Response to Referee Comments:

We would like to express our gratitude to Ref #1 for the detailed comments and suggestions which helped to improve the manuscript significantly. Our point-by-point responses are posted below, with the reviewer's comments being quoted first and our response (R) below each comment.

General Comments

This study addresses the underlying factors that may influence the spatial and seasonal distribution of the prokaryotic communities and nutrient dynamics along the Rajang River, South China. Although the results of this study are valid and interesting, there are several points that need to be addressed.

1) Dividing sampling cruises into "wet season" and "dry season" may be more beneficial than referring to them individually. Authors mentioned both wet and dry seasons in the Methodology section (section 2.1), however, sampling cruises associated with each are lacking.
R: We agree that it would be beneficial to classify the sampling cruises into "dry season" and "wet season", however as the two "wet" seasons also differ in terms of its microbial community composition, we kept the individual cruises in order to prevent confusion between the two wet seasons. We have clarified the 'classification' of the three cruises as wet or dry season in the method section (it now reads: The August 2016 cruise (colored red) is classified as the dry season based on the lower mean rainfall value as compared to the other two (March 2017 and September 2017), in which the both are classified as the wet season (refer to Sup. Fig. 1).

2) The site map (Figure 1A) currently shows sampling points throughout the river with source types (Figure 1B), but illustration of anthropogenic activities along the river is missing. It will be helpful to add these as it's not clear which sections of the river are impacted by which activities.
R: Thank you for this. For the anthropogenic activities, the data was extracted from a report done by Wetlands International (2015) and is more a qualitative description. This description was then used for the classification of land use. The Fig 1(B) was intended only to show the zones of peatlands and not for the anthropogenic activities. We have added a third map which contains the requested information (Fig. 1C).

3) Additional statistical analysis, such as PERMANOVA, may be used to infer the impact of anthropogenic activities (e.g. human settlements,effluents, transportation and sand dredging) and source types on beta diversity. Much of the Results and Discussion sections revolve around alpha diversity indices but very little is mentioned about beta diversity.
R: Thank you for pointing this out. Beta diversity was in fact calculated and used for the discussion, however, obviously, not clearly pointed out. For example, the plotting of nMDS via PRIMER includes Kruskal-Wallis calculations (Kruskal stress formula: 1; Minimum stress: 0.01; 2-d: Minimum stress 0.18 occurred 21 times). Furthermore, the resemblance matrix was calculated using the Bray-Curtis dissimilarity measure. We have included this information in the methods section to reflect its inclusion in our analyses. Additional models have been run for the impact of land use on the microbial communities

and the discussion extended to address this point. New supplementary figures have been added as well (10-12)

4) Potential functionality inferred from PICRUST showed clear distinction between samples when comparing source type. It would be interesting to see if potential functionality differed too such an extent when samples are compared by anthropogenic perturbations.

R: Thank you for the suggestion. While comparing the potential functionality of the anthropogenic perturbations, there was not much variation across the different anthropogenic activities, hence this was not included in the results and discussion.

5) The general flow of the Discussion section needs improvement. The significance and contribution of the study will have a bigger impact when the Discussion is presented clear and logically. Also, the authors should double check the tense (present/past/passive) for each section.

R: Thank you for pointing this out. We have rearranged the discussion section whereby the bulk of the discussion was the drivers of microbial community composition and was separated into 3 sections, i.e. spatial and environmental drivers, seasonal drivers and anthropogenic drivers.

6) Recheck format of in-text references. Not all citations are written in the same format.

R: Thank you. We have checked through the in-text reference and changed those that have errors.

Specific Comments

1.0 Introduction p. 4 paragraph 6: The authors aimed to study microbial diversity and potential function in the Rajang River. Although this study is the first to investigate microbial diversity along a freshwater-marine gradient, with a tropical peatland component, the importance of the river in Malaysia and clear objectives need to stated.

