JOURNAL OF DEGRADED AND MINING LANDS MANAGEMENT
Volume 10, Number 3 (April 2023):4305-4313, doi:10.15243/jdmlm.2023.103.4305
ISSN: 2339-07 6X (p); 2502-2458 (e), www.jdmlm.ub.ac.id

Research Article

Mercury-resistant biofilm-forming bacteria and local plants in phytoremediation of small-scale gold mine tailings in Lombok Island, Indonesia

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Article history:
Received 13 September 2022
Accepted 12 December 2022
Published 1 April 2023

Keywords:
biofilm-forming bacteria
gold mine tailings
local plants
phytoremediation
soil pollution

Abstract

Small-scale gold mining is one of the sectors that contribute to the world's largest mercury contamination through the tailings it produces. Many efforts have been made to reduce mercury concentrations from tailings, one of which is by utilizing a combination of plants and bacteria. This study aimed to analyze the combination of mercury-resistant biofilm-forming bacteria and local plants in the phytoremediation of small-scale gold mine tailings. This study used ten plant species divided into three groups and three biofilm-forming mercury-resistant bacteria (*Bacillus toyonensis*, *Burkholderia cepacia*, and *Microbacterium chocolatum*). Parameters observed included plant biomass, total chlorophyll, plant mercury content and media. The results showed that adding bacteria to each plant in the treatment had a different effect. Some plants with the addition of biofilm-forming bacteria had a higher wet weight than others. However, the addition of bacteria was not effective in increasing plant dry weight. The combination of biofilm-forming bacteria in the first and second plant groups reduced tailings mercury concentrations better than without the addition of bacteria. The combination of plants and bacteria in the third group gave higher mercury concentrations in the medium and plant. This study showed that the addition of biofilm-forming bacteria could lead to increased remediation by plants. The second plant group treatment with a combination of *Plucea indica, Paspalum conjugatum*, and *Sesbania sesban* plants was the most effective in reducing tailings mercury content.

To cite this article: Nurfitriani, S., Arisoesilaningsih, E. and Nuraini, Y. 2023. Mercury-resistant biofilm-forming bacteria and local plants in phytoremediation of small-scale gold mine tailings in Lombok Island, Indonesia. Journal of Degraded and Mining Lands Management 10(3):4305-4313, doi:10.15243/jdmlm.2023.103.4305.

Introduction

Human activities cause an increase in the content of heavy metal elements in the soil. Activities that can increase the heavy metals include mining and smelting, burning fossil fuels, using fertilizers and pesticides for agriculture, and disposal of household waste (Chibuike and Obiora, 2014). Artisanal and small-scale gold mining (ASGM) is one of the mining sub-sectors that contribute significantly to increasing the mercury content in the soil through the tailings it produces. The total mercury pollution produced from ASGM ranges from 410-1400 tons and contributes to 37% of global mercury emissions annually (Hagan et al., 2015; Esdaile and Chalker, 2018). These activities increase the mercury content in the soil to levels that are harmful to human, animal and plant life (Chibuike and Obiora, 2014).

Plants have a significant role in ecosystems (Quijas et al., 2012). Some plants are known to be able to clean soil contaminated with heavy metals. The utilization of plants to clean up environmental pollution is called phytoremediation. Plants in phytoremediation clean the soil by absorbing and storing it in the plant body (Gupta et al., 2016). Various plants are known to have the potential for phytoremediation (Tangahu et al., 2011). However, local plants from mining sites are known to have a higher potential in phytoremediation. *Shorea leprosula, Hevea brasiliensis, Anacardium occidentale, Archidendron pauciflorum, Vitex pinnata, Alstonia scholaris* and *Dillenia suffruticosa* from small-scale gold mining areas in West Kalimantan, which are known to be able to remediate mercury with Bioconcentration Factor (BF)>1 (Ekyastuti et al., 2016). Other local and invasive plants such as West India marsh grass or "kumpai" grass (*Hymenachne acutigluma* (Steud.) Gilliland), elephant grass (*Pennisetum purpureum* Schumach) and reed grass (*Saccharum spontaneum* L.) are potential pioneer vegetation for phytoremediation of soils after tin mining (Khodijah et al., 2019).

