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Microbial diversity of biofilm in spent nuclear fuel storage pond

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ABSTRACT

Spent nuclear fuel (SNF) storage is a facility requiring careful attention to prevent corrosion, with microbial-induced corrosion (MIC) being a significant contributing factor. While previous investigations have genetically identified total microbes and sulfate-reducing bacteria (SRB) in SNF storage ponds, similar studies have highlighted the dominance of Proteobacteria and Firmicutes in these environments. Therefore, this study aimed to identify the diversity of bacteria biofilm in SNF storage ponds through a metagenomic approach, with a specific focus on those potentially causing MIC. The results showed that the rack had the highest number of taxa based on taxonomic identification. The bacteria community on the rack, at the phylum level, was dominated by Proteobacteria (34.04%), Firmicutes (24.96%), and Chloroflexi (20.52%). Chloroflexi constituted the majority of the bacteria community on the pool floor (91.09%), while on the pool walls, Chloroflexi (45.17%) and Proteobacteria (44.02%) dominated almost equally. Metabolic pathway analysis further confirmed the activity of MIC in biofilm by the presence of MIC-related pathways. These findings contribute novel insights into the microbial composition of biofilms in SNF storage ponds, providing a foundation for future studies on the prevention and management of MIC in such facilities.

1. INTRODUCTION

A spent nuclear fuel (SNF) storage pond is an extreme environment for the life of microorganisms due to the high levels of radiation and radioactive substances in the form of fission products [1,2]. Bacteria communities in spent fuel ponds with the potential to form biofilm pose a risk of increasing microbial-induced corrosion (MIC) in the pool liner material and increasing the risk of fission product leakage [3,4]. This can result in environmental pollution and radiation exposure for workers and the surrounding community.

Several studies have identified the presence of microbial communities in nuclear waste storage facilities. For instance, research in French and Belgian facilities detected Actinobacteria, Bacteroidetes, and Proteobacteria as dominant groups [5,6]. Another study in Slovakia identified microorganisms capable of accumulating radioactive isotopes [7].

Rahayu *et al.* [8] initiated a similar study in Indonesia examining the presence of bacteria in the interim storage for spent fuel (ISSF) pond. This study revealed the presence of potentially corrosive acid-producing microorganisms. Further research by Rahayu *et al.* [8] at the same facility was conducted to detect and quantify the amount of sulfate-reducing bacteria (SRB) in pond biofilm. However, these studies provided limited information on taxonomic identification.

Therefore, this study aimed to use a metagenomic approach in determining microbial diversity and MIC potential in spent fuel storage ponds. By integrating findings from similar studies and expanding upon them with detailed metagenomic analysis, this research offers novel insights into the microbial dynamics within nuclear fuel storage facilities and their implications for MIC.

2. MATERIALS AND METHODS

The study was carried out at the ISSF, Directorate of Management of Nuclear Facility, National Research and Innovation Agency (BRIN), BJ Habibie Science and Technology Serpong Area.

2.1. Sample Preparation

Biofilm sample preparation was achieved using a stick designed as a swab test device. The swab was taken from three locations, namely on

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the pool walls, floor, and SNF racks (Fig. 1), with each measurement conducted twice for replication. The swab test results were placed into a closed plastic vial in a cool box for 1 hour.

2.2. Environmental DNA Extraction and Sequencing

DNA of the water and swab sample was isolated using the CTAB/SDS method. Furthermore, the isolates were amplified in the 16s rRNA gene, particularly in the V3-V4 hypervariable region. Polymerase chain reaction (PCR) reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Sequencing libraries were generated using NEBNext® UltraTM DNA Library Pre Kit for Illumina following the manufacturer's recommendation, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Aligned Bioanalyzer 2100 system. Finally, the library was sequenced using the Illumina platform, and 250 bp paired-end reads were generated.

2.3. Data Analysis

Microbiome DNA sequencing data obtained from Illumina (FASTQ files) was analyzed with Ubuntu-based tools called QIIME2 ver. 2021.11 [9]. QIIME2 is an open-source software platform for the analysis of microbiome DNA sequencing data. Through this program, raw sequences were imported and trimmed to remove non-biological sequences. Trimmed sequences were then denoised [10], and assigned to the taxonomic category by referring to SILVA (ver. 138) database [11]. From the assigned sequences, alpha diversity data (Shannon-Wiener index (H'), Simpson's diversity (D), Margalef index (SR), and Chao1) were obtained.

QIIME2 output is further passed to RStudio to be visualized into a bar plot and Venn diagram; and to the PICRUSt2 program. PICRUSt2 is a bioinformatic tool that uses marker gene sequences alone to predict metabolic pathways. Subsequently, the output from PICRUSt2 was visualized using RStudio into heatmaps. QIIME2 and PICRUSt2 are often used together to gain a more comprehensive understanding of the microbiome.

