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16870 / **SOPIALENA et al.** / Assessment of nematode and microbial diversity and screening of entomopathogens in revegetated post-coal-mining land in Kutai Kartanegara District, East Kalimantan, Indonesia

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sopialena sopialena:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Entomophatogenic Microbia Diversity at Revegation of Ex-Coal Mining Land". Complete your revision with a Table of Responses containing your answers to reviewer comments (for multiple comments) or enable Track Changes.

Our decision is: Revisions Required

Entomophatogenic nematodes and microbia diversity at revegetation land of post-coal mining in East Kalimantan

Comment [U1]: Is it really only entomopathogenic nematodes? What about entomopathogenic fungi and bacteria? Or are on entomopathogenic nematodes observed?

Comment [U2]: Was the diversity of microbes the soil also observed and counted in addition to those that function as entomopathogenic microb

Abstract. Post-mining management of coal should be followed by revegetation. The success of revegetation activities is largely determined by the fertility status of the soil which consists of physical, chemical and biological fertility components. Biological fertility is indicated by the presence of microbes and numbers of soil microbes. The study aims to identify entomophatogenic nematodes and soil microbes in land of ex-coal mining with palm oil vegetation in Kutai Kartanegara District and to analyze the relationships between the diversity of entomophatogenic nematodes-soil microbes and the chemical fertility status of the land. As a control, the non-coal mining area, a rubber plantation, was investigated. The results showed that the microbial diversity in post-coal mining is diverse with the non-mining land. The soil fungi and bacteria diversity and density in ex-coal mining was considerably lower compared to the control, non-mining land. The microbial (bacteria and fungi) diversity and density reduced in the deeper layer of soil either in post-coal mining or non-mining land, in which the microbes decrease as the depth of the soil layer increases. On the contrary to the soil fungi, soil bacteria, soil nematodes and entomopathogenic nematodes were noticed more abundant in the post-mining than in the non-mining land, in term of diversity and density. The nematodes were also observed more in the deeper layer of soil. Variations in the soil nematode communities might be due to the distinct characteristics of soil at different layers. The fertility of soil was observed lower in the ex-coal mining land characterized by several soil physicochemical properties, in which lower soil pH, basic saturation, soil organic carbon (SOC) and nutrient content were examined, causing the lower microbial community compared to non-mining land.

.

Keywords: Entomophatogenic nematodes, soil microbia, diversity, revegetation area, post coal mining area, Kutai Kartanegara

INTRODUCTION

Coal mining activities in Kutai Kartanegara Regency, East Kalimantan, have experienced rapid growth in recent years, with both large-scale and small-scale operations, including those with mining authorities. However, this expansion has

come at a significant cost to the environment, resulting in widespread environmental damage. This damage manifests in the form of arid and infertile post-mining lands or artificial "craters" with unclear purposes. While some efforts have been made to rehabilitate these ex-coal mining lands, they remain sporadic and insufficient, allowing environmental degradation to outpace recovery efforts.

Rehabilitating these lands is essential to mitigate the adverse environmental impacts of coal mining. The success of land recovery can be evaluated by assessing the diversity of soil microbes, which serves as an indicator of biological fertility. Soil microbes play a pivotal role in converting potential chemical fertility into actual fertility. Moreover, their activities gradually enhance the physical properties of the soil, further indicating improved physical fertility (Cui et al., 2021). However, the restoration of ex-coal mining lands faces numerous challenges, including the need to address soil degradation, erosion, and biodiversity loss resulting from mining activities (Liu et al., 2010; Singh and Singh, 2017). To address these challenges, it is crucial to consider the role of soil microbes, including entomopathogenic microbia, which are an integral part of soil ecosystems (Boucias, D. G., & Pendland, 2018).

Entomopathogenic microbia, which encompass various taxonomic groups, including bacteria, fungi, nematodes, and viruses, are known to regulate insect populations and play a vital role in ecological processes (Eilenberg et al., 2001) However, their presence and diversity in mining-affected areas can be influenced by factors such as soil type, climate, and land management practices (Rosenheim et al., 2018). Consequently, understanding and managing the diversity of these microorganisms are critical for the success of revegetation efforts. One of the most remarkable contributions of entomopathogenic microbia during revegetation is their role as natural pest controllers (Vega et al., 2009). These microorganisms serve as natural enemies of various insect pests that could harm newly planted vegetation (Shrestha et al, 2020) By infecting and eliminating these pests, entomopathogens help regulate insect populations and protect the restored vegetation (Rozpądek et al., 2019). In regions where entomopathogens thrive, the risk of pest outbreaks is reduced, safeguarding the overall health of the revegetated land (Koskella et al, 2020) Moreover, the presence of entomopathogenic microbia indirectly benefits plant health during revegetation endeavors (Castrillo et al, 2017) By reducing insect herbivory through the control of pest populations, these microorganisms support plant establishment and growth (Lacey et al., 2015). Healthy and robust plant growth, in turn, stabilizes soil, prevents erosion, and enhances biodiversity, all of which are crucial for the overall success of revegetation projects (Xu et al, 2020) Entomopathogenic microbia, including fungi and bacteria, are natural antagonists of insects and play a pivotal role in regulating insect populations in terrestrial ecosystems. While extensively studied for their potential in biological pest control in agriculture, their utilization in ecological restoration, particularly in ex-coal mining land revegetation, remains a relatively unexplored area, and a promising avenue for research and practical implementation. The diversity and abundance of entomopathogenic microbia are subject to environmental factors (Wakil et al, 2018). Factors such as soil physicochemical properties which can fluctuate significantly in mining-affected areas, play pivotal roles in shaping microbial community structure (Mudrak et al, 2021)).

The specific objectives of this study are to identify entomopathogenic nematodes and soil microbes associated with ex-coal mining areas, explore the roles of these identified microbes, and analyze the relationships between entomopathogenic nematodes-soil microbe diversity and the chemical fertility levels of the research site.

MATERIALS AND METHODS

Experimental site

The study was conducted at two locations, in post-coal mining and non-mining areas, in the Pendingin Subdistrict of Sanga-Sanga, in Kutai Kartanegara District, East Kalimantan Province, Indonesia. The post-coal mining area located at the coordinate S 00040'29.7 "E 117016'09.8") has been revegetated with oil palm plantation. The non-mining area, as a control, located at the coordinate S 00041'41.3 "E 117015'54.9", is cultivated with rubber plant.

Sample collection

The sampling method used in this study refers to the standard method of ISPM (International Standards for Phytosanitary Measure), which is an international standard guide to conduct surveys in the field. Soil samples were taken randomly at ten sampling point in each experimental sites. The soil samples were taken at four levels of depth from soil surface, 0-30 cm, 30-60 cm, 60-80 cm, 80-100 cm. After removing the gravel, and plant debris, the soil samples then were composited and taken 1 kg for microbial extraction and identification especially for fungi, bacteria, and nematodes in laboratory. In addition, the fertility status of the soil were also analyzed by identifying the chemical and physical soil properties.

Fungal and Bacterial Isolation

Comment [U3]: Why refer directly to nemato entomopathogens? Do the abundant fungi, bacte and other microbes in the soil function as entomopathogens? What about microbes that ar not entomopathogens? Which are more abundar in the soil? It is best to start with the case of man plant pest problems in ex-mining vegetation, so t environmentally friendly treatments are needed, and one of them is using entomopathogenic microbes.

Comment [U4]: Is it only influenced by these factors? What about the interaction between pla and microbes through root exudates? Which is th most important factor, that or the presence of pl roots on soil microbes?

Comment [U5]: Are only entomopathogenic microbes useful for plant health?

Comment [U6]: Are only insect pests found in the crops grown on the land? What about disease Which is more dominant between pests and diseases? Why is this the case?

Comment [U7]: Is there a lot of bacteria in th former mining area? Bacteria only grow in soil wi neutral or alkaline conditions, while mined land is different.

Comment [U8]: Not only nematodes, but also fungi and bacteria

Comment [U9]: The soil microbes identified were all tested with caterpillars?

Comment [U10]: check

Comment [U11]: Is mined land only found in this area? What about other areas? Can it be considered representative of East Kalimantan as the title above?

Comment [U12]: What tools are used for soil sampling?

Comment [U13]: From the same sampel? Fer soil status is not only determined by chemical and physical characteristics, but also by biological characteristics.

Comment [U14]: What about the identificatio Whose reference was used to identify? Was the work repeated and how many times was it repeated? How is population density calculated a by whom?

For fungal isolation, 10 grams of soil samples were dissolved in a liter of distilled water. An ose needle was dipped in the soil solution, and then inoculated on the Potato Dextrose Agar (PDA) media. A week after, the microbes were isolated and observed Mycobacteria observations were carried out using microscope to identify the type, color, and shape of bacteria, as well as its colony following the procedure of Hucker's with minor modification (Hucker, 1921) Fungal identification was conducted by observing the morphological characteristics of fungi using a microscope**.** Conidia density was calculated using the *Neubauer* type *haemocytometer*.

Bacteria isolation was carried out by dissolving 10 grams of soil samples in a liter of distilled water. An ose needle was dipped in the soil solution, and then inoculated on the Nutrient Agar (NA) media. Two days after, the microbes were isolated and observed bacteria observations were carried out using microscope to identify the type, color, and shape of bacteria, as well as its colony following the procedure of Hucker's with minor modification (Hucker, 1921).

Entomopathogen fungi and bacteria was selected using *Tenebrio mollitor* L., the Hong Kong caterpillar larvae aged 30-40 days (Figure 1.) One milliliter of each fungi and bacteria isolates were inoculated to *Tenebrio mollitor* L. Only fungi and bacteria that can cause mortality to *Tenebrio mollitor* L was considered in the data of this study to screen the entomopathogen bacteria and fungi.

Figure 1. *Tenebrio mollitor* L (*Steinernema*: blacken brown; *Heterorhabditis*: blacken red)

Nematodes Isolation

Nematodes isolation was carried out using *Baermann funnel method* with minor modification. This method is considered as the most effective in nematode isolation (van den Hoogen etal., 2020). A silicone hose wasattached to the funnel. The end of the hose is tied using a Gauze rubber band and placed on the top of the funnel. The Baermann-funnel apparatus was installed in a horizontal position without buckling the silicon hose. Fifty grams of soil for each sample was placed on the filter paperthen wrapped the soil to cover its entire surface. The water was added slowly into the funnel to submerge the sampel, until the sample saturate, to create a moist environment that stimulate nematodes to migrate toward the water source. The funnel was incubated for 3x24 hours and the water released from the bottom of the funnel. The water was collected in a film tube / fial. The collected water then was passed through a fine sieve to separate the nematodes from the water and debris. Formalin (5%) was added as much as 1 drop and fixed for 15 minutes. Entomopathogenic nematodes from soil were carried out by insect trap using *Tenebrio mollitor*. Entomopathogenic nematode trapping was carried out using T. molitor larvae ± 150 g of moist soil is placed in a plastic container containing 3-5 larvae. The container is then inverted so that the larvae are covered with soil, then placed at room temperature (Figure 1).

After 3-4 days, if the larvae show death, the larval cadaver is removed and rinsed with sterilized distilled water. Entomopathogenic nematode trapping was then continued using the White trap method to release entomopathogenic nematodes from the cadaver's body. Nematodes were observed and calculated using a stereo microscope. Identification of soil nematodes and entomopathogenic nematodes genus were carried out by morphological observations.

Soil fertility analysis

Samples were analyzed for their chemical and physical properties including organic C, total N, available P (Bray), available K (Morgan), Ca^{++}, Mg^{++}, K^{+}, Na^{+}, CEC (Cation Exchange Capacity); Base saturation (BS) Al saturation, pH and soil texture.

Data analysis:

Data collected were analyzed descriptively to find out the role of soil microbes in supporting the recovery of post-coal mining land and the relationship between the diversity of soil microbes and the chemical fertility status of the study site.

Comment [U15]: Were soil samples taken fro all four soil depths mixed first or separately?

Comment [U16]: Separate from isolation.

Comment [U17]: Idem. Is the Hong Kong caterpillar test done after microbial identification before? If after microbial identification, it should inoculated to the caterpillar not the soil but the microbes.

Comment [U18]: What is the procedure and according to whom?

RESULTS

Entomophatogenic Fungi diversity and density

Higher entomopathogenic fungi diversity was observed in non-coal-mining compared to ex-coal mining land, in which five fungi genera was found in non-coal mining, and four genera in ex-coal mining (Figure 2, Table 1). The entomophatogenic fungi diversity in ex-coal mining and non-mining land were different in every soil layer observed in this study (Table 1). As an example, *Penicillium* sp. which was found in every soil layer of post coal mining land was not present in non-mining land. On the other hand, *Mucor* sp. which was found in non-mining land was absence in coal mining land. In addition, Aspergillus was observes as abundant fungi which was found in almost all soil layer either in ex-coal mining land or in non-mining land. Besides its diversity, the fungal density in non-coal-mining was also considerably higher (ranging from 4.2 - 9.6x10^6 cfu.g^{-1}) than that of ex-coal mining land (ranging from 1.4 - 5.1x10^6 cfu.g^{-1}) in every soil depth layer. The deeper the soil layer, the fewer Entomophatogenic fungal populations was detected.

Table 1. Entomopathogenic fungi diversity and density at different soil depth layer in ex-coal mining and non-coal mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

	Soil depth layer	Fungal Population cfu/g	Genera/species
	Non-mining land		
1.	0-30	7.2 x 10^5	*Aspergillus* *Trichoderma* *Penicillium*
2.	30-60	5.4 x 10^5	*Aspergillus* *Trichoderma* *Penicillium*
3.	60-80	3.2 x 10^5	*Trichoderma* *Phytium* *Penicillium*
4.	80-100	2.6 x 10^5	*Aspergillus* *Penicillium*
	Post-coal-mining land		
1.	0-30	3.8 x 10^5	*Aspergillus* *Trichoderma* sp.
2.	30-60	2.2 x 10^5	*Aspergillus niger* *Trichoderma*
3.	60-80	1.8 x 10^5	*Aspergillus* sp. *Mucor* sp.
4.	80-100	1.4 x 10^5	*Aspergillus niger* *Aspergillus flavus*.

Comment [U19]: What do the numbers refer to? Not in the table

Comment [U20]: idem

Comment [U21]: Population density

Comment [U22]: Are all these genera tested Hong Kong caterpillars? Identification should be down to specie not genera or families

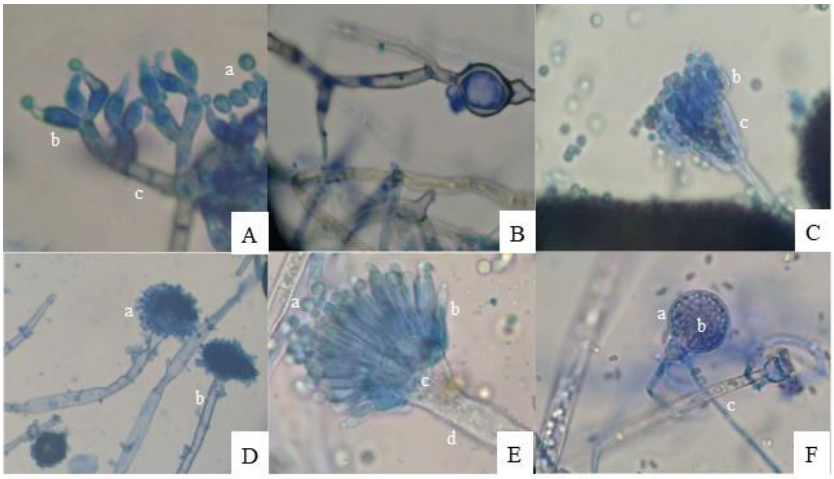

Figure 2. Entomopathogen Fungi A. *Trichoderma: conidia (a), hialin (b), conidiofor (c); B. Phytium: oogonium (a), hyphae (b); C. Penicillium: conidium (a), phialide (b), metula (c), stipe (d); D. A. niger: conidia (a), conidiofor (b); E. A. flavus: conidia (a), phialide (b), vesicle (c), stipe (d); F. Mucor* sp.*: sporangium (a), sporangiofore (b), hyphae (c)*

Enthomopatogenic Bacteria

Two family of Enthomopatogenic bacteria, *Azotobacteraceae* and *Bacillaceae* were observed in both non-coal-mining and ex-coal mining land (Figure 3). However, in the deeper layer of subsoil of ex-coal mining land, only *Azotobacteraceae* was present, either in 60-80 cm or in 80-100 cm. Interestingly, all of the Enthomopatogenic bacteria in the family of *Azotobacteraceae* in this study was noticed as coccus gram positive bacteria, except in ex-coal mining land, there were coccus gram negative *Azotobacteraceae* in the soil depth of 30-60 cm and 60-80 cm. The bacteria population was denser in non-coal-mining ($4.2 – 9.6x10^6$) compared to ex-coal mining land ($1.4 – 5.1x10^6$). The bacteria population was decreased in line with the soil depth layer in the two sites of experiment (Table 2).

Table 2. Entomopathogenic bacteria diversity and density at different soil depth layer in ex-coal mining and non-coal mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Bacterial Population cfu/g	Family E	F
	Non-mining land			
1.	0-30	9.6×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*	
2.	30-60	5.5×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*	
3.	60-80	4.5×10^6	- Coccus, gram: (+) *Azotobacteraceae* - bacil, gram (+): *Bacillaceae*	
4.	80-100	4.2×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*	
	Post-coal-mining land			
1.	0-30	5.1×10^6	- Coccus, gram (+): *Azotobacteraceae* - Basil, gram (+): *Bacillaceae*	
2.	30-60	4.9×10^6	- Coccus, gram: (+) Azotobacteraceae - Basil, gram (+): *Bacillaceae*	
3.	60-80	1.6×10^6	- Coccus, gram: (+): Azotobacteraceae	

Comment [U23]: idem

Comment [U24]: Identification should be dov to specie not genera or families

4.	80-100	1.4×10^6	- Coccus, gram: (+): Azotobacteraceae

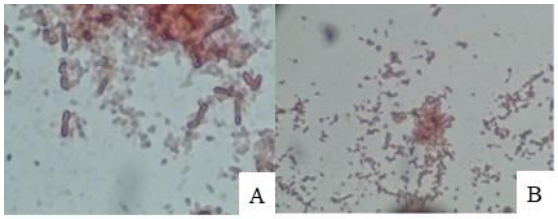

Figure 3. Bacteria (left to right: A. *Bacillaceae,* and B. *Azotobacteraceae*)

Enthomopatogenic Nematoda

In total, two genus of enthomopatogenic nematodes (Steinernema and Heterorhabditis) and thirteen nematodes associates with entomopathogenic nematodes in soil rhizosphere, namely *Heterodera, Meloidogyne, Tylenchus, Pratlyenchus, Radopholus, Hoplolaimus, Rotylenchus, Rotylenchulus, Dorylaimus, Xiphinema, Rhabditis and Longidorus* was isolated in this study (Figure 4). Among the fifteen genus, nine genera were found in non-coal mining, and ten genera in ex-coal mining land (Table 3). Parallel with nematode diversity, the number of nematode populations is also higher in ex-coal mining compared to non-coal mining land. It was also observed that the deeper the soil layer, the higher the number of isolated entomopathogenic nematodes, both found in non-mining and ex-coal mining land (Table 3).

Table 3. Entomopathogenic nematode diversity and density at different soil depth layer in ex-coal mining and non-coal mining land in Pendingin, Sanga-Sanga Subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Nematoda Population/ kg soil	Genus
	Non-mining land		
1.	0-30	1.1×10^2	*Heterodera* *Meloidogyne* *Tylenchus* *Heterorhabditis*
2.	30-60	1.2×10^2	*Heterorhabditis* *Pratlyenchus* *Heterodera* *Radopholus* *Hoplolaimus*
3.	60-80	4×10^2	*Pratylenchus* *Heterodera*
4.	80-100	2×10^2	*Rotylenchus* *Rotylenchulus* *Dorylaimus*
	Post-coal-mining land		
1.	0-30	5.1×10^2	*Heterodera* *Xiphinema*
2.	30-60	7.0×10^2	*Heterorhabditis* *Steinernema* *Radopholus* *Rhabditis*
3.	60-80	8.0×10^2	*Steinernema* *Hoplolaimus*

Comment [U25]: Identification should be dov to specie not genera or families. Are these nematodes from the caterpillar test?

			Rotylenchus
4.	80-100	9.2×10^2	*Pratylenchus* *Longidorus*

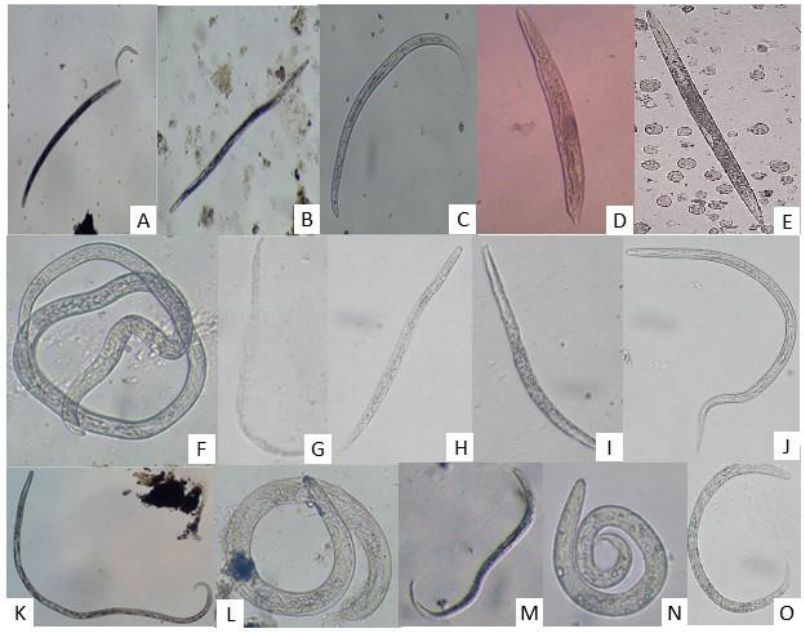

Figure 4. Nematodes (left to right: A. *Hoplolaimus,* B. *Pratylenchus*, C. *Tylenchus,* D. *Heterorhabditis,* E. *Steinernema,* F. *Dorylaimus,* G. *Xiphinema,* H. *Heterodera,* I. *Rhabditis,* J. *Radopholus,* K. *Longidorus,* L. *Rotylenchus,* M. *Rotylenchulus,* N. *Helycotylenchus,* O. *Aphelenchus*)

Soil physicochemical properties

In general, soil nutrient content in non-mining land was higher compared to post-coal mining (Table 4). There were also variation of the nutrient content in different soil depth layer. Most of the nutrient were available more in the subsoil part, compared to topsoil, as observed in available K, Ca++. Mg++, K++, and Na+, as well as base saturation in non-mining land. On the contrary, that phenomenon were in the reverse condition in post-coal mining land.

Aluminium content seemed to be higher in the post-coal mining than that of the non-mining land. The aluminium content tends to decrease as the soil layer gets deeper on non-coal mining land, but tends to be higher on the deeper soil layers on post-coal mining land. The pH status in both of non-mining and post-coal mining land was in the range of 4.02-4.9 and 3.19-4.19, respectively, showing the higher acidity of post-coal mining land.

Soil texture is determined by the proportion of mineral components in the soil. Among all these mineral particles, silt is a dominant soil texture compared to clay and sand, either in non-mining and post-coal mining land, in the top soil or subsoil. However, the relative proportions of different mineral particles (sand, silt, and clay) were varied in each soil layer resulted in distinct soil texture.

Table 4. Soil fertility on two Land Location in Pendingin Village, Sanga-sanga District, Kutai Kartanegara Regency

No.	Soil Parameter	Unit	Non-coal Mining Land	Ex-coal Mining Land

			R1 0-30	R2 30-60	R3 60-80	R4 80-100	S1 0-30	S2 30-60	S3 60-80	S4 80-100
1.	Carbon organic	%	7.65	4.64	4.14	3.41	3.80	3.74	1.38	0.86
2.	Nitrogen total	%	0.41	0.24	0.51	0.14	0.17	0.15	0.16	0.19
3.	Ratio C/N	-	18.66	19.33	8.12	24.36	22.35	24.93	8.63	4.53
4.	Available P (Bray1)	Ppm	19.39	12.60	13.38	20.26	5.11	4.03	0.96	4.12
5.	Available K tersedia (Morgan)	Ppm	110.45	214.54	170.21	234.33	209.22	81.56	82.09	143.28
6.	Al^{3+} (Morgan)		4.00	2.20	2.10	3.10	2.00	6.80	5.10	8.40
7.	H^{+} (Morgan)		3.46	1.80	1.70	2.66	1.66	6.20	4.46	4.26
8.	Ca^{++}	meq/100 g	0.63	1.67	0.83	1.19	1.95	0.79	0.52	0.61
9.	Mg^{++}	meq/100 g	0.48	1.25	0.68	0.98	1.01	0.48	0.37	0.49
10.	K^{+}	meq/100 g	0.30	0.65	0.48	0.91	0.67	0.20	0.26	0.40
11.	Na^{+}	meq/100 g	0.04	0.38	0.28	0.29	0.34	0.11	0.02	0.12
12.	CEC	meq/100 g	8.91	7.95	6.07	9.13	8.16	8.58	10.73	14.28
13.	BS	%	16.3	49.7	37.4	36.9	48.7	18.4	10.9	11.3
14.	Al Saturation	%	44.89	27.67	34.60	33.95	26.21	46.64	47.53	58.82
15.	pH	–	4.02	4.62	4.54	4.90	3.56	3.35	4.19	3.19
16.	Clay	%	14.77	36.54	20.07	23.21	28.72	16.54	23.94	32.85
17.	Silt	%	34.48	58.18	55.30	35.43	67.66	64.75	33.70	35.54
18.	Sand	%	50.75	5.28	24.63	41.36	3.62	18.71	42.36	31.61
19.	Texture		SiCL	SiCL	SiL	L	SiCL	SiL	L	CL

DISCUSSION

The most concern of land degradation impact in post-mining area in agriculture sector is the reduction of biodiversity and soil fertility. Mining activities often lead to the destruction of natural habitats and ecosystems result in the deracination and decline of plant and animal species, leading to a loss of biodiversity which further negatively affects agricultural productivity. In addition, post-mining land undergoes physical and chemical changes that render it unsuitable for agriculture, since it reducing soil porosity and aeration due to the alteration of soil structure, lacks the organic matter and nutrients due to the topsoil removal, introducing contaminants including heavy metals and toxic chemicals, reducing organic matter and soil nutrients, altering the pH of the soil, making it more acidic or alkaline, which can be detrimental to plant growth (Prince et al., 2018; Janečková et al, 2023).

Efforts to address the loss of arable land in post-mining areas involve comprehensive reclamation and rehabilitation practices that aim to restore soil fertility, structure, water-holding capacity and repair the quality of the environment and ecosystem as mentioned in the regulation issued by the Minister of Energy and Mineral Resources Regulation 7/2014 and Regional Regulation East Kalimantan 8/2013. These efforts may include topsoil replacement, planting vegetation, and ongoing monitoring to ensure the land can support agriculture practices. To achieve normal criteria for fertile soil supporting agricultural activities, maintenance of microbial community structures is very important.

Entomopathogen microbial diversity and density

Microorganisms play an important roles in various ecosystems and biological processes. One of the crucial roles of microorganisms in agriculture is as biological control agents. Biological control is a pest management method through the use of natural enemies, to suppress the pest population. The use of biological control is considered as the most prudent manner to control pest since it can reduced reliance on chemical pesticides, specific pest targeting, long-term pest management, environmentally friendly, cost-effective, safe for human health, and supporting the sustainable agriculture (Boro et al, 2022)

In this study, the microorganisms observed are focused on entomothogenic nematodes and its associated with soil bacteria, fungi, and nematode. Entomopathogen nematodes used as biological control specifically for suppressing insect

population. Insects are the largest and most diverse group of organisms included as one of the most common and economically significant types of plant pests (Schoonhoven et al., 2005; García-Lara, 2016). Insect pests cause significant damage and lead to productivity reduction of any crop plant species (Oleivera et al., 2013; Manosathiyadevan, 2017). Therefore, implementing effective and sustainable insect pest management is crucial for achieving a crop production system that optimizes the use of natural resources, preserve the environment, and maximizes output in a sustainable manner. The use of entomopathogen as biological control for reducing insect population considered as the most effective sustainable pest management.

In this study, it was clearly observed that the entomopathogenic nematodes and soil microbial diversity in post-coal mining is diverse with the non-mining land. The soil fungi diversity and density in post-coal mining was considerably lower compared to the non-mining land (Table 1). This situation was not only observed in fungi, but also in soil bacteria (Table 2), showing a low biodiversity of microorganisms in post-coal mining even after reclamation process. A significant reduction of microbial diversity due to coal mining practices after land restoration was also observed in the previous study conducted by de Quadros et al. (2016) In their study, the post-coal mining land has been restored for several years (3-19 years) by replacing the soil layers, applying limestone to raise the pH to 6.0, and revegetating the land with grasses. Even after a completion of reclamation process in many years, the composition of the microbial diversity and biomass of post-mined sites was significantly lower from the control (unaltered sites). A similar results were also found in different studies comparing the microbial diversity and density in reclamation and re-vegetation post-coal mine with undisturbed sites resulted in the significant declines in microbial community structure and diversity (Li et al, 2014; Upadhyay et al., 2014) as also showed in this current study.

A reduction of microbial (bacteria and fungi) diversity and density was also observed in the deeper layer of soil either in post-coal mining or non-mining land (Table 1 and Table 2), in which the microbes decrease as the depth of the soil layer increases. A similar remark was also reported in a study of Upadhyay et al., (2016) revealed that the rhizospheric soils showed greater diversity in both bacteria and fungi compared to the non-rhizospheric soil. This outcome is supported by the recent studies concluded that soil microbial diversity decreasing along with the soil depth (Wang et al, 2021; Guo et al., 2021)

It is well known that the topsoil is the most biologically active and favorable layer for microorganisms compared to the subsoil layer. The topsoil contains the highest concentration of organic matter, providing food source and nutrients for microorganisms (Bahram et al., 2018) In addition, the uppermost layer of soil or also known as A-horizon is well-aerated and has a suitable moisture content, moderate temperature providing a suitable environment for a wide range of microorganisms (N. Liu et al., 2019; Aquado et al, 2023). Furthermore, there is an interaction between microorganisms and plant roots which are more abundant in the top layer of soil. Plant roots release root exudates and provide energy source for many soil microorganisms, thereby contributing to a more active microbial community (Sasse et al., 2018; Zhalnina et al, 2018; Zhou et al, 2020) Therefore, the vegetation types and plant species will influence the soil microbial diversity and community composition (Šourková et al., 2005; Li et al., 2013).

