Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 16, Issue 1, 2026, 23
https://doi.org/10.33263/BRIAC161.023

Comparison of Biofouling Bacterial Community on Wood and Cement Concrete Surface at Tanjung Mas Semarang Port Using eDNA Metabarcoding

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Received: 8.08.2025; Accepted: 27.10.2025; Published: 15.02.2026

Abstract: Tanjung Emas Port Semarang is one of the places where massive biofouling occurs, especially on wood and concrete surfaces, due to colonization and attachment of microbial communities. The aim of this research is to determine the diversity of bacteria that cause biofouling on wood and cement concrete surfaces at Tanjung Mas Port, Semarang, using eDNA 16S rRNA metabarcoding. Biofilm samples from Tanjung Mas Port waters were used for DNA extraction and amplification of the 16S rRNA V3-V4 region, followed by sequencing and construction of an eDNA Metabarcoding library. Sequence analysis was performed in QIIME 2, RStudio DADA2, and Phyloseq. The results showed that a greater variety of genera was found on cement concrete surfaces compared to wood. The cement concrete surface was dominated by genus *Erythrobacter*, while on the wood surface, it was dominated by *Photobacterium*. Metabolic pathway analysis shows that bacteria in cement, concrete, and wood carry out extensive aerobic and anaerobic respiration, fatty acid and amino acid biosynthesis, and energy generation for biofilm formation.

Keywords: cement concrete; wood; bacteria; biofilm; metabarcoding; quorum sensing.

1. Introduction

Marine biofouling on man-made structures caused by microbial biofilms is a serious challenge in Tanjung Mas dock infrastructure. As the busiest port area in Central Java, Tanjung Mas handles heavy traffic on domestic and international routes [1]. High service traffic affects nutrient cycling and pollution in the area [2,3]. This transaction result increased organism growth, especially for pollutant-living organisms [4]. The organic and non-organic waste from the ship and port can contaminate hulls and the environment [5,6]. The use of cement makes concrete surfaces sterile and inhibits the growth of organisms [7,8]. At the same time, a wooden structure seems more sustainable but has weaknesses, such as limited capacity and capability

for complex construction [9,10]. Man-made infrastructures forced nature to activate self-cleaning and preserve the environment with the attachment of organisms like biofouling [11].

Initiation of biofouling, through the formation of biofilms by microorganisms such as bacteria, fungi, and microalgae, provides a foundation for macroorganisms, such as barnacles, to attach [12]. The accumulation of biofouling organisms not only endangers the structural safety of marine infrastructure but also causes economic losses, environmental damage, and reduced operational efficiency [13]. International ports that have international shipping and trade traffic must immediately address the problem of biofouling in an environmentally friendly and sustainable manner [14]. Strategy formulation can be done through various stages. The aim of the research is to test the hypothesis that there will be differences in the types of bacteria that cause fouling, which are substrate-specific on cement concrete and wood surfaces. Problem analysis, understanding biofouling, formulating potential solutions, and testing solutions require serious commitment [15]. Understanding bacterial biofilms is needed to find out the initial cause of biofouling [16]. The use of methods such as next-generation sequencing (NGS) helps to more quickly understand the composition of biofilm communities [17]. It is hoped that a prevention strategy using a potential quorum-sensing (QS) inhibitor approach can be a solution to the biofouling problem.

Biofilm formation on submerged surfaces occurs in several stages [18]. Exposure to bacteria in seawater forms a biofilm layer consisting of organic molecules such as lipids, polysaccharides, and proteins. Changing the physicochemical properties of the material surface so that it can be easily attached to microbes [19,20]. Primary colonies of bacteria attach to the surface of the material using structures such as pili, flagella, and extracellular polymeric substances (EPS) [21]. The matrix of polysaccharides, proteins, and nucleic acids that form EPS also helps stabilize the biofilm structure and protect organisms from environmental changes [22].

2. Materials and Methods

2.1. Sample collection.

The sampling location consists of three points in the Tanjung Mas Port area, Semarang. Biofilm microbiome samples were taken from two material surfaces, wood and cement concrete. Biofilm samples were collected from cement concrete and wood surfaces at the Tanjung Mas Port pier in Semarang at a depth of one meter. Triplicate samples were collected from each surface during the sampling procedure. All swab samples were placed into a sterile, labeled container immediately. Tubes were kept at 4°C using portable coolers during fieldwork and subsequently transferred to a laboratory refrigerator within 4 hours. DNA extraction was performed using the Zymo Quick-DNA/RNA Miniprep Kit [23]. DNA extraction was followed by library preparation using the V3-V4 region amplified 16S rRNA gene with a length of 470 bp. DNA quality and quantity were analysed using a Nanodrop by measuring the 260/280 ratio (DNA/protein) and the 260/230 ratio (DNA/humic acid) to assess contamination by protein and humic acid, respectively.

