BIODIVERSITAS
Volume 24, Number 10, October 2023
Pages: 5391-5398

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241021

Amplicon-based sequencing revealed potential microbiologically influenced corrosion in the interim storage for spent fuel of RSG-GAS, Serpong, Indonesia

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Manuscript received: 13 June 2023. Revision accepted: 18 October 2023.

Abstract. *Sugoro I, Fadila DSR, Pikoli MR, Hermanto FE, Ramadhan F, Shalsabilla SE, Cici A, Rijal MS, Rahayu DS, Puspito MJ, Haribowo DR, Tetriana D. 2023. Amplicon-based sequencing revealed potential microbiologically influenced corrosion in the interim storage for spent fuel of RSG-GAS, Serpong, Indonesia. Biodiversitas 24: 5391-5398.* Interim storage of spent fuel (ISSF) corrosion can negatively impact nuclear power plant maintenance, specifically concerning safety, financial costs, and regulatory compliance. Besides chemical reactions, microbiologically influenced corrosion (MIC) also primarily occurred. Sulfate-reducing bacteria (SRB) plays a significant role in the development of MIC. The quantitative of MIC research has never been conducted in the ISSF - Multipurpose nuclear reactor of G.A. Siwabessy (RSG-GAS). So, this study aims to monitor the potential MIC development in the ISSF of RSG-GAS pool and canal through bacterial diversity analysis using total plate count (TPC) and metagenomic approach. TPC results showed that SRB was identified in the canal of ISSF but not in the pool site. However, metagenomics analysis revealed higher diversity measures in the pool than in the canal sites. *Staphylococcus* sp. was the most abundant species in both sites, with a relative abundance greater than 85%. The number of SRB and MIC-related bacteria was higher in the canal than in the pool, both from TPC and metagenomic analysis. Several SRB taxa were identified, such as *Desulfobulbus mediterraneus*, *Desulfobulbus* sp., *Desulfofustis* sp., *Desulfovibrio* sp., and *Truepera* sp. The potential MIC development also strengthens by diversity-based metabolic pathway prediction, which mainly displayed sulfur oxidation and sulfate reduction pathways in the canal system.

Keywords: Bacterial diversity, biocorrosion, ISSF, MIC, SRB

INTRODUCTION

The spent nuclear fuel (SNF) will be stored in the interim storage of spent fuel (ISSF) upon being used in a reactor or power plant. The ISSF mainly builds as a water basin (Ojovan and Lee 2014). Wet storage lets the fuel securely disperse heat while allowing dangerous radionuclides to decay naturally (Bagwell et al. 2018). The ISSF facility for Multipurpose Reactor-G. A. Siwabessy (RSG-GAS) was built as pool and canal (Sugoro et al. 2022). Long-term storing of SNF from nuclear research reactors in pool and canal creates the potential for corrosion (Karley et al. 2022). These conditions may be caused by several microbes that highly adapt to oligotrophic environment at ISSF (Karley et al. 2022). This microbes-associated corrosion is known as microbiologically influenced corrosion (MIC) (Diler et al. 2023), highly adaptive microbes, involve in MIC progression mainly classified as sulfate-reducing bacteria (SRB) (Lavanya 2022). The growth inhibition of SRB is essential step as a part of maintenance of the ISSF facility.

Spent fuel has fission products bordered with stainless steel coatings. The quality of the water in the spent fuel storage pond, according to the International Atomic Energy Agency (IAEA) in 2011, has a pH value of 6-8, a conductivity value of <10 µS/cm, a TDS value of <1350 mg/L, a temperature of <45°C, and has a maximum bacterial density of 1000 CFU/mL. Water in ISSF pools must be free from microorganisms such as microalgae and bacteria that live in a radioactive environment. Moreover, microalgae can cause corrosion to stainless steel, an ISSF component. Microalgae at ISSF facility of RSG-GAS are dominated by the genus *Chlorella* sp. (Chlorophyceae). Bacteria associated with algae can induce corrosion-affected MIC (Sugoro et al. 2022), and bacteria that has an essential role in corrosion is SRB. SRB is a type of bacteria that can live in an environment with little or no oxygen; this bacterium can convert sulfates into sulfites so they can

corrode (Wahyu 2015). Corrosion to material degradation in the environment due to the presence of a biofilm layer attached to the metal surface, forming a diameter on the surface and exposed on the surface so that it can cause local corrosion (Rahayu and Puspito 2017).