On the contrary to the fungi and bacteria, entomopathogen nematodes were noticed more abundant in the post-mining than in the non-mining land, in term of diversity and density. The nematodes were also observed more in the deeper layer of soil (Table 3). Most of the studies observed that the vertical distribution of nematode communities is decreasing from the topsoil layer to the sub soil layer(Lazarova et al., 2004)(Cheng et al, 2021) (Suyadi et al. 2021). However, it is also explained that the distinct characteristics of soil at different layers cause variations in soil nematode communities (Liu et al., 2022). It means that differences in soil nematode diversity and density in different soil depths might be influenced by soil variables. In a study of (Ilieva-Makulec et al., 2015)), the number of nematode diversity varied in different experimental sites, in which the nematode generic richness is higher in the topsoil, while in different habitat, the nematode diversity was significantly lower in the topsoil and the highest in the subsoil. The nematode abundance and distribution are influenced by many factors, such as soil properties, climate and environmental conditions, land use and management, plant species and root exudates, predation and competition, succession and ecosystem development (Ilieva-Makulec et al., 2015; Zhang, 2020; Cheng et al, 2021; Li et al., 2022)

Microbial life is always directly affected by changes in the soil. On ex-mining land changes in soil (physical, chemical, and biological) occur drastically, so that microbes must adapt to the new environment, or become extinct (Helbig et al., 2008). Microbial life in the soil plays an important role in controlling the stability of the soil ecosystem. Factors that influence microbial community structure in the soil are soil and plant types, and soil management. In conditions of a stable soil ecosystem, the pathogenic microbial suppressants can be suppressed naturally (Sopialena & Palupi, 2017). Ecological soil fertility management gives more hope in realizing a sustainable agricultural system, by paying attention to the interaction between microbes and soil as well as microbes and plants by the influence of time and space in the soil.

Relation of soil physicochemical properties and microbial community

Soil is a medium for plant growth and also acts as an environment for microbial growth, so that the soil conditions determine the quality of growing soil microbes and plants. The status of soil, often referred to as soil health or soil quality, is of utmost importance to the existence and functioning of microbial communities in the soil. The interactions between soil microbes and plants play a crucial role in soil health and fertility. Fertile soil typically is a supply of organic matter for microbial activity. Soil status encompasses a wide range of physical, chemical, and biological properties that

Comment [U26]: It would be good to discuss why there are different characters in mined and unmined soils. Furthermore, it is associated with presence of microbes.

influence the habitat and conditions for soil microorganisms. A healthy soil status with sufficient organic matter and nutrient availability ensures that microorganisms have the resources they need to thrive (Kaur et al., 2022) The presence of high organic matter in soil is characterized by several physicochemical parameters, such as pH levels, cation exchange capacity (CEC), soil organic carbon (SOC) content, and nutrient content especially nitrogen, phosphorus, and potassium (Sangita Changdeo Dandwate, 2020)

In this study, the soil acidity is included as acid soil either in post-coal mining or non-mining land with minor variation in every soil depth layer. However, it is examined that soil pH in the post-mining land was considerably lower, in the range of 3.19-4.19, compared to non-mining in the range of 4.02-4.9. Related to the lower pH in post-mining land, aluminium content is also higher in this land than that of the non-mining (Table 4) since the acidic soils enhance aluminum solubility and availability. Furthermore, soil pH is positively correlated with another soil property, basic saturation (BS), which was observed higher in the non-mining land (Table 4).

Soil pH strongly influences microbial communities. Acidic or alkaline soils can limit the diversity and activity of microorganisms, impacting nutrient availability and overall soil fertility (Fierer and Jackson, 2006; Rousk et al., 2010). Soil pH is a critical factor that affects the activity and diversity of soil microorganisms (Lauber et al., 2009). Different microbial species have specific pH preferences, and soil pH can influence their metabolic activities. Soil pH outside the optimal range for specific microbes can limit their growth and activity (Wang et al, 2019). Maintaining an appropriate pH level is essential for a diverse and active microbial community.

Soil organic matter in the non-mining land was presumably higher in this study, characterized by the higher soil organic carbon (SOC) and nutrient content than that of the post-coal mining land, beside the pH level (Table 4). Higher organic matter are associated with high SOC, as carbon is a fundamental component of organic materials, mainly occurs via the microbial breakdown, partial decay, and conversion of deceased organic materials (Lefèvre et al, 2017) This activity co-related with nutrient availability, as organic matter decomposes, it releases nutrients into the soil, contributing to the soils overall fertility and health (Gerke, 2022)

All over, soil physicochemical properties play a significant role in shaping the composition and activity of the microbial community (Brahmaprakash, 2021). Changes in these properties can influence the composition and activity of the microbial community, subsequently impacting nutrient cycling, organic matter decomposition, and overall soil fertility (Leiva et al, 2020; Kaur et al., 2022) as clearly shown in this study. A higher microbial community was observed in the higher physicochemical of soil properties, which was shown in the non-mining land, compared to the post-coal mining land. Fertile soil is characterized by a well-balanced and diverse microbial community that supports plant growth by providing essential nutrients and improving soil structure. The composition and diversity of the soil microbial community are important factors in maintaining soil health which further supporting plant growth and health (Wang & Li, 2023).

Microbial life in the soil plays an important role in controlling the stability of the soil ecosystem (Gattinger, 2008) The increasing microbial population (both types and numbers) causes the dynamics of the soil to be better and become naturally healthy (Bertola et al., 2021). The ability to change soil biological properties in a positive direction can increase the population of microbes, included entomopathogen microbial, that benefit plants, and make plants grow healthy without the need for the use of artificial fertilizers and pesticides.

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CONCLUSION

The microbial diversity in post-coal mining is diverse with the non-mining land. The entomopathogenic fungi and bacteria diversity and density in ex-coal mining was considerably lower compared to the control, non-mining land. The microbial (bacteria and fungi) diversity and density reduced in the deeper layer of soil either in post-coal mining or non-mining land, in which the microbes decrease as the depth of the soil layer increases. On the contrary to the fungi and bacteria, entomopathogen nematodes were noticed more abundant in the post-mining than in the non-mining land, in term of diversity and density. The nematodes were also observed more in the deeper layer of soil. Variations in the soil nematode communities might be due to the distinct characteristics of soil at different layers. The fertility of soil was observed lower in the ex-coal mining land characterized by several soil physicochemical properties, in which lower soil pH, basic saturation, soil organic carbon (SOC) and nutrient content were examined, causing the lower microbial community compared to non-mining land.

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[biodiv] Editor Decision

2024-06-25 11:15 AM

sopialena sopialena:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Entomophatogenic Microbia Diversity at Revegation of Ex-Coal Mining Land". **Complete your revision with a Table of Responses containing your answers to reviewer comments (for multiple comments) and/or enable Track Changes**.

Our decision is: Revisions Required

Reviewer A:

Comments:

1. Some lines are confusing so correct them.

2. Some references are missing in the text, so correct them.

3. Did you performed triplicates for soil depth layer, if yes then you should write their mean and SD or SE.

4. Read your paper very carefully and make the possible corrections.

Recommendation: See Comments

Biodiversitas Journal of Biological Diversity

Entomophatogenic nematodes and microbia diversity at revegetation land of post-coal mining in East Kalimantan

Abstract. Post-mining management of coal should be followed by revegetation. The success of revegetation activities is largely determined by the fertility status of the soil which consists of physical, chemical and biological fertility components. Biological fertility is indicated by the presence and numbers of soil microbes. This study aims to identify the diversity of nematodes and soil microbes on revegetated ex-coal mining land with palm oil vegetation in Kutai Kartanegara District, screen for entomopathogenic nematodes and microbes, and analyze their relationships with the chemical fertility status of the land. As a control, a non-mining land was also investigated.. The results showed that the microbial diversity and density (including fungi and bacteria) in the post-coal mining area were considerably lower compared to the control, non-mining land.. The soil fungi and bacteria diversity and density in ex-coal mining was considerably lower compared to the control, non-mining land. Additionally, microbial diversity and density decreased in the deeper layer, both in the post- mining and non-mining lands.. In contrast, soil nematodes were more abundant in the post-mining land, and were also more plentiful in the deeper soil layers. Among the nematodes and microbes studied, only *Heterorhabditis* and *Steinernema* (nematodes); *Trichoderma* (fungi); and *Bacillaceae* (bacteria) were identified as entomopathogens, causing death in Tenebrio molitor L. larvae.l. Overall, the soil fertility in the post-coal mining land was observed to be lower,characterized by decreasedsoil pH, basic saturation, soil organic carbon (SOC), and nutrient content, which contributed to a reduced microbial community compared to non-mining land.

Comment [U27]: This conclution is the opposite to the other conclution

Comment [Me28]: It has been revisedIt has been revised

Keywords: Entomophatogenic, nematodes, soil microbia, post-coal mining, Kutai Kartanegara

INTRODUCTION

Coal mining activities in Kutai Kartanegara Regency, East Kalimantan, have experienced rapid growth in recent years, with both large-scale and small-scale operations, including those with mining authorities. However, this expansion has

come at a significant cost to the environment, resulting in widespread environmental damage. This damage manifests in the form of arid and infertile post-mining lands or artificial "craters" with unclear purposes. While some efforts have been made to rehabilitate these ex-coal mining lands, they remain sporadic and insufficient, allowing environmental degradation to outpace recovery efforts.

Rehabilitating these lands is essential to mitigate the adverse environmental impacts of coal mining. The success of land recovery can be evaluated by assessing the diversity of soil microbes, which serves as an indicator of biological fertility. Soil microbes play a pivotal role in converting potential chemical fertility into actual fertility. Moreover, their activities gradually enhance the physical properties of the soil, further indicating improved physical fertility (Cui et al., 2021). However, the restoration of ex-coal mining lands faces numerous challenges, including the need to address soil degradation, erosion, and biodiversity loss resulting from mining activities (Liu et al., 2010; Singh and Singh, 2017). To address these challenges, it is crucial to consider the role of soil microbes, including entomopathogenic microbia, which are an integral part of soil ecosystems (Boucias, D. G., & Pendland, 2018).

Entomopathogenic microbia, which encompass various taxonomic groups, including bacteria, fungi, nematodes, and viruses, are known to regulate insect populations and play a vital role in ecological processes (Eilenberg et al., 2001) However, their presence and diversity in mining-affected areas can be influenced by factors such as soil type, climate, and land management practices (Rosenheim et al., 2018). Consequently, understanding and managing the diversity of these microorganisms are critical for the success of revegetation efforts. One of the most remarkable contributions of entomopathogenic microbia during revegetation is their role as natural pest controllers (Vega et al., 2009). These microorganisms serve as natural enemies of various insect pests that could harm newly planted vegetation (Shrestha et al, 2020) By infecting and eliminating these pests, entomopathogens help regulate insect populations and protect the restored vegetation (Rozpądek et al., 2019). In regions where entomopathogens thrive, the risk of pest outbreaks is reduced, safeguarding the overall health of the revegetated land (Koskella et al, 2020) Moreover, the presence of entomopathogenic microbia indirectly benefits plant health during revegetation endeavors (Castrillo et al, 2017) By reducing insect herbivory through the control of pest populations, these microorganisms support plant establishment and growth (Lacey et al., 2015). Healthy and robust plant growth, in turn, stabilizes soil, prevents erosion, and enhances biodiversity, all of which are crucial for the overall success of revegetation projects (Xu et al, 2020) Entomopathogenic microbia, including fungi and bacteria, are natural antagonists of insects and play a pivotal role in regulating insect populations in terrestrial ecosystems. While extensively studied for their potential in biological pest control in agriculture, their utilization in ecological restoration, particularly in ex-coal mining land revegetation, remains a relatively unexplored area, and a promising avenue for research and practical implementation. The diversity and abundance of entomopathogenic microbia are subject to environmental factors (Wakil et al, 2018). Factors such as soil physicochemical properties which can fluctuate significantly in mining-affected areas, play pivotal roles in shaping microbial community structure (Mudrak et al, 2021)).

The specific objectives of this study are to identify entomopathogenic nematodes and soil microbes associated with ex-coal mining areas, explore the roles of these identified microbes, and analyze the relationships between entomopathogenic nematodes-soil microbe diversity and the chemical fertility levels of the research site.

MATERIALS AND METHODS

Experimental site

The study was conducted at two locations, in post-coal mining and non-mining areas, in the Pendingin Subdistrict of Sanga-Sanga, in Kutai Kartanegara District, East Kalimantan Province, Indonesia. The post-coal mining area located at the coordinate S 00040'29.7 "E 117016'09.8") has been revegetated for two years with oil palm plantation. The non-mining area, as a control, located at the coordinate S 00041'41.3 "E 117015’54.9", is cultivated with rubber plant.

Comment [U29]: How long has oil palm been planted?

Comment [Me30]: 2 years. It has been added

Comment [U31]: Why do non-mining area choose rubber instead of palm oil, so that the comparison is the same? Aren't there many palm plantations in Kalimantan?

Comment [Me32]: We can not found the non mining land with palm oil plantation, in the same research sites. If we use the non-mining land with palm oil plantation in different sub-district or district, the gap of environmental factors could b greater

Sample collection

The sampling method used in this study refers to the standard method of ISPM (International Standards for Phytosanitary Measure), which is an international standard guide to conduct surveys in the field. Soil samples were taken randomly at ten sampling point in each experimental sites. The soil samples were taken at four levels of depth from soil surface, 0-30 cm, 30-60 cm, 60-80 cm, 80-100 cm. After removing the gravel, and plant debris, the soil samples then were composited and taken 1 kg for microbial extraction and identification especially for fungi, bacteria, and nematodes in laboratory. In addition, the fertility status of the soil were also analyzed by identifying the chemical and physical soil properties.

Fungal and Bacterial Isolation

For fungal isolation, 10 grams of soil samples were dissolved in a liter of distilled water. An ose needle was dipped in the soil solution, and then inoculated on the Potato Dextrose Agar (PDA) media. A week after, the microbes were isolated and observed Mycobacteria observations were carried out using microscope to identify the type, color, and shape of bacteria, as well as its colony following the procedure of Hucker's with minor modification (Hucker, 1921) Fungal identification was conducted by observing the morphological characteristics of fungi using a microscope**.** Conidia density was calculated using the *Neubauer* type *haemocytometer*.

Bacteria isolation was carried out by dissolving 10 grams of soil samples in a liter of distilled water. An ose needle was dipped in the soil solution, and then inoculated on the Nutrient Agar (NA) media. Two days after, the microbes were isolated and observed bacteria observations were carried out using microscope to identify the type, color, and shape of bacteria, as well as its colony following the procedure of Hucker's with minor modification (Hucker, 1921).

Entomopathogen fungi and bacteria was selected using *Tenebrio mollitor* L., the Hong Kong caterpillar larvae aged 30-40 days (Figure 1.) One milliliter of each fungi and bacteria isolates were inoculated to *Tenebrio mollitor* L. Only fungi and bacteria that can cause mortality to *Tenebrio mollitor* L was considered in the data of this study to screen the entomopathogen bacteria and fungi.

Figure 1. *Tenebrio mollitor* L (*Steinernema*: blacken brown; *Heterorhabditis*: blacken red)

Nematodes Isolation

Nematodes isolation was carried out using *Baermann funnel method* with minor modification. This method is considered as the most effective in nematode isolation (van den Hoogen etal., 2020). A silicone hose wasattached to the funnel. The end of the hose is tied using a Gauze rubber band and placed on the top of the funnel. The Baermann-funnel apparatus was installed in a horizontal position without buckling the silicon hose. Fifty grams of soil for each sample was placed on the filter paperthen wrapped the soil to cover its entire surface. The water was added slowly into the funnel to submerge the sampel, until the sample saturate, to create a moist environment that stimulate nematodes to migrate toward the water source. The funnel was incubated for 3x24 hours and the water released from the bottom of the funnel. The water was collected in a film tube / fial. The collected water then was passed through a fine sieve to separate the nematodes from the water and debris. Formalin (5%) was added as much as 1 drop and fixed for 15 minutes. Entomopathogenic nematodes from soil were carried out by insect trap using *Tenebrio mollitor*. Entomopathogenic nematode trapping was carried out using T. molitor larvae ± 150 g of moist soil is placed in a plastic container containing 3-5 larvae. The container is then inverted so that the larvae are covered with soil, then placed at room temperature (Figure 1).

After 3-4 days, if the larvae show death, the larval cadaver is removed and rinsed with sterilized distilled water. Entomopathogenic nematode trapping was then continued using the White trap method to release entomopathogenic nematodes from the cadaver's body. Nematodes were observed and calculated using a stereo microscope. Identification of soil nematodes and entomopathogenic nematodes genus were carried out by morphological observations.

Comment [U33]: Why does it have to be incubated for 3 days, the standard is 1 day.

Comment [Me34]: Nematodes isolation using *Baermann funnel* method could be conducted fr 16-72 hours. As an example in the study of Mohanapriya and Ragini 2023; Johnson 2015; etc

Soil fertility analysis

Samples were analyzed for their chemical and physical properties including organic C, total N, available P (Bray), available K (Morgan), Ca^{++}, Mg^{++}, K^{+}, Na^{+}, CEC (Cation Exchange Capacity); Base saturation (BS) Al saturation, pH and soil texture.

Data analysis:

Data collected were analyzed descriptively to find out the role of soil microbes in supporting the recovery of post-coal mining land and the relationship between the diversity of soil microbes and the chemical fertility status of the study site.

RESULTS

Entomophatogenic Fungi diversity and density

Higher entomopathogenic fungi diversity was observed in non-coal-mining compared to ex-coal mining land, in which five fungi genera was found in non-coal mining, and four genera in ex-coal mining (Figure 2, Table 1). The entomophatogenic fungi diversity in ex-coal mining and non-mining land were different in every soil layer observed in this study (Table 1). As an example, *Penicillium* sp. which was found in every soil layer of post coal mining land was not present in non-mining land. On the other hand, *Mucor* sp. which was found in non-mining land was absence in coal mining land. In addition, Aspergillus was observes as abundant fungi which was found in almost all soil layer either in ex-coal mining land or in non-mining land. Besides its diversity, the fungal density in non-coal-mining was also considerably higher (ranging from 4.2 - 9.6x10^6 cfu.g^{-1}) than that of ex-coal mining land (ranging from 1.4 - 5.1x10^6 cfu.g^{-1}) in every soil depth layer. The deeper the soil layer, the fewer Entomophatogenic fungal populations was detected.

Table 1. Entomopathogenic fungi diversity and density at different soil depth layer in ex-coal mining and non-coal mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

	Soil depth layer	Fungal Population cfu/g	Genera/species
	Non-mining land		
1.	0-30	7.2 x 10^5	*Aspergillus* *Trichoderma* *Penicillium*
2.	30-60	5.4 x 10^5	*Aspergillus* *Trichoderma* *Penicillium*
3.	60-80	3.2 x 10^5	*Trichoderma* *Phytium* *Penicillium*
4.	80-100	2.6 x 10^5	*Aspergillus* *Penicillium*
	Post-coal-mining land		
1.	0-30	3.8 x 10^5	*Aspergillus* *Trichoderma* sp.
2.	30-60	2.2 x 10^5	*Aspergillus niger* *Trichoderma*
3.	60-80	1.8 x 10^5	*Aspergillus* sp. *Mucor* sp.
4.	80-100	1.4 x 10^5	*Aspergillus niger* *Aspergillus flavus*.

Comment [U35]: just select the species

Comment [Me36]: For some fungi, we can no define the species

Comment [U37]: Please write according to writing rules (*Aspergillus* sp.)

Comment [Me38]: For some fungi, we can no define the species

Figure 2. Entomopathogen Fungi A. *Trichoderma: conidia (a), hialin (b), conidiofor (c); B. Phytium: oogonium (a), hyphae (b); C. Penicillium: conidium (a), phialide (b), metula (c), stipe (d); D. A. niger: conidia (a), conidiofor (b); E. A. flavus: conidia (a), phialide (b), vesicle (c), stipe (d); F. Mucor* sp.*: sporangium (a), sporangiofore (b), hyphae (c)*

Enthomopatogenic Bacteria

Two family of Enthomopatogenic bacteria, *Azotobacteraceae* and *Bacillaceae* were observed in both non-coal-mining and ex-coal mining land (Figure 3). However, in the deeper layer of subsoil of ex-coal mining land, only *Azotobacteraceae* was present, either in 60-80 cm or in 80-100 cm. Interestingly, all of the Enthomopatogenic bacteria in the family of *Azotobacteraceae* in this study was noticed as coccus gram positive bacteria, except in ex-coal mining land, there were coccus gram negative *Azotobacteraceae* in the soil depth of 30-60 cm and 60-80 cm. The bacteria population was denser in non-coal-mining ($4.2 – 9.6x10^6$) compared to ex-coal mining land ($1.4 – 5.1x10^6$). The bacteria population was decreased in line with the soil depth layer in the two sites of experiment (Table 2).

Table 2. Entomopathogenic bacteria diversity and density at different soil depth layer in ex-coal mining and non-coal mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Bacterial Population cfu/g	Family
	Non-mining land		
1.	0-30	9.6×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*
2.	30-60	5.5×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*
3.	60-80	4.5×10^6	- Coccus, gram: (+) *Azotobacteraceae* - bacil, gram (+): *Bacillaceae*
4.	80-100	4.2×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*
	Post-coal-mining land		
1.	0-30	5.1×10^6	- Coccus, gram (+): *Azotobacteraceae* - Basil, gram (+): *Bacillaceae*
2.	30-60	4.9×10^6	- Coccus, gram: (+) Azotobacteraceae - Basil, gram (+): *Bacillaceae*
3.	60-80	1.6×10^6	- Coccus, gram: (+): Azotobacteraceae

4.	80-100	1.4×10^6	- Coccus, gram: (+): Azotobacteraceae

Figure 3. Bacteria (left to right: A. *Bacillaceae,* and B. *Azotobacteraceae*)

Enthomopatogenic Nematoda

In total, two genus of enthomopatogenic nematodes (Steinernema and Heterorhabditis) and thirteen nematodes associates with entomopathogenic nematodes in soil rhizosphere, namely *Heterodera, Meloidogyne, Tylenchus, Pratlyenchus, Radopholus, Hoplolaimus, Rotylenchus, Rotylenchulus, Dorylaimus, Xiphinema, Rhabditis and Longidorus* was isolated in this study (Figure 4). Among the fifteen genus, nine genera were found in non-coal mining, and ten genera in ex-coal mining land (Table 3). Parallel with nematode diversity, the number of nematode populations is also higher in ex-coal mining compared to non-coal mining land. It was also observed that the deeper the soil layer, the higher the number of isolated entomopathogenic nematodes, both found in non-mining and ex-coal mining land (Table 3).

Table 3. Entomopathogenic nematode diversity and density at different soil depth layer in ex-coal mining and non-coal mining land in Pendingin, Sanga-Sanga Subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Nematoda Population/ kg soil	Genus
	Non-mining land		
1.	0-30	1.1×10^2	*Heterodera* *Meloidogyne* *Tylenchus* *Heterorhabditis*
2.	30-60	1.2×10^2	*Heterorhabditis* *Pratlyenchus* *Heterodera* *Radopholus* *Hoplolaimus*
3.	60-80	4×10^2	*Pratylenchus* *Heterodera*
4.	80-100	2×10^2	*Rotylenchus* *Rotylenchulus* *Dorylaimus*
	Post-coal-mining land		
1.	0-30	5.1×10^2	*Heterodera* *Xiphinema*
2.	30-60	7.0×10^2	*Heterorhabditis* *Steinernema*

			Radopholus *Rhabditis*
3.	60-80	8.0×10^2	*Steinernema* *Hoplolaimus* *Rotylenchus*
4.	80-100	9.2×10^2	*Pratylenchus* *Longidorus*

Figure 4. Nematodes (left to right: A. *Hoplolaimus,* B. *Pratylenchus*, C. *Tylenchus,* D. *Heterorhabditis,* E. *Steinernema,* F. *Dorylaimus,* G. *Xiphinema,* H. *Heterodera,* I. *Rhabditis,* J. *Radopholus,* K. *Longidorus,* L. *Rotylenchus,* M. *Rotylenchulus,* N. *Helycotylenchus,* O. *Aphelenchus*)

Soil physicochemical properties

In general, soil nutrient content in non-mining land was higher compared to post-coal mining (Table 4). There were also variation of the nutrient content in different soil depth layer. Most of the nutrient were available more in the subsoil part, compared to topsoil, as observed in available K, Ca++. Mg++, K++, and Na+, as well as base saturation in non-mining land. On the contrary, that phenomenon were in the reverse condition in post-coal mining land.

Aluminium content seemed to be higher in the post-coal mining than that of the non-mining land. The aluminium content tends to decrease as the soil layer gets deeper on non-coal mining land, but tends to be higher on the deeper soil layers on post-coal mining land. The pH status in both of non-mining and post-coal mining land was in the range of 4.02-4.9 and 3.19-4.19, respectively, showing the higher acidity of post-coal mining land.

Soil texture is determined by the proportion of mineral components in the soil. Among all these mineral particles, silt is a dominant soil texture compared to clay and sand, either in non-mining and post-coal mining land, in the top soil or subsoil. However, the relative proportions of different mineral particles (sand, silt, and clay) were varied in each soil layer resulted in distinct soil texture.

Table 4. Soil fertility on two Land Location in Pendingin Village, Sanga-sanga District, Kutai Kartanegara Regency

No.	Soil Parameter	Unit	Non-coal Mining Land				Ex-coal Mining Land			
			R1 0-30	R2 30-60	R3 60-80	R4 80-100	S1 0-30	S2 30-60	S3 60-80	S4 80-100
1.	Carbon organic	%	7.65	4.64	4.14	3.41	3.80	3.74	1.38	0.86
2.	Nitrogen total	%	0.41	0.24	0.51	0.14	0.17	0.15	0.16	0.19
3.	Ratio C/N	-	18.66	19.33	8.12	24.36	22.35	24.93	8.63	4.53
4.	Available P (Bray1)	Ppm	19.39	12.60	13.38	20.26	5.11	4.03	0.96	4.12
5.	Available K tersedia (Morgan)	Ppm	110.45	214.54	170.21	234.33	209.22	81.56	82.09	143.28
6.	Al^{3+} (Morgan)		4.00	2.20	2.10	3.10	2.00	6.80	5.10	8.40
7.	H^{+} (Morgan)		3.46	1.80	1.70	2.66	1.66	6.20	4.46	4.26
8.	Ca^{++}	meq/100 g	0.63	1.67	0.83	1.19	1.95	0.79	0.52	0.61
9.	Mg^{++}	meq/100 g	0.48	1.25	0.68	0.98	1.01	0.48	0.37	0.49
10.	K^{+}	meq/100 g	0.30	0.65	0.48	0.91	0.67	0.20	0.26	0.40
11.	Na^{+}	meq/100 g	0.04	0.38	0.28	0.29	0.34	0.11	0.02	0.12
12.	CEC	meq/100 g	8.91	7.95	6.07	9.13	8.16	8.58	10.73	14.28
13.	BS	%	16.3	49.7	37.4	36.9	48.7	18.4	10.9	11.3
14.	Al Saturation	%	44.89	27.67	34.60	33.95	26.21	46.64	47.53	58.82
15.	pH	–	4.02	4.62	4.54	4.90	3.56	3.35	4.19	3.19
16.	Clay	%	14.77	36.54	20.07	23.21	28.72	16.54	23.94	32.85
17.	Silt	%	34.48	58.18	55.30	35.43	67.66	64.75	33.70	35.54
18.	Sand	%	50.75	5.28	24.63	41.36	3.62	18.71	42.36	31.61
19.	Texture		SiCL	SiCL	SiL	L	SiCL	SiL	L	CL

DISCUSSION

The most concern of land degradation impact in post-mining area in agriculture sector is the reduction of biodiversity and soil fertility. Mining activities often lead to the destruction of natural habitats and ecosystems result in the deracination and decline of plant and animal species, leading to a loss of biodiversity which further negatively affects agricultural productivity. In addition, post-mining land undergoes physical and chemical changes that render it unsuitable for agriculture, since it reducing soil porosity and aeration due to the alteration of soil structure, lacks the organic matter and nutrients due to the topsoil removal, introducing contaminants including heavy metals and toxic chemicals, reducing organic matter and soil nutrients, altering the pH of the soil, making it more acidic or alkaline, which can be detrimental to plant growth (Prince et al., 2018; Janečková et al, 2023).

Efforts to address the loss of arable land in post-mining areas involve comprehensive reclamation and rehabilitation practices that aim to restore soil fertility, structure, water-holding capacity and repair the quality of the environment and ecosystem as mentioned in the regulation issued by the Minister of Energy and Mineral Resources Regulation 7/2014 and Regional Regulation East Kalimantan 8/2013. These efforts may include topsoil replacement, planting vegetation, and ongoing monitoring to ensure the land can support agriculture practices. To achieve normal criteria for fertile soil supporting agricultural activities, maintenance of microbial community structures is very important.

Entomopathogen microbial diversity and density

Microorganisms play an important roles in various ecosystems and biological processes. One of the crucial roles of microorganisms in agriculture is as biological control agents. Biological control is a pest management method through the use of natural enemies, to suppress the pest population. The use of biological control is considered as the most prudent manner to control pest since it can reduced reliance on chemical pesticides, specific pest targeting, long-term pest

Comment [U39]: one of the microorganisms found was *Trichoderma* sp., which acts as an antagonist and also as a biodecomposer.
one of the microorganisms found was Trichoderm sp., which acts as an antagonist and also as a biodecomposer.

Comment [Me40]: Yes, in this study we found that Trichoderma as entomopathogenic fungi

management, environmentally friendly, cost-effective, safe for human health, and supporting the sustainable agriculture (Boro et al, 2022)

In this study, the microorganisms observed are focused on entomothogenic nematodes and its associated with soil bacteria, fungi, and nematode. Entomopathogen nematodes used as biological control specifically for suppressing insect population. Insects are the largest and most diverse group of organisms included as one of the most common and economically significant types of plant pests (Schoonhoven et al., 2005; García-Lara, 2016). Insect pests cause significant damage and lead to productivity reduction of any crop plant species (Oleivera et al., 2013; Manosathiyadevan, 2017). Therefore, implementing effective and sustainable insect pest management is crucial for achieving a crop production system that optimizes the use of natural resources, preserve the environment, and maximizes output in a sustainable manner. The use of entomopathogen as biological control for reducing insect population considered as the most effective sustainable pest management.

In this study, it was clearly observed that the entomopathogenic nematodes and soil microbial diversity in post-coal mining is diverse with the non-mining land. The soil fungi diversity and density in post-coal mining was considerably lower compared to the non-mining land (Table 1). This situation was not only observed in fungi, but also in soil bacteria (Table 2), showing a low biodiversity of microorganisms in post-coal mining even after reclamation process. A significant reduction of microbial diversity due to coal mining practices after land restoration was also observed in the previous study conducted by de Quadros et al. (2016) In their study, the post-coal mining land has been restored for several years (3-19 years) by replacing the soil layers, applying limestone to raise the pH to 6.0, and revegetating the land with grasses. Even after a completion of reclamation process in many years, the composition of the microbial diversity and biomass of post-mined sites was significantly lower from the control (unaltered sites). A similar results were also found in different studies comparing the microbial diversity and density in reclamation and re-vegetation post-coal mine with undisturbed sites resulted in the significant declines in microbial community structure and diversity (Li et al, 2014; Upadhyay et al., 2014) as also showed in this current study.