2.2. Sequencing and data processing.

Sequencing was performed on the MGI platform DNBSeq G-400 machine. The sequencing data were analyzed using QIIME2 software version 2023.9. Data were then filtered to remove low-quality reads and then grouped into operational taxonomic units (OTUs) based

on their sequence similarity to the SILVA 138 SSU 99NR reference. Base sequence quality control was also performed using the program https://nephele.niaid.nih.gov/data import. Phyloseq RStudio 2024.04.2 was used for data analysis and visualization [24]. A minimum of 79,849 reads per sample was targeted and rarified to ensure sufficient coverage for downstream taxonomic and functional analysis [25].

2.3. Diversity analysis.

Alpha diversity indices (observed richness, Shannon, and Inverse Simpson indices) were calculated using the Kruskal-Wallis statistical comparison. Beta diversity was assessed through permutational multivariate analysis of variance (PERMANOVA) to determine differences in community composition. PERMANOVA was used to measure the effect size and significance of beta diversity for a grouping variable. Non-metric multidimensional scaling (NMDS) analysis was used to visualize community composition. NMDS will maximize the rank-based correlation between the original distances, as well as the distances between samples in the new reduced ordination space [24].

2.4. Functional prediction.

Functional pathways were predicted using PICRUSt2 v2.5.3 to examine potential metabolic activities in each biofilm type, focusing on pathways such as aerobic respiration, pyruvate fermentation, and fatty acid biosynthesis [26].

3. Results and Discussion

3.1. Microbial diversity and composition.

The six samples that were collected from two different surfaces produced 5.230 OTU after quality check and filtration. Data processing with rarefaction continued with analysis of alpha diversity comparison of observed OTU, Shannon, and inverse Simpson index as shown in Table 1.

Table 1. Alpha diversity index.

Materials		**Observed richness**	**Shannon diversity**	**Inverse simpson**
Cement concrete	SB	728 ± 43.8	5.36 ± 9.63	125 ± 15.3
Wood	SK	708 ± 53.4	5.18 ± 1.04	100 ± 10.8

The alpha diversity index showed that cement concrete (SB) samples had higher diversity and richness indices than wood (SK). Beta diversity analysis in Table 2 determines the structural differences of the microbial community between the two surfaces [27]. Rarified data continue with analysis of beta diversity index using PERMANOVA statistic method and non-metric multidimensional scaling (NMDS) to give a broader explanation of sample description.

Table 2. Beta diversity analysis result.

	Permutation dispersion			**Permanova**	
	F	N. Perm	Pr(>F)	F	p- value
Groups	0.113	719	0.6014	0.4925	0.9*

PERMANOVA analysis showed homogeneous microbial communities between cement concrete and wood samples. However, within the phylum, family, and class, some mi-

crobes exhibit very specific types on both wood and concrete surfaces. The microbial community on an object's surface is influenced by the nature of the substrate. The microbial communities on cement concrete and wood surfaces should differ, with cement concrete generally exhibiting higher diversity [28,29]. However, over time, the formation of marine biofilms tends to bring microbial diversity closer together regardless of substrate type, suggesting little long-term impact of different substrate properties on microbial community composition [30].

3.2. Taxonomic abundance patterns.

A total of 5.230 OTU and 700.576 reads were assigned to the SILVA 99NR classifier. We filtered the results to overview the top 2% OTU abundance. The taxonomic assignment revealed 26 genera belonging to 23 families, 9 classes, and 8 phyla, all of which were identified across all samples.

Proteobacteria dominated both biofilms, contributing more than half of the total community. Cyanobacteria were identified with a slightly higher presence on wood than on cement concrete. The phylum Bacteroidota was identified in both materials, as shown in Figure 2. Typically, the most abundant phylum in marine biofilms is Proteobacteria, which is followed by Cyanobacteria. However, factors such as nutrients, substrate characteristics, and salinity influence the relative abundance of each phylum [31]. Proteobacteria, Cyanobacteria, and Bacteroidetes are the most abundant phyla found in marine biofilms on artificial surfaces such as cement, concrete, and wood [28].