Plants grown in environmental conditions with heavy metal stress modify the environment in which they grow extensively to help them obtain nutrients, utilize water, protect against physical disturbance and soil cohesion, and release complex exudate materials (Ebert et al., 2007). Plants used in phytoremediation often interact with microbes to reduce stress due to heavy metals (Mishra et al., 2017). A large number of plant-associated microorganisms develop to attach to plants, live in plant cells, and around infected plants (Ebert et al., 2007). The presence of microbes in the soil also helps to increase plant growth, improve soil quality, and detoxify and remove heavy metals from the soil (Mishra et al., 2017).

Several rhizosphere microorganisms, one of which is bacteria, play a key role in managing stress due to heavy metals in plants. Bacteria accumulate, detoxify, and convert heavy metals into other less harmful forms to plants, thereby increasing plant health (Mishra et al., 2017). These bacteria affect plant health and productivity (Bogino et al., 2013). The microbial community in the rhizosphere also secretes extracellular polymeric substances (EPS) such as polysaccharides, glycoproteins, lipopolysaccharides, and soluble peptides, which have many anion functional groups and help remove or restore the soil rhizosphere of heavy metals through biosorption (Ayangbenro and Babalola, 2017). EPS production by some PGP (plant growth promoting) microbes induces the formation of biofilms in response to exposure to toxic heavy metals. The formation of bacterial biofilms in plants is a symbiotic response. Biofilms can interact with plants in the form of commensalism symbiosis, such as *Agrobacterium tumefaciens* (Bogino et al., 2013; Heidin et al., 2014).

This study aimed to analyze the combination of mercury-resistant biofilm-forming bacteria and local plants in the phytoremediation of small-scale gold mine tailings.

Materials and Methods

Plant preparation

This study used ten plant species that grow in tailing disposal sites and around small-scale gold mining from various regions. Four plants, i.e., beluntas (*Pluchea indica*), buffalo grass or "papaitan" (*Paspalum conjugatum*), *Fimbristyis dichotoma* (L.) Vahl and "Jayanti" (*Sesbania sesban*), were obtained from areas around small-scale gold mining in Jonggat, Pujut and Sekotong Districts, Lombok Island, Indonesia. Selection of plants based on the highest mercury content in the tissue. A total of five other plants, i.e., "purun danau" (*Lepironia articulata*), water bamboo (*Equisetum hyemale*), water spinach or "kangkung" (*Ipomoea carnea*), *Sorghum timorense*, and *Themeda arundinacea* were selected based on literature studies on plants that can adapt in the gold mining area, and *Lygodium circinnatum* was chosen based on the high utilization of plants for crafts in Lombok. The plants used in this study were grown in polybags until their growth was uniform. The plants were watered every day. The plants used had the same weight and height (Sudarsan et al., 2018).

Bacterial preparation

The bacteria used in this study were mercury-resistant biofilm-forming bacteria isolated from gold mine tailings in Lombok. The bacterial species used were *Bacillus toyonensis, Burkholderia cepacia*, and *Microbacterium chocolatum* (Nurfitriani et al., 2022). Bacterial isolates were grown in LB (Luria Bertani)-broth medium up to 2 x 10^8 CFU mL^{-1}, and 1 mL of suspension from each isolate was taken and added to new LB-broth medium to form a consortium. The consortium of biofilm-forming bacteria was grown on LB-broth medium and incubated for 24 hours until it reached a density of 2 x 10^8 CFU mL^{-1} (Liu et al., 2019). The bacterial consortium was applied to the plants at 5 mL $plant^{-1}$ according to the treatment (Hernández-Montiel et al., 2017).

Phytoremediation potential of mercury using a combination of plants and bacteria

The experiment was carried out in a greenhouse of the Faculty of Agriculture, Brawijaya University, using polybags. The medium in the experiment was tailings collected from a gold processing site in the Jonggat District of Lombok Island, Indonesia, with a tailing age of 2-3 years and a mercury content of 23.2-42.1 ppm. The fine tailings were weighed and put into polybags, 2.5 kg $polybag^{-1}$. The plants in the second phase of the research were grouped into three gropus. The combination of plants in the first plant group (T1) represents a water or wetland ecosystem, namely *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea*. The combination of plants in the second plant group (T2) represents the terrestrial ecosystem found in Lombok and consists of local plants in the field, namely *P. indica, P. conjugatum*, and *S. sesban*. The combination of plants in the third plant group (T3) represents an extremely dry, sandy and nutrient-poor terrestrial ecosystem, namely *L. circinnatum, S. timorense*, and *T. arundinacea*. The treatment in the second phase of the research treatment is presented in Table 1.

Table 1. Treatments tested in this study.

