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ORIGINAL RESEARCH

Assessing soil bacterial community response to organophosphate pesticides in agricultural field of Yogyakarta, Java, Indonesia

ANNA RAKHMAWATI[1*], BERNADETTA OCTAVIA[1], SUHARTINI[1], TIEN AMINATUN[1]

[1]Department of Biology Education, Faculty of Mathematics and Natural Sciences, Universitas Negeri Yogyakarta, Yogyakarta, Indonesia

Abstract. Soil contamination by pesticides is one of the world's most pressing environmental issues. The widespread use of Organophosphate pesticides (OPPs) in agriculture has led to biological diversity changes. The indigenous bacterial community played significant roles in the remediation of soil contaminated with OPPs. This study examines the overall bacterial community composition of three agricultural fields in Yogyakarta, Java, Indonesia, that were exposed to OPPs. The agricultural field was divided into zones near the beach, residential, and mountainous. Sequencing 16S rRNA amplicon fragments used to analyze the soil bacterial community. It was discovered that Proteobacteria, Actinobacteria, and Firmicutes comprised the majority of the bacterial community. In addition, the samples contain a high relative abundance of *Bacillus*, *Bradyrhizobium*, *Chryseobacterium*, *Cystobacter*, *Microvirga*, and *Burkholderia*. The high alpha diversity indexes suggest that the agricultural soil microbiome provides important ecological services and may harbor a wide variety of bacteria and genes with biotechnological applications. The physicochemical soil characteristics are also correlated with the bacterial community structure. The findings can be used to develop bioremediation strategies that employ native microbes to clean and restore agricultural soil contaminated with OPPs.

Keywords: 16S rRNA, agricultural soil, bacterial diversity, organophosphate pesticides (OPPs), Proteobacteria

INTRODUCTION

Pesticides are typically used to increase agricultural productivity, and their various physicochemical properties as major pollutants that drive environmental change [1] and biodiversity [2] are a major causes of these effects. Organophosphate pesticides (OPPs) are most commonly used to eradicate pests and disease vectors in agriculture, homes, and public health. OPPs are widely used in global agriculture because they have a short half-life [3], are relatively inexpensive, easily accessible, and are effective against various pests [4]. The removal of OPPs is influenced by the chemical structure, functional groups, morphology, and competitive effects of the compounds [5]. In addition, physical, chemical, and biological processes have affected the soil mobility of OPPs. These compounds can be transported to lower horizons or remain in the topsoil layer. Moreover, it can damage ecosystems and community health over time [6]. The irreversible inhibition of the acetylcholinesterase (AChE) enzyme, essential for the neurotransmission of signals and whose suppression impairs the respiratory tract and neuromuscular transmission, is the primary toxicological effect of these pesticides are exposed on living organisms [7].

The OPPs'exposure harms the flora, fauna, and ecosystem. OPPs can influence soil microorganisms' biochemical and physiological behavior and their metabolic events [8]. Inhibiting microbial functions, decreasing the number of microbial groups, and altering the diversity, composition, and structure of soil microbial communities are among the ecotoxicological effects of pesticide on soil ecosystems [9]. Increased microbial population and adapted strains result from persistent pesticide use [10]. The persistence of pesticides in agricultural soil can influence the development of multidrug resistance among soil microorganisms [11]. When multiple pesticides are applied to agricultural soils' ecosystems,

*Corresponding Author:
anna_rakhmawati@uny.ac.id

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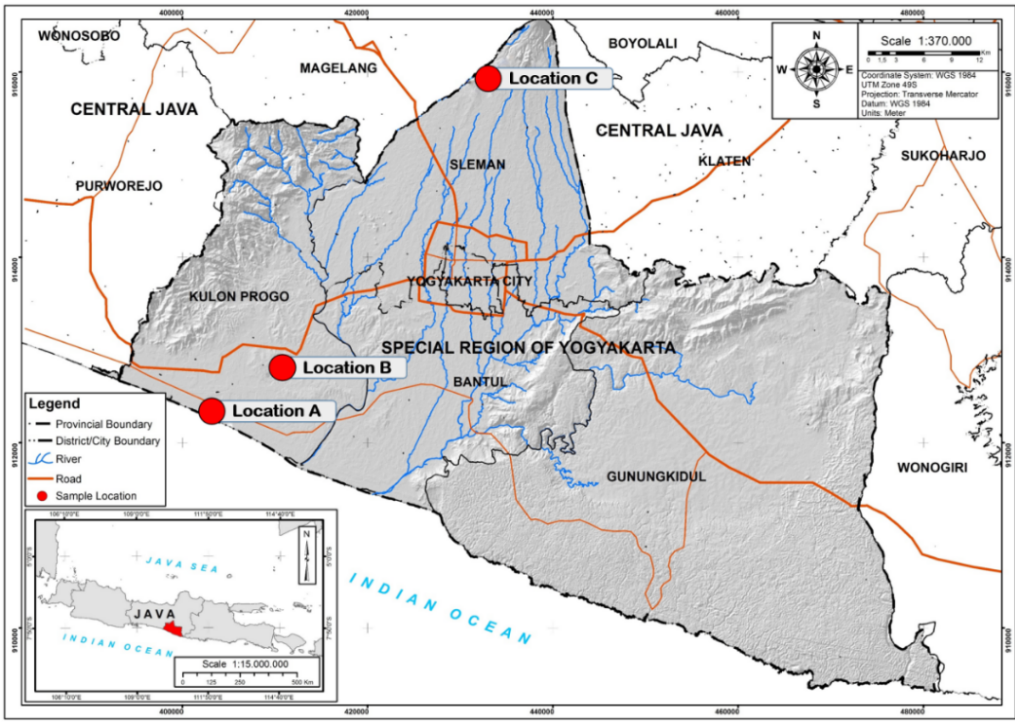


