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In Situ Bioremediation Strategies for the Recovery of Mercury-contaminated Land in Abandoned Traditional Gold Mines in Indonesia

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Abstract. Traditional gold mining activities release mercury into the environment, creating a major concern for the Indonesian governments today. *In situ* bioremediation, which draws on the activities of indigenous soil bacteria for the recovery of mercury-contaminated land, has never been conducted intensively in the country. This research set out to determine the most efficient *in situ* bioremediation strategy for this purpose. It took place in Mandor Village, Landak Regency, Kalimantan Barat-Indonesia. During the experiment, four groups of sampling plots were made into triplicate and given various treatments: a. nutrient addition, b. aeration, c. pH neutralization, and d. without nutrient addition and aeration as a control. pH neutralization was conducted in all sampling plots by adding lime until soil pH of ±7 was achieved. The experiment was performed during both rainy and dry seasons to determine the influence of seasonal weather. Total mercury levels of each plot were measured on day 0, 30, 60, 90, and 120, and the effects of treatments and time on mercury depletion were analyzed by two-way ANOVA ($P<0.05$), followed by a post hoc test to identify the best treatment and optimum time for in situ bioremediation. The results showed that the best time to conduct this bioremediation was in the rainy season by applying nutrient addition and aeration for 90 days on soils with neutral pH; these stimulations could remove ±89.6% of the mercury. This bioremediation technique is a novel technological approach in land recovery that local governments can adopt to restore soils contaminated with mercury from traditional gold mining.

Key words: bioremediation technique; in situ; indigenous bacteria; sampling plot

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INTRODUCTION

Local communities have been mining gold sporadically and traditionally using very simple technology without regard to environmental sustainability. These activities use mercury to bind gold and, thereby, release mercury into the environment (Mirdat et al., 2013). Mercury is a highly toxic heavy metal (Tchounwou et al., 2014) that makes most of the land formerly used for traditional gold mining cannot be re-utilized.

There have been various remediation efforts applied to recover the normal functions of the soil and allow further utilization by the communities (Hardiani et al., 2011). Phytoremediation is among the most widely used land recovery methods (Ali et al., 2013). However, contaminated marginal soils limit vegetation growth and even cause plant death (Zhang et al., 2012).

Prior scholars have confirmed that remediation using microorganisms can naturally reduce or remove unwanted compounds from soils, mud, groundwater, and surface water until the environment is clean (Abatenh et al., 2017). Several types of microorganisms are known to have the ability to remove different pollutants, including heavy metals, from the environment (Alhasawi et al., 2015). Compared to physical methods, this removal technique is more environmentally friendly. Also, for heavy metal pollutants in relatively low presence, it offers more advantages, including low cost and in situ application (Sinha et al., 2012). The utilization of microorganism activities requires information on the condition of local habitats (Rodrigues et al., 2015). In situ land bioremediation is a proven strategy for overcoming hydrocarbon pollutants in the environment (Suja et al., 2014) using many different approaches, including biostimulation with indigenous microorganisms, nutrient addition, and aeration; bioaugmentation by introducing certain microbial cultures that use/consume pollutants; and the combination of both (Cerqueira et al., 2011).

This research, during the field experiment, applied nutrient addition and aeration and factored in environmental factors, such as pH and water moisture, to improve the activity of indigenous microorganisms—a variable of bioremediation technique. It was designed to determine the most *in situ* bioremediation technique with the best performance in recovering mercury-contaminated land. Also, it will add to the knowledge about this specific technique that remains under-researched and provide local governments with the basis for determining optimum bioremediation strategy for former traditional gold mines in Indonesia.

METHODS

This research was conducted in Mandor Village, Mandor District, Landak Regency, Kalimantan Barat Province, Indonesia, from April to July 2019.

Experimental Design

The bioremediation technique was examined using a plot test. Each plot was 1 m^2 in size, and for triplicate measurements, plots were placed in a row with a distance interval of ± 1.5-2 m, producing samples with relatively homogeneous physical-chemical-biological properties (Suja et al., 2014). These plots were then categorized according to rows and given different treatments (Table 1). The experiment factored in seasonal conditions: sample plots in the dry season were conditioned by creating a structure with transparent roofs, while the ones in the rainy season remained open.