A reduction of microbial (bacteria and fungi) diversity and density was also observed in the deeper layer of soil either in post-coal mining or non-mining land (Table 1 and Table 2), in which the microbes decrease as the depth of the soil layer increases. A similar remark was also reported in a study of Upadhyay et al., (2016) revealed that the rhizospheric soils showed greater diversity in both bacteria and fungi compared to the non-rhizospheric soil. This outcome is supported by the recent studies concluded that soil microbial diversity decreasing along with the soil depth (Wang et al, 2021; Guo et al., 2021)

It is well known that the topsoil is the most biologically active and favorable layer for microorganisms compared to the subsoil layer. The topsoil contains the highest concentration of organic matter, providing food source and nutrients for microorganisms (Bahram et al., 2018) In addition, the uppermost layer of soil or also known as A-horizon is well-aerated and has a suitable moisture content, moderate temperature providing a suitable environment for a wide range of microorganisms (N. Liu et al., 2019; Aquado et al, 2023). Furthermore, there is an interaction between microorganisms and plant roots which are more abundant in the top layer of soil. Plant roots release root exudates and provide energy source for many soil microorganisms, thereby contributing to a more active microbial community (Sasse et al., 2018; Zhalnina et al, 2018; Zhou et al, 2020) Therefore, the vegetation types and plant species will influence the soil microbial diversity and community composition (Šourková et al., 2005; Li et al., 2013).

On the contrary to the fungi and bacteria, entomopathogen nematodes were noticed more abundant in the post-mining than in the non-mining land, in term of diversity and density. The nematodes were also observed more in the deeper layer of soil (Table 3). Most of the studies observed that the vertical distribution of nematode communities is decreasing from the topsoil layer to the sub soil layer(Lazarova et al., 2004)(Cheng et al, 2021) (Suyadi et al. 2021). However, it is also explained that the distinct characteristics of soil at different layers cause variations in soil nematode communities (Liu et al., 2022). It means that differences in soil nematode diversity and density in different soil depths might be influenced by soil variables. In a study of (Ilieva-Makulec et al., 2015)), the number of nematode diversity varied in different experimental sites, in which the nematode generic richness is higher in the topsoil, while in different habitat, the nematode diversity was significantly lower in the topsoil and the highest in the subsoil. The nematode abundance and distribution are influenced by many factors, such as soil properties, climate and environmental conditions, land use and management, plant species and root exudates, predation and competition, succession and ecosystem development (Ilieva-Makulec et al., 2015; Zhang, 2020; Cheng et al, 2021; Li et al., 2022)

Microbial life is always directly affected by changes in the soil. On ex-mining land changes in soil (physical, chemical, and biological) occur drastically, so that microbes must adapt to the new environment, or become extinct (Helbig et al., 2008). Microbial life in the soil plays an important role in controlling the stability of the soil ecosystem. Factors that influence microbial community structure in the soil are soil and plant types, and soil management. In conditions of a stable soil ecosystem, the pathogenic microbial suppressants can be suppressed naturally (Sopialena & Palupi, 2017). Ecological soil fertility management gives more hope in realizing a sustainable agricultural system, by paying attention to the interaction between microbes and soil as well as microbes and plants by the influence of time and space in the soil.

Relation of soil physicochemical properties and microbial community

Soil is a medium for plant growth and also acts as an environment for microbial growth, so that the soil conditions determine the quality of growing soil microbes and plants. The status of soil, often referred to as soil health or soil quality, is of utmost importance to the existence and functioning of microbial communities in the soil. The interactions between soil microbes and plants play a crucial role in soil health and fertility. Fertile soil typically is a supply of organic matter for microbial activity. Soil status encompasses a wide range of physical, chemical, and biological properties that influence the habitat and conditions for soil microorganisms. A healthy soil status with sufficient organic matter and nutrient availability ensures that microorganisms have the resources they need to thrive (Kaur et al., 2022) The presence of high organic matter in soil is characterized by several physicochemical parameters, such as pH levels, cation exchange capacity (CEC), soil organic carbon (SOC) content, and nutrient content especially nitrogen, phosphorus, and potassium (Sangita Changdeo Dandwate, 2020)

In this study, the soil acidity is included as acid soil either in post-coal mining or non-mining land with minor variation in every soil depth layer. However, it is examined that soil pH in the post-mining land was considerably lower, in the range of 3.19-4.19, compared to non-mining in the range of 4.02-4.9. Related to the lower pH in post-mining land, aluminium content is also higher in this land than that of the non-mining (Table 4) since the acidic soils enhance aluminum solubility and availability. Furthermore, soil pH is positively correlated with another soil property, basic saturation (BS), which was observed higher in the non-mining land (Table 4).

Soil pH strongly influences microbial communities. Acidic or alkaline soils can limit the diversity and activity of microorganisms, impacting nutrient availability and overall soil fertility (Fierer and Jackson, 2006; Rousk et al., 2010). Soil pH is a critical factor that affects the activity and diversity of soil microorganisms (Lauber et al., 2009). Different microbial species have specific pH preferences, and soil pH can influence their metabolic activities. Soil pH outside the optimal range for specific microbes can limit their growth and activity (Wang et al, 2019). Maintaining an appropriate pH level is essential for a diverse and active microbial community.

Soil organic matter in the non-mining land was presumably higher in this study, characterized by the higher soil organic carbon (SOC) and nutrient content than that of the post-coal mining land, beside the pH level (Table 4). Higher organic matter are associated with high SOC, as carbon is a fundamental component of organic materials, mainly occurs via the microbial breakdown, partial decay, and conversion of deceased organic materials (Lefèvre et al, 2017) This activity co-related with nutrient availability, as organic matter decomposes, it releases nutrients into the soil, contributing to the soils overall fertility and health (Gerke, 2022)

All over, soil physicochemical properties play a significant role in shaping the composition and activity of the microbial community (Brahmaprakash, 2021). Changes in these properties can influence the composition and activity of the microbial community, subsequently impacting nutrient cycling, organic matter decomposition, and overall soil fertility (Leiva et al, 2020; Kaur et al., 2022) as clearly shown in this study. A higher microbial community was observed in the higher physicochemical of soil properties, which was shown in the non-mining land, compared to the post-coal mining land. Fertile soil is characterized by a well-balanced and diverse microbial community that supports plant growth by providing essential nutrients and improving soil structure. The composition and diversity of the soil microbial community are important factors in maintaining soil health which further supporting plant growth and health (Wang & Li, 2023).

Microbial life in the soil plays an important role in controlling the stability of the soil ecosystem (Gattinger, 2008) The increasing microbial population (both types and numbers) causes the dynamics of the soil to be better and become naturally healthy (Bertola et al., 2021). The ability to change soil biological properties in a positive direction can increase the population of microbes, included entomopathogen microbial, that benefit plants, and make plants grow healthy without the need for the use of artificial fertilizers and pesticides.

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CONCLUSION

The microbial diversity in post-coal mining is diverse with the non-mining land. The entomopathogenic fungi and bacteria diversity and density in ex-coal mining was considerably lower compared to the control, non-mining land. The microbial (bacteria and fungi) diversity and density reduced in the deeper layer of soil either in post-coal mining or non-mining land, in which the microbes decrease as the depth of the soil layer increases. On the contrary to the fungi and bacteria, entomopathogen nematodes were noticed more abundant in the post-mining than in the non-mining land, in term of diversity and density. The nematodes were also observed more in the deeper layer of soil. Variations in the soil nematode communities might be due to the distinct characteristics of soil at different layers. The fertility of soil was observed lower in the ex-coal mining land characterized by several soil physicochemical properties, in which lower soil pH, basic saturation, soil organic carbon (SOC) and nutrient content were examined, causing the lower microbial community compared to non-mining land.

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Comment [U41]: written under Lazarova et a
2004

Comment [Me42]: We have deleted Lazarova
al., 2004, to reduce the old references

Comment [U43]: put them in alphabetical or

Comment [Me44]: Yes thank you very much
the revised version of the manuscript, the
references have been order alphabetically

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Comment [U45]: put them in alphabetical or

Comment [Me46]: Yes thank you very much the revised version of the manuscript, the references have been order alphabetically

- **Round 3**

- **Round 4**

- **Round 5**

- **Round 6**

Round 6 Status Submission accepted.

Notifications

- [biodiv] Editor Decision2024-01-17 10:46 PM
- [biodiv] Editor Decision2024-06-06 06:15 AM
- [biodiv] Editor Decision2024-06-25 11:15 AM
- [biodiv] Editor Decision2024-07-17 02:30 PM

Reviewer's Attachments

- **Search**

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Revisions

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Review Discussions

- **Add discussion**

Name	From	Last Reply	Replies
Uncorrected proof	ddianti1 2024-07-08 02:54 PM	-	0
BILLING	dewinurpratiwi 2024-07-09 12:12 AM	-	0

Notifications

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[biodiv] Editor Decision

2024-06-25 11:15 AM

sopialena sopialena:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Entomophatogenic Microbia Diversity at Revegation of Ex-Coal Mining Land". **Complete your revision with a Table of Responses containing your answers to reviewer comments (for multiple comments) and/or enable Track Changes**.

Our decision is: Revisions Required

Reviewer A:

Comments:

1. Some lines are confusing so correct them.

2. Some references are missing in the text, so correct them.

3. Did you performed triplicates for soil depth layer, if yes then you should write their mean and SD or SE.

4. Read your paper very carefully and make the possible corrections.

Recommendation: See Comments

Assessment of nematode and microbial diversity and screening of entomopathogens in revegetated post-coal-mining land in Kutai Kartanegara District, East Kalimantan, Indonesia

Abstract. Post-coal-mining management should strive toward site revegetation. The success of such revegetation efforts is largely determined by the soil's fertility status, which encompasses physical, chemical, and biological components. Biological fertility is indicated by the presence and abundance of soil microbes and nematodes. The objective of this study was to identify the diversity of nematodes and soil microbes in ex-coal-mining land revegetated with oil palm in Kutai Kartanegara District (East Kalimantan, Indonesia), to screen for entomopathogenic nematodes and microbes, and to analyze their relationships with the chemical fertility status of the land. As a control, non-mining land was also investigated. The results showed that microbial diversity and density (including fungi and bacteria) in the ex-coal-mining area were significantly lower compared to the control. Additionally, a decrease in microbial diversity and density was found in deeper soil layers of both post-mining and non-mining lands. In contrast, soil nematodes were more abundant in post-mining land, both in terms of diversity and density, and were also more plentiful in the deeper soil layers. Among the nematodes and microbes studied, only *Heterorhabditis* and *Steinernema* (nematodes), *Trichoderma* (fungi), and *Bacillaceae* (bacteria) were identified as entomopathogens, causing death in *Tenebrio molitor* L. larvae. Overall, soil fertility in ex-coal-mining land was observed to be lower, characterized by decreased soil pH, basic saturation, soil organic carbon (SOC), and nutrient content, which contributed to a reduced microbial community compared to non-mining land.

Keywords: Entomopathogen, nematodes, soil microbes, post-coal mining, Kutai Kartanegara, *Trichoderma*.

INTRODUCTION

Coal mining activities in Kutai Kartanegara District, East Kalimantan, Indonesia, have experienced rapid growth in recent years, with both large-scale and small-scale operations, including those with mining authorities. However, this expansion has come at a significant cost to the environment, resulting in widespread damage. This damage manifests in the form of arid and infertile post-mining lands or artificial "craters" with unclear purposes. While some efforts have been made to rehabilitate these ex-coal-mining lands, they remain sporadic and insufficient, allowing environmental degradation to outpace recovery efforts.

Rehabilitating these lands is essential to mitigating the adverse environmental impacts of coal mining. The success of land recovery can be evaluated by assessing the diversity of soil microbes, which serves as an indicator of biological fertility. Soil microbes play a pivotal role in converting potential chemical fertility into actual fertility. Moreover, their activities gradually enhance the physical properties of the soil, further improving physical fertility (Cui et al. 2021). However, the restoration of ex-coal-mining lands faces numerous challenges, including the need to address soil degradation, erosion, and biodiversity loss resulting from mining activities (Ahirwal et al. 2016). To address these challenges, it is crucial to consider the role of soil microbes, including entomopathogenic microbes, which are an integral part of soil ecosystems (Boucias and Pendland 2018).

The roles of nematodes and microbes in ex-mining lands are fundamental to ecological restoration efforts, improving soil health, supporting plant growth, and maintaining ecological balance. These microorganisms can significantly impact the success of revegetation and restoration projects in post-mining landscapes. The presence of nematodes and microbes in the soil benefits plant health during revegetation endeavors (Guo et al. 2021; Li et al. 2022). Furthermore, these microorganisms support plant establishment and growth. Healthy and robust plant growth, in turn, stabilizes the soil, prevents erosion, and enhances biodiversity, all of which are important for the overall success of revegetation

projects (Mensah 2015). The presence of microbial diversity in mining-affected areas can be influenced by factors such as soil type, climate, land management practices, as well as the host plants' root exudate composition (Santoyo 2022; Sasse et al. 2017). Consequently, understanding and managing the diversity of these microorganisms are critical for the success of revegetation efforts.

Entomopathogenic microbes, which encompass various taxonomic groups, including bacteria, fungi, nematodes, and viruses, are known to regulate insect populations and play a vital role in ecological processes (Deka et al. 2021). One of the most remarkable contributions of entomopathogenic microbes during revegetation is their role as natural pest controllers (Vega et al. 2009). These microorganisms serve as natural enemies of various insect pests that could harm newly planted vegetation (Shrestha et al. 2020). By infecting and eliminating these pests, entomopathogens help regulate insect populations and protect the restored vegetation (Rozpądek et al. 2019). In regions where entomopathogens thrive, the risk of pest outbreaks is reduced, safeguarding the overall health of the revegetated land (Koskella et al. 2020). Entomopathogenic microbes, such as fungi and bacteria are natural antagonists of insects and play a pivotal role in regulating insect populations in terrestrial ecosystems. While extensively studied for their potential in biological pest control in agriculture, their utilization in ecological restoration, particularly in ex-coal-mining land revegetation, remains a relatively unexplored area, and a promising avenue for research and practical implementation. The diversity and abundance of entomopathogenic microbes are subject to environmental factors (Fatu et al. 2020). Factors such as soil physicochemical properties, which can fluctuate significantly in mining-affected areas, play pivotal roles in shaping microbial community structure (Mudrak et al. 2021).

The objective of this study was to identify the diversity of nematodes and soil microbes on revegetated ex-coal-mining land, to investigate for entomopathogenic nematodes and microbes, and to analyze their relationships with the chemical fertility status of the land.

MATERIALS AND METHODS

Experimental sites

The study was conducted at two locations, in post-coal mining and non-mining areas, in the Pendingin Subdistrict of Sanga-Sanga, in Kutai Kartanegara District, East Kalimantan Province, Indonesia. Oil palm plantations were planted in a post-coal mining area (located at S 00040'29.7" E 117016'09.8"), while rubber plantations were cultivated in a non-mining area (located at S 00041'41.3" E 117015'54.9") used as a control.

Sample collection

The sampling method used in this study was the standard method of ISPM (International Standards for Phytosanitary Measure). Soil samples were taken randomly at ten sampling points at each experimental site. The soil samples were taken at four levels of depth (0-30 cm, 30-60 cm, 60-80 cm, and 80-100 cm) from the soil surface. After removing the gravel and plant debris, soil samples were composited for each level of soil depth, and 1 kg of soil was collected for microbial extraction and identification of fungi, bacteria, and nematodes in the laboratory. In addition, physicochemical properties of soil were also analyzed in this study.

Fungal and bacterial isolation and identification

For both fungal and bacterial isolation, 10 g of soil samples were dissolved in a liter of distilled water. Then 1 ml of soil solution was taken and diluted in steps from 10^{-1} to 10^{-3} to determine the CFU value of each sample, by repeating 2 times each dilution from three dilution levels. An inoculating needle was dipped in the soil solution, and then inoculated on Potato Dextrose Agar (PDA) medium to culture fungi, while another inoculating needle was dipped in the same solution and applied to Nutrient Agar (NA) medium to culture bacteria. After one week of culturing for fungi, or two days for bacteria, the microbes were isolated and observed under a microscope to identify the type, color, and shape of individual bacteria and fungi, as well as that of their colonies, following Hucker's procedure with minor modifications (Hucker 1921). The number of microorganisms (fungi and bacteria) present in each test sample as a weighted average of two consecutive dilutions was calculate using the following equation (FDA 2001):

$$N = \frac{\sum C}{[(1\ x\ n_1) +\ (0{,}1\ x\ n_2)\ x\ (d)}$$

N = Number of colonies per ml or g of product
ΣC = Sum of all colonies on all plates counted
n1 = Number of plates in first dilution counted
n2 = Number of plates in second dilution counted

d = Dilution from which the first counts were obtained

Entomopathogenic fungal and bacterial screening

Entomopathogenic fungi and bacteria were selected using mealworm (*Tenebrio molitor* L.) larvae 30-40 days of age (Figure 1). One milliliter of each fungal and bacterial isolate was inoculated to mealworms. Only fungi and bacteria that could cause mortality to mealworms were considered entomopathogens in the present study.

Figure 1. Mealworm (*Tenebrio molitor* L.) larvae at 30-40 days of age.

Isolation and identification of nematodes

Nematode isolation was carried out using the Baermann funnel method with minor modifications. This method was considered the most effective in nematode isolation (van den Hoogen et al. 2020). Briefly, a silicone hose was attached to a funnel, then the end of the hose was tied using a gauze rubber band and placed on top of the funnel. The Baermann funnel apparatus was installed in a horizontal position without buckling the silicon hose. Fifty grams of soil for each sample was placed on the filter paper, and the soil was wrapped to cover its entire surface. The water was added slowly into the funnel to submerge the sample, until the sample was saturated, to create a moist environment that stimulated nematodes to migrate toward the water source. The funnel was incubated for 3×24 hours and the water released from the bottom of the funnel. The water was collected in a film tube/vial. The collected water then was passed through a fine sieve to separate the nematodes from the water and debris. Up to 1 drop of formalin (5%) was added and fixed for 15 minutes.

Entomopathogenic nematode screening

Entomopathogenic nematodes were screened by bait trap using mealworms. The mealworm larvae (3-5 larvae) were placed in a plastic container and mixed with ~150 g of moist soil containing nematodes. The container was then inverted so that the larvae were covered with soil, then placed at room temperature.

After 3-4 days, if larval mortality was observed, the larval cadaver was removed and rinsed with sterilized distilled water. Entomopathogenic nematode trapping was then conducted using the White trap method to release entomopathogenic nematodes from the cadaver's body. Nematodes were observed and counted using a stereo microscope. Identification of soil nematodes and entomopathogenic nematodes to genus level was carried out through morphological observations.

Physicochemical analysis of soil

Samples were analyzed for their chemical and physical properties, including organic C, total N, available P (Bray), available K (Morgan), Ca^{++}, Mg^{++}, K^{+}, Na^{+}, CEC (Cation Exchange Capacity), base saturation (BS), Al saturation, pH, and soil texture. The soil physicochemical analysis was conducted based on the general procedure developed in the Soil Laboratory, Faculty of Agriculture, Mulawarman University, Samarinda, Indonesia.

Data analysis

Data collected were analyzed descriptively to uncover the role of soil microbes in supporting the recovery of post-coal-mining land and relationship between the diversity of soil microbes and chemical fertility status of the study site.

RESULTS AND DISCUSSION

Fungal diversity and density

Result showed that higher fungal diversity was observed in the non-mining land compared to the ex-mining land, with five fungal genera found in non-mining, and four genera in ex-mining soil (Figure 2 and Table 1). The fungal diversity in

ex-coal mining and non-mining land was different in every soil layer observed in this study (Table 1). *Penicillium* sp., which was found in every soil layer of post coal-mining land, was not present in the non-mining land soil. On the other hand, *Mucor* sp., which was found in the non-mining land soil, was absent in coal-mining land soil. In addition, *Aspergillus* was found abundant in almost all soil layers in both ex-coal-mining and non-mining land. Fungal density in non-mining land soil was also significantly higher (ranging from 2.6-7.2×10^5 cfu.g^{-1}) than in soil of ex-coal-mining land (ranging from 1.4-3.8×10^5 cfu.g^{-1}) at every soil depth layer. The deeper the soil layer, the fewer fungal populations were found. Among all the fungi identified in the ex-mining and non-mining land, only *Trichoderma* was screened as an entomopathogenic fungi. *Trichoderma* was observed in the digestive system of deceased *T. molitor* mealworm larvae.

Table 1. Fungal diversity and density at different soil depth layers, and screening of entomopathogenic fungi in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara District.

S. No.	Soil Depth Layers (cm)	Fungal Population Density cfu/g	Genera/Species
	Non-mining land		
1.	0-30	7.2 x 10^5	*Aspergillus* *Trichoderma** *Penicillium*
2.	30-60	5.4 x 10^5	*Aspergillus* *Trichoderma** *Penicillium*
3.	60-80	3.2 x 10^5	*Trichoderma** *Pythium* *Penicillium*
4.	80-100	2.6 x 10^5	*Aspergillus* *Penicillium*
	Post-coal-mining land		
1.	0-30	3.8 x 10^5	*Aspergillus* *Trichoderma **
2.	30-60	2.2 x 10^5	*Aspergillus niger* *Trichoderma**
3.	60-80	1.8 x 10^5	*Aspergillus* sp. *Mucor* sp.
4.	80-100	1.4 x 10^5	*Aspergillus niger* *Aspergillus flavus*

*Entomopathogenic fungi.

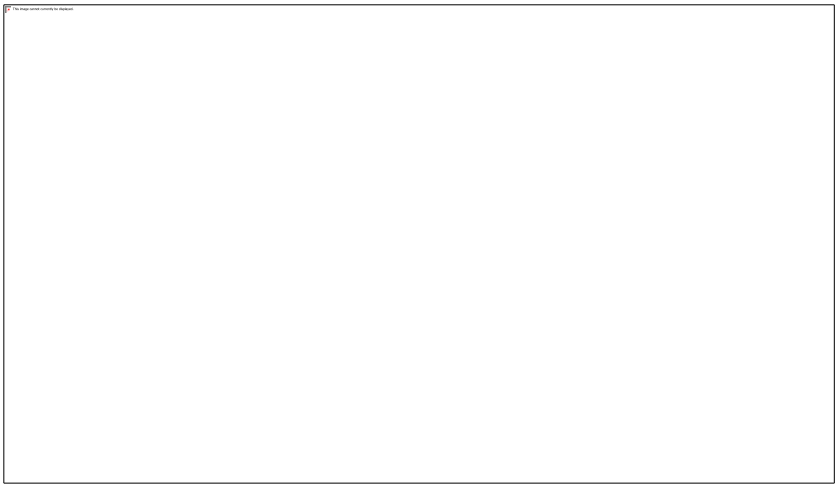

Figure 2. Fungal diversity: A. *Trichoderma*: conidia (a), hyalin (b), conidiophore (c); B. *Pythium*: oogonium (a), hyphae (b); C. *Penicillium*: conidium (a), phialide (b), metula (c), stipe (d); D. *A. niger*: conidia (a), conidiophore (b); E. *A. flavus*: conidia (a), phialide (b), vesicle (c), stipe (d); F. *Mucor* sp.: sporangium (a), sporangiophore (b), hyphae (c).

Entomopathogenic bacteria

Two bacterial families, *Azotobacteraceae* and *Bacillaceae*, were observed in the soils of both non-mining and ex-coal-mining land (Figure 3). However, only *Azotobacteraceae* was present in the deeper subsoil layers (60-80 cm or 80-100 cm) of the ex-coal-mining land . Interestingly, all *Azotobacteraceae* observed in this study were identified as coccus Gram-positive bacteria, except in the ex-coal-mining land, in which coccus Gram-negative *Azotobacteraceae* were found at the soil depths of 30-60 cm and 60-80 cm. The bacterial population was denser in non-coal-mining ($4.2\text{-}9.6\times10^6$) compared to ex-coal-mining land ($1.4\text{-}5.1\times10^6$). Bacterial population decreased along with soil depth at both experimental sites (Table 2). In this study, only *Bacillaceae* bacteria, observed in the digestive system of deceased *T. molitor* larvae, were identified as entomopathogenic bacteria in both ex-mining and non-mining lands.

Table 2. Bacterial diversity and density at different soil depth layers, and screening of entomopathogenic bacteria in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara District.

S. No.	Soil Depth Layers (cm)	Bacterial Population Density cfu/g	Family
	Non-mining land		
1.	0-30	9.6 x 10^6	- Coccus, Gram (+), *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
2.	30-60	5.5 x 10^6	- Coccus, Gram (+), *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
3.	60-80	4.5 x10^6	- Coccus, Gram (+) *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
4.	80-100	4.2 x 10^6	- Coccus, Gram (+): *Azotobacteraceae* - Bacilli, Gram (+): *Bacillaceae*
	Post-coal-mining land		
1.	0-30	5.1 x 10^6	- Coccus, Gram (+); *Azotobacteraceae* - Bacilli, Gram (+); *Bacillaceae**
2.	30-60	4.9 x 10^6	- Coccus, Gram (+) Azotobacteraceae - Bacilli, Gram (+); *Bacillaceae**
3.	60-80	1.6 x 10^6	- Coccus, Gram (+); Azotobacteraceae
4.	80-100	1.4 x 10^6	- Coccus, Gram (+); Azotobacteraceae

*Entomopathogenic bacteria.

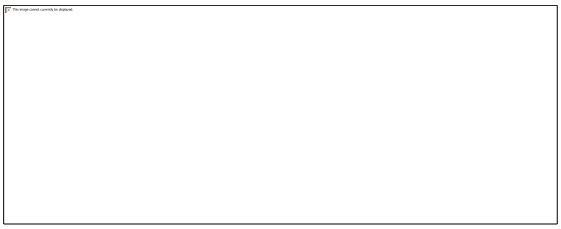

Figure 3. Bacteria (left to right: A. *Bacillaceae*, and B. *Azotobacteraceae*).

Entomopathogenic nematodes

A high genetic diversity of nematodes was observed in both non-mining and ex-mining land. Among the fifteen genera identified (Figure 4), six—namely *Heterodera*, *Heterorhabditis*, *Hoplolaimus*, *Pratylenchus*, *Radopholus*, and *Rotylenchus*were present in both non-mining and ex-coal-mining lands. Conversely, *Dorylaimus*, *Meloidogyne*, *Rotylenchulus*, and *Tylenchus* were found only in non-mining area, while *Longidorus*, *Rhabditis*, *Steinernema*, and *Xiphinema* were exclusive to ex-mining land. The number of nematode populations was notably higher in ex-coal-mining compared to non-mining land. It was also observed that the deeper the soil layer, the greater the number of isolated nematodes, as found in both non-mining and ex-coal-mining land (Table 3). Despite the high nematode diversity, only *Heterorhabditis* and *Steinernema* were identified as entomopathogenic nematodes.

Table 3. Nematode diversity and density at different soil depth layers, and screening of entomopathogenic nematodes in ex-mining and non-mining land in Pendingin, Sanga-Sanga Subdistrict, Kutai Kartanegara District.

S. No.	Soil Depth Layers (cm)	Nematodes Population/ kg soil	Genus

	Non-mining land		
1.	0-30	1.1 x10^2	*Heterodera* *Meloidogyne* *Tylenchus* *Heterorhabditis**
2.	30-60	1.2 x 10^2	*Heterorhabditis** *Pratlyenchus* *Heterodera* *Radopholus* *Hoplolaimus*
3.	60-80	4 x 10^2	*Pratylenchus* *Heterodera*
4.	80-100	2 x 10^2	*Rotylenchus* *Rotylenchulus* *Dorylaimus*
	Post-coal-mining land		
1.	0-30	5.1 x 10^2	*Heterodera* *Xiphinema*
2.	30-60	7.0 x 10^2	*Heterorhabditis** *Steinernema* *Radopholus* *Rhabditis*
3.	60-80	8.0 x 10^2	*Steinernema** *Hoplolaimus* *Rotylenchus*
4.	80-100	9.2 x 10^2	*Pratylenchus* *Longidorus*

*Entomopathogenic nematodes.

Figure 4. Nematodes (left to right: A. *Hoplolaimus*, B. *Pratylenchus*, C. *Tylenchus*, D. *Heterorhabditis*, E. *Steinernema*, F. *Dorylaimus*, G. *Xiphinema*, H. *Heterodera*, I. *Rhabditis*, J. *Radopholus*, K. *Longidorus*, L. *Rotylenchus*, M. *Rotylenchulus*, N. *Helicotylenchus*, O. *Aphelenchus*).

Physicochemical properties of soil

In general, soil nutrient content was higher in non-mining land compared to post-coal mining land (Table 4). There was also a variation in the nutrient content at different soil depth layers. Most of the nutrients were more abundant in the subsoil compared to the topsoil, as observed in the available K, Ca++. Mg++, K++, and Na+, as well as base saturation in non-mining land. In contrast, the reverse phenomenon was observed in post-coal-mining land.

Aluminum content was found to be higher in post-coal-mining than non-mining land. Moreover, the aluminum content tended to decrease with increasing soil depth on non-coal-mining land, while tending to be higher in the deeper soil layers on post-coal-mining land. The pH status of non-mining and post-coal-mining land was in the range of 4.02-4.9 and 3.19-4.19, respectively, showing higher acidity in post-coal-mining land.

Soil texture was determined by the proportion of mineral components in the soil. Among these mineral particles, silt, rather than clay or sand, was the dominant soil texture in topsoil and subsoil of both non-mining and post-coal-mining land. However, the relative proportions of different mineral particles (sand, silt, and clay) varied in each soil layer, resulting in distinct soil textures.

Table 4. Physicochemical properties of soil in non-mining and ex-coal-mining land in Kutai Kartanegara District, East Kalimantan.

No.	Soil Parameters	Unit	Non-coal Mining Land				Ex-coal Mining Land			
			R1 0-30	R2 30-60	R3 60-80	R4 80-100	S1 0-30	S2 30-60	S3 60-80	S4 80-100
1.	Carbon organic	%	7.65	4.64	4.14	3.41	3.80	3.74	1.38	0.86
2.	Nitrogen total	%	0.41	0.24	0.51	0.14	0.17	0.15	0.16	0.19
3.	Ratio C/N	-	18.66	19.33	8.12	24.36	22.35	24.93	8.63	4.53
4.	Available P (Bray1)	Ppm	19.39	12.60	13.38	20.26	5.11	4.03	0.96	4.12
5.	Available K tersedia (Morgan)	Ppm	110.45	214.54	170.21	234.33	209.22	81.56	82.09	143.28
6.	Al^{3+} (Morgan)		4.00	2.20	2.10	3.10	2.00	6.80	5.10	8.40

7.	H^+ (Morgan)		3.46	1.80	1.70	2.66	1.66	6.20	4.46	4.26
8.	Ca^{++}	meq/100 g	0.63	1.67	0.83	1.19	1.95	0.79	0.52	0.61
9.	Mg^{++}	meq/100 g	0.48	1.25	0.68	0.98	1.01	0.48	0.37	0.49
10.	K^+	meq/100 g	0.30	0.65	0.48	0.91	0.67	0.20	0.26	0.40
11.	Na^+	meq/100 g	0.04	0.38	0.28	0.29	0.34	0.11	0.02	0.12
12.	CEC	meq/100 g	8.91	7.95	6.07	9.13	8.16	8.58	10.73	14.28
13.	BS	%	16.3	49.7	37.4	36.9	48.7	18.4	10.9	11.3
14.	Al Saturation	%	44.89	27.67	34.60	33.95	26.21	46.64	47.53	58.82
15.	pH	–	4.02	4.62	4.54	4.90	3.56	3.35	4.19	3.19
16.	Clay	%	14.77	36.54	20.07	23.21	28.72	16.54	23.94	32.85
17.	Silt	%	34.48	58.18	55.30	35.43	67.66	64.75	33.70	35.54
18.	Sand	%	50.75	5.28	24.63	41.36	3.62	18.71	42.36	31.61
19.	Texture*		SiCL	SiCL	SiL	L	SiCL	SiL	L	CL

Note: *Soil Texture, SiCL= Silty Clay Loam; SiL= Silty Loam; CL= Clay Loam; L=Loam.