Research related to MIC has previously been carried out, e.g., at ISSF in the Savannah River Site (SRS) from 2001 to 2020, a series of monitoring phases have been undertaken. These studies revealed that the populations of MIC-related bacteria such as oligotrophic bacteria and SRB were detected to increase during that year with an average abundance of 2.90 and 0.19 CFU/mL, respectively (Veytskin et al. 2023). The research was also conducted by Smart (2014) on Swedish nuclear fuels. The study shows that anaerobic bacteria such as *Desulfovibrio ferrophilus* are capable of living and are found to be the most dominant and able to metabolize iron or metal directly. Investigations on the microbial population inside a nuclear facility have been performed worldwide to mitigate MIC development (Balamurugan et al. 2011; Smart et al. 2014; Bagwell et al. 2018; Petit et al. 2020). This monitoring was investigated in the ISSF of RSG-GAS based on qualitative of SRB test (Rahayu and Puspito 2017) and algae diversity (Sugoro et al. 2022). Quantitative MIC analysis using the total plate count (TPC) method for SRB total at ISSF-RSG GAS must be conducted and supported by metagenomic research using Next-Generation Sequencing (NGS) to determine bacterial abundance. NGS methods make it possible to identify potential taxa that can initiate or accelerate MICs directly from environmental samples.

MATERIALS AND METHODS

Water sampling at Interim Storage of Spent Fuel (ISSF)

The ISSF-RSG GAS facility is in the B.J. Habibie Science and Technology Center, National Research and Innovation Agency (BRIN), Serpong, Indonesia. Sampling was conducted in August 2022 at ISSF in pool and canal at 14 points here, points 1-9 are located in the pool area, and points 10-14 are in the canal area (Figure 1). The sample point was recommended by BRIN because there was a biofilm in the water of pool and canal. Furthermore, water samples were taken to a depth of ±6 m using a horizontal water sampler.

Chemical-physical analysis of ISSF water

Light intensity was measured using a digital lux meter LX 1010B, while the pH, temperature, total dissolved solids (TDS), and electrical conductivity (EC) were estimated with a multiparameter instrument, Hanna Instruments 9811-5 (Hanna Instruments Inc. Woonsocket, RI, USA). Subsequently, dissolved oxygen (DO) was evaluated with Hanna DO-5510, and water radioactivity using an STHF-R Water Proof High Dose Rate Probe by inserting the probe into the water with guidance from the IAEA (IAEA 2011) in "Good Practices for Water Quality Management".

Total colony determination

The pour plate method performed bacterial analysis and SRB, where a sample of 100 µL was inoculated into a sterile Petri dish. About 20 mL of NA and Postgate B media were poured into a saucer filled with samples, and homogenization occurred by turning the cup to form the number 8. Furthermore, the NA and Postgate media was stored in the incubator and anaerobic jar. Anaerobic conditions are obtained by flowing CO_2 into the jar for 5 min. Bacterial colonies were counted after two days, and SRB colonies are counted after 14 days of incubation.