Aeration (Plots B and C) was carried out by plowing the soils to a depth of 50 cm (Suja et al., 2014), and the soil pH was neutralized (pH 7.00) by adding lime. Throughout the dry season, soil moisture was maintained to mimic the natural condition by watering once every week.

Table 1. Treatments Applied to the Sampling Plots during the Field Experiment

Plots in Row	Treatments
A	Nutrient addition: NPK 300 g/l/m^2 and cow dung 1 kg/m^2, without aeration (with pH treatment).
B	Without nutrient addition and with aeration (with pH treatment)
C	Nutrient addition: NPK 300 g/l/m^2 and cow dung 1 kg/m^2, with aeration (with pH treatment).
D	Without nutrient addition and aeration (with pH treatment)

Bioremediation experiment

The bioremediation experiment involved stimulating the activities of soil bacteria (**Table 1**). Samples were collected by drilling boreholes using a hand drill to depths of 0-50 cm from the ground surface (Pimmata et al., 2013). The total mercury concentration of each plot was measured using a mercury analyzer. Changes in soil pH were monitored to maintain this variable at neutral. Bacterial activities in the bioremediation in every treatment were observed based on the number of cells (bacterial population) in a particular time unit: Days 0, 30, 60, 90, and 120 in the rainy and dry seasons. The bacterial population of every treatment was cultured in the NA medium using the pour plate method and incubated at room temperature to promote growth. Afterward, the population size of bacterial colonies was observed microscopically and enumerated by Gram staining (Winardi et al., 2019).

Statistical Analysis

Throughout each season, the effects of treatment (stimulation), bioremediation time, and treatment-time interaction on the reduction of pollutant levels in soils were analyzed using the two-way ANOVA, with a significant interaction marked by $P<0.05$. A post hoc test was intended to determine the best treatment and optimum bioremediation time that generated the highest bioremediation performance in the experiment.

RESULTS AND DISCUSSION

This research compares the results of different bioremediation treatments in rainy and dry seasons, which were later tested for the effects of seasonal weather on bioremediation performance. Treatments and time producing optimum bioremediation results and performance were determined as the best *in-situ* bioremediation strategy.

(a). Sampling Plots in the Rainy Season (b). Sampling Plots in the Dry Season

Figure 1. The condition of the sampling plots in the rainy season (a) and dry season (b)

Over 120 days of observation in the rainy season, all treatments were able to reduce total mercury concentrations at the rates of 60-66%. During this season, mercury levels showed a decreasing trend from Day 30 until Day 90 but had signs of an increase on Day 120. Such an increase was attributed to mercury-carrying surface runoff produced by rain. Rainwater

oxidizes highly volatile mercury (Hg^o) into its highly reactive form (Hg^{2+}) that persists in soils (Mori et al., 2013). Meanwhile, after 120 days, bacterial activities continued to decline due to nutrient depletion over the rainy seasons. The availability of nutrients affects soil microbial activities (Perelo, 2010).

Throughout the rainy season, the oxidation process will add Hg^{2+} to the naturally occurring Hg^{2+} in soils and, thus, elevate the overall concentration of Hg^{2+}. The Hg^{2+} oxidized by Hg^o tends to persist in a relatively long time in soils (Couto et al., 2012). Depending on environmental factors, soil microbial activities will reduce Hg^{2+} back into Hg^o (Kardena et al., 2020).

The most significant mercury depletion in 120 days was in Plot C, which was treated with nutrient addition and plowing (Table 2.). Adequately added nutrient and oxygen into the soil stimulated the activities of soil bacteria that transform Hg^{2+} into its volatile form, Hg^o. These stimulations basically accelerate the process of pollutant removal (Perelo, 2010). Aeration, nutrient addition, and pH neutralization (Plot C) advance soil microbial activities in transforming Hg^{2+} back to volatile Hg^o and, thereby, lower the total mercury concentration in soils (Dash & Das, 2012).

