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Effect of biochar on microbial population in heavy metal contaminated soil for 23 years

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ABSTRACT

Soil microbial populations and activities have been repeatedly reported to be severely affected by high concentrations of heavy metals. However, little of this information comes from tropical soil. The fungal and bacterial populations in tropical soils contaminated with heavy metals were observed in a laboratory study. Soils that have been amended once with different rates of heavy-metal-containing waste (0-60 Mg ha^{-1}) in 1998 (23 years ago) were used in this study. We then treated the contaminated soils with different rates of biochar (0-10 Mg ha^{-1}). Biochar is known to significantly reduce heavy metal contaminants through various immobilization reactions. The soil-biochar mixtures were allowed to equilibrate at the soil field water capacity, maintained by a common water reservoir beneath the soil-biochar mixtures, for 4 weeks. After this period, the soil fungal and bacterial populations were counted. The results of the present study showed that high soil levels of Cu and Zn significantly enhanced the fungal population. In contrast, the bacterial population was not affected by the presence of Cu and Zn. In the highly contaminated pots, the addition of biochar significantly enhanced the population of soil fungi (identified as *Aspergillus* sp.), but it did not affect the population of bacteria. The results of the study suggest that biochar application led to significant enhancement of the population of *Aspergillus* sp. in pots with high soil Cu and Zn levels, most likely through improved habitat conditions provided by biochar's porous structure, which could be leveraged in bioremediation efforts for heavy metal-contaminated soils.

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1. INTRODUCTION

Several laboratory experiments have demonstrated that the population and activities of soil microorganisms are affected by the presence of high amounts of heavy metals (Jamal et al., 2013; Sun et al., 2023; Xie et al., 2016). Most researchers have observed that an increase in heavy metal levels lowers the soil microbial population and its activities. For example, the activity of soil microorganisms was reduced in Pb-contaminated soils (Sun et al., 2023). Some other researchers, on the contrary, observed that several heavy metals stimulated an increase in microbial population and activity (Arif et al., 2016). The changes in the soil microbial population and activities may eventually affect various important soil properties and processes, among which are the soil acidity, chelate concentrations, enzymatic activities (Qu et al., 2020), organic matter decomposition, mineral weathering processes (Ribeiro et al., 2020) organic acid excretion (Dietz et al., 2020; Salam & Sriyani, 2019; Wu et al., 2018; Yang et al., 2019), land use change (Meena & Rao, 2021), and chemical fertilizers (Khalisha et al., 2022).

Some researchers have reported soil pollution by heavy metals from various regions worldwide. The increase in human activities, such as industrial growth and urbanization, has led to the release of pollutants into the environment (Timothy & Williams, 2019). Heavy metal concentrations were found to be significantly higher in urban recreational areas (Popova, 2016; Sodango et al., 2018; Yang et al., 2018), industrial zones (Adejoh, 2016; Aksu, 2015; Salam, Pakpahan, et al., 2021), mining sites (Sun et al., 2019; Wei et al., 2015), and coastal areas (Febriansyah et al., 2021; Yunus et al., 2020). The destructive effects of heavy metals were also observed in soils polluted with heavy metals (Sun et al., 2023; Xie et al., 2016). Xie et al. (2016) reported that heavy metal

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pollution may cause adverse effects on microbial communities and activities, as well as an impact on plant growth and genetic structure. The soil microorganism community and population were lowered in soil contaminated by Pb (Sun et al., 2023). The decrease was even more significant with the increase in more toxic elements, such as Ag and Pb. Salam (2014) suggested that high levels of heavy metals can reduce the population of soil microorganisms, which in turn may decrease the activity of soil enzymes. Since soil microorganisms are primary producers of these enzymes, this decline is detrimental to soil health. Many of these microbes are beneficial for plant growth (Setiawati et al., 2023).