Figure 1. Location of the soil sampling sites location A (-7,9425560,110,1534620), location B (-7.897964,110,180508); and location C (-7,6131567,110,3802683).

the risk to soil microorganisms is greater than when a single pesticide is used [12].

Due to the fact that OPPs spray and leach into the soil, with the majority of this spray accumulating on agricultural land, many researchers have proposed that the soil's bacterial consortium and the enzymes they produce can be used as pesticide degradation agents. The soil's physicochemical and biological conditions defined its quality. The application of OPPs to agricultural land is directly related to variations in soil quality. This change is typically reflected by shifts in the soil's physicochemical properties and microbial indices. This study analyzed the bacterial community of agricultural soil in Yogyakarta, Java, Indonesia using 16S rRNA based metagenome. Principally, 16S rRNA gene sequencing is used to profile population diversity. The estimate of bacterial diversity derived from the metagenome analysis demonstrates that the genetic makeup of different species is distinct. Moreover, in metagenomics, the structure and sequence of an organism's DNA are discovered, thereby resolving problems associated with ecosystem functions, climate change, and biodiversity loss. The unique physicochemical characteristics of various agricultural soils may affect bacterial life forms. Therefore, it is a promising source of microbes and genes with biotechnological value.

However, only some studies describe the microbiome's composition, ecology, and dynamics in Java, Indonesia of the agricultural soil exposed to OPPs. This study centered on the agricultural land near the beach, residential areas, and mountainous region. For this project, it has been determined that profenofos are the most prevalent OPPs type in the area. The duration of OPP application, the cultivated plant, pesticide residues, and the physicochemical soil were evaluated. The objective of this research was to gain a better understanding of how the dynamics of bacterial composition change under different agricultural soil conditions. In addition, it provides valuable direction for future research on the efficacy of pesticide degradation agents.

METHODOLOGY

Study area and soil sampling

In April 2022, soil samples were taken from three agricultural land in Yogyakarta, Java, Indonesia (Figure 1). All areas exposed to organophosphate pesticides, such as profenofos for approximately two months with daily spraying. Location A was near the beach, location B was close to a residence, and location C was on the highlands, to assess the differences in metagenome and physicochemical soil characteristics. Bacil (*Ocimum bacilicum*) and chili (*Capsicum annum*) were grown at location A, while red onion (*Allium cepa*), mustard

(*Brassica rapa*) and chili (*Capsicum annum*) were grown at locations B and C, respectively. Four hundred fifty grams of soil were collected from a depth of 20 cm depth for each of the three soil samples. The soil was thoroughly mixed, and then 50 g was placed in a sterile 50 ml tube and stored at ± 30 °C before being transported for additional bacterial analysis using 16S rRNA sequence data. With the remaining 400 g, pesticide residues and soil physicochemical properties were analyzed.

DNA extraction, amplification, and sequencing

The DNA extraction was conducted in accordance with a modified protocol by Abundo et al. (2021). Following the manufacturer's instructions, the ZymoBIOMICS™ DNAMiniprep Kit was used to isolate the DNA genome from the soil sample (Qiagen, Hilden, Germany). The gDNA samples were then sent to Novogene Bioinformatics Technology Co., Ltd (Tianjin, China) for amplicon sequencing. The procedure for DNA amplification and sequencing were adapted from previous study by Zhang et al. (2021). Qubit fluorometric quantitation was then used to assess the extracted DNA' quality. Analysis of bacterial community was carried out using Illumina HiSeq 2500 PE250. The replicated metagenomic DNA were pooled and the hypervariable V3-V4 regions of the bacterial 16S rRNA gene were amplified using universal primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') primer pair to generate an amplicon library. Phusion High-Fidelity PCR master mix (New England Biolabs master mix was used in every PCR reactions. The loading buffer was mixed with PCR products and run on 2% agarose gel electrophoresis for detection the bands. For further experiments, samples with bright main bands in the 400–450 bp range were chosen. PCR products were mixed at equal density ratios. The mixed PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing library preparation were generated using NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified via Qubit and QPCR, was analysed by Illumina platform. The library quality was assessed using a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Finally, 250 bp paired-end reads were generated after the library was sequenced using an Illumina platform.