Table 2. Mercury concentrations at the sampling plots in the rainy season

Plots	Treatments	Total Mercury Concentration (µg/kg)				
		Day 0	Day 30	Day 60	Day 90	Day 120
A.	Nutrient addition without plowing	128.68	12.28	53.38	47.12	32.32
B.	Without nutrient addition, with soil plowing	189.41	49.72	20.42	11.04	36.75
C.	Nutrient addition and soil plowing	307.02	23.35	12.37	21.92	42.05
D.	Control (without nutrient addition and without soil plowing)	192.30	133.56	40.13	8.15	74.24

Surface flow ensures oxygen availability and creates a more even distribution of nutrients and pollutants (Li et al., 2015). There is a relationship between high soil microbial activity and a decrease in pollutant concentrations under possible environmental conditions (Groudev et al., 2010), including proper nutrient availability, soil moisture, and pH. High microbial activity was indicated by the large population of soil bacteria, especially in Plot C, where soil bacteria had multiplied more significantly than in other plots for every period of bioremediation, from Day 0 until Day 90. On Day 120, the largest bacterial population was found in Plot B because the soil bacteria had developed high adaptability to the treatment, no nutrient addition, from the start. Adaptability is strongly dependent on the condition of the local environment in which the bacteria live (Rodrigues et al., 2015).

Observation during the rainy season found that the bacterial population increased, primarily until Day 30, but decreased in the next observation period due to lack of nutrients and oxygen. Plots with nutrient addition (A and C) showed a relatively larger bacterial population than those without nutrient addition (Table 3). In this context, nutrients are a growth-limiting factor (Groudev et al., 2010).

The reduction of mercury content in soils involves a metal transformation process. In this research, mercury-contaminated soil bioremediation converted mercury from reactive Hg^{2+} to volatile Hg^o using microbial activities. Microorganism-induced metal transformation in this process aims at converting active metals in contaminated soils to inactive ones (Sathendra et al., 2018). Microorganisms can remove and/or change the form of metals (Smitha et al., 2017).

Table 3. The population size of soil microbes in the rainy season

Plots	Treatments	Bacterial Population (cfu/gr soil) x (10^3)				
		Day 0	Day 30	Day 60	Day 90	Day 120
A.	Nutrient addition without plowing	48.0	290	160	40	40
B.	Without nutrient addition, with soil plowing	70.0	190	20	90	80
C.	Nutrient addition and soil plowing	79.0	380	220	170	20
D.	Control (without nutrient addition and without soil plowing)	64.0	70	10	20	20

Table 4. The decrease in mercury concentrations in the rainy season

Plots	Treatments	Reduction of Mercury Concentrations (%)				
		Day 0	Day 30	Day 60	Day 90	Day 120
A.	Nutrient addition without plowing	0	90.4	58.5	63.4	74.9
B.	Without nutrient addition, with soil plowing	0	73.8	89.2	94.2	80.6
C.	Nutrient addition and soil plowing	0	92.4	96	92.9	86.3
D.	Control (without nutrient addition and without soil plowing)	0	30.5	79.1	95.8	61.4

The depletion of pollutant levels (%) reflects the performance of the bioremediation technique applied (Table 4). In the rainy season, such performance is determined by microbial activity, which is influenced by the availability of nutrients and oxygen in the soil (Febria et al., 2016). The plots treated with nutrient addition (Plots A and C) had relatively better bioremediation performance than the plots without such treatment (Plots B and D). Similarly, the plots treated with plowing, which re-introduces oxygen into soils, showed a significantly higher bioremediation performance than those that were not plowed but received enough oxygen supply from surface runoff. Nutrients in the rainy season were, however, adequate for only 30 days, during which nutrients were distributed relatively fast both horizontally on the soil surface and vertically as a result of surface flow. Consequently, bioremediation showed good performance in the first 30 days (Figure 2).