Since heavy metals negatively impact various soil properties and processes, it is essential to mitigate the adverse effects of heavy metals on soil microorganisms. The easiest way is by lowering the heavy metal concentration through several mechanisms that may increase the soil adsorption capacity concerning heavy metals by the addition of some soil amendments (El-Maghrabi & Mikhail, 2014; Hayyat et al., 2016; Lahori et al., 2020; Lekfeldt et al., 2017; Patra et al., 2020; Salam et al., 2022; Selvi et al., 2019; Siddika & Parveen, 2022; Wang et al., 2022; Xiao et al., 2017; Zhang et al., 2021). One of the methods is the use of biochar (Baquy et al., 2022; Chen et al., 2018; Komkiene & Baltrenaite, 2016; Salam, 2022; Siddika & Parveen, 2022; Sukartono et al., 2022; Taraqqi-A-Kamal et al., 2021; Wang et al., 2018; Zhang et al., 2021). Biochar possesses several key properties that render it to effectively remediate soils contaminated with heavy metals. These include a substantial surface area, various reactive functional groups, an increase in soil pH, and high capacity for cation exchange. When applied to heavy metal-polluted soils, biochar can significantly lower the levels of these contaminants through various immobilization processes. These reactions include precipitation, ion exchange, physical sorption, chemisorption, and complexation. Consequently, biochar can decrease heavy metal bioavailability, mobility, and toxicity to living organisms. Additionally, biochar enhances overall soil health and promotes plant growth (Baquy et al., 2022; Ebido et al., 2021).

However, limited research has been reported on the above phenomena in tropical soils, which exhibit significantly different properties compared to those of temperate soils, particularly in terms of their pedogenesis, such as temperature and precipitation rates. Most research reports were also related to plants, animals, and humans (Aprile & De Bellis, 2020; Arif et al., 2016; Asati et al., 2016; Khodijah et al., 2019). The researchers also evaluated the effect of heavy metal-containing waste amendments on the growth of the plant, revealing significant increases in both the root and shoot growth of elephant grass. A strong correlation was observed between the levels of copper (Cu) and zinc (Zn) in the soil and the corresponding growth measurements. The findings show that both root and shoot growth, as well as the absorption of copper (Cu) and zinc (Zn) by elephant grass, are influenced by the concentrations of these metals present in the soil (Salam, Hidayatullah, et al., 2021). Although heavy metals can be harmful to plants and microorganisms, the impact on soil microorganisms is especially concerning, as these populations are vital for maintaining soil health and fertility. To explore this issue further, we then studied the fungal and bacterial populations in tropical soils contaminated with heavy metal-containing industrial waste for 23 years.

2. MATERIALS AND METHODS

2.1. Study site and material descriptions

The experiment was conducted in the glasshouse of the Faculty of Agriculture, University of Lampung, Bandar Lampung, Indonesia (5°22’11.38’’S, 105°14’25”E). Soil samples were taken from a field experiment on applying industrial waste with a high level of Cu and Zn. These experiments have been ongoing since 1998, located in Sidosari, Natar, South Lampung, Indonesia (50°20'13 ''S, 105°14'38 '' E). The soil type in Sidosari, Natar, is Ultisols (Salam, Pakpahan, et al., 2021). Three different bulk samples of soils treated with industrial waste at the rate of 0 (Control), 15 (Low Metal Level), and 60 Mg ha^{-1} (High Metal Level) collected from Sidosari, Natar, South Lampung, (from a 0–15 cm depth) were used in this lab trial. All soil samples (0-15 cm depth) were thoroughly mixed after air-drying and then finely ground to pass through a 2 mm sieve. Gravimetric measurement was used to determine the soil water content (at 105 °C, 24 hours) for treatment material calculation. Table 1 presents some of the soil's chemical and physical properties. A simple pyrolizer was used to prepare biochar from rice husk. Charring was conducted for 4 to 5 hours. Biochar was air-dried after charring and measured for its water content (Fig. 1).

2.2. Experimental design and soil analysis

To evaluate the response of soil microorganism population on the effect of heavy metals and biochar, 200 g air-dry samples of the heavy-metal-contaminated soils and air-dry biochar were thoroughly mixed on clean plastic sheets and placed into clean plastic pots. All mixtures in pots were then incubated for four weeks at the soil water content at field capacity. The soil water field capacity was maintained by connecting the soil-biochar masses in pots to a common water reservoir located beneath the pots, which were placed on a wooden board through cotton wicks. The water reserve in the common reservoir was daily maintained to ensure the soil–biochar mixture had sufficient water moisture at field water capacity (Fig. 2). The pot construction, along with the capillary system, was put in a shaded room in a glasshouse.

