Effect of Inorganic Fertilizer Application on Soil Microbial Diversity in an Oil Palm Plantation

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Excessive fertilizer applications in oil palm plantations are conventionally done to increase the oil yield, but they result in high production cost and environmental pollution. There have been only separate reports on the effects of fertilizer application on soil physical, chemical characteristics, and microbial biodiversity. Therefore, this study was conducted to determine the correlation between soil characteristics and soil microbial biodiversity in oil palm plantation after long-term frequent chemical fertilizer application compared with secondary soil, using molecular methods of polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and MiSeq. Secondary forest soil was chosen as the control. The results showed that after 25 years of fertilizer application, the total nitrogen and organic carbon contents decreased from low to very low scale, indicating soil infertility condition. Reduction of *Firmicutes* was related to suppression of soil borne diseases, and *Bacteroidetes* which is an indicator of soil health were both almost eliminated after 25 years of fertilizer application. In conclusion, long-term inorganic fertilizer application reduced the soil nitrogen, and organic carbon, altered beneficial microbes in the soil.

Keywords: Firmicutes; Bacteroidetes; Inorganic fertilizer application; Oil palm plantation; Soil microbial biodiversity

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INTRODUCTION

The second largest area of oil palm plantation in the world is in Malaysia. In 2010, roughly 5.2 Mha or 16% of the country's total land area was occupied by oil palm plantation (Gunarso *et al.* 2013). Almost 60% of the total forests turned to oil palm plantations were primary forest in Malaysia (Wicke *et al.* 2011). The limited areas available for oil palm plantation in Malaysia have contributed to the widespread use of a cropping method in the cultivation of palm oil. The impacts of the restricted land region and numerous other influences have induced uninterrupted agriculture such as soil physicochemical degradation and altered microbial profiles. Several of studies have documented the depletion effect of microbial populations. However, the connection between soil productivity and microbial diversity remains uncertain (Berry *et al.* 2010). Frequently agricultural activities lead to changes on soil properties (top soil and soil compaction) and at the same time reduction in biodiversity (soil microbes). Soil

productivity and soil microbes are sensitive to the changes in the soil (Agnieszka *et al.* 2012).

Some studies have shown that the microbial community profile and structure are key determinants of soil health, and these can be affected by various agricultural management practices including frequent fertilizer applications, pesticides, crop rotation, tillage, and use of machinery (Dorr *et al.* 2012). Long-term continuous cropping contributes to inhibition of plant development and severe soil-borne diseases (Yang *et al.* 2012; Liu *et al.* 2014). Various factors have added impediments towards the induction of continuous cropping, including physicochemical degradation of soil characteristics and the build-up of soil-borne pathogens (Fuentes *et al.* 2009; Huang *et al.* 2013). Bacteria is the most diverse and abundant class of soil microorganisms that act as indicators of plant health (Gans *et al.* 2005). Bacteria play key roles as soil-borne pathogens and biocontrol agents. Laborious and costly methods were used previously to determine the dominant soil microbial groups (Roesch *et al.* 2007). Recently, next-generation sequencing (NGS) technology, such as the Illumina Miseq sequencing method, have been used, and these offer an unparalleled potential for fast efficiency and greater visibility in soil bacterial population and profile.

In this study, four replications of soil from an oil palm plantation at the first cycle of a 25-year planting history in the south of Peninsular Malaysia were compared with secondary forest soil within two depth of soil (0 to 15 cm and 15 to 30 cm). The effects of inorganic fertilizer used for continuous-cropping on soil physiochemical characteristics and soil microbial diversity were studied. The objectives were to study the correlation between soil physiochemical properties and soil health to elucidate changes in soil bacterial communities of oil palm plantation under the continuous-cropping system for one plantation cycle.

EXPERIMENTAL

Materials

The soil samples were collected (Fig. 1) for field sites of oil palm plantation and secondary forest at Serting Hilir Negeri Sembilan. Field sites were situated in the enrichment planting area (Latitude 2.935113°, Longitude 102.464371°). The soil series was named as Kechor Soil Series and categorized under Typic Kandiudults according to Paramananthan (2020). This soil was developed over non-accreting sub-recent alluvium (T2) with fine, kaolinitic, isohyperthermic, and yellow in colour. It has not more than 30% silt content, and the drainage class was categorised as well draining. Generally, the cation exchange capacity (CEC) was not more than 16 cmol(+) kg^{-1} clay. Clay decreased by more than 20% from maximum within 100 cm from soil surface. The comparative area was categorized as Secondary Forest (Latitude 2.938261°, Longitude 102.461535°). This area was selected to represent non-disturbed soil that had not been subjected to oil cultivation activity. In fact, this area can be considered as riparian reserves or riverine buffer zone, since by law no activity or human interference should take place within 10 meters from a river on the right or left side (Barclay *et al.* 2017). The downside of this point is that there are no established soil maps to refer. However, the distance is only 471 meters from the test plot, and the structure of the soil was similar to that of the trial site. No other option was available, since all the area has been developed either for oil palm cultivation or housing for people. Urea, rock phosphate, muriate of potash, and kieserite types of fertilizer have been used at field sites of oil palm plantation. The amount of fertilizer used is generally different between immature and mature oil palm trees, with four applications per year. Climatic conditions were in the dry area with rain accumulation of 1500 to 1800 mm per year.

Soil samples were collected in January 2014 after running the field experiment planted for 25 years with oil palm. The sampling times were chosen to ensure that there were minimal effects of the most recent fertilizer schedule from the FELDA plantation on the secondary forest at Jalan Kampung Lui (Selatan Serting Hilir, Negeri Sembilan).

Experimental design and soil sampling

The experiment at each site was designed using a randomized complete block design (RCBD) with four times treatment and four blocks. Soil samples from each of the four plots were collected from 0 to 15 cm and 15 to 30 cm depth of soil by taking 16 sub-samples with an auger and subsequently pooling the subsamples from each plot. The treatment plots were labelled as Plot 1 (P1), Plot 2 (P2), Plot 3 (P3), Plot 4 (P4), fertilized area (FA), and unfertilized area (UFA) and with the secondary forest as a control. The soil was spread on a table overnight to dry at room temperature and was sieved through a 5 mm sieve prior to storage at -20 °C in a freezer.

Soil properties and nutrients

The pH was determined using a pH meter (HANNA HI9812). About 20 g of soil sample was mixed with 50 mL distilled water and left overnight prior to soil pH determination (MS, 1980). The total nitrogen and phosphorus contents of the soil samples were determined using a Block-Digester and Auto-Analyser (CNS Analyser, LECO Model – Based on Dumas) according to the SIRIM standard method for soil chemical analysis (MS, 1980). The total organic carbon content of the soil was determined using the Walkely and Black titration method (Gelman *et al.* 2012). The cation exchange capacity (CEC) was determined by leaching the soil samples with ammonium acetate using the colorimetric method (Mehlich 1980). The analysis of soil exchangeable cation (K, Ca, Mg,) was conducted by leaching the soil samples using 1 M ammonium acetate pH 7.

Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis

Total metagenomic DNA was extracted from 10 g soil using a MoBio Power soil DNA isolation kit (MoBio Laboraries Inc., Carlsbad, Ca, USA) following the manufacturer's protocol. Total DNA quality and concentration were measured by the Nano Drop ND-2000 spectrophotometric method (Nano Drop Technologies, Thermo Scientific, USA) and by 1% gel electrophoresis. The PCR was carried out by amplifying the variable region (V3 region) of the 16S rDNA using forward and reverse primers. The PCR amplifications were performed in 50 μL containing 1.0 μL template DNA, 25 μL Ex-Taq DNA polymerase (Takara Shuzo, Japan), 20 μL ultrapure water (Millipore), and 2 μL of each primer: 357F-with a 40-bp GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3') and 518R (5'- ATT ACC GCG GCT GCT GG-3'). The PCR amplification was done following the method of Zainudin *et al*. (2014). The DGGE was performed by loading the PCR product into the well of a 1.5-mm-thick vertical denaturing 8% acrylamide gel with a gradient from 30% to 60%. One hundred percent of the denaturant corresponded to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed at 200 V at 60 °C for 5 h. After electrophoresis, the gel was stained with gelred (1 mg/L) and viewed with the Gel Doc XR+ System (Biorad laboratories, USA). The denaturing gradient gel electrophoresis (DGGE) band from each lane was excised from the gel with a sterile pipette tip.