No	Treatment code	Description
1	T1B0	Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea*
2	T1B1	Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea* + biofilm-forming bacteria
3	T2B0	Combination of *P. indica, P. conjugatum*, and *S. sesban*
4	T2B1	Combination of *P. indica, P. conjugatum, and S. sesban* + biofilm-forming bacteria
5	T3B0	Combination of *L. circinnatum, S. timorense*, and *T. arundinacea*
6	T3B1	Combination of *L. circinnatum, S. timorense*, and *T. arundinacea* + biofilm-forming bacteria

The six treatments were arranged in a randomized block design and grouped based on the number of destructive observations. Each treatment was repeated three times with six times destructive observations, so there were 108 experimental units. The destructive observation was made every two weeks, so the experimental time was 12 weeks (Adji, 2005). Parameters measured were the amount of leaf chlorophyll, plant biomass (plant fresh weight and dry weight), plant mercury content, and medium (tailing) mercury content.

Data analysis

Statistical data analysis was performed using PAST 4.06 software. Single-factor data were analyzed using the Analysis of Variance (ANOVA) or the F test with a significant level of $p \leq 0.5$. Data with multiple factors were analyzed using the Multivariate Analysis of Variance (MANOVA). Data that could not be analyzed using ANOVA and MANOVA were analyzed descriptively using the Principal Component Analysis (PCA). The PCA simplifies the complexity of the interrelationships between many observed variables to a relatively small number while still explaining most of the diversity of the original variables (Hendro et al., 2012). Differences between treatments were analyzed by the Duncan Multiple Range Test (DMRT) at a significant level of $p \leq 0.5$.

Results and Discussion

Plant biomass

The average fresh weight of plants after planting in tailing medium with biofilm-forming bacteria and without the addition of bacteria was much lower than the initial plant weight (Figure 1). *I. carnea, S. sesban*, and *S. timorense* had higher post-planting weights in the treatment with the addition of biofilm-forming bacteria compared to the initial weight. The addition of biofilm-forming bacteria also increased the fresh weight of *L. articulata, E. hyemale, I. carnea, S. timorense*, and *L. circinatum* compared without the addition of bacteria. This study showed that adding biofilm-forming bacteria could increase plant resistance to environmental stress and reduce plant tissue damage. The results of this study are in line with the research of Haque et al. (2020), who showed that tomatoes grown and treated with biofilm-forming PGPR (Plant Growth-Promoting Rhizobacteria) showed a higher antioxidant defense system and less tissue damage than plants that were not inoculated under water stress conditions. The addition of biofilm-forming bacteria to barley was also able to reduce the damaging effect of salinity on several growth criteria, such as seedling length, wet and dry weight, and relative water concentration, compared to without the addition of bacteria (Kasim et al., 2016). However, in this study, the addition of bacteria had a different impact on the dry weight of each plant.

The effect of adding biofilm-forming bacteria on dry weight and determining the best plant growth was analyzed using Principal Component Analysis (PCA). The analysis was carried out by comparing all treatments at destructive observation times 1 (2 weeks after planting = WAP) and 5 (10 WAP). The PCA results (Figure 2) showed that the addition of biofilm-forming bacteria to each plant group did not increase the dry weight compared to that without the addition of bacteria. This is shown by the treatment area with the addition of biofilm, which is below the X-axis in the graph (Figure 2), while the area without the addition of biofilm-forming bacteria is above. Based on Figure 2, the addition of biofilm-forming bacteria has not been effective in increasing plant dry weight in each treatment unit.