DISCUSSION

The greatest concern regarding land degradation impacts of post-mining areas for the agriculture sector is the reduction of biodiversity and soil fertility. Mining activities often lead to the destruction of natural habitats and ecosystems, resulting in the deracination and decline of plant and animal species, and leading to a loss of biodiversity that also negatively affects agricultural productivity. In addition, post-mining land undergoes physical and chemical changes that render it unsuitable for agriculture, such as reduced soil porosity and aeration due to the alteration of soil structure, a lack of organic matter and nutrients due to the topsoil removal, introduced contaminants such as heavy metals and toxic chemicals, and altered soil pH—more acidic or alkaline—which can all be detrimental to plant growth (Prince et al. 2018; Janečková et al. 2023).

Efforts to address the loss of arable land in post-mining areas involve comprehensive reclamation and rehabilitation practices that aim to restore soil fertility, structure, and water-holding capacity, and to repair the quality of the environment and ecosystem, as mentioned in the regulation issued by the Minister of Energy and Mineral Resources Regulation 7/2014 and Regional Regulation East Kalimantan 8/2013 (Yulianingrum 2023). These efforts may include topsoil replacement, revegetation, and ongoing monitoring to ensure the land can support agriculture practices. Furthermore, to achieve normal criteria for fertile soil supporting agricultural activities, maintenance of microbial community structures is very important.

Microbial entomopathogen diversity and density

In this study, it was observed that the nematode and soil microbial diversity in post-coal-mining land clearly differed to that in the non-mining land. Fungal diversity and density in post-coal-mining land soil was considerably lower compared to the non-mining land. This situation was also observed in soil bacteria, which showed low biodiversity in post-coal-mining land even after the completion of the reclamation process. A significant reduction of microbial diversity due to coal mining practices after land restoration was also observed in a study conducted by de Quadros et al. (2016). In their study, post-coal-mining land had undergone different durations of restoration (3-19 years), which involved replacing the soil layers, applying limestone to raise the pH to 6.0, and revegetating the land with grasses. Even after the completion of the reclamation process after many years, the composition of the microbial diversity and biomass of post-mined sites was significantly lower than that of the control (unaltered sites). Similar results were also found in different studies comparing the microbial diversity and density in reclaimed and revegetated post-coal-mining sites with that in undisturbed sites, both of which reported significant declines in microbial community structure and diversity (Li et al. 2014; Upadhyay et al. 2014).

A reduction of microbial (bacterial and fungal) diversity and density was also observed in the deeper layers of soil in both the post-coal-mining and non-mining-land, in which the microbes decreased as the depth of the soil layer increased. A similar observation was studied by Upadhyay et al. (2014), who reported that rhizospheric soils showed greater diversity in both bacteria and fungi compared to non-rhizospheric soil. This outcome is supported by recent studies concluding that soil microbial diversity decreases along with the soil depth (Wang et al. 2021; Guo et al. 2021)

It is well known that topsoil is the most biologically active and favorable layer for microorganisms. Compared to the subsoil layer, topsoil contains the highest concentration of organic matter, providing food and nutrients to microorganisms (Bahram et al. 2018). In addition, the uppermost layer of soil, also known as A-horizon, is well-aerated and has adequate moisture content and moderate temperatures, providing a suitable environment for a wide range of microorganisms (Liu et al. 2019; Aquado et al. 2023). Furthermore, there is an interaction between microorganisms and plant roots, which are more abundant in the top layer of soil. Plant roots release root exudates and provide energy to many soil microorganisms, thereby contributing to a more active microbial community (Zhalnina et al. 2018; Zhou et al.2020). The vegetation types, plant species, and climate all influence the soil microbial diversity and community composition (Li et al. 2022; Sopialena and Palupi 2017).

One of the key roles of microorganisms in agriculture is as agents of biological control, a process that harnesses natural enemies to suppress pest populations. The use of biological control agents is considered the most prudent manner of controlling pests as it can reduce our reliance on chemical pesticides, and because it is a long-term, specific pest targeting, environmentally friendly, cost-effective approach that is safe for human health and supports sustainable agriculture (Boro et al. 2022). Therefore, besides observing the genetic diversity and density of the microorganisms in the ex-mining and non-mining land, this study also focused on screening entomopathogenic nematodes and microbes as potential biological control agents for suppressing insect populations. Insects are the largest and most diverse group of organisms included as one of the most common and economically significant types of plant pests (García-Lara 2016), causing significant damage to many crop plant species (Manosathiyadevan 2017).

Among all microbes, only *Trichoderma* (fungi) and *Bacillaceae* (bacteria) were identified as entomopathogenic microorganisms in the present study, in both ex-mining and non-mining lands. *Trichoderma* can indirectly act as an entomopathogen by producing insecticidal secondary metabolites, antifeedant compounds, and repellent metabolites (Poveda 2021). This is due to *Trichoderma*'s ability to produce metabolites such as citric acid (Vinale et al. 2012), as well as enzymes like urease, cellulase, glucanase, and chitinase (Carlile et al. 2001), which can negatively affect insects and even cause death when they enter the body. On the other hand, bacteria from the *Bacillaceae* family, particularly *Bacillus thuringiensis*, have received significant attention as entomopathogens. This bacterium is capable of producing proteinaceous protoxin crystals with insecticidal properties. When ingested by insects, these crystals disintegrate in the stomach and are broken down by host proteases to produce an active poison known as endotoxin, which ultimately leads to insect death (Deka et al. 2021).

In contrast to the fungi and bacteria, nematodes were found to be more abundant in the post-mining than in the non-mining land in terms of their diversity and density. Additionally, more nematodes were observed in the deeper soil layers. Most related studies have observed that the vertical distribution of nematode communities decreases from the topsoil layer to the subsoil layer (Cheng et al. 2021; Suyadi et al. 2021). This variation in soil nematode communities may be explained by the distinct characteristics of soils at different layers (Liu et al. 2022). The differences in soil nematode diversity and density at different soil depths observed in this study might be influenced by soil variables. The amount of nematode diversity varied in different experimental sites (Ilieva-Makulec et al. 2015). Results showed that nematode generic richness was higher in the topsoil of one site, while in a different habitat, nematode diversity was significantly lower in the topsoil and highest in the subsoil. Overall, it is likely that nematode abundance and distribution are influenced by many factors, such as soil properties, climate and environmental conditions, land use and management, plant species and root exudates, predation and competition, and succession and ecosystem development (Ilieva-Makulec et al. 2015; Zhang 2020; Cheng et al. 2021; Li et al. 2022).

Nematodes may be classified into three main groups: saprophages (living on dead organic matter), predators (feeding on small animals, including other nematodes), and parasites (infesting insects, animals, human, fungi and higher plants) (Pant et al. 1998). Eleven of the fifteen nematodes identified in this study, namely *Heterodera*, *Meloidogyne*, *Tylenchus*, *Pratlyenchus*, *Radopholus*, *Hoplolaimus*, *Rotylenchus*, *Rotylenchulus*, *Xiphinema*, and *Longidorus* were plant-parasitic nematodes. *Meloidogyne*, *Radopholus*, and *Rotylenchulus* were the top three genera of plant-parasite nematodes observed in East Kalimantan (Suyadi and Rosfiansyah 2017). *Dorylaimus* and *Rhabditis*, on the other hand, were non-plant-parasitic nematodes. *Dorylaimus* functions as an omnivore in the rhizosphere, and *Rhabditis* as a bacterivore. In this study, only *Heterorhabditis* and *Steinernema* were identified as entomopathogenic nematodes, as also observed in the previous studies (Bhat et al., 2020; Julià et al., 2023).

Comment [AKG47]: Correct it or removed it. They observed nematodes in which sample?

Comment [Me48]: We have corrected the sentence.

Relation of soil physicochemical properties and microbial community

The physicochemical properties of post-mining soil were considerably different from those of non-mining soil in this study. The different characteristics of post-mining land arise from the significant alterations in soil properties, structure, and chemical composition due to mining activities. The mining process disrupts the natural soil structure, leading to soil compaction, which reduces pore spaces and affects water infiltration and root penetration (Yuningsih et al. 2021). Additionally, the removal or burying of topsoil (due to improper reclamation processes) results in a significant reduction in the organic content of the soil (especially in the root growth layer), leading to nutrient loss and further diminishing soil fertility. Furthermore, the mining process can expose rocks and minerals that, when weathered, can alter the soil's pH, leading to acid mine drainage and significantly lowering the soil pH (Herman et al. 2024). These conditions make the soil

ecosystem in post-mining land unconducive for microbial communities, which contrasts with the conditions in non-mining land.

Soil is a medium for plant growth and also acts as an environment for microbial growth, determining the quality of growing soil microbes and plants. The interactions between soil microbes and plants play a crucial role in soil health and fertility. Fertile soil is typically a source of organic matter for microbial activity. Soil status encompasses a wide range of physical, chemical, and biological properties that influence the habitat and conditions for soil microorganisms. A healthy soil status with sufficient organic matter and nutrient availability ensures that microorganisms have the resources they need to thrive (Kaur et al. 2022) The presence of high organic matter in soil is characterized by several physicochemical parameters, such as pH levels, cation exchange capacity (CEC), soil organic carbon (SOC) content, and nutrient content, especially of nitrogen, phosphorus, and potassium (Dandwate 2020)

It was also observed that soils in both post-coal mining and non-mining land were found to be acidic, with minor variations at every soil depth layer. However, soil pH in the post-mining land was considerably lower, in the range of 3.19-4.19, compared to that in the non-mining land (4.02-4.9). Related to the lower pH in post-mining land was the aluminum content, which was higher than that in the non-mining land as acidic soils enhance aluminum solubility and availability. Furthermore, soil pH is positively correlated with another soil property, basic saturation (BS), which was observed to be higher in the non-mining land.

Soil pH strongly influences microbial communities. Acidic or alkaline soils can limit the diversity and activity of microorganisms, impacting nutrient availability and overall soil. It is a critical factor that affects the activity and diversity of soil microorganisms. Different microbial species have specific pH preferences, and soil pH can influence their metabolic activities. Soil pH outside the optimal range for specific microbes can limit their growth and activity (Wang et al. 2019). Therefore, maintaining an appropriate pH level is essential for a diverse and active microbial community.

Soil organic matter was higher in non-mining land in this study, as characterized by the higher soil organic carbon (SOC) and nutrient content than that of post-coal-mining land, as well as by the pH level. Higher organic matter content is associated with high SOC, which, as carbon is a fundamental component of organic materials, mainly occurs via the microbial breakdown, partial decay, and conversion of deceased organic materials (Lefèvre et al. 2017) This activity is co-related with nutrient availability; that is, as organic matter decomposes, it releases nutrients into the soil, contributing to the soil's overall fertility and health (Gerke 2022)

Overall, soil physicochemical properties play a significant role in shaping the composition and activity of the microbial community (Brahmaprakash 2021). A larger microbial community was observed alongside the higher physicochemical soil properties of the non-mining land, rather than in the post-coal-mining land. Changes in these properties can influence the composition and activity of microbial community, subsequently impacting nutrient cycling, organic matter decomposition, and overall soil fertility (Leiva et al. 2020; Kaur et al. 2022). Fertile soil is characterized by a well-balanced and diverse microbial community that supports plant growth by providing essential nutrients and improving soil structure. The composition and diversity of the soil microbial community are important factors in maintaining soil health that also support plant growth and health (Wang and Li 2023). The increasing microbial population (both in diversity and abundance) causes the dynamics of the soil to improve and become healthier naturally (Bertola et al. 2021). The ability to change soil biological properties in a positive direction can increase the population of microbes, including entomopathogenic microbes, which benefit plant growth and reduce the need for artificial fertilizer and pesticide use.

CONCLUSION

The results showed that microbial diversity in post-coal-mining land differed from that in non-mining control. The entomopathogenic fungal and bacterial diversity and density in ex-coal-mining soil was considerably lower compared to that in the non-mining land. The microbial (bacterial and fungal) diversity and density were lower in deeper layers of soil in both post-coal-mining and non-mining land, and tended to decrease soil layer depth increased. In contrast to fungi and bacteria, entomopathogenic nematodes were observed to be more abundant in the post-mining than in non-mining land in terms of their diversity and density. Additionally, nematodes were more abundant in the deeper layers of soil. Soil fertility was found to be lower in ex-coal-mining land, as characterized by several soil physicochemical properties, of which the lower soil pH, basic saturation, soil organic carbon (SOC) and nutrient content detected likely supported a smaller microbial community compared to the non-mining land.

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Assessment of nematode and microbial diversity and screening of entomopathogens in revegetated post-coal-mining land in Kutai Kartanegara District, East Kalimantan, Indonesia

Abstract. Post-coal-mining management should strive toward site revegetation. The success of such revegetation efforts is largely determined by the soil's fertility status, which encompasses physical, chemical, and biological components. Biological fertility is indicated by the presence and abundance of soil microbes and nematodes. The objective of this study was to identify the diversity of nematodes and soil microbes in ex-coal-mining land revegetated with oil palm in Kutai Kartanegara District (East Kalimantan, Indonesia), to screen for entomopathogenic nematodes and microbes, and to analyze their relationships with the chemical fertility status of the land. As a control, non-mining land was also investigated. The results showed that microbial diversity and density (including fungi and bacteria) in the ex-coal-mining area were significantly lower compared to the control. Additionally, a decrease in microbial diversity and density was found in deeper soil layers of both post-mining and non-mining lands. In contrast, soil nematodes were more abundant in post-mining land, both in terms of diversity and density, and were also more plentiful in the deeper soil layers. Among the nematodes and microbes studied, only *Heterorhabditis* and *Steinernema* (nematodes), *Trichoderma* (fungi), and *Bacillaceae* (bacteria) were identified as entomopathogens, causing death in *Tenebrio molitor* L. larvae. Overall, soil fertility in ex-coal-mining land was observed to be lower, characterized by decreased soil pH, basic saturation, soil organic carbon (SOC), and nutrient content, which contributed to a reduced microbial community compared to non-mining land.

Keywords: Entomopathogen, nematodes, soil microbes, post-coal mining, Kutai Kartanegara, *Trichoderma*.

INTRODUCTION

Coal mining activities in Kutai Kartanegara District, East Kalimantan, Indonesia, have experienced rapid growth in recent years, with both large-scale and small-scale operations, including those with mining authorities. However, this expansion has come at a significant cost to the environment, resulting in widespread damage. This damage manifests in the form of arid and infertile post-mining lands or artificial "craters" with unclear purposes. While some efforts have been made to rehabilitate these ex-coal-mining lands, they remain sporadic and insufficient, allowing environmental degradation to outpace recovery efforts.

Rehabilitating these lands is essential to mitigating the adverse environmental impacts of coal mining. The success of land recovery can be evaluated by assessing the diversity of soil microbes, which serves as an indicator of biological fertility. Soil microbes play a pivotal role in converting potential chemical fertility into actual fertility. Moreover, their activities gradually enhance the physical properties of the soil, further improving physical fertility (Cui et al. 2021). However, the restoration of ex-coal-mining lands faces numerous challenges, including the need to address soil degradation, erosion, and biodiversity loss resulting from mining activities (Ahirwal et al. 2016). To address these challenges, it is crucial to consider the role of soil microbes, including entomopathogenic microbes, which are an integral part of soil ecosystems (Boucias and Pendland 2018).

The roles of nematodes and microbes in ex-mining lands are fundamental to ecological restoration efforts, improving soil health, supporting plant growth, and maintaining ecological balance. These microorganisms can significantly impact the success of revegetation and restoration projects in post-mining landscapes. The presence of nematodes and microbes in the soil benefits plant health during revegetation endeavors (Guo et al. 2021; Li et al. 2022). Furthermore, these microorganisms support plant establishment and growth. Healthy and robust plant growth, in turn, stabilizes the soil, prevents erosion, and enhances biodiversity, all of which are important for the overall success of revegetation projects (Mensah 2015). The presence of microbial diversity in mining-affected areas can be influenced by factors such as soil type, climate, land management practices, as well as the host plants' root exudate composition (Santoyo 2022; Sasse et al. 2017). Consequently, understanding and managing the diversity of these microorganisms are critical for the success of revegetation efforts.

Entomopathogenic microbes, which encompass various taxonomic groups, including bacteria, fungi, nematodes, and viruses, are known to regulate insect populations and play a vital role in ecological processes (Deka et al. 2021). One of the most remarkable contributions of entomopathogenic microbes during revegetation is their role as natural pest controllers (Vega et al. 2009). These microorganisms serve as natural enemies of various insect pests that could harm newly planted vegetation (Shrestha et al. 2020). By infecting and eliminating these pests, entomopathogens help regulate insect populations and protect the restored vegetation (Rozpądek et al. 2019). In regions where entomopathogens thrive, the risk of pest outbreaks is reduced, safeguarding the overall health of the revegetated land (Koskella et al. 2020). Entomopathogenic microbes, such as fungi and bacteria are natural antagonists of insects and play a pivotal role in regulating insect populations in terrestrial ecosystems. While extensively studied for their potential in biological pest control in agriculture, their utilization in ecological restoration, particularly in ex-coal-mining land revegetation, remains a relatively unexplored area, and a promising avenue for research and practical implementation. The diversity and abundance of entomopathogenic microbes are subject to environmental factors (Fatu et al. 2020). Factors such as soil physicochemical properties, which can fluctuate significantly in mining-affected areas, play pivotal roles in shaping microbial community structure (Mudrak et al. 2021).

The objective of this study was to identify the diversity of nematodes and soil microbes on revegetated ex-coal-mining land, to investigate for entomopathogenic nematodes and microbes, and to analyze their relationships with the chemical fertility status of the land.

MATERIALS AND METHODS

Experimental sites

The study was conducted at two locations, in post-coal mining and non-mining areas, in the Pendingin Subdistrict of Sanga-Sanga, in Kutai Kartanegara District, East Kalimantan Province, Indonesia. Oil palm plantations were planted in a post-coal mining area (located at S 00040'29.7" E 117016'09.8"), while rubber plantations were cultivated in a non-mining area (located at S 00041'41.3" E 117015'54.9") used as a control.

Sample collection

The sampling method used in this study was the standard method of ISPM (International Standards for Phytosanitary Measure). Soil samples were taken randomly at ten sampling points at each experimental site. The soil samples were taken at four levels of depth (0-30 cm, 30-60 cm, 60-80 cm, and 80-100 cm) from the soil surface. After removing the gravel and plant debris, soil samples were composited for each level of soil depth, and 1 kg of soil was collected for

microbial extraction and identification of fungi, bacteria, and nematodes in the laboratory. In addition, physicochemical properties of soil were also analyzed in this study.

Fungal and bacterial isolation and identification

For both fungal and bacterial isolation, 10 g of soil samples were dissolved in a liter of distilled water. Then 1 ml of soil solution was taken and diluted in steps from 10^{-1} to 10^{-3} to determine the CFU value of each sample, by repeating 2 times each dilution from three dilution levels. An inoculating needle was dipped in the soil solution, and then inoculated on Potato Dextrose Agar (PDA) medium to culture fungi, while another inoculating needle was dipped in the same solution and applied to Nutrient Agar (NA) medium to culture bacteria. After one week of culturing for fungi, or two days for bacteria, the microbes were isolated and observed under a microscope to identify the type, color, and shape of individual bacteria and fungi, as well as that of their colonies, following Hucker's procedure with minor modifications (Hucker 1921). The number of microorganisms (fungi and bacteria) present in each test sample as a weighted average of two consecutive dilutions was calculate using the following equation (FDA 2001):

$$N = \frac{\sum C}{[(1\ x\ n_1) +\ (0{,}1\ x\ n_2)\ x\ (d)}$$

N = Number of colonies per ml or g of product
ΣC = Sum of all colonies on all plates counted
n1 = Number of plates in first dilution counted
n2 = Number of plates in second dilution counted
d = Dilution from which the first counts were obtained

Entomopathogenic fungal and bacterial screening

Entomopathogenic fungi and bacteria were selected using mealworm (*Tenebrio molitor* L.) larvae 30-40 days of age (Figure 1). One milliliter of each fungal and bacterial isolate was inoculated to mealworms. Only fungi and bacteria that could cause mortality to mealworms were considered entomopathogens in the present study.

Figure 1. Mealworm (*Tenebrio molitor* L.) larvae at 30-40 days of age.

Isolation and identification of nematodes

Nematode isolation was carried out using the Baermann funnel method with minor modifications. This method was considered the most effective in nematode isolation (van den Hoogen et al. 2020). Briefly, a silicone hose was attached to a funnel, then the end of the hose was tied using a gauze rubber band and placed on top of the funnel. The Baermann funnel apparatus was installed in a horizontal position without buckling the silicon hose. Fifty grams of soil for each sample was placed on the filter paper, and the soil was wrapped to cover its entire surface. The water was added slowly into the funnel to submerge the sample, until the sample was saturated, to create a moist environment that stimulated nematodes to migrate toward the water source. The funnel was incubated for 3×24 hours and the water released from the bottom of the funnel. The water was collected in a film tube/vial. The collected water then was passed through a fine sieve to separate the nematodes from the water and debris. Up to 1 drop of formalin (5%) was added and fixed for 15 minutes.

Entomopathogenic nematode screening

Entomopathogenic nematodes were screened by bait trap using mealworms. The mealworm larvae (3-5 larvae) were placed in a plastic container and mixed with ~150 g of moist soil containing nematodes. The container was then inverted so that the larvae were covered with soil, then placed at room temperature.

After 3-4 days, if larval mortality was observed, the larval cadaver was removed and rinsed with sterilized distilled water. Entomopathogenic nematode trapping was then conducted using the White trap method to release entomopathogenic nematodes from the cadaver's body. Nematodes were observed and counted using a stereo microscope. Identification of soil nematodes and entomopathogenic nematodes to genus level was carried out through morphological observations.

Physicochemical analysis of soil

Samples were analyzed for their chemical and physical properties, including organic C, total N, available P (Bray), available K (Morgan), Ca^{++}, Mg^{++}, K^{+}, Na^{+}, CEC (Cation Exchange Capacity), base saturation (BS), Al saturation, pH, and soil texture. The soil physicochemical analysis was conducted based on the general procedure developed in the Soil Laboratory, Faculty of Agriculture, Mulawarman University, Samarinda, Indonesia.

Data analysis

Data collected were analyzed descriptively to uncover the role of soil microbes in supporting the recovery of post-coal-mining land and relationship between the diversity of soil microbes and chemical fertility status of the study site.

RESULTS AND DISCUSSION

Fungal diversity and density

Result showed that higher fungal diversity was observed in the non-mining land compared to the ex-mining land, with five fungal genera found in non-mining, and four genera in ex-mining soil (Figure 2 and Table 1). The fungal diversity in ex-coal mining and non-mining land was different in every soil layer observed in this study (Table 1). *Penicillium* sp., which was found in every soil layer of post coal-mining land, was not present in the non-mining land soil. On the other hand, *Mucor* sp., which was found in the non-mining land soil, was absent in coal-mining land soil. In addition, *Aspergillus* was found abundant in almost all soil layers in both ex-coal-mining and non-mining land. Fungal density in non-mining land soil was also significantly higher (ranging from $2.6\text{-}7.2\times10^5$ cfu.g^{-1}) than in soil of ex-coal-mining land (ranging from $1.4\text{-}3.8\times10^5$ cfu.g^{-1}) at every soil depth layer. The deeper the soil layer, the fewer fungal populations were found. Among all the fungi identified in the ex-mining and non-mining land, only *Trichoderma* was screened as an entomopathogenic fungi. *Trichoderma* was observed in the digestive system of deceased *T. molitor* mealworm larvae.

Table 1. Fungal diversity and density at different soil depth layers, and screening of entomopathogenic fungi in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara District.

S. No.	Soil Depth Layers (cm)	Fungal Population Density cfu/g	Genera/Species
	Non-mining land		
1.	0-30	7.2×10^5	*Aspergillus* *Trichoderma** *Penicillium*
2.	30-60	5.4×10^5	*Aspergillus* *Trichoderma** *Penicillium*
3.	60-80	3.2×10^5	*Trichoderma** *Pythium* *Penicillium*
4.	80-100	2.6×10^5	*Aspergillus* *Penicillium*
	Post-coal-mining land		
1.	0-30	3.8×10^5	*Aspergillus* *Trichoderma **
2.	30-60	2.2×10^5	*Aspergillus niger* *Trichoderma**

3.	60-80	1.8 x 10^5	*Aspergillus* sp. *Mucor* sp.
4.	80-100	1.4 x 10^5	*Aspergillus niger* *Aspergillus flavus*

*Entomopathogenic fungi.

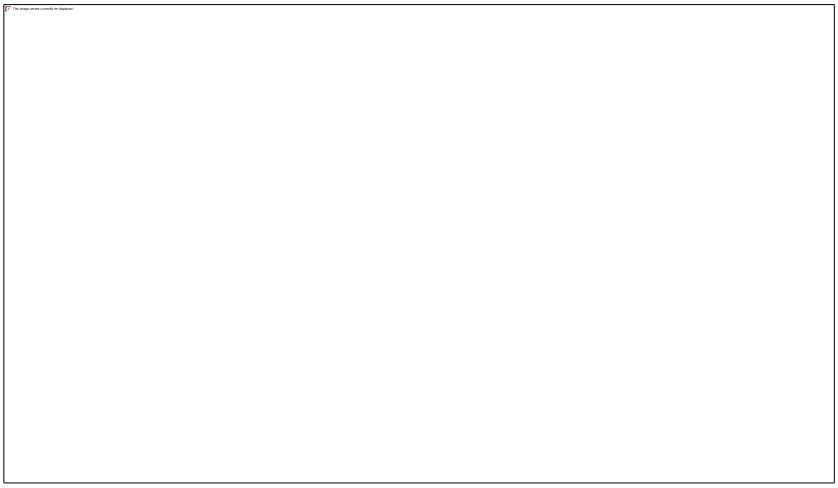

Figure 2. Fungal diversity: A. *Trichoderma*: conidia (a), hyalin (b), conidiophore (c); B. *Pythium*: oogonium (a), hyphae (b); C. *Penicillium*: conidium (a), phialide (b), metula (c), stipe (d); D. *A. niger*: conidia (a), conidiophore (b); E. *A. flavus*: conidia (a), phialide (b), vesicle (c), stipe (d); F. *Mucor* sp.: sporangium (a), sporangiophore (b), hyphae (c).

Entomopathogenic bacteria

Two bacterial families, *Azotobacteraceae* and *Bacillaceae*, were observed in the soils of both non-mining and ex-coal-mining land (Figure 3). However, only *Azotobacteraceae* was present in the deeper subsoil layers (60-80 cm or 80-100 cm) of the ex-coal-mining land . Interestingly, all *Azotobacteraceae* observed in this study were identified as coccus Gram-positive bacteria, except in the ex-coal-mining land, in which coccus Gram-negative *Azotobacteraceae* were found at the soil depths of 30-60 cm and 60-80 cm. The bacterial population was denser in non-coal-mining ($4.2\text{-}9.6\times10^6$) compared to ex-coal-mining land ($1.4\text{-}5.1\times10^6$). Bacterial population decreased along with soil depth at both experimental sites (Table 2). In this study, only *Bacillaceae* bacteria, observed in the digestive system of deceased *T. molitor* larvae, were identified as entomopathogenic bacteria in both ex-mining and non-mining lands.

Table 2. Bacterial diversity and density at different soil depth layers, and screening of entomopathogenic bacteria in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara District.

S. No.	Soil Depth Layers (cm)	Bacterial Population Density cfu/g	Family
	Non-mining land		
1.	0-30	9.6 x 10^6	- Coccus, Gram (+), *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
2.	30-60	5.5 x 10^6	- Coccus, Gram (+), *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
3.	60-80	4.5 x10^6	- Coccus, Gram (+) *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
4.	80-100	4.2 x 10^6	- Coccus, Gram (+): *Azotobacteraceae* - Bacilli, Gram (+): *Bacillaceae*
	Post-coal-mining land		
1.	0-30	5.1 x 10^6	- Coccus, Gram (+); *Azotobacteraceae* - Bacilli, Gram (+); *Bacillaceae**
2.	30-60	4.9 x 10^6	- Coccus, Gram (+) Azotobacteraceae - Bacilli, Gram (+); *Bacillaceae**
3.	60-80	1.6 x 10^6	- Coccus, Gram (+); Azotobacteraceae
4.	80-100	1.4 x 10^6	- Coccus, Gram (+); Azotobacteraceae

*Entomopathogenic bacteria.

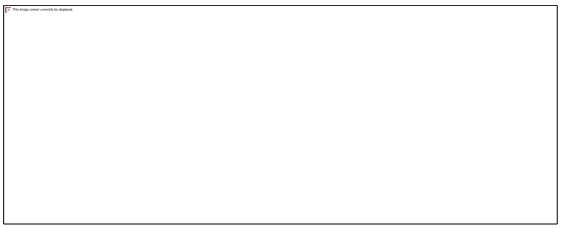

Figure 3. Bacteria (left to right: A. *Bacillaceae*, and B. *Azotobacteraceae*).

Entomopathogenic nematodes

A high genetic diversity of nematodes was observed in both non-mining and ex-mining land. Among the fifteen genera identified (Figure 4), six—namely *Heterodera*, *Heterorhabditis*, *Hoplolaimus*, *Pratylenchus*, *Radopholus*, and *Rotylenchus*were present in both non-mining and ex-coal-mining lands. Conversely, *Dorylaimus*, *Meloidogyne*, *Rotylenchulus*, and *Tylenchus* were found only in non-mining area, while *Longidorus*, *Rhabditis*, *Steinernema*, and *Xiphinema* were exclusive to ex-mining land. The number of nematode populations was notably higher in ex-coal-mining compared to non-mining land. It was also observed that the deeper the soil layer, the greater the number of isolated nematodes, as found in both non-mining and ex-coal-mining land (Table 3). Despite the high nematode diversity, only *Heterorhabditis* and *Steinernema* were identified as entomopathogenic nematodes.

Table 3. Nematode diversity and density at different soil depth layers, and screening of entomopathogenic nematodes in ex-mining and non-mining land in Pendingin, Sanga-Sanga Subdistrict, Kutai Kartanegara District.