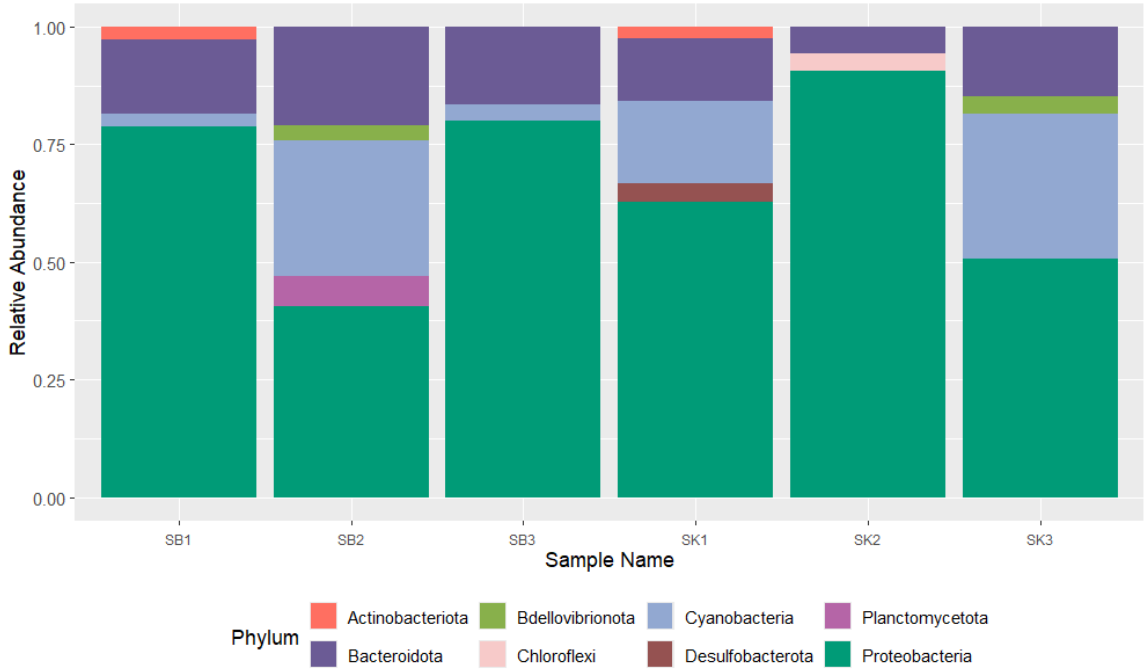

Figure 1. Phylum-level bacteria composition in cement concrete (SB) and wood (SK) material surface with 2% abundance.

The genus *Vibrio* and *Pleurocapsa*_PCC-7319 dominated the community on both material surfaces. The genus *Vibrio* and *Pleurocapsa*_PCC-7319 had different grouping arrangements. The genus *Vibrio* belongs to the family Vibrionaceae within the phylum Proteobacteria, while *Pleurocapsa*_PCC-7319 is a member of the family Xenococcaceae within the phylum Cyanobacteria. The genus *Ruegeria*, which belongs to the family Rhodobacteriaceae and the phylum Proteobacteria, also showed dominance in both communities. There are many discoveries of the genus *Vibrio* in research on bacterial biodiversity in marine biofilms. This is due to

its role as an early colonizer of surfaces, followed by the presence of the genus Pleurocapsa, a filamentous cyanobacterium that plays a very important role in biofilm formation [32-34].

Based on Figures 1-4, it can be seen that there are differences in several microbial members that are very specific to wood and cement concrete. At the phylum level, Planctomycota is only found on cement concrete surfaces, while Desulfobacterota and Chloroflexi are only found on wood surfaces. At the class level, Dehalococcoidia is only found on wood surfaces, while Phycissphaerea is only found on cement concrete surfaces. Cyclobacteriaceae is a family specific to cement concrete, while the SAR202 clade and Shewanellaceae are found only on wood.