R: Thank you for highlighting this. The last paragraph of the introduction was combined with the last few sentences from the previous paragraph. This paragraph now reads: "This study focuses on the Rajang River, which is the longest river in Malaysia and one of the most socio-economically important peat-draining rivers in South East Asia. It transports large amounts of terrestrial material (Müller-Dum et al., 2019) experiences two monsoonal seasons (Sa'adi et al., 2017) and is subjected to anthropogenic disturbances (Gaveau et al., 2016; Miettinen et al., 2016). Thus, it is fundamental to take into consideration both seasonal and anthropogenic influences on the microbial communities of the Rajang River. Given the rapid development in Sarawak and the importance of microbes in several biogeochemical processes in the Rajang river (Jiang et al., 2019; Martin et al., 2018; Müller-Dum et al., 2019; Zhu et al. 2019), it is imperative to study the microbial communities to enable future predictions and management responses. The Rajang river offers the opportunity to study the microbial diversity along a river to sea continuum and at the same time assess influence of natural conditions such as seasons (dry vs. wet), different soil types (peat vs. mineral soil), as well as anthropogenic disturbances (e.g human settlements and plantations) on microbial succession. This study aims to investigate (1) the microbial community structure, diversity and probable function across wet and dry seasons in order to (2) understand the underlying factors that may influence the spatial and seasonal distribution of the prokaryotic communities and the nutrient dynamics involved in the Rajang River."

2.0 Methodology 2.1 Study area and sampling strategy p. 5 line 136-139: "According to Wetlands International (2015), the land surrounding the study sites is characterized by a range of anthropogenic activities, ranging from oil palm and sago plantations to human settlements as well as transportation and sand dredging activities (Fig. 1(B))." This is not clear from Fig 1B. Colours are associated with forested or non-forested lands, however, the map does not depict the different anthropogenic activities along the river.

R: Thank you for this. For the anthropogenic activities, the data was extracted from a report done by Wetlands International (2015) and is more a qualitative description. This description was then used for the classification of land use. The Fig 1(B) was intended only to show the zones of peatlands and not for the anthropogenic activities. We have added a new figure highlighting the anthropogenic activities (1C).

2.1 Pyrosequencing and Bioinformatics Analyses. Change "Pyrosequencing" to "Illumina sequencing" in the subheading. The authors did not perform 454-pyrosequencing but Illumina sequencing. Did ACE also extract DNA from samples? If that's the case, the authors should mention this in the beginning of this section.

R: Agreed. "Pyrosequencing" was changed to "Illumina sequencing". Yes, ACE also extracted the DNA samples. This information was placed at the sentence before section 2.2.

3.1 2.4 Statistical Analyses and distLM model. The authors used db-RDA to determine the impact of environmental variables on microbial diversity. The same method can be used to determine which parameters have an influence on specific bacterial taxa. Likewise, Spearman/Pearson correlations can be drawn between environmental parameters and taxa. The information inferred from these additional analyses can help the authors to link certain taxa to specific source types or pollution sources.

R: We agree that this would be a good addition to the existing discussion and have carried out the suggested analyses. Spearman's ranking on the major taxa does support the key role of salinity shaping the microbial diversity.

I also suggest that anthropogenic inputs should be divided into the following categories: human settlements, effluents (from both palm oil and sago plantations), transportation and sand dredging. Variation partitioning, if possible, may then be used to determine which anthropogenic input, or source type, had the biggest impact on bacterial diversity along the river.

R: Thank you for this suggestion. We did indeed use variation partitioning for the distLM models. Unfortunately we do not think that we have sufficient data points from areas affected by sand dredging to be included in the model. Transportation of logs and sand by boats can be observed throughout the whole river, making it difficult to distinguish its impact between different sites.

3.0 Results 3.3 Bacterial Distribution according to source type and cruise This section may be improved by organizing it into the following paragraphs: Mention the dominant taxa and their relative abundances. The author mentioned this in the Discussion section (section 4.1 line 333-335) but not in the Results section.

R: Agreed, this was moved to the results section 3.3.

Which taxa (dominant or specialized) were more predominant at specific source types and/or seasons?