S. No.	Soil Depth Layers (cm)	Nematodes Population/ kg soil	Genus

	Non-mining land		
1.	0-30	1.1 x10^2	*Heterodera* *Meloidogyne* *Tylenchus* *Heterorhabditis**
2.	30-60	1.2 x 10^2	*Heterorhabditis** *Pratlyenchus* *Heterodera* *Radopholus* *Hoplolaimus*
3.	60-80	4 x 10^2	*Pratylenchus* *Heterodera*
4.	80-100	2 x 10^2	*Rotylenchus* *Rotylenchulus* *Dorylaimus*
	Post-coal-mining land		
1.	0-30	5.1 x 10^2	*Heterodera* *Xiphinema*
2.	30-60	7.0 x 10^2	*Heterorhabditis** *Steinernema* *Radopholus* *Rhabditis*
3.	60-80	8.0 x 10^2	*Steinernema** *Hoplolaimus* *Rotylenchus*
4.	80-100	9.2 x 10^2	*Pratylenchus* *Longidorus*

*Entomopathogenic nematodes.

Figure 4. Nematodes (left to right: A. *Hoplolaimus*, B. *Pratylenchus*, C. *Tylenchus*, D. *Heterorhabditis*, E. *Steinernema*, F. *Dorylaimus*, G. *Xiphinema*, H. *Heterodera*, I. *Rhabditis*, J. *Radopholus*, K. *Longidorus*, L. *Rotylenchus*, M. *Rotylenchulus*, N. *Helicotylenchus*, O. *Aphelenchus*).

Physicochemical properties of soil

In general, soil nutrient content was higher in non-mining land compared to post-coal mining land (Table 4). There was also a variation in the nutrient content at different soil depth layers. Most of the nutrients were more abundant in the subsoil compared to the topsoil, as observed in the available K, Ca++. Mg++, K++, and Na+, as well as base saturation in non-mining land. In contrast, the reverse phenomenon was observed in post-coal-mining land.

Aluminum content was found to be higher in post-coal-mining than non-mining land. Moreover, the aluminum content tended to decrease with increasing soil depth on non-coal-mining land, while tending to be higher in the deeper soil layers on post-coal-mining land. The pH status of non-mining and post-coal-mining land was in the range of 4.02-4.9 and 3.19-4.19, respectively, showing higher acidity in post-coal-mining land.

Soil texture was determined by the proportion of mineral components in the soil. Among these mineral particles, silt, rather than clay or sand, was the dominant soil texture in topsoil and subsoil of both non-mining and post-coal-mining land. However, the relative proportions of different mineral particles (sand, silt, and clay) varied in each soil layer, resulting in distinct soil textures.

Table 4. Physicochemical properties of soil in non-mining and ex-coal-mining land in Kutai Kartanegara District, East Kalimantan.

No.	Soil Parameters	Unit	Non-coal Mining Land				Ex-coal Mining Land			
			R1 0-30	R2 30-60	R3 60-80	R4 80-100	S1 0-30	S2 30-60	S3 60-80	S4 80-100
1.	Carbon organic	%	7.65	4.64	4.14	3.41	3.80	3.74	1.38	0.86
2.	Nitrogen total	%	0.41	0.24	0.51	0.14	0.17	0.15	0.16	0.19
3.	Ratio C/N	-	18.66	19.33	8.12	24.36	22.35	24.93	8.63	4.53
4.	Available P (Bray1)	Ppm	19.39	12.60	13.38	20.26	5.11	4.03	0.96	4.12
5.	Available K tersedia (Morgan)	Ppm	110.45	214.54	170.21	234.33	209.22	81.56	82.09	143.28
6.	Al^{3+} (Morgan)		4.00	2.20	2.10	3.10	2.00	6.80	5.10	8.40

7.	H^+ (Morgan)		3.46	1.80	1.70	2.66	1.66	6.20	4.46	4.26
8.	Ca^{++}	meq/100 g	0.63	1.67	0.83	1.19	1.95	0.79	0.52	0.61
9.	Mg^{++}	meq/100 g	0.48	1.25	0.68	0.98	1.01	0.48	0.37	0.49
10.	K^+	meq/100 g	0.30	0.65	0.48	0.91	0.67	0.20	0.26	0.40
11.	Na^+	meq/100 g	0.04	0.38	0.28	0.29	0.34	0.11	0.02	0.12
12.	CEC	meq/100 g	8.91	7.95	6.07	9.13	8.16	8.58	10.73	14.28
13.	BS	%	16.3	49.7	37.4	36.9	48.7	18.4	10.9	11.3
14.	Al Saturation	%	44.89	27.67	34.60	33.95	26.21	46.64	47.53	58.82
15.	pH	–	4.02	4.62	4.54	4.90	3.56	3.35	4.19	3.19
16.	Clay	%	14.77	36.54	20.07	23.21	28.72	16.54	23.94	32.85
17.	Silt	%	34.48	58.18	55.30	35.43	67.66	64.75	33.70	35.54
18.	Sand	%	50.75	5.28	24.63	41.36	3.62	18.71	42.36	31.61
19.	Texture*		SiCL	SiCL	SiL	L	SiCL	SiL	L	CL

Note: *Soil Texture, SiCL= Silty Clay Loam; SiL= Silty Loam; CL= Clay Loam; L=Loam.

DISCUSSION

The greatest concern regarding land degradation impacts of post-mining areas for the agriculture sector is the reduction of biodiversity and soil fertility. Mining activities often lead to the destruction of natural habitats and ecosystems, resulting in the deracination and decline of plant and animal species, and leading to a loss of biodiversity that also negatively affects agricultural productivity. In addition, post-mining land undergoes physical and chemical changes that render it unsuitable for agriculture, such as reduced soil porosity and aeration due to the alteration of soil structure, a lack of organic matter and nutrients due to the topsoil removal, introduced contaminants such as heavy metals and toxic chemicals, and altered soil pH—more acidic or alkaline—which can all be detrimental to plant growth (Prince et al. 2018; Janečková et al. 2023).

Efforts to address the loss of arable land in post-mining areas involve comprehensive reclamation and rehabilitation practices that aim to restore soil fertility, structure, and water-holding capacity, and to repair the quality of the environment and ecosystem, as mentioned in the regulation issued by the Minister of Energy and Mineral Resources Regulation 7/2014 and Regional Regulation East Kalimantan 8/2013 (Yulianingrum 2023). These efforts may include topsoil replacement, revegetation, and ongoing monitoring to ensure the land can support agriculture practices. Furthermore, to achieve normal criteria for fertile soil supporting agricultural activities, maintenance of microbial community structures is very important.

Microbial entomopathogen diversity and density

In this study, it was observed that the nematode and soil microbial diversity in post-coal-mining land clearly differed to that in the non-mining land. Fungal diversity and density in post-coal-mining land soil was considerably lower compared to the non-mining land. This situation was also observed in soil bacteria, which showed low biodiversity in post-coal-mining land even after the completion of the reclamation process. A significant reduction of microbial diversity due to coal mining practices after land restoration was also observed in a study conducted by de Quadros et al. (2016). In their study, post-coal-mining land had undergone different durations of restoration (3-19 years), which involved replacing the soil layers, applying limestone to raise the pH to 6.0, and revegetating the land with grasses. Even after the completion of the reclamation process after many years, the composition of the microbial diversity and biomass of post-mined sites was significantly lower than that of the control (unaltered sites). Similar results were also found in different studies comparing the microbial diversity and density in reclaimed and revegetated post-coal-mining sites with that in undisturbed sites, both of which reported significant declines in microbial community structure and diversity (Li et al. 2014; Upadhyay et al. 2014).

A reduction of microbial (bacterial and fungal) diversity and density was also observed in the deeper layers of soil in both the post-coal-mining and non-mining-land, in which the microbes decreased as the depth of the soil layer increased. A similar observation was studied by Upadhyay et al. (2014), who reported that rhizospheric soils showed greater diversity in both bacteria and fungi compared to non-rhizospheric soil. This outcome is supported by recent studies concluding that soil microbial diversity decreases along with the soil depth (Wang et al. 2021; Guo et al. 2021)

It is well known that topsoil is the most biologically active and favorable layer for microorganisms. Compared to the subsoil layer, topsoil contains the highest concentration of organic matter, providing food and nutrients to microorganisms (Bahram et al. 2018). In addition, the uppermost layer of soil, also known as A-horizon, is well-aerated and has adequate moisture content and moderate temperatures, providing a suitable environment for a wide range of microorganisms (Liu et al. 2019; Aquado et al. 2023). Furthermore, there is an interaction between microorganisms and plant roots, which are more abundant in the top layer of soil. Plant roots release root exudates and provide energy to many soil microorganisms, thereby contributing to a more active microbial community (Zhalnina et al. 2018; Zhou et al.2020). The vegetation types, plant species, and climate all influence the soil microbial diversity and community composition (Li et al. 2022; Sopialena and Palupi 2017).

One of the key roles of microorganisms in agriculture is as agents of biological control, a process that harnesses natural enemies to suppress pest populations. The use of biological control agents is considered the most prudent manner of controlling pests as it can reduce our reliance on chemical pesticides, and because it is a long-term, specific pest targeting, environmentally friendly, cost-effective approach that is safe for human health and supports sustainable agriculture (Boro et al. 2022). Therefore, besides observing the genetic diversity and density of the microorganisms in the ex-mining and non-mining land, this study also focused on screening entomopathogenic nematodes and microbes as potential biological control agents for suppressing insect populations. Insects are the largest and most diverse group of organisms included as one of the most common and economically significant types of plant pests (García-Lara 2016), causing significant damage to many crop plant species (Manosathiyadevan 2017).

Among all microbes, only *Trichoderma* (fungi) and *Bacillaceae* (bacteria) were identified as entomopathogenic microorganisms in the present study, in both ex-mining and non-mining lands. *Trichoderma* can indirectly act as an entomopathogen by producing insecticidal secondary metabolites, antifeedant compounds, and repellent metabolites (Poveda 2021). This is due to *Trichoderma*'s ability to produce metabolites such as citric acid (Vinale et al. 2012), as well as enzymes like urease, cellulase, glucanase, and chitinase (Carlile et al. 2001), which can negatively affect insects and even cause death when they enter the body. On the other hand, bacteria from the *Bacillaceae* family, particularly *Bacillus thuringiensis*, have received significant attention as entomopathogens. This bacterium is capable of producing proteinaceous protoxin crystals with insecticidal properties. When ingested by insects, these crystals disintegrate in the stomach and are broken down by host proteases to produce an active poison known as endotoxin, which ultimately leads to insect death (Deka et al. 2021).

In contrast to the fungi and bacteria, nematodes were found to be more abundant in the post-mining than in the non-mining land in terms of their diversity and density. Additionally, more nematodes were observed in the deeper soil layers. Most related studies have observed that the vertical distribution of nematode communities decreases from the topsoil layer to the subsoil layer (Cheng et al. 2021; Suyadi et al. 2021). This variation in soil nematode communities may be explained by the distinct characteristics of soils at different layers (Liu et al. 2022). The differences in soil nematode diversity and density at different soil depths observed in this study might be influenced by soil variables. The amount of nematode diversity varied in different experimental sites (Ilieva-Makulec et al. 2015). Results showed that nematode generic richness was higher in the topsoil of one site, while in a different habitat, nematode diversity was significantly lower in the topsoil and highest in the subsoil. Overall, it is likely that nematode abundance and distribution are influenced by many factors, such as soil properties, climate and environmental conditions, land use and management, plant species and root exudates, predation and competition, and succession and ecosystem development (Ilieva-Makulec et al. 2015; Zhang 2020; Cheng et al. 2021; Li et al. 2022).

Nematodes may be classified into three main groups: saprophages (living on dead organic matter), predators (feeding on small animals, including other nematodes), and parasites (infesting insects, animals, human, fungi and higher plants) (Pant et al. 1998). Eleven of the fifteen nematodes identified in this study, namely *Heterodera*, *Meloidogyne*, *Tylenchus*, *Pratlyenchus*, *Radopholus*, *Hoplolaimus*, *Rotylenchus*, *Rotylenchulus*, *Xiphinema*, and *Longidorus* were plant-parasitic nematodes. *Meloidogyne*, *Radopholus*, and *Rotylenchulus* were the top three genera of plant-parasite nematodes observed in East Kalimantan (Suyadi and Rosfiansyah 2017). *Dorylaimus* and *Rhabditis*, on the other hand, were non-plant-parasitic nematodes. *Dorylaimus* functions as an omnivore in the rhizosphere, and *Rhabditis* as a bacterivore. In this study, only *Heterorhabditis* and *Steinernema* were identified as entomopathogenic nematodes, as also observed in the previous studies (Bhat et al., 2020; Julià et al., 2023).

Comment [AKG53]: Correct it or removed it. They observed nematodes in which sample?

Comment [Me54]: We have corrected the sentence.

Relation of soil physicochemical properties and microbial community

The physicochemical properties of post-mining soil were considerably different from those of non-mining soil in this study. The different characteristics of post-mining land arise from the significant alterations in soil properties, structure, and chemical composition due to mining activities. The mining process disrupts the natural soil structure, leading to soil compaction, which reduces pore spaces and affects water infiltration and root penetration (Yuningsih et al. 2021). Additionally, the removal or burying of topsoil (due to improper reclamation processes) results in a significant reduction in the organic content of the soil (especially in the root growth layer), leading to nutrient loss and further diminishing soil fertility. Furthermore, the mining process can expose rocks and minerals that, when weathered, can alter the soil's pH, leading to acid mine drainage and significantly lowering the soil pH (Herman et al. 2024). These conditions make the soil

ecosystem in post-mining land unconducive for microbial communities, which contrasts with the conditions in non-mining land.

Soil is a medium for plant growth and also acts as an environment for microbial growth, determining the quality of growing soil microbes and plants. The interactions between soil microbes and plants play a crucial role in soil health and fertility. Fertile soil is typically a source of organic matter for microbial activity. Soil status encompasses a wide range of physical, chemical, and biological properties that influence the habitat and conditions for soil microorganisms. A healthy soil status with sufficient organic matter and nutrient availability ensures that microorganisms have the resources they need to thrive (Kaur et al. 2022) The presence of high organic matter in soil is characterized by several physicochemical parameters, such as pH levels, cation exchange capacity (CEC), soil organic carbon (SOC) content, and nutrient content, especially of nitrogen, phosphorus, and potassium (Dandwate 2020)

It was also observed that soils in both post-coal mining and non-mining land were found to be acidic, with minor variations at every soil depth layer. However, soil pH in the post-mining land was considerably lower, in the range of 3.19-4.19, compared to that in the non-mining land (4.02-4.9). Related to the lower pH in post-mining land was the aluminum content, which was higher than that in the non-mining land as acidic soils enhance aluminum solubility and availability. Furthermore, soil pH is positively correlated with another soil property, basic saturation (BS), which was observed to be higher in the non-mining land.

Soil pH strongly influences microbial communities. Acidic or alkaline soils can limit the diversity and activity of microorganisms, impacting nutrient availability and overall soil. It is a critical factor that affects the activity and diversity of soil microorganisms. Different microbial species have specific pH preferences, and soil pH can influence their metabolic activities. Soil pH outside the optimal range for specific microbes can limit their growth and activity (Wang et al. 2019). Therefore, maintaining an appropriate pH level is essential for a diverse and active microbial community.

Soil organic matter was higher in non-mining land in this study, as characterized by the higher soil organic carbon (SOC) and nutrient content than that of post-coal-mining land, as well as by the pH level. Higher organic matter content is associated with high SOC, which, as carbon is a fundamental component of organic materials, mainly occurs via the microbial breakdown, partial decay, and conversion of deceased organic materials (Lefèvre et al. 2017) This activity is co-related with nutrient availability; that is, as organic matter decomposes, it releases nutrients into the soil, contributing to the soil's overall fertility and health (Gerke 2022)

Overall, soil physicochemical properties play a significant role in shaping the composition and activity of the microbial community (Brahmaprakash 2021). A larger microbial community was observed alongside the higher physicochemical soil properties of the non-mining land, rather than in the post-coal-mining land. Changes in these properties can influence the composition and activity of microbial community, subsequently impacting nutrient cycling, organic matter decomposition, and overall soil fertility (Leiva et al. 2020; Kaur et al. 2022). Fertile soil is characterized by a well-balanced and diverse microbial community that supports plant growth by providing essential nutrients and improving soil structure. The composition and diversity of the soil microbial community are important factors in maintaining soil health that also support plant growth and health (Wang and Li 2023). The increasing microbial population (both in diversity and abundance) causes the dynamics of the soil to improve and become healthier naturally (Bertola et al. 2021). The ability to change soil biological properties in a positive direction can increase the population of microbes, including entomopathogenic microbes, which benefit plant growth and reduce the need for artificial fertilizer and pesticide use.

CONCLUSION

The results showed that microbial diversity in post-coal-mining land differed from that in non-mining control. The entomopathogenic fungal and bacterial diversity and density in ex-coal-mining soil was considerably lower compared to that in the non-mining land. The microbial (bacterial and fungal) diversity and density were lower in deeper layers of soil in both post-coal-mining and non-mining land, and tended to decrease soil layer depth increased. In contrast to fungi and bacteria, entomopathogenic nematodes were observed to be more abundant in the post-mining than in non-mining land in terms of their diversity and density. Additionally, nematodes were more abundant in the deeper layers of soil. Soil fertility was found to be lower in ex-coal-mining land, as characterized by several soil physicochemical properties, of which the lower soil pH, basic saturation, soil organic carbon (SOC) and nutrient content detected likely supported a smaller microbial community compared to the non-mining land.

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Comment [AKG55]: Check the journal name again.

Comment [Me56]: Thank you very much, we have corrected it

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Comment [AKG57]: Check the number again

Comment [Me58]: Thank you very much, we have corrected it

- **Round 2**

- **Round 3**

- **Round 4**

- **Round 5**

- **Round 6**

Round 6 Status Submission accepted.

Notifications

- [biodiv] Editor Decision2024-01-17 10:46 PM
- [biodiv] Editor Decision2024-06-06 06:15 AM
- [biodiv] Editor Decision2024-06-25 11:15 AM
- [biodiv] Editor Decision2024-07-17 02:30 PM

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Uncorrected proof	ddianti1 2024-07-08 02:54 PM	-	0
BILLING	dewinurpratiwi 2024-07-09 12:12 AM	-	0

Notifications

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[biodiv] Editor Decision

2024-07-17 02:30 PM

SOPIALENA, ROSFIANSYAH, ANDI SURYADI:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Assessment of nematode and microbial diversity and screening of entomopathogens in revegetated post-coal-mining land in Kutai Kartanegara District, East Kalimantan, Indonesia".

Our decision is to: Accept Submission

LOA link https://smujo.id/loa/16870

Best Regards,
Team Support Smujo.id

Biodiversitas Journal of Biological Diversity

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Assessing nematode and microbial diversity and screening for entomopathogens on revegetated post-coal-mining land: Case study in Kutai Kartanegara Regency, East Kalimantan

Abstract. Post-coal-mining management should strive toward site revegetation. The success of such revegetation efforts is largely determined by the soil's fertility status, which encompasses physical, chemical, and biological components. Biological fertility is indicated by the presence and abundance of soil microbes and nematodes. This study aims to identify the diversity of nematodes and soil microbes on ex-coal-mining land revegetated with oil palm in Kutai Kartanegara Regency (East Kalimantan, Indonesia), screen for entomopathogenic nematodes and microbes, and analyze their relationships with the chemical fertility status of the land. As a control, non-mining land was also investigated. The results showed that the microbial diversity and density (including fungi and bacteria) in the ex-coal-mining area were considerably lower compared to that in the control. Additionally, microbial diversity and density decreased in the deeper soil layers of both the post-mining and non-mining lands. In contrast, soil nematodes were more abundant in the post-mining land, both in terms of diversity and density, and were also more plentiful in the deeper soil layers. Among the nematodes and microbes studied, only *Heterorhabditis* and *Steinernema* (nematodes), *Trichoderma* (fungi), and *Bacillaceae* (bacteria) were identified as entomopathogens, causing death in *Tenebrio molitor* L. larvae. Overall, the soil fertility in the ex-coal-mining land was observed to be lower, characterized by decreased soil pH, basic saturation, soil organic carbon (SOC), and nutrient content, which contributed to a reduced microbial community compared to non-mining land.

Keywords: Entomopathogen, nematodes, soil microbes, post-coal mining, Kutai Kartanegara

INTRODUCTION

Coal mining activities in Kutai Kartanegara Regency, East Kalimantan, Indonesia, have experienced rapid growth in recent years, with both large-scale and small-scale operations, including those with mining authorities. However, this expansion has come at a significant cost to the environment, resulting in widespread damage. This damage manifests in the form of arid and infertile post-mining lands or artificial "craters" with unclear purposes. While some efforts have been made to rehabilitate these ex-coal-mining lands, they remain sporadic and insufficient, allowing environmental degradation to outpace recovery efforts.

Rehabilitating these lands is essential to mitigating the adverse environmental impacts of coal mining. The success of land recovery can be evaluated by assessing the diversity of soil microbes, which serves as an indicator of biological fertility. Soil microbes play a pivotal role in converting potential chemical fertility into actual fertility. Moreover, their activities gradually enhance the physical properties of the soil, further improving physical fertility (Cui et al., 2021). However, the restoration of ex-coal-mining lands faces numerous challenges, including the need to address soil degradation, erosion, and biodiversity loss resulting from mining activities (Ahirwal et al., 2016). To address these challenges, it is crucial to consider the role of soil microbes, including entomopathogenic microbes, which are an integral part of soil ecosystems (Boucias and Pendland, 2018).

The roles of nematodes and microbes in ex-mining lands are fundamental to ecological restoration efforts, improving soil health, supporting plant growth, and maintaining ecological balance. These microorganisms can significantly impact the success of revegetation and restoration projects in post-mining landscapes. The presence of nematodes and microbes in the soil benefits plant health during revegetation endeavors (Guo et al., 2021; Li et al., 2022). Furthermore, these microorganisms support plant establishment and growth. Healthy and robust plant growth, in turn, stabilizes the soil, prevents erosion, and enhances biodiversity, all of which are crucial for the overall success of revegetation projects (Mensah, 2015). The presence of microbial diversity in mining-affected areas can be influenced by factors such as soil type, climate, land management practices, as well as the host plants' root exudate composition (Santoyo, 2022; Sasse et al., 2017). Consequently, understanding and managing the diversity of these microorganisms are critical for the success of revegetation efforts.

Entomopathogenic microbes, which encompass various taxonomic groups, including bacteria, fungi, nematodes, and viruses, are known to regulate insect populations and play a vital role in ecological processes (Deka et al., 2021) One of the most remarkable contributions of entomopathogenic microbes during revegetation is their role as natural pest controllers (Vega et al., 2009). These microorganisms serve as natural enemies of various insect pests that could harm newly planted vegetation (Shrestha et al., 2020). By infecting and eliminating these pests, entomopathogens help regulate insect populations and protect the restored vegetation (Rozpądek et al., 2019). In regions where entomopathogens thrive, the risk of pest outbreaks is reduced, safeguarding the overall health of the revegetated land (Koskella et al., 2020). Entomopathogenic microbes such as fungi and bacteria are natural antagonists of insects and play a pivotal role in regulating insect populations in terrestrial ecosystems. While extensively studied for their potential in biological pest control in agriculture, their utilization in ecological restoration, particularly in ex-coal-mining land revegetation, remains a relatively unexplored area, and a promising avenue for research and practical implementation. The diversity and abundance of entomopathogenic microbes are subject to environmental factors (Fatu et al., 2020). Factors such as soil physicochemical properties, which can fluctuate significantly in mining-affected areas, play pivotal roles in shaping microbial community structure (Mudrak et al., 2021).

The specific objectives of this study are to identify the diversity of nematodes and soil microbes on revegetated ex-coal-mining land, screen for entomopathogenic nematodes and microbes, and analyze their relationships with the chemical fertility status of the land.

MATERIALS AND METHODS

Experimental site

The study was conducted at two locations, in post-coal mining and non-mining areas, in the Pendingin Subdistrict of Sanga-Sanga, in Kutai Kartanegara Regency, East Kalimantan Province, Indonesia. The post-coal mining area (located at S 00040'29.7" E 117016'09.8") had been revegetated with an oil palm plantation, while the non-mining area used as a control (located at S 00041'41.3" E 117015'54.9") was cultivated with rubber plants.

Sample collection

The sampling method used in this study was the standard method of ISPM (International Standards for Phytosanitary Measure), which is an international standard guide for conducting field surveys. Soil samples were taken randomly at ten sampling points in each experimental site. The soil samples were taken at four levels of depth from the soil surface: 0-30 cm, 30-60 cm, 60-80 cm, and 80-100 cm. After removing the gravel and plant debris, the soil samples were composited for each level of soil depth, and 1 kg of soil was collected for microbial extraction and identification of fungi, bacteria, and nematodes in the laboratory. In addition, the soil physicochemical properties were analyzed in this study.

Fungal and bacterial isolation and identification

For both fungal and bacterial isolation, 10 g of soil samples were dissolved in a liter of distilled water. An inoculating needle was dipped in the soil solution, and then inoculated on Potato Dextrose Agar (PDA) medium to culture fungi, while another inoculating needle was dipped in the same solution and applied to Nutrient Agar (NA) medium to culture bacteria. After one week of culturing for fungi, or two days of culturing for bacteria, the microbes were isolated and observed under a microscope to identify the type, color, and shape of individual bacteria and fungi, as well as that of their colonies, following Hucker's procedure with minor modifications (Hucker, 1921). Fungal conidia density was calculated using a Neubauer hemocytometer.

Entomopathogenic fungal and bacterial screening

Entomopathogenic fungi and bacteria were selected using mealworm (*Tenebrio molitor* L.) larvae 30-40 days of age (Figure 1). One milliliter of each fungal and bacterial isolate was inoculated to mealworms. Only fungi and bacteria that could cause mortality to mealworms were considered entomopathogens in this study.

Figure 1. Mealworm (*Tenebrio molitor* L.) larvae at 30-40 days of age

Nematode isolation and identification

Nematode isolation was carried out using the Baermann funnel method with minor modifications. This method is considered the most effective in nematode isolation (van den Hoogen et al., 2020). Briefly, a silicone hose was attached to a funnel, then the end of the hose was tied using a gauze rubber band and placed on top of the funnel. The Baermann funnel apparatus was installed in a horizontal position without buckling the silicon hose. Fifty grams of soil for each sample was placed on the filter paper, and the soil was wrapped to cover its entire surface. The water was added slowly into the funnel to submerge the sample, until the sample was saturated, to create a moist environment that stimulated nematodes to migrate toward the water source. The funnel was incubated for 3×24 hours and the water released from the bottom of the funnel. The water was collected in a film tube/vial. The collected water then was passed

through a fine sieve to separate the nematodes from the water and debris. Up to 1 drop of formalin (5%) was added and fixed for 15 minutes.

Entomopathogenic nematode screening

Entomopathogenic nematodes were screened by bait trap using mealworms (Figure 1). The mealworm larvae (3-5 larvae) were placed in a plastic container and mixed with ~150 g of moist soil containing nematodes. The container was then inverted so that the larvae were covered with soil, then placed at room temperature.

After 3-4 days, if larval mortality was observed, the larval cadaver was removed and rinsed with sterilized distilled water. Entomopathogenic nematode trapping was then conducted using the White trap method to release entomopathogenic nematodes from the cadaver's body. Nematodes were observed and counted using a stereo microscope. Identification of soil nematodes and entomopathogenic nematodes to genus level was carried out through morphological observations.

Soil physicochemical analysis

Samples were analyzed for their chemical and physical properties, including organic C, total N, available P (Bray), available K (Morgan), Ca^{++}, Mg^{++}, K^{+}, Na^{+}, CEC (Cation Exchange Capacity), base saturation (BS), Al saturation, pH, and soil texture. The soil physicochemical analysis was conducted based on the general procedure developed in the Soil Laboratory, Faculty of Agriculture, Mulawarman University, Samarinda, Indonesia.

Data analysis

Data collected were analyzed descriptively to uncover the role of soil microbes in supporting the recovery of post-coal-mining land and the relationship between the diversity of soil microbes and the chemical fertility status of the study site.

RESULTS

Fungal diversity and density

Higher fungal diversity was observed in the non-mining land compared to the ex-mining land, with five fungal genera found in non-mining, and four genera in ex-mining soil (Figure 2, Table 1). The fungal diversity in ex-coal mining and non-mining land were different in every soil layer observed in this study (Table 1). For example, *Penicillium* sp., which was found in every soil layer of post coal-mining land, was not present in the non-mining land soil. On the other hand, *Mucor* sp., which was found in the non-mining land soil, was absent in coal-mining land soil. In addition, the fungi *Aspergillus* was found to be abundant, and present in almost all soil layers in both ex-coal-mining and non-mining land. As with its diversity, the fungal density in non-mining land soil was also considerably higher (ranging from $2.6\text{-}7.2\times10^5$ cfu.g^{-1}) than that of ex-coal-mining land soil (ranging from $1.4\text{-}3.8\times10^5$ cfu.g^{-1}) at every soil depth layer. The deeper the soil layer, the fewer fungal populations were detected. Among all the fungi identified in the ex-mining and non-mining land, only *Trichoderma* was screened as an entomopathogenic fungi. *Trichoderma* was observed in the digestive system of deceased *T. molitor* mealworm larvae.

Table 1. Fungal diversity and density at different soil depth layers, and screening of entomopathogenic fungi in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

	Soil depth layer	Fungal population density cfu/g	Genera/species
	Non-mining land		
1.	0-30	7.2×10^5	*Aspergillus* *Trichoderma** *Penicillium*
2.	30-60	5.4×10^5	*Aspergillus* *Trichoderma** *Penicillium*
3.	60-80	3.2×10^5	*Trichoderma** *Pythium* *Penicillium*
4.	80-100	2.6×10^5	*Aspergillus* *Penicillium*
	Post-coal-mining land		
1.	0-30	3.8×10^5	*Aspergillus*

			Trichoderma sp.*
2.	30-60	2.2 x 10^5	*Aspergillus niger* *Trichoderma**
3.	60-80	1.8 x 10^5	*Aspergillus* sp. *Mucor* sp.
4.	80-100	1.4 x 10^5	*Aspergillus niger* *Aspergillus flavus*.

*Entomopathogenic fungi that successfully kill mealworms by entering through their digestive system (acting as a stomach poison).

Figure 2. Fungal diversity: A. *Trichoderma*: conidia (a), hyalin (b), conidiophore (c); B. *Pythium*: oogonium (a), hyphae (b); C. *Penicillium*: conidium (a), phialide (b), metula (c), stipe (d); D. *A. niger*: conidia (a), conidiophore (b); E. *A. flavus*: conidia (a), phialide (b), vesicle (c), stipe (d); F. *Mucor* sp.: sporangium (a), sporangiophore (b), hyphae (c)

Entomopathogenic bacteria

Two bacterial families, *Azotobacteraceae* and *Bacillaceae*, were observed in the soils of both non-mining and ex-coal-mining land (Figure 3). However, in the deeper subsoil layers (60-80 cm or 80-100 cm) of the ex-coal-mining land, only *Azotobacteraceae* was present. Interestingly, all of the *Azotobacteraceae* bacteria observed in this study were identified as coccus Gram-positive bacteria, except in the ex-coal-mining land, in which coccus Gram-negative *Azotobacteraceae* were found at the soil depths of 30-60 cm and 60-80 cm. The bacterial population was denser in non-coal-mining ($4.2\text{-}9.6\times10^6$) compared to ex-coal-mining land ($1.4\text{-}5.1\times10^6$). The bacterial population decreased along with the soil depth in both experimental sites (Table 2). In this study, only *Bacillaceae* bacteria, observed in the digestive system of deceased *T. molitor* larvae, were identified as entomopathogenic bacteria in both ex-mining and non-mining lands.