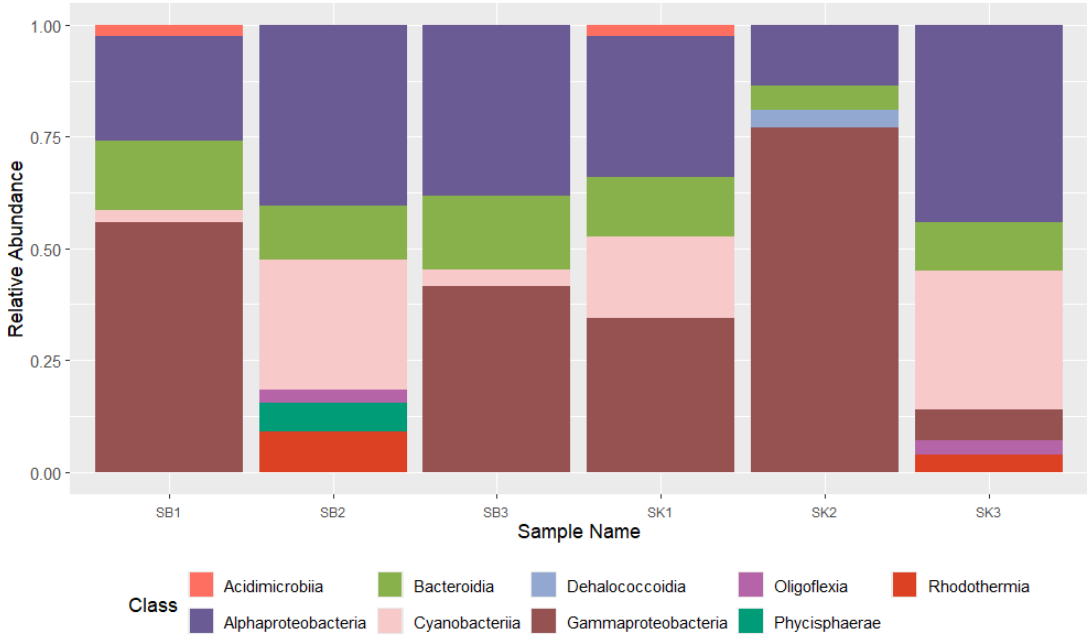

Figure 2. Class-level bacteria composition in cement concrete (SB) and wood (SK) material surface with 2% abundance.

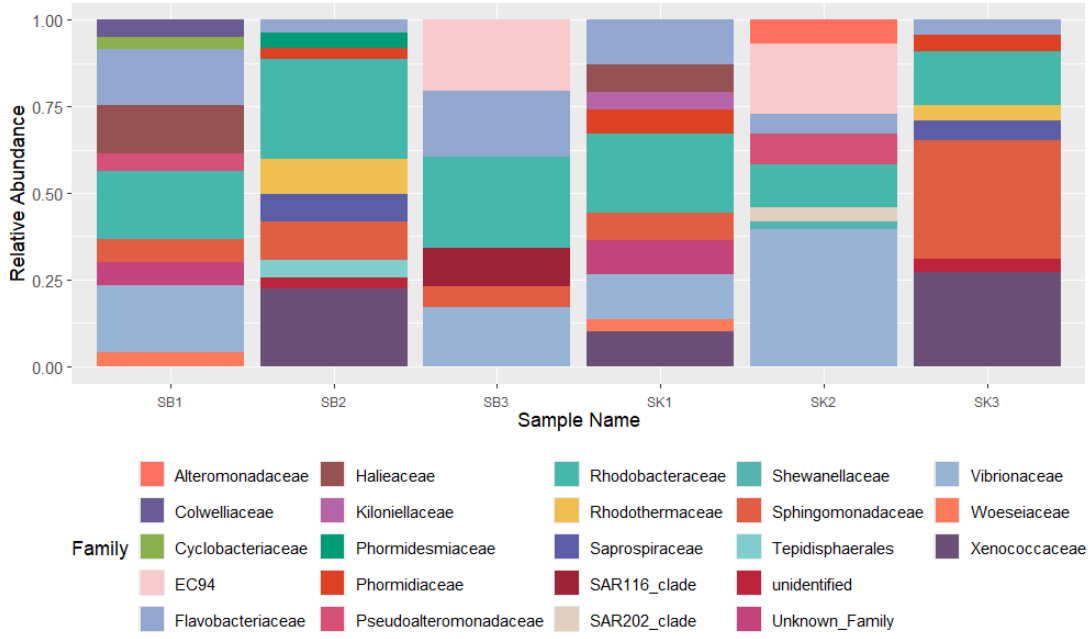

Figure 3. Family-level bacteria composition in cement concrete (SB) and wood (SK) material surface with 2% abundance.