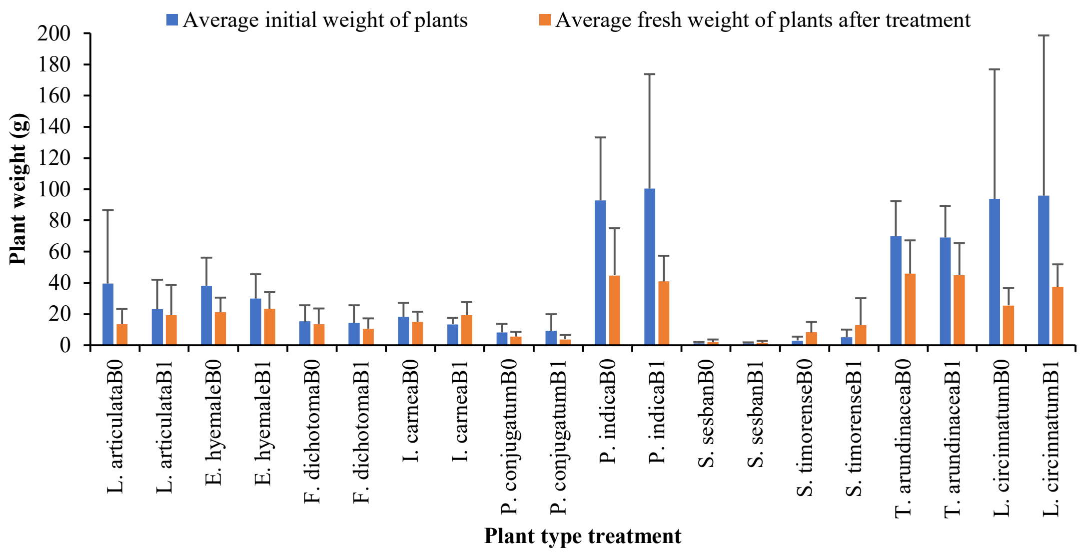

Figure 1. Comparison of fresh weight of plants before and after planting treatment in tailings containing mercury. B0: without the addition of biofilm-forming bacteria; B1: with the addition of biofilm-forming bacteria.

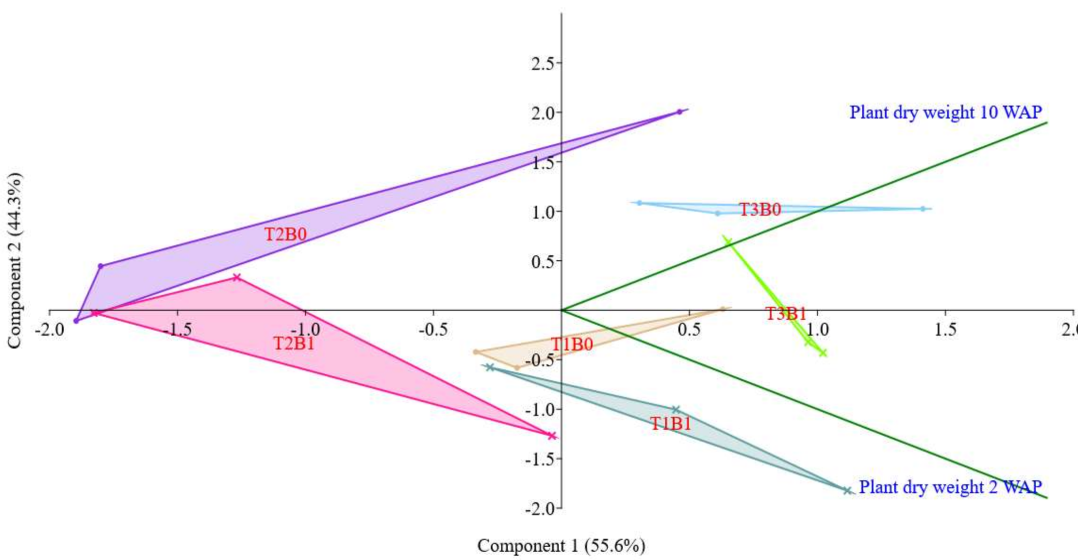

Figure 2. Comparison of plant dry weight between destructive times. T1B0: Combination of E. *hyemale, F. dichotoma, L. articulata,* and *I. carnea*; T1B1: Combination of E. *hyemale, F. dichotoma, L. articulata*, and *I. carnea* + biofilm-forming bacteria; T2B0: Combination of *P. indica, P. conjugatum*, and *S. sesban*; T2B1: Combination of *P. indica, P. conjugatum*, and *S. sesban* + biofilm-forming bacteria; T3B0: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea*; T3B1: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea* + biofilm-forming bacteria.

Plant dry weight reflects the accumulation of organic compounds that plants successfully synthesize from inorganic compounds, especially water and carbon dioxide. Nutrients that have been absorbed contribute to the addition of plant dry weight. Dry weight is also a reflection of the efficiency of absorption and utilization of available solar radiation by the plant canopy (Apsari et al., 2018). Mercury stress causes growth inhibition resulting in low plant dry weight. The biofilm produced by the bacteria was able to reduce the impact of mercury exposure on some of the test plants so that the plants had a higher dry weight compared to the treatment without the addition of biofilm-producing bacteria. Biofilms protect plants from mercury by enveloping plant roots by forming a physical barrier. In general, the physical barrier components of biofilms are water, bacterial cells, and an exopolysaccharide matrix (Bogino et al., 2013).