The ANOVA results confirmed the effects of time and treatment on mercury reduction (sig<0.05) and a correlation between time-treatment interaction and mercury reduction in the rainy season (sig<0.05). The latter asserts that treatment as a variable does not independently affect the decrease in mercury concentration but jointly with another variable, bioremediation time. Based on their interaction, the optimum time and best treatment were able to be determined.

The post hoc test of bioremediation time revealed that on Day 90, the reduction in mercury concentrations was the highest, i.e., 93.12%. For this reason, 90 days are concluded as the optimum bioremediation time (t_{opt}) during the rainy season. This relatively long removal period is inseparable from the activity of mercury-resistant soil bacteria in transforming mercury. These bacteria have the genes of mer operons, i.e., mer A, which produces the mercuric reductase enzyme to reduce Hg^{2+} to Hg^{o} (Purkan et al., 2017). Time determines the length of interaction between microorganisms and pollutants that can increase the adaptability and the ability of microorganisms to remove contaminants (Rodrigues et al., 2015). Bacteria with high adaptability to environments with severe heavy metal contamination are assumed to be effective in reducing heavy metals (Rezekikasari et al., 2018).

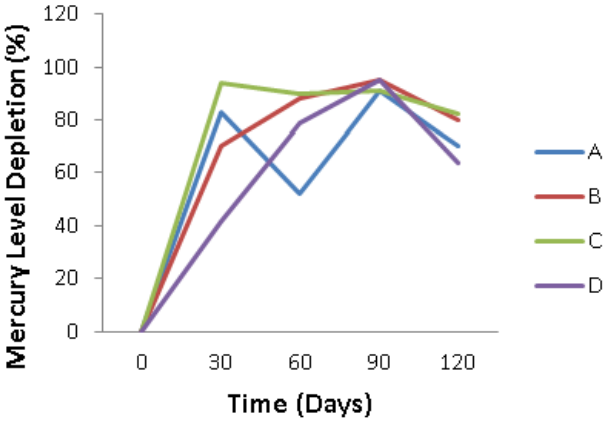

Figure 2. The decrease in mercury concentrations in the rainy season

The post hoc test of all treatments showed that Plot C had the most significant mercury transformation during the rainy season. Therefore, these plot's stimulations are deemed the best treatment for the season because they removed up to 89.6% mercury found in soils. Groudev et al. (2014), through a study of bioremediation of soils contaminated by metal pollutants other than mercury, found that bioremediation removes the presence of Fe (by 80%), Mn (76%), Zn (69%), Cu (68%), and Cd (> 50%). The proven effect of treatment on Hg transformation shows that a decrease in this metal concentration is attributed to not only the process of washing by surface runoff but also microorganism activity. Besides, nutrient and oxygen (aeration) addition in neutral pH maximizes the reduction of total mercury levels (Lors et al., 2012). Consequently, the treatments given during the rainy season basically affect microorganism activities in transforming total mercury in soils.

Similarly, throughout the 120 days of observation in the dry season, all treatments successfully lowered mercury levels in soils by up to 50-84%, but the most substantial reduction was observed on Day 30 (Table 5).

Table 5. Mercury concentrations of the sample plots in the dry season

Plots	Treatments	Total Mercury Concentration (µg/kg)				
		Day 0	Day 30	Day 60	Day 90	Day 120
A.	Nutrient addition without plowing	118.90	7.28	31.55	33.93	51.26
B.	Without nutrient addition, with soil plowing	215.05	29.53	50.94	25.42	69.93
C.	Nutrient addition and soil plowing	126.72	26.24	42.80	31.67	45.4
D.	Control (without nutrient addition and without soil plowing)	254.58	81.38	124.37	62.85	41.75

In the soil, mercury can appear in the form of Hg^o that is volatile (Connor et al., 2019). During the dry season, mercury-resistant bacteria were found in large populations in the early observation period (Day 30) mostly because Hg^o in the soil evaporated naturally. The oxidation of Hg^o to Hg^{2+} is unavoidable because sandy soils are rich in oxygen, thus accelerating mercury oxidation (Mori et al., 2013). However, this oxidation only involves a relatively small amount of Hg^o compared to the one occurring in the rainy season. Together with the naturally occurring Hg^{2+} in the soil, the Hg^{2+} produced in the oxidation will be reactive and very difficult to transform back to Hg^o because of low microbial activity in the dry season; it is evident from how the stimulations could not significantly reduce the levels of mercury in soils (Table 5).

