

SOIL BACTERIAL BIODIVERSITY IN DEVELOPMENT OF SECONDARY LOGGED-OVER FOREST TO OIL PALM PLANTATION IN MINERAL SOIL OF BELAGA, SARAWAK

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ABSTRACT

Conversion of forest to oil palm typically results in significant land changes within the soil, for example, extensive damage to the top soil, soil compaction and erosion. Such changes are often related to reductions within the biodiversity and abundance of the soil microbial communities. By using 16S rDNA and denaturing gradient gel electrophoresis, we investigated the microbial composition between oil palm plantations, at various stages of planting in mineral soil of Belaga, Sarawak in Malaysia, and forest ecosystem (biodiversity strip). Bacterial composition within biodiversity strips of secondary logged-over forests was mostly dominated by unclassified bacteria, Acidobacteria, and Actinobacteria followed by α -Proteobacteria whereas oil palm management areas were most commonly consisted of unclassified bacteria, Firmicutes, β -Proteobacteria, Actinobacteria, Acidobacteria and Chloroflexi. In a newly developed oil palm plantation, pH factor with the addition of fertiliser during land clearing altered the bacteria composition, affecting several phyla especially Acidobacteria, and with increasing pH, the population showed an abundance of Firmicutes, β -Proteobacteria, γ -Proteobacteria, Chloroflexi, and Bacteroidetes. Shifts in the bacterial community of the oil palm plantation, especially with the increasing number of the phylum Firmicutes, β -Proteobacteria, Bacteroidetes and the decreasing number of α -Proteobacteria caused by the slight increase of pH values indicated that changes of soil properties introduced by field management (e.g., fertiliser application) somehow affects bacterial composition. Nevertheless, the method of intercropping oil palm with black pepper and pineapple, during oil palm aged 4.5 years, showed increasing emergence of minor phyla from Gemmatimonadetes, Planctomycetes, Chlamydiae and Cyanobacteria, which contributes to the diverse variations of new phylum to the microbial diversity of the oil palm area indicating this method as a sustainable system for the oil palm management. Overall, there is a need for further research in using sustainable methods such as a suitable intercropping system that can positively improve soil fertility and a balanced long-term usage of biofertilisers and chemical fertilisers, which can enhance the biodiversity of the bacterial community and contributes to the sustainable development of oil palm plantations.

Keywords: microbial biodiversity, 16S rDNA, denaturing gradient gel electrophoresis, oil palm plantation.

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INTRODUCTION

Soil microbes are associated with biochemical processes of the soil including organic matter deterioration, humus development and nutrient cycle that played an important role in conserving

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soil productivity (Curtis *et al.*, 2002). Conversion of secondary forest to agricultural cultivation such as oil palm plantations can cause rapid changes in the soil microbial populations and activities. Oil palm is becoming the world's most rapidly expanding tropical crop, with increased expansion of oil palm planted area in Malaysia from 5.74 million hectares in 2016 to 5.81 million hectares in 2017 (Kushairi *et al.*, 2018). The development to oil palm plantations has led to conversion of tropical forest, logged-over forest and peatlands (Koh *et al.*, 2011; Carlson *et al.*, 2012). Most of these soil microbial populations are sensitive to the soil changes; therefore, some microbiological parameters such as microbial biodiversity, maybe used as an indicator for soil quality (Fierer *et al.*, 2007a). Microbes decomposing carbon-based litter are a vital constituent of global biogeochemical cycles (Burgin *et al.*, 2011; van der Heijden *et al.*, 2008; Zhang *et al.*, 2013), breaking down complicated organic materials and releasing CO₂ to the atmosphere via respiration and causing nutrients accessible to promote primary production (Dubinsky *et al.*, 2010; Nielsen *et al.*, 2011;).

Conventional methods for microbial identification based on phenotypic characteristics (*e.g.* morphology and biochemistry) are generally less accurate in identifying species. Development of molecular and genomic tools have recently opened a window to assess the diversity and composition of these communities (Caporaso *et al.*, 2011; Fierer *et al.*, 2007b). Most target the *16S rRNA* gene by polymerase chain reaction (PCR) amplification and subsequent analysis using clone libraries (He *et al.*, 2006), denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993), terminal restriction fragment length polymorphism (T-RFLP) (Fierer *et al.*, 2007b) and the advancement of next generation sequencing (NGS). NGS or high-throughput sequencing has become a powerful sequencing tool which led to the establishment in the field of 'metagenomics'. Metagenomics is the genomic analysis of microbes by direct DNA extraction and DNA cloning from an assemblage of microorganisms (Oulas *et al.*, 2015). NGS consists of different platforms, such as Roche 454 pyrosequencing and Illumina/Solexa where, Roche 454 pyrosequencing has increase its average read lengths to ~500 base pairs compared to Illumina which only has ~150 base pairs.

Microbial diversity, in the structural and compositional changes among communities sampled, has been used to evaluate the impact of land use change in a wide range of studies (Ding *et al.*, 2013; Suleiman *et al.*, 2013). In this research, we aim to investigate the microbial composition between oil palm plantations, at various stages of planting in mineral soil of Belaga, Sarawak, in Malaysia and forest ecosystem by using 16S rDNA and denaturing gradient gel electrophoresis. The information acquired from these molecular tools,

can evaluate the changes in soil or environment and determine the main drivers of change in microbial diversity (Wakelin *et al.*, 2008). This knowledge can also be used to classify the taxa and indicators for monitoring the effect of land use change.

MATERIALS AND METHOD

Soil Sampling

Sampling for microbial biodiversity study at Sungai Asap, Belaga, Sarawak was conducted at four sites namely Biodiversity Strip 1 (S1, hilly secondary jungle), Strip 2 (S2, disturbed secondary jungle), Strip 3 (S3, riparian area) and oil palm (OP) planted area to investigate the bacterial composition between oil palm cultivated and uncultivated areas (Figure 1). Sampling was carried out at respective GPS points from depth of 0-15 cm (Table 1). The distance between each GPS point for OP was approximately from 40 m to 200 m on average whereas biodiversity strips sampling points were about 100 m apart. A total of 40 soil samples were collected from hilly secondary jungle (10), disturbed secondary jungle (10), riparian area (10) and oil palm plantation (10). Four soil replicates were collected from each GPS sampling point. All of the four replicates of soil samples from the same sampling point were pooled together and homogenised. Fifty grams of soil samples were collected in 50 ml sterile falcon tubes and transported in an ice box and subsequently kept at 10°C prior to laboratory analysis. Oil palm area was intercropped with pepper and pineapple during July 2012. Sampling for Strip 3 was stopped after fourth sampling (OP 2.5 years).

Extraction of DNA from Soil

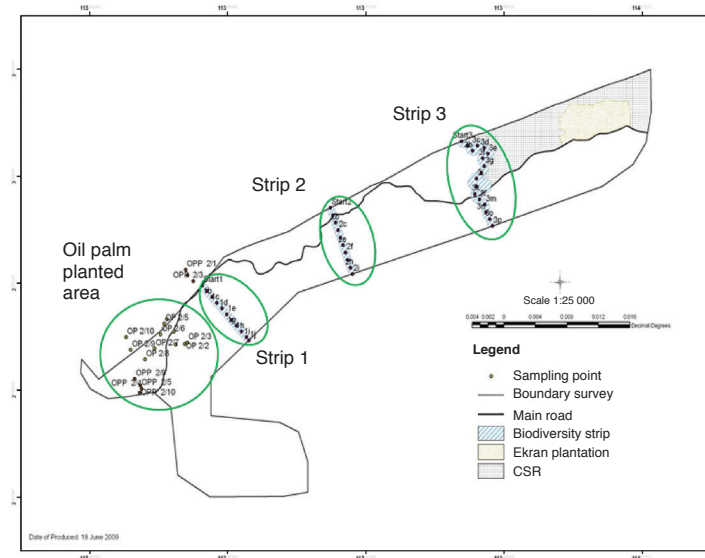
Microbial DNA were extracted directly from soil, using GeneMatrix Soil DNA Purification kit (EURx Ltd., Poland) (Agnieszka *et al.*, 2012). DNA were quantified using Nanophotometer (Implen GMBH, Germany) with absorbance ratio of A260/A280 for measurement of nucleic acids.