Mercury content in growing medium

Small-scale gold mining solid waste in this study was used as a planting medium for local plants. The results showed that the concentration of mercury decreased by 12 WAP. The effect of using a combination of plants with or without the addition of biofilm-forming bacteria on the mercury concentration of growing medium containing mercury was inconsistent and gave fluctuating results (results not shown). The fluctuating value of soil mercury content in the treatment with the addition of biofilm-forming bacteria can be caused by various factors. According to Dranguet et al. (2017), various physical, chemical, and biological factors related to the surrounding environment and the biofilm itself can affect the process of Hg accumulation in the biofilm. Biofilm-specific factors will also significantly affect the bioavailability of mercury in medium, such as speciation and transformation of mercury in the biofilm matrix, interactions between various microorganisms that inhabit the biofilm, and variations in the main physicochemical characteristics within the biofilm. Speciation and transformation of mercury in the biofilm matrix are important factors that act as bioavailability modifying factors. This is because the mercury species around the microorganisms embedded in the biofilm matrix will determine the interaction of mercury and microorganisms (Dranguet et al., 2017). The results of analysis using PCA showed that the lowest mercury concentration in the treatment medium with the addition of biofilm-forming bacteria was T2B1, followed by T3B1. The T1B1 treatment in this study had the least effect compared to other treatments (Figure 3). In the treatment without the addition of biofilm-forming bacteria, the lowest mercury concentration was found in T3B0. These results indicated that the addition of biofilm-forming bacteria was only effective in reducing mercury in the medium in second plant groups, namely *P. conjugatum, S. sesban*, and *P. indica*. The third plant groups, namely *T. arundinacea, S. timorense*, and *L. circinatum* had a good ability to reduce the concentration of mercury in the medium. This can be seen from the low concentration of mercury in the medium at 10 WAP and 12 WAP, even without the addition of biofilm-forming bacteria (Figure 3).

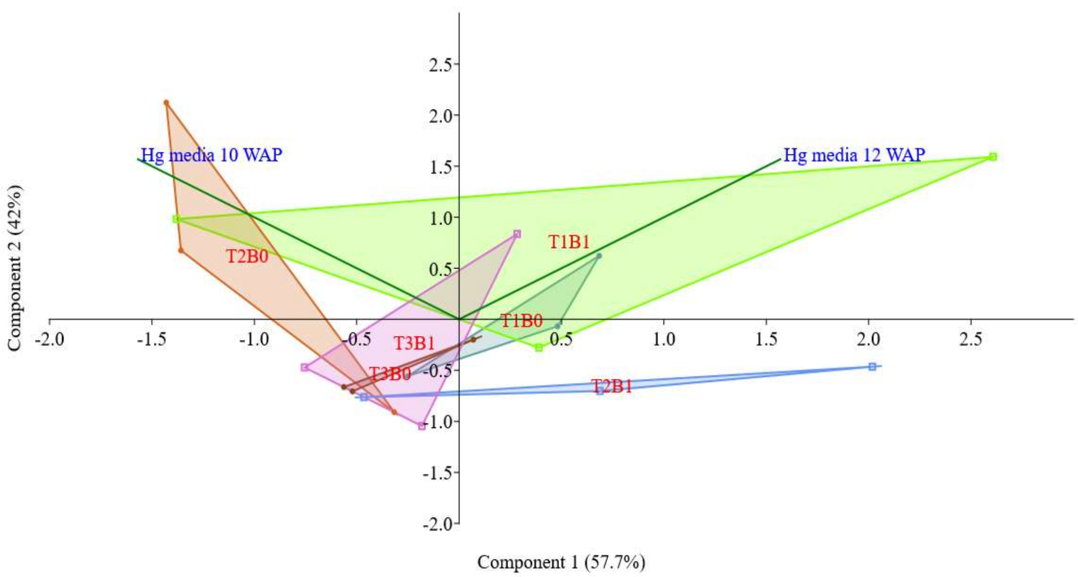

Figure 3. Results of PCA analysis of mercury concentrations in the planting medium at 10 and 12 WAP. T1B0: Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea*; T1B1: Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea* + biofilm-forming bacteria; T2B0: Combination of *P. indica, P. conjugatum*, and *S. sesban*; T2B1: Combination *of P. indica, P. conjugatum*, and *S. sesban* + biofilm-forming bacteria; T3B0: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea*; T3B1: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea* + biofilm-forming bacteria.