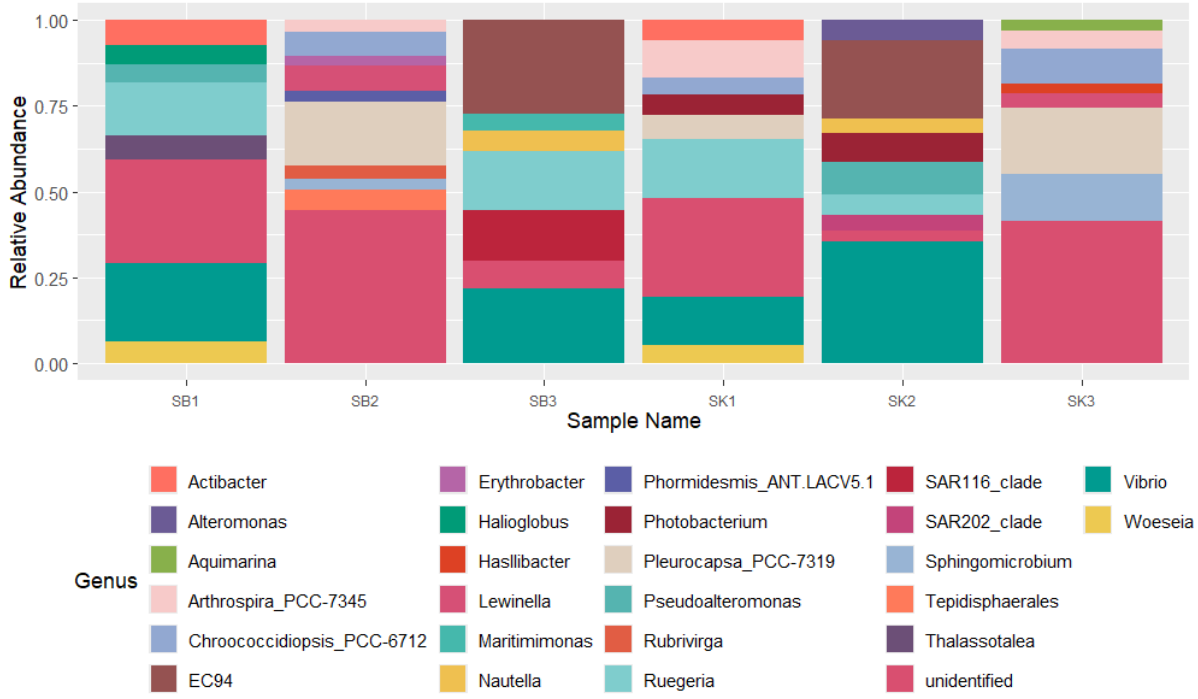

Figure 4. Genus-level bacteria composition in cement concrete (SB) and wood (SK) material surface with 2% abundance.

Identification of bacterial composition on cement concrete and wood surfaces using metabarcoding provides a broad overview of the dominant bacterial taxa inhabiting these materials, as shown in Figure 4 and Table 3. There are several genera that specifically grow on cement concrete surfaces, including *Erythrobacter*, *Halioglobus*, *Hasllibacter*, *Maritimimonas*, *Nautella*, *Phormidesmis*, and *Tepisdisphaerales*. Genus specific to wood surfaces are *Aquimarina*, *Photobacterium*, and *Rubrivirga*. Most of the dominant genera were found in both materials shown in Figures 5 and 6.

Table 3. List of top 25 biofilm genera identified through the amplicon eDNA metabarcoding method

