The Diversity and Resistance of Microbial Community under Mercury Contamination in Paddy Soils

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Article Info	ABSTRACT
	Traditional gold mining may cause mercury pollution in rice fields, resulting in a significant decrease in rice grain yields, as well as socio-economic and health issues. Bioremediation using indigenous microbes is a promising method to alleviate mercury contamination. Thus, it is necessary to explore the diversity and ability of microbes to remediate the contamination. This study investigated the indigenous microbes in mercury-contaminated soils collected from paddy fields around a gold mining area in Sukabumi Regency, Indonesia. Six soil samples were collected from three locations at the dry season; two paddy fields and a tailing pond located next to gold amalgamation machinery. Physicochemical analysis and total mercury concentration measurement of the soil samples were performed immediately after sampling. Bacteria in soil samples were cultured on Nutrient Agar and its colonies were counted after 24 hours of cultivation. Morphological characterization of the colonies was observed under a light microscope. Bacterial community composition was investigated using 16S rRNA amplicon sequencing. The resistance of bacteria to the mercury was tested by inoculating the mixed cultures in Mueller-Hinton agar supplemented with $HgCl_2$ in concentrations of 10, 30, and 50 ppm. Bacterial colonies appeared higher in the soil sample with the lowest total mercury concentration. However, microbial community compositions were more diverse in the soil with medium mercury contamination. Furthermore, the microbial community cultured from tailing soil, despite lower bacterial diversity, performed mercury resistance up to 50 ppm. Upcoming studies should be conducted to investigate further potential indigenous microbial communities for reducing mercury contamination in soils.

INTRODUCTION

Mercury (Hg) is included in the WHO 2020 list of ten chemicals with public health concerns (WHO, 2020). Mercury contamination is globally widespread through atmospheric transportation, deposited in soil, water, and biota, and exposed to humans through dermal contact, inhalation, and oral consumption (Gworek, Dmuchowski & Baczewska-Dąbrowska, 2020). In humans, mercury toxicity may cause acute mental and physical health disturbances (Jyothi & Farook, 2020). Anthropological activities, such as coal-fueled combustion and artisanal gold mining, are the primary source of mercury pollution in the surrounding environments (USEPA, 2021). In developing countries, such as Indonesia, artisanal gold mining most possibly pollutes

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the surrounding areas, which in many cases are agricultural soils (i.e., paddy fields) or house yards (Astuti, Mallongi & Rauf, 2021; Hindersah et al., 2018; Novirsa et al., 2019; Suhadi et al., 2021). In many studies (i.e. Hindersah et al., 2018; Novirsa et al., 2019; Saragih et al., 2021), the mercury contamination in the paddy soils in Indonesia has extremely exceeded the national permissible limit of maximum 0.5 mg/kg.

Bioremediation using indigenous microbes has been proposed to alleviate mercury deposition in agricultural soils as the most environmentally and economically feasible approach (Sangwan & Dukare, 2018). Indigenous microbes naturally inhabit the contaminated sites, indicating their resistance to the pollutant and their potential as bioremediation agents (Tarfeen et al., 2022). In addition, the capacity of microbes to adapt to an environment with a high concentration of heavy metals indicates their potential application to reduce the contamination level (Igiri et al., 2018).

Studies regarding the bioremediation of numerous pollutants using indigenous microbial communities have been reported many times (Khomarbaghi et al., 2019; Miao et al., 2021; Rahmeh et al., 2021; Safonov et al., 2018). However, the potential of indigenous microbes for mercury bioremediation is predominantly emphasized in the utilization of single strains (Dash & Das, 2014; Kotwal et al., 2018; Pushkar, Sevak & Singh, 2019; Saranya et al., 2017). Therefore, exploration of the soil microbial communities and their potential as an in-situ bioremediation agent is essential to remediate polluted soils. This study is aimed to investigate the microbial community composition and variation through the sequencing of 16S rRNA gene amplicons. This high-throughput sequencing technology has provided a powerful tool to explore the complexity of microbial communities and their shifting profiles under the threat of contamination.

MATERIAL & METHODS

Sukabumi Regency was chosen as the sampling site as a previous study reported high mercury concentration in paddy field soils around artisanal gold amalgamation facilities in this region (Saragih et al., 2021). Two sampling plots were purposely selected in Mangun Jaya village (7°11'44.6"S, 106°37'19.2"E) and Mekar Mukti village (7°13'39.1"S, 106°35'02.2"E), Waluran district (Figure 1). Soil samples were taken from each plot following McPherson et al. (2018) with modification. Additionally, sludge from the tailing pond (TP) in Mangun Jaya was taken for comparison. Samples were stored in sterile plastic bags and kept in cool condition (4°C).

The pH and humidity of soil and sludge samples were measured directly in the field using a portable pH meter and moisture tester (Takemura DM-5, Japan). Organic C, total N, available P and K, cation exchange capacity, and salinity were determined by the soil analysis laboratory of the Soil Research Center (Bogor, Indonesia) according to standard protocol (Sulaeman, Suparto & Eviati, 2005). Total mercury in the samples was measured with the thermal decomposition method using a direct mercury analyzer MA-3000 (NIC, Japan) following EPA Method 7473.