3. RESULTS AND DISCUSSION

The analysis with QIIME2 showed that the rack, in general, had the highest alpha diversity index compared to the other two samples (Table 1). Diversity was measured using the Shannon-Wiener index (H'), Simpson's diversity (D), Margalef index (SR), and Chao1. All of the indices showed a similar pattern, with the highest number occurring in the rack and the lowest on the pool wall. This showed that the rack was high in richness and evenness [12].

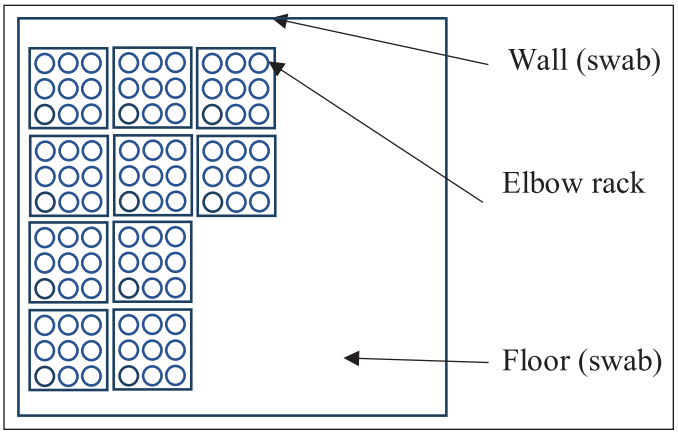

Figure 1. Water and swab biofilm sampling position.

Comparison of diversity between samples was measured through Bray–Curtis dissimilarity and weighted Unifrac, which considered species abundance (Table 2). From the calculation, it was found that the rack had a significant difference compared to the other samples (0.72 and 0.66). However, when phylogenetics was considered, the wall-rack difference (0.27) was less significant compared to diversity (0.3). Beta diversity analysis further showed that the rack had significant diversity among two other samples.

The relative abundance of bacteria on the racks, floor, and walls of the pool is shown in Figure 2. Taxa with abundance values >0.2% (phylum) and >0.09% (genus) are shown in the graph, along with those grouped under "other". The results of taxonomic identification confirmed that the rack had the largest number of taxa at the phylum, family, and genus levels. Meanwhile, the smallest number was found on the floor. Among the total 26 phyla identified, 10 had the highest relative abundance in each community (Fig. 2A). The bacteria community on the rack, at the phylum level, was dominated by Proteobacteria (34.04%), followed by Firmicutes (24.96%), and Chloroflexi (20.52%). On the other hand, Chloroflexi made up most of the bacteria community on the pool floor (91.09%). On the pool walls, Chloroflexi (45.17%) and Proteobacteria (44.02%) dominated almost equally. These groups of bacteria accelerated the corrosion process indirectly through electron transfer and biofilm formation [13]. In forming biofilm, Proteobacteria function as initial colonizers on the metal surfaces, followed by other bacteria [14,15]. Over time, oxygen consumption and acid production by *Clostridia* (phylum: Firmicutes) form stratification of microenvironments [16,17].

In the lower level of taxa (Fig. 2B), the community on the rack had the highest evenness in abundance, characterized by the highest component in the form of "other species" (40.6%). Meanwhile, on the pond floor, the dominance of the phylum Chloroflexi was reflected in the high abundance of *Ktedonobacter* sp. which constituted more than half of the community (61.6%). *Ktedonobacter* sp. was also the dominant bacteria on the pool walls (33.8%), followed by *Acidibacter* sp. (21.6%) from the phylum Firmicutes. The presence of acidophilic

Table 1. Alpha diversity indices of rack, floor, and wall pool.

Sample	Shannon-Wiener	Simpson	Chao1	Margalef
Rack	6.16	0.95	811.88	72.39
Floor	3.79	0.87	333.76	28.06
Wall	4.64	0.91	451.59	38.81

Table 2. Bray–Curtis and weighted unifrac dissimilarity matrix of rack, floor, and wall pool.