The pots for the incubation experiment were set up using a two-factor experimental layout based on a randomized block design, i.e, polluted soils and biochar. Soils were those treated with heavy metal-containing waste at 0 (Control), 15 (Low Metal), and 60 Mg waste ha^{-1} (High Metal) as reported in Salam, Pakpahan, et al. (2021). The rice husk biochar comprised three levels: 0, 5, and 10 Mg ha^{-1}. Each treatment was replicated three times, which was assumed as a block. The design provided a total of 27 experimental units.

The soil-mixture samples were taken after the four weeks of incubation time and analysed for the soil Cu and Zn extracted by 1 N HNO_3 (Flame AAS), soil organic C (Walkley and Black method), pH (pH electrode), and the fungal and bacterial population.

Table 1. The properties of the soils used in the experiment prior to biochar application

Metal Level	Waste	pH	CEC	Sand	Silt	Clay	Organic C	Cu	Zn
	Mg ha^{-1}		cmol(+) kg^{-1}	……………. % ……………			g kg^{-1}	…. mg kg^{-1} ….	
Control	0	5.61	8.6	34.4	34.5	31.1	16.1	44.6	33.8
Low Metal	15	5.71	11.2	35.3	33.8	30.9	22.0	39.5	32.3
High Metal	60	5.64	11.0	33.6	33.7	32.7	17.1	63.1	45.1

Remarks: average of three replicates

Figure 1. Biochar production equipment

The method used to calculate the total number of soil bacteria and fungi involved growing bacteria and fungi from dilutions in petri dishes using the spread plate count method. The soil extraction was performed using the Dilution Plating Method (Mwangi & Lelei, 2023). The bacterial population was observed using the spread plate method on nutrient agar (NA), incubated for 4 days, and then the total colonies that appeared on the surface of the agar were counted using a Quebec Colony counter. However, for total fungi colonies, it was grown on Potato Dextrose Agar (PDA) with an incubation period of 7 days. The formula for calculating the population of bacteria and fungi was as Equation 1 (Mwangi & Lelei, 2023).

$Total\ Population\ (CFU)\ g$ soil^{-1} = $total\ colony\ dillution\ factor\ x\ soil\ dry$-weight .... [1]

2.3. Data analysis

Analysis of variance (ANOVA) was used to evaluate the data, and treatment comparisons were subsequently made using 5% significance level of Honest Significant Difference - (HSD) test. Additionally, the Relationship between variables was also determined through Principal Component Analysis (PCA), whether they are related to each other or independent (Abdi & Williams, 2010).

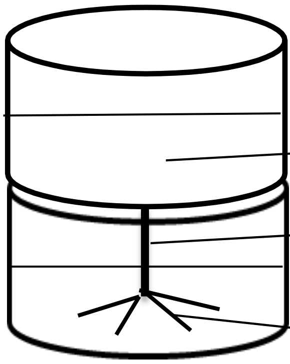

Figure 2. The pot construction was used to evaluate the effect of heavy metals and biochar on the soil microbial population.

3. RESULTS

3.1. Soil heavy metal levels

The analysis of variance revealed that the effect of heavy metal waste amendment was significant on the soil levels of Cu and Zn at the 5% significance level (Table 2). Significant variations in Cu and Zn levels were found between high-metal pots and the control, as well as low-metal pots. The soil analysis results indicated that the residual levels of copper (Cu) in the high heavy metal pots, ranging from 63.1 to 68.2 mg kg^{-1}, showed a statistically significant improvement than those observed in the control pots, which had levels between 44.6 and 48.1 mg kg^{-1}. Similarly, the residual levels of zinc (Zn) in the high heavy metal pots ranged from 45.1 to 49.3 mg kg^{-1}, also significantly surpassing the control pots, which recorded levels of 32.7 to 35.3 mg kg^{-1} (Table 3). When compared to the control pots, the increases in Cu and Zn levels in the high heavy metal pots were 41.5% and 39.0%, respectively. In contrast, the levels of Cu and Zn in the low heavy metal pots were relatively similar to those in the control pots.

3.2. Changes in fungal and bacterial population after 23 years

Table 3 presents the findings of this study, indicating that the population of fungi in high heavy metal pots (3.72 – 4.17 CPU g^{-1}) was significantly higher than in control pots (2.67 – 3.58 CPU g^{-1}), increasing by about 14 – 28%. However, there was no effect of waste amendment on the bacterial population (Table 2).