In order to observe and quantify cultivable bacteria inhabiting soil samples, 1 mL of diluted sample was inoculated onto a Petri dish containing Nutrient Agar media in triplicates for each sample. After 24 hours of incubation at 37°C, the number of colonies (CFU/mL) was counted. The characteristics of the microbial colonies, such as color, shape, elevation, and margin, were determined and photographed under a stereo microscope SZH10 (Olympus, Japan) following Cappucino & Sherman (2014). Colonies with different characteristics were counted as colony morphotypes.

Bacterial community composition in soil samples of PF1, PF2, and TP was performed by the bacterial 16S rRNA gene sequencing. Amplification of 16S rRNA gene was performed using primer set 63F and 1387R (Marchesi et al., 1998). Sequencing libraries were generated

Fig. 1. Sampling sites in Mekar Mukti (PF1) and Mangun Jaya (PF1 and TP), Waluran district, Sukabumi regency

using NEBNext® UltraTM DNA Library Prep Kit for Illumina® (New England Biolabs, USA). The library quality was assessed on the Qubit @ 2.0 Fluorometer (Thermo Scientific, USA) and Bioanalyzer 2100 (Agilent, USA). The library was sequenced on an HiSeq 2500 platform (Illumina, USA). High-quality raw tags were filtered using the QIIME platform (Caporas et al. 2010), and compared with the reference database using the UCHIME algorithm (Edgar et al. 2011). Multiple sequence alignments between different OTUs were performed using MUSCLE (Edgar 2004). Alpha diversity was calculated with QIIME version 1.7.0 and displayed with R software version 2.15.3. A cluster tree at the phylum level was constructed based on the Unweighted Pair-group Method with Arithmetic Mean (UPGMA) using QIIME software (Version 1.7.0).

The mercury resistance of the microbial community from each sample was assessed by measuring the Minimum Inhibitory Concentration (MIC) of $HgCl_2$ following Pérez-Valdespino et al. (2013) with modification. One mL of diluted sample was cultured in 100 mL Luria Bertani broth for 24 hours at 37°C, and the cultivated cultured was swabbed onto the Petri dish filled with Mueller-Hinton agar media supplemented with $HgCl_2$ with the concentration of 10 ppm, 30 ppm, and 50 ppm. At the end of the incubation period for 24 hours at 37°C, the intensities of colonies grown in media with $HgCl_2$ were quantified by comparing to the ones without $HgCl_2$ (control).

RESULTS AND DISCUSSION

Soil samples collected from two paddy fields have neutral pH (6.7-6.9), which is advantageous for rice cultivation (Krisnawati and Bowo 2019). Meanwhile, the sludge sample was found to be slightly alkalic (8.2) as a result of the use of calcium oxide (CaO) in the gold purifying process commonly performed by the gold miners in the study sites (Syafei et al. 2020). Furthermore, soil pH is influenced by soil properties and water content in the soil (Neina 2019) and will affect the biomass of soil microorganisms (Mohd-Aizat et al. 2014).

Regarding soil moisture, the samples collected from paddy fields (PF1 and PF2) have

considerably low humidity (20-30%) due to the dry season. Meanwhile, the moisture of the sludge sample (TP) was much higher (60%) due to the high content of wastewater. According to Olaniran, Balgobind, and Pillay (2013), soil humidity affects the mobility and bioavailability of heavy metals, as the water increases the interaction of metal compounds and organic materials, thus affecting the ability of the microbes to metabolize the pollutants.

Another soil property that acts on the type and number of inhabiting microbes is electrical conductivity (Kim et al. 2016), determined by the concentration of dissolved salts or salinity. The electrical conductivity of soil samples in this study correlated with the salinity, as shown in Table 1. These two parameters have interchangeable effects on the soil microbes (Wang et al. 2020). Furthermore, many studies have reported the effect of salinity on the soil microbial community, i.e., Rath et al. (2019) indicated that bacterial community composition is consistently shifted with the increase of salt tolerance, meanwhile Zhang et al. (2019) implied that the increase in salinity might decrease the microbial biomass.

PCA of physical parameters and mercury concentration (Figure 2) reveals that heavily mercury-contaminated site clearly differs from low and medium mercury-contaminated sites. Carbon (C), nitrogen (N), and phosphorus (P) are the essential factors in the microbial community composition and biomass level (Naylor, McClure, and Jansson 2022). According to Prescott (2002), the ratio of C, N, and P as much as 100:10:1 is optimum for microbial growth. Our soil samples almost have an optimum C:N ratio (9:1 – 12:1), while the potential P measured in the samples was varied, with the highest P concentration is in sample TP. Oliverio et al. (2020) reported that soils with a low level of P are more suitable for oligotrophic microbes, while high P soils are ideal for the copiotrophic taxa.

