniculata* in the identified species, constituting 3.09% of the community. *Pseudomonas* genus, plays a key role in marine biofouling due to its adaptability and metabolic diversity. Notably, this bacterium produces extracellular polymeric substances (EPS), creating a slimy matrix for surface adhesion and biofilm formation (Maes *et al.*, 2019). As *P. geniculata* adheres to submerged surfaces, the secreted EPS facilitates the development of biofilms—complex communities of microorganisms crucial in marine biofouling. The bacterium's proficiency in initial biofilm formation underscores its contribution to the early stages of surface fouling in aquatic environments. *P. geniculata*, a recognized species, opens avenues for understanding its specific ecological roles and contributions to the overall microbial community dynamics (Wu *et al*., 2022). The prevalence of unidentified species suggests the potential for uncovering novel microbial diversity within Tanjung Mas seawater, highlighting the importance of advanced sequencing techniques for future taxonomic exploration (Wu *et al*., 2020). Future studies also need to incorporate multiple samples over time, allowing for a more thorough examination of microbial community dynamics. It is important to note that the findings from our single sample offer a specific snapshot of the microbial landscape at the time of collection and serve as a starting point for further investigations into the broader patterns of microbial diversity in the region.

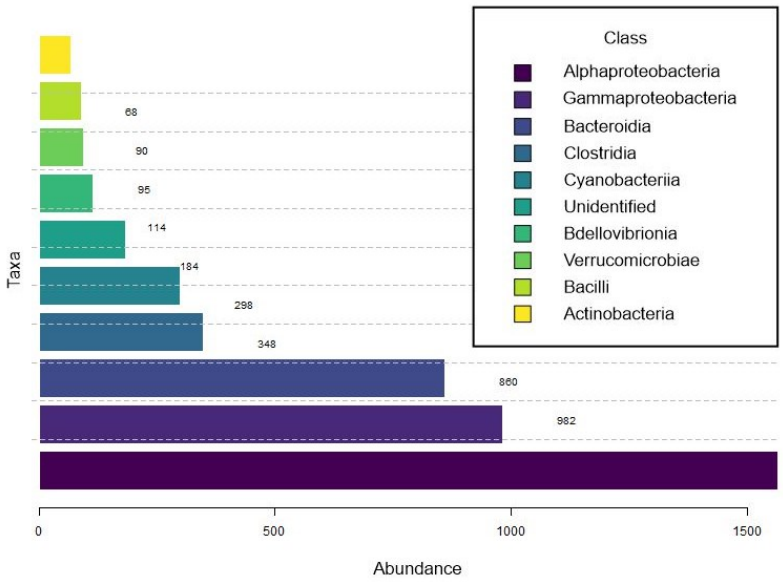

Figure 2. Bar plot depicting top 10 class-level distribution in Seawater Tanjung Mas

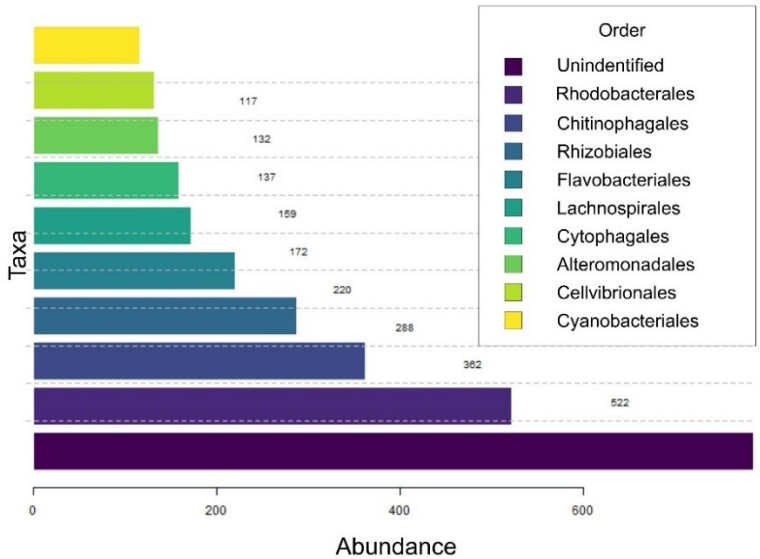

Figure 3. Bar plot showcasing top 10 order-level distribution in Seawater Tanjung Mas.