Bioinformatics analysis

Bioinformatics analysis was performed for the interpretation of biological data by using a variety of software tools and particular algorithms were performed by [13] and [14] procedures. Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. The data from each paired-end sequenced reading were combined using the FLASH software. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME (V1.7.0) software. High-quality tags were obtained by filtering the raw tags using the UCHIME algorithm and

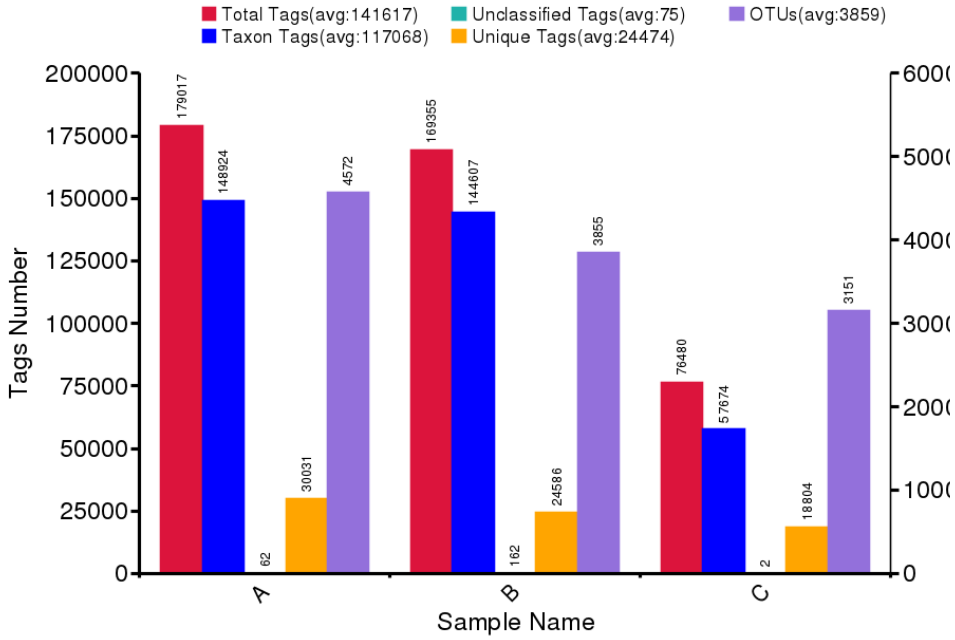


Figure 2. Summarization of the tags and OTUs number of sample location A, B, and C.