Moreover, the concentration of potassium (K), as described by Luo et al. (2016), was one of the critical factors of soil enzymatic activities, which in turn influence microbial functions and

Table 1. Soil physicochemical profile of the samples from paddy fields (PF1 and PF2), and tailing pond (TP)

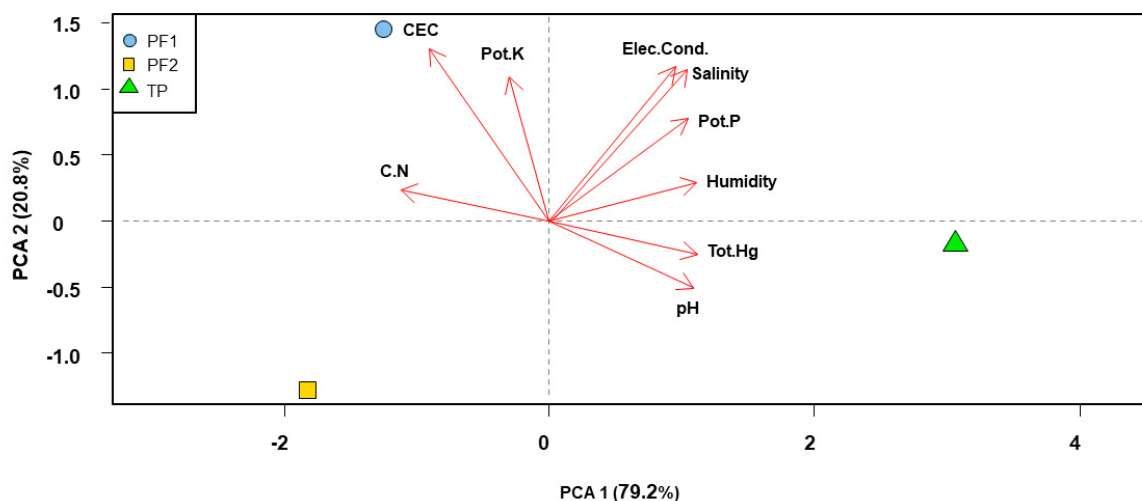| Sample ID	pH	Humidity (%)	Electrical Conductivity (dS/m)	Salinity (mg/L)	C/N	Potential P (mg per 100 g)	Potential K (mg per 100 g)	CEC (cmol/kg)
PF1	6.7	30	0.344	173	12	245.56	6,438.82	30.32
PF2	6.9	20	0.104	53	12	108.37	1,385.90	16.88
TP	8.2	60	0.488	244	9	411.05	572.44	3.62

Fig. 2. Principal Component Analysis (PCA) of physical parameters and mercury concentrations in soil

nutrient cycling. Sample PF1 has a relatively high level of potential K, which might indicate higher microbial-favoring enzymatic activities. The accessibility of these essential nutrients to the soil organisms is associated with the soil's cation exchange capacity (CEC). A study by Liddicoat et al. (2018) showed that soil CEC is a critical factor in microbial diversity, as the higher the soil CEC higher the accessible nutrient. Sample PF1 has the highest soil CEC, indicating higher exchangeable nutrients, and thus higher microbial activity than the other samples.

As expected, the total mercury concentration in the paddy field samples was much lower than in the sample from the tailing pond. Even though the PF2 sample was taken from a paddy field next to a tailing pond, the mercury contamination was not much higher compared to the PF1 sample, which was taken from a paddy field located quite far from the pollution source. We suppose that sampling at the dry season may affect the mercury concentration in the PF2 sample. Our results are opposite to those of Saragih et al. (2021), who reported a total mercury concentration of 1.60-2.97 ppm in the paddy soils collected during the rainy season near the sampling plots of this study. We argue that sunlight intensity may decrease mercury concentration in the soil through the volatilization process. Osterwalder et al. (2019) reported that mercury volatilization, when the divalent mercury (Hg^{2+}) is converted into elemental mercury (Hg^0), in the soil is much more likely to happen as a function of solar radiation, soil temperature, and microbial activity. Moreover, O'Connor et al. (2019) suggested that mercury contamination in soil is a complex system influenced by many factors, including meteorological factors as well as soil properties, such as soil pH and microbial composition.

Profiling cultivable bacterial isolates in contaminated soils is crucial as the initial approach to obtain bacterial culture for bioremediation. In this study, the characterization of cultivable bacteria in mercury-contaminated soil samples was done through colony enumeration and morphotyping. Morphology of bacterial colonies are listed in Table 2 and shown separately in Figure 3.

Notable difference number of cultivable bacterial colonies in the two paddy field soil samples is congruent with the increased concentration of total mercury measured from those two samples. On the other hand, bacterial community composition in sample PF2 was more diverse than those of in sample PF1. This may indicate that the PF2 sample is preferable to nurture bacterial richness, despite its slightly higher mercury concentration and lower soil moisture. Meanwhile, the sample TP was estimated to have the lowest number of cultivable bacteria.

The revelation of the whole species within a microbial community remains a challenge to date (Vitorino and Bessa 2018). However, the exploration of microbial communities to describe the overall diversity of microorganisms has been made possible due to the advance in the metagenomics approach (Nam et al. 2023). In terms of the bacterial community, the use of high-throughput next-generation sequencing in combination with the taxonomic classification of 16S rRNA genes maximizes the bacterial species identification with high-resolution power (Nam et al. 2023).