In this section, the authors acknowledge a higher Cyanobacterial abundance for the September 2017 marine and brackish peat samples. In the Discussion section they refer to the higher Cyanobacterial counts as "blooms" without prior testing (e.g. chlorophyll-a) as a proxy measure to confirm algal blooms. Since Cyanobacteria are more pronounced during warmer months, and anthropogenic activities close to the sampling areas can cause nutrient input and thus proliferation of Cyanobacteria, how valid is the assumption/statement made in the manuscript without additional measurements?

R: Thank you for pointing this out. There were two measurements for validating cyanobacterial abundance. One was the respiration experiment, which showed that there was greater respiration as compared to oxygen production. The other was phytoplankton identification via pigments using a software (CHEMTAX). Only two sampling cruises were available for the phytoplankton identification, which unfortunately for September 2017 is unavailable. However, between the dry (August 2016) and wet (March 2017) seasons, the wet season did indeed show greater counts of *Cyanobacteria*.

Alpha Diversity Indices: How did the authors calculate the effect of land use and source type on alpha diversity indices? This was not mentioned in the Methodology section.

R: The information "The alpha diversity was calculated using the estimate_richness function embedded within the plot_richness function found within the phyloseq package utilizing R (v.3.5.3)." is now included in Section 2.2

Instead of comparing indices between cruises, the authors can make comparisons between seasons (e.g. compare the entire wet season with the dry season).

R: Referring to the earlier explanation, we kept the individual cruises as the two wet seasons exhibited different microbial community composition, which warrants two separate cruises instead of the entire wet season.

Discussion: I recommend that the authors re-write certain paragraphs of the Discussion section so that it may have a bigger impact on readers. Instead of naming all the different types of taxa in the river, focus on the important ones and what their roles are. How does the environment and different inputs (source types and anthropogenic activities) impact these taxa?

R: Thank you for highlighting this. The sections were further subdivided whereby the main discussion was focused on the factors determining bacterial community composition and was subdivided into three components which are (4.2.1 Spatial and environmental drivers, 4.2.2 Seasonality as a driver of microbial community composition and 4.2.3 Land-use change and anthropogenic drivers.

Possible pathogenic bacteria and/or anthropogenic influence and land-use change. Was Flavobacterium the only potential pathogen identified? I would suggest to start the paragraph with anthropogenic influence and land-use change. A second, shorter paragraph can discuss the potential pathogens

R: The possible pathogenic bacteria identified were part of the CFB group. We grouped together the other information regarding *Proteobacteria* and *Bacteroidetes* as part of the paragraph relating to land use change and anthropogenic drivers.

C) Technical comments

1.0 Introduction p. 3: Combine paragraphs 2 and 5. Both are discussing lotic environments and nutrient cycling; it will thus make more sense to combine these two.

Response: Agreed, the two paragraphs were combined. This paragraph now reads: "Lotic environments are the interface between soil and aquatic environments and aquatic environments as terrestrial environments seed microbes into the adjacent water column due to flowing waters (Crump et al., 2012). Until not long ago, rivers were thought to be passive channels in the global and regional determination of carbon (C) and weathering products until it became clear that rivers regulate for example the transfer of nutrients from land to coastal areas (Smith and Hollibaugh, 1993). Several studies have shown that bacteria are key players in nutrient processing in freshwater systems (Cotner and Biddanda, 2002; Findlay, 2010; Madsen, 2011). Zhang et al. (2018a) stated that the organic matter composition is strongly modified by bacteria as well as its resistance to degradation. Bacteria strongly influence the fluvial organic matter, hence playing a role in carbon cycle (Dittmar et al., 2001) and recent studies in the Rajang river have demonstrated that as indicated by high concentrations of D-form amino acids (Zhu et al., 2019). Moreover, it was demonstrated by Jiang et al. (2019) that Dissolved Organic Nitrogen was reduced to NH4+ via mineralization and ammonification, again highlighting the biogeochemical activity and the importance of microbes in the Rajang River. Until now, there has, however, been no study on their diversity yet; a gap that this study aims to fill. Thus, it is essential to understand the dynamics and structure of microbial communities in them to assess their contribution towards biogeochemical fluxes such as carbon and nitrogen (Battin et al., 2008; Raymond et al., 2013), as well as phosphate cycling (Hall et al., 2013). In addition, the fluxes as well as transformations of organic matter as well as nutrients in aquatic systems are environmentally driven by parameters such as temperature or the availability of nutrients in these ecosystems (Welti et al., 2017). In turn, various gradients (i.e physical, chemical, hydrological or even biological) contribute to the changes in the microbial diversity and distribution living within the lotic environments (Zeglin, 2015)."