PCR Amplification for Bacterial 16s rDNA

Universal primers used were forward (f) with GC-clamps, 341f (5'-cgc-ccg-ccg-cgc-gcg-gcg-ggc-ggg-gcg-ggg-gca-cgg-ggg-gcc-tac-gg-agg-cag-cag-3') and reverse primers 907r (5'-ccc-cgt-caa-ttc-att-tga-gtt-t-3') (Muyzer *et al.*, 1993).

Denaturing Gradient Gel Electrophoresis (DGGE) for Bacterial 16s rDNA

PCR products were electrophoresed on 6% (w/v) polyacrylamide (37.5:1; acrylamide:bisacrylamide) and separated with a denaturing gradient of 40% to



Note: (a) Oil palm plantation (OPP), (b) Strip 1 - S1, (c) Strip 2 - S2 and (d) Strip 3 - S3.

Figure 1. Sampling points studied in Sungai Asap, Belaga, Sarawak.

TABLE 1. GLOBAL POSITIONING SYSTEM (GPS) SAMPLING POINTS OF SITES STUDIED IN BELAGA, SARAWAK

No.	Sites	Forest type	Sampling points	Location on GPS
1.	Oil palm cultivated area	Lowlands	1	N, 2° 59' 6.71" E, 113° 57' 11.976"
			2	N, 2° 59' 7.0" E, 113° 57' 16.0"
			3	N, 2° 59' 7.5" E 113° 57' 17.2"
			4	N 2° 59' 17" E 113° 57' 7.9"
			5	N 2° 59' 15.2" E 113° 57' 6.7"
			6	N 2° 59' 10.6" E 113° 57' 5.3"
			7	N 2° 59' 5.3" E 113° 57' 2.2"
			8	N 2° 59' 00.8" E 113° 56' 57.6"
			9	N 2° 59' 4.6" E 113° 56' 52"
			10	N 2° 59' 09.4" E 113° 57' 49.2"
2.	Biodiversity Strip 1	Secondary jungle, hilly area, undisturbed	1	N 2° 59' 28.5" E 113° 57' 26.338"
			2	N 2° 59' 26.11" E 113° 57' 28.541"
			3	N 2° 59' 23.754" E 113° 57' 30.779"
			4	N 2° 59' 21.434" E 113° 57' 33.052"
			5	N 2° 59' 19.028" E 113° 57' 35.238"
			6	N 2° 59' 16.756" E 113° 57' 37.539"
			7	N 2° 59' 14.371" E 113° 57' 39.769"
			8	N 2° 59' 12.021" E 113° 57' 42.013"
			9	N 2° 59' 9.692" E 113° 57' 44.276"
			10	N 2° 59' 8.438" E 113° 57' 45.432"
3.	Biodiversity Strip 2	Secondary jungle, disturbed	1	N 3° 0' 2.328" E 113° 58' 23.176"
			2	N 2° 59' 59.381" E 113° 58' 23.874"
			3	N 2° 59' 56.343" E 113° 58' 25.041"
			4	N 2° 59' 53.296" E 113° 58' 26.183"
			5	N 2° 59' 50.247" E 113° 58' 27.321"
			6	N 2° 59' 47.204" E 113° 58' 28.474"
			7	N 2° 59' 44.154" E 113° 58' 29.616"
			8	N 2° 59' 41.099" E 113° 58' 30.752"
			9	N 2° 59' 38.036" E 113° 58' 31.859"
			10	N 2° 59' 35.442" E 113° 58' 32.754"
4.	Biodiversity Strip 3	Riparian area	1	N 3° 0' 27.726" E 113° 113° 59' 25.363"
			2	N 3° 0' 25.628" E 113° 113° 59' 27.742"
			3	N 3° 0' 27.69" E 113° 113° 59' 30.025"
			4	N 3° 0' 26.761" E 113° 113° 59' 33.044"
			5	N 3° 0' 24.38" E 113° 113° 59' 34.681"
			6	N 3° 0' 22.522" E 113° 113° 59' 32.335"
			7	N 3° 0' 19.337" E 113° 113° 59' 33.059"
			8	N 3° 0' 16.558" E 113° 113° 59' 31.706"
			9	N 3° 0' 14.364" E 113° 113° 59' 29.502"
			10	N 3° 0' 11.12" E 113° 113° 59' 29.518"

80% (100% denaturant corresponds to 7 M urea and 40% [vol/vol] deionised formamide) run at 60 V, 16 hr.

Recovery and Purification of DNA

DNA bands from DGGE were cut out using a sterile blade and collected in a 1.5 ml microcentrifuge tube containing 50 μ l ddH₂O. For DNA elution, the excised gel with DNA were incubated overnight at 4°C. Eluted DNA was purified using QIAquick gel extraction kit (QIAGEN, Inc., Valencia, CA), according to the kit's protocol.

PCR Amplification of Purified Band from DGGE

DNA from the DGGE gel successfully excised, clean and purified was amplified again using PCR with forward (F) primers 16S rDNA, without GC-clamps (GC). PCR products from agarose gel were again cut and purified using QIAquick Gel Extraction Kit, as mentioned above. The purified DNA was sequenced in both directions with the same primers without GC as used in the PCR amplification step above.

Sequencing Analysis

The PCR products were sent to First Base Laboratories (Malaysia) for sequencing analysis. Nucleotide-nucleotide basic logic alignment search tool (BLASTn) service of the NCBI GenBank database was used to identify the nearest relatives of the partially sequenced 16S rDNA genes of excised dominant bands.

Statistical Analysis and Biodiversity Indices

The Shannon-Weaver Biodiversity index is a common diversity index which increases with species number and is highest when the mass is distributed more evenly over the species (Hill, 1973). The Berger-Parker Dominance index expresses the proportional importance of the most abundant species (the dominant species). It is a numerical measure of the most abundant species in the formula, $D = N_{max} / N$, where N_{max} is the number of individuals in the most abundant species, and N is the total number of individuals in the sample (Hill, 1973). Statistics for the significant difference was done by means of one-way analysis of variance (ANOVA) and post hoc Tukey test ($p < 0.05$). Principal component analysis (PCA) with Pearson correlation, was used using Excel combined XLStat for correlation analysis.

RESULTS AND DISCUSSION

Biodiversity Indices

Time-course of Shannon-Weaver bacterial biodiversity index showed an increase after clean-

clearing to early planting of oil palm, from October 2008 to July 2009, from 7.423 to 7.627, respectively (Table 2). Shannon-Weaver indices for biodiversity strip was high during early planting (July 2009). Further increase in index for the OP planted area, was observed when oil palm was from 2.5 years old (April 2010) to 3.5 years old (June 2011), with the increase index value from 7.773 to 8.323, respectively. At 4.5 years and 5.5 years, the biodiversity index slightly fluctuated but remained steadily at index 8.279 (Table 2). Berger-Parker Dominancy index showed generally microbial diversity increased as the age of the oil palm increased to 5.5 years (Table 3). This is supported by Dilly *et al.* (2004), which documented that the increase in Shannon-Weaver Biodiversity index indicates that in general, microbial diversity intensifies as the decomposition of organic material progresses as these provide nutrient rich environment for the microbes to thrive. Indices from the Shannon-Weaver Biodiversity index showed index values from OP area were significantly different from that of biodiversity strips until June 2013 (Table 2).

Prevalence Percentage of Soil Prokaryotic Phyla

Percentage occurrence of phyla levels in Figures 2 to 7, indicated that, before clean-clearing (October 2008) until palm reached 5.5 years (June 2013), unclassified bacteria was the dominant phylum amongst the prokaryotic population in all sites including the oil palm cultivated area. The unclassified bacteria group are species of bacteria which are not assigned to any classification of genus or species by the taxonomy classifier or BlastN, based on their sequence similarities/blasts. Before clean-clearing (October 2008) Acidobacteria group was the second most dominant in the OP area, while the Actinobacteria, Firmicutes, and α -Proteobacteria group were found in all biodiversity strip areas (Figure 2). In OP cultivated area, the phyla consisted of 10 groups compared to 5-7 phylum groups in strip areas. The presence of phylum beta, gamma and delta proteobacteria were observed in the OP area but they were not found in the biodiversity strips area.

Eight months after clean-clearing in July 2009, there was an abundance of Firmicutes (25%) in OP cultivated area, followed by Acidobacteria (6%) and β -Proteobacteria (6%) (Figure 3). Actinobacteria was predominant in biodiversity strips, S1, S2 and S3 with 15%, 19% and 11%, respectively (Figure 3). In the 1.5-year old OP area, phylum decreased to nine groups, compared to 11 phylum groups in strip areas. In OP cultivated area, the abundance of Firmicutes seemed to be related with the abundance of organic materials after clean-clearing and also with the increased application of fertiliser CIRP (rock phosphate) used in March and April 2009.