Table 2. Bacterial diversity and density at different soil depth layers, and screening of entomopathogenic bacteria in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Bacterial population density cfu/g	Family
	Non-mining land		
1.	0-30	9.6 x 10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae**
2.	30-60	5.5 x 10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae**

3.	60-80	4.5 x10^6	- Coccus, gram: (+) *Azotobacteraceae* - bacil, gram (+): *Bacillaceae**
4.	80-100	4.2 x 10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*
	Post-coal-mining land		
1.	0-30	5.1 x 10^6	- Coccus, gram (+): *Azotobacteraceae* - Basil, gram (+): *Bacillaceae**
2.	30-60	4.9 x 10^6	- Coccus, gram: (+) Azotobacteraceae - Basil, gram (+): *Bacillaceae**
3.	60-80	1.6 x 10^6	- Coccus, gram: (+): Azotobacteraceae
4.	80-100	1.4 x 10^6	- Coccus, gram: (+): Azotobacteraceae

*Entomopathogenic bacteria that successfully kill mealworms by entering through their digestive system (acting as a stomach poison).

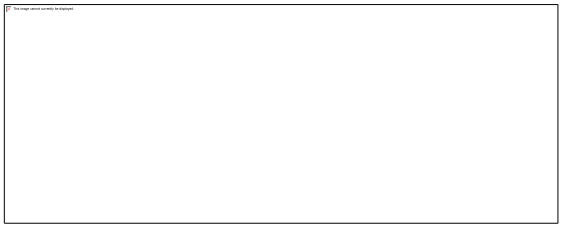

Figure 3. Bacteria (left to right: A. *Bacillaceae*, and B. *Azotobacteraceae*)

Entomopathogenic nematodes

A high genetic diversity of nematodes was observed in both non-mining and ex-mining land (Table 3). Among the fifteen genera identified (Figure 4), six—namely *Heterodera*, *Heterorhabditis*, *Hoplolaimus*, *Pratylenchus*, *Radopholus*, and *Rotylenchus*—were present in both non-mining and ex-coal-mining lands. Conversely, *Dorylaimus*, *Meloidogyne*, *Rotylenchulus*, and *Tylenchus* were found only in non-mining area, while *Longidorus*, *Rhabditis*, *Steinernema*, and *Xiphinema* were exclusive to ex-mining land. The number of nematode populations was notably higher in ex-coal-mining compared to non-mining land. It was also observed that the deeper the soil layer, the greater the number of isolated nematodes, as found in both non-mining and ex-coal-mining land (Table 3). Despite the high nematode diversity identified in this study, only *Heterorhabditis* and *Steinernema* were screened as entomopathogenic nematodes.

Table 3. Nematode diversity and density at different soil depth layers, and screening of entomopathogenic nematodes in ex-mining and non-mining land in Pendingin, Sanga-Sanga Subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Nematoda Population/ kg soil	Genus
	Non-mining land		
1.	0-30	1.1 x10^2	*Heterodera* *Meloidogyne* *Tylenchus* *Heterorhabditis**
2.	30-60	1.2 x 10^2	*Heterorhabditis** *Pratlyenchus* *Heterodera* *Radopholus* *Hoplolaimus*
3.	60-80	4 x 10^2	*Pratylenchus* *Heterodera*
4.	80-100	2 x 10^2	*Rotylenchus*

			Rotylenchulus *Dorylaimus*
	Post-coal-mining land		
1.	0-30	5.1 x 10^2	*Heterodera* *Xiphinema*
2.	30-60	7.0 x 10^2	*Heterorhabditis** *Steinernema* *Radopholus* *Rhabditis*
3.	60-80	8.0 x 10^2	*Steinernema** *Hoplolaimus* *Rotylenchus*
4.	80-100	9.2 x 10^2	*Pratylenchus* *Longidorus*

*Entomopathogenic nematodes obtained from the cadavers of deceased mealworms

Figure 4. Nematodes (left to right: A. *Hoplolaimus*, B. *Pratylenchus*, C. *Tylenchus*, D. *Heterorhabditis*, E. *Steinernema*, F. *Dorylaimus*, G. *Xiphinema*, H. *Heterodera*, I. *Rhabditis*, J. *Radopholus*, K. *Longidorus*, L. *Rotylenchus*, M. *Rotylenchulus*, N. *Helicotylenchus*, O. *Aphelenchus*)

Soil physicochemical properties

In general, soil nutrient content was higher in non-mining land compared to post-coal mining land (Table 4). There was also variation in the nutrient content at different soil depth layers. Most of the nutrients were more abundant in the subsoil compared to the topsoil, as observed in the available K, Ca++. Mg++, K++, and Na+, as well as base saturation in non-mining land. In contrast, the reverse phenomenon was observed in post-coal-mining land.

Aluminum content seemed to be higher in the post-coal-mining than the non-mining land. Moreover, the aluminum content tended to decrease with increasing soil depth on non-coal-mining land, while tending to be higher in the deeper soil layers on post-coal-mining land. The pH status of the non-mining and post-coal-mining land was in the range of 4.02-4.9 and 3.19-4.19, respectively, showing higher acidity in the post-coal-mining land.

Soil texture is determined by the proportion of mineral components in the soil. Among these mineral particles, silt, rather than clay or sand, was the dominant soil texture in the topsoil and subsoil of both the non-mining and post-coal-mining land. However, the relative proportions of different mineral particles (sand, silt, and clay) varied in each soil layer, resulting in distinct soil textures.

Table 4. Soil physicochemical properties of the non-mining and ex-coal-mining land in Kutai Kartanegara Regency, East Kalimantan

No.	Soil Parameter	Unit	Non-coal Mining Land				Ex-coal Mining Land			
			R1 0-30	R2 30-60	R3 60-80	R4 80-100	S1 0-30	S2 30-60	S3 60-80	S4 80-100
1.	Carbon organic	%	7.65	4.64	4.14	3.41	3.80	3.74	1.38	0.86
2.	Nitrogen total	%	0.41	0.24	0.51	0.14	0.17	0.15	0.16	0.19
3.	Ratio C/N	-	18.66	19.33	8.12	24.36	22.35	24.93	8.63	4.53
4.	Available P (Bray1)	Ppm	19.39	12.60	13.38	20.26	5.11	4.03	0.96	4.12
5.	Available K tersedia (Morgan)	Ppm	110.45	214.54	170.21	234.33	209.22	81.56	82.09	143.28
6.	Al^{3+} (Morgan)		4.00	2.20	2.10	3.10	2.00	6.80	5.10	8.40
7.	H^{+} (Morgan)		3.46	1.80	1.70	2.66	1.66	6.20	4.46	4.26
8.	Ca^{++}	meq/100 g	0.63	1.67	0.83	1.19	1.95	0.79	0.52	0.61
9.	Mg^{++}	meq/100 g	0.48	1.25	0.68	0.98	1.01	0.48	0.37	0.49
10.	K^{+}	meq/100 g	0.30	0.65	0.48	0.91	0.67	0.20	0.26	0.40
11.	Na^{+}	meq/100 g	0.04	0.38	0.28	0.29	0.34	0.11	0.02	0.12
12.	CEC	meq/100 g	8.91	7.95	6.07	9.13	8.16	8.58	10.73	14.28
13.	BS	%	16.3	49.7	37.4	36.9	48.7	18.4	10.9	11.3
14.	Al Saturation	%	44.89	27.67	34.60	33.95	26.21	46.64	47.53	58.82
15.	pH	–	4.02	4.62	4.54	4.90	3.56	3.35	4.19	3.19
16.	Clay	%	14.77	36.54	20.07	23.21	28.72	16.54	23.94	32.85
17.	Silt	%	34.48	58.18	55.30	35.43	67.66	64.75	33.70	35.54
18.	Sand	%	50.75	5.28	24.63	41.36	3.62	18.71	42.36	31.61
19.	Texture		SiCL	SiCL	SiL	L	SiCL	SiL	L	CL

DISCUSSION

The greatest concern regarding land degradation impacts of post-mining areas for the agriculture sector is the reduction of biodiversity and soil fertility. Mining activities often lead to the destruction of natural habitats and ecosystems, resulting in the deracination and decline of plant and animal species, and leading to a loss of biodiversity that also negatively affects agricultural productivity. In addition, post-mining land undergoes physical and chemical changes that render it unsuitable for agriculture, such as reduced soil porosity and aeration due to the alteration of soil structure, a lack of organic matter and nutrients due to the topsoil removal, introduced contaminants such as heavy metals and toxic chemicals, and altered soil pH—more acidic or alkaline—which can all be detrimental to plant growth (Prince et al., 2018; Janečková et al., 2023).

Efforts to address the loss of arable land in post-mining areas involve comprehensive reclamation and rehabilitation practices that aim to restore soil fertility, structure, and water-holding capacity, and to repair the quality of the environment and ecosystem, as mentioned in the regulation issued by the Minister of Energy and Mineral Resources Regulation 7/2014 and Regional Regulation East Kalimantan 8/2013 (Yulianingrum 2023). These efforts may include topsoil replacement, revegetation, and ongoing monitoring to ensure the land can support agriculture practices. Furthermore, to achieve normal criteria for fertile soil supporting agricultural activities, maintenance of microbial community structures is very important.

Microbial entomopathogen diversity and density

In this study, it was observed that the nematode and soil microbial diversity in post-coal-mining land clearly differed to that in the non-mining land. For example, the soil fungal diversity and density in post-coal-mining land was considerably lower compared to the non-mining land (Table 1). This situation was also observed in soil bacteria (Table 2), which showed low biodiversity in post-coal-mining land even after the completion of the reclamation process. A significant reduction of microbial diversity due to coal mining practices after land restoration was also observed in a study conducted by de Quadros et al. (2016). In their study, the post-coal-mining land had undergone different durations of restoration (3-19 years), which involved replacing the soil layers, applying limestone to raise the pH to 6.0, and revegetating the land with grasses. Even after the completion of the reclamation process after many years, the composition of the microbial diversity and biomass of post-mined sites was significantly lower than that of the control (unaltered sites). Similar results were also found in different studies comparing the microbial diversity and density in reclaimed and revegetated post-coal-mining sites with that in undisturbed sites, both of which reported significant declines in microbial community structure and diversity (Li et al., 2014; Upadhyay et al., 2014), as also shown in the current study.

A reduction of microbial (bacterial and fungal) diversity and density was also observed in the deeper layers of soil in both the post-coal-mining and non-mining-land (Table 1 and Table 2), in which the microbes decreased as the depth of the soil layer increased. A similar observation was reported in the study of Upadhyay et al. (2014), who reported that rhizospheric soils showed greater diversity in both bacteria and fungi compared to non-rhizospheric soil. This outcome is supported by recent studies concluding that soil microbial diversity decreases along with the soil depth (Wang et al., 2021; Guo et al., 2021)

It is well known that the topsoil is the most biologically active and favorable layer for microorganisms. Compared to the subsoil layer, the topsoil contains the highest concentration of organic matter, providing food and nutrients to microorganisms (Bahram et al., 2018). In addition, the uppermost layer of soil, also known as the A-horizon, is well-aerated and has adequate moisture content and moderate temperatures, providing a suitable environment for a wide range of microorganisms (Liu et al., 2019; Aquado et al., 2023). Furthermore, there is an interaction between microorganisms and plant roots, which are more abundant in the top layer of soil. Plant roots release root exudates and provide energy to many soil microorganisms, thereby contributing to a more active microbial community (Zhalnina et al., 2018; Zhou et al., 2020). Therefore, the vegetation types, plant species, and climate all influence the soil microbial diversity and community composition (Li et al., 2022; Sopialena and Palupi, 2017).

One of the key roles of microorganisms in agriculture is as agents of biological control, a process that harnesses natural enemies to suppress pest populations. The use of biological control agents is considered the most prudent manner of controlling pests as it can reduce our reliance on chemical pesticides, and because it is a long-term, specific pest targeting, environmentally friendly, cost-effective approach that is safe for human health and supports sustainable agriculture (Boro et al., 2022). Therefore, besides observing the genetic diversity and density of the microorganisms in the ex-mining and non-mining land, this study also focused on screening entomopathogenic nematodes and microbes as potential biological control agents for suppressing insect populations. Insects are the largest and most diverse group of organisms included as one of the most common and economically significant types of plant pests (García-Lara, 2016), causing significant damage to many crop plant species (Manosathiyadevan, 2017).

Among all microbes, only *Trichoderma* (fungi) and *Bacillaceae* (bacteria) were identified as entomopathogenic microorganisms in this study, present in both ex-mining and non-mining lands. *Trichoderma* can indirectly act as an entomopathogen by producing insecticidal secondary metabolites, antifeedant compounds, and repellent metabolites (Poveda, 2021). This is due to *Trichoderma*'s ability to produce metabolites such as citric acid (Vinale et al., 2012), as well as enzymes like urease, cellulase, glucanase, and chitinase (Carlile et al., 2001), which can negatively affect insects and even cause death when they enter the body. On the other hand, bacteria from the *Bacillaceae* family, particularly *Bacillus thuringiensis*, have received significant attention as entomopathogens. This bacterium is capable of producing proteinaceous protoxin crystals with insecticidal properties. When ingested by insects, these crystals disintegrate in the stomach and are broken down by host proteases to produce an active poison known as endotoxin, which ultimately leads to insect death (Deka et al., 2021).

In contrast to the fungi and bacteria, nematodes were found to be more abundant in the post-mining than in the non-mining land in terms of their diversity and density. Additionally, more nematodes were observed in the deeper soil layers (Table 3). Most related studies have observed that the vertical distribution of nematode communities decreases from the topsoil layer to the subsoil layer (Cheng et al., 2021; Suyadi et al., 2021). This variation in soil nematode communities may be explained by the distinct characteristics of soils at different layers (Liu et al., 2022); that is, the differences in soil nematode diversity and density at different soil depths observed in this study might be influenced by soil variables. In a study by Ilieva-Makulec et al. (2015), the amount of nematode diversity varied in different experimental sites. Specifically, the nematode generic richness was higher in the topsoil of one site, while in a different habitat, the nematode diversity was significantly lower in the topsoil and highest in the subsoil. Overall, it is likely that nematode abundance and distribution are influenced by many factors, such as soil properties, climate and environmental conditions, land use and management, plant species and root exudates, predation and competition, and succession and ecosystem development (Ilieva-Makulec et al., 2015; Zhang, 2020; Cheng et al., 2021; Li et al., 2022)

Nematodes may be classified into three main groups: saprophages (living on dead organic matter), predators (feeding on small animals, including other nematodes), and parasites (infesting insects, animals, human, fungi and higher plants) (Pant et al., 1998). Among the fifteen nematodes identified in this study, *Heterodera*, *Meloidogyne*, *Tylenchus*, *Pratlyenchus*, *Radopholus*, *Hoplolaimus*, *Rotylenchus*, *Rotylenchulus*, *Xiphinema*, and *Longidorus* were plant-parasitic nematodes. *Meloidogyne*, *Radopholus*, and *Rotylenchulus* were the top three genera of plant-parasite nematodes observed in East Kalimantan (Suyadi & Rosfiansyah, 2017). *Dorylaimus* and *Rhabditis*, on the other hand, were non-plant-parasitic nematodes; *Dorylaimus* functions as an omnivore in the rhizosphere, and *Rhabditis* as a bacterivore. In this study, only *Heterorhabditis* and *Steinernema* were screened as entomopathogenic nematodes, as also observed in previous studies (Bhat et al., 2020; Julià et al., 2023).

Relation of soil physicochemical properties and microbial community

The physicochemical properties of post-mining soil were considerably different from those of the non-mining soil in this study (Table 4). The different characteristics of post-mining land arise from the significant alterations in soil properties, structure, and chemical composition due to mining activities. The mining process disrupts the natural soil structure, leading to soil compaction, which reduces pore spaces and affects water infiltration and root penetration (Yuningsih et al., 2021). Additionally, the removal or burying of topsoil (due to improper reclamation processes) results in a significant reduction in the organic content of the soil (especially in the root growth layer), leading to nutrient loss and further diminishing soil fertility. Furthermore, the mining process can expose rocks and minerals that, when weathered, can alter the soil's pH, leading to acid mine drainage and significantly lowering the soil pH (Herman et al., 2024). These conditions make the soil ecosystem in post-mining land unconducive for microbial communities, which contrasts with the conditions in non-mining land.

Soil is a medium for plant growth and also acts as an environment for microbial growth, determining the quality of growing soil microbes and plants. The interactions between soil microbes and plants play a crucial role in soil health and fertility. Fertile soil is typically a source of organic matter for microbial activity. Soil status encompasses a wide range of physical, chemical, and biological properties that influence the habitat and conditions for soil microorganisms. A healthy soil status with sufficient organic matter and nutrient availability ensures that microorganisms have the resources they need to thrive (Kaur et al., 2022) The presence of high organic matter in soil is characterized by several physicochemical parameters, such as pH levels, cation exchange capacity (CEC), soil organic carbon (SOC) content, and nutrient content, especially of nitrogen, phosphorus, and potassium (Dandwate, 2020)

In this study, the soil was determined to be acidic in both the post-coal mining and non-mining land, with minor variations at every soil depth layer. However, soil pH in the post-mining land was considerably lower, in the range of 3.19-4.19, compared to that in the non-mining land (4.02-4.9). Related to the lower pH in post-mining land was the aluminum content, which was higher than that in the non-mining land (Table 4) as acidic soils enhance aluminum solubility and availability. Furthermore, soil pH is positively correlated with another soil property, basic saturation (BS), which was observed to be higher in the non-mining land (Table 4).

Soil pH strongly influences microbial communities. Acidic or alkaline soils can limit the diversity and activity of microorganisms, impacting nutrient availability and overall soil. Soil pH is a critical factor that affects the activity and diversity of soil microorganisms. Different microbial species have specific pH preferences, and soil pH can influence their metabolic activities. Soil pH outside the optimal range for specific microbes can limit their growth and activity (Wang et al., 2019). Therefore, maintaining an appropriate pH level is essential for a diverse and active microbial community.

Soil organic matter was presumably higher in the non-mining land in this study, as characterized by the higher soil organic carbon (SOC) and nutrient content than that of the post-coal-mining land, as well as by the pH level (Table 4). Higher organic matter content is associated with high SOC, which, as carbon is a fundamental component of organic materials, mainly occurs via the microbial breakdown, partial decay, and conversion of deceased organic materials (Lefèvre et al., 2017) This activity is co-related with nutrient availability; that is, as organic matter decomposes, it releases nutrients into the soil, contributing to the soil's overall fertility and health (Gerke, 2022)

Overall, soil physicochemical properties play a significant role in shaping the composition and activity of the microbial community (Brahmaprakash, 2021). Changes in these properties can influence the composition and activity of the microbial community, subsequently impacting nutrient cycling, organic matter decomposition, and overall soil fertility (Leiva et al., 2020; Kaur et al., 2022) as clearly shown in this study. A larger microbial community was observed alongside the higher physicochemical soil properties of the non-mining land, rather than in the post-coal-mining land. Fertile soil is characterized by a well-balanced and diverse microbial community that supports plant growth by providing essential nutrients and improving soil structure. The composition and diversity of the soil microbial community are important factors in maintaining soil health that also support plant growth and health (Wang and Li, 2023). The increasing microbial population (both in diversity and abundance) causes the dynamics of the soil to improve and become healthier naturally (Bertola et al., 2021). The ability to change soil biological properties in a positive direction can increase the population of

microbes, including entomopathogenic microbes, which benefit plant growth and reduce the need for artificial fertilizer and pesticide use.

CONCLUSION

The microbial diversity in post-coal-mining land differed from that in the non-mining control. The entomopathogenic fungal and bacterial diversity and density in ex-coal-mining soil was considerably lower compared to that in the non-mining land. The microbial (bacterial and fungal) diversity and density were lower in the deeper layers of soil in both the post-coal-mining and non-mining land, and tended to decrease as the depth of the soil layer increased. In contrast to the fungi and bacteria, entomopathogenic nematodes were observed to be more abundant in the post-mining than in the non-mining land in terms of their diversity and density. Additionally, the nematodes were more abundant in the deeper layers of soil. Observed variations in the soil nematode communities might be due to the distinct characteristics of the soil at different layers. The fertility of soil was found to be lower in the ex-coal-mining land, as characterized by several soil physicochemical properties, of which the lower soil pH, basic saturation, soil organic carbon (SOC) and nutrient content detected likely supported a smaller microbial community compared to the non-mining land.

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Assessing nematodes and microbial diversity and screening for entomopathogen on revegetated post-coal mining land: case study in Kutai Kartanegara Regency, East Kalimantan

Abstract. Post-mining management of coal should be followed by revegetation. The success of these revegetation efforts is largely determined by the soil's fertility status, which encompasses physical, chemical, and biological components. Biological fertility is indicated by the presence and numbers of soil microbes and nematodes. This study aims to identify the diversity of nematodes and soil microbes on revegetated ex-coal mining land with palm oil vegetation in Kutai Kartanegara District, screen for entomopathogenic nematodes and microbes, and analyze their relationships with the chemical fertility status of the land. As a control, a non-mining land was also investigated. The results showed that the microbial diversity and density (including fungi and bacteria) in the post-coal mining area were considerably lower compared to the control, non-mining land. Additionally, microbial diversity and density decreased in the deeper soil layers, both in the post-mining and non-mining lands. In contrast, soil nematodes were more abundant in the post-mining land, both in terms of diversity and density, and were also more plentiful in the deeper soil layers. Among the nematodes and microbes studied, only *Heterorhabditis* and *Steinernema* (nematodes); *Trichoderma* (fungi); and *Bacillaceae* (bacteria) were identified as entomopathogens, causing death in Tenebrio molitor L. larvae. Overall, the soil fertility in the post-coal mining land was observed to be lower, characterized by decreased soil pH, basic saturation, soil organic carbon (SOC), and nutrient content, which contributed to a reduced microbial community compared to non-mining land.

Keywords: Entomophatogen, nematodes, soil microbes, post-coal mining, Kutai Kartanegara

INTRODUCTION

Coal mining activities in Kutai Kartanegara Regency, East Kalimantan, have experienced rapid growth in recent years, with both large-scale and small-scale operations, including those with mining authorities. However, this expansion has come at a significant cost to the environment, resulting in widespread environmental damage. This damage manifests in the form of arid and infertile post-mining lands or artificial "craters" with unclear purposes. While some efforts have been made to rehabilitate these ex-coal mining lands, they remain sporadic and insufficient, allowing environmental degradation to outpace recovery efforts.

Rehabilitating these lands is essential to mitigate the adverse environmental impacts of coal mining. The success of land recovery can be evaluated by assessing the diversity of soil microbes, which serves as an indicator of biological fertility. Soil microbes play a pivotal role in converting potential chemical fertility into actual fertility. Moreover, their activities gradually enhance the physical properties of the soil, further indicating improved physical fertility (Cui et al., 2021). However, the restoration of ex-coal mining lands faces numerous challenges, including the need to address soil degradation, erosion, and biodiversity loss resulting from mining activities (Ahirwal et al, 2016). To address these challenges, it is crucial to consider the role of soil microbes, including entomopathogenic microbia, which are an integral part of soil ecosystems (Boucias and Pendland, 2018).

The roles of nematodes and microbes in ex-mining lands are fundamental to ecological restoration efforts, improving soil health, supporting plant growth, and maintaining ecological balance. They can significantly impact the success of revegetation and restoration projects in post-mining landscapes. The presence of nematodes and microbes in the soil benefit plant health during revegetation endeavors (Guo et al., 2021; Li et al., 2022). These microorganisms support plant establishment and growth. Healthy and robust plant growth, in turn, stabilizes soil, prevents erosion, and enhances biodiversity, all of which are crucial for the overall success of revegetation projects (Mensah, 2015). The presence of microbial diversity in mining-affected areas can be influenced by factors such as soil type, climate, land management practices, as well as the host plants root exudates composition (Santoyo, 2022; Sasse et al., 2017). Consequently, understanding and managing the diversity of these microorganisms are critical for the success of revegetation efforts.

Entomopathogenic microbes which encompass various taxonomic groups, including bacteria, fungi, nematodes, and viruses, are known to regulate insect populations and play a vital role in ecological processes (Deka et al., 2021) One of the most remarkable contributions of entomopathogenic microbial during revegetation is their role as natural pest

controllers (Vega et al., 2009). These microorganisms serve as natural enemies of various insect pests that could harm newly planted vegetation (Shrestha et al, 2020). By infecting and eliminating these pests, entomopathogens help regulate insect populations and protect the restored vegetation (Rozpądek et al., 2019). In regions where entomopathogens thrive, the risk of pest outbreaks is reduced, safeguarding the overall health of the revegetated land (Koskella et al, 2020). Entomopathogenic microbial, including fungi and bacteria, are natural antagonists of insects and play a pivotal role in regulating insect populations in terrestrial ecosystems. While extensively studied for their potential in biological pest control in agriculture, their utilization in ecological restoration, particularly in ex-coal mining land revegetation, remains a relatively unexplored area, and a promising avenue for research and practical implementation. The diversity and abundance of entomopathogenic microbial are subject to environmental factors (Fatu et al, 2020). Factors such as soil physicochemical properties which can fluctuate significantly in mining-affected areas, play pivotal roles in shaping microbial community structure (Mudrak et al, 2021).

The specific objectives of this study are to identify the diversity of nematodes and soil microbes on revegetated ex-coal mining land, screen for entomopathogenic nematodes and microbes, and analyze their relationships with the chemical fertility status of the land.

MATERIALS AND METHODS

Experimental site

The study was conducted at two locations, in post-coal mining and non-mining areas, in the Pendingin Subdistrict of Sanga-Sanga, in Kutai Kartanegara District, East Kalimantan Province, Indonesia. The post-coal mining area located at the coordinate S 00040'29.7 "E 117016'09.8") has been revegetated with oil palm plantation. The non-mining area, as a control, located at the coordinate S 00041'41.3 "E 117015’54.9", is cultivated with rubber plant.

Sample collection

The sampling method used in this study refers to the standard method of ISPM (International Standards for Phytosanitary Measure), which is an international standard guide to conduct surveys in the field. Soil samples were taken randomly at ten sampling point in each experimental sites. The soil samples were taken at four levels of depth from soil surface, 0-30 cm, 30-60 cm, 60-80 cm, 80-100 cm. After removing the gravel, and plant debris, the soil samples then were composited for each level of soils’s depth and taken 1 kg for microbial extraction and identification especially for fungi, bacteria, and nematodes in laboratory. In addition, the the soil physicochemical properties were also analyzed in this study.

Fungal and Bacterial Isolation and Identification

For fungal isolation, 10 grams of soil samples were dissolved in a liter of distilled water. An ose needle was dipped in the soil solution, and then inoculated on the Potato Dextrose Agar (PDA) media. A week after, the microbes were isolated and observed Mycobacteria observations were carried out using microscope to identify the type, color, and shape of bacteria, as well as its colony following the procedure of Hucker's with minor modification (Hucker, 1921) Fungal identification was conducted by observing the morphological characteristics of fungi using a microscope**.** Conidia density was calculated using the *Neubauer* type *haemocytometer*.

Bacteria isolation was carried out by dissolving 10 grams of soil samples in a liter of distilled water. An ose needle was dipped in the soil solution, and then inoculated on the Nutrient Agar (NA) media. Two days after, the microbes were isolated and observed bacteria observations were carried out using microscope to identify the type, color, and shape of bacteria, as well as its colony following the procedure of Hucker's with minor modification (Hucker, 1921).

Entomopathogenic fungal and bacterial screening

Entomopathogen fungi and bacteria was selected using mealworms (*Tenebrio mollitor* L.), larvae aged 30-40 days (Figure 1.) One milliliter of each fungus and bacteria isolates were inoculated to mealworms. Only fungi and bacteria that can cause mortality to mealworms was considered in the data of this study to screen the entomopathogen bacteria and fungi.

Figure 1. *Mealworms, Tenebrio mollitor* L. larvae 30-40 days

Nematodes Isolation and identification

Nematodes isolation was carried out using *Baermann funnel method* with minor modification. This method is considered as the most effective in nematode isolation (van den Hoogen etal., 2020). A silicone hose wasattached to the funnel. The end of the hose is tied using a Gauze rubber band and placed on the top of the funnel. The Baermann-funnel apparatus was installed in a horizontal position without buckling the silicon hose. Fifty grams of soil for each sample was placed on the filter paperthen wrapped the soil to cover its entire surface. The water was added slowly into the funnel to submerge the sampel, until the sample saturate, to create a moist environment that stimulate nematodes to migrate toward the water source. The funnel was incubated for 3x24 hours and the water released from the bottom of the funnel. The water was collected in a film tube / fial. The collected water then was passed through a fine sieve to separate the nematodes from the water and debris. Formalin (5%) was added as much as 1 drop and fixed for 15 minutes.

Entomopathogenic nematodes screening

Entomopathogenic nematodes were screened by bait trap using mealworms (Figure 1). The mealworms larvae (3-5 larvae) were placed in a plastic container and mixed with ± 150 g of moist soil containing nematodes. The container is then inverted so that the larvae are covered with soil, then placed at room temperature.

After 3-4 days, if the larvae show death, the larval cadaver is removed and rinsed with sterilized distilled water. Entomopathogenic nematode trapping was then continued using the White trap method to release entomopathogenic nematodes from the cadaver's body. Nematodes were observed and calculated using a stereo microscope. Identification of soil nematodes and entomopathogenic nematodes genus were carried out by morphological observations.

Soil physicochemical analysis

Samples were analyzed for their chemical and physical properties including organic C, total N, available P (Bray), available K (Morgan), Ca^{++}, Mg^{++}, K^{+}, Na^{+}, CEC (Cation Exchange Capacity); Base saturation (BS) Al saturation, pH and soil texture. The soil physicochemical analysis was conducted based on the general procedure developed in the Soil Laboratory, Faculty of Agriculture, Mulawarman University, Samarinda, Indonesia.

Data analysis:

Data collected were analyzed descriptively to find out the role of soil microbes in supporting the recovery of post-coal mining land and the relationship between the diversity of soil microbes and the chemical fertility status of the study site.

RESULTS

Fungi diversity and density

Higher fungi diversity was observed in non-mining compared to ex-mining land, in which five fungi genera was found in non-mining, and four genera in ex-mining (Figure 2, Table 1). The fungi diversity in ex-coal mining and non-mining land were different in every soil layer observed in this study (Table 1). As an example, *Penicillium* sp. which was found in every soil layer of post coal mining land was not present in non-mining land. On the other hand, *Mucor* sp. which was found in non-mining land was absence in coal mining land. In addition, Aspergillus was observed as abundant fungi which was found in almost all soil layer either in ex-coal mining land or in non-mining land. Besides its diversity, the fungal density in non-mining was also considerably higher (ranging from 2.6-7.2 x 10^5 cfu.g^{-1}) than that of ex-coal mining land (ranging from 1.4-3.8 x 10^5 cfu.g^{-1}) in every soil depth layer. The deeper the soil layer, the fewer fungal populations were detected. Among all the fungi identified in the ex-mining and non-mining land, only *Trichoderma* was screened as entomopathogenic fungi. *Tricodherma* was observed in the digestive system of deceased mealworms, the larvae of T. Mollitor.