p. 4 line 93-93: Due to their high diversity and fast generation time, microbial communities are the first responders to environmental changes

R: Agreed and changed to recommended sentence.

p. 4 line 96: Liao et al. (2019) showed that p. 4 line 97: delete "further" p. 4 line 97: Bruland et al. (2008) demonstrated that the

R: Agreed, removed "further"

p. 4 line 99-102: "Thus, as the Rajang River experiences two monsoonal seasons (Sa'adi et al., 2017) and is subjected to anthropogenic disturbances (Gaveau et al., 2016; Miettinen et al., 2016), it is thus fundamental to take into consideration both seasonal and anthropogenic influences on the microbial communities of the Rajang River." This forms part of the aim and objective and should rather move to last paragraph

R: Agreed, this was changed to better reflect the aim and importance of the Rajang River.

p. 4 line 115: delete "hypothesized"

R: Agreed, removed "hypothesized"

p. 4 line 120-121: as well as anthropogenic disturbances (e.g. human settlements and plantations) on microbial succession.

R: Agreed, changed to recommended sentence.

p. 4 line 121-122: Delete "Linear models are

used to examine the relationship between the microbial community structure and their environment."

R: Agreed to remove sentence as it was already explained in methodology.

2.0 Methodology 2.1 Study area and sampling strategy p. 5 line 130: The region:

R: Agreed. Changed to "The"

p.5 line 134: small tributaries

R: Agreed, changed from "distributaries" to "tributaries"

p. 5 line 142: Which months were associated with the wet and dry season, respectively?

R: The following sentence was extracted from the caption of Sup. Fig. 1. " The August 2016 cruise (colored red) is classified as the dry season based on the lower mean rainfall value as compared to the other two (March 2017 and September 2017), in which the both are classified as the wet season." to be placed in-text in the methodology for ease of reference

p. 5 line 149: Approximately 250 – 500 mL of water: : :.

R: Agreed, changed to "approximately"

p. 5 line 153-156: A total of 117 filters were recovered (1 x 3.0 _m filter was discarded due to contamination) and immediately stored at - 20_C.

R: Agreed. The sentence was changed as recommended.

2.2 Pyrosequencing and Bioinformatics Analyses p. 5 line 160: Briefly, fastq files generated: : :

R: Agreed. "In short" changed to "Briefly"

p. 6 line 161: quality trimmed with fastqc, primer sequeces: : :.

R: Agreed, changed from "processed" to "quality trimmed"

p. 6 line 162-163: High quality sequences were subsequently processed using the Mothur pipeline.

R: Agreed. Changed to the recommended sentence.

p. 6 line 164: SILVA database

R: Agreed, "alignment" changed to "database"

p. 6 line 171: potential functional genes

R: Agreed, added "potential"

2.3 Physico-chemical Data and Geochemical Analyses p. 6 line 179: in-between the Cruises

R: Agreed, added "-"

p. 6 line 189-191: Belawai samples (2_13'47.16"N, 111_12'19.04"E) were used in an incubation experiment to study the net primary productivity and respiration rate of the Rajang River. Technical triplicates were incubated in both light and dark set-ups (Refer to Supp. Table 1 for details).

R: Agreed, the sentence was modified to the recommended sentence.