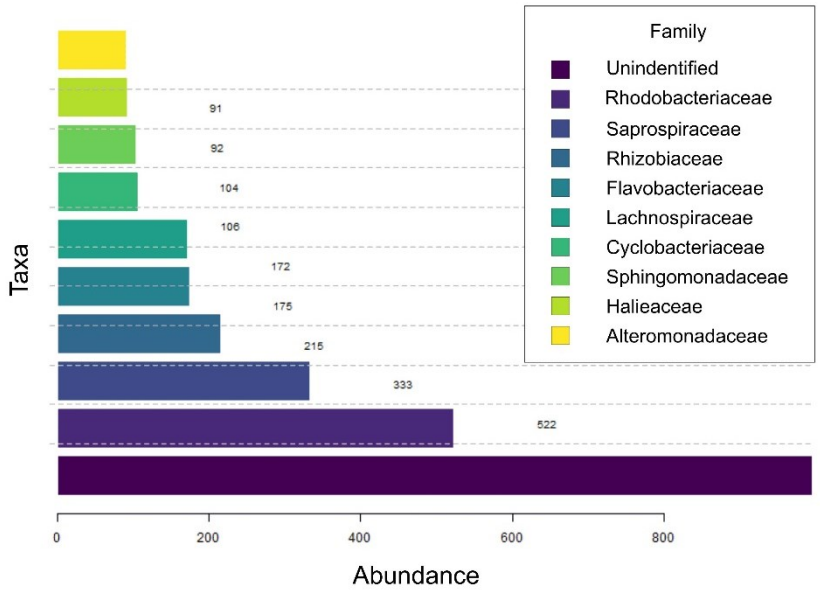

Figure 4. Bar plot illustrating top 10 family-level distribution in Seawater Tanjung Mas.

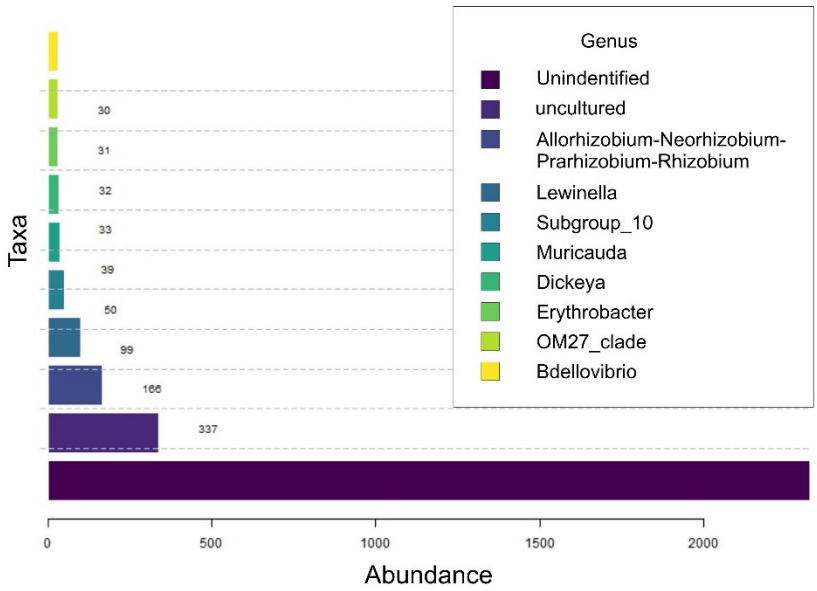

Figure 5. Bar plot highlighting top 10 genus-level diversity in Seawater Tanjung Mas.