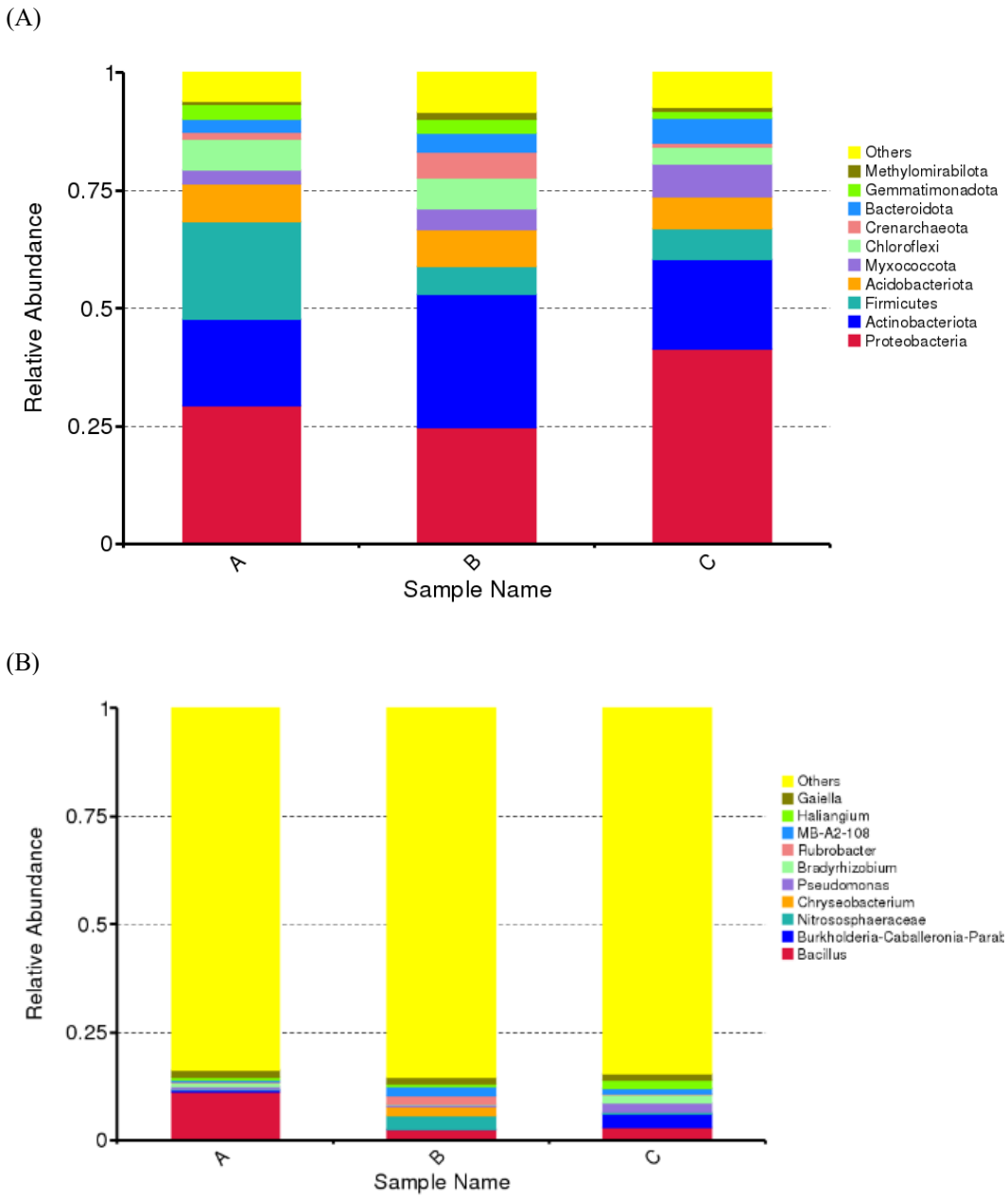
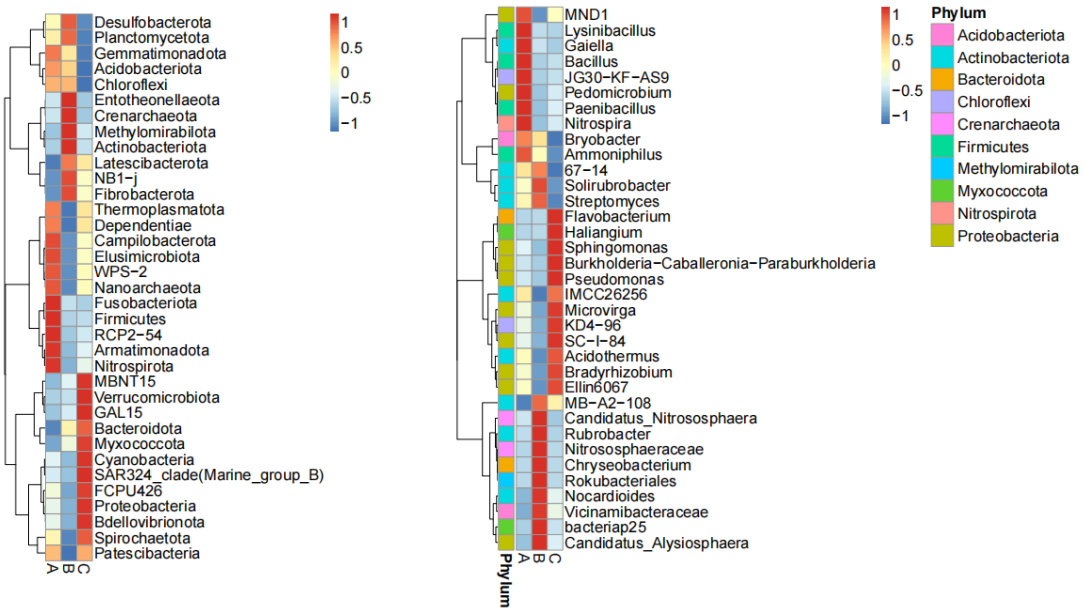


Figure 3. Taxa relative abundance in phylum (a) and genus (b) at sample location A, B, and C.

clustered into operational taxonomical units (OTU) using a cutoff percentage of bases with the quality score > 20 and error rate < 0.01 (Q20). The clean tags were compared to databases (Gold database) using the UCHIME algorithm to detect chimera sequences which were then removed to obtain effective tags. Sequenced data (effective tags) were then analyzed using the UPARSE software. Sequences with a similarity ≥ 97% were grouped into the same OTU. After that, the SILVA 132 database (https:// www.arb-silva.de/) was compared to each OTU annotate species at each taxonomic rank. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data. The annotation results were then used to calculate the Shannon-Weiner index (relative abundance) and its effect on data distribution using principal coordinate analysis (PCoA). The Shannon-Weiner relative abundance and index were calculated using the R software and the Minitab 19 software was used for the PCoA.

Soil properties

The soil samples physicochemical properties were examined at Faculty of Geography Universitas Gadjah Mada. The soil pH, texture,



a b

Figure 4. Taxonomic abundance cluster heatmap in phylum (a) and genus (b) at sample location A, B, and C. The colors indicate the relative abundance of taxa, ranging from blue (low) to red (high).

organic matter (OM), organic carbon (OC), cation exchange capacity (CEC), total nitrogen (TN), available phosphorus (AP) and available potassium (AK) were measured.

Organophosphate pesticides analysis

Organophosphate pestisides (OPPs) analysis was observed to detect pesticide residues in soil. Entire samples were packed at Integrated Research and Testing Laboratory (LPPT) Universitas Gadjah Mada and send to PT Saraswanti Indo Genetech (SIG) Laboratory in Bogor, Indonesia with EN 15662:2018 or internal number 18-12-2/MU/SMM-SIG procedure. Solid/cold liquid extraction, one of the most commonly used sorbent techniques in analyzing pesticide residues was used. This extraction was followed by the dispersive SPE purification Liquid Chromatography coupled with mass spectrometry (LC-MS / MS) ABSciex QTRAP 4500 (SIG/FNA/ALB/IN-0100) and UPLC Waters Acquity I Class (SIG/FNA/ALB/IN-0179) to identify different substances within a test sample.