Sample	Rack	Floor	Wall
Bray–Curtis			
Rack	0		
Floor	0.72	0	
Wall	0.66	0.51	0
Unweighted unifrac			
Rack	0		
Floor	0.46	0	
Wall	0.27	0.30	0

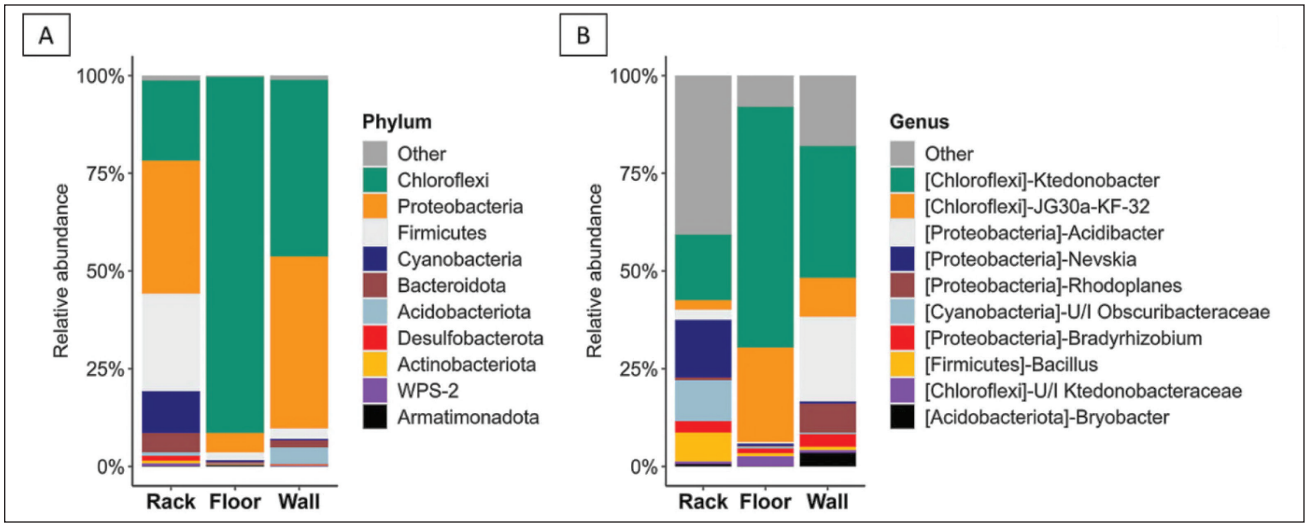

Figure 2. Biofilm microbial composition at the phylum (A) and genus (B) level.

bacteria in pool water with a neutral pH (~6) confirmed the existence of a microenvironment in biofilm. *Acidibacter* can reduce iron and corrode Fe ions from metal surfaces, potentially inhabiting the middle to deep layers of biofilm, while the top layers are inhabited by phototrophic microorganisms such as *Rhodoplanes* [18–21].

Desulfobacterota had very low abundance in all three samples (0.16%–1.23%) despite being in the top 10. SRB such as *Desulvubrio* are capable of initiating corrosion in the deepest anaerobic layer of biofilm [22,23]. One example of *Desulvubrio, Desulfovibrio ferrophilus* able to thrive in exclusive populations and directly metabolizes metallic iron of pond material [24].

The findings of bacterial diversity from several comparable research in different sites are broadly similar to the result of this study. Research at the SNF Cofrentes Nuclear Power storage ponds (Spain) successfully identified bacterial biofilms as Proteobacteria, Firmicutes, and Actinobacteria that were resistant to radionuclide exposure [25]. Gammaproteobacterium was also found to be dominating the BR2 reactor pool in Belgium [26]. Bacterial biofilms that thrive under low nutrient and chronic radioactivity environments were also identified in Research Centre Facilities in Kalpakkam, India [2]. On the contrary, a wider study discovered two groups of fungi as the main contaminant of nuclear fuel storage ponds in Brazil [27]. Another study in Magnox storage ponds, UK, successfully identified a single cyanobacterial genus as the dominant contaminant [28].

Venn diagram directly compared the taxonomic diversity of each sample in Figure 3. Among the 340 genera detected, 113 were similar across the three locations, as the sample from the wall had the fewest unique taxa, while those from the rack had the highest, with up to 106 unique genera (62%). The Venn diagram further confirmed that the bacterial community of the rack had the highest diversity compared to other locations. It comprised distinct genera with similarly low abundance (~2%). The high level of both richness and evenness confirmed the diversity indices measurement. Several factors could cause the differences in the diversity of bacteria communities from the three samples. The high variety of bacteria taxa on the rack may be caused by the immense exposure to radioactivity (560.40 mSv/hour). This leads to the growth prevention of a single species, resulting in the appearance of various species with limited abundance.