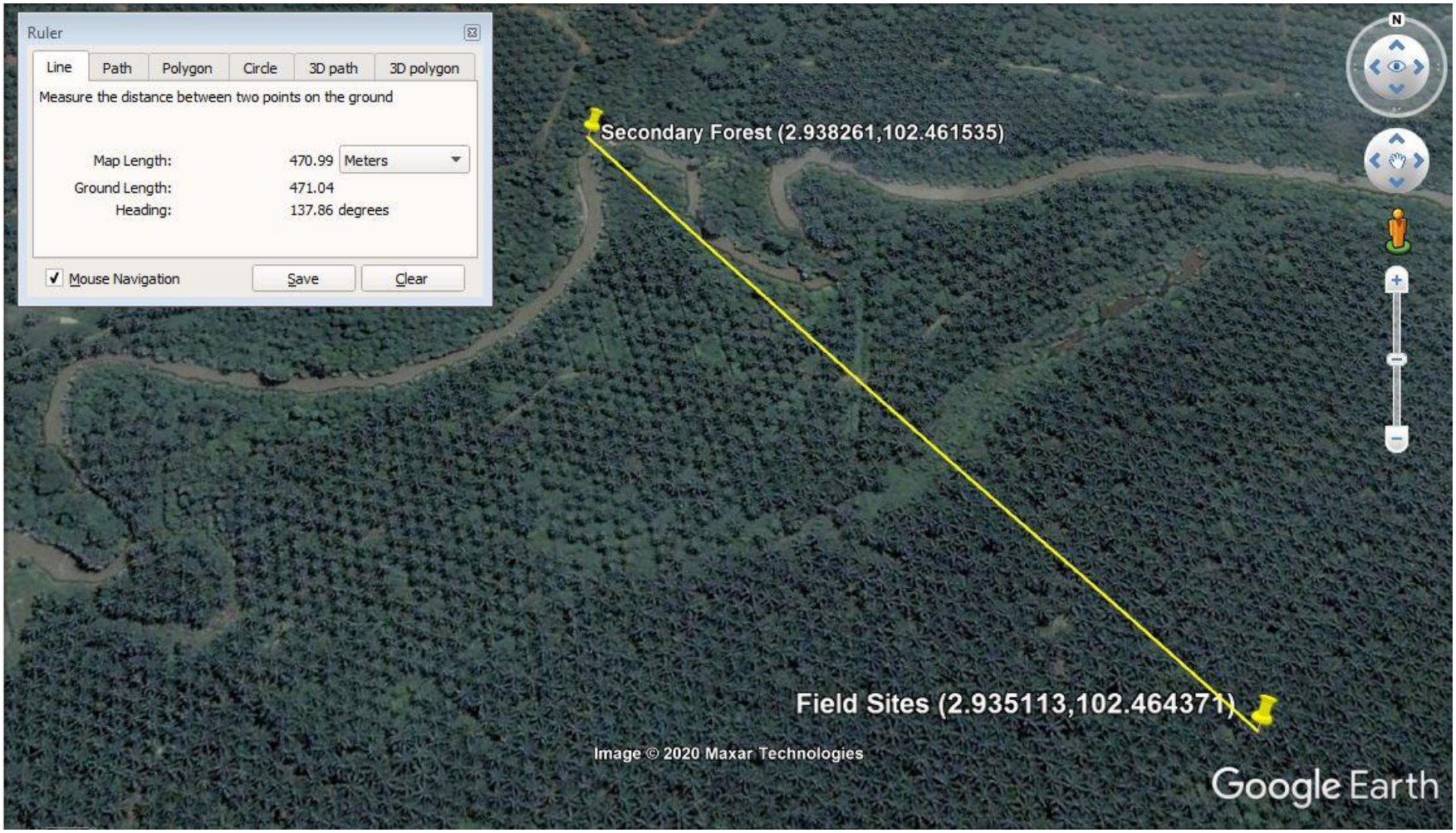

Fig. 1. Visualization of field sites of oil palm plantation and secondary forest using Google Earth

Each of the excised gel was transferred into a 1.5 mL tube containing 50 μL tris-EDTA buffer (pH 8.0). The tube was then incubated overnight to elute the DNA from the gel. The eluted DNA fragment was amplified.

High-throughput 16S rRNA gene sequencing

The extracted DNA used primers V4-V5 region, forward primer 515F (5′-GTGCCAGCMGCCGCGG-3′) and reverse primer 907R (5′CCGTCAATTCMTTT-RAGTTT-3′). The 25 μL of PCR reaction was comprised of 10x Taq buffer, Taq polymerase (Bio Labs), 20 μM of forward and reverse primer, 2 mM dNTP, 25 mM $MgSO_4$ (Toyobo), and 50 ng of cDNA template. The amplification of the DNA sample was done using an initial denaturation temperature at 94 °C for 3 min, followed by 35 cycles consisting of denaturation at 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. Following this, the amplicons were purified using Macherey-Nagel, (Duren, Germany). Once the concentrations of the purified PCR products were determined using a Qubit dsDNA HS Assay Kit (Life Technologies, Oregon, (USA), they were processed using Nextera XT DNA library preparation kit according to the Illumina protocol, followed by the sequencing process using the MiSeq sequencing system (Illumina, San Diego, CA). The high-throughput MiSeq data were processed and analysed using QIIME v.1.9.0. (Caporaso *et al.* 2010). The raw paired-end reads were assembled using the PANDA seq tool, followed by a trimming process to remove low quality and ambiguous reads. The high-quality reads were clustered into operational taxonomic units (OTUs) with 97% sequence similarity using the *de novo* OTU picking pipeline. The UCLUST v1.2.22q (DeSantis *et al.* 2006) software was used to classify each representative sequence prior to aligning the sequences against the Green genes database v13.8 using the PyNAST program (DeSantis *et al.* 2006; Caporaso *et al.* 2010). The rarefied OTU tables for the alpha diversity measurement, and rarefaction curves by Shannon diversity metric were used. The beta diversity was analysed by principal coordinate analysis (PCoA) and cJackknife beta-diversity with UPGMA for cluster.

Statistical analyses

The data for soil properties and nutrient depletion were analyzed by using analysis of variance (ANOVA) with the aid of the SAS software windows version 9.1 (SAS, 2007). Turkey analysis at $p \leq 0.05$ was used to test for significant differences between the treatments. The Tukey's honestly significant difference test for all pairwise comparisons were calculated after ANOVA to compare the treatment means. Statistical analysis of the DGGE profiles using their gel images that were converted, normalized, and digitized using BLAST searches. The neighbour-joining method (Saitou and Nei 1987), evolutionary distance using maximum composite likelihood (Tamura *et al.* 2004), and evolutionary were analysed in MEGA X (Kumar *et al.* 2018). QIIME analysis versionV1.9.0, using OUT clustering for alpha and beta diversity (parallel uclust_ref method), and standard UCLUST used *de novo* OUT at the 97% similarity level. Alpha diversity estimated with Chaol and ACE indexes, and diversity Shannon. Principal coordinate analysis (PCoA) (Gower 1966) were used to determine the relationship between bacterial taxa within phylum and the sampling area.

RESULTS AND DISCUSSION

The effects of inorganic fertilizer after 25 years of application on an oil palm plantation were investigated. Table 1 shows that the pH, organic carbon, and cation exchange capacity (CEC) were significantly different between plots at $p < 0.05$.

Samples at plots P1, P2, P3, and P4 with two different depths 0 to 15 cm and 15 to 30 cm at the fertilized and unfertilized areas had the same pH value of 4.5 reduction. However, the pH was 4.6 at the secondary forest soil, with a reduction of only 0.1. Previous research at Sarawak physiochemical properties between planted and non-planted (secondary forest) in oil palm plantation showed the same result with respect to pH (Hamzah *et al.* 2009). The value of organic carbon can be reduced by long-term cropping (Pan *et al.* 2011). Soil organic carbon is the main component in soil organic matter as indicator for soil health (Johns 2017). The CEC and the electrical charge of some of the soil components that contributed to the CEC were affected by the pH of the soil. The results of the CEC on Table 1 show significant increase when the pH was decreased. The secondary forest soil value was 3.50 compared with those of P1 to P4 with different depths whose values were about 4.75 until 5.83. The CEC refers to the amount of negative charges available on the surface of the soil particles and indicated the potential of the soil to hold plant nutrients. Therefore, the CEC of the soil was directly affected by the amount and frequency of fertilizer application.

Macronutrient elements included N, P, K, calcium (Ca), magnesium (Mg), and sulphur (S), and micronutrient elements included copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) (Dhaliwal *et al.* 2019). Furthermore, the macronutrients after 25 years of inorganic fertilizer being applied showed significant difference at $p \leq 0.05$. Table 3 showed decreases after 25 years' application of inorganic fertilizer from total nitrogen, total phosphorus, and potassium. The total nitrogen content was 0.12% at the secondary forest, and it decreased between 0.03% and 0.05% after 25-years inorganic fertilizer application. Classification of soil nutrient status for oil palm by Goh (1997) is total nitrogen and organic carbon drop from low in oil secondary forest to very low after 25-years inorganic fertilizer application. Data indicated low total nitrogen because of plant uptake and leaching (Arora and Juo 1982; Cobo *et al.* 2002). Factors contributing to the losses of organic carbon may include land clearing and subsequent crop cultivation. An important finding regarding nitrogen between secondary forest soil and after 25 years inorganic fertilizer application involved nitrogen stored in organic matter. Soil organic content is prominent in the forest because N immobilization by trees (Watanabe *et al.* 2015). Macro and micro-nutrient elements increased after 25-years application of inorganic fertilizer such as CEC, available P, calcium, magnesium, manganese, and iron. The CEC is an indicator nutrient adsorption in organo-mineral complexes, in particular preservation by electrostatic basic of cation such as K, Mg, Ca and Na (Zech *et al*. 1997). Table 3 shows that the micronutrients boron (B), iron (F), manganese (Mn), zinc (Z), and molybdenum $(Mo)^{+}$ contents were significantly different only between plots.