The high-throughput sequencing of 16S rRNA amplicons yielded 137,080–140,180 effective sequence tags in all three samples after filtering out low-quality reads and removing sequences corresponding to adapters, barcodes, primers, and chimeras. The effective tags with 97% similarity were clustered as the operational taxonomic units (OTUs), with a total number of 1,162; 807; and 606 OTUs, for PF1, PF2, and TP, respectively. Among these OTUs, 152 OTUs were shared between the samples, while the unique OTUs to each sample were 743, 331 and 183 OTUs, for PF1, PF2, and TP, respectively.

Table 3 shows the alpha diversity of each sample according to Shannon index, Simpson index, and Chao index. Shannon and Simpson diversity indexes are commonly used in bacterial diversity measurement based on operational taxonomic units (OTUs), in which Shannon index gives a higher weight to rare species, whereas Simpson index emphasizes more to the species

Table 2. Bacterial colony enumeration and morphotype based on colony appearances

Sample ID	Mean (SD) of colony population (x10^3 CFU/mL)	Colony morphotype					
		Colony ID	Margin	Color	Elevation	Texture	Shape
PF1	36.58 (22.20)	1a	Undulate	Opaque	Flat	Brittle	Irregular
		1b	Undulate	Milky	Flat	Moist	Round
		1c	Entire	Opaque	Convex	Slimy	Round
		1d	Undulate	White	Flat	Translucent	Round
		1e	Filamentous	White	Umbote	Translucent	Filamentous
		1f	Entire	White	Umbote	Translucent	Round
		1g	Lobate	Opaque	Flat	Moist	Spindle
		1h	Undulate	Orange	Flat	Translucent	Irregular
		1i	Filamentous	White	Pulvinate	Shiny	Rhizoid
		1j	Lobate	Milky	Raised	Slimy	Irregular
		1k	Entire	Opaque	Flat	Translucent	Round
		1l	Entire	Milky	Convex	Shiny	Round
		1m	Entire	Yellow	Raised	Translucent	Round
		1n	Lobate	Opaque	Raised	Translucent	Round
		1o	Lobate	Opaque	Umbote	Translucent	Irregular
		1p	Undulate	Opaque	Umbote	Moist	Curled
		1q	Undulate	Opaque	Flat	Moist	Round
		1r	Entire	Milky	Flat	Shiny	Round
PF2	2.85 (0.95)	2a	Undulate	White	Umbote	Slimy	Round
		2b	Undulate	Opaque	Umbote	Slimy	Round
		2c	Undulate	White	Flat	Brittle	Irregular
		2d	Entire	Opaque	Flat	Matte	Round
		2e	Undulate	Opaque	Convex	Translucent	Round
		2f	Entire	White	Curled	Dry	Round
		2g	Entire	White	Convex	Shiny	Round
		2h	Entire	Milky	Convex	Moist	Round
		2i	Lobate	White	Umbote	Shiny	Irregular
		2j	Undulate	Opaque	Pulvinate	Shiny	Irregular
		2k	Curled	White	Umbote	Moist	Irregular
		2l	Filamentous	Opaque	Flat	Matte	Round
		2m	Entire	Opaque	Umbote	Moist	Round
		2n	Undulate	White	Curled	Dry	Round
		2o	Lobate	Opaque	Flat	Moist	Irregular
		2p	Filamentous	White	Umbote	Moist	Rhizoid
		2q	Lobate	White	Umbote	Moist	Curled
		2r	Entire	White	Umbote	Translucent	Curled
TP	0.50 (0.08)	3a	Entire	Opaque	Convex	Shiny	Round
		3b	Curled	White	Umbote	Slimy	Irregular
		3c	Lobate	Opaque	Flat	Translucent	Round
		3d	Undulate	White	Umbote	Moist	Round
		3e	Undulate	White	Convex	Shiny	Round
		3f	Curled	Opaque	Flat	Matte	Irregular
		3g	Undulate	Opaque	Flat	Brittle	Irregular
		3h	Lobate	White	Umbote	Translucent	Irregular
		3i	Filamentous	White	Umbote	Translucent	Round

with more frequency in a sample (Nagendra 2002). Meanwhile, Chao index focuses on the low-abundance species in order to estimate the number of missing species (Anne 1984).