Another factor that can affect the biofilm community is the attachment position and material. The horizontal surface of the floor may cause sedimentation of planktonic (non-motile) bacteria [29,30]. Additionally, stainless steel, as a negatively charged (hydrophilic) material, also tends to be infested with non-motile bacteria including *Ktedonobacter* sp. [29,31,32]. Despite taking the rack samples from a horizontal surface, no dominance of any species was observed. The total number of bacteria on the rack was significantly lower than on the walls, which was taken from vertical surfaces. This indicates that planktonic sedimentation did not occur. The disparity may be attributed to the rough surface of the rack, which reduced bacteria attachment and growth on stainless steel [33].

Biofilm level of development can also cause differences between the community structure of the sample. As biomass increased, the old mature biofilm became homogenous, while the younger biofilm still had a diverse range of species [34]. The domination of *Ktedonobacter* in the old biofilm communities was attributed to the ability to thrive in a wide range of oxygen levels [29,35].

The presence of other microorganisms can also influence the structure of the biofilm community. The previous study by Sugoro *et al.* [36] identified the presence of microalgae in pool water at the same facility with corrosive and radioactive resistance potential. Green algae were also found to survive high iron toxicity due to the high phosphate concentration [37].

The top eight predicted pathways are depicted in the heatmap in the log10 scale (Fig. 4). The predicted common metabolism pathway ranged from log10 = 4.9 until 5.3. The pathways related to respiration, biosynthesis, and fermentation were the most dominant (Fig. 4A). The study identified MIC-related pathways with roughly even distribution in all sites. The top MIC-related pathway was assimilative sulfate reduction (Fig. 4B). Aside from being the most common for many bacteria to obtain sulfur, this process also produces intermediate compounds used as substrates for SRB [38].

The abundance of the pathway did not differ significantly between the rack and wall samples. However, on the floor, it was observed that anaerobic hydrogen oxidation and assimilative nitrate reduction were lower compared to the other two samples. The nitrate-reduction pathway indirectly contributes to MIC by oxidizing iron spontaneously

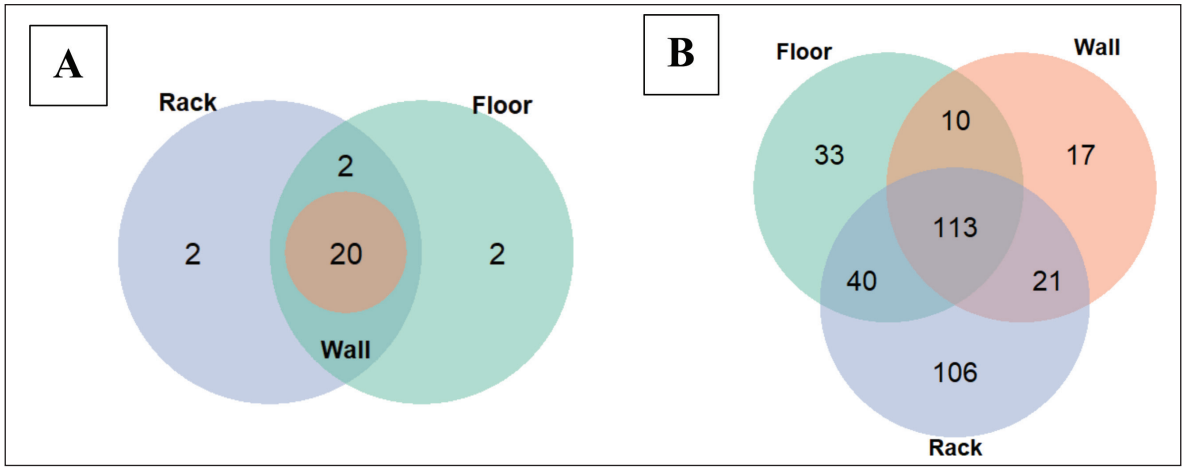

Figure 3. Depiction of unique and common OTUs among samples from different sites at the phylum (A) and genus (B) levels.

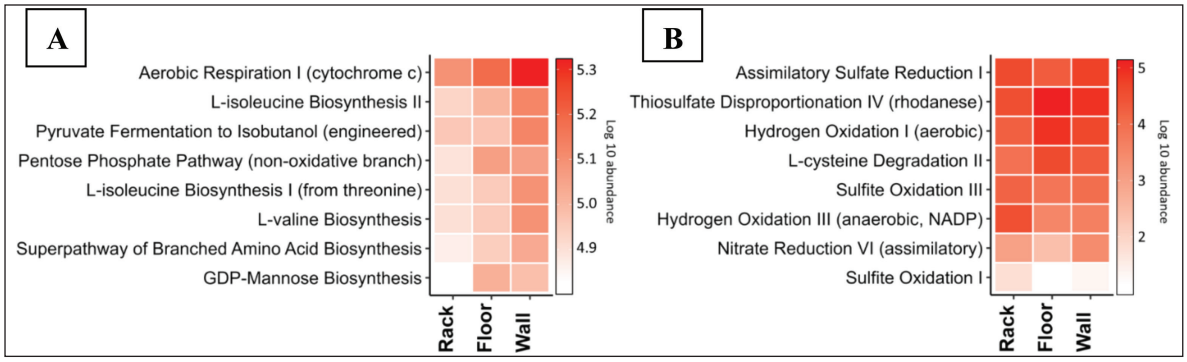

Figure 4. The abundance of predicted metabolic pathways of various bacteria from the three samples at the general scope (A) and associated MIC (B).