In order to investigate the effects of inorganic fertilizer to the soil structure, the particle size distributions of various samples were studied (Table 4). The results showed that the soil properties (course sand and fine sand) in terms of particle size distributions were significantly different within plots and in the secondary forest soil. Soil structure disruption on cropland was observed compare secondary forest because useful of mechanical machine during land preparation and harvesting process (Watanabe *et al.* 2015).

The DGGE profiles for the plots after 25 years of inorganic fertilizer applications and forest soil are shown in Fig. 2. Figure 2A shows first DGGE with different sample between fertilizer areas, unfertilized areas, and secondary forest with two different depths with labelled a, b, c, d, e, and f. Figure 2B shows the second DGGE to reconfirm the band from first DGGE. Data clearly showed that sample secondary forest at 0 to 15 cm labelled as 'e' had eight thick bands (1, 2, 3, 4, 5, 6, 7 and 8).

Table 1. Soil Properties Composition between Secondary Forest Soil and Soil at Oil Palm Plantation after 25 Years of Inorganic Fertilizer Application in Malaysia

Plot	Area	Depth (cm)	pH (1:2.5)	Org C (%)	CEC (%)
P1	Fertilizer area	0-15	4.48±0.01b	1.11±0.24abc	5.99±0.40a
	Fertilizer area	15-30	4.48±0.05b	0.87±0.40bc	5.43±1.04ab
	Unfertilized area	0-15	4.46±0.05b	0.71±0.17bc	5.76±0.24a
	Unfertilized area	15-30	4.46±0.05b	0.92±0.32bc	5.91±0.31ab
P2	Fertilizer area	0-15	4.54±0.01ab	0.64±0.25c	5.07±0.66a
	Fertilizer area	15-30	4.54±0.01ab	0.62±0.21c	5.14±0.50ab
	Unfertilized area	0-15	4.51±0.05b	1.15±0.75bc	5.61±0.67a
	Unfertilized area	15-30	4.51±0.05b	0.84±0.42bc	5.25±0.65ab
P3	Fertilizer area	0-15	4.48±0.01b	1.13±0.37c	4.80±0.82a
	Fertilizer area	15-30	4.48±0.01b	0.67±0.28c	4.70±0.28ab
	Unfertilized area	0-15	4.46±0.01b	1.19±0.27c	5.43±0.66a
	Unfertilized area	15-30	4.40±0.05b	0.90±0.35c	5.34±0.30ab
P4	Fertilizer area	0-15	4.43±0.06b	1.07±0.54c	5.14±0.40a
	Fertilizer area	15-30	4.41±0.05b	0.63±0.25c	4.91±0.46ab
	Unfertilized area	0-15	4.46±0.05b	0.95±0.05c	5.71±0.24a
	Unfertilized area	15-30	4.46±0.05b	0.95±0.39c	5.31±0.49ab
Soil Forest		0-15	4.70±0.10a	2.30±0.44ab	3.57±0.87c
		15-30	4.58±0.10ab	2.74±0.74a	4.22±1.11c
Treatment			***	***	***
Depth			NSD	NSD	NSD
Treatment*Depth			NSD	NSD	NSD

NSD: Not significantly different statistically not significantly at p ≥0.05; ***: statistically highly significant at p ≤0.001; **: statistically significant at p ≤0.01; *: statistically significant at p ≤0.05; (n:4)

Table 2. Soil Macronutrients Composition between Secondary Forest Soil and Soil at Oil Palm Plantation after 25 Years of Inorganic Fertilizer Application in Malaysia

Plot	Area	Depth (cm)	Total N	P (mg/kg)		K	Ca	Mg
			(%)	Total	Avail	Exch cmol(+)/kg		
P1	Fertilizer area	0-15	0.06±0.03ab	217.63±59.90a	5.72±0.40ab	0.11±0.01b	1.12±0.13ab	0.31±0.08a
	Fertilizer area	15-30	0.07±0.03ab	179.38±21.10a	5.83±1.04a	0.12±0.04b	1.00±0.11ab	0.27±0.07a
	Unfertilized area	0-15	0.06±0.02ab	154.13±42.80a	5.72±0.24ab	0.11b±0.01b	1.00±0.18ab	0.21±0.12a
	Unfertilized area	15-30	0.06±0.02ab	189.88±49.43a	5.83±0.31a	0.12±0.01b	1.10± 0.18ab	0.30±0.13a
P2	Fertilizer area	0-15	0.04±0.02ab	85.13±43.11a	5.72±0.66ab	0.11±0.04b	1.14±0.04ab	0.33±0.07a
	Fertilizer area	15-30	0.05±0.02ab	85.13±41.48a	5.83±0.50a	0.12±0.02b	1.10±0.06ab	0.30±0.06a
	Unfertilized area	0-15	0.04±0.02ab	153.88±78.37a	5.72±067ab	0.11±0.03b	1.22±0.40ab	0.55±0.34a
	Unfertilized area	15-30	0.04±0.02ab	117.13±85.95a	5.83±0.65a	0.12±.02b	1.44±0.12b	0.39±0.11a
P3	Fertilizer area	0-15	0.03±0.02b	137.13 ±22.84a	5.72±0.83ab	0.11±0.03b	1.20±0.11ab	0.40±0.17a
	Fertilizer area	15-30	0.02±0.01b	81.38±49.86a	5.83±0.28a	0.12±0.05b	1.00± 0.09ab	0.27±0.08a
	Unfertilized area	0-15	0.05±0.03ab	128.13±77.09a	5.72±0.66ab	0.11±0.05b	1.20±0.22ab	0.40±0.16a
	Unfertilized area	15-30	0.04±0.02ab	123.13±65.66a	5.83±0.30a	0.12±0.04b	1.20±0.19ab	0.32±0.15a
P4	Fertilizer area	0-15	0.03±0.01a	141.88±41.98a	5.72±0.40ab	0.11±0.02b	1.20±0.20ab	0.38±0.18a
	Fertilizer area	15-30	0.03±0.01b	73.13±13.72a	5.83±0.46a	0.12±0.02b	1.00±0.12ab	0.24±0.11a
	Unfertilized area	0-15	0.06±0.04ab	117.88±37.26a	5.72±0.24ab	0.11±0.03b	1.20±0.17ab	0.31±0.03a
	Unfertilized area	15-30	0.02±0.02b	107.13±42.59a	5.83±0.49a	0.12±0.03b	1.11±0.20ab	0.3±0.19a
Soil Forest		0-15	0.12±0.03a	136.00 ±24.74a	3.13±0.50c	0.28±0.02a	1.00±0.20ab	0.23±0.04a
		15-30	0.12±0.03a	157.75±60.57a	3.13±1.73c	0.28±0.02a	0.62±0.06b	0.21±0.04a
Treatment			***	NSD	***	***	**	NSD
Depth			NSD	NSD	NSD	NSD	*	NSD
Treatment*Depth			NSD	NSD	NSD	NSD	NSD	NSD

NSD: Not significantly different statistically not significantly at p ≥0.05; ***: statistically highly significant at p ≤0.001; **: statistically significant at p ≤0.01; *: statistically significant at p ≤0.05; (n:4)

Table 3. Soil Micronutrients Composition between Secondary Forest Soil and Soil at Oil Palm Plantation After 25 Years Inorganic Fertilizer Application in Malaysia