As expected, the bacterial diversity and relative abundance are lower in samples with relatively high mercury concentrations. Compared to another study by (Fatimawali et al. 2020),
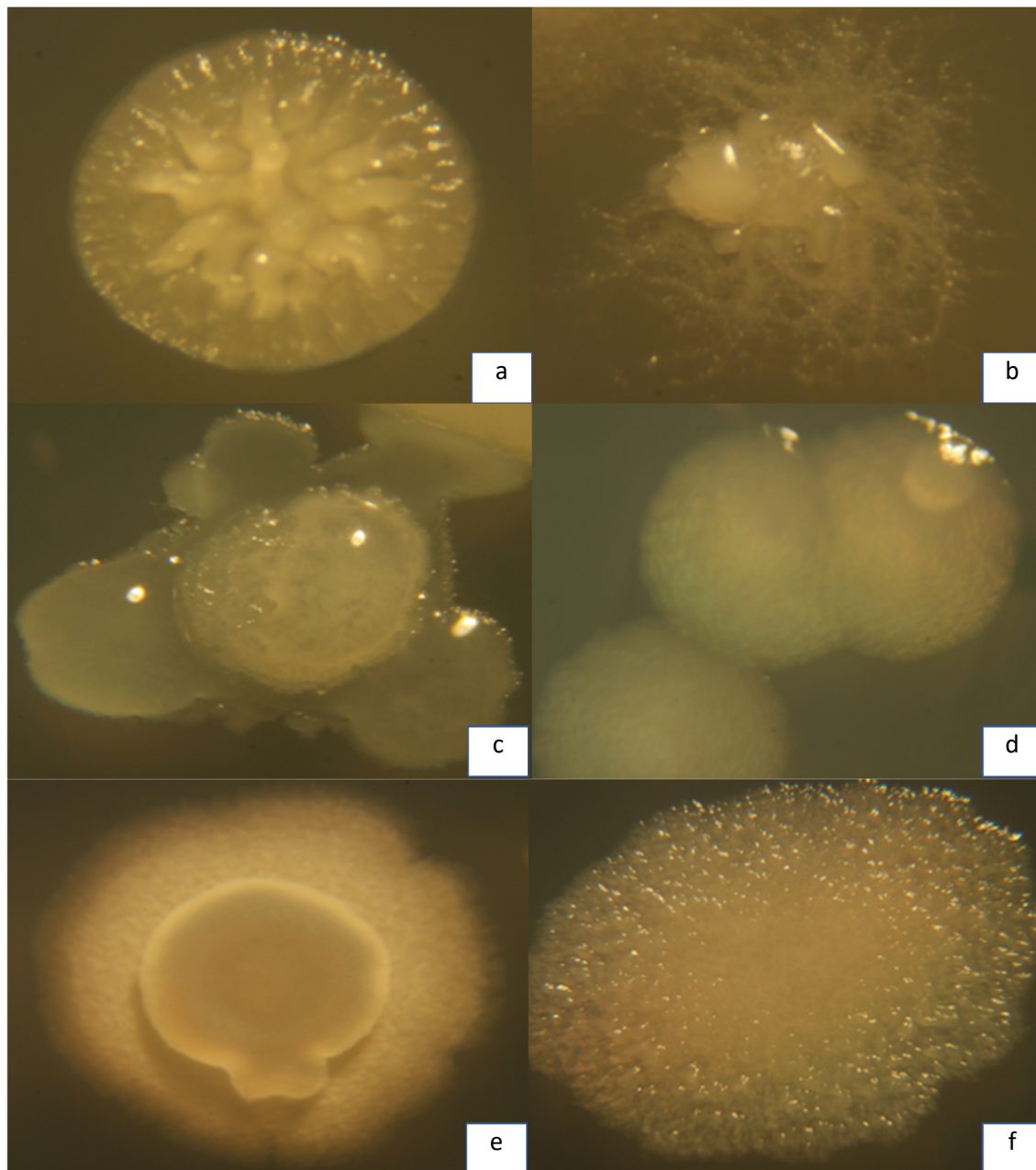
Fig. 3. Microbial colony appearance: a) colony no. 1f, b) colony no. 1i, c) colony no. 2j, d) colony no. 2o, e) colony no. 3f, f) colony no. 3g

Table 3. Bacterial alpha diversity calculation using three different indexes ([a]Konopiński, 2020; [b]Simpson, 1949; [c]Chao, 1984)

Sample ID	OTUs	Unique OTUs	Shannon (H)[a]	Simpson (D)[b]	Chao[c]
PF1	1,162	743	7.322	0.987	1,187.404
PF2	807	331	6.769	0.969	814.154
TP	606	183	3.237	0.803	595.321

the Shannon and Simpson indexes were calculated in the range of 2.836-3.665 and 0.825-0.951, respectively, under mercury contamination with the concentration of 3.98-230 ppm, which is much higher than the mercury contamination in this study. Moreover, a study by Ji et al. (2018) reported that the concentration of mercury contamination was one factor that

significantly influenced the soil microbial community, as shown by the decrease of Chao and Shannon indexes when the mercury concentration was higher.

In total, ten taxa (apart from 'others') were found in the three soil samples, annotated as Proteobacteria, Patescibacteria, Chloroflexi, Actinobacteria, Dependentiae, Acidobacteria, Bacteroidota, Gemmatimonodota, Firmicutes, and Verrucomicrobia. Proteobacteria was the most abundant (>50% of relative abundance) phylum in all samples, and it is worth noting that the relative abundance of this phylum was higher as the mercury concentration increased (Figure 3). The dominance of Proteobacteria was also reported by Zheng et al. (2022); however, the mercury concentration was negatively correlated to this phylum, in contrast to this recent study. The Proteobacteria was found to be dominant in soils contaminated with hydrocarbon (Labbé et al. 2007), uranium (Yan, Luo, and Zhao 2016), and heavy metals (Hemmat-Jou et al. 2018; Zhao et al. 2019). Furthermore, a study by Caracciolo et al. (2015) implied that Proteobacteria was typically the most abundant phylum found in good-quality soil.