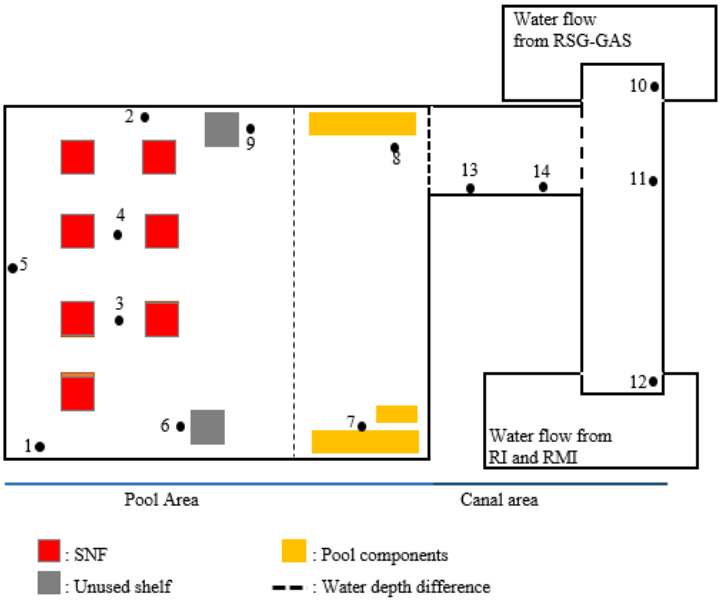

Figure 1. Sampling point map from the ISSF of RSG-GAS, Serpong, Indonesia. The sample site no. 1-9 represented the pool, while the sampling site no. 10-14 were the canal

Environmental DNA extraction and sequencing

One liter water sample was filtered with filter paper (Whatman 7141-104). The obtained filtrate was then isolated by ZymoBIOMICS DNA Miniprep Kit (Zymo Research). DNA quality was assessed by Nanodrop, Qubit Fluorometer, and targeted 16S rRNA gene amplification. A qualified DNA sample was then sequenced by NovaSeq Illumina with a gen 16S region V3-V4 target as a taxonomic barcode (Hermanto et al. 2021).

Sequence analysis, diversity measurement and functional pathway prediction

Sequence and diversity analysis were performed by QIIME2 2021.4 pipeline (Bolyen et al. 2019). Briefly, raw sequences were imported and trimmed by q2-cutadapt to remove non-biological sequences (Martin 2011). Trimmed sequences were then denoised using DADA2 (Callahan et al. 2016), followed by taxonomic assignment using q2-classifier with classify-Sklearn option (Bokulich et al. 2018) referring to SILVA version 138 as taxonomic database (Glöckner et al. 2017). Assigned sequences were then directed into alpha diversity analysis using Shannon Wiener, Simpson and Chao1 index. Subsequently, assigned sequences were exported along with the abundance data to obtain the predicted pathway metagenome. The pathway metagenome prediction was performed by PICRUSt2 using a standard parameter setting (Douglas et al. 2020), and the data from the MetaCyc pathway was analyzed to predict the relative abundance of the predicted functional pathway.

RESULTS AND DISCUSSION

Total bacteria and sulfate-reducing bacteria

Total colony enumerations showed that bacteria were found in all the sampling site with sites no. 6 and 10 having the greatest CFU/mL (Table 1). However, the average number of CFU is plausible and still meets IAEA requirements, i.e., below 1000 CFU/mL (IAEA 2011). The high number of bacteria in site no. 6 occurred due to calmer water flow than other sites, while no. 10 is the point of transfer of spent fuel from RSG-GAS to ISSF, allowing the presence of a high density of bacteria. Regarding SRB, most sampling sites have detected the presence of SRB, with sites no. 10 and 13 showing the highest number (50-60 CFU/mL). These biofilm formation in those sampling sites supports this event.

The physicochemical parameters may also influence the growth of SRB in the sampling sites. Although the physicochemical parameters have met the IAEA criteria, those conditions may lead to the development of microalgae. It can also enhance algae growth through complex communication mechanisms and nutrient exchange (Philippot et al. 2013). Dong et al. (2020) found that the algae *Spirulina platensis* was associated with the bacterium *Halomonas titanicae*, inducing corrosion in stainless steel. Microalgae dissolve available organic carbon for bacteria; in return, the bacteria supply the microalgae with carbon dioxide and some minerals (Han et al. 2016; Muchammad et al. 2013). This symbiotic mechanism will accelerate biofilm formation and leads to MIC. The development of MIC in ISSF - RSG GAS has the correlation with the microalgae. Sugoro et al. (2022) found that the water of the ISSF-RGS GAS pool and canal has detected microalgae. This condition can initiate corrosion on pool walls and canals because symbiotic bacteria and microalgae can initiate the biofilm that cause MIC.