Mercury content in plants

The T3B1 treatment gave a different response to plant mercury content than the T1B1 and T2B1 treatments (Figure 4). Nevertheless, the value of mercury concentration in plants in the T1B1 treatment was lower than T2B1. This suggests that biofilms adhering to root surfaces have different effects on plants. The increased concentration of mercury accumulation in the third group of plants with the addition of biofilm-forming bacteria (T3B1) could be due to the role of bacteria in breaking down metal compounds and the very high plant tolerance. The results of this study indicate that mercury concentrations in the plant are affected by the addition of biofilm-forming bacteria, species, and combinations of plants. Mercury accumulation in the second plant group, namely *P. conjugatum*, *S. sesban*, and *P. indica* are known as accumulator plants and capable of accumulating several types of heavy metals. *S. sesban* plants are accumulators of cadmium (Cd), lead (Pb), and zinc (Zn) (Gupta et al., 2011; Varun et al., 2017). *P. conjugatum* plants have been known as accumulators of mercury (Hg), lead (Pb), arsenic (As), copper (Co), tin (Sn), and zinc (Zn) (Ashraf et al., 2011; Hamim et al., 2017). *P. indica* is an accumulator of copper (Cu), zinc (Zn), and lead (Pb) (Soraya et al., 2019).

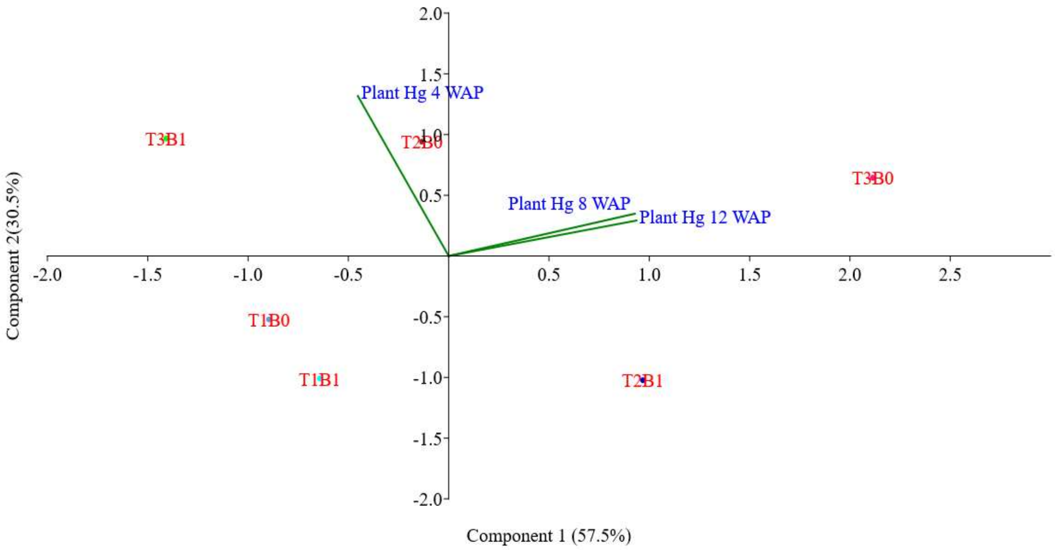

Figure 4. Results of PCA analysis of mercury concentrations in plants. T1B0: Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea*; T1B1: Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea* + biofilm-forming bacteria; T2B0: Combination of *P. indica, P. conjugatum*, and *S. sesban;* T2B1: Combination of *P. indica, P. conjugatum*, and *S. sesban* + biofilm-forming bacteria; T3B0: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea*; T3B1: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea* + biofilm-forming bacteria.

The addition of biofilm-forming bacteria to pots containing *L. circinnatum, S. timorense*, and *T. arundinacea* plants made it possible to increase the amount of uptake by plants. This is thought to be related to the ability of biofilm-forming bacteria to break down various toxic contaminants into simpler compounds (Sharma, 2022).

Biofilms provided a protective effect against Hg stress in the first and third plant groups (T1 and T3). The first and third plant groups are a combination of plants found in various mining locations and have different habitats. Mercury can be very toxic in this group of plants. The addition of biofilm-forming bacteria protects plant groups one and three by adhering to the surface of plant roots. Biofilms form on root surfaces very quickly. Bacterial cells adhere to the root surface and gradually fill the empty spaces on the root surface that are not colonized with bacterial cells or microcolonies (Zhou and Gao, 2019).