Table 2. The impact of heavy metal pollution in soils and the use of biochar on soil chemical and microbial properties

Treatment Factor[a]	pH	Organic C	Cu	Zn	Fungi	Bacteria
K	ns[b]	*	ns	ns	*	**
W	*	*	*	*	*	ns
B	ns	ns	ns	ns	ns	ns
WB	ns	ns	ns	ns	**	ns

Remarks: [a]K = treatment blocks, W = waste, B = biochar; [b]ns = non-significant at 5%

Table 3. The soil chemical and microbial properties of heavy-metal polluted soils treated with biochar

Metal Level	Waste	Biochar	pH	Organic C	Cu	Zn	Fungi	Bacteria
	.... Mg ha^{-1}			.. g kg^{-1} ..	..... mg kg^{-1}		...... log CPU g^{-1}...	
Control	0	0	5.61	16.0	44.6	33.8	3.58	5.10
		5	5.62	18.1	44.9	32.7	3.48	4.17
		10	5.64	21.0	48.1	35.3	2.67	5.06
Low	15	0	5.71	22.1	39.5	32.3	2.48	4.10
		5	5.80	25.0	36.8	31.0	2.97	3.88
		10	5.64	23.0	35.9	30.4	3.33	4.72
High	60	0	5.64	17.1	63.1	45.1	3.72	4.88
		5	5.63	19.1	68.2	49.3	3.38	3.92
		10	5.65	17.2	63.1	46.2	4.17	3.96

Table 4. The impact of heavy metal pollution in soils and the use of biochar on soil pH, organic carbon, and fungal population

Waste Level	pH	Organic C	Fungi
...... Mg ha^{-1}		...... g kg^{-1}	...... CFU g^{-1} ......
0	5.62 a	18.1 a	3.24 a[a]
15	5.72 b	23.1 b	2.94 a
60	5.63 a	17.2 a	3.75 b
HSD 5%	0.06	1.0	0.59

Remarks: [a] Different letters in the same column denote a significant difference at the 5% HSD test level

The ANOVA in Table 2 revealed that the waste amendment had a significant influence on the fungal population, but not on the bacterial population. The population of fungi in high-metal pots was significantly higher than that in the control and low-metal pots (Table 4). The population of fungi in high-metal pots was 13.6% higher than that in the control and 21.7% higher than that in low-metal pots (Table 4). Based on observations on agar medium, the character of the fungus that grew dominantly in the treatment applied was the fungal genus Aspergillus (Fig. 3).

3.3. Effect of biochar on selected soil properties and microbial population

The presence of biochar did not alter the soil pH, organic carbon, soil Cu and Zn levels, or the population of fungi and bacteria (Table 2). However, the population of fungi was significantly affected by the interaction of waste and biochar. The ANOVA shown previously in Table 3 demonstrates a significant interaction effect between waste and biochar on the population of fungi, but not on the population of bacteria, as well as on the soil pH, organic C, and Cu and Zn levels (Table 3). The HSD test showed that treating high-level biochar (10 Mg ha^{-1}) with high metal pots resulted in the highest logarithmic population of fungi, i.e., 4.17 CFU g^{-1}, which was significantly higher than that in the treatment of heavy metal control without biochar (0 Mg ha^{-1}), i.e., 3.58. However, it had a similar effect to 5 Mg ha^{-1}, i.e., 3.3 CFU g^{-1}, and caused no differences in low and high metal pots (Table 5). Fungus *Aspergillus* sp. was observed at all levels of heavy metals and biochar. *Aspergillus* sp. is a member of the phylum Ascomycota. The identified *Aspergillus* sp. in various combinations of biochar levels and heavy-metal-polluted soils is depicted in Figure 3.

Principal Component Analysis (PCA) is an analysis for measuring the relationship among various observation variables. If the results of each component show a relationship with each other indicated by lines forming an angle of less than 90 degrees, conversely, if the system formed by two variables creates an angle greater than 90 degrees, then there is no relationship between the two variables. Based on Principal Component Analysis (PCA) (Fig. 4), there was a close correlation between soil Cu and Zn levels with fungal population, but not with bacterial population. Additionally, other variables, such as soil pH and organic carbon, were not closely correlated with the soil levels of Cu and Zn.