TABLE 2. SHANNON-WEAVER BIODIVERSITY INDICES FOR SOIL PROKARYOTES ON MINERAL SOIL SAMPLED FROM SUNGAI ASAP, BELAGA, SARAWAK

Sites	OPP	S1	S2	S3
Oct. 2008 (logged-over forest)	7.423 ± 0.043 ^a	7.001±0.056 ^b	6.789±0.014 ^c	6.276±0.01 ^d
July 2009 (OP 1.5 yr)	7.627±0.008 ^a	8.26 ± 0.023 ^b	8.002±0.014 ^c	8.070±0.011 ^c
April 2010 (OP 2.5 yr)	7.773±0.02 ^a	7.322± 0.018 ^b	7.337±0.041 ^c	8.003±0.024 ^d
June 2011 (OP 3.5 yr)	8.323±0.02 ^a	8.003± 0.012 ^b	7.710±0.015 ^c	-
July 2012 (OP 4.5 yr)	8.173±0.018 ^a	8.064± 0.013 ^b	7.939±0.01 ^c	-
June 2013 (OP 5.5 yr)	8.279±0.018 ^a	8.141± 0.014 ^b	8.064±0.016 ^c	-

Note: OP - oil palm area, OPP - oil palm plantation, S1- Biodiversity Strip 1, S2 - Biodiversity Strip 2, and S3 - Biodiversity Strip 3. Indices were compared using Tukey's Method. Shannon-Weaver Biodiversity indices with the different letters are significantly different at $p<0.05$.

TABLE 3. BERGER-PARKER DOMINANCE INDICES FOR SOIL PROKARYOTES ON MINERAL SOIL SAMPLED FROM SUNGAI ASAP, BELAGA, SARAWAK

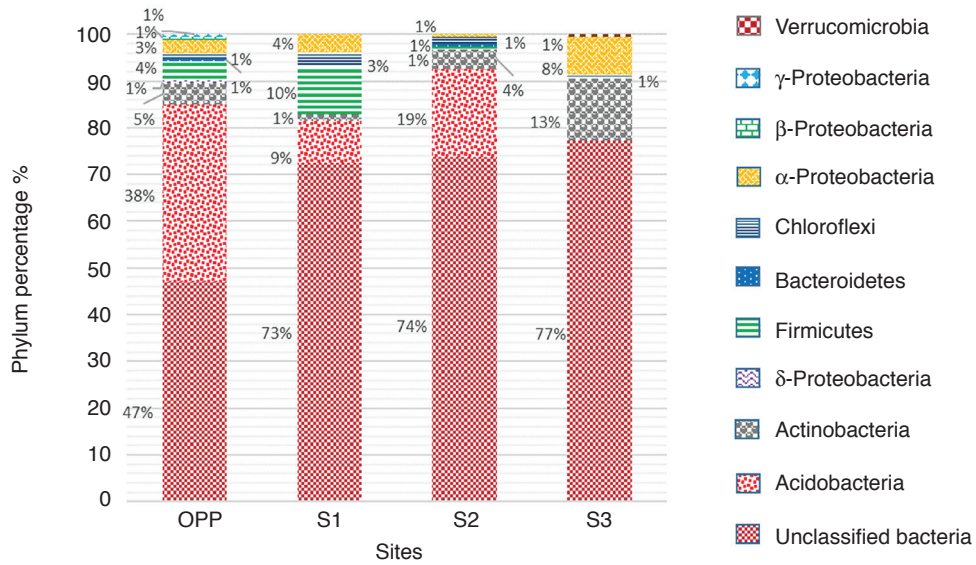
Sites	OPP	S1	S2	S3
Oct. 2008 (logged-over forest)	0.5742±0.014 ^a	0.5822±0.019 ^b	0.6628±0.053 ^c	0.5885±0.075 ^d
July 2009 (OP 1.5 yr)	0.3958±0.011 ^a	0.2863±0.027 ^b	0.3173±0.019 ^c	0.5202±0.016 ^d
April 2010 (OP 2.5 yr)	0.4735±0.029 ^a	0.3545±0.019 ^b	0.4148±0.04 ^c	0.4894±0.027 ^d
June 2011 (OP 3.5 yr)	0.5570±0.027 ^a	0.5874±0.016 ^b	0.6600±0.021 ^c	-
July 2012 (OP 4.5 yr)	0.4718±0.022 ^a	0.4807±0.014 ^a	0.4887±0.018 ^b	-
June 2013 (OP 5.5 yr)	0.3684±0.018 ^a	0.4681±0.016 ^b	0.6301±0.014 ^c	-

Note: OP - oil palm area, OPP - oil palm plantation, S1 - Biodiversity Strip 1, S2 - Biodiversity Strip 2, and S3 - Biodiversity Strip 3. Indices were compared using Tukey's Method. Berger-Parker Dominance indices with the different letters are significantly different at $p<0.05$.

Firmicutes such as orders Bacillales and unclassified Firmicutes thriving in this area specifically enriched the level of phosphorus in plants with the capability to solubilise insoluble inorganic phosphate (mineral phosphate) compounds (Hayat *et al.*, 2010).

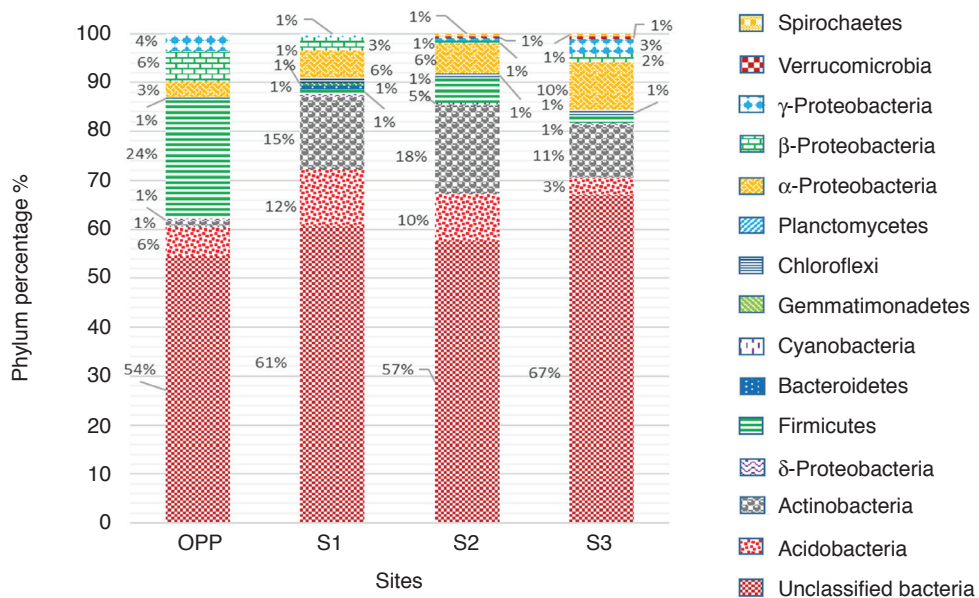
Eight to 12 months later, when the oil palm was 2.5 to 3.5 years (April 2010 to June 2011), unclassified bacteria dominated the population from 51%-58% in all sites. Another predominant phylum, Actinobacteria dominated 12%-35% of the population in April 2010 (Figure 4), whereas Acidobacteria dominated about 14%-15% in June 2011 (Figure 5) in all sites. In OP area of 2.5 years,

phylum consisted of 10 groups compared to nine phylum groups of strip areas. There was an increase in the phylum Firmicutes and β -Proteobacteria group when palms were at 4.5 and 5.5 years, respectively (Figures 6 and 7). MPOB F1 fertiliser (1.75 kg palm⁻¹) was added during May, one to two months before soil was sampled in July 2012 and June 2013 (Table 5), which may increase the occurrence of these two phyla. The phylum Firmicutes is usually involve in nitrogen fixation in plants, and also as a phosphate solubilising bacteria, specifically enhancing the phosphorus level of plants (Hayat *et al.*, 2010). Meanwhile, β -Proteobacteria are



Note: (a) OPP - oil palm cultivable area, (b) S1 - Strip 1, (c) S2 - Strip 2 and (d) S3 - Strip 3.