Plot	Area	Depth (cm)	B (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Zn (mg/kg)	Mo (mg/L)
P1	Fertilizer area	0-15	1.83±0.23abc	182.28±11.26a	953.08±139.10a	1.96±0.07ab	1.95±2.41a
	Fertilizer area	15-30	1.27±0.40c	175.92±13.51a	789.42±227.84abc	1.93±0.13ab	1.31±2.61b
	Unfertilized area	0-15	1.62±0.49abc	179.25±7.25a	814.94±59.21abc	1.92±0.02ab	1.63±1.04b
	Unfertilized area	15-30	1.67±0.17abc	173.18±5.29a	856.63±105.18ab	1.95±0.05ab	2.50±1.44b
P2	Fertilizer area	0-15	1.66±0.18abc	195.70±13.74a	655.64±103.21abc	1.65±0.03ab	1.73±1.21b
	Fertilizer area	15-30	1.64±0.13abc	199.43±24.37a	647.55±97.12abc	1.61±0.12b	2.07±1.44b
	Unfertilized area	0-15	2.00±0.45abc	180.85±28.11a	899.84±357.52a	1.76±0.16ab	2.29±2.00b
	Unfertilized area	15-30	1.60±0.30bc	202.17±31.09a	615.95±179.10abc	1.59±0.12b	0.95±1.89b
P3	Fertilizer area	0-15	1.55±0.19bc	180.91±8.01a	844.73±144.54ab	1.79±0.07ab	1.20±1.97b
	Fertilizer area	15-30	1.31±0.20b	186.79±31.92a	618.47±124.84abc	1.69±0.08ab	2.46±1.99b
	Unfertilized area	0-15	1.61±0.15bc	169.63±45.87a	821.21±69.08abc	1.76±0.20ab	1.01±1.26b
	Unfertilized area	15-30	1.45±0.14c	190.98±37.73a	725.10±89.79abc	1.75±0.20ab	1.90±2.11b
P4	Fertilizer area	0-15	1.54±0.31bc	185.76±47.73a	782.10±224.38abc	1.85±0.16ab	3.18±2.67ab
	Fertilizer area	15-30	1.50±0.30b	166.63±38.40ab	619.47±116.10abc	1.75±0.06ab	3.07±2.41b
	Unfertilized area	0-15	1.65±0.17abc	185.11±24.84a	873.86±92.60a	1.82±0.05ab	1.84±2.21b
	Unfertilized area	15-30	1.22±0.25c	176.90±22.38a	797.43±120.80abc	1.79±0.05ab	1.30±1.53b
Soil Forest		0-15	2.43±0.26a	70.07±27.60bc	290.75±70.87c	1.84±0.18ab	5.02±0.82a
		15-30	2.43±0.24a	49.02±22.34c	332.08±55.51bc	2.10±0.26a	5.83±1.64a
Treatment			***	***	***	*	**
Depth			**	NSD	**	NSD	NSD
Treatment*Depth			NSD	NSD	NSD	NSD	NSD

NSD: Not significantly different statistically not significantly at p ≥0.05; ***: statistically highly significant at p ≤0.001; **: statistically significant at p ≤0.01; *: statistically significant at p ≤0.05; (n:4)

Table 4. Soil Particle-Size Distribution between Secondary Forest Soil and Soil at Oil Palm Plantation After 25 Years Inorganic Fertilizer Application in Malaysia

Plot	Area	Depth (cm)	Particle-Size Distribution			
			Clay (%)	Slit (%)	Coarse sand (%)	Fine Sand (%)
P1	Fertilizer area	0-15	48.42±0.79a	36.00±1.04ab	11.41±0.95abc	15.05±1.70b
	Fertilizer area	15-30	50.16±2.73a	36.35±2.21a	10.59±3.14ab	13.78±1.20b
	Unfertilized area	0-15	48.46±0.95a	35.68±0.70ab	11.91±1.70abc	14.83±1.20b
	Unfertilized area	15-30	48.38±0.40a	35.23±0.26abc	12.04±1.64abc	15.25±1.37b
P2	Fertilizer area	0-15	48.58±2.94a	26.93±2.06bcd	11.61±2.44abc	12.40±2.11b
	Fertilizer area	15-30	47.55±3.13a	29.63±2.73bcd	12.74±2.85ab	12.43±3.63b
	Unfertilized area	0-15	48.73±2.76a	29.33±1.10bcd	12.11±2.42abc	12.08±1.38b
	Unfertilized area	15-30	47.45±3.56a	31.23±2.91abcd	12.04±2.79abc	11.33±1.44b
P3	Fertilizer area	0-15	47.48±2.61a	21.40±3.06cd	13.34±4.27a	11.23±2.57b
	Fertilizer area	15-30	46.45±3.24a	21.48±3.76cd	14.84±2.77a	11.40±1.78b
	Unfertilized area	0-15	45.33±5.42a	20.00±4.86d	14.89±5.51a	13.25±3.18b
	Unfertilized area	15-30	45.33±4.19a	21.20±5.07d	13.94±3.82a	12.08±3.23b
P4	Fertilizer area	0-15	46.18±2.40a	21.60±4.32cd	12.29±3.16abc	11.73±4.10b
	Fertilizer area	15-30	48.20±2.00a	19.28±3.79d	12.29±2.97abc	13.68±1.98b
	Unfertilized area	0-15	45.80±2.14a	20.00±2.61d	13.06±2.97a	14.28±0.67b
	Unfertilized area	15-30	45.60±0.37a	20.00±1.36d	14.11±2.57a	10.85±7.34b
Soil Forest		0-15	43.50±0.91a	35.00±10.02abc	2.27±0.55c	28.23±2.38a
		15-30	43.80±1.39a	41.02±6.62a	2.70±0.38bc	25.70±1.48a
Treatment			NSD	***	***	***
Depth			NSD	NSD	NSD	NSD
Treatment*Depth			NSD	NSD	NSD	NSD

NSD: Not significantly different statistically not significantly at p ≥0.05; ***: statistically highly significant at p ≤0.001; **: statistically significant at p ≤0.01; *: statistically significant at p ≤0.05; (n:4)

Another sample only had thick bands on number '1' and '2'. Second DGGE as Fig. 1B reconfirms the appearance of all eight bands. Therefore, the impact of frequent fertilizer applications on soil microbial diversity compared to that of the secondary forest was clearly realized. Soil bacterial diversity differed between each of the depths (0 to 15 cm and 15 to 30 cm) of the fertilized and the unfertilized areas in the oil palm plantations, indicating that both treatments affected the microbial diversity in the soil. Detailed analysis of each sample showed that the samples were predominated by different microbial species of each phylum (Fig. 3). The variations of the predominant bacterial species in the secondary forest and also in the oil palm plantation after 25 years of inorganic fertilizer application could be due to the difference in environmental conditions and substrate characteristics, which promoted the growth of certain bacterial species. In this study, the phylogenetic relationships showed that the soils were predominated mainly by *Actinobacteria, Firmicutes*, and *Proteobacteria* phyla (Fig. 2.)

Figure 4 shows the relative abundances of the various bacterial communities of the samples. Dominant bacterial phyla at relative abundance >1% were *Acidobacteria, Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, Firmicutes*, and *Chloroflexi* for all samples. The oil palm plantation soil is mainly dominated in the order by *Acidobacteria* > *Proteobacteria* > *Actinobacteria* > *Firmicutes* > *Bacteroidetes* > *Chloroflexi* whereas the order was *Firmicutes* > *Bacteroidetes* > *Chloroflexi* > *Planctomycetes* for secondary forest. The results are comparable to the previous studies, which showed that soil in oil palm plantation is dominated mostly by all of these bacterial phyla (Lee-Cruz *et al.* 2013; Berkelmann *et al.* 2020; Tang *et al.* 2020). The results showed that long term continuous cropping decreased the *Bacteroides* and *Firmicutes* phyla of the oil palm plantation. In comparison to the secondary forest, the soil after 25 years of inorganic fertilizer applications had higher relative abundances of *Acidobacteria, Proteobacteria, and Actinobacteria* but lower abundances of *Firmicutes.* On the other hand, *Firmicutes, Bacteroidetes*, and *Chloroflexi* phyla were found to be highly abundant in the secondary forest soil. Previous study by Chaudhari *et al.* (2020) showed that high abundance of *Firmicutes* was found in the soils that are rich in organic matter, while the soils with low pH increase the *Acidobacteria* abundance. Therefore, higher abundance of *Acidobacteria* and *Chloroflexi* over *Firmicutes* could be attributed to the lower soil organic matter and low pH of soil after 25 years of inorganic fertilizer as compared to the secondary forest soil (Table 1).

The results also are in agreement to the previous study, which reported that *Acidobacteria* were highly abundance in oil palm plantation than primary forest soils and their variations were due to the low carbon content and pH of the soil (Lee-Cruz *et al.* 2013; Wood *et al.* 2017). The relative abundance of the top 20 classified bacterial genera showed notable variations between the secondary forest soil and soil after 25 years application of inorganic fertilizer (Fig. 3). Soil physicochemical properties and microbial community structure are mainly affected by different fertilizer management, as indicated by Tang *et al.* (2020), whereby the application of organic and inorganic fertilizer significantly alter the soil microbial diversity and activities of the paddy field. The three-dimensional principal coordinates analysis (Fig. 4) showed phyla *Firmicutes* (secondary forest 0 to 15 cm and 15 to 30 cm) and phyla *Acidobacteria* (fertilizer area 0 to 15 cm). Bacterial beta diversity represented using principal coordinate analysis indicated that the bacterial communities were diverse among the samples. Table 5 shows that the Archaea phylum was found to be highly abundant for both samples (0 to 15 cm and 15 to 30 cm depth levels) of the secondary forest soil. This phylum consisted of genera related to *Methanobacterium, Methanobrevibacter, Methanosphaera, Candidatus, Methanolinea, Methanosaeta, Methanomethyloyorans, Methanosarcina, Methanomassiliicocus,* and *vadin CA11*. The dominant bacteria closely related to the

phylum *Acidobacteria* were *Acidobacterium*, *Bryocella*, *Edaphobacteria*, *Telmatobacter*, *Terriglobus*, *Candidatus koribacteria*, *Geothrix*, and *Candidatus Solibacter*. The dominant bacteria related to the phylum *Actinobacteria* were *Actinotalea*, *Actinomyces*, *Actinoalloteichus*, *Actinokineospora*, *Arsenicicoccus*, *Actinomycetales*, *Actinocatenispora*, *Actinoplanes*, *Arthrobacter*, *Agromyces*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Catellatospora*, *Clavibacter*, *Cryocola*, *Curtobacterium*, and *Candidatus Microthrix*.