The second most abundant phylum was Patescibacteria (sample PF1), Chloroflexi (sample PF2), and Actinobacteriota (sample TP). The relative abundance of Patescibacteria was smaller in samples with higher mercury concentration. Meanwhile, Actinobacteriota appeared to be consistent in all the samples regardless of the contamination level. The effect of mercury concentration was different to the abundance of each phylum, indicating that the contamination might favor several bacteria groups with a potential tolerance, and subsequently inhibit the intolerant groups. In this study, mercury contamination decreased the bacterial diversity but made the composition of microorganisms in the soil more concentrated.

At the genus level, a total of 456 genera were obtained from the three samples. Among the whole genera, there were 94 genera present in all samples; 114 genera, 45 genera, and 40 genera were present only in samples PF1, PF2, and TP, respectively. The most abundant genus in PF1, PF2, and TP was Aquicella (12.21%), Sphingomonas (25.13%), and Porphyrobacter (37.46%), respectively. Aquicella was reported to be tolerant to hydrocarbon contaminants (Dasgupta, Saikia, and Handique 2018), but no studies have been found on the potential of this genus as pollutant-reducing bacteria. Sphingomonas is a nitrogen-fixing bacterium (Videira et al. 2009), and was reported to render the ability to degrade a broad range of chemical pollutants (Asaf et al. 2020). Furthermore, Porphyrobacter belongs to the aerobic anoxygenic phototrophic bacteria cluster (Liu et al. 2017), and several members of this genus are capable of degrading organic
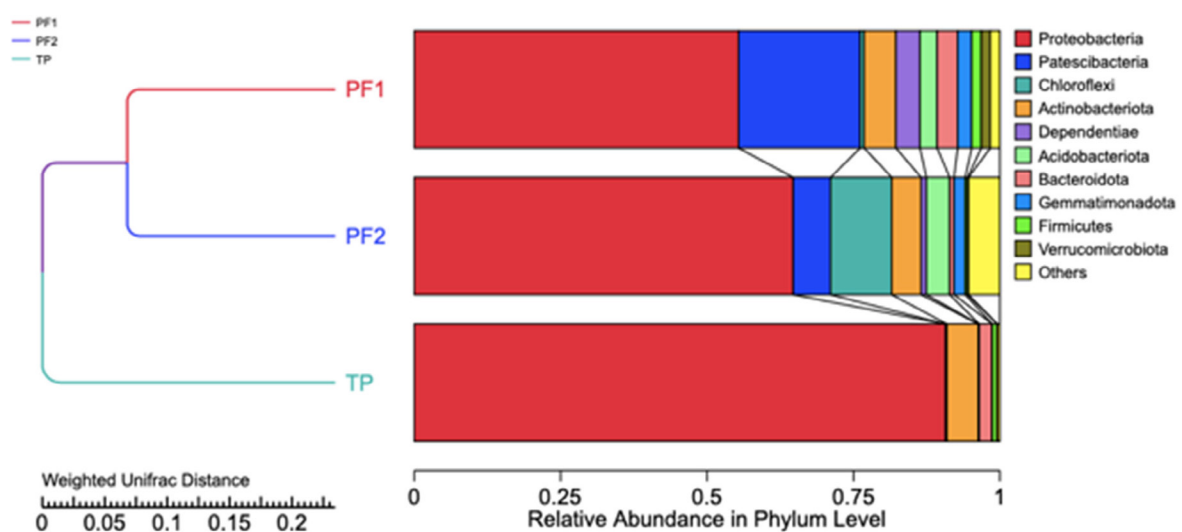