Figure 2. Prevalence of soil bacterial phyla at Belaga from October 2008 for biodiversity strips versus oil palm sampled from Sungai Asap, Belaga, Sarawak.



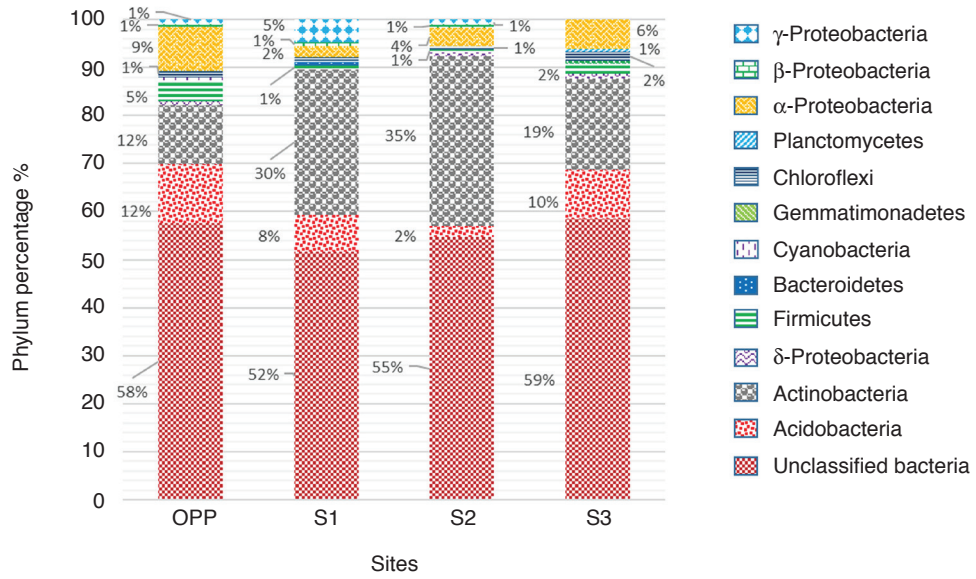
Note: (a) OPP - oil palm aged 1.5 years, (b) S1 - Strip 1, (c) S2 - Strip 2 and (d) S3 - Strip 3.

Figure 3. Prevalence of soil bacterial phyla at Belaga from July 2009 for biodiversity strip versus oil palm sampled from Sungai Asap, Belaga, Sarawak.

chemoorganotrophs and colonises plant roots acting as nitrogen fixers (Hilton *et al.*, 2013). At 4.5 years, intercropping in the OP area with peppers and pineapple caused an increased in phylum diversity of the OP area, with 15 phyla compared to 10-11 phyla in strip areas, where OP showed an increase of minor phylum groups.

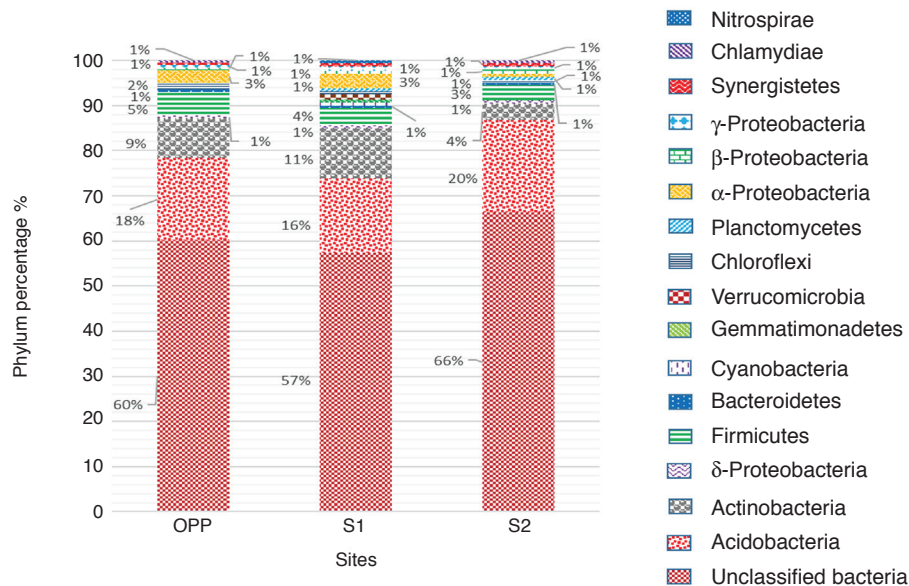
Table 4 indicates that the number of species increased steadily from logged over, after clearing, and under oil palms aged 1.5, 2.5, 3.5, 4.5 and 5.5 years (October 2008 until July 2013) with

values of 85, 159, 116, 186, 293 and 230, respectively. As for the biodiversity Strip 2 the number of species fluctuates and remained the lowest until July 2013. The reduced species number in Strip 2 was caused by the disturbance of the natives colonising, hunting and burning hills for growing paddy and oil palm in the area during July 2009. Towards palms age of 4.5 and 5.5 years the oil palm area exhibited high species number of β-Proteobacteria, Bacteroidetes Firmicutes and Chloroflexi compared to strips area (Table 4). According to a study by Fierer



Note: (a) OPP - oil palm aged 2.5 years, (b) S1 - Strip 1, (c) S2 - Strip 2 and (d) S3 - Strip 3.

Figure 4. Prevalence of soil bacterial phyla at Belaga from April 2010 for biodiversity strips versus oil palm sampled from Sungai Asap, Belaga, Sarawak.



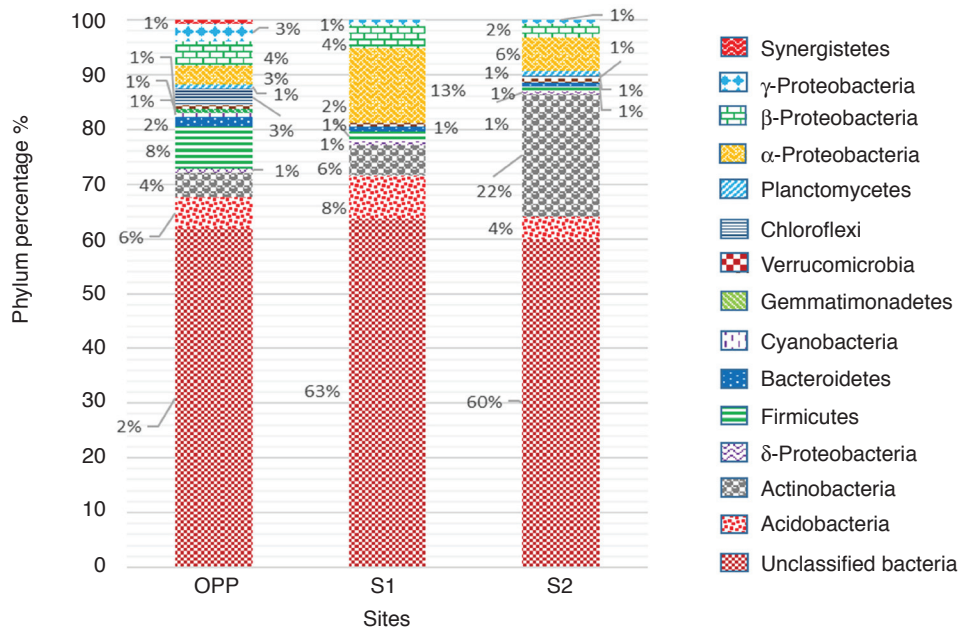
Note: (a) OPP - oil palm aged 3.5 years, (b) S1 - Strip 1, (c) S2 - Strip 2 and (d) S3 - Strip 3.

Figure 5. Prevalence of soil bacterial phyla at Belaga from June 2011 for biodiversity strips versus oil palm sampled from Sungai Asap, Belaga, Sarawak.

(2007a), the available carbon (C) in the soil was positively correlated with the relative abundance of Bacteroidetes and Betaproteobacteria. During these years, there were also appearances of several minor phyla, namely, Gemmatimonadetes, Planctomycetes, Chlamydiae, Nitrospirae and Cyanobacteria which made up a more diverse microbial population in the OP cultivated area (Table 4).