Figure 6 shows the heat-maps for the secondary forest and the soil after 25 years fertilizer application in the oil palm plantation. The bacterial abundance significantly decreased with increasing years of the oil palm plantation. Fierer *et al.* (2007) found that soil microbial diversity had almost been completely eradicated after decades of intense agricultural practices in the tall grass prairies of the United States. Similarly, continuous cultivation of cucumber and potatoes caused a reduction in soil bacterial community richness and diversity (Xiang *et al.* 2014). The soil microbial abundance and diversity play important roles in soil quality, function, and soil ecosystem sustainability. Hence, the loss of soil microbial abundance and diversity might be contributing to the poor growth of plants in continuous cropping systems. During the long years of inorganic fertilizer application to the soil, *Firmicutes* and *Bacteroidetes* decreased, whereas *Actinobacteria* and *Proteobacteria* were increased. A previous study by Xiang *et al.* (2014) pointed out that the *Bacteroidetes* phylum could be a very useful indicator for determining soil health in the vanilla monoculture system and in the culture of black pepper. Moreover, the relative abundance of *Firmicutes* corresponded with soil-borne disease suppression (Mendes *et al.* 2011). Phylum *Chloroflexus*, an indicator for organic carbon, decreased significantly after 25 years application of inorganic fertilizer.

In this study, the microbial community analyses using DGGE and MiSeq were used to observe the side effects of long-term applications of inorganic fertilizer on soil bacterial diversity. As reported earlier, the soil microbial community is essential for maintaining soil health and quality (Garbeva *et al.* 2006). Microbial activity can be affected by bioavailability of macro and micro-nutrients in soil. In addition, nutrient composition in soil will influence microbial activity. The DGGE profiles showed major variations of microbial community compositions in various types of soil samples. Molecular fingerprinting techniques, including PCR-DGGE analysis, have become popular for assessing diversity, structural composition, and dynamics of microbial communities (Nocker *et al.* 2007*).* Although PCR-DGGE allows the rapid assessment of the microbial community's complete structure and the identification of the dominant species, this analytical technique has some limitations. In addition, recovering DNA sequence information from an excised gel band ultimately requires cloning. Only PCR products with relatively small fragment sizes can be separated (up to 500 bp), and due to the limited success of direct sequencing from an electrophoresed gel band, cloning of amplified 16S rRNA genes is required.

Understanding the correlation between soil properties and microbial diversity is the key to sustainable productivity in oil palm plantations as a continuously cropping system in Malaysia. Application of inorganic fertilizer decreased soil pH in many studies. In this study, long-term fertilizer application in oil palm plantation only revealed a small decline of pH. The pH from secondary forest soil was reduced by only 0.1 (from 4.6 to 4.5) after 25 years of fertilizer application. Repeated applications of inorganic fertilizer decreased the soil pH (Liu *et al.* 2012). Long-term applications of inorganic fertilizer might be the factor leading to soil stress and acidification in oil palm plantations.

Table 5. Percentage of Relative Abundance on Genus of OTU Effect on Treatments

Kingdom	Phylum	Related Genus	Percentage of relative abundance					
			FA0	FA30	UFA0	UFA30	SF0	SF30
Archaea	*Euryarchaeota*	*Methanobacterium*	-	-	-	-	0.099	0.369
Archaea	*Euryarchaeota*	*Methanobrevibacter*	-	-	-	-	0.001	0.018
Archaea	*Euryarchaeota*	*Methanosphaera*	-	-	-	-	-	0.001
Archaea	*Euryarchaeota*	*CandidatusMethanoregula*	-	-	-	-	0.003	-
Archaea	*Euryarchaeota*	*Methanolinea*	-	-	-	-	0.006	0.003
Archaea	*Euryarchaeota*	*Methanospirillum*	-	-	-	-	0.009	0.01
Archaea	*Euryarchaeota*	*Methanosaeta*	-	-	-	-	0.164	0.096
Archaea	*Euryarchaeota*	*Methanomethylovorans*	-	-	-	-	-	0.006
Archaea	*Euryarchaeota*	*Methanosarcina*	-	-	-	-	0.001	0.003
Archaea	*Euryarchaeota*	*Methanomassiliicoccus*	-	-	-	-	0.004	0.017
Archaea	*Euryarchaeota*	*vadinCA11*	-	-	-	-	0.001	0.004
Bacteria	*Acidobacteria*	*Acidobacterium*	0.001	-	0.003	-	-	-
Bacteria	*Acidobacteria*	*Bryocella*	-	-	-	0.002	-	-
Bacteria	*Acidobacteria*	*Edaphobacter*	0.065	0.088	0.295	0.196	0.001	0.001
Bacteria	*Acidobacteria*	*Telmatobacter*	-	-	0.006	-	-	-
Bacteria	*Acidobacteria*	*Terriglobus*	0.009	0.007	0.031	0.035	-	-
Bacteria	*Acidobacteria*	*CandidatusKoribacter*	1.223	0.703	1.507	1.935	0.007	0.003
Bacteria	*Acidobacteria*	*Geothrix*	0.003	0.002	0.006	-	-	-
Bacteria	*Acidobacteria*	*CandidatusSolibacter*	2.511	2.529	3.629	2.664	0.016	0.025
Bacteria	*Actinobacteria*	*Actinotalea*	0.012	0.005	0.009	0.004	0	0.003
Bacteria	*Actinobacteria*	*Actinomyces*	0.103	0.035	0.057	0.028	0.001	-
Bacteria	*Actinobacteria*	*Actinoalloteichus*	0.001	-	-	0.002	-	-
Bacteria	*Actinobacteria*	*Actinokineospora*	0.001	-	0.003	-	-	-
Bacteria	*Actinobacteria*	*Arsenicicoccus*	0.001	0.005	-	0.002	-	-
Bacteria	*Actinobacteria*	*Actinomycetales*	0.001	-	-	-	-	-
Bacteria	*Actinobacteria*	*Actinocatenispora*	-	-	-	0.002	-	-
Bacteria	*Actinobacteria*	*Actinoplanes*	0.005	-	0.006	-	-	-
Bacteria	*Actinobacteria*	*Arthrobacter*	0.247	0.342	0.279	0.081	-	-
Bacteria	*Actinobacteria*	*Agromyces*	0.007	0.002	0.006	0.009	-	-
Bacteria	*Actinobacteria*	*Brevibacterium*	0.004	-	-	-	-	-
Bacteria	*Actinobacteria*	*Cellulomonas*	0.038	0.021	0.013	0.013	0.001	-
Bacteria	*Actinobacteria*	*Corynebacterium*	0.007	-	-	-	-	0.003
Bacteria	*Actinobacteria*	*Catellatospora*	0.001	-	-	0.002	-	-
Bacteria	*Actinobacteria*	*Clavibacter*	0.002	-	-	-	-	-
Bacteria	*Actinobacteria*	*Cryocola*	0.22	0.1	0.17	0.183	-	-
Bacteria	*Actinobacteria*	*Curtobacterium*	0.062	0.051	0.088	0.044	-	-
Bacteria	*Actinobacteria*	*Candidatus Microthrix*	0.001	-	-	0.002	-	-

FA0: Fertilizer areas 0-15 cm **FA30**: Fertilizer areas 15-30 cm
UFA0: Unfertilized areas 0-15 **UFA30**: Unfertilized areas 15-30 cm
SF0: Secondary forest 0-15 cm **SF30**: Secondary forest 15-30 cm