Phylum	Class	Family	Genus	Reads	Read proportion
Bacteroidota	Bacteroidia	Flavobacteriaceae	*Actibacter*	6163	1%
Bacteroidota	Bacteroidia	Flavobacteriaceae	*Aquimarina*	7561	2%
Bacteroidota	Bacteroidia	Flavobacteriaceae	*unidentified*	6059	1%
Bacteroidota	Bacteroidia	Saprospiraceae	*Lewinella*	13232	3%
Bacteroidota	Rhodothermia	Rhodothermaceae	*Rubrivirga*	6348	1%
Bacteroidota	Rhodothermia	Rhodothermaceae	*unidentified*	10762	2%
Bdellovibrionota	Oligoflexia	unidentified	*unidentified*	8569	2%
Cyanobacteria	Cyanobacteriia	Phormidiaceae	*Arthrospira_PCC-7345*	14517	3%
Cyanobacteria	Cyanobacteriia	Xenococcaceae	*Chroococcidiopsis_PCC-6712*	21086	5%
Cyanobacteria	Cyanobacteriia	Xenococcaceae	*Pleurocapsa_PCC-7319*	47288	10%
Planctomycetota	Phycisphaerae	Tepidisphaerales	*Tepidisphaerales*	7975	2%
Proteobacteria	Alphaproteobacteria	Rhodobacteraceae	*Nautella*	7808	2%
Proteobacteria	Alphaproteobacteria	Rhodobacteraceae	*Ruegeria*	27395	6%
Proteobacteria	Alphaproteobacteria	Rhodobacteraceae	*unidentified*	61313	14%
Proteobacteria	Alphaproteobacteria	SAR116_clade	*SAR116_clade*	7178	2%
Proteobacteria	Alphaproteobacteria	Sphingomonadaceae	*Erythrobacter*	9263	2%
Proteobacteria	Alphaproteobacteria	Sphingomonadaceae	*Sphingomicrobium*	20274	4%
Proteobacteria	Alphaproteobacteria	Sphingomonadaceae	*unidentified*	34738	8%
Proteobacteria	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*	9548	2%
Proteobacteria	Gammaproteobacteria	EC94	*EC94*	33683	7%

Phylum	Class	Family	Genus	Reads	Read proportion
Proteobacteria	Gammaproteobacteria	Halieaceae	*unidentified*	5937	1%
Proteobacteria	Gammaproteobacteria	Pseudoalteromonadaceae	*Pseudoalteromonas*	10963	2%
Proteobacteria	Gammaproteobacteria	Unknown_Family	*unidentified*	7664	2%
Proteobacteria	Gammaproteobacteria	Vibrionaceae	*Photobacterium*	11887	3%
Proteobacteria	Gammaproteobacteria	Vibrionaceae	*Vibrio*	55299	12%

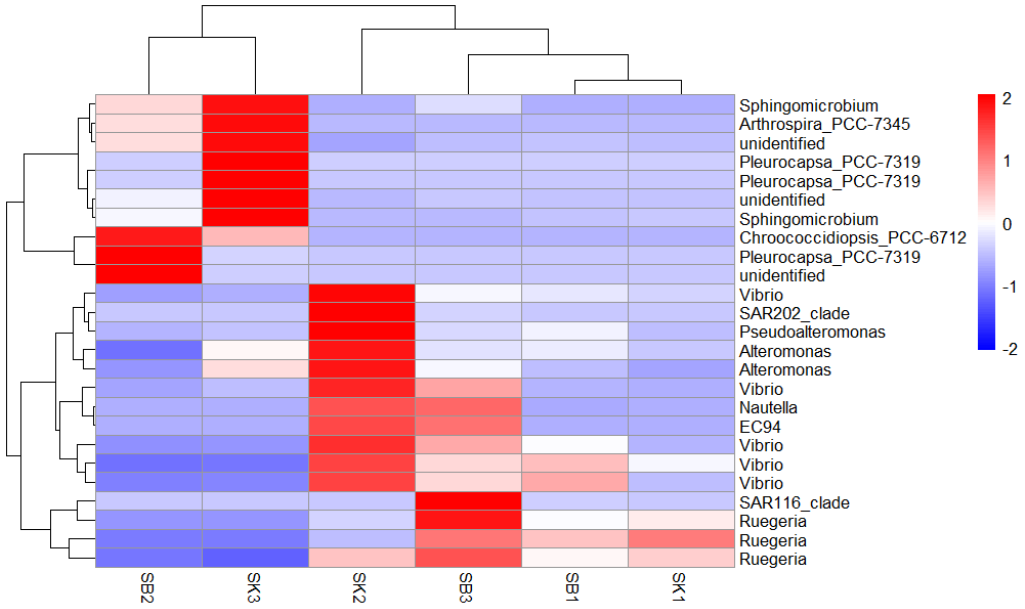

Figure 5. Biofilm assigned OTU read abundance and site/depth cluster analysis. Site codes: SB= Cement concrete, SK = Wood.