RESULTS AND DISCUSSION

Metagenome sequencing and assembly

Operational Taxonomic Units (OTUs) were identified by clustering all samples' Effective Tags with 97% identity to examine each sample's microbial community composition. Total tags as the number of effective tags. Taxon tags means the number of annotated tags. Unclassified Tags means the number of unannotated tags. Unique Tags means the number of tags with a frequency of 1 and only occurs in one sample (Figure 2). A total of 424,852 16S rRNA gene sequence fragments might be grouped in all metagenomes and clustered into 11,578 OTUs sequencing of the soil samples.

To create the distribution histogram of relative abundance of taxa, choose the top ten for each taxonomic rank from each sample (Phylum, Class, Order, Family, Genus) using the results of the taxonomic annotation. The relative abundance of taxa in the phylum and genus was illustrated to provide a visual representation of the taxa with higher relative abundance and proportion in various classification levels of each sample (Figure 3). Location A was mainly dominated by Proteobacteria followed by Firmicutes, and Actinobacteria. A different pattern was observed in location B which was dominated by Actinobacteria and followed by Proteobacteria. The most apparent abundance at Location C was Proteobacteria attended by Actinobacteria. It has been shown that Proteobacteria, Actinobacteria, and Firmicutes were consistently the phyla with the highest relative abundance in all soil samples. This result indicated that these phyla occur in various natural habitat due to their diverse ecological, metabolic, also biochemical capabilities and activities. They are known to be globally distributed mainly in soils [15]. In line with results from former studies performed Proteobacteria dominance in agricultural field

Table 1. Alpha diversity indices

Location	OTUs	Shannon	Simpson	Chao1	ACE	Good_coverage
A	3951	9.611	0.993	4897.143	4879.518	0.981
B	3340	9.466	0.995	4136.453	4095.842	0.985
C	2903	9.710	0.997	3059.826	3069.927	0.994

[16], lateritic soil [17], and Firmicutes in soils contaminated with e-waste [18], mine-tailing sites [19], sulfometuron-methyl contaminated soil [20]. Furthermore, these phyla dominate because they can utilize complex substrates, particularly those not access to other microbial groups, and can grow in lower supplement environmental elements [21].

At genera level, *Bacillus, Bradyrhizobium,* and *Microvirga* were dominated at location A. High abundant genera in location B consisted of *Chryseobacterium*, *Microvirga*, and *Cystobacter*. In location C, *Bradyrhizobium*, *Burkholderia*, and *Bacillus* dominated. The present study demonstrated the dominance of *Bacillus* at agricultural soil near beach and highland zones. *Bacillus* is a large, heterogeneous group of Gram positive bacteria with wide phenotypic diversity. The previous investigation isolated *Bacillus* from hot spring soil [22]; mined-out soil [23], beach soil [24], rhizosphere [25]. Moreover, this genus is known as potential organochlorinated [26], chlorphyrifos [27], and organophosphate [28] pesticide degraders. Other dominant taxa are *Bradyrhizobium*, a Gram-negative soil bacteria, many of which fix nitrogen, can be isolated from contaminated soil [29] can degrade herbicides [30]. *Microvirga* can accumulate heavy metal [31]; be dominant in wastewater [32], and insecticide degrader [33]. *Chryseobacterium* is an organochlorine pesticides degrader [34]. The myxobacterium *Cystobacter* dominance at multi-metal contaminated acidic soil [35]. *Burkholderia* can be founded from heavy metal and pesticide contaminated soil [36], also pesticide contaminated water [37]. Furthermore, this genera is potential for remediation of hydrocarbon [38]; persistent organic pollutants (POPs) [39]; diverse groups of pesticide [40]; and poly-contaminant [41].

The heatmap was drawn in line with the abundance of information on the top 35 genera of all samples. Furthermore, it can be checked whether the samples with similar processing are clustered. The similarity and differences between samples can also be observed (Figure 4). The heatmap analysis showed that each location harbored a specific microbial community, which was unique and different.

When applied to the analysis of bacterial community diversity within a sample, alpha-diversity can reflect the richness and diversity of each sample's bacterial communities. OTUs, Chao, ACE, Shannon, and Simpson as well as other alpha-diversity were evaluated (Table 1). The average of OTU species was 3398. In addition, the species richness estimators ACE and Chao performed better than the OTUs that were previously reported. Both Chao and ACE show the rate of lost species in the observed samples and signifying the presence of undetermined tags. However, the coverage reported in this study was more than 98% for all samples that indicate the completeness of the sample observation in terms of species richness. The Shannon and Simpson diversity indices were estimated based on observed OTUs. These