Ratiko et al. (2020) stated that a good level of radioactivity value in the pool and canal water was <0.025 mSv/hour. The ISSF canal meets the radioactive level, whereas there are 7 points in the pools below the radioactivity value. Meanwhile, the value can influence the diversity of species residing in ISSF facilities. SRB tends to maintain its diversity by forming spores capable of reducing sulfates. Therefore, it affects the metabolism of crucial defense mechanisms and has thicker cell walls to aid long-term survival in environments that are dominant against pressure, radiation, and heat (Haynes et al. 2018).

Table 1. Assessment of chemical, physical, and biological factors

Point*	Parameters								
	T (°C)	pH	DO (mg/L)	TDS (mg/L)	Conductivity (µS/cm)	Light (lux)	Radioactivity (mSv/hour)	Average of TPC (CFU/mL)	
								Bacteria	SRB
1	25.8	6.9	5.2	0	1.02	8000	108.80	0	0
2	25.8	6.9	5.2	0	1.02	6000	6822.00	500	0
3	25.8	6.9	5.2	0	1.02	4000	7118.00	0	0
4	25.8	6.9	5.2	0	1.02	4000	6488.00	10	0
5	25.8	6.9	5.2	0	1.02	4000	62.18	40	0
6	25.8	6.9	5.2	0	1.02	4000	0.00007	1000	0
7	25.8	6.9	5.2	0	1.02	4000	2.14	500	0
8	25.8	6.9	5.2	0	1.02	4000	0.00001	20	0
9	25.8	6.9	5.2	0	1.02	4000	282.20	100	0
10	25.0	7.18	5.3	0	1.9	4000	0.00001	1000	50
11	25.0	7.18	4.5	0	1.9	22000	0.00001	500	0
12	25.0	7.18	3.9	0	1.9	4000	0.00008	40	0
13	25.9	7.18	4.0	0	1.9	32000	0.00001	700	60
14	25.0	7.18	4.0	0	1.9	40000	0.00001	600	0

Note: points 1-9 are part of the pool, while points 10-14 are part of the canal on the ISSF

The microorganisms can occur due to changing the conditions of chemical physics and causing MIC, which starts from forming Aqueous hydrogen sulfide H_2S(aq). Various sulfur compounds in water, such as sulfates and sulfur, contain organic compounds such as proteins and amino acids. Sulfates can be reduced to sulfides by SRB, and reactions mainly occur in biofilms under anaerobic conditions below the water's surface. Furthermore, SRB relies on organic substances available for nutrients, including biodegradable organic substances present in waste or formed from the fermentation of carbohydrates, proteins, and lipids (Wu et al. 2020).

Bacterial diversity based on metagenomic analysis

Several diversity indices were used to describe the diversity of bacteria using the Shannon, Simpson, and Chao1 Indexes. Shannon and Simpson's values indicate that pool and canal water have moderate species diversity. The Chao1 index shows the abudance of species the community in the pool and canal of the ISSF. All indices described that the pool site had more diverse and even bacteria than the canal site (Table 2). This condition needs future assessment and attention since the degree of corrosion has negatively correlated with microbial diversity (Tang et al. 2019). Thus, the MIC may develop faster at the canal than at the pool site. According to the identified taxa, at the phylum level, the number of observed taxa did not differ between the pool and canal (Figure 2A). Nevertheless, several observed families in those two sites confirmed the diversity indices measurements (Figure 2B). The pool site had more observed phyla and families than the Canal site, along with relatively lower domination of certain taxa (Figures 2C and 2D). Firmicutes was the most abundant phylum with a relative abundance higher than 75%, followed by proteobacteria, Actinobacteria, etc. Desulfobacterota was found in both sites, although the abundance of this phylum is lower than the others (Figure 2C). At the family level, Staphylococcaceae became the most abundant family. As described at the phylum level, SRB-related families have a low abundance and are not visualized in the bar chart (Figure 2D).