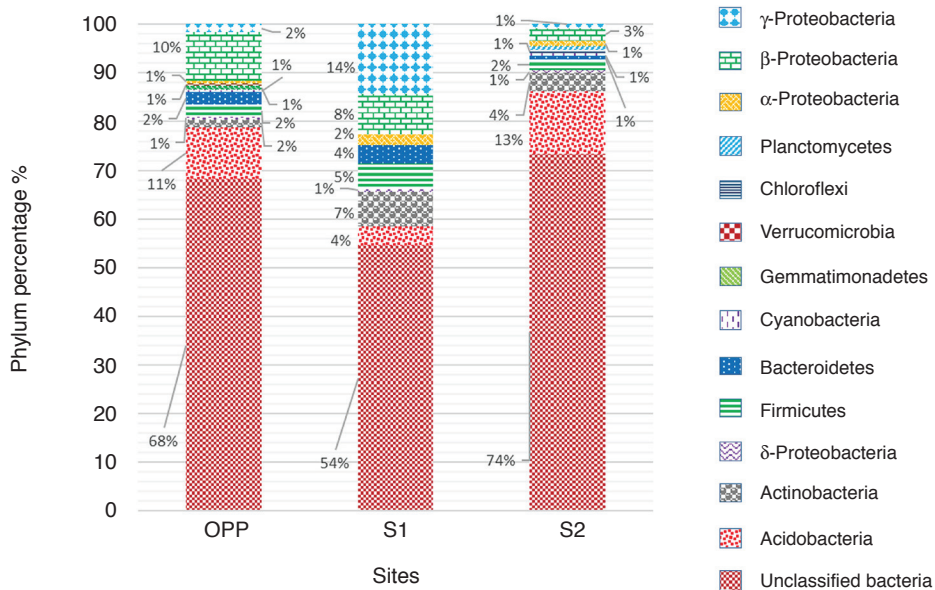
Table 4 shows that species of Actinobacteria remained mostly prevalent in biodiversity strips area until 2013. Actinobacteria are prevalent in forest

as they are attracted to abundant degrading organic materials like cellulose, lignin and chitin (Schafer *et al.*, 2010). Acidobacteria were predominant at all sites and has been reported to be involved in microbial decomposition of lignocellulosic plant biomass (Eichorst *et al.*, 2011). When the palms were at 4.5 to 5.5 years old, γ -Proteobacteria was prevalent in oil palm plantation, as it plays an important role in the decomposition of numerous soil C compounds. (Cleveland *et al.*, 2007). The α -Proteobacteria were mostly abundant in soil ecosystems rich in organic



Note: (a) OPP - oil palm aged 4.5 years, (b) S1 - Strip 1, (c) S2 - Strip 2 and (d) S3 - Strip 3.

Figure 6. Prevalence of soil bacterial phyla at Belaga from July 2012 for biodiversity strips versus oil palm sampled from Sungai Asap, Belaga, Sarawak.



Note: (a) OPP - oil palm aged 5.5 years, (b) S1 - Strip 1, (c) S2 - Strip 2 and (d) S3 - Strip 3.

Figure 7. Prevalence of soil bacterial phyla at Belaga from June 2013 for biodiversity strips versus oil palm sampled from Sungai Asap, Belaga, Sarawak.

materials, playing a role as a nitrogen fixer but the group slightly decreased in OP area probably due to the slightly higher pH and low rainfall (Shange *et al.*, 2012). During intercropping in 2012 to 2013, it was observed that major species of unclassified bacteria, Acidobacteria, γ -Proteobacteria, β -Proteobacteria and Bacteroidetes increased in species number (Table 4) in the OP areas. There was also presence of minor phyla δ -Proteobacteria, Verrucomicrobia, Gemmatimonadetes, Planctomycetes, Synergistetes

and Cyanobacteria compared to earlier planting of OP.

Bacterial Community Composition Based on the Prevalent Phylum and Order Level under Different Ages of Oil Palm

The analysis of the bacterial community composition (without the phylum unclassified bacteria) based on the prevalent phylum and order

TABLE 4. TOTAL PROKARYOTIC SPECIES IN BELAGA, SARAWAK

Sites	Phylum	2008	2009	2010	2011	2012	2013
S1	Unclassified bacteria	3	6	6	9	20	12
S2		4	6	7	5	14	12
S3		3	4	6	-	-	-
OPP		6	8	11	8	19	21
S1	Acidobacteria	5	10	6	11	5	6
S2		3	6	6	7	12	22
S3		-	6	6	-	-	-
OPP		7	6	9	15	10	18
S1	Actinobacteria	5	51	31	35	56	41
S2		18	64	14	13	75	42
S3		16	58	47	-	-	-
OPP		14	10	28	67	49	27
S1	α -Proteobacteria	9	14	9	23	144	18
S2		3	2	33	5	55	18
S3		3	32	19	-	-	-
OPP		16	35	37	28	60	15
S1	Firmicutes	32	5	12	20	16	27
S2		2	5	2	5	6	8
S3		-	13	4	-	-	-
OPP		30	75	11	22	42	4
S1	γ -Proteobacteria	3	11	18	3	5	48
S2		2	6	4	15	1	5
S3		-	29	4	-	-	-
OPP		2	8	17	8	35	34
S1	β -Proteobacteria	-	16	3	5	30	53
S2		-	1	5	10	16	25
S3		-	21	2	-	-	-
OPP		2	12	4	4	40	59
S1	δ -Proteobacteria	-	4	-	1	2	2
S2		1	1	1	1	2	3
S3		-	2	1	-	-	-
OPP		2	1	2	1	4	8
S1	Chloroflexi	5	7	2	3	1	-
S2		5	4	2	2	52	-
S3		5	1	6	-	-	-
OPP		5	3	9	9	13	-
S1	Bacteroidetes	-	6	2	3	2	31
S2		1	-	-	2	4	4
S3		-	-	-	-	-	-
OPP		1	-	-	9	53	9
S1	Verrucomicrobia	-	-	-	1	1	-
S2		-	2	-	-	1	-
S3		1	1	-	-	-	-
OPP		-	1	-	1	5	1
S1	Gemmatimonadetes	-	4	-	1	-	-
S2		-	-	-	-	-	-
S3		-	-	1	-	-	-
OPP		-	-	-	-	5	2
S1	Planctomycetes	-	-	-	2	3	-
S2		-	-	-	5	-	1
S3		-	-	1	-	-	-
OPP		-	-	-	-	1	-
S1	Spirochaetes	-	-	-	-	-	-
S2		-	2	-	-	-	-
S3		-	2	-	-	-	-
OPP		-	-	-	-	-	-

TABLE 4. TOTAL PROKARYOTIC SPECIES IN BELAGA, SARAWAK (continued)

Sites	Phylum	2008	2009	2010	2011	2012	2013
S1	Synergistetes	-	-	-	3	-	-
S2		-	-	-	2	-	-
S3		-	-	-	-	-	-
OPP		-	-	-	1	2	-
S1	Chlamydiae	-	-	-	-	-	-
S2		-	-	-	1	-	-
S3		-	-	-	-	-	-
OPP		-	-	-	12	-	-
S1	Cyanobacteria	-	-	-	5	-	-
S2		-	-	-	1	-	2
S3		-	-	-	-	-	-
OPP		-	-	1	-	2	2
S1	Nitrospirae	-	-	-	1	-	-
S2		-	-	-	1	-	-
S3		-	-	-	-	-	-
OPP		-	-	-	1	-	-
S1	Total	62	130	89	117	288	238
S2		39	99	74	80	191	145
S3		27	169	98	-	-	-
OPP		85	159	116	186	293	230

Note: OPP - oil palm plantation, S1- Biodiversity Strip 1, S2 - Biodiversity Strip 2, and S3 - Biodiversity Strip 3.