Potential discovery of microbial diversity at Tanjung Mas Port

Understanding the dynamic community structure, composition, and functional prediction of the bacterial community at Tanjung Mas Port, Semarang, is very important to manage marine biofouling. Hopefully, the use of the eDNA metabarcoding method will result in obtaining a comprehensive data on microbial diversity relating with biofouling. The taxonomic analysis of Seawater Tanjung Mas revealed a surprising presence of Rhizobiaceae, constituting 6.42% of the microbial community, challenging conventional expectations of its terrestrial association. Recent studies, such as Aoki *et al*. (2022), have provided insights into the ecological significance and metabolic potential of Rhizobiaceae in marine environments, particularly in nutrient cycling dynamics. This unexpected occurrence may prompts further exploration into Rhizobiaceae's potential contributions to biofilm formation and marine biofouling processes, as its adaptability to marine niches suggests implications for these crucial phenomena. Concurrently, the identification of *P. geniculata*, constituting 3.09% of the community, underscores its significance in marine biofouling. *Pseudomonas*, known for its versatility and metabolic diversity, plays a pivotal role

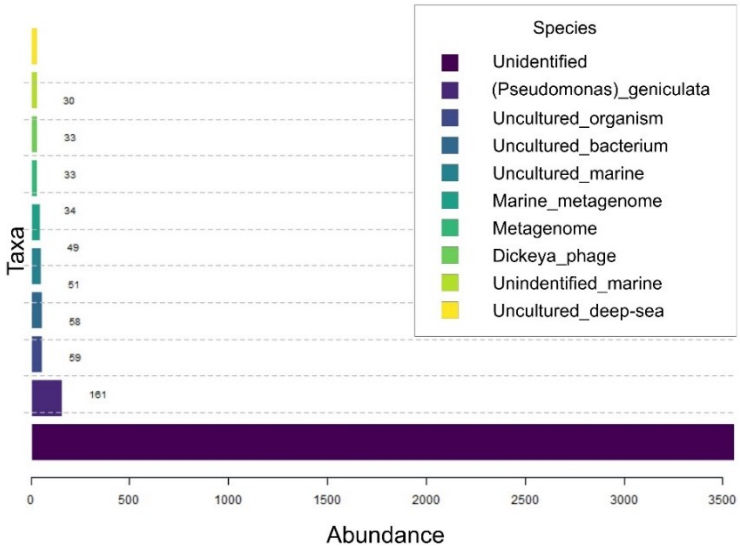

Figure 6. Bar plot demonstrating top 10 species-level distribution in Seawater Tanjung Mas.

in biofilm formation through the production of extracellular polymeric substances (EPS) (Maes *et al.*, 2019). The proficiency of *P. geniculata* in initiating biofilm formation highlights its role in the early stages of surface fouling in aquatic environments (Ouyang *et al.*, 2016). This dual discovery of Rhizobiaceae and *Pseudomonas geniculata* in Tanjung Mas seawater offers a unique opportunity to unravel their specific ecological roles in biofilm-mediated biofouling phenomena, contributing to a more nuanced understanding of microbial interactions in marine ecosystems. Further research is essential to unveil the distinctive attributes of these microbial players and their impact on marine biofouling dynamics.

Conclusion

The exploration of Tanjung Mas Port microbial communities through eDNA metabarcoding had found the dominant taxon lays on Proteobacteria, Bacteroidota, and Firmicutes. Alphaproteobacteria and Gammaproteobacteria emerge at the Class level showcasing their versatility and influence on ecosystem dynamics. The Order and Family levels revealing the prevalence of Rhodobacterales, Chitinophagales, and Rhizobiales, along with families like Rhodobacteraceae, Saprospiraceae, and Rhizobiaceae. These taxonomic entities contribute in nutrient cycling, organic matter degradation, and overall ecosystem stability.

Acknowledgement

The authors gratefully acknowledge the support of Diponegoro University in providing grant No. 609-81/UN7.D2/PP/VIII/2023 which is gratefully acknowledged. We would like to thank Naufal Sebastian Anggoro who has contributed to the study especially acquisition of data, supplying documents/ photographs or analysis and interpretation of data and also involved in drafting the manuscript, Hermin Pancasakti Kusumaningrum in establishing research design and helping in drafting the manuscript and revising it critically for important intellectual content, Muhammad Zainuri Wijanarka and Tony Hadibarata in revising it critically for important intellectual content.