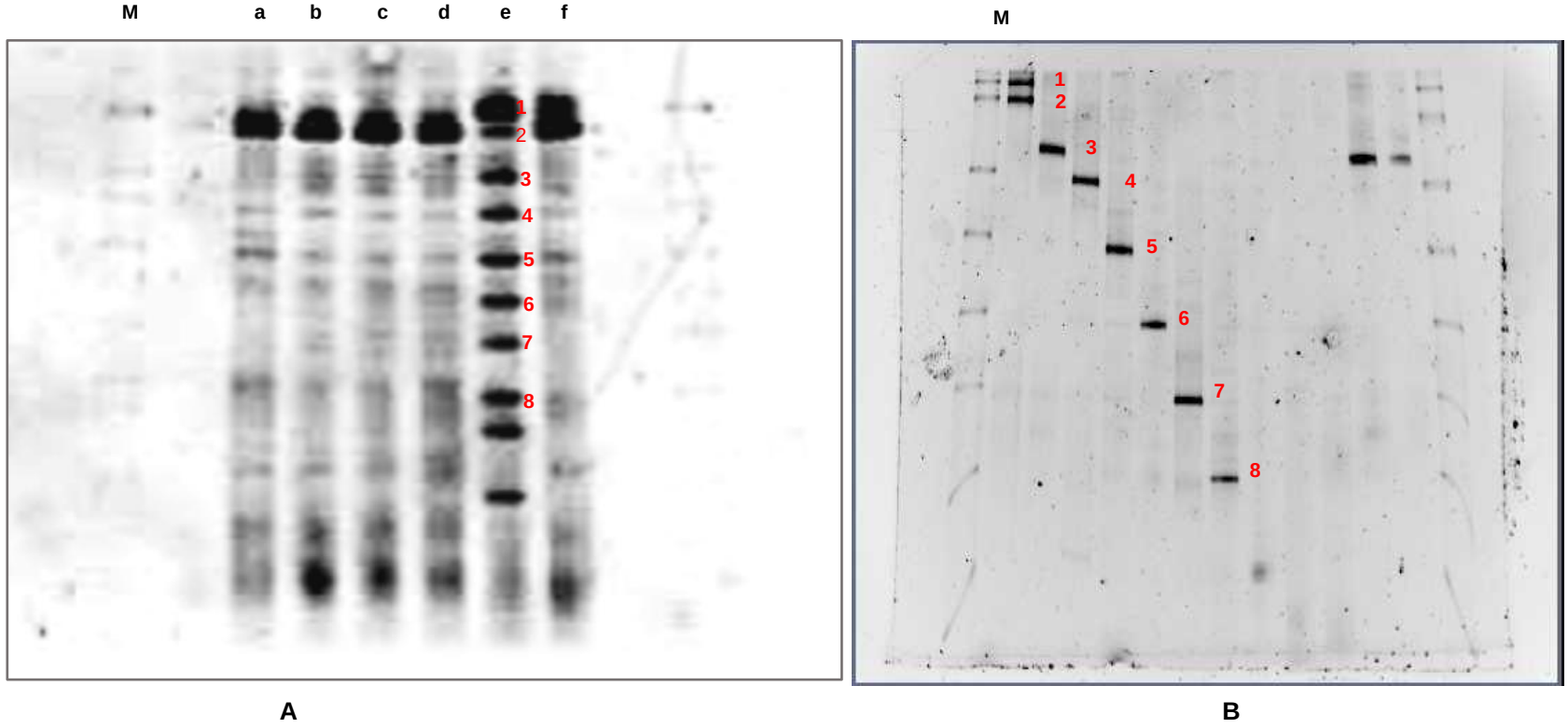

Fig. 2A. The DGGE profiles 16S rRNA genes from representative surrounding soils of the secondary forest and of the oil palm plantation after 25 years application of inorganic fertilizer; and **Fig. 2B.** Secondary DGGE of sample e. The soil samples were from two different depths, 0 - 5 cm and 15 - 30 cm. The arrow indicates the direction of the electrophoresis and the percentages of the DNA denaturant.
M: Marker; **a**: Fertilizer areas 0 to 15 cm; **b**: Fertilizer areas 15 to 30 cm; **c**: Unfertilized areas 0 to 15 **d**: Unfertilized areas 15-30 cm **e**: Secondary forest 0 to 15 cm **f**: Secondary forest 15 to 30 cm

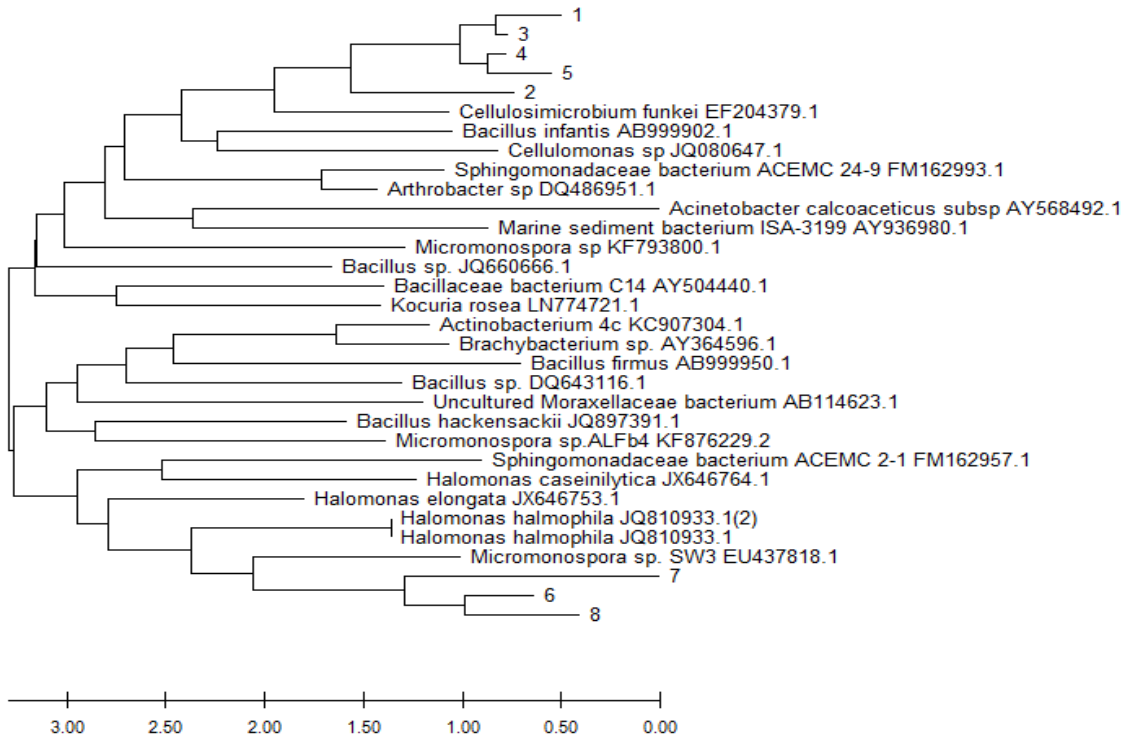

Fig. 3. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987) of the most abundant 16S rDNA sequences originating from secondary forest soil and from oil palm plantation soil after 25 years application of inorganic fertilizer to various closely related sequences obtained from BLAST searches. The optimal tree is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. This analysis involved 32 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1590 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.* 2018)