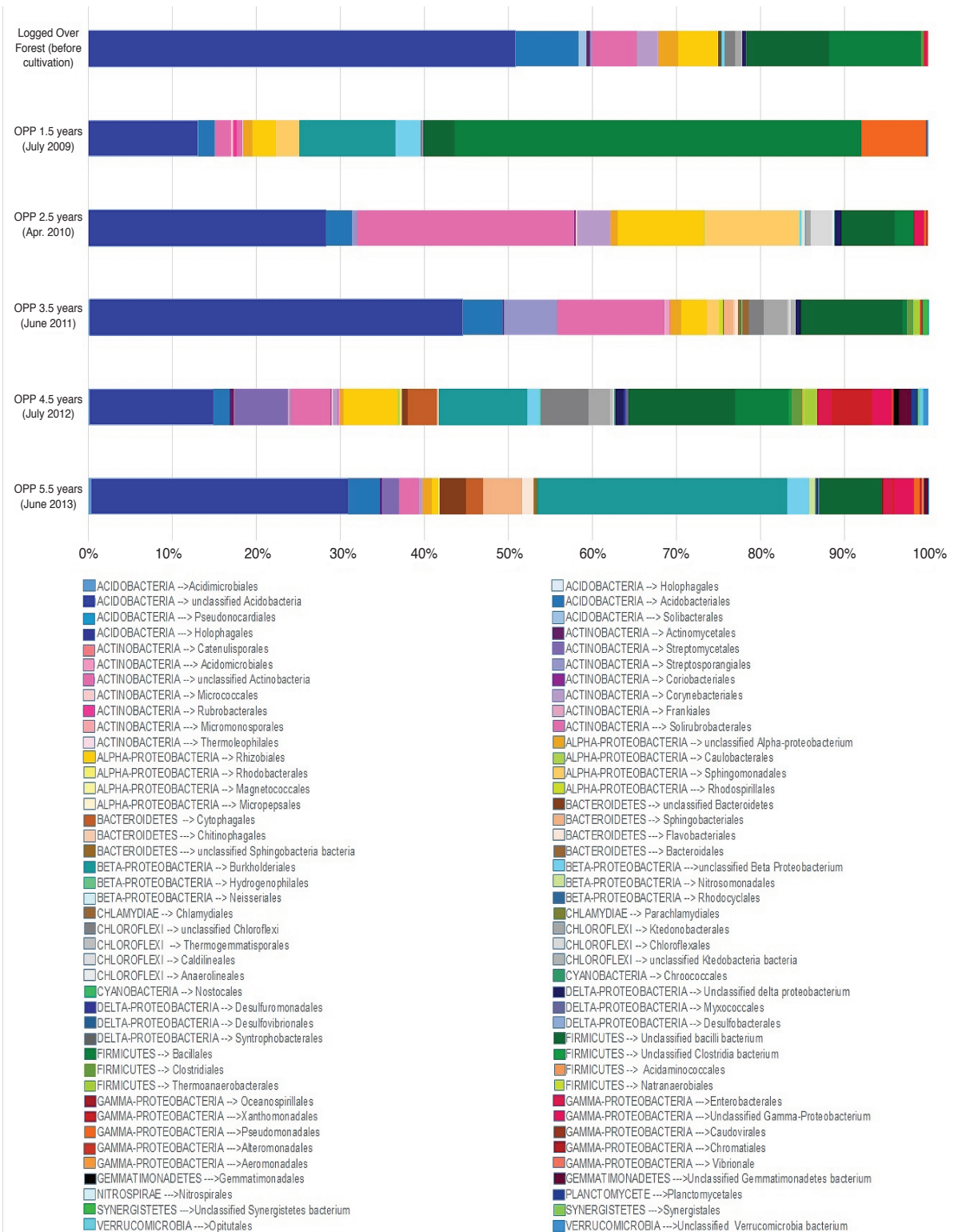
Palms age: 2008 - logged-over forest, 2009 - 1.5 years, 2010 - 2.5 years, 2011 - 3.5 years, 2012 - 4.5 years and 2013 - 5.5 years.

within the oil palm plantation revealed that the soil bacterial communities were diversified between the different ages of oil palm planting (Figure 8). Comparison of microbial order level revealed that in the logged-over forest (before cultivation) the most abundant was the unclassified Acidobacteria at 51% higher than in the oil palm planting areas. Other orders under the phylum Acidobacteria are Acidobacteriales (7%) and Solibacteriales (2%). Solibacteriales was only available in the logged-over forest area and was lacking in the oil palm planting area. Several studies show that Acidobacteria are mostly abundant in forest soils than in agricultural areas. Acidobacteria are oligotrophic microbes mostly dominant in carbon poor soils such as in forest soils as agricultural soil are rich in carbon and nitrogen (Nemergut *et al.*, 2010). The proportion of Acidobacteria was described to be considerably lower in nutritional rich rhizosphere than in bulk soil, confirming their oligotrophic existence in the logged-over forest soil (Kielak *et al.*, 2009). Recently the abundance of Acidobacteria was discovered to be negatively correlated with the nitrogen level in fertiliser (Fierer *et al.*, 2012). The order Bacillales from Firmicutes was the second most abundant at 10% among the bacterial order before cultivation of oil palm.

After the land-clearing of the forest and planting of 1.5 year old palms, the order Bacillales was the most abundant at 48% for the phylum Firmicutes, followed by Burkholderiales, 12% for β -Proteobacteria and a significant increase of the

phylum γ -Proteobacteria order Pseudomonadales at 6%. The abundance of these phyla maybe correlated to the richness of soil C content after land-clearing confirming a study by Trivedi *et al.* (2016) that, soil C in soil corresponded with the relative abundance of Firmicutes, Verrucomicrobia, and Proteobacteria. The CIRP fertiliser applied during March to April 2009 which enhanced the phosphorus (P) level may also increase the abundance of Firmicutes with its ability to solubilise insoluble inorganic phosphate.

However, land-cleared activities also reduced the population of several phyla that were present in logged-over forest such as, Acidobacteria, Actinobacteria, α -Proteobacteria and Chloroflexi. Acidobacteria was often associated with slightly higher pH in agricultural ecosystems compared to natural ecosystems (Rodrigues *et al.*, 2013) hence the decrease in Acidobacteria population in 1.5 years old oil palm (Table 4). The α -Proteobacteria displayed a negative correlation with the level of phosphorus in the soil. The genus *Bradyrhizobium*, in the order Rhizobiales of the phylum α -Proteobacteria, was also strongly and negatively correlated with the level of P in the soil (Herman *et al.*, 2017). The group Chloroflexi prevails in nutritional poor soils (Will *et al.*, 2010) and other oligotrophic ecosystems. Actinobacteria which is usually the most abundant phyla in all types of cultivated area, reduced in population in the clean-cleared areas due to the slight increase of pH to 4.8 (Table 5). The order Solirubrobacterales of Actinobacteria which were only present after clean-clearing, were considered



Note: The unclassified bacteria phylum was not included.

Figure 8. Bacterial community composition based on the prevalent phylum and order level under different ages of oil palm (OP).

as a biomarker to determine the effects on land use since the order seemed to favour physical disturbance where its abundance was usually related to the cultivated system (Shange *et al.*, 2012).

When the palms were at 2.5 years, within the Actinobacteria, unclassified Actinobacteria and

Corynebacteriales exhibited higher abundances at 25% and 7%, respectively. The phylum Actinobacteria produces numerous types of peroxidases (Le Roes-Hill *et al.*, 2011) in which most are lignin peroxidases, accelerating humification and composting through hydrolysis of lignin into humic acid derivatives

in a carbon and lignocellulosic rich environment. Among α -Proteobacteria, Sphingomonadales was the most abundant with 12% and Rhizobiales, with 10%. Sphingomonadales were more abundant in oil palm, possibly because this group preferred cultivation and the low C/N ratio of the oil palm plantations (Kuramae *et al.*, 2012).

When the oil palm reached 3.5 years, the phyla Acidobacteria orders increased for unclassified Acidobacteria, 43% and Acidobacteriales at 4%, due to the slight acidic pH of the soil at pH 4.5 (Table 5). The low pH may be due to the decomposition of organic matter in the surrounding area of the oil palm due to the accumulation of dried fronds and empty fruit bunches. Organic matter decomposed by microbes was converted directly into organic acid, carbon dioxide (CO₂) and water forming carbonic acid, which eventually resulted in more acidic soils. Within the phylum Actinobacteria, the order unclassified Actinobacteria increased at 25% in abundance and there was presence of orders, Streptosporangiales and Pseudonocardiales with 7% and 5% abundance, respectively. The species in the order Streptosporangiales seems to originate from rare species of Actinobacteria and have the potential to produce new antibiotics (Lazzarini *et al.*, 2000). Some Actinobacterial groups, from orders Frankiales and Pseudonocardiales are considered N₂ fixing bacteria (Rughoft *et al.*, 2016). Two other phyla with increasing presence were Chloroflexi with the order, Ktedonobacteriales, 3% and Bacteroidetes with the order Sphingobacteriales, at 2%. The presence of Bacteroidetes under cultivated systems may be associated with their capability in utilising organic matter and colonising soils shortly after crops are planted (Acosta-Martínez *et al.*, 2008) whereas Chloroflexi thrived in oligotrophic ecosystems and the abundance of this photosynthetic phylum in oil palm plantation could be elucidated by the open canopy of the plantation which has greater exposure to sunlight than forest soils (Nacke *et al.*, 2014).