References

Abellan-Schneyder, I., Matchado, M.S., Reitmeier, S., Sommer, A., Sewald, Z., Baumbach, J., List, M. & Neuhaus, K. 2021. Primer, pipelines, parameters: issues in 16S rRNA gene sequencing, *Msphere*. 6(1): 1110-1128. https://doi.org/10.1128/mSphere.01202-20

Alves, L. M. C., De Souza, J. A. M., de Mello Varani, A. & de Macedo Lemos, E.G. 2014. The family rhizobiaceae. by E. Rosenberg, EF DeLong, S. Lory, *et al*. Berlin, Heidelberg: *Springer*, 419-437. https://doi.org/10.1007/978-3-642-30197-1_297

Aoki, M., Nakahara, N., Kusube, M. & Syutsubo, K. 2022. Metagenome-Assembled Genome Sequence of Marine Rhizobiaceae sp. Strain MnEN-MB40S, Obtained from Manganese-Oxidizing Enrichment Culture. *Microbiol. Resour. Announc.*, 11(9): e00645-22. https://doi.org/10.1128/mra.00645-22

Behzad, H., Gojobori, T. & Mineta, K. 2015. Challenges and opportunities of airborne metagenomics. *Genome Biol. Evol*., 7(5): 1216–1226. https://doi.org/10.1093/gbe/evv064

Dang, H., Klotz, M.G., Lovell, C.R. & Sievert, S.M. 2019. The responses of marine microorganisms, communities and ecofunctions to environmental gradients. *Front. Microbiol.,* 10: p.115. https://doi.org/10.3389/fmicb.2019.00115

Frates, E.S., Spietz, R.I., Silverstein, M.R., Girguis, P., Hatzenpichler, R. & Marlow, J.J. 2023. Natural and anthropogenic carbon input affect microbial activity in salt marsh sediment. *Front. Microbiol*., 14: 1–20. https://doi.org/10.3389/fmicb.2023.1235906

Fu, G.Y., Yu, X.Y., Zhang, C.Y., Zhao, Z., Wu, D., Su, Y., Wang, R.J., Han, S.B., Wu, M. & Sun, C. 2017. Mesorhizobium oceanicum sp. nov., isolated from deep seawater. *Int. J. Syst. Evol. Microbiol.* 67(8): 2739-2745. https://doi.org/10.1099/ijsem.0.002009

Gillan, D. C., Pan, H., Roulez, A. & Wattiez, R. 2023. The metaphenome of a calaminiferous soil. *Microbe*. 1: 100002. https://doi.org/10.1016/j.microb.2023.100002

Husna, I. E., Anggoro, S., Rachim, H.R., Subagiyo, S. & Ighwerb, M.I. 2022. Bacteriological study of ballast water at Tanjung Emas Port, Semarang. *Ilmu Kelautan: Indonesian Journal of Mararine Science,* 27(1): 45-52. https://doi.org/10.14710/ik.ijms.27.1.45-52

Igiehon, N.O. & Babalola, O.O.. 2018. Rhizosphere microbiome modulators: Contributions of nitrogen fixing bacteria towards sustainable agriculture. *Int. J. Environ. Res. Public Health*. 15(4): p.574. https://doi.org/10.3390/ijerph15040574

Karimi, E., Slaby, B.M., Soares, A.R., Blom, J., Hentschel, U. & Costa, R. 2018. Metagenomic binning reveals versatile nutrient cycling and distinct adaptive features in alphaproteobacterial symbionts of marine sponges. *FEMS Microbiol Ecol*. 94(6):1–18. https://doi.org/10.1093/femsec/fiy074

Kieft, B., Li, Z., Bryson, S., Crump, B. C., Hettich. R., Pan, C., Mayali, X. & Mueller, R. S. 2018. Microbial community structure-function relationships in Yaquina Bay estuary reveal spatially distinct carbon and nitrogen cycling capacities. *Front. Microbiol*., 9: 1–15. https://doi.org/10.3389/fmicb.2018.01282