Table 1. Fungi diversity and density at different soil depth layer, and screening of entomopathogenic fungi in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

	Soil depth layer	Fungal population density cfu/g	Genera/species
	Non-mining land		
1.	0-30	7.2×10^5	*Aspergillus* *Trichoderma** *Penicillium*
2.	30-60	5.4×10^5	*Aspergillus* *Trichoderma** *Penicillium*
3.	60-80	3.2×10^5	*Trichoderma** *Phytium* *Penicillium*
4.	80-100	2.6×10^5	*Aspergillus* *Penicillium*
	Post-coal-mining land		
1.	0-30	3.8×10^5	*Aspergillus* *Trichoderma* sp.*
2.	30-60	2.2×10^5	*Aspergillus niger* *Trichoderma**
3.	60-80	1.8×10^5	*Aspergillus* sp. *Mucor* sp.
4.	80-100	1.4×10^5	*Aspergillus niger* *Aspergillus flavus*.

*Entomopathogenic fungi that successfully kill mealworms by entering through their digestive system (acting as a stomach poison).

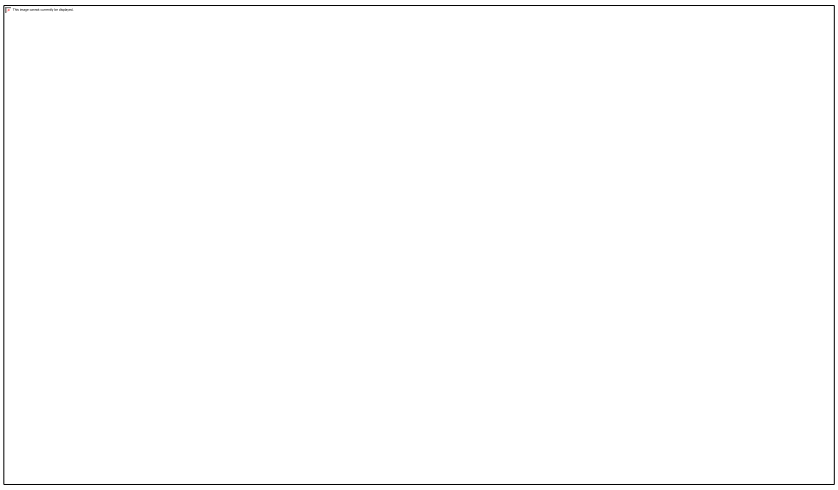

Figure 2. Fungi diversity: A. *Trichoderma: conidia (a), hialin (b), conidiofor (c); B. Phytium: oogonium (a), hyphae (b); C. Penicillium: conidium (a), phialide (b), metula (c), stipe (d); D. A. niger: conidia (a), conidiofor (b); E. A. flavus: conidia (a), phialide (b), vesicle (c), stipe (d); F. Mucor* sp.*: sporangium (a), sporangiofore (b), hyphae (c)*

Enthomopatogenic Bacteria

Two family of bacteria, *Azotobacteraceae* and *Bacillaceae* were observed in both non-mining and ex-coal mining land (Figure 3). However, in the deeper layer of subsoil of ex-coal mining land, only *Azotobacteraceae* was present, either in 60-80 cm or in 80-100 cm. Interestingly, all of the bacteria in the family of *Azotobacteraceae* in this study was noticed as coccus gram positive bacteria, except in ex-coal mining land, there were coccus gram negative *Azotobacteraceae* in the soil depth of 30-60 cm and 60-80 cm. The bacteria population was denser in non-coal-mining ($4.2 – 9.6x10^6$) compared to ex-coal mining land ($1.4 – 5.1x10^6$). The bacteria population was decreased in line with the soil depth layer in the two sites of experiment (Table 2). In this study, only *Bacillaceae* bacteria, observed in the digestive system of deceased *T. Mollitor* larvae, were identified as entomopathogenic bacteria in both ex-mining and non-mining lands.

Table 2. Bacteria diversity and density at different soil depth layer, and screening of entomopathogenic bacteria in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Bacterial population density cfu/g	Family
	Non-mining land		
1.	0-30	9.6×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae**
2.	30-60	5.5×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae**
3.	60-80	4.5×10^6	- Coccus, gram: (+) *Azotobacteraceae* - bacil, gram (+): *Bacillaceae**
4.	80-100	4.2×10^6	- Coccus, gram (+): *Azotobacteraceae* - Bacil, gram (+): *Bacillaceae*
	Post-coal-mining land		
1.	0-30	5.1×10^6	- Coccus, gram (+): *Azotobacteraceae* - Basil, gram (+): *Bacillaceae**
2.	30-60	4.9×10^6	- Coccus, gram: (+) Azotobacteraceae - Basil, gram (+): *Bacillaceae**
3.	60-80	1.6×10^6	- Coccus, gram: (+): Azotobacteraceae
4.	80-100	1.4×10^6	- Coccus, gram: (+): Azotobacteraceae

*Entomopathogenic bacteria that successfully kill mealworms by entering through their digestive system (acting as a stomach poison).

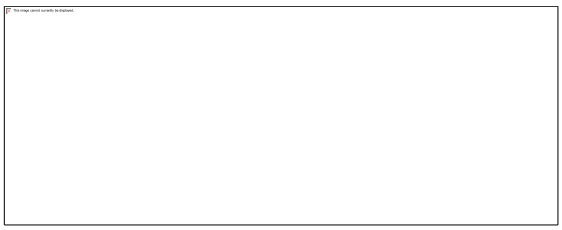

Figure 3. Bacteria (left to right: A. *Bacillaceae,* and B. *Azotobacteraceae*)

Enthomopatogenic Nematoda

A high genetic diversity of nematodes was observed in both non-mining and ex-mining lands (Table 3). Among the fifteen genera (Figure 4), six namely *Heterodera*, *Heterorhabditis*, *Hoplolaimus*, *Pratlyenchus*, *Radopholus*, and *Rotylenchus* were present in both non-mining and ex-coal mining lands.. Conversely, *Dorylaimus*, *Meloidogyne*, *Rotylenchulus*, and *Tylenchus* were found only in non-mining area, while *Longidorus*, *Rhabditis*, *Steinernema*, and *Xiphinema* were exclusive to ex-mining land. The number of nematode populations was notably higher in ex-coal mining compared to non- mining land. It was also observed that the deeper the soil layer, the greater the number of isolated nematodes, found in both non-

mining and ex-coal mining lands (Table 3). Among all the nematodes diversity identified in this study, only *Heterorhabditis* and *Steinernema* were screened as entomopathogenic nematodes.

Table 3. Nematode diversity and density at different soil depth layer, and screening of entomopathogenic nematode in ex-mining and non-mining land in Pendingin, Sanga-Sanga Subdistrict, Kutai Kartanegara Regency

No	Soil Depth layer	Nematoda Population/ kg soil	Genus
	Non-mining land		
1.	0-30	1.1 x10^2	*Heterodera* *Meloidogyne* *Tylenchus* *Heterorhabditis**
2.	30-60	1.2 x 10^2	*Heterorhabditis** *Pratlyenchus* *Heterodera* *Radopholus* *Hoplolaimus*
3.	60-80	4 x 10^2	*Pratylenchus* *Heterodera*
4.	80-100	2 x 10^2	*Rotylenchus* *Rotylenchulus* *Dorylaimus*
	Post-coal-mining land		
1.	0-30	5.1 x 10^2	*Heterodera* *Xiphinema*
2.	30-60	7.0 x 10^2	*Heterorhabditis** *Steinernema* *Radopholus* *Rhabditis*
3.	60-80	8.0 x 10^2	*Steinernema** *Hoplolaimus* *Rotylenchus*
4.	80-100	9.2 x 10^2	*Pratylenchus* *Longidorus*

*Entomopathogenic nematodes obtained from the cadavers of deceased mealworms

Figure 4. Nematodes (left to right: A. *Hoplolaimus,* B. *Pratylenchus*, C. *Tylenchus,* D. *Heterorhabditis,* E. *Steinernema,* F. *Dorylaimus,* G. *Xiphinema,* H. *Heterodera,* I. *Rhabditis,* J. *Radopholus,* K. *Longidorus,* L. *Rotylenchus,* M. *Rotylenchulus,* N. *Helycotylenchus,* O. *Aphelenchus*)

Soil physicochemical properties

In general, soil nutrient content in non-mining land was higher compared to post-coal mining (Table 4). There was also variation of the nutrient content in different soil depth layer. Most of the nutrient were available more in the subsoil part, compared to topsoil, as observed in available K, Ca++. Mg++, K++, and Na+, as well as base saturation in non-mining land. On the contrary, that phenomenon was in the reverse condition in post-coal mining land.

Aluminium content seemed to be higher in the post-coal mining than that of the non-mining land. The aluminium content tends to decrease as the soil layer gets deeper on non-coal mining land, but tends to be higher on the deeper soil layers on post-coal mining land. The pH status in both of non-mining and post-coal mining land was in the range of 4.02-4.9 and 3.19-4.19, respectively, showing the higher acidity of post-coal mining land.

Soil texture is determined by the proportion of mineral components in the soil. Among all these mineral particles, silt is a dominant soil texture compared to clay and sand, either in non-mining and post-coal mining land, in the top soil or subsoil. However, the relative proportions of different mineral particles (sand, silt, and clay) were varied in each soil layer resulted in distinct soil texture.

Table 4. Soil physicochemical properties of the non-mining and ex-coal-mining land in Kutai Kartanegara Regency, East Kalimantan

No.	Soil Parameter	Unit	Non-coal Mining Land				Ex-coal Mining Land			
			R1 0-30	R2 30-60	R3 60-80	R4 80-100	S1 0-30	S2 30-60	S3 60-80	S4 80-100
1.	Carbon organic	%	7.65	4.64	4.14	3.41	3.80	3.74	1.38	0.86
2.	Nitrogen total	%	0.41	0.24	0.51	0.14	0.17	0.15	0.16	0.19
3.	Ratio C/N	-	18.66	19.33	8.12	24.36	22.35	24.93	8.63	4.53

4.	Available P (Bray1)	Ppm	19.39	12.60	13.38	20.26	5.11	4.03	0.96	4.12
5.	Available K tersedia (Morgan)	Ppm	110.45	214.54	170.21	234.33	209.22	81.56	82.09	143.28
6.	Al^{3+} (Morgan)		4.00	2.20	2.10	3.10	2.00	6.80	5.10	8.40
7.	H^{+} (Morgan)		3.46	1.80	1.70	2.66	1.66	6.20	4.46	4.26
8.	Ca^{++}	meq/100 g	0.63	1.67	0.83	1.19	1.95	0.79	0.52	0.61
9.	Mg^{++}	meq/100 g	0.48	1.25	0.68	0.98	1.01	0.48	0.37	0.49
10.	K^{+}	meq/100 g	0.30	0.65	0.48	0.91	0.67	0.20	0.26	0.40
11.	Na^{+}	meq/100 g	0.04	0.38	0.28	0.29	0.34	0.11	0.02	0.12
12.	CEC	meq/100 g	8.91	7.95	6.07	9.13	8.16	8.58	10.73	14.28
13.	BS	%	16.3	49.7	37.4	36.9	48.7	18.4	10.9	11.3
14.	Al Saturation	%	44.89	27.67	34.60	33.95	26.21	46.64	47.53	58.82
15.	pH	–	4.02	4.62	4.54	4.90	3.56	3.35	4.19	3.19
16.	Clay	%	14.77	36.54	20.07	23.21	28.72	16.54	23.94	32.85
17.	Silt	%	34.48	58.18	55.30	35.43	67.66	64.75	33.70	35.54
18.	Sand	%	50.75	5.28	24.63	41.36	3.62	18.71	42.36	31.61
19.	Texture		SiCL	SiCL	SiL	L	SiCL	SiL	L	CL

DISCUSSION

The most concern of land degradation impact in post-mining area in agriculture sector is the reduction of biodiversity and soil fertility. Mining activities often lead to the destruction of natural habitats and ecosystems result in the deracination and decline of plant and animal species, leading to a loss of biodiversity which further negatively affects agricultural productivity. In addition, post-mining land undergoes physical and chemical changes that render it unsuitable for agriculture, since it reducing soil porosity and aeration due to the alteration of soil structure, lacks the organic matter and nutrients due to the topsoil removal, introducing contaminants including heavy metals and toxic chemicals, reducing organic matter and soil nutrients, altering the pH of the soil, making it more acidic or alkaline, which can be detrimental to plant growth (Prince et al., 2018; Janečková et al, 2023).

Efforts to address the loss of arable land in post-mining areas involve comprehensive reclamation and rehabilitation practices that aim to restore soil fertility, structure, water-holding capacity and repair the quality of the environment and ecosystem as mentioned in the regulation issued by the Minister of Energy and Mineral Resources Regulation 7/2014 and Regional Regulation East Kalimantan 8/2013 (Yulianingrum 2023). These efforts may include topsoil replacement, planting vegetation, and ongoing monitoring to ensure the land can support agriculture practices. To achieve normal criteria for fertile soil supporting agricultural activities, maintenance of microbial community structures is very important.

Entomopathogen microbial diversity and density

In this study, it was clearly observed that the nematodes and soil microbial diversity in post-coal mining is diverse with the non-mining land. The soil fungi diversity and density in post-coal mining was considerably lower compared to the non-mining land (Table 1). This situation was not only observed in fungi, but also in soil bacteria (Table 2), showing a low biodiversity of microorganisms in post-coal mining even after reclamation process. A significant reduction of microbial diversity due to coal mining practices after land restoration was also observed in the previous study conducted by de Quadros et al. (2016). In their study, the post-coal mining land has been restored for several years (3-19 years) by replacing the soil layers, applying limestone to raise the pH to 6.0, and revegetating the land with grasses. Even after a completion of reclamation process in many years, the composition of the microbial diversity and biomass of post-mined sites was significantly lower from the control (unaltered sites). A similar results were also found in different studies comparing the microbial diversity and density in reclamation and re-vegetation post-coal mine with undisturbed sites resulted in the significant declines in microbial community structure and diversity (Li et al, 2014; Upadhyay et al., 2014) as also showed in this current study.

A reduction of microbial (bacteria and fungi) diversity and density was also observed in the deeper layer of soil either in post-coal mining or non-mining land (Table 1 and Table 2), in which the microbes decrease as the depth of the soil layer

increases. A similar remark was also reported in a study of Upadhyay et al., (2014) revealed that the rhizospheric soils showed greater diversity in both bacteria and fungi compared to the non-rhizospheric soil. This outcome is supported by the recent studies concluded that soil microbial diversity decreasing along with the soil depth (Wang et al, 2021; Guo et al., 2021)

It is well known that the topsoil is the most biologically active and favorable layer for microorganisms compared to the subsoil layer. The topsoil contains the highest concentration of organic matter, providing food source and nutrients for microorganisms (Bahram et al., 2018). In addition, the uppermost layer of soil or also known as A-horizon is well-aerated and has a suitable moisture content, moderate temperature providing a suitable environment for a wide range of microorganisms (Liu et al., 2019; Aquado et al, 2023). Furthermore, there is an interaction between microorganisms and plant roots which are more abundant in the top layer of soil. Plant roots release root exudates and provide energy source for many soil microorganisms, thereby contributing to a more active microbial community (Zhalnina et al, 2018; Zhou et al, 2020). Therefore, the vegetation types, plant species, and climated will influence the soil microbial diversity and community composition (Li et al., 2022; Sopialena and Palupi, 2017).

One of the crucial roles of microorganisms in agriculture is as biological control agents, suppress the pest population, using natural enemies. The use of biological control is considered as the most prudent manner to control pest since it can reduced reliance on chemical pesticides, specific pest targeting, long-term pest management, environmentally friendly, cost-effective, safe for human health, and supporting the sustainable agriculture (Boro et al, 2022). Therefore, besides observing the genetic diversity and density of the microorganisms in the ex-mining and non-mining land, this study also focus on screening entomopathogenic nematodes and microbial, as biological control suppressing insect population. Insects are the largest and most diverse group of organisms included as one of the most common and economically significant types of plant pests (García-Lara, 2016), causing significant damage of any crop plant species (Manosathiyadevan, 2017).

Among all microbes, only *Trichoderma* (fungi) and *Bacillaceae* (bacteria) were identified as entomopathogenic microorganisms in this study, present in both ex-mining and non-mining lands. *Trichoderma* can indirectly act as an entomopathogen by producing insecticidal secondary metabolites, antifeedant compounds, and repellent metabolites (Poveda, 2021). This is due to Trichoderma's ability to produce metabolites such as citric acid (Vinale et al., 2012), as well as enzymes like urease, cellulase, glucanase, and chitinase (Carlile et al., 2001), which can negatively affect insects and even cause death when they enter the body. On the other hand, bacteria from the *Bacillaceae* family, particularly *Bacillus thuringiensis*, have received significant attention as entomopathogens. This bacterium is capable of producing proteinaceous protoxin crystals with insecticidal properties. When ingested by insects, these crystals disintegrate in the stomach and are broken down by host proteases to produce an active poison known as endotoxin, which ultimately leads to insect death (Deka et al., 2021).

On the contrary to the fungi and bacteria, nematodes were noticed more abundant in the post-mining than in the non-mining land, in term of diversity and density. The nematodes were also observed more in the deeper layer of soil (Table 3). Most of the studies observed that the vertical distribution of nematode communities is decreasing from the topsoil layer to the sub soil layer (Cheng et al, 2021; Suyadi et al. 2021). However, it is also explained that the distinct characteristics of soil at different layers cause variations in soil nematode communities (Liu et al., 2022). It means that differences in soil nematode diversity and density in different soil depths might be influenced by soil variables. In a study of (Ilieva-Makulec et al., 2015), the number of nematode diversity varied in different experimental sites, in which the nematode generic richness is higher in the topsoil, while in different habitat, the nematode diversity was significantly lower in the topsoil and the highest in the subsoil. The nematode abundance and distribution are influenced by many factors, such as soil properties, climate and environmental conditions, land use and management, plant species and root exudates, predation and competition, succession and ecosystem development (Ilieva-Makulec et al., 2015; Zhang, 2020; Cheng et al, 2021; Li et al., 2022)

Nematodes may be classified into three main groups: saprophagous (living on dead organic matter), predators (feeding on small animals, including other nematodes), and parasites (infesting insects, animals, human, fungi and higher plants) (Pant et al., 1998). Among the fifteen nematodes identified in this study, *Heterodera, Meloidogyne, Tylenchus, Pratlyenchus, Radopholus, Hoplolaimus, Rotylenchus, Rotylenchulus, Xiphinema*, and *Longidorus* were plant-parasitic nematodes. *Meloidogyne, Radhopholus,* and *Rotylenchulus* were the top three genera of plant-parasite nematodes observed in East Kalimantan (Suyadi & Rosfiansyah, 2017). *Dorylaimus* and *Rhabditis*, on the other hand, were non-plant-parasitic nematodes; *Dorylaimus* functions as an omnivore in the rhizosphere, and *Rhabditis* as a bacteriovore. In this study, only *Heterorhabditis* and *Steinernema* were screened as entomopathogenic nematodes, as also observed in previous studies (Bhat et al., 2020; Julià et al., 2023).

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Relation of soil physicochemical properties and microbial community

The physicochemical properties of post-mining soil are considerably different from those of non-mining land (Table 4). The different characteristics of post-mining land arise from the significant alterations in soil properties, structure, and

chemical composition due to mining activities. The mining process disrupts the natural soil structure, leading to soil compaction, which reduces pore spaces and affects water infiltration and root penetration (Yuningsih et al., 2021). Additionally, the removal or burying of topsoil (due to improper reclamation processes) results in a significant reduction in the organic content of the soil (especially in the root growth layer), leading to nutrient loss and further diminishing soil fertility. Furthermore, the mining process can expose rocks and minerals that, when weathered, can alter the soil's pH, leading to acid mine drainage and significantly lowering the soil pH (Herman et al., 2024). These conditions make the soil ecosystem in post-mining land unconducive for microbial communities, which contrasts with the conditions in non-mining land.

Soil is a medium for plant growth and also acts as an environment for microbial growth, determining the quality of growing soil microbes and plants. The interactions between soil microbes and plants play a crucial role in soil health and fertility. Fertile soil typically is a supply of organic matter for microbial activity. Soil status encompasses a wide range of physical, chemical, and biological properties that influence the habitat and conditions for soil microorganisms. A healthy soil status with sufficient organic matter and nutrient availability ensures that microorganisms have the resources they need to thrive (Kaur et al., 2022) The presence of high organic matter in soil is characterized by several physicochemical parameters, such as pH levels, cation exchange capacity (CEC), soil organic carbon (SOC) content, and nutrient content especially nitrogen, phosphorus, and potassium (Dandwate, 2020)

In this study, the soil acidity is included as acid soil either in post-coal mining or non-mining land with minor variation in every soil depth layer. However, it is examined that soil pH in the post-mining land was considerably lower, in the range of 3.19-4.19, compared to non-mining in the range of 4.02-4.9. Related to the lower pH in post-mining land, aluminium content is also higher in this land than that of the non-mining (Table 4) since the acidic soils enhance aluminum solubility and availability. Furthermore, soil pH is positively correlated with another soil property, basic saturation (BS), which was observed higher in the non-mining land (Table 4).

Soil pH strongly influences microbial communities. Acidic or alkaline soils can limit the diversity and activity of microorganisms, impacting nutrient availability and overall soil. Soil pH is a critical factor that affects the activity and diversity of soil microorganisms. Different microbial species have specific pH preferences, and soil pH can influence their metabolic activities. Soil pH outside the optimal range for specific microbes can limit their growth and activity (Wang et al, 2019). Maintaining an appropriate pH level is essential for a diverse and active microbial community.

Soil organic matter in the non-mining land was presumably higher in this study, characterized by the higher soil organic carbon (SOC) and nutrient content than that of the post-coal mining land, beside the pH level (Table 4). Higher organic matter are associated with high SOC, as carbon is a fundamental component of organic materials, mainly occurs via the microbial breakdown, partial decay, and conversion of deceased organic materials (Lefèvre et al, 2017) This activity co-related with nutrient availability, as organic matter decomposes, it releases nutrients into the soil, contributing to the soils overall fertility and health (Gerke, 2022)

All over, soil physicochemical properties play a significant role in shaping the composition and activity of the microbial community (Brahmaprakash, 2021). Changes in these properties can influence the composition and activity of the microbial community, subsequently impacting nutrient cycling, organic matter decomposition, and overall soil fertility (Leiva et al, 2020; Kaur et al., 2022) as clearly shown in this study. A higher microbial community was observed in the higher physicochemical of soil properties, which was shown in the non-mining land, compared to the post-coal mining land. Fertile soil is characterized by a well-balanced and diverse microbial community that supports plant growth by providing essential nutrients and improving soil structure. The composition and diversity of the soil microbial community are important factors in maintaining soil health which further supporting plant growth and health (Wang and Li, 2023). The increasing microbial population (both types and numbers) causes the dynamics of the soil to be better and become naturally healthy (Bertola et al., 2021). The ability to change soil biological properties in a positive direction can increase the population of microbes, included entomopathogen microbial, that benefit plants, and make plants grow healthy without the need for the use of artificial fertilizers and pesticides.

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CONCLUSION

The microbial diversity in post-coal mining is diverse with the non-mining land. The entomopathogenic fungi and bacteria diversity and density in ex-coal mining was considerably lower compared to the control, non-mining land. The microbial (bacteria and fungi) diversity and density reduced in the deeper layer of soil either in post-coal mining or non-mining land, in which the microbes decrease as the depth of the soil layer increases. On the contrary to the fungi and bacteria, entomopathogen nematodes were noticed more abundant in the post-mining than in the non-mining land, in term of diversity and density. The nematodes were also observed more in the deeper layer of soil. Variations in the soil nematode communities might be due to the distinct characteristics of soil at different layers. The fertility of soil was

observed lower in the ex-coal mining land characterized by several soil physicochemical properties, in which lower soil pH, basic saturation, soil organic carbon (SOC) and nutrient content were examined, causing the lower microbial community compared to non-mining land.

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Assessment of nematode and microbial diversity and screening of entomopathogens in revegetated post-coal-mining land in Kutai Kartanegara District, East Kalimantan, Indonesia

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Abstract. *Sopialena, Rosfiansyah, Suryadi A. 2024. Assessment of nematode and microbial diversity and screening of entomopathogens in revegetated post-coal-mining land in Kutai Kartanegara District, East Kalimantan, Indonesia. Biodiversitas 25: 2921-2930.* Post-coal-mining management should strive toward site revegetation. The success of such revegetation efforts is largely determined by the soil's fertility status, which encompasses physical, chemical, and biological components. Biological fertility is indicated by the presence and abundance of soil microbes and nematodes. The objective of this study was to identify the diversity of nematodes and soil microbes in ex-coal-mining land revegetated with oil palm in Kutai Kartanegara District (East Kalimantan, Indonesia), to screen for entomopathogenic nematodes and microbes, and to analyze their relationships with the chemical fertility status of the land. As a control, non-mining land was also investigated. The results showed that microbial diversity and density (including fungi and bacteria) in the ex-coal-mining area were significantly lower compared to the control. Additionally, a decrease in microbial diversity and density was found in deeper soil layers of both post-mining and non-mining lands. In contrast, soil nematodes were more abundant in post-mining land, both in terms of diversity and density, and were also more plentiful in the deeper soil layers. Among the nematodes and microbes studied, only *Heterorhabditis* and *Steinernema* (nematodes), *Trichoderma* (fungi), and *Bacillaceae* (bacteria) were identified as entomopathogens, causing death in *Tenebrio molitor* L. larvae. Overall, soil fertility in ex-coal-mining land was observed to be lower, characterized by decreased soil pH, basic saturation, Soil Organic Carbon (SOC), and nutrient content, which contributed to a reduced microbial community compared to non-mining land.

Keywords: Entomopathogen, Kutai Kartanegara, nematodes, post-coal mining, soil microbes, *Trichoderma*

INTRODUCTION

Coal mining activities in Kutai Kartanegara District, East Kalimantan, Indonesia, have experienced rapid growth in recent years, with both large-scale and small-scale operations, including those with mining authorities. However, this expansion has come at a significant cost to the environment, resulting in widespread damage. This damage manifests in the form of arid and infertile post-mining lands or artificial "craters" with unclear purposes. While some efforts have been made to rehabilitate these ex-coal-mining lands, they remain sporadic and insufficient, allowing environmental degradation to outpace recovery efforts.

Rehabilitating these lands is essential to mitigating the adverse environmental impacts of coal mining. The success of land recovery can be evaluated by assessing the diversity of soil microbes, which serves as an indicator of biological fertility. Soil microbes play a pivotal role in converting potential chemical fertility into actual fertility. Moreover, their activities gradually enhance the physical properties of the soil, further improving physical fertility (Cui et al. 2021). However, the restoration of ex-coal-mining lands faces numerous challenges, including the need to address soil degradation, erosion, and biodiversity loss resulting from mining activities (Ahirwal et al. 2016). To address these challenges, it is crucial to consider the role of soil microbes, including entomopathogenic microbes, which are an integral part of soil ecosystems (Boucias and Pendland 2018).

The roles of nematodes and microbes in ex-mining lands are fundamental to ecological restoration efforts, improving soil health, supporting plant growth, and maintaining ecological balance. These microorganisms can significantly impact the success of revegetation and restoration projects in post-mining landscapes. The presence of nematodes and microbes in the soil benefits plant health during revegetation endeavors (Guo et al. 2021; Li et al. 2022). Furthermore, these microorganisms support plant establishment and growth. Healthy and robust plant growth, in turn, stabilizes the soil, prevents erosion, and enhances biodiversity, all of which are important for the overall success of revegetation projects (Mensah 2015). The presence of microbial diversity in mining-affected areas can be influenced by factors such as soil type, climate, land management practices, as well as the host plants' root exudate composition (Sasse et al. 2017; Santoyo 2022). Consequently, understanding and managing the diversity of these microorganisms are critical for the success of revegetation efforts.

Entomopathogenic microbes, which encompass various taxonomic groups, including bacteria, fungi, nematodes, and viruses, are known to regulate insect populations and play a vital role in ecological processes (Deka et al. 2021). One of the most remarkable contributions of entomopathogenic

microbes during revegetation is their role as natural pest controllers (Vega et al. 2009). These microorganisms serve as natural enemies of various insect pests that could harm newly planted vegetation (Shrestha et al. 2020). By infecting and eliminating these pests, entomopathogens help regulate insect populations and protect the restored vegetation (Rozpądek et al. 2019). In regions where entomopathogens thrive, the risk of pest outbreaks is reduced, safeguarding the overall health of the revegetated land (Koskella et al. 2020). Entomopathogenic microbes, such as fungi and bacteria are natural antagonists of insects and play a pivotal role in regulating insect populations in terrestrial ecosystems. While extensively studied for their potential in biological pest control in agriculture, their utilization in ecological restoration, particularly in ex-coal-mining land revegetation, remains a relatively unexplored area, and a promising avenue for research and practical implementation. The diversity and abundance of entomopathogenic microbes are subject to environmental factors (Fatu et al. 2020). Factors such as soil physicochemical properties, which can fluctuate significantly in mining-affected areas, play pivotal roles in shaping microbial community structure (Mudrak et al. 2021).

The objective of this study was to identify the diversity of nematodes and soil microbes on revegetated ex-coal-mining land, to investigate for entomopathogenic nematodes and microbes, and to analyze their relationships with the chemical fertility status of the land.

MATERIALS AND METHODS

Experimental sites

The study was conducted at two locations, in post-coal mining and non-mining areas, in the Pendingin Subdistrict of Sanga-Sanga, in Kutai Kartanegara District, East Kalimantan Province, Indonesia. Oil palm plantations were planted in a post-coal mining area (00°40'29.7"S 117°16'09.8"E), while rubber plantations were cultivated in a non-mining area (00°41'41.3"S 117°15’54.9"E) used as a control.

Sample collection

The sampling method used in this study was the standard method of ISPM (International Standards for Phytosanitary Measure). Soil samples were taken randomly at ten sampling points at each experimental site. The soil samples were taken at four levels of depth (0-30 cm, 30-60 cm, 60-80 cm, and 80-100 cm) from the soil surface. After removing the gravel and plant debris, soil samples were composited for each level of soil depth, and 1 kg of soil was collected for microbial extraction and identification of fungi, bacteria, and nematodes in the laboratory. In addition, physicochemical properties of soil were also analyzed in this study.