4. DISCUSSION

The present study revealed that after a long-term period (23 years), the concentrations of copper and zinc in high heavy metal pots were approximately 1.40 times those in control pots. In accordance to the results in the current study,

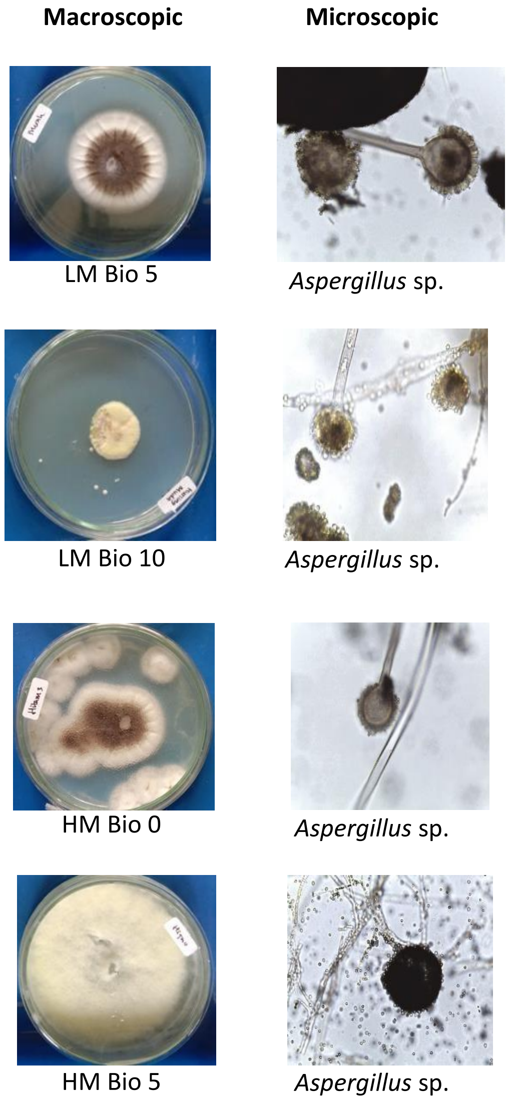

Figure 3. Fungal species identified in biochar-treated metal-polluted soils

previous studies in the same field site by others also reported that Cu and Zn in high metal soils were considerably higher than the values observed in low metal and control soils (Salam, 2022; Salam & Ginanjar, 2018; Salam, Hidayatullah, et al., 2021; Salam et al., 2022; Silva et al., 2021). The results of the present study also showed that treating high-level heavy metal-contaminated soils (60 Mg ha^{-1}) with biochar did not reduce the fungal population. However, the concentrations of Cu in the soil ranged from 63 to 68 mg kg^{-1}, and Zn ranged from 45.1 to 46.2 mg kg^{-1} in the soils. Studies reported that the toxic effects of heavy metals on fungal growth and development varied between species (Gadd, 2016).

Furthermore, Arnebrant et al. (1987) stated that *Fusarium* sp., *Alternaria* sp., and *Epicoccum* sp. were sensitive to soil Zn at a concentration of 57 mg kg^{-1}. In the present study, *Aspergillus* sp. was detected in all levels of heavy metals and biochar (Fig. 3). Congeevaram et al. (2007) reported that different species of *Aspergillus*, *Penicillium*, *Phanerochaete*, *Rhizopus*, and *Trichoderma* are efficient bioremediating agents. This fungus can alter or mitigate the toxicity of metal contaminants through pH changes, biosorption, and bioaccumulation (Anand et al., 2006). Fungi produce intracellular and extracellular enzymes to attract metals. They may have an active transport process of metal ions outside the cell by chelating the enzymatic transformation of metal ions and producing metal-binding compounds (Gonzalez-Chavez et al., 2002).