At 4.5 years old, intercropping of pineapple and black pepper with oil palm showed increasing emergence of variability in phylum groups. With increasing pH of 4.9, the population showed a rise in abundance of Firmicutes, β -Proteobacteria, γ -Proteobacteria, Chloroflexi, Bacteroidetes, and an increase in minor phylum of Verrucomicrobia, Gemmatimonadetes, Cyanobacteria and Planctomycete. The most abundant orders for Firmicutes were unclassified Bacilli, 13%, followed by β -Proteobacteria with order Burkholderiales, 11%, the phylum Gammaproteobacteria with order Xanthomonadales, 7% and Bacteroidetes with order Cytophagales at 5%. The most abundant minor phylum was Chloroflexi with the order, unclassified Chloroflexi at 4%, Gemmatimonadetes, with order unclassified Gemmatimonadetes, 2%,

Verrucomicrobia with unclassified Verrucomicrobia, 2%, δ -Proteobacteria with unclassified Deltaproteobacteria, 2% and the emerging phylum Cyanobacteria and Planctomycete each with 1% abundance. There was also a presence of order Streptomycetales from Actinobacteria at 8% abundance. Genera from *Streptomyces* spp. were the most abundant indicating possible signs of plant pathogens in the areas as these species have the ability to synthesise metabolites such as antibiotics in the presence of antagonistic microbes (Vaz Jauri *et al.*, 2013). The phylum β -Proteobacteria showed an increase in abundance during oil palm at 4.5 and 5.5 years of age (Figure 8). The betaproteobacterial Burkholderiales seemed more abundant in oil palm plantations. The genera *Burkholderia* is one of the most important bacterial groups for nitrogen fixation in tropical soils, as a number of species are known to be plant associated nitrogen fixing bacteria (Estrada-De Los Santos *et al.*, 2001). Within γ -Proteobacteria, Xanthomonadales were the predominant order, however, their abundance was not significantly different between forest and agricultural ecosystems (Schneider *et al.*, 2015). Nemergut *et al.* (2010) revealed that the abundance of Bacteroidetes increased in fertilised agricultural soil compared to forest soils, as this group was positively correlated with soil pH.

The important minor phylum, Chloroflexi are bacteria that is widely abundant in the terrestrial and aquatic ecosystems (Fierer *et al.*, 2012) and the ecophysiological role of Chloroflexi is often associated with the nitrogen cycle, such as, nitrogen fixation and nitrite oxidation (Sorokin *et al.*, 2012). Another important minor phylum, Cyanobacteria is also a nitrogen fixer, increases phosphorous availability and releasing auxins, promoting plant growth (Sharma *et al.*, 2011). The number of photosynthetic Cyanobacteria increased in the oil palm plantations, probably due to the more exposed canopy of the oil palm plantations compared to rainforest, resulting in increased levels of light on the ground subsequently supporting growth of photosynthetically active bacteria. The fungi-like Myxococcales of the phylum δ -Proteobacteria, which is more abundant in the agricultural soils is another indication of increased utilisation of organic fertilisation (dung) within managed agricultural systems (Brenner *et al.*, 2005).

The minor phylum Gemmatimonadetes are ubiquitous in soil, however, they are reliant on moisture availability and studies reported members of this phylum are well adapted to low moisture environments (DeBruyn *et al.* 2011). Intercropping during these times may also increase population of functional rhizosphere soil bacteria, indicating a possible relation between increase yields of intercropping crops and microbes in comparison to monocropping (Yang *et al.*, 2016). This is evident

TABLE 5. LOCATIONS AND PHYSICO-CHEMICAL PROPERTIES OF SAMPLES STUDIED IN BELAGA, SARAWAK

Sites	Oil palm area (OP)	Biodiversity Strip 1 (S1)	Biodiversity Strip 2(S2)	Biodiversity Strip 3(S3)
Land type	Lowlands	Undisturbed secondary jungle, hilly area	Disturbed secondary jungle	Riparian area
Sampling year	October 2008* (logged-over forest)			
Rainfall		420 mm ³		
Moisture content	16.4 %	20.0%	4.3%	24.6%
pH	4.7	4.5	4.7	5.1
Sampling year	July 2009 (1.5 years oil palm)			
Rainfall		140 mm ³		
Moisture content	22.7%	35%	33.0%	16.0%
pH	4.8	4.4	4.3	5.1
Fertiliser application	March-April CIRP 0.5 kg	-	-	-
Sampling year	April 2010 (2.5 years oil palm)			
Rainfall		344.43 mm ³		
Moisture content	22.1%	40.3%	43.1%	31.5%
pH	4.7	4.6	4.2	4.6
Fertiliser application	May-June MPOB F1 0.8 kg	-	-	-
Sampling year	June 2011 (3.5 years oil palm)			
Rainfall		158.76 mm ³		
Moisture content	28.6%	34.6%	37.2%	-
pH	4.5	4.3	4.3	-
Fertiliser application	Feb-May-Aug-Nov MPOB F1 1.75 kg	-	-	-
Sampling year	July 2012 (4.5 years oil palm)			
Rainfall		302.770 mm ³		
Moisture content	26.9%	46.6%	43.1%	-
pH	4.9	4.5	4.2	-
Fertiliser application	Feb-May-Aug-Nov MPOB F1 1.75 kg	-	-	-
Sampling year	June 2013 (5.5 years oil palm)			
Rainfall		98.89 mm ³		
Moisture content	24.3%	36.3%	33.0%	-
pH	4.6	4.4	4.1	-
Fertiliser application	Feb-May-Aug-Nov MPOB F1 1.75 kg			

in the increase of microbial species number and variants shown, from 4.5 years to 5.5 years. (Table 4).

Under dry season during June 2013, the numbers of Actinobacteria was reduced, suggesting that increases in respiration caused by high temperatures (Luo, 2007) may be negatively affecting this phylum as they are well-known to be sensitive to increased CO₂ (Goodfellow and Williams, 1983). Furthermore, at 5.5 years old, the slight abundance of Acidobacteria might be the cause of reduced population in Actinobacteria, as these two phyla share the same niches. The slight increase in the presence of Nitrosomonadales at 2% abundance, a well-known, ammonia-oxidising bacteria (Shen *et al.*,

2012), also correlated with the increased treatment of fertiliser in intensively managed plantations in oil palm of 5.5 years old. The β -Proteobacteria was the phylum with the most increase in abundance of up to 30%, mostly from the order Burkholderiales. Burkholderiales has a range of roles in soil functions, such as, plant growth promoters (Estrada-De Los Santos *et al.*, 2001), nitrogen fixers (Gyaneshwar *et al.*, 2011) and biocontrol agents against plant pathogens. The phylum Bacteroidetes also increased to 10% abundance which played a role in plant growth promotion (Soltani *et al.*, 2010) and cellulose degradation (Verkhovtseva *et al.*, 2007), amongst other functions.