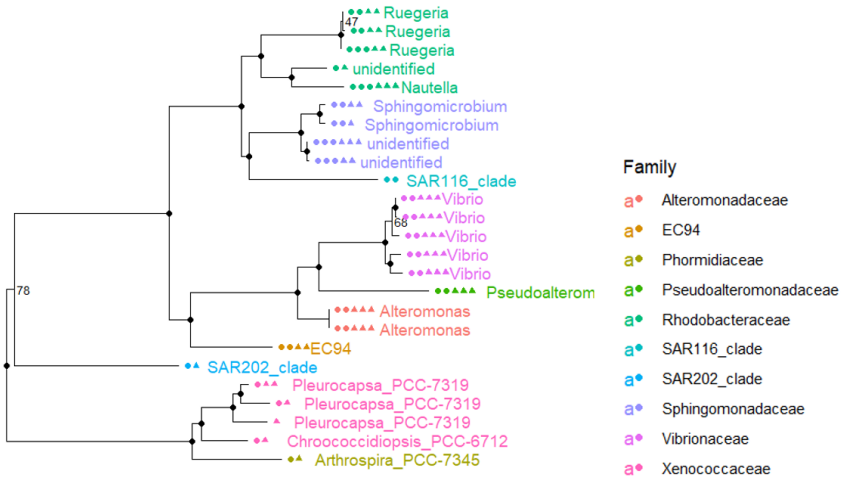

Figure 6. Neighbor-joining phylogenetic tree based on OTU sequences produced through amplicon eDNA metabarcoding from 2 sites in Tanjung Mas Port (Site_Name: ● SB (Cement concrete), ▲ SK (Wood)).

3.3. Functional prediction.

Functional predictions indicated 16 common and essential metabolic pathways in both materials, as shown in Figure 7. The dominance of metabolic pathways for aerobic respiration (PWY-3781) and pyruvate fermentation (PWY-7663; PWY-7111) indicated a stable gas-exchange mechanism in biofilms. Other identified metabolic pathways include organic degradation, such as fatty acid biosynthesis (FASYN-ELONG-PWY; PWY-7094) and other metabolisms (PWY-5101; VALSYN-PWY; PWY-5973; PWYG-321; BCAAS-PWY) that support biofilm growth

and resilience. Formation of marine biofilm is strongly influenced by multiple metabolic pathways, particularly involving pyruvate fermentation and anaerobic fermentation, that have a significant role in biofilm formation [35,36]. Marine biofilms typically form under anaerobic conditions, relying on pyruvate metabolism, fatty acid biosynthesis, and other essential metabolic processes to develop and maintain their structure [37].

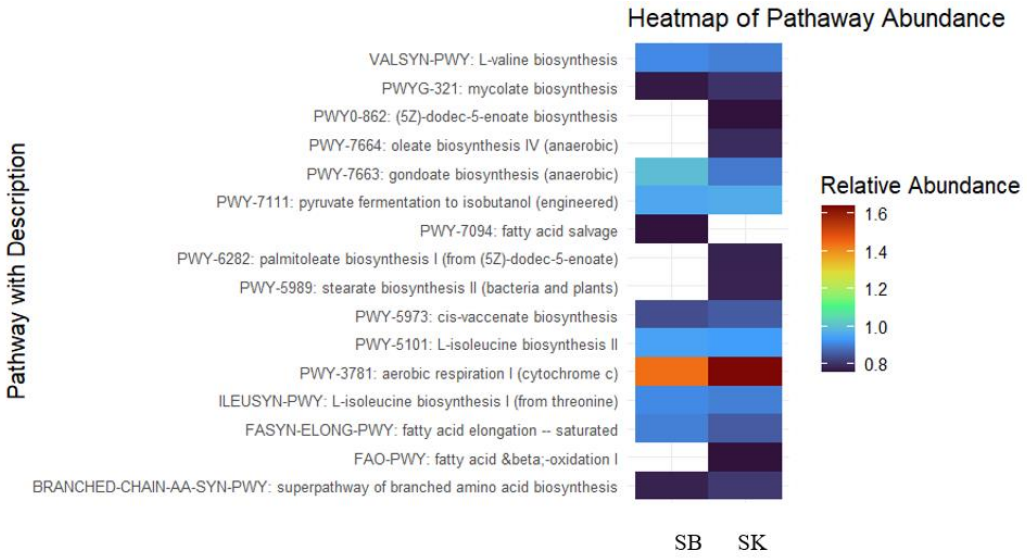

Figure 7. Biofilm assigned OTU read metabolic pathway abundance and site/depth cluster analysis. Site codes: SB = Cement concrete, SK =Wood.