Plant chlorophyll content

Chlorophyll is a green leaf substance found in all green plants as the main photosynthetic pigment (Hendriyani et al., 2018). The chlorophyll content in plants is influenced by several things, including the plant growth phase, excess or deficiency of nutrients and water, temperatures that are too low and too high, and other environmental stresses (Song and Banyo, 2011; Hendriyani et al., 2018). Mercury in tailings causes a low total chlorophyll content in the test plants. Total chlorophyll in the test plants ranged from 0.30-2.39 ug g^{-1} (Figure 5), with an average of 0.88 ug g^{-1}. The highest concentration of chlorophyll in this study was still low compared to the average chlorophyll from the leaves of plants growing in forest communities in China. Research conducted by Li et al. (2018) on the leaves of 823 plant species from nine forest communities stretching from cold to tropical zones in China showed that the total chlorophyll content of plants ranged from 1.20-22.58 mg g^{-1} with an average of 5.97 mg g^{-1}. The lowest chlorophyll concentrations were found in *F. dichotoma* plants with values of 0.30 and 0.36 ug g^{-1}. Low chlorophyll concentration in plants will cause chlorosis. The chlorosis formed is a symptom indicating that mercury poisoning has occurred in plants (Muddarisna et al., 2013). The addition of biofilm-forming bacteria could only increase the total chlorophyll concentration in several plant species, namely *L. articulate, F. dichotoma*, and *S. sesban* (Figure 5). The highest total chlorophyll concentration in the treatment with the addition of biofilm-forming bacteria was found in *S. sesban* plants, with a percentage of 18.4% higher than in the treatment without the addition of biofilm-forming bacteria. The results of this study indicate that the addition of biofilm-forming bacteria is not effective in increasing and reducing chlorophyll damage as a result of mercury uptake by plants.

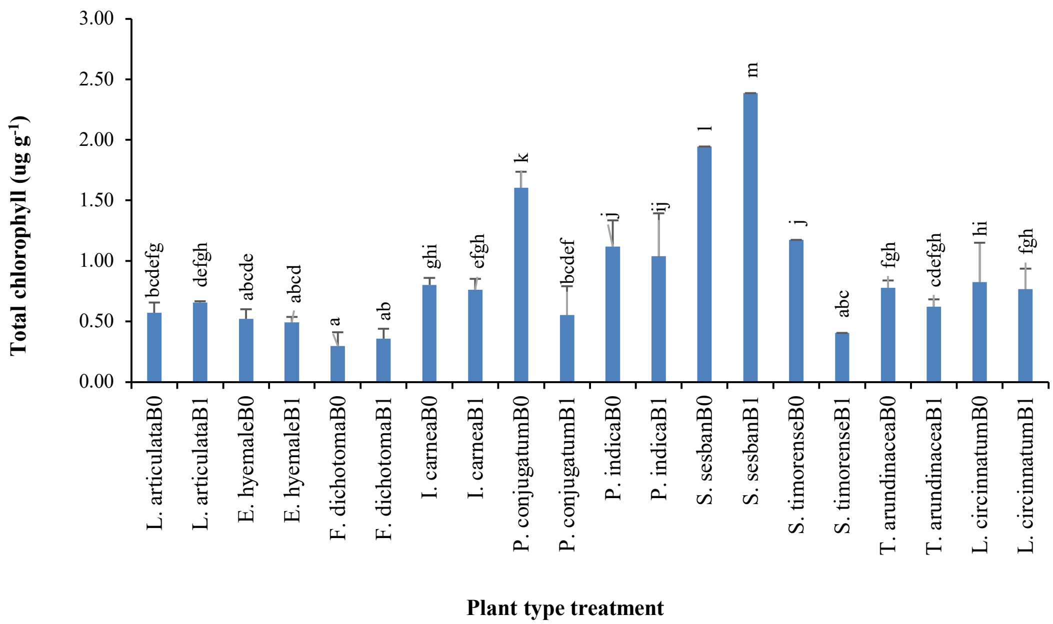

Figure 5. Total chlorophyll concentration of plants. B0: without the addition of biofilm-forming bacteria; B1: with the addition of biofilm-forming bacteria. Numbers followed by different letters indicate significant differences between treatments based on the Least Significant Difference (LSD) test at the 5% level.