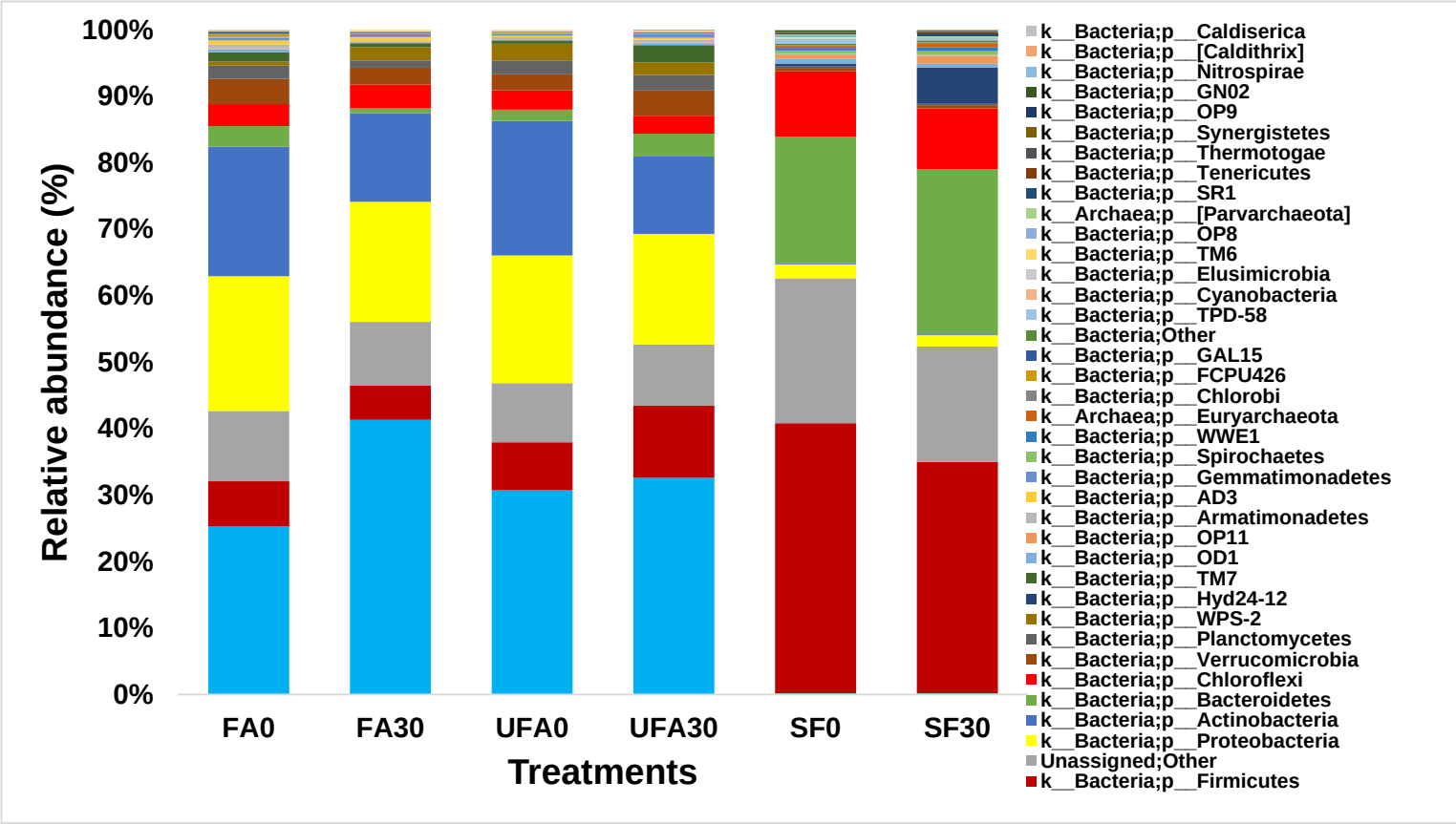

Fig. 4. Distribution of partial sequences of bacterial 16S rRNA genes from secondary forest soil and from oil palm plantation soil after 25 years application of inorganic fertilizer at the phylum level with a threshold level of 97.
FA0: Fertilizer areas 0 to 15 cm; **FA30**: Fertilizer areas 15 to 30 cm; **UFA0**: Unfertilized areas 0 to 15; **UFA30**: Unfertilized areas 15 to 30 cm; **SF0**: Secondary forest 0 to 15 cm; **SF30**: Secondary forest 15 to 30 cm

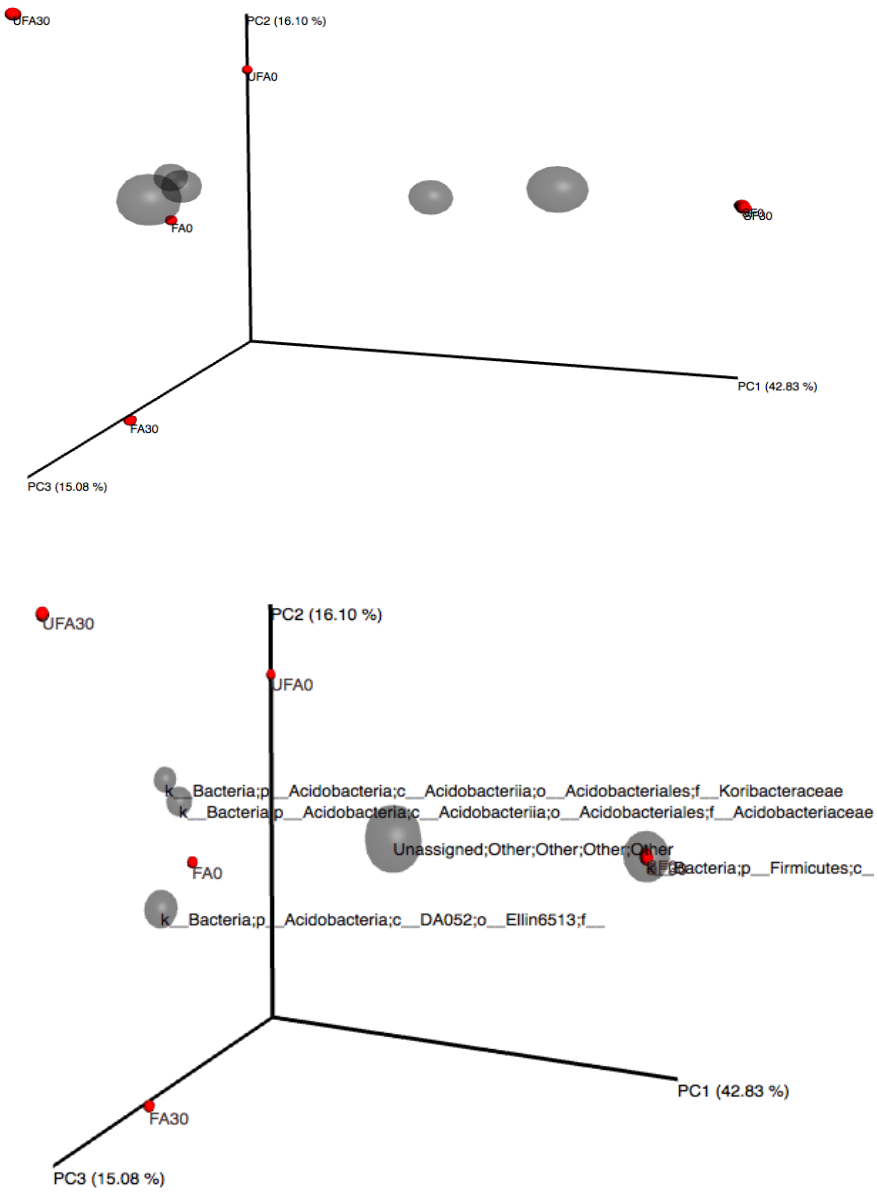

Fig. 5. Three-dimensional principal coordinates analysis plots showing the connection of datasets using the Bray-Curtis method.
FA0: Fertilizer areas 0 to 15 cm; **FA30**: Fertilizer areas 15 to 30 cm;
UFA0: Unfertilized areas 0 to 15; **UFA30**: Unfertilized areas 15 to 30 cm
SF0: Secondary forest 0 to 15 cm; **SF30**: Secondary forest 15 to 30 cm