Li, Y., Jing, H., Xia, X., Cheung, S., Suzuki, K. & Liu, H. 2018. Metagenomic insights into the microbial community and nutrient cycling in the western subarctic Pacific Ocean. *Front. Microbiol.,* 9: p.623. https://doi.org/10.3389/fmicb.2018.00623

Liu, Y., Wang, R.P., Ren, C., Lai, Q.L. & Zeng, R.Y. 2015. *Rhizobium marinum* sp. nov., a malachite-green-tolerant bacterium isolated from seawater. *Int. J. Syst. Evol. Microbiol* 65(Pt_12): 4449-4454. https://doi.org/10.1099/ijsem.0.000593

Maes, S., Vackier, T., Nguyen Huu, S., Heyndrickx, M., Steenackers, H., Sampers, I., Raes, K., Verplaetse, A. & De Reu, K. 2019. Occurrence and characterisation of biofilms in drinking water systems of broiler houses. *BMC microbiology*, 19: 1-15. https://doi.org/10.1186/s12866-019-1451-5

Mahmoud, M.A. & Magdy, M. 2021. Metabarcoding profiling of microbial diversity associated with trout fish farming. *Scientific Reports*. 11(1): p.421. https://doi.org/10.1038/s41598-020-80236-x

Monaghan, E.A., Freel, K.C. & Rappé, M.S. 2020. Isolation of SAR11 marine bacteria from cryopreserved seawater. *Msystems*, 5(6): 1110-1128. https://doi.org/10.1128/mSystems.00954-20.

Moulton, O.M., Altabet, M.A., Beman, J.M., Deegan, L.A., Lloret, J., Lyons, M.K., Nelson, J.A. & Pfister, C.A. 2016. Microbial associations with macrobiota in coastal ecosystems: patterns and implications for nitrogen cycling. *Front. Ecol. Environ.*, 14(4): 200-208. https://doi.org/10.1002/fee.1262

Palit, K., Rath, S., Chatterjee, S. & Das, S. 2022. Microbial diversity and ecological interactions of microorganisms in the mangrove ecosystem: Threats, vulnerability, and adaptations. *Environ. Sci. Pollut. Res.,* 29(22): 32467-32512. https://doi.org/10.1007/s11356-022-19048-7

Pavlovska, M., Prekrasna, I., Dykyi, E., Zotov, A., Dzhulai, A., Frolova, A., Slobodnik, J. & Stoica, E. 2021. Niche partitioning of bacterial communities along the stratified water column in the Black Sea. *Microbiologyopen*. 10(3):1–24. https://doi.org/10.1002/mbo3.1195

Pohlner, M., Dlugosch, L., Wemheuer, B., Mills, H., Engelen, B., Reese, B.K. 2019. The majority of active Rhodobacteraceae in marine sediments belong to uncultured genera: A molecular approach to link their distribution to environmental conditions. *Front. Microbiol*., 10:

1–16. https://doi.org/10.3389/fmicb.2019.00659

Pollet, T., Berdjeb, L., Garnier, C., Durrieu, G., Le Poupon, C., Misson, B. & Briand, J.F. 2018. Prokaryotic community successions and interactions in marine biofilms: The key role of Flavobacteriia. *FEMS. Microbiol. Ecol*. 94(6): 1–13. https://doi.org/10.1093/femsec/fiy083

Puspitasari, I., Trianto, A. & Supriyanto, J. 2020 Eksplorasi Bakteri Pendegradasi Minyak dari Perairan Pelabuhan Tanjung Mas, Semarang. *J Mar Res*. 9(3): 281–8. https://doi.org/10.14710/jmr.v9i3.27606

Ouyang, J., Sun, F., Feng, W., Sun, Y., Qiu, X., Xiong, L., & Chen, Y. 2016. Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in *Pseudomonas aeruginosa*. *J. Appl. Microbiol.*, 120(4): 966-974. https://doi.org/10.1111/jam.13073