Fungal and bacterial isolation and identification

For both fungal and bacterial isolation, 10 g of soil samples were dissolved in a liter of distilled water. Then 1 ml of soil solution was taken and diluted in steps from 10^{-1} to 10^{-3} to determine the CFU value of each sample, by repeating 2 times each dilution from three dilution levels. An inoculating needle was dipped in the soil solution, and then inoculated on Potato Dextrose Agar (PDA) medium to culture fungi, while another inoculating needle was dipped in the same solution and applied to Nutrient Agar (NA) medium to culture bacteria. After one week of culturing for fungi, or two days for bacteria, the microbes were isolated and observed under a microscope to identify the type, color, and shape of individual bacteria and fungi, as well as that of their colonies, following Hucker's procedure with minor modifications (Hucker 1921). The number of microorganisms (fungi and bacteria) present in each test sample as a weighted average of two consecutive dilutions was calculate using the following equation (Matuurin and Peeler 2001):

$$N = \frac{\sum C}{[(1\ x\ n_1) + (0{,}1\ x\ n_2)\ x\ (d)}$$

Where: N = Number of colonies per ml or g of product; ΣC = Sum of all colonies on all plates counted; n1 = Number of plates in first dilution counted; n2 = Number of plates in second dilution counted; d = Dilution from which the first counts were obtained

Entomopathogenic fungal and bacterial screening
Entomopathogenic fungi and bacteria were selected using mealworm (*Tenebrio molitor* L.) larvae 30-40 days of age (Figure 1). One milliliter of each fungal and bacterial isolate was inoculated to mealworms. Only fungi and bacteria that could cause mortality to mealworms were considered entomopathogens in the present study.

Isolation and identification of nematodes
Nematode isolation was carried out using the Baermann funnel method with minor modifications. This method was considered the most effective in nematode isolation (van den Hoogen et al. 2020).

Figure 1. Mealworm (*Tenebrio molitor* L.) larvae at 30-40 days of age

Briefly, a silicone hose was attached to a funnel, then the end of the hose was tied using a gauze rubber band and placed on top of the funnel. The Baermann funnel apparatus was installed in a horizontal position without buckling the silicon hose. Fifty grams of soil for each sample was placed on the filter paper, and the soil was wrapped to cover its entire surface. The water was added slowly into the funnel to submerge the sample, until the sample was saturated, to create a moist environment that stimulated nematodes to migrate toward the water source. The funnel was incubated for 3×24 hours and the water released from the bottom of the funnel. The water was collected in a film tube/vial. The collected water then was passed through a fine sieve to separate the nematodes from the water and debris. Up to 1 drop of formalin (5%) was added and fixed for 15 minutes.

Entomopathogenic nematode screening
Entomopathogenic nematodes were screened by bait trap using mealworms. The mealworm larvae (3-5 larvae) were placed in a plastic container and mixed with ~150 g of moist soil containing nematodes. The container was then inverted so that the larvae were covered with soil, then placed at room temperature.

After 3-4 days, if larval mortality was observed, the larval cadaver was removed and rinsed with sterilized distilled water. Entomopathogenic nematode trapping was then conducted using the White trap method to release entomopathogenic nematodes from the cadaver's body. Nematodes were observed and counted using a stereo microscope. Identification of soil nematodes and entomopathogenic nematodes to genus level was carried out through morphological observations.

Physicochemical analysis of soil
Samples were analyzed for their chemical and physical properties, including organic C, total N, available P (Bray), available K (Morgan), Ca^{++}, Mg^{++}, K^{+}, Na^{+}, CEC (Cation Exchange Capacity), Base Saturation (BS), Al saturation, pH, and soil texture. The soil physicochemical analysis was conducted based on the general procedure developed in the Soil Laboratory, Faculty of Agriculture, Universitas Mulawarman, Samarinda, Indonesia.

Data analysis
Data collected were analyzed descriptively to uncover the role of soil microbes in supporting the

recovery of post-coal-mining land and relationship between the diversity of soil microbes and chemical fertility status of the study site.

RESULTS AND DISCUSSION

Fungal diversity and density

Result showed that higher fungal diversity was observed in the non-mining land compared to the ex-mining land, with five fungal genera found in non-mining, and four genera in ex-mining soil (Figure 2 and Table 1). The fungal diversity in ex-coal mining and non-mining land was different in every soil layer observed in this study (Table 1). *Penicillium* sp., which was found in every soil layer of post coal-mining land, was not present in the non-mining land soil. On the other hand, *Mucor* sp., which was found in the non-mining land soil, was absent in coal-mining land soil. In addition, *Aspergillus* was found abundant in almost all soil layers in both ex-coal-mining and non-mining land. Fungal density in non-mining land soil was also significantly higher (ranging from 2.6-7.2×10^5 cfu.g^{-1}) than in soil of ex-coal-mining land (ranging from 1.4-3.8×10^5 cfu.g^{-1}) at every soil depth layer. The deeper the soil layer, the fewer fungal populations were found. Among all the fungi identified in the ex-mining and non-mining land, only *Trichoderma* was screened as an entomopathogenic fungi. *Trichoderma* was observed in the digestive system of deceased *T. molitor* mealworm larvae.

Table 1. Fungal diversity and density at different soil depth layers, and screening of entomopathogenic fungi in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara District, Indonesia

Soil depth layers (cm)	Fungal population density (cfu/g)	Genera/species
Non-mining land		
0-30	7.2 x 10^5	*Aspergillus, Trichoderma*, Penicillium*
30-60	5.4 x 10^5	*Aspergillus, Trichoderma*, Penicillium*
60-80	3.2 x 10^5	*Trichoderma*, Pythium, Penicillium*
80-100	2.6 x 10^5	*Aspergillus, Penicillium*
Post-coal-mining land		
0-30	3.8 x 10^5	*Aspergillus, Trichoderma **
30-60	2.2 x 10^5	*Aspergillus niger, Trichoderma**
60-80	1.8 x 10^5	*Aspergillus* sp., *Mucor* sp.
80-100	1.4 x 10^5	*Aspergillus niger, Aspergillus flavus*

Note: *Entomopathogenic fungi

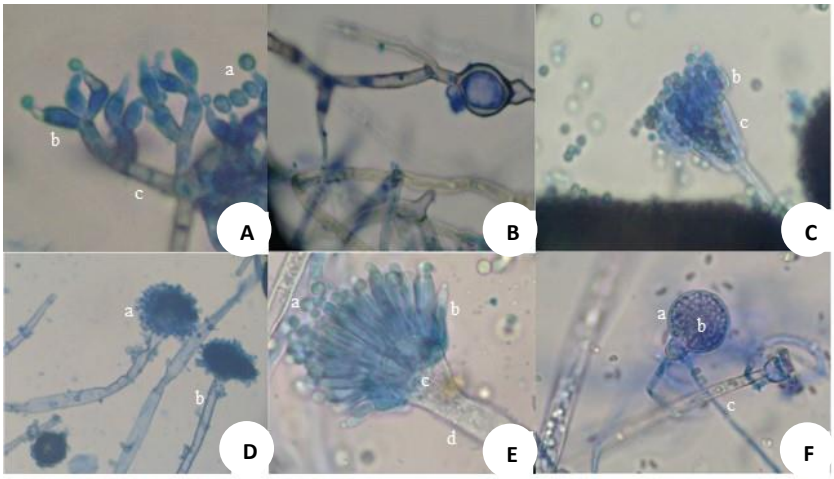

Figure 2. Fungal diversity: A. *Trichoderma*: conidia (a), hyalin (b), conidiophore (c); B. *Pythium*: oogonium (a), hyphae (b); C. *Penicillium*: conidium (a), phialide (b), metula (c), stipe (d); D. *A. niger*: conidia (a), conidiophore (b); E. *A. flavus*: conidia (a), phialide (b), vesicle (c), stipe (d); F. *Mucor* sp.: sporangium (a), sporangiophore (b), hyphae (c)

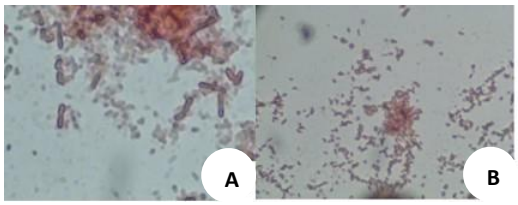

Figure 3. Bacteria (left to right: A. Bacillaceae, and B. Azotobacteraceae)

Entomopathogenic bacteria

Two bacterial families, *Azotobacteraceae* and *Bacillaceae*, were observed in the soils of both non-mining and ex-coal-mining land (Figure 3). However, only *Azotobacteraceae* was present in the deeper subsoil layers (60-80 cm or 80-100 cm) of the ex-coal-mining land. Interestingly, all *Azotobacteraceae* observed in this study were identified as coccus Gram-positive bacteria, except in the ex-coal-mining land, in which coccus Gram-negative *Azotobacteraceae* were found at the soil depths of 30-60 cm and 60-80 cm. The bacterial population was denser in non-coal-mining ($4.2\text{-}9.6\times10^6$) compared to ex-coal-mining land ($1.4\text{-}5.1\times10^6$). Bacterial population decreased along with soil depth at both experimental sites (Table 2). In this study, only *Bacillaceae* bacteria, observed in the digestive system of deceased *T. molitor* larvae, were identified as entomopathogenic bacteria in both ex-mining and non-mining lands.

Entomopathogenic nematodes

A high genetic diversity of nematodes was observed in both non-mining and ex-mining land. Among the fifteen genera identified (Figure 4), six—namely *Heterodera*, *Heterorhabditis*, *Hoplolaimus*, *Pratylenchus*, *Radopholus*, and *Rotylenchus*were present in both non-mining and ex-coal-mining lands. Conversely, *Dorylaimus*, *Meloidogyne*, *Rotylenchulus*, and *Tylenchus* were found only in non-mining area, while *Longidorus*, *Rhabditis*, *Steinernema*, and *Xiphinema* were exclusive to ex-mining land. The number of nematode populations was notably higher in ex-coal-mining compared to non-mining land. It was also observed that the deeper the soil layer, the greater the number of isolated nematodes, as found

in both non-mining and ex-coal-mining land (Table 3). Despite the high nematode diversity, only *Heterorhabditis* and *Steinernema* were identified as entomopathogenic nematodes.

Physicochemical properties of soil

In general, soil nutrient content was higher in non-mining land compared to post-coal mining land (Table 4). There was also a variation in the nutrient content at different soil depth layers. Most of the nutrients were more abundant in the subsoil compared to the topsoil, as observed in the available K, Ca++. Mg++, K++, and Na+, as well as base saturation in non-mining land. In contrast, the reverse phenomenon was observed in post-coal-mining land.

Aluminum content was found to be higher in post-coal-mining than non-mining land. Moreover, the aluminum content tended to decrease with increasing soil depth on non-coal-mining land, while tending to be higher in the deeper soil layers on post-coal-mining land. The pH status of non-mining and post-coal-mining land was in the range of 4.02-4.9 and 3.19-4.19, respectively, showing higher acidity in post-coal-mining land.

Soil texture was determined by the proportion of mineral components in the soil. Among these mineral particles, silt, rather than clay or sand, was the dominant soil texture in topsoil and subsoil of both non-mining and post-coal-mining land. However, the relative proportions of different mineral particles (sand, silt, and clay) varied in each soil layer, resulting in distinct soil textures.

Table 2. Bacterial diversity and density at different soil depth layers, and screening of entomopathogenic bacteria in ex-mining and non-mining land in Pendingin, Sanga-Sanga subdistrict, Kutai Kartanegara District, Indonesia

Soil depth layers (cm)	Bacterial population density cfu/g	Family
Non-mining land		
0-30	9.6 x 10^6	- Coccus, Gram (+), *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
30-60	5.5 x 10^6	- Coccus, Gram (+), *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
60-80	4.5 x10^6	- Coccus, Gram (+) *Azotobacteraceae* - Bacilli, Gram (+), *Bacillaceae**
80-100	4.2 x 10^6	- Coccus, Gram (+): *Azotobacteraceae* - Bacilli, Gram (+): *Bacillaceae*
Post-coal-mining land		
0-30	5.1 x 10^6	- Coccus, Gram (+); *Azotobacteraceae* - Bacilli, Gram (+); *Bacillaceae**
30-60	4.9 x 10^6	- Coccus, Gram (+) Azotobacteraceae - Bacilli, Gram (+); *Bacillaceae**
60-80	1.6 x 10^6	- Coccus, Gram (+); Azotobacteraceae
80-100	1.4 x 10^6	- Coccus, Gram (+); Azotobacteraceae

Note: *Entomopathogenic bacteria

Table 3. Nematode diversity and density at different soil depth layers, and screening of entomopathogenic nematodes in ex-mining and non-mining land in Pendingin, Sanga-Sanga Subdistrict, Kutai Kartanegara District, Indonesia

Soil depth layers (cm)	Nematodes Population/ kg soil	Genus
Non-mining land		
0-30	1.1 x10^2	*Heterodera, Meloidogyne, Tylenchus, Heterorhabditis**
30-60	1.2 x 10^2	*Heterorhabditis*, Pratlyenchus, Heterodera, Radopholus, Hoplolaimus*
60-80	4 x 10^2	*Pratylenchus, Heterodera*
80-100	2 x 10^2	*Rotylenchus, Rotylenchulus, Dorylaimus*
Post-coal-mining land		
0-30	5.1 x 10^2	*Heterodera, Xiphinema*
30-60	7.0 x 10^2	*Heterorhabditis*, Steinernema, Radopholus, Rhabditis*
60-80	8.0 x 10^2	*Steinernema*, Hoplolaimus, Rotylenchus*
80-100	9.2 x 10^2	*Pratylenchus, Longidorus*

Note: *Entomopathogenic nematodes

Discussion

The greatest concern regarding land degradation impacts of post-mining areas for the agriculture sector is the reduction of biodiversity and soil fertility. Mining activities often lead to the destruction of natural habitats and ecosystems, resulting in the deracination and decline of plant and animal species, and leading to a loss of biodiversity that also negatively affects agricultural productivity. In addition, post-mining land undergoes physical and chemical changes that render it unsuitable for agriculture, such as reduced soil porosity and aeration due to the alteration of soil structure, a lack of organic matter and nutrients due to the topsoil removal, introduced contaminants such as heavy metals and toxic chemicals, and altered soil pH—more acidic or alkaline—which can all be detrimental to plant growth (Prince et al. 2018; Janečková et al. 2023).

Efforts to address the loss of arable land in post-mining areas involve comprehensive reclamation and rehabilitation practices that aim to restore soil fertility, structure, and water-holding capacity, and to repair the quality of the environment and ecosystem, as mentioned in the regulation issued by the Minister of Energy and Mineral Resources Regulation 7/2014 and Regional Regulation East Kalimantan 8/2013 (Yulianingrum 2023). These efforts may include topsoil replacement, revegetation, and ongoing monitoring to ensure the land can support agriculture practices. Furthermore, to achieve normal criteria for fertile soil supporting agricultural activities, maintenance of microbial community structures is very important.

Microbial entomopathogen diversity and density

In this study, it was observed that the nematode and soil microbial diversity in post-coal-mining land clearly differed to that in the non-mining land. Fungal diversity and density in post-coal-mining land soil

was considerably lower compared to the non-mining land. This situation was also observed in soil bacteria, which showed low biodiversity in post-coal-mining land even after the completion of the reclamation process. A significant reduction of microbial diversity due to coal mining practices after land restoration was also observed in a study conducted by de Quadros et al. (2016). In their study, post-coal-mining land had undergone different durations of restoration (3-19 years), which involved replacing the soil layers, applying limestone to raise the pH to 6.0, and revegetating the land with grasses. Even after the completion of the reclamation process after many years, the composition of the microbial diversity and biomass of post-mined sites was significantly lower than that of the control (unaltered sites). Similar results were also found in different studies comparing the microbial diversity and density in reclaimed and revegetated post-coal-mining sites with that in undisturbed sites, both of which reported significant declines in microbial community structure and diversity (Li et al. 2014; Upadhyay et al. 2014).

A reduction of microbial (bacterial and fungal) diversity and density was also observed in the deeper layers of soil in both the post-coal-mining and non-mining-land, in which the microbes decreased as the depth of the soil layer increased. A similar observation was studied by Upadhyay et al. (2014), who reported that rhizospheric soils showed greater diversity in both bacteria and fungi compared to non-rhizospheric soil. This outcome is supported by recent studies concluding that soil microbial diversity decreases along with the soil depth (Guo et al. 2021; Wang et al. 2021).

It is well known that topsoil is the most biologically active and favorable layer for microorganisms. Compared to the subsoil layer, topsoil contains the highest concentration of organic matter, providing food and nutrients to microorganisms (Bahram et al. 2017). In addition, the uppermost layer of soil, also known as A-horizon, is well-aerated and has adequate moisture content and moderate temperatures, providing a suitable environment for a wide range of microorganisms (Liu et al. 2019; Aguado et al. 2023).

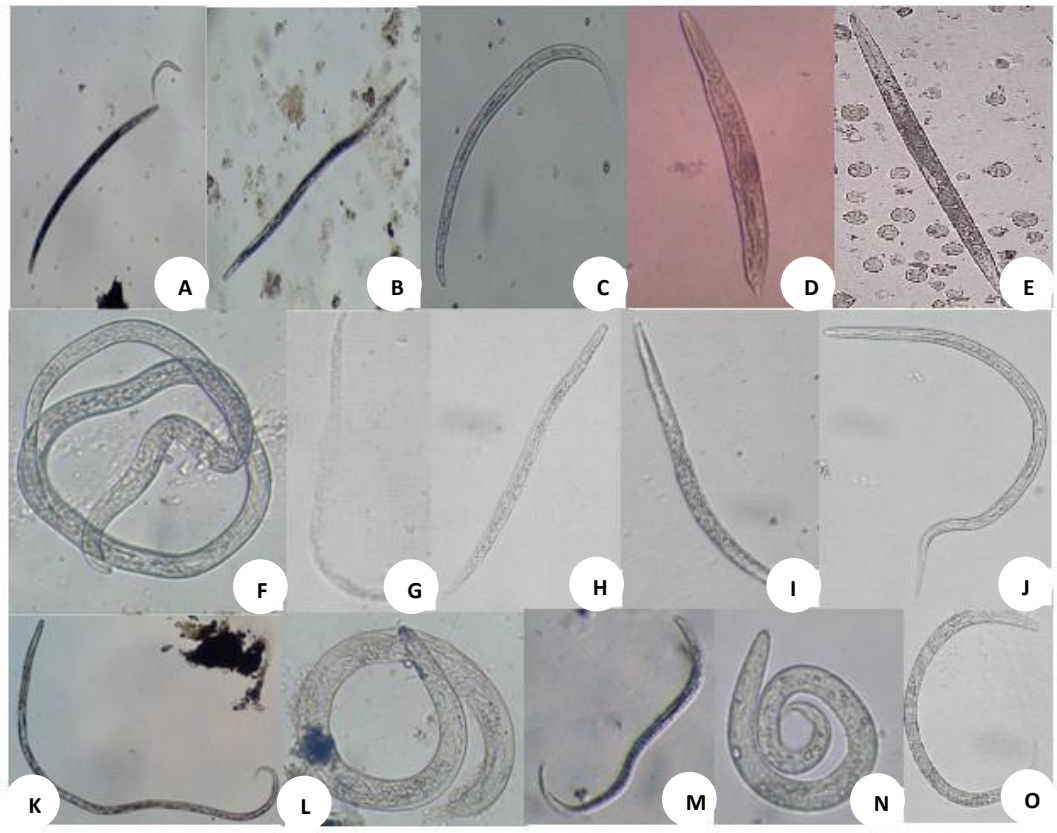

Figure 4. Nematodes (left to right: A. *Hoplolaimus*, B. *Pratylenchus*, C. *Tylenchus*, D. *Heterorhabditis*, E. *Steinernema*, F. *Dorylaimus*, G. *Xiphinema*, H. *Heterodera*, I. *Rhabditis*, J. *Radopholus*, K. *Longidorus*, L. *Rotylenchus*, M. *Rotylenchulus*, N. *Helicotylenchus*, O. *Aphelenchus*)

Table 4. Physicochemical properties of soil in non-mining and ex-coal-mining land in Kutai Kartanegara District, East Kalimantan, Indonesia

Soil parameters	Units	Non-coal mining land				Ex-coal mining land			
		R1 0-30	R2 30-60	R3 60-80	R4 80-100	S1 0-30	S2 30-60	S3 60-80	S4 80-100
Carbon organic	%	7.65	4.64	4.14	3.41	3.80	3.74	1.38	0.86
Nitrogen total	%	0.41	0.24	0.51	0.14	0.17	0.15	0.16	0.19
Ratio C/N	-	18.66	19.33	8.12	24.36	22.35	24.93	8.63	4.53

Available P (Bray1)	Ppm	19.39	12.60	13.38	20.26	5.11	4.03	0.96	4.12
Available K Available (Morgan)	Ppm	110.45	214.54	170.21	234.33	209.22	81.56	82.09	143.28
Al^{3+} (Morgan)		4.00	2.20	2.10	3.10	2.00	6.80	5.10	8.40
H^{+} (Morgan)		3.46	1.80	1.70	2.66	1.66	6.20	4.46	4.26
Ca^{++}	meq/100 g	0.63	1.67	0.83	1.19	1.95	0.79	0.52	0.61
Mg^{++}	meq/100 g	0.48	1.25	0.68	0.98	1.01	0.48	0.37	0.49
K^{+}	meq/100 g	0.30	0.65	0.48	0.91	0.67	0.20	0.26	0.40
Na^{+}	meq/100 g	0.04	0.38	0.28	0.29	0.34	0.11	0.02	0.12
CEC	meq/100 g	8.91	7.95	6.07	9.13	8.16	8.58	10.73	14.28
BS	%	16.3	49.7	37.4	36.9	48.7	18.4	10.9	11.3
Al Saturation	%	44.89	27.67	34.60	33.95	26.21	46.64	47.53	58.82
pH	–	4.02	4.62	4.54	4.90	3.56	3.35	4.19	3.19
Clay	%	14.77	36.54	20.07	23.21	28.72	16.54	23.94	32.85
Silt	%	34.48	58.18	55.30	35.43	67.66	64.75	33.70	35.54
Sand	%	50.75	5.28	24.63	41.36	3.62	18.71	42.36	31.61
Texture*		SiCL	SiCL	SiL	L	SiCL	SiL	L	CL

Note: *Soil Texture, SiCL = Silty Clay Loam; SiL = Silty Loam; CL = Clay Loam; L = Loam

Furthermore, there is an interaction between microorganisms and plant roots, which are more abundant in the top layer of soil. Plant roots release root exudates and provide energy to many soil microorganisms, thereby contributing to a more active microbial community (Zhalnina et al. 2018; Zhou et al. 2020). The vegetation types, plant species, and climate all influence the soil microbial diversity and community composition (Li et al. 2022; Sopialena and Palupi 2017).

One of the key roles of microorganisms in agriculture is as agents of biological control, a process that harnesses natural enemies to suppress pest populations. The use of biological control agents is considered the most prudent manner of controlling pests as it can reduce our reliance on chemical pesticides, and because it is a long-term, specific pest targeting, environmentally friendly, cost-effective approach that is safe for human health and supports sustainable agriculture (Boro et al. 2022). Therefore, besides observing the genetic diversity and density of the microorganisms in the ex-mining and non-mining land, this study also focused on screening entomopathogenic nematodes and microbes as potential biological control agents for suppressing insect populations. Insects are the largest and most diverse group of organisms included as one of the most

common and economically significant types of plant pests (García-Lara 2016), causing significant damage to many crop plant species (Manosathiyadevan 2017).

Among all microbes, only *Trichoderma* (fungi) and *Bacillaceae* (bacteria) were identified as entomopathogenic microorganisms in the present study, in both ex-mining and non-mining lands. *Trichoderma* can indirectly act as an entomopathogen by producing insecticidal secondary metabolites, antifeedant compounds, and repellent metabolites (Poveda 2021). This is due to *Trichoderma*'s ability to produce metabolites such as citric acid (Vinale et al. 2012), as well as enzymes like urease, cellulase, glucanase, and chitinase (Carlile et al. 2001), which can negatively affect insects and even cause death when they enter the body. On the other hand, bacteria from the Bacillaceae family, particularly *Bacillus thuringiensis*, have received significant attention as entomopathogens. This bacterium is capable of producing proteinaceous protoxin crystals with insecticidal properties. When ingested by insects, these crystals disintegrate in the stomach and are broken down by host proteases to produce an active poison known as endotoxin, which ultimately leads to insect death (Deka et al. 2021).

In contrast to the fungi and bacteria, nematodes were found to be more abundant in the post-mining than in the non-mining land in terms of their diversity and density. Additionally, more nematodes were observed in the deeper soil layers. Most related studies have observed that the vertical distribution of nematode communities decreases from the topsoil layer to the subsoil layer (Cheng et al. 2021). This variation in soil nematode communities may be explained by the distinct characteristics of soils at different layers (Liu et al. 2022). The differences in soil nematode diversity and density at different soil depths observed in this study might be influenced by soil variables. The amount of nematode diversity varied in different experimental sites (Ilieva-Makulec et al. 2015). Results showed that nematode generic richness was higher in the topsoil of one site, while in a different habitat, nematode diversity was significantly lower in the topsoil and highest in the subsoil. Overall, it is likely that nematode abundance and distribution are influenced by many factors, such as soil properties, climate and environmental conditions, land use and management, plant species and root exudates, predation and competition, and succession and ecosystem development (Ilieva-Makulec et al. 2015; Zhang 2020; Cheng et al. 2021; Li et al. 2022).

Nematodes may be classified into three main groups: saprophages (living on dead organic matter), predators (feeding on small animals, including other nematodes), and parasites (infesting insects, animals, human, fungi and higher plants) (Pant et al. 1998). Eleven of the fifteen nematodes identified in this study, namely *Heterodera*, *Meloidogyne*, *Tylenchus*, *Pratlyenchus*, *Radopholus*, *Hoplolaimus*, *Rotylenchus*, *Rotylenchulus*, *Xiphinema*, and *Longidorus* were plant-parasitic nematodes. *Meloidogyne*, *Radopholus*, and *Rotylenchulus* were the top three genera of plant-parasite nematodes observed in East Kalimantan (Suyadi and Rosfiansyah 2017). *Dorylaimus* and *Rhabditis*, on the other hand, were non-plant-parasitic nematodes. *Dorylaimus* functions as an omnivore in the rhizosphere, and *Rhabditis* as a bacterivore. In this study, only *Heterorhabditis* and *Steinernema* were identified as entomopathogenic nematodes, as also observed in the previous studies (Bhat et al. 2020; Julià et al. 2023).

Relation of soil physicochemical properties and microbial community

The physicochemical properties of post-mining soil were considerably different from those of non-mining soil in this study. The different characteristics of post-mining land arise from the significant alterations in soil properties, structure, and chemical composition due to mining activities. The mining process disrupts the natural soil structure, leading to soil compaction, which reduces pore spaces and affects water infiltration and root penetration (Hermansyah et al. 2021). Additionally, the removal or burying of topsoil (due to improper reclamation

processes) results in a significant reduction in the organic content of the soil (especially in the root growth layer), leading to nutrient loss and further diminishing soil fertility. Furthermore, the mining process can expose rocks and minerals that, when weathered, can alter the soil's pH, leading to acid mine drainage and significantly lowering the soil pH (Herman et al. 2024). These conditions make the soil ecosystem in post-mining land unconducive for microbial communities, which contrasts with the conditions in non-mining land.

Soil is a medium for plant growth and also acts as an environment for microbial growth, determining the quality of growing soil microbes and plants. The interactions between soil microbes and plants play a crucial role in soil health and fertility. Fertile soil is typically a source of organic matter for microbial activity. Soil status encompasses a wide range of physical, chemical, and biological properties that influence the habitat and conditions for soil microorganisms. A healthy soil status with sufficient organic matter and nutrient availability ensures that microorganisms have the resources they need to thrive (Kaur et al. 2022). The presence of high organic matter in soil is characterized by several physicochemical parameters, such as pH levels, Cation Exchange Capacity (CEC), Soil Organic Carbon (SOC) content, and nutrient content, especially of nitrogen, phosphorus, and potassium (Dandwate 2020).

It was also observed that soils in both post-coal mining and non-mining land were found to be acidic, with minor variations at every soil depth layer. However, soil pH in the post-mining land was considerably lower, in the range of 3.19-4.19, compared to that in the non-mining land (4.02-4.9). Related to the lower pH in post-mining land was the aluminum content, which was higher than that in the non-mining land as acidic soils enhance aluminum solubility and availability. Furthermore, soil pH is positively correlated with another soil property, Basic Saturation (BS), which was observed to be higher in the non-mining land.

Soil pH strongly influences microbial communities. Acidic or alkaline soils can limit the diversity and activity of microorganisms, impacting nutrient availability and overall soil. It is a critical factor that affects the activity and diversity of soil microorganisms. Different microbial species have specific pH preferences, and soil pH can influence their metabolic activities. Soil pH outside the optimal range for specific microbes can limit their growth and activity (Wang et al. 2019). Therefore, maintaining an appropriate pH level is essential for a diverse and active microbial community.

Soil organic matter was higher in non-mining land in this study, as characterized by the higher Soil Organic Carbon (SOC) and nutrient content than that of post-coal-mining land, as well as by the pH level. Higher organic matter content is associated with high SOC, which, as carbon is a fundamental component of organic materials, mainly occurs via the microbial breakdown, partial decay, and conversion of deceased organic materials (Lefèvre et al. 2017). This activity is co-related with nutrient availability; that is, as organic matter decomposes, it releases nutrients into the soil, contributing to the soil's overall fertility and health (Gerke 2022).

Overall, soil physicochemical properties play a significant role in shaping the composition and activity of the microbial community (Dasgupta and Brahmaprakash 2021). A larger microbial community was observed alongside the higher physicochemical soil properties of the non-mining land, rather than in the post-coal-mining land. Changes in these properties can influence the composition and activity of microbial community, subsequently impacting nutrient cycling, organic matter decomposition, and overall soil fertility (Leiva et al. 2020; Kaur et al. 2022). Fertile soil is characterized by a well-balanced and diverse microbial community that supports plant growth by providing essential nutrients and improving soil structure. The composition and diversity of the soil microbial community are important factors in maintaining soil health that also support plant growth and health (Wang and Li 2023).

The increasing microbial population (both in diversity and abundance) causes the dynamics of the soil to improve and become healthier naturally (Bertola et al. 2021). The ability to change soil biological properties in a positive direction can increase the population of microbes, including entomopathogenic microbes, which benefit plant growth and reduce the need for artificial fertilizer and pesticide use.

In conclusion, the results showed that microbial diversity in post-coal-mining land differed from that in non-mining control. The entomopathogenic fungal and bacterial diversity and density in ex-coal-mining soil was considerably lower compared to that in the non-mining land. The microbial (bacterial and fungal) diversity and density were lower in deeper layers of soil in both post-coal-mining and non-mining land, and tended to decrease soil layer depth increased. In contrast to fungi and bacteria, entomopathogenic nematodes were observed to be more abundant in the post-mining than in non-mining land in terms of their diversity and density. Additionally, nematodes were more abundant in the deeper layers of soil. Soil fertility was found to be lower in ex-coal-mining land, as characterized by several soil physicochemical properties, of which the lower soil pH, basic saturation, Soil Organic Carbon (SOC) and nutrient content detected likely supported a smaller microbial community compared to the non-mining land.

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