Table 2. Diversity index of pools and canals on the ISSF

Sample	Shannon Wiener Index	Simpson Index	Chao1 Index
Pool	2.6813	0.4251	944.9161
Canal	2.2398	0.3554	847.0806

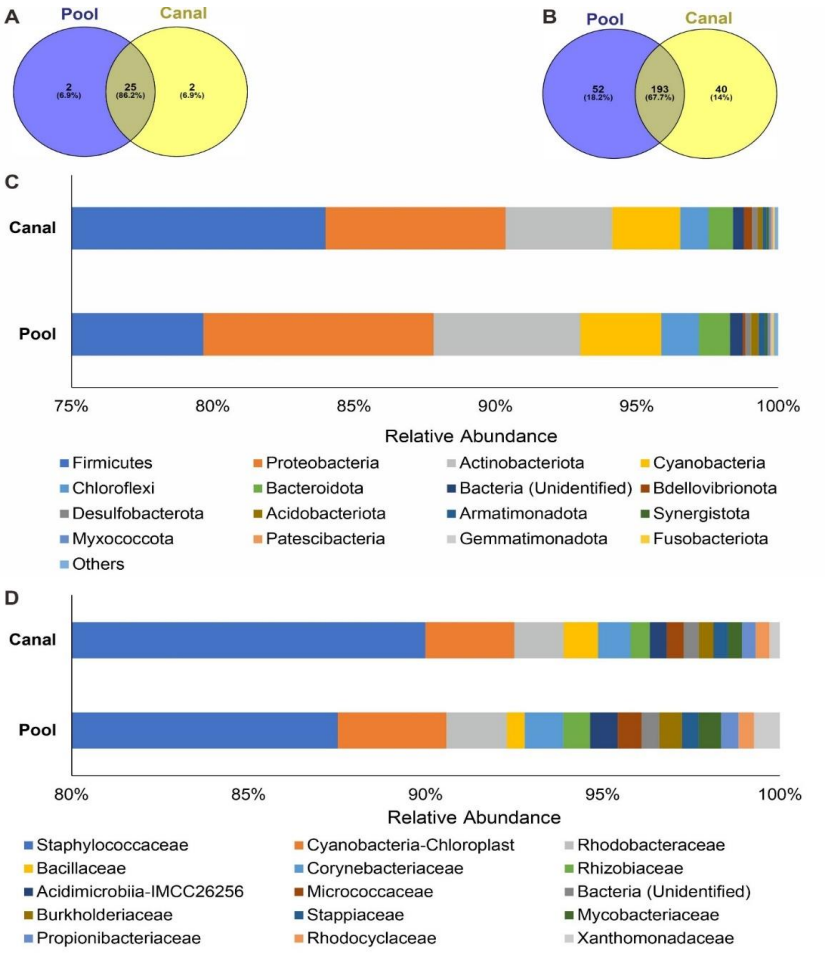

Figure 2. The diversity of microbes in the pool and canal is based on the total observed taxa and relative abundances. Venn diagrams describe the comparison of total observed phylum (A) and family (B) in both sites, while the bar chart represents the relative abundance of observed phyla (C) and the top 15 most abundance families in both sites (D)