Fig. 4. The bacterial community composition of the three samples at the phylum level depicted as UPGMA cluster tree
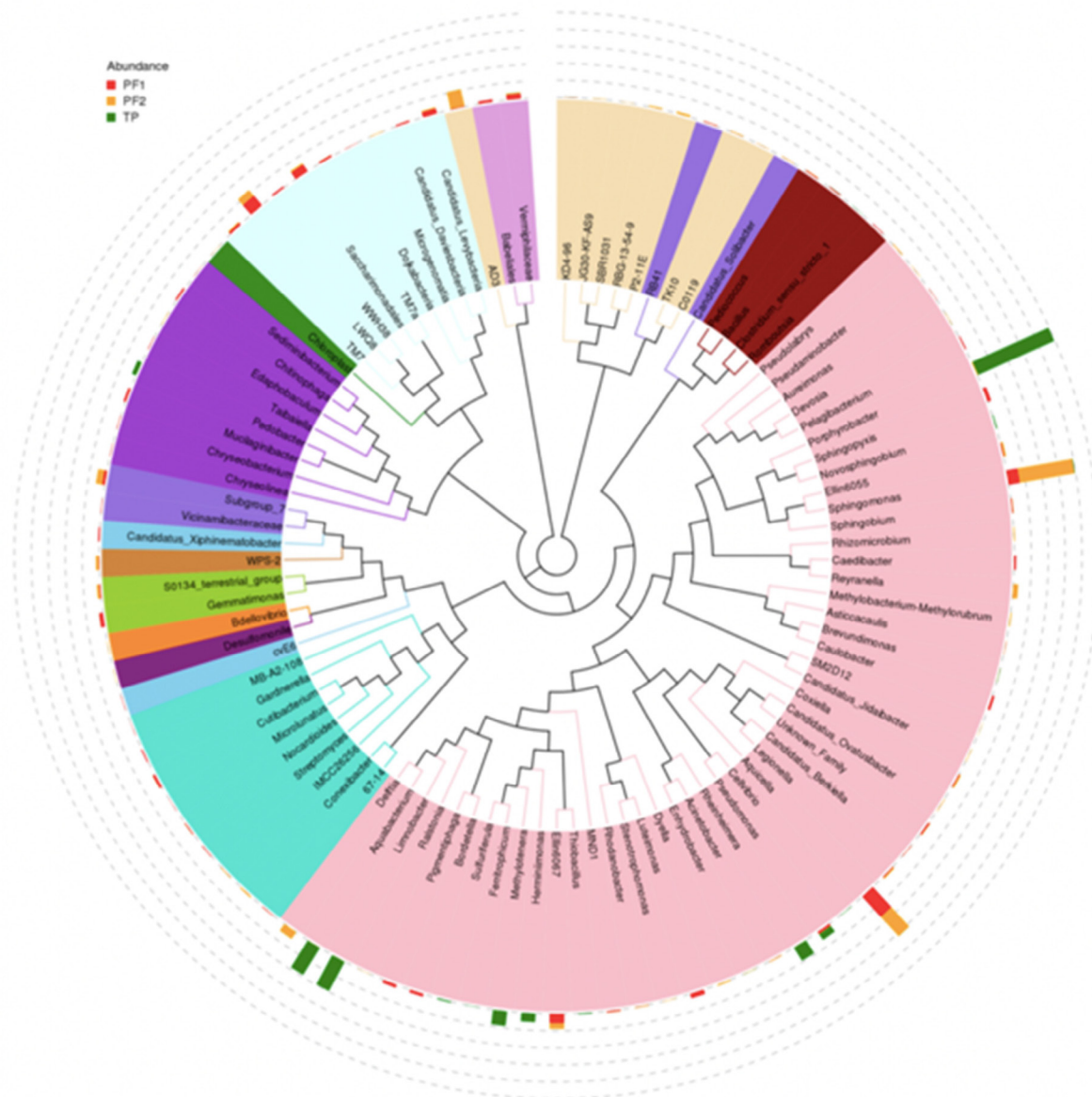
Fig. 5. The evolutionary tree of 100 most-abundant genera in three samples

pollutants (Hiraishi 2003).

The microbial community of all samples were proven to be very resistant to HgCl$_2$ 10 ppm, as indicated by a dense growth of the colonies (Figure 5). However, when the HgCl$_2$ concentration was increased up to 30 and 50 ppm, the Petri dishes of PF1 and PF2 did not show any sign of microbial growth, implying the minimum inhibitory concentration (MIC) of the pollutant to the microbial communities in these samples. On the other hand, moderate growth was found in a Petri dish of TP sample with HgCl$_2$ 30 and 50 ppm, indicating the resistance of the microbial community in this sample to the higher concentrations of mercury.