The application of biochar creates microhabitats through its particles and pores, promoting the colonization of fungi and other filamentous microbes (Paneque et al., 2016). In addition, due to biochar's distinct mode of action, it can influence microbial metabolism in diverse ways, causing measurable shifts in microbial community taxonomy composition (Khodadad et al., 2011). These could be the reasons why *Aspergillus* sp. was found in all pots with varying levels of heavy metals and biochar in this study. However, it was not the case for the population of soil bacteria. Moreover, this study also demonstrated that biochar mitigated the adverse effects of Cu on soil microorganisms, particularly on fungi. These results could be related to the high specific surface area of biochar, which provides a suitable habitat for microorganisms. The pore structure and volume of biochar increase water retention, soil CEC, and nutrient availability in soils (Lu et al., 2015). Heavy metal contamination can lead to various alterations in microbial community structures. Despite significant changes in many chemical and biological properties of the soil, many native microorganisms persist within the microbial community (Pérez-de-Mora et al., 2006). Over time, soils contaminated with heavy metals tend to favor microorganisms that are specifically capable of adapting to the polluted environment (Kozdrój & van Elsas, 2001).

Previous studies at the same trial site demonstrated that higher soil levels of Cu and Zn significantly affected the growth of different plant species. The soil Cu and Zn levels correlate well with the inhibition of growth of thorny amaranth, mustard, Napier grass, lettuce, land spinach, and water spinach (Salam et al., 2022; Silva et al., 2021) in high-metal soils. Unlike in the case of plant growth, this phenomenon was not observed in the fungal and bacterial populations (Table 2). The measured population of bacteria was not affected by the presence of high levels of Cu and Zn (Tables 2 & 3). It indicates that bacteria were most likely persistent in the high residual concentrations of heavy metals in the soil. The levels of heavy metals, approximately 69–70 mg kg^{-1} of Cu and 30–50 mg kg^{-1} of Zn, were likely still below the level that could have adverse effects on the soil bacterial population.

In contrast, these levels of heavy metals significantly increased the populations of fungi by 13,6 % (Tables 2, 3, & 4). In short, the presence of copper and zinc in the soil stimulated fungal growth but not bacterial growth. Currently, some reports suggest that soil microorganisms can adapt to elevated and even toxic levels of heavy metals and other xenobiotics in the soil (Kozdrój, 1995). However, numerous studies have shown that the build up of heavy metals in soils can have toxic effects on soil microorganisms (Pawlowska & Charvat, 2004), leading to disruptions in their diversity, population sizes, and overall activities within the soil microbial communities (Kelly et al., 2003; Šmejkalová et al., 2003).

Table 5. The effect of biochar on the fungal population in soils polluted with heavy metals

Biochar Level	Waste Level		
	0 (Control)	15 (Low Metal)	60 (High Metal)
……. Mg ha^{-1} …….	…………………………….………. log CFU g^{-1} …………………………………….		
0	3.58 B b	2.48 A a	3.72 A b[a]
5	3.48 AB a	2.97 A a	3.38 Aa
10	2.67 A a	3.33 A ab	4.17 A b
HSD 5%		0.89	

Remarks: [a]Different capital letters in one column and lowercase letters in another denote a significant difference at the 5% HSD test