Rädecker, N., Pogoreutz, C., Voolstra, C.R., Wiedenmann, J. & Wild, C. 2015. Nitrogen cycling in corals: the key to understanding holobiont functioning?. *Trends Microbiol.,* 23(8): 490-497. https://doi.org/10.1016/j.tim.2015.03.008

Rea, M.C., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P.D., Shanahan, F., Kiely, B., Hill, C. & Ross, R.P. 2011. Effect of broad-and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proc. Natl. Acad. Sci. USA.* 108 (supplement_1):4639-4644. https://doi.org/10.1073/pnas.1001224107

Teeling, H., Fuchs, B. M., Bennke, C.M., Krüger, K., Chafee, M., Kappelmann, L., R einjes, G., Waldmann, J., Quast, C., Glockner, F.O., Lucas, J., Wichels, A., Gerdts, G., Karen, W.H. & Amann, R.I. 2016. Recurring patterns in bacterioplankton dynamics during coastal spring algae blooms. *elife*. 5: e11888. https://doi.org/10.7554/eLife.11888

Thijs, S., Op De Beeck, M., Beckers, B., Truyens, S., Stevens, V., Van Hamme, J. D., Weyens, N. & Vangronsveld, J. 2017. Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. *Fronti. Microbiol.*, 8: p.494. https://doi.org/10.3389/fmicb.2017.00494

Tjahjono, A. & Handoko, W. 2018. The implementation of ballast water management in Port of Tanjung Emas semarang: Strategy and model. *AACL Bioflux*. 11(4): 1231–1247.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G.H., Geniez, P., Pont, D., Argillier, C., Baudoin, J.M., Peroux, T., Crivelli, A.J., Olivier, A., Acqueberge, M., Le Brun, M., Møller, P.R., Willerslev, E. & Dejean, T. 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*. 25 (4): 929-942. https://doi.org/10.1111/mec.13428

Wong, W.W., Greening, C., Shelley, G., Lappan, R., Leung, P.M., Kessler, A., Winfrey, B., Poh, S.C. & Cook, P. 2021. Effects of drift algae accumulation and nitrate loading on nitrogen cycling in a eutrophic coastal sediment. *Sci. Total Environ.,* 790: p.147749. https://doi.org/10.1016/j.scitotenv.2021.147749

Wu, C., Wu, X., Chen, S. & Wu, D. 2020. A Newly Discovered Humic-Reducing Bacterium, *Pseudomonas geniculata* PQ01, Isolated From Paddy Soil Promotes Paraquat Anaerobic Transformation. *Front. Microbiol*. 11: 1–12. https://doi.org/ 10.3389/fmicb.2020.02003

Wu, J., Danko, D., Afshinnekoo, E,, Bezdan, D,, Bhattacharyya, M, Castro-Nallar, E,E., Chmielarczyk, A., Hazrin-Chong, N.H., Deng, Y., Dias-Neto, E., Frolova, A., Mason-Buck, G., Iraola, G., Jang, S., Łabaj, P., Lee, P.K.H., Nieto-Caballero, M., Osuolale, O.O., Ouzounis, C.A., Perlin, M.H. & Shi, T. 2022. Annotating unknown species of urban microorganisms on a global scale unveils novel functional diversity and local environment association. *Environ. Res.* 207: p.112183. https://doi.org/10.1016/j.envres.2021.112183

Zhou, Z., Tran, P. Q., Kieft, K. & Anantharaman, K. 2020. Genome diversification in globally distributed novel marine Proteobacteria is linked to environmental adaptation. *The ISME journal*. 14(8): 2060-2077. http://doi.org/10.1038/s41396-020-0669-4

Zoppini, A., Bongiorni, L., Ademollo, N., Patrolecco, L., Cibic, T., Franzo, A., Melita, M., Bazzaro, M. & Amalfitano, S. 2020. Bacterial diversity and microbial functional responses to organic matter composition and persistent organic pollutants in deltaic lagoon sediments. *Estuar. Coast. Shelf Sci.,* 233: p.106508. https://doi.org/10.1016/j.ecss.2019.10650