The resistance of microbes to mercury contamination in the environment is made possible by the presence of mer genes (Lin, Yee, and Barkay 2011). Mer genes in principle are classified into two groups, the narrow-spectrum mer that control the resistance to inorganic mercury, and the broad-spectrum mer determines the resistance to organic mercury (methylmercury and phenylmercury) besides the inorganic mercury, and these genes has varied position in the
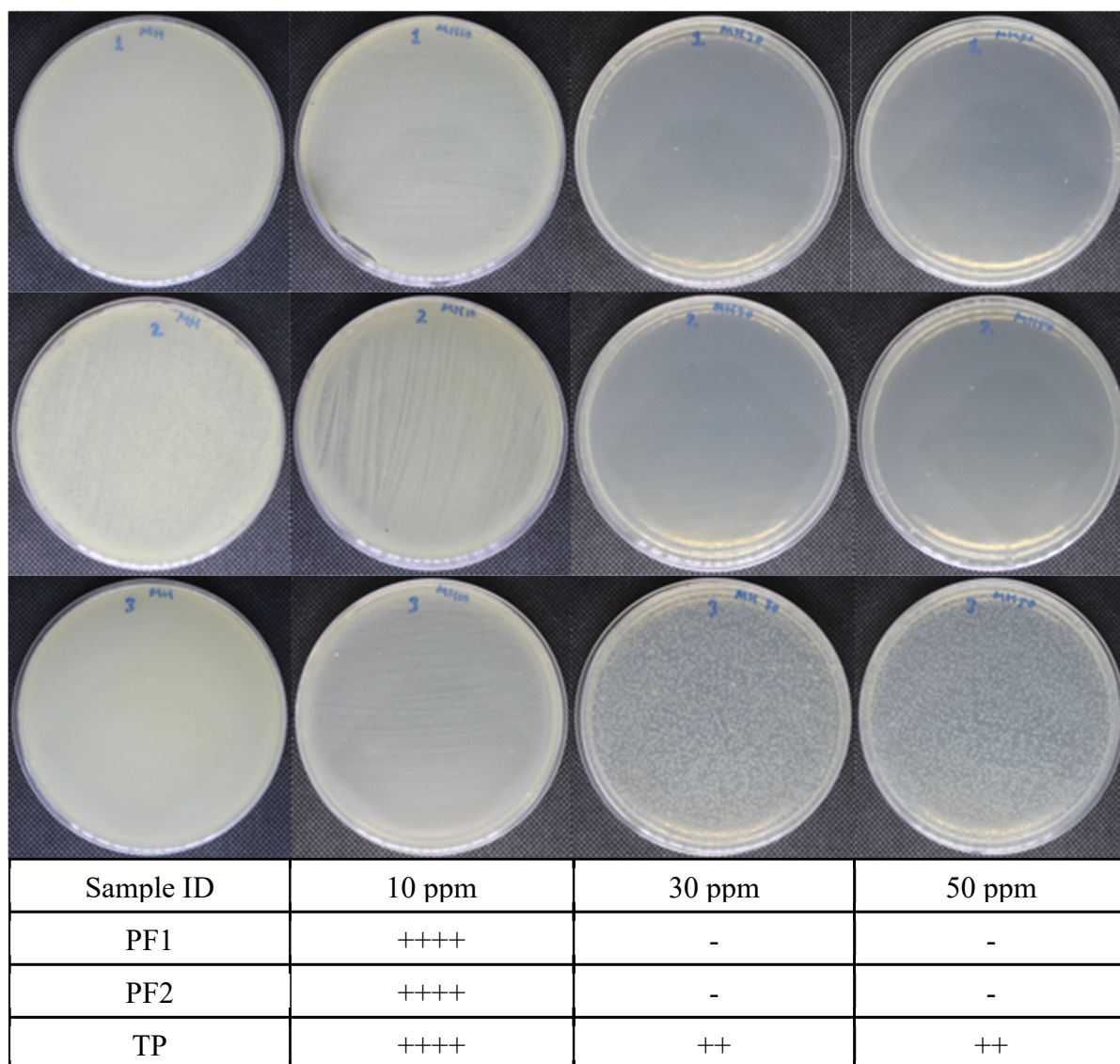
Sample ID	10 ppm	30 ppm	50 ppm
PF1	++++	-	-
PF2	++++	-	-
TP	++++	++	++

Fig. 6. The intensity of microbial colony growth on Mueller-Hinton agar media supplemented with $HgCl_2$ (left to right: control, 10 ppm, 30 ppm, 50 ppm; up to down: PF1, PF2, TP). The resistant values were quantified by comparing the intensity of colony growth in media with and without $HgCl_2$. "-" means no growth observed, "+" means the growth is <25%, "++" means the growth is 25-50%, "+++" means the growth is 50-75%, and "++++" means the growth is >75%

genome, including plasmid and genomic DNA (Priyadarshanee et al. 2022). All these genes code for specific proteins/enzymes and carry out Hg removal from the inhabiting environment. The central mechanism in the microbial mercury detoxification system is the mercuric reductase (MerA) protein to catalyze the reduction of Hg^{2+} to volatile Hg^0, which is controlled by the merA gene (Nascimento and Chartone-Souza 2003). A recent study by Pereira-García et al. (2024) confirms the expression of MerA gene by bacteria cells in the presence of mercury. Naguib, El-Gendy, and Khairalla (2018) claim the distribution of mer genes along with different microbial populations through horizontal and vertical gene transfer. The mobility of such genes has been a crucial property of the resistance of microbial communities in order to be survived in an environment with increasing mercury contamination (Barkay, Miller, and Summers 2003).

The expansion of the diversity of taxa that carry the mer system clearly points to the need to characterize the Hg transformation activities of a larger and more diverse range of organisms. The insights obtained in this study will improve our understanding of the ecology of microorganisms that resistant to Hg in the environment and their potential uses in bioremediation.

CONCLUSION

This study shows that mercury contamination of the agricultural soil alters soil physicochemistry and drives bacterial richness and numbers. The presence of mercury may suppress bacterial diversity, meaning that bacteria which tolerate mercury up to a certain degree may become the dominant one. The resistance to mercury contamination was clearly performed by the bacterial communities in this study, indicating the potential of using indigenous bacteria for in-situ bioremediation. Further study is needed to investigate the capability of the indigenous bacterial community to reduce the mercury concentration from contaminated sites.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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