The number of observed taxa at the genus-species level also confirmed the diversity indices. The pool site had more observed and unique taxa than the canal (Figure 3B). *Staphylococcus* sp. was the most abundant species in both sites, with a relative abundance greater than 85%. This taxon was previously found in the ISSF from past studies (Chicote et al. 2005; Sarró et al. 2007; Karley et al. 2019) and could form a biofilm even at an oligotrophic environment such as water in ISSF (Karley et al. 2019), suggesting a possible contribution in the development of MIC. Moreover, some microalgae such as Cyanobacteria and *Picochlorum* sp. also identified, supporting an aforementioned assumption about the contribution microalgae in the biofilm formation to induce MIC (Figure 3A). *Picochlorum* sp. is categorized as an adaptive microalgae in various environmental stress conditions. The adaptive ability of this microalgae was gained through horizontal gene transfer from prokaryotes (Foflonker et al. 2018). Furthermore, other species such as *Thermomonas* sp. (Huang et al. 2021), *Bacillus* sp. (Giacobone et al. 2011; Zaidi et al. 2021), *Ruegeria* sp. (Smith et al. 2019), and *Limnobacter* sp. (Wang et al. 2012) were also discovered in this study (Figure 3A) and may associated with the occurrence of MIC in the ISSF as they were considered as SRB and mostly observed in the corrosive samples or environment according to previous studies. The occurrence of those taxa in both sites should be addressed in future mitigation on the prevention of MIC.