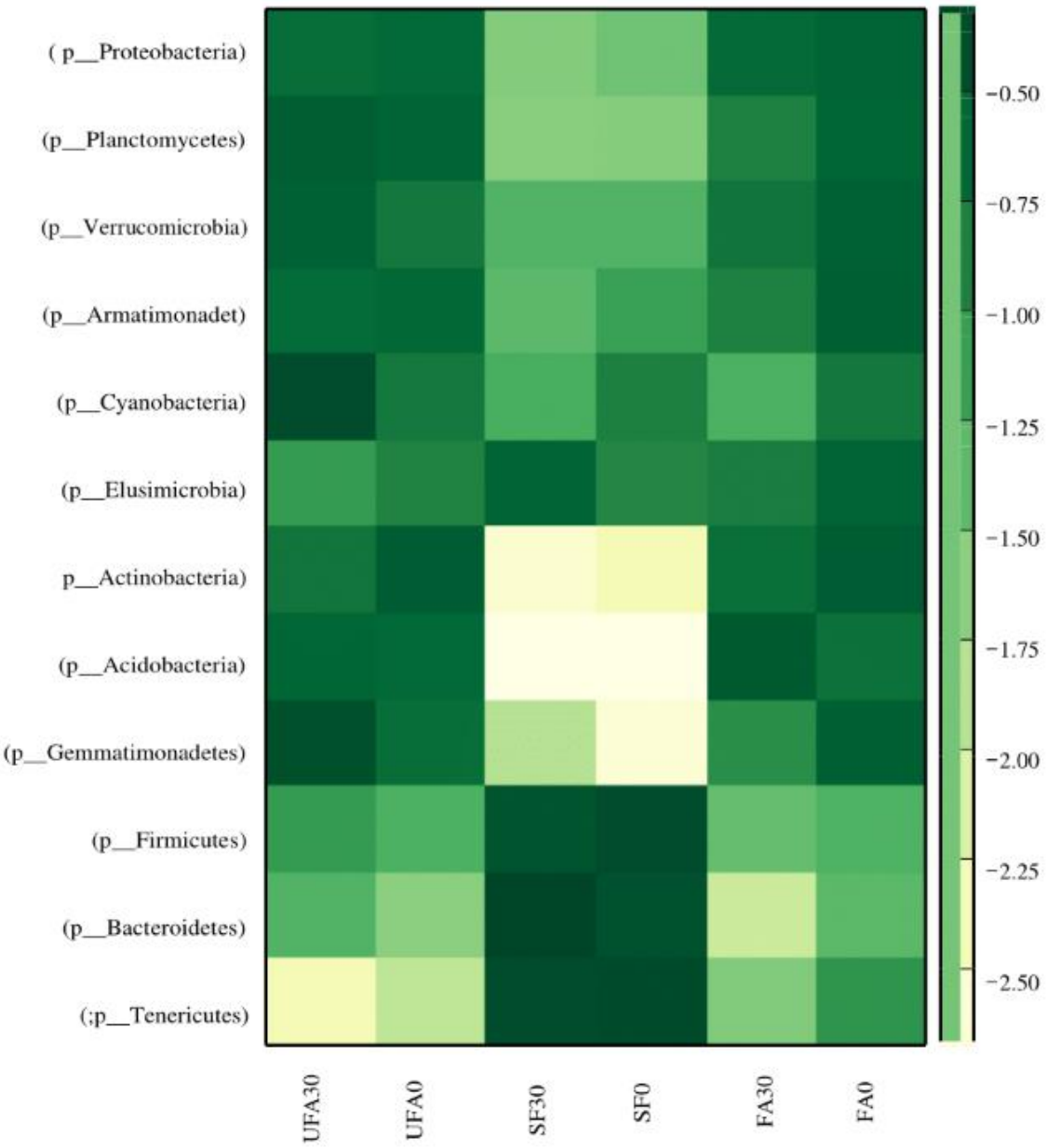

Fig. 6. Heat-map of the secondary forest soil and of oil palm plantation soil after 25 years application of inorganic fertilizer.
FA0: Fertilizer areas 0 to 15 cm **FA30**: Fertilizer areas 15 to 30 cm
UFA0: Unfertilized areas 0 to 15cm **UFA30**: Unfertilized areas 15 to 30 cm
SF0: Secondary forest 0 to 15 cm **SF30**: Secondary areas 15 to 30cm

Due to the current lack of land space in Malaysia, oil palm is cultivated in acidic soils, to which inorganic fertilizer is added, resulting in leaching and hydrological conditions during rainy seasons (Comte *et al.* 2013). Classification of soil nutrient status (Goh *et al.* 1997) showed that CEC at 6 cmol/kg and total N content lower than 0.08 % were very prevalent in oil palm plantation. It was demonstrated in this study that when soil acidity increased, more H^+ ions were attached to the soil and at the same time, the available CEC was decreased. The CEC is an indicator of the potential nutrient adsorption by organic mineral complexes, particularly the retention of basic cations (Zech *et al.* 1997). From the results previously reported as support data, the continuous cropping often resulted in a decline in soil organic matter (Luo *et al*. 2015). Long-term application of inorganic fertilizer in oil palm plantation results in macronutrient element contents decreases (N, total P, and K) and increases (total available P, Ca, and M). Micronutrient elements such as B, Mn, Zn, and Mo contents were also decreased, with an increase only in Fe. The results showed that long-term fertilizer application, especially of N, had acidifying effects that causes a decrease in pH (Fig. 4).

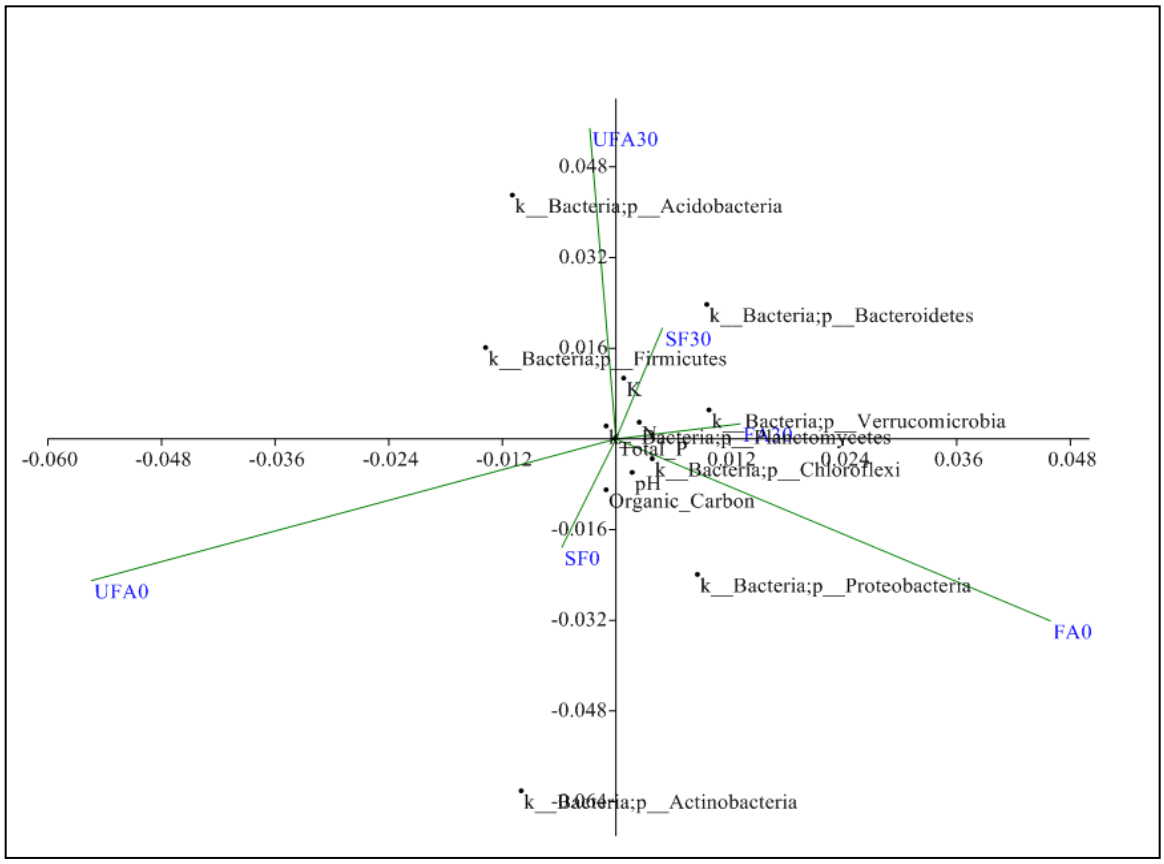

Fig. 7. Principal coordinate analysis of secondary forest soil and soil after 25 years of inorganic fertilizer application.

FA0: Fertilizer areas 0 to 15 cm
FA30: Fertilizer areas 15 to 30 cm
UFA0: Unfertilized areas 0 to 15cm
UFA30: Unfertilized areas 15 to 30 cm
SF0: Secondary forest 0 to 15 cm
SF30: Secondary areas 15 to 30cm

The Fe content increases were indicators of the acidic soil condition. This confirmed earlier findings that most N containing fertilizers tended to acidify the soil (Belay *et al*. 2002). In another study, the high levels of variations observed in the organic content, available P, Ca, Mg, and K could be attributed to the oil palm's impact on the soil, as no fertilization had been carried out on this field for over fifteen years (Ogeh and Osiomwan 2012). Soil acidity can cause distraction in nutrient absorption. The size distribution of soil particles showed big differences between the secondary forest soil and the plantation soil after 25 years application in terms of the percentages of slit, coarse sand, and fine sand. Deceases in the percentage of clay and increases in the percentages of coarse sand, and fine sand were also observed. These observations might be due to the management practices of the oil palm plantation including the usage of machines for fruit harvesting and for cutting grass. Changes in soil properties, macro and micro-nutrient element effect on soil microbial diversity in oil palm plantation. Phyla *Firmicutes*, *Bacteroides* and *Chloroflexi* highly in secondary forest soil switch to *Acidobacteria, Proteobacteria, and Actinobacteria* in oil palm plantation after long term continuous cropping. Figure 7 shows the principal coordinate analysis of the bacterial treatments. The results showed that the soil organic carbon content was significantly correlated with the secondary forest samples. The samples also positively correlated with the *Bacteroidetes* phylum. On the other hand, the long-term fertilized soil was significantly correlated with the *Firmicutes, Chloroflexi*, and *Proteobacteria* phyla. As described earlier, these communities particularly the *Firmicutes* play important roles for maintaining soil health.

CONCLUSIONS

1. Important findings in soil properties as a consequence of long-term continuous cropping included decreases in total nitrogen and organic C valued from secondary forest. The changes were attributed to land clearing and subsequent plant uptake. The relationship between cation exchange capacity (CEC) and organic C extent were influenced by pH of amount and frequency of fertilizer application. The Fe contents was highly elevated, indicating the stressed condition of the soil.
2. Long-term application of inorganic fertilizer in oil palm plantation switch and altered microbial communities in secondary forest. *Firmicutes* as a suppressor of soil-borne diseases and *Bacteroidetes* as an indicator of soil health were eliminated in the soil of the oil palm plantation. By contrast, increasing contents of the phyla *Acidobacteria*, *Protobacteria*, and *Actinobacteria* were observed in soil from the oil palm plantation.

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