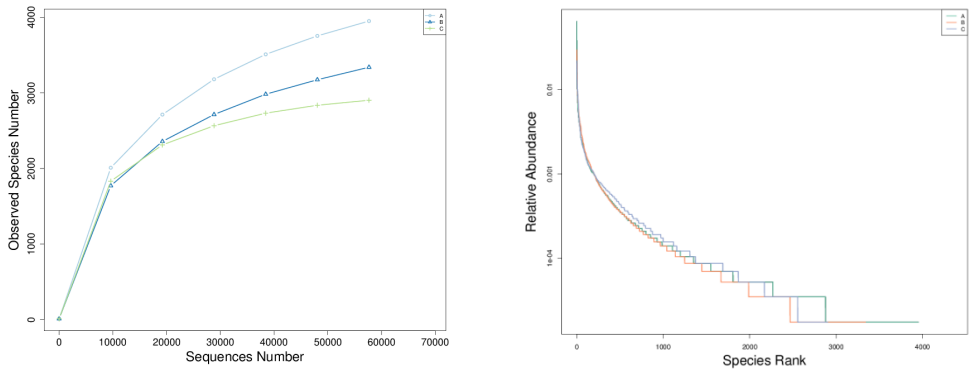


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Figure 5. Rarefaction curves and Rank abundance curves at location A, B, and C.

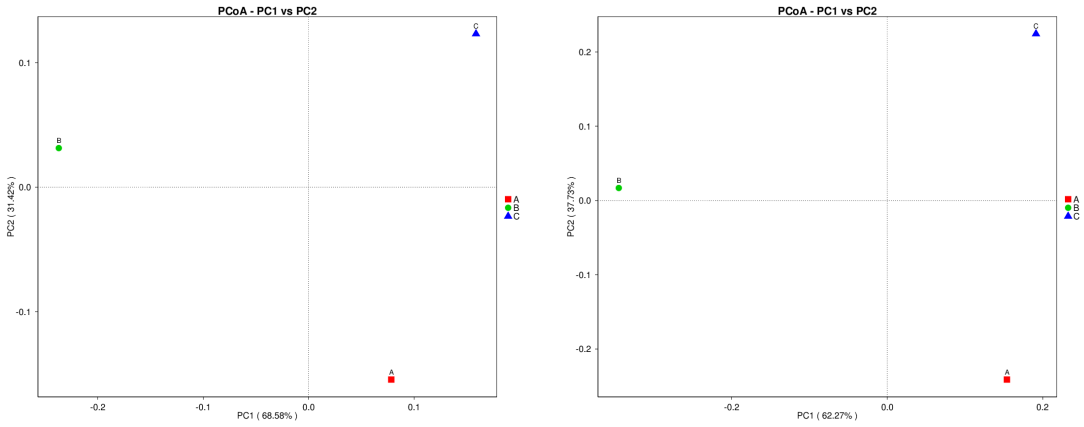

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Figure 6. PCoA based on (a) Weighted Unifrac (b) Unweighted Unifrac distance.

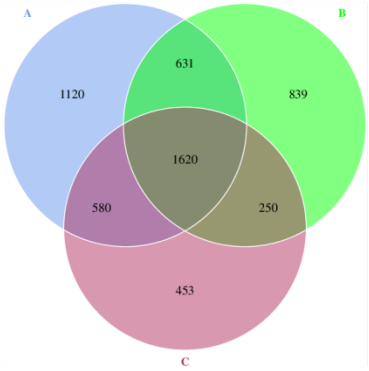


Figure 7. Venn diagram OTUs clustering from location A, B, and C

indices provide information regarding the community structure within samples. Shannon index was higher in location C, followed location A, and B, respectively. These findings showed maximum bacterial richness within three samples.

Rarefaction curves and rank abundance curves were used to assess the biodiversity of three samples. The rarefaction curves measuring of alpha-diversity indicated location A to have higher species richness compared to locations B and C, respectively (Figure 5). Principal coordinates analysis (PCoA) is an ordination technique, which picks up the main elements and structures from reduced multi-dimensional data series of eigenvalues and eigenvectors. The technique has the advantage over PCA that each ecological distance can be investigated. Weighted Unifrac and Unweighted Unifrac are calculated to assist the PCoA analysis (Figure 6). Clustering using Principal coordinate analysis (PCoA) biplot indicated that the bacterial communities at sites A, B, and C were clustered separately. Different compositions of plant and physicochemical Higher OTUs indices of bacterial communities near the beach than residence and highland sites. The non-urbanized area was overrepresented by genera of ubiquitous microbes that act in the maintenance of environments. On the contrary, the urbanized metagenome was rich in genera pathogenic to humans [42].

Following the analysis result of OTUs clustering and research requirements, the normalized table of OTU considered both the common and unique information for three samples and generating Venn diagram. The 1620 OTUs in the Venn diagram are common among all samples. Location A shares 631 with location B, and 530 OTUs with location C. Moreover, location B and C share 250 OTUs. However, there are 1220, 839, and 453 unique OTUs in location A, B, and C, respectively (Figure 7). The 16S rRNA gene is commonly employed in microbiome studies as well as in bacterial phylogenetics and species identification. The 16S rRNA gene-based phylogeny representatives of all bacterial genera from location near beach (A), residence (B), and highland (C) (Figure 8). By plotting each taxon at location A, B, and C, we are able to demonstrate that OPPs tolerance are widely dispersed across the bacterial tree of life. It was apparent that *Bacillus* was dominated at location A, B, and C.