Effectiveness of addition of biofilm-forming bacteria: viewed from the relationship between parameters

The total chlorophyll content and plant biomass (dry weight) were strongly related to the mercury concentration of the medium and plants. This is because the mercury content in the planting medium can be absorbed by plants which causes decreased growth, stunted plants, and chlorosis (Chibuike and Obiora, 2014). Analysis using PCA was carried out to determine the relationship between parameters with each plant in one group and the relationship between parameters and treatment in the study. The results of the analysis show that each observed parameter is not closely related to total chlorophyll (Figure 6). T2B1 treatment was able to reduce the concentration of medium mercury and plant mercury content, as well as increase the total chlorophyll concentration of each plant. However, the T1B1 treatment was able to reduce mercury concentrations in the medium and plant better than the T2B1 treatment. Even so, plant growth in the T1B1 treatment was lower than that of T2B1. This is evidenced by the total chlorophyll content and low dry weight. Based on the results of PCA analysis, it was known that the group of plants with the addition of biofilm treatment that gave the best results was T2B1 (a combination of *P. conjugatum, S. sesban,* and *P. indica*).

Conclusion

The addition of biofilm-forming bacteria had a different effect on the three groups of plants. The effectiveness of the combination of bacteria and various plant groups in the remediation of mercury (seen from the mercury content of tailings) is also very different. However, based on this study, the success of phytoremediation can be achieved by using a combination of biofilm-forming bacteria and plant group 2. Several activities or other steps need to be carried out to increase the effectiveness of the combination of biofilm-forming bacteria and *P. indica, P. conjugatum,* and *S. sesban* plants in phytoremediation mercury. Tailings filtration using biofilm-forming bacteria in wetland systems can be applied before the phytoremediation stage using a combination of bacteria and plants to increase remediation effectiveness.

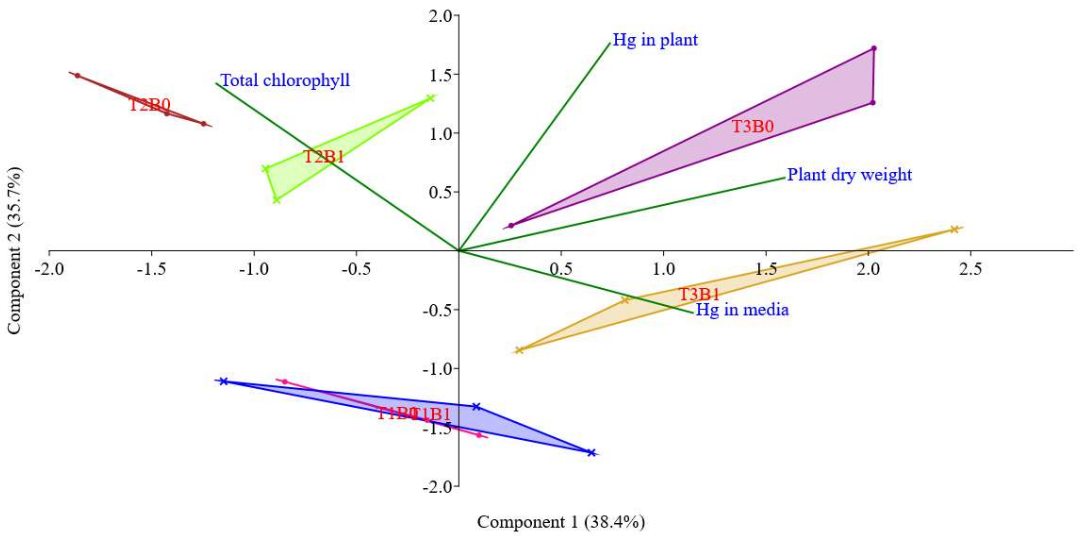

Figure 6. The relationship between each treatment and each parameter in the study. T1B0: Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea*; T1B1: Combination of *E. hyemale, F. dichotoma, L. articulata*, and *I. carnea* + biofilm-forming bacteria; T2B0: Combination *of P. indica, P. conjugatum*, and *S. sesban*; T2B1: Combination of *P. indica, P. conjugatum*, and *S. sesban* + biofilm-forming bacteria; T3B0: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea*; T3B1: Combination of *L. circinnatum, S. timorense*, and *T. arundinacea* + biofilm-forming bacteria.

Acknowledgements

The first author thanks the Directorate General of Higher Education of Indonesia for supporting the study through the PMDSU (Master leading to PhD Program) scholarship. The valuable technical assistance and support from the staff of the Biology Laboratory of the Faculty of Mathematics and Natural Sciences and the Experimental Field of the Faculty of Agriculture, Brawijaya University, are highly appreciated.

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