Relationships between Bacterial Community Structure with Different Oil Palm Age

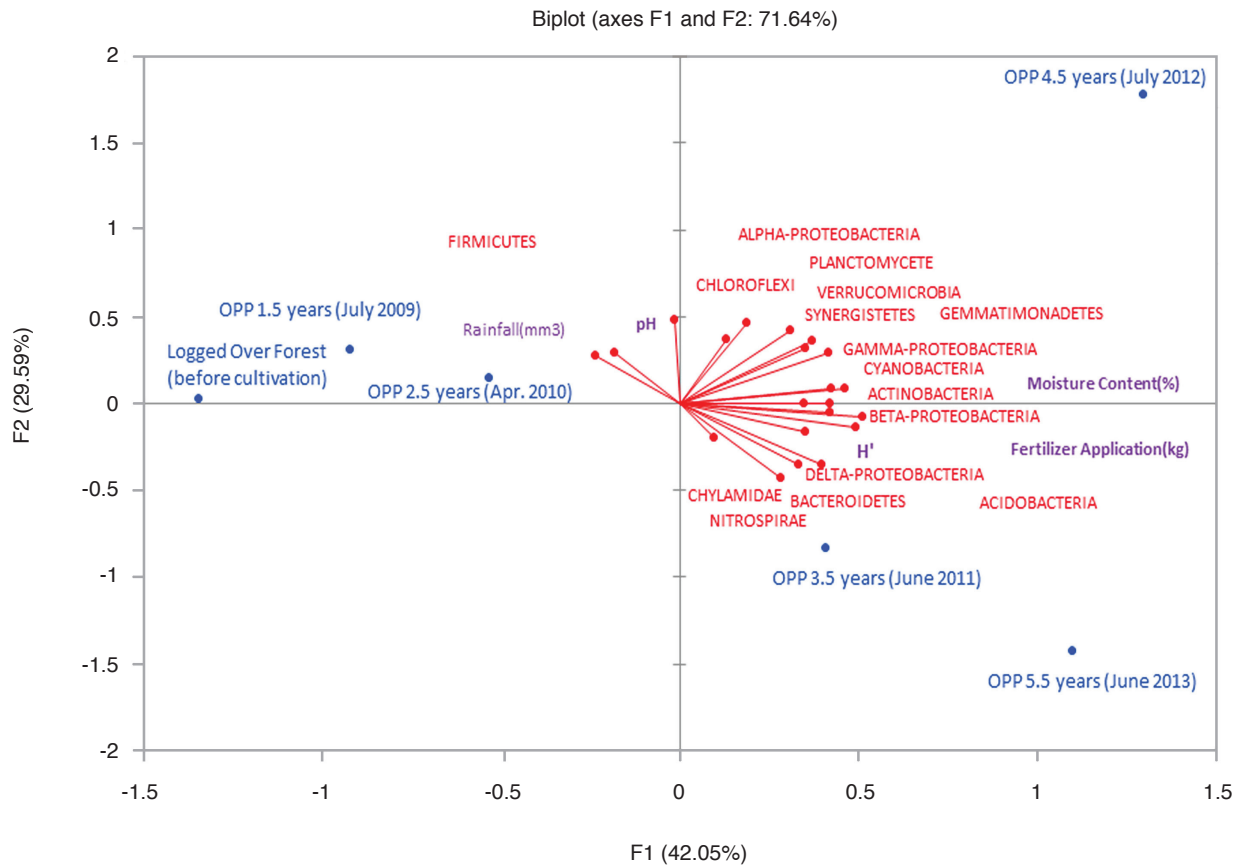
In the PCA, the analysis on the data collected on microbial community structure, biodiversity index and physico-chemical properties was carried out to see the correlation between age of oil palm, soil properties and prevalent types of microbial community present. The first and second principal components explain the substantial variation between these soil samples with prevalent phylum and soil properties, occurring in the oil palm soil (F1 varied by 42.05% while F2 varied by 29.59%). Microbial community structure and abundance with soil physico-chemical properties were correlated with the major axes of the different oil palm age (Figure 9). The upper right hand quadrant shows the increased abundance of phylum Synergistetes, Actinobacteria, Cyanobacteria, Gemmatimonadetes, Chloroflexi, Verrucomicrobia Planctomycete, Gammaproteobacteria and Alphaproteobacteria in 4.5-year old oil palms area. These groups are positively correlated with soil moisture content. Another positive correlation is with the phylum groups in the 3.5 and 5.5 years old oil palm, where this quadrant exhibits the increase abundance of Betaproteobacteria, Bacteroidetes, Acidobacteria, Deltaproteobacteria, Nitrospirae and Chytridiomycota. The abundance of these bacteria phylae are correlated with fertiliser applications (kg). Shannon-Weaver index (H'), had greater values in 3.5 and 5.5 years old oil palm. The last quadrant on the upper left consisting of early age oil palm of logged-over forest, 1.5 and 2.5 years old oil palm was only predominant with the phylum Firmicutes in correlation with rainfall and pH. Logged-over forest, furthestmost to the left had the least value in microbial biodiversity (H'). Overall, the clusters in PCA corresponds to the relative abundances of phylum occurring in the different oil palm age areas associated with soil parameters pH, moisture content, rainfall and fertiliser regime.

In this present study, it was shown that the bacterial composition in biodiversity strips, secondary logged-over forest was mostly predominated by Acidobacteria, followed by Actinobacteria and α -Proteobacteria whereas oil palm management areas towards oil palm aged 5.5 years were most commonly consisted of Firmicutes, β -Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes and Chloroflexi. In the oil palm plantation, there seem to be shifts in the bacterial community especially with the increasing number of the phylum Firmicutes, β -Proteobacteria, Bacteroidetes and decreasing number of α -Proteobacteria. The changes in abundance of these phylae is from the slight increase of pH values where it might be the reason for the most prominent observed changes within the Firmicutes,

β -Proteobacteria and α -Proteobacteria. Several studies indicated that soil bacterial community shifts after rainforest conversion were caused by fertiliser application, liming and reduction of plant diversity (Abdi *et al.*, 2016; Val-Moraes *et al.*, 2016; Liu *et al.*, 2017). We also detected the nitrification-related phylum Nitrospirae with genus Nitrospirales in oil palm aged 3.5 years, where this genus is exclusively found in the oil palm plantations (Lücker *et al.*, 2010; Ma *et al.*, 2013). This genus signifies higher degrees of nitrification in oil palm soils compared to rainforest, probably caused by fertiliser application and correspondingly nitrogen input, leading to more favourable conditions for nitrifiers (Ma *et al.*, 2013; Quan *et al.*, 2016). The fertiliser regime increased certain bacteria population such as Firmicutes which was prevalent in the oil palm area with 1.5-year old palms where the application of fertiliser increased the pH and phosphate level in the soil (Table 5).

The bacterial community composition data revealed that the phyla responding to agricultural land use were associated with soil parameters such as, pH, fertiliser regime and rainfall. In a newly developed oil palm plantation, pH factors affected several phyla especially Acidobacteria, where a slight increase would reduce its abundance whereas with increasing pH, the population showed an abundance of Firmicutes, β -Proteobacteria, γ -Proteobacteria, Chloroflexi, and Bacteroidetes. Slight disturbance in pH and addition of fertiliser during land-clearing for newly developed oil palm plantation altered the bacteria composition where Firmicutes largely dominated the 1.5-year oil palm area. The biodiversity index and bacterial community composition gradually increased in variation after land-clearing. Rainfall influenced the phylum group Actinobacteria during the dry season in June 2013, where higher temperature increased CO_2 in which this group is recognised to be sensitive to high levels of CO_2 (Goodfellow and Williams, 1983).

In agricultural soils, changes of soil physico-chemical factors introduced by field management such as repeated fertiliser application often increases the salt content in the soil, hence, altering the pH of the surrounding environment, and in consequence, affecting bacterial composition and probably changes in bacterial functioning. Even though this is the case, nutrient inputs by fertiliser improves overall plant growth, increases accumulation of soil organic matter leading to improvement in soil quality (Smith *et al.*, 2016). There is a need for further research in using sustainable methods such as a suitable intercropping system that can positively improve soil fertility and a balanced long-term usage of biofertilisers and chemical fertilisers, which can enhance the biodiversity of the bacterial community and contributes to the sustainable development of agro-ecosystems.



Note: H' - Shannon-Weaver Biodiversity index.

Figure 9. Relationships between microbial community structure with physico-chemical properties in different oil palm age in Belaga, Sarawak from principal components analysis (PCA) using Pearson correlation.

CONCLUSION

In summary, our study revealed that soil physico-chemical factors, especially pH, fertiliser application, rainfall and incorporation of intercropping systems were the key factors explaining the variance in bacterial communities, in the field management of oil palm. Shifts in the bacterial community especially with the increasing number of the phylum Firmicutes, β -Proteobacteria, Bacteroidetes and the decreasing number of α -Proteobacteria caused by the slight increase of pH values indicated that changes of soil properties introduced by field management (e.g., fertiliser application) somehow affects bacterial composition. Nevertheless, the sustainable method of intercropping oil palm with black pepper and pineapple, showed increasing emergence of variability in minor phylum groups which indicates this method as a sustainable system in the oil palm management. Overall, however, upcoming researches should also focus on functional changes related with shifts in the bacterial composition with developed methods in soil metagenomics or next generation sequencing, something not addressed in

this study. There's also a need to explore researches in sustainable methods in field management such as intercropping to improve fertility and balanced usage of organic or biofertiliser with chemical fertiliser to enhance the biodiversity of bacterial community which contributes to the sustainable development of oil palm plantations.

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