Cluster analysis of three samples was performed with the Unweighted Pair-group Method with Arithmetic Mean (UPGMA) dendrogram. Among all samples, location B separately and location A collected close to the beach clustered more closely to C samples from the highland. The soil samples clustered together comparatively showed higher similarity (Figure 9).

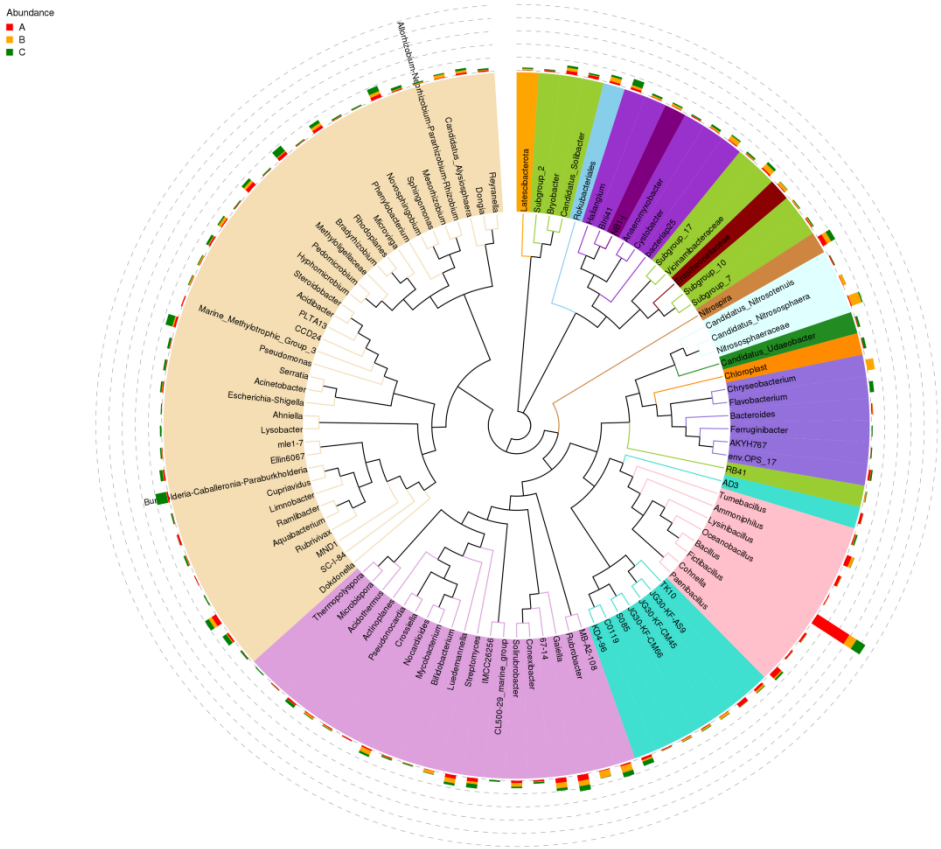
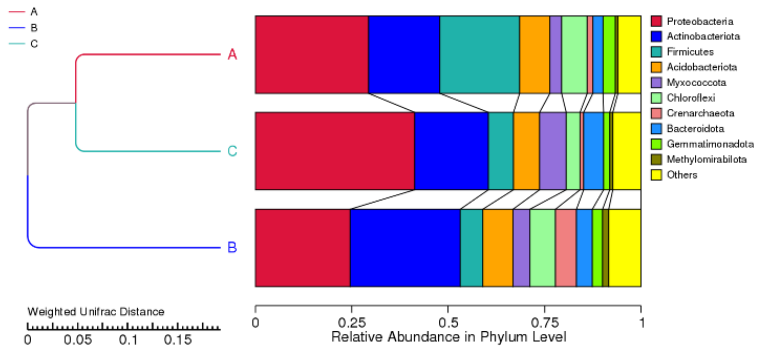


Figure 8. 16S rRNAgene-based phylogeny showing representatives of all bacterial genera in soil from location A (red bar), B (orange bar) and C (green bar).

(a)



(b)

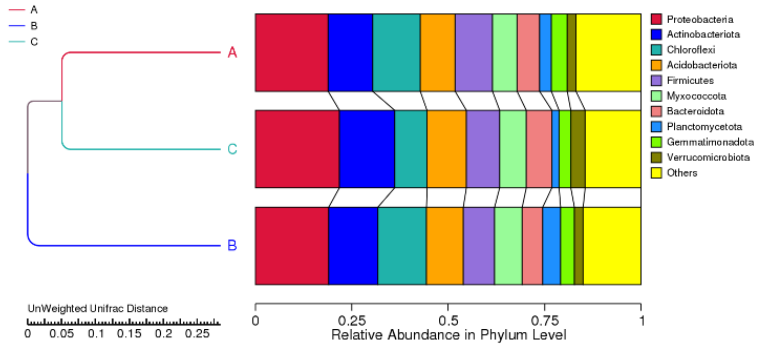


Figure 9. UPGMA cluster tree based on (a) Weighted Unifrac (b) Unweighted Unifrac distance