[39]. The low anaerobic oxidation of hydrogen can be explained by the low abundance of SRB, such as *Desulfovibrio* sp. These bacteria use hydrogen oxidation to acquire electrons in the absence of oxygen [40].

The floor sample demonstrated a significantly higher level of aerobic hydrogen oxidation, possibly due to Chloroflexi being the hydrogenase-producing bacteria [41,42]. The presence of genes encoding this enzyme is directly proportional to the corrosion rate [43,44].

Among the three samples, dissimilative sulfate reduction I, the metabolism SRB used in respiration, was not detected. However, some parts of this pathway (sulfite oxidation I and III) were found in different abundances. Another study found that hydrogen sulfide (H_2S) in certain concentrations can increase pitting corrosion on stainless steel [45]. This compound is regularly produced by bacteria through the breaking down of cysteine [46]. In this study, the cysteine degradation pathway was found to be evenly distributed among the samples.

Bacterial corrosion is a complex, non-linear process that involves a community of microorganisms in biofilm. Generally, bacteria in the biofilm exchange electrons with the metal surface, generating an electric current that can accelerate the corrosion process. Besides that, acids and free radicals as metabolic byproducts also pose a threat to corrosion [2]. SRB considered as main contributor to corrosion due to their ability to produce H_2S. This compound reacts with Fe ions on pool material to form insoluble iron sulfide (FeS). In addition, SRB also cause corrosion directly by taking electrons from metal surfaces [47].

These data provide evidence that in the SNF pool, there is a potential for MIC development either in the rack, floor, or wall. Therefore, prevention strategies are needed to avoid metal degradation in the facility before biofilm matures. After maturation, microorganisms detach and move to join other biofilm communities [48]. Mature biofilm has a high level of cell adhesion and cohesion due to the presence of the EPS matrix. Therefore, once biofilm forms, killing or mechanically removing it from the surface is challenging.

Besides mechanical removal, chemical cleaning can be used to efficiently mitigate bacterial corrosion. Organic biocides in place of biocides are used to prevent bacterial growth [49], and quorum sensing inhibitors (QSIs) are also used to interfere with cellular communication between microorganisms in biofilms [50].

Further studies are needed to determine the actual metabolic activity of microorganisms. The metabolic pathway prediction as an *in-silico* method is limited to a relational database, which might be out of date. In addition, the corrosion rate needed for each site should be measured to observe the correlation between the presence of microbes and the occurrence of MIC.

4. CONCLUSION

This study employed a metagenomic approach to explore the microbial diversity of biofilms in a SNF storage pond, focusing on microorganisms associated with MIC. The results revealed significant bacterial diversity on the rack surface, with Proteobacteria, Firmicutes, and Chloroflexi being the most abundant, while Chloroflexi dominated the microbial communities on the pool floor and walls. These findings indicate that different surfaces within the SNF storage pond harbor distinct microbial communities influenced by factors such as radiation exposure, surface material, and environmental conditions. The presence of MIC-related pathways across all biofilm samples suggests a potential risk for MIC throughout the storage facility, underscoring the importance of monitoring and managing microbial communities to prevent corrosion and ensure the long-term integrity of these critical infrastructures. Understanding the microbial composition and activity within these biofilms is crucial for developing effective strategies to mitigate corrosion. Future research should focus on the actual metabolic activities of these microorganisms and measure the corrosion rates associated with different biofilm communities, involving in-depth studies of metabolic pathways and gene expressions. Additionally, exploring advanced chemical and mechanical cleaning methods, as well as the use of QSIs could provide robust solutions to prevent and manage biofilm formation and MIC in these critical environments. The findings contribute to the broader scientific understanding of microbial dynamics in extreme environments and their impact on industrial materials, providing a foundation for future studies aimed at mitigating MIC in nuclear storage facilities. By integrating metagenomic techniques with traditional microbiological methods, we can enhance our ability to monitor and control microbial populations, protecting nuclear storage infrastructure from microbial degradation and ensuring environmental safety and the longevity of these facilities.

5. ACKNOWLEDGMENTS

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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