During the dry season, the population size of microorganisms rose until Day 30 and later decreased until Day 120 due to a lack of nutrients, oxygen, and moisture in the environment after thirty days of observation. Day 30 also marked the highest bioremediation performance at all sampling plots (Figure 3), and the role of microbial activity in reducing pollutants was categorically smaller than that of natural evaporation of mercury. Nutrient and oxygen addition to Plot C created a suitable environment for optimum microbial growth, resulting in a relatively large population size, but did not significantly lower mercury concentrations than other treatments (Tables 6 and 7). Here, soil moisture is believed to be the other factor that substantially influences soil microbial activities (Donlon & Bauder, 2012). Meanwhile, treatments without nutrient addition and aeration (Plot D) allowed the growth of a large number of bacterial populations. Some microbes have genetic equipment that can keep them alive and even grow optimally in extreme environments (Zeng et al., 2017).

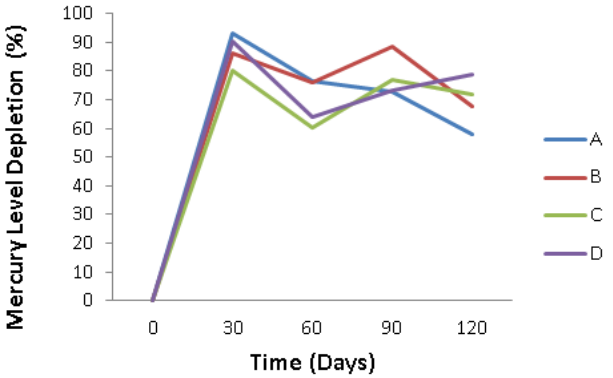

Figure 3. The decrease in mercury concentrations in the dry season

Table 6. The population size of soil bacteria in the dry season

Plots	Treatments	Bacterial Population (cfu/gr soil) x (10^3)				
		Day 0	Day 30	Day 60	Day 90	Day 120
A.	Nutrient addition without plowing	> 300	360	230	20	15
B.	Without nutrient addition, with soil plowing	58	119	60	60	140
C.	Nutrient addition and soil plowing	15.0	430	200	220	320
D.	Control (without nutrient addition and without soil plowing)	16.0	250	20	110	220

Table 7. The decrease in mercury concentrations in the dry season

Plots	Treatments	Bioremediation performance (%)				
		Day 0	Day 30	Day 60	Day 90	Day 120
A.	Nutrient addition without plowing	0	93.4	73.5	71.5	56.9
B.	Without nutrient addition, with soil plowing	0	86.2	76.3	84.2	67.5
C.	Nutrient addition and soil plowing	0	79.3	62.2	75	71.8
D.	Control (without nutrient addition and without soil plowing)	0	68	51.1	75.3	83.6

Since low soil moisture inhibits microbial growth, the activities of soil bacteria in removing mercury through the mechanism: $Hg^{2+} \rightarrow Hg^{o}$, become low. The amount of Hg^{2+} converted to Hg^{o} is relatively small, meaning that mercury reduction through this mechanism is insignificant. Here, mercury removal instead occurs as a result of direct evaporation at the beginning of the bioremediation without the influence of microbial activity. In the context of metal pollution, in situ bioremediation depends on the metabolic capacity of indigenous microbes and environmental conditions, including soil moisture (Rodrigues et al., 2015). Water content in the soil acts not only as an oxidizer that can change Hg^{o} to Hg^{2+} but also as an environmental factor that supports microbial activity (Dash & Das, 2012). It is crucial in the life, growth, and metabolic activity of soil microbes (Fahruddin, 2014), meaning that seasonal conditions are a vital determinant of bioremediation performance.