3.4. Community identification and explanation.

Identification of bacterial composition on cement concrete and wood surfaces using metabarcoding provides a broad overview of the dominant bacterial taxa inhabiting these materials. The cement concrete surface was dominated by genus *Erythrobacter*, while on the wood surface, it was dominated by *Photobacterium*. A greater variety of genera was found on cement concrete surfaces compared to wood. The results obtained are in accordance with previous research, which found some microorganisms in biofilms on permanently or intermittently immersed concrete [38].

The genera *Vibrio* and *Ruegeria*, identified as dominant in the phylum Proteobacteria, are potential key initiators of biofilm attachment on both surfaces. Through the diguanylate cyclase (DGC) and tdaC gene that synthesize cyclic-di-GMP (c-di-GMP) and the antibiotic tropodithietic acid (TDA), *Ruegeria* can initiate biofilm formation [39-41]. The genus *Vibrio* was identified as more abundant on wood surfaces than on cement concrete due to its ability to degrade organic matter aerobically and anaerobically [42]. The genus *Vibrio* produces extracellular enzymes (EE) such as lignin peroxidase (LiP), catalase-peroxidase (KatG), and decolorizing peroxidase (DyP). The genus *Vibrio* can degrade wood through the mechanism of lignin depolymerization and oxidation [43]. Lignocellulose can be broken down into carbon dioxide and water under aerobic conditions, and into carbon dioxide, methane, and water under anaerobic conditions [44, 45]. The phylum Cyanobacteria includes the genus *Pleurocapsa*, which has phototrophic abilities. The genus *Pleurocapsa* can bind carbon and produce oxygen through photosynthesis, thereby creating favorable conditions for aerobic and heterotrophic microbial species in biofilms [46]. The dominance of Proteobacteria in both materials, as shown in Table 3, may be due to their greater flexibility in adaptation and metabolism [47]. Proteobacteria can thrive in marine environments with varying nutrients [48, 49].

Differences in the surface characteristics of cement concrete and wood are due to their composition, surface properties, and environmental interactions. In terms of hardness and durability, cement concrete has a stronger surface compared to wood [50]. Cement concrete is also easier to clean than wood [51, 52]. Although the two differ in characteristics, the results showed no differences in the biofilm bacterial community, suggesting that similar treatments may be needed to prevent biofouling.

Mitigation of biofilm formation has been achieved through various methods, such as chemical biocide treatments with Tributyltin (TBT), which has been banned, physical cleaning by friction, or biological synthesis on surfaces resembling fish skin or aquatic plants [53]. The development of antifouling in recent years has shown progress by categorizing it as antifouling with low toxicity or no toxicity [54, 55]. In the future, antifouling needs to move towards environmentally friendly and sustainable development. One strategy is to develop natural inhibitors to prevent biofilm formation by Vibrio, Ruegeria, and Pleurocapsa, providing an environmentally friendly and sustainable antifouling approach. The genera *Vibrio*, *Ruegeria*, and *Pleurocapsa* are Gram-negative bacteria that have similar inhibitory potential. Potential natural inhibitor agents must at least be able to produce secondary metabolite compounds such as phenolics, quinones, flavonoids, alkaloids, terpenoids, and polyacetylenes [56-58]. The research provides the potential to develop antifouling measures against biofilm formation on cement concrete and wood, based on the specific and dominant taxa identified.

4. Conclusions

Biofilm communities on both materials showed the presence of several genera. A greater variety of genera was found on cement concrete surfaces compared to wood. The results of the relative abundance analysis showed that the cement concrete surface was dominated by the genus Erythrobacter, while the wood surface was dominated by *Photobacterium*.

Author Contributions

Conceptualization, H.P.K.; methodology, N.S.A. and H.P.K.; formal analysis, N.S.A., B.C.W., and E.P.; resources, H.P.K., T.H., and M.Z.; writing—original draft preparation, H.P.K. and N.S.A.; writing—review and editing, H.P.K., T.H., N.S.A., B.C.W., and E.P.; funding acquisition, H.P.K. and M.Z. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data of this study are available from the corresponding author upon reasonable request.

Funding

This research was funded by Diponegoro University for its support in providing grant No. 609-81/UN7.D2/PP/VII/2024, which was conveyed with gratitude.

Acknowledgments

The authors are grateful to the UPT Integrated Laboratory of Diponegoro University for DNA extraction and sequencing analysis, and the Biotechnology Study Program Laboratory for e-DNA analysis.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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