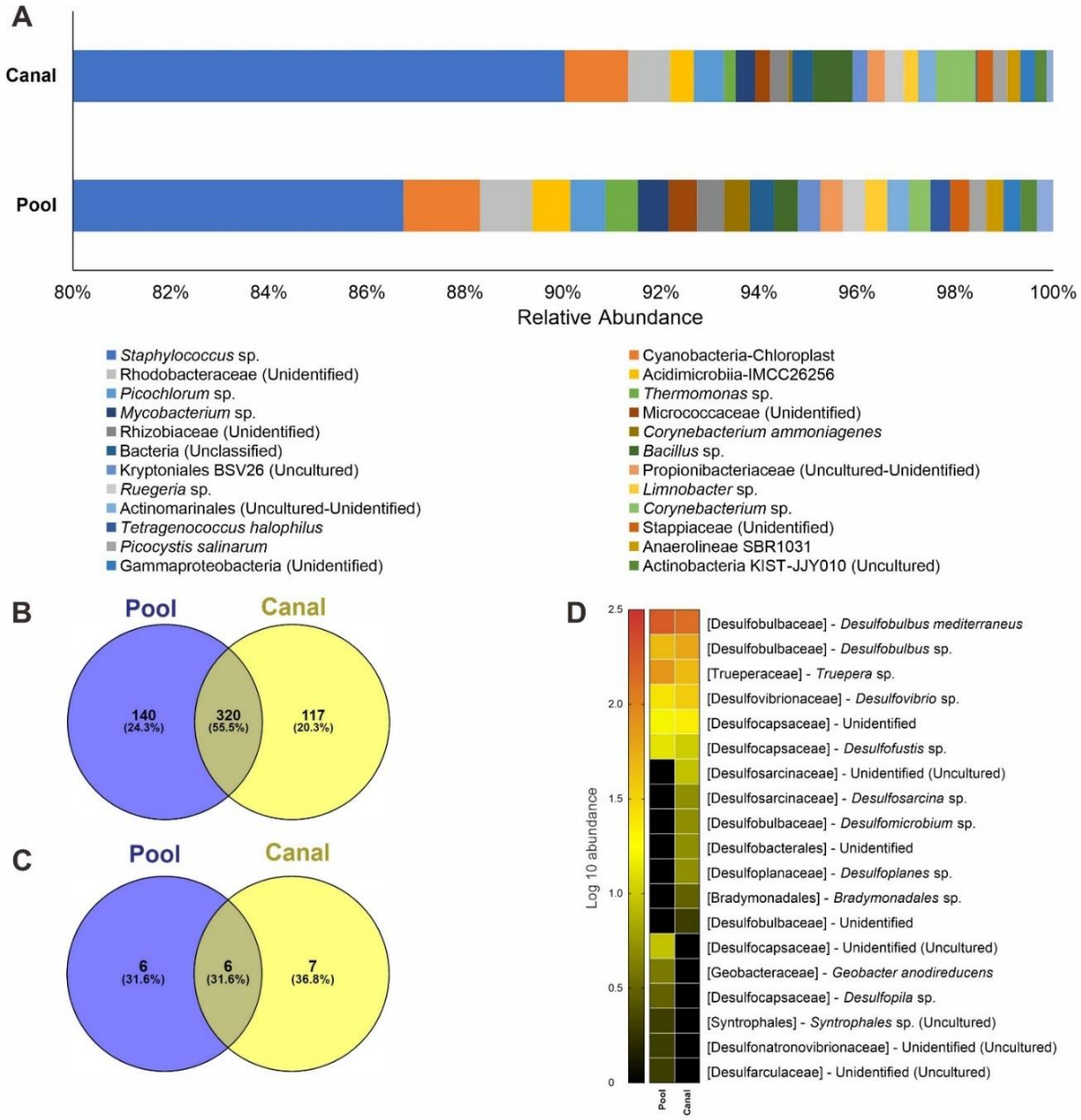

Figure 3. The comparison of diversity assessment of the microbes in the pool and canal at the genus-species level. The top 25 identified genus-species were visualized in the bar chart (A). The Venn diagrams also compared the total observed taxa at the genus-species level (B) and the total observed Deinococcota and Desulfobacterota species (C). Also, the abundance of genus-species from Deinococcota and Desulfobacterota groups was represented in heatmap (D)

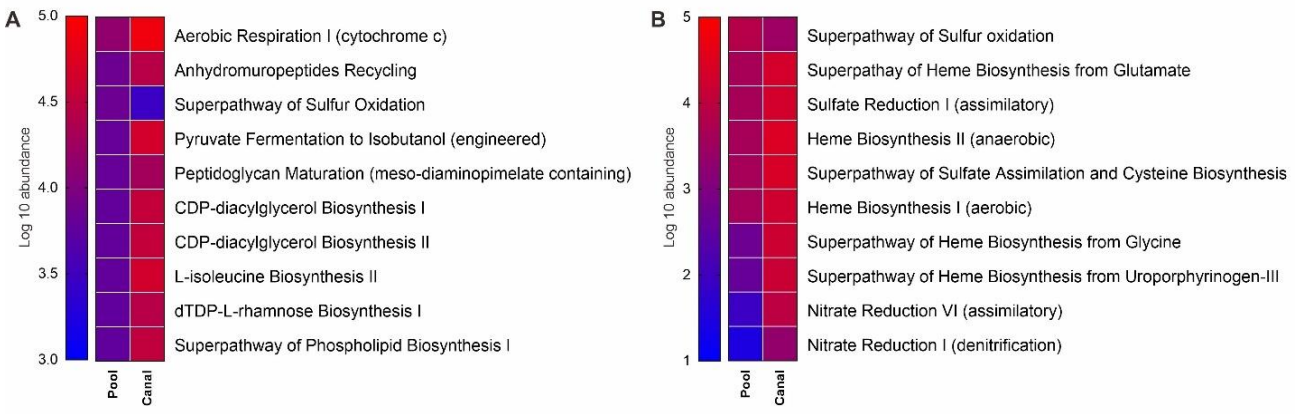

Figure 4. The bacterial diversity data shows the abundance of predicted metabolic pathways in the pool and canal site**s.** Only the top 10 most abundance pathways (A) and corrosion-related pathways (B) were visualized in the heatmap

The most known SRB from the identified taxa comes from Deinococcota and Desulfobacterota (Trivedi et al. 2020). Thus, in depth analysis was also performed according to the diversity of genus- and species-related to those two phyla. The number of identified taxa from Deinococcota and Desulfobacterota was higher in the canal than in the pool (Figure 3C). This confirms the TPC result and the biofilm formation in the Canal sites, particularly sampling sites 10 and 13. Only one genus was observed from Deinococcota, i.e., *Truepera* sp. This taxon is known as the radioactive resistant species (Albuquerque et al. 2005), so the finding in the current experiment is plausible. Other observed SRBs are identified as *Desulfobulbus mediterraneus*, *Desulfobulbus* sp., *Desulfofustis* sp., and *Desulfovibrio* sp., which were observed both in the pool and the canal (Figure 3D). Moreover, a previous study also revealed that *Desulfobulbus* sp., *Desulfofustis* sp., and *Desulfovibrio* sp. were the abundance of sulfate-reducer bacteria in the corrosive oil reservoir (Li et al. 2017). Those taxa's presence should be considered to perform mitigative policy against MIC in the ISSF.