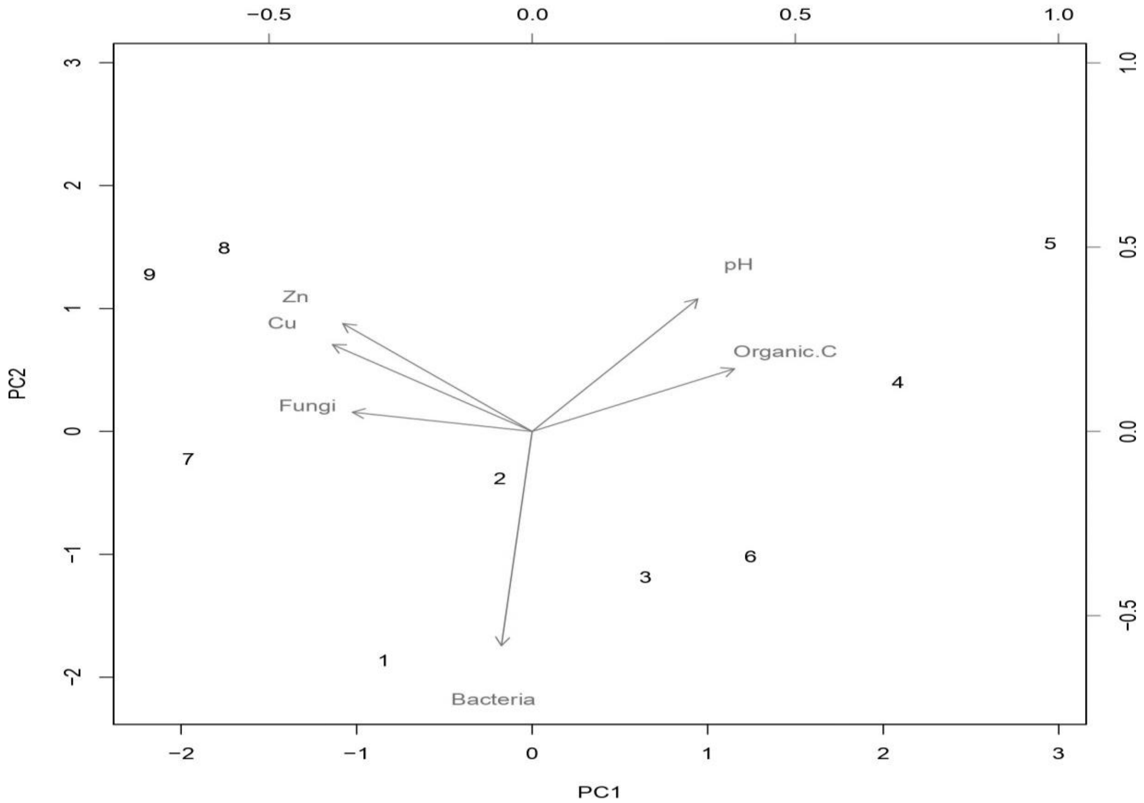

Figure 4. Principal Component Analysis (PCA) from soil chemical and biological properties after 23 years amended once with a heavy metal-containing industrial waste

Furthermore, in line with previous studies (Salam, 2022; Salam & Ginanjar, 2018; Salam, Pakpahan, et al., 2021; Salam et al., 2022). The results of this study revealed that the presence of biochar did not affect heavy metal levels in these soils (Tables 2 & 3). This study also found that the addition of biochar did not affect the soil bacterial population. In contrast to the bacteria population, the fungi population showed a significant increase due to the presence of biochar (Tables 2, 3, & 5).

The addition of 10 Mg biochar ha^{-1} enhanced the fungal population increased from a range of 2.67 – 3.58 under control conditions to 3.72 – 4.17 CFU g^{-1}, or an increase of 35.97 %. However, a field study observed that the application of sugarcane biochar to soils resulted in an increase in bacterial populations alongside a reduction in actinomycetes and fungi (Nie et al., 2018). On the other hand, another study found that when biochar was applied, there was a notable enhancement in fungal community diversity, while bacterial diversity significantly declined (Song et al., 2020). The addition of biochar could have improved soil structure and enhanced soil aeration, potentially supplying more oxygen (O_2) to support fungal growth.

5. CONCLUSIONS

This long-term study demonstrates that soils contaminated with elevated levels of Cu and Zn (approximately 1.4 times higher than control soils) maintain high residual metal concentrations even after 23 years. The fungal population, identified as *Aspergillus* sp., significantly increased after a prolonged period of 23 years, particularly in response to high levels of copper (Cu) and zinc (Zn) in the soil. In contrast, the bacterial population showed no effect from the elevated Cu and Zn levels, consistent with previous research findings. This suggests that fungal populations were stimulated by higher concentrations of Cu and Zn, indicating that different microbes respond uniquely to heavy metal stress. The current study revealed that adding biochar did not affect the levels of heavy metals in the soil. However, biochar significantly increased the population of fungi, particularly *Aspergillus* spp., in soils with high concentrations of copper

(Cu) and zinc (Zn). Though biochar did not reduce soil metal concentrations, it improved habitat conditions for fungi, which could be leveraged in bioremediation efforts. Meanwhile, the reactions of fungal and bacterial communities to heavy metals and biochar differ notably, emphasizing the importance of considering microbial community composition when designing remediation approaches. Furthermore, the resilience of *Aspergillus* spp. highlights their potential role in bioremediation strategies for soils contaminated with heavy metals.

Declaration of Competing Interest

The authors declare that no competing financial or personal interests may appear to influence the work reported in this paper.

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