Table 2. Soil properties of study location

Location	Texture (%)			pH	Organic Matter (%)	Organic C (%)	Cation Exchange Capacity (me/100g)	Total N (%)	Available P (ppm)	Available K (ppm)
	Sand	Dust	Clay							
A	86.80	3.07	10.13	5.60	1.93	1.12	13.10	0.18	57.66	342
B	8.73	33.39	57.88	6.40	2.07	1.19	77.53	0.17	13.78	10
C	70.37	19.22	10.41	6.13	1.97	1.14	18.87	0.23	21.84	2266

Soil physicochemical properties

The physiochemical soil properties are listed in Table 2. The sand was dominated at location A and C. Meanwhile, location B was dominated by clay. Soil pH varied between 5.60 and 6.40 in three location. It was observed higher in Organic Matter (OM), Organic Carbon (OC), and Cation Exchange Capacity (CEC) at location B. Nevertheless, at location C, Total Nitrogen (TN) and Available Potassium (AK) higher than location A and B. Available Phosphat (AP) at location A larger than at location C and B, respectively.

The environmental conditions, physicochemical properties of soils, the nature of pesticide, and other factors have an impact on the degradation kinetics of pesticides, which is closely linked to their persistence in soils. Moreover, higher organic matter content (OMC) in soils could increase the number of microorganisms. The chemical degradation of OMC and the degradation activities of microbial enzyme were both affected by soil pH. It is suggested that pH is one of the best predictors in determining the composition of bacterial communities universally. It was found that both edaphic components and geochemical properties are important in structuring bacterial community composition [43]. Usually, conditions with a neutral pH make it easier for microorganisms to grow and keep their activities going. Because the pH of the soil is 7.1, which encourages bacterial growth, pesticides are degraded more quickly than in other soils.

The number of microorganisms, for instance, may rise in soils with a higher OMC content. OMC has the greatest impact on molecular pesticides mobility followed by the clay content. The greater clay content of the soil generally indicates less sandy content, a lower proportion of large pores, a larger specific surface area per volume of soil, and a higher affinity in adsorption of soils for pesticides, thus resulting in lesser mobility of pesticides. There were complicated dynamics of the residues between sediments and water, reflecting the complex interactions [44]. Therefore, the improved pesticides concentration degradation is mainly due to the increased activity of microorganism [45]. When stressing on more recent studies, as shown how different properties of the amendment or soil conditions can affect the microbial community.

Cation exchange capacity (CEC) is one of the most important concepts in soil fertility, and it is an important method to measure the number of cations that can be retained on surfaces of soil. The natural environment is composed of a large number and diversity of microorganisms with many physiological properties and different tolerance to pollutants [46]. It was found that the contents of ammonium, nitrate, and phosphorus represented the highest loading values in the ordination based on the distance between metagenomes, eventhough the edaphic variables that were measured can bot be used to explain the variation in the microbial composition [47].

Organophosphate pestisides residues

OPPs residues i.e azinphos methyl, chlorphyriphos, chlorphyriphos methyl, diazinon, dichlorvos, dimethoate, disulfoton, etrimfos, fenamiphos, fenitrotion, fenthion, malathion, methacrifros, methyl pirimiphos, methidathion, monotocrotophos, phosalon, phosphamidon, and profenofos were tested. The result showed there is no OPPs residues were detected by LC-MS/MS. No residues were detected in all soil samples means that the residual content of OPPs residues in all soil samples is below the detection threshold of the tool. This can also be caused by place sampling be caused by the active ingredient of the pesticide that is no longer present in 20 cm depth of soil. Climate change, exposure time, and OPPs concentration significantly impact the evaluation of OPPs residue. As a result, this demonstrates that various sensors have been developed to detect pesticides, but their detection limit and linear range limitations make them imperfect. Additionally, the high cost of some of the sensors makes it difficult for anyone ant anywhere. Furthermore, more research is required to enhance the pesticide detection sensor [48]. The impact of water flow on the fate of the pesticide and soil

microorganisms in soil have been effected pesticide existance [49]. Based on the number of chemicals present in the environment and other variables such as pH, temperature, and nutrients, much more effort to understand multiple stressors are needed [50].

CONCLUSION

Proteobacteria, Actinobacteria, and Firmicutes were found to be the main composition of the bacterial community from agricultural fields exposed to organophosphate pesticides. In addition, the samples contain high relative abundance of *Bacillus*, *Bradyrhizobium*, *Chryseobacterium*, *Cystobacter*, *Microvirga*, and *Burkholderia*. We also demonstrated that bacterial community structure correlates with the physicochemical soil characteristics i.e. texture, pH, organic matter, organic carbon, nitrogen total, cation exchange capacity, available phosphorus, and available potassium. This finding is important as a foundation for developing organophosphate pesticides-contaminated sites bioremediation systems by using unculturable bacteria which may be applied to various conditions.

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