Diversity-based metabolic pathways

Pathways related to respiration and building blocks biosynthesis were the most dominant pathways (top 10 most abundance). Those pathways ranged from respiration, fermentation, phospholipid biosynthesis, peptidoglycan maturation, etc. Interestingly, there was a Superpathway of Sulfur Oxidation in the top 10 most abundance pathways (Figure 4A), indicating the existence of Sulfate Oxidizing Bacteria (SOB). The Superpathway of Sulfur Oxidation constitutes a biochemical route responsible for converting sulfur compounds into sulfate (Campbell et al. 2019). SOB, a specialized bacterial group, harness the capability to oxidize sulfur compounds for energy acquisition and contributes to a corrosion of sewerage and wastewater treatment facilities through the action of Biogenic Sulfuric Acid (BSA) (Gao and Fan 2023). This form of deterioration is facilitated by Sulfur/Sulfide Oxidizing Bacteria (SOB) that mediate the conversion of sulfur compounds into sulfuric acid, thereby instigating the process of BSA corrosion (Gao and Fan 2023; Huber et al. 2016). In addition, together with SRB, SOB plays a role in oxidizing hydrogen sulfide produced by SRB to sulfuric acid that can corrodes the materials (Tian et al. 2017). Moreover, there were also discovered MIC-related pathways in both sites. Sulfur oxidation became the most abundant MIC-related pathway. MIC-related such as heme biosynthesis, Sulfate reduction, Sulfate assimilation, and nitrate reduction were observed in greater abundance at the canal than in the pool (Figure 4B). Heme acts as the cofactor during the Sulfate reduction process, particularly by heme-containing cytochromes (Tripathi et al. 2021). In addition, nitrate reduction also accelerates corrosion through extracellular electron transfer and nitrite formation as an intermediate product (Liu et al. 2021). Therefore, this data provides evidence of the more advanced MIC development in the canal than in the pool water reservoir.

Although the microbial population was observed in the pool and the canal reservoir, the number of SRB and MIC-related bacteria was higher in the canal than in the pool from TPC and metagenomic analysis. Among several species, *Staphylococcus* sp., *Picochlorum* sp., *D. mediterraneus*, *Truepera* sp., *Desulfovibrio* sp., and *Desulfofustis* sp. may become the key bioindicators in the development of MIC at ISSF station. Metabolic pathway prediction also revealed a higher abundance of corrosion-related pathways in the canal reservoir. Therefore, the canal may undergo more severe MIC development and need more attention to prevent or slow corrosion. Besides, although the TPC method was useful in predicting the probable number of SRB, the metagenomic analysis should accompany the method better to understand the potential MIC occurrence in the ISSF facility.

In conclusion, there is a potential impact of MIC on the waters from the ISSF pools and canals, so strategies are needed to reduce the risk of corrosion on the surface of walls and shelves of nuclear spent fuel. One alternative is to replace the biocide with the new active compound, clean the wall surface, check the water filter, and monitor water quality. In addition, metagenomic data in the form of bacterial abundance and metabolic pathways shows that

bacteria have potential as bioprospecting agents that can be used for research in the fields of health as radioprotector agents and the environment as bioremediation agents.

ACKNOWLEDGEMENTS

We would like to thank the National Research and Innovation Agency for funding, and the Integrated Laboratory Centre, Biology Laboratory, Universitas Islam Negeri Syarif Hidayatullah Jakarta, Indonesia for this research's physical-chemical tools.

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