The results of ANOVA on bioremediation time, treatment, and mercury transformation showed that only variable 'time' affected the reduction of mercury levels. On the contrary, the treatment given throughout the experiment did not significantly affect mercury removal. The post hoc tests on variable 'time' showed that 30 days was the optimum time to achieve the most significant reduction in mercury levels (87.4%) during the dry season.

Another factor of bioremediation performance is the type of bacteria used. In this research, the bacterial consortium growing and working in the rainy season was different from during the dry season (Table 8). The morphological test of bacterial cultures in Plot C found three shapes of cells: bacillus, coccus, and diplococcus. Based on gram staining, bacteria that played a part in the rainy season were all gram-positive, while the ones in the dry season were gram-positive and negative. During the study, bacterial diversity was high, signifying a good soil quality after the treatment (Marou et al., 2018).

Table 8. The morphology of indigenous bacterial cultures in the rainy and dry seasons

Seasons	Gram-Staining Results	Cell Shapes	Bacterial Cultures
Rainy	Gram-positive	bacillus	*Bacillus subtilis*
	Gram-positive	coccus	*Staphylococcus epidermidis*
	Gram-positive	diplococcus	*Stomatococcus* sp.
	Gram-positive,	bacillus	*Bacillus cereus*
Dry	Gram-positive,	bacillus	*Bacillus subtilis*
	Gram-positive,	bacillus	*Bacillus subtilis*
	Gram-negative,	bacillus	*Pseudomonas cepasia*
	Gram-positive,	bacillus	*Bacillus subtilis*
	Gram-positive,	diplococcus	*Stomatococcus* sp.
	Gram-positive,	coccus	*Micrococcus* sp.
	Gram-positive,	bacillus	*Bacillus cereus*
	Gram-negative,	bacillus	*Proteus mirabilis*
	Gram-positive,	diplococcus	*Stomatococcus* sp.
	Gram-positive,	coccus	*Staphylococcus*
	Gram-positive,	bacillus	*Bacillus subtilis*

The biotransformation rate of heavy metals depends on, among others, microbial activity, availability of nutrients, and the environmental determinants of these factors (Dewi et al., 2019), namely soil moisture and pH. Water can increase microbial activity in reducing Hg^{2+} to Hg^{o} that is volatile. At the same time, neutral pH creates a condition that prevents mercury ions and heavy metals that are reactive in the soil from dissolving (Mirdat et al., 2013) and alleviates extreme physical, chemical, and biological envi-

ronments to enable and optimize microbial activity in transforming mercury in the soil. Microbes can generally grow at good rates at pH 6.8-8.0 (Kurniati et al., 2016). A bioremediation study using *Bacillus cereus* and pH variation by Sinha et al. (2012) found that a lower percentage of mercury removal coincided with a lower pH. Too acidic soils reduce the contribution of bacteria in removing mercury from soils.

Stimulations, in the form of nutrient addition, aeration, and pH neutralization (near-neutral), resulted in the maximum decrease in mercury concentration in the study. Physical, chemical, and biological conditions in the environment need to be regulated to support the bioremediation process, which is an interaction between environmental factors (temperature, pH, soil moisture, and aeration) and biological factors (microorganisms) (Groudev et al., 2010). Many approaches can be used to enhance microorganism activity in bioremediation, including (1) optimization of oxygen and nutrient availability and (2) control of pH, humidity, and temperature (Dashti et al., 2015).

Nutrient addition, plowing, and pH neutralization triggered an increase in the activity of indigenous bacteria in the rainy season for 90 days. These stimulations are, thereby, the best bioremediation strategy in reducing mercury concentrations in abandoned mines, which is still not widely conducted in Indonesia. This bioremediation technique is a novel technological approach to restoring abandoned land contaminated with mercury after being used for traditional gold mining practices. It is expected to provide a recommendation for the local government in bioremediating such land in Indonesia.

CONCLUSION

Indigenous bacteria are an excellent metal-removing agent for the *in situ* bioremediation of mercury-contaminated soils. The best time to conduct bioremediation is the rainy season, with stimulations including nutrient addition, aeration by plowing, and pH neutralization by adding lime for 90 days.

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