2.4 Statistical Analyses and distLM model p. 6 line 195-197: to determine if the various terrestrial source types or different land use impacted bacterial community composition.
R: Agreed, sentence structure was changed to the recommended.

p. 7 line 199: what type of normalization method was used?
R: The following sentence was added: "using the "Normalise Variables" function in the PRIMER 7 software".

p. 7 line 202-204: "The authors would like to note that the distLM models are based on only the August 2016 and March 2017 cruise as there was a lack of physico-chemical data from the September 2017 cruise due to malfunctioning equipment." Delete this sentence, no need to mention this twice, at the end of the paragraph (lines 205-208) is sufficient
R: Agreed, the sentence was removed.

p. 7 line 215: A total of 74,690 high quality bacterial sequences: : :.
R: Agreed and changed as recommended.

3.2 Shifts in bacterial community structure p. 7 line 223-224: Delete this sentence, it's Redundant
R: Agreed, the sentence was removed.

p. 7 line 230: August 2016 (dry season) samples
R: Agreed, added "(dry season)" to the sentence.

p. 7 line 231: September 2017 (wet season) samples
R: Agreed, added "(wet season)" to the sentence.

p. 7 line 231: There were clear overlaps between samples from:
R: Agreed, changed from "there are apparent" to "there were clear"

p. 7 line 232-233: We also observed a gradual shift in bacterial composition from mineral soils and freshwater peat towards brackish and marine samples.
R: Agreed, sentence was changed accordingly as recommended.

3.3 Bacterial Distribution according to source type and cruise p. 8 line 240: Delete "Fig 3 show that"
R: Removed as recommended but added (Fig. 3) to the end of the sentence.

3.4 Alpha Diversity Indices p. 8 line 263-264: Rewrite the sentence
R: Sentence was rewritten as "For the September 17 cruise, we observed increased values of Chao1 across the brackish peat, freshwater peat as well as mineral soils."

p. 8 line 265: microbial communities varied significantly along the different source types
R: Agreed, changed sentence as recommended.
p. 8 line 266: to be higher than that of March 2017:
R: Agreed, changed from "found in" to "of"

p. 9 line 276: Authors are referring to"upstream" samples in this sentence, which samples are these? They did not clearly differentiate between upstream and downstream samples in the Methodology section which is causing confusion in subsequent text.

R: Agreed. Added explanation at the end of the text: (i.e. Human Settlement, Oil Palm and Sago Plantation, Oil Palm Plantation and Secondary Forest).

p. 9 line 289-290: Potential KEGG pathways between (i) marine and brackish peat, and (ii) freshwater peat and mineral soil were similar. There were differences between source types and seasons

R: Agreed, the recommended sentence provided more clarity.

p. 9 line 290-292: Delete this sentence. It's part of Discussion

R: Agreed and removed.

p. 9 line 301: Dissolved Inorganic Phosphate (10.57%)

R: removed "at" and added parenthesis to "10.57%".

p. 9 line 304: Delete "lastly"

R: Agreed.

p. 9 line 305: Dissolved Inorganic Nitrogen (4.29%) respectively made up the

R: Agreed, changed from "(4.29%, respectively)" to "(4.29%) respectively

p. 10 line 308-309: Move this sentence to p. 9 line 300: "Marginal DistLM was performed in order to gauge the extent of physicochemical parameters or environmental variables accounting for a compelling proportion of variation in the bacterial communities. Significant vectors of environmental variables ($R2>0.3892$, $P<0.001$) were 308 calculated based on a linear model (DistLM) and plotted against the bacterial community composition as shown in Fig 7. Salinity was the single best predictor variable

R: Agreed and changed.

p. 10 line 311-320: The distLM model clustered samples from the August 2016 cruise separately from the March 2017 samples. Brackish peat, as well as marine samples from August 2016, correlated more strongly with salinity, irrespective of land use. On the contrary, the March 2017 samples were found to cluster separately with DO. In addition, the August 2016 mineral soil samples correlated with silicate.

R: Agreed and changed to suggested sentences.

p. 10 line 332: Delete this subheading and move subheading 4.2 to 4.1

R: Agreed, the remaining labels were corrected accordingly.

p. 10 line 335-342: in varying abundances, indicating high variation within the system. The majority of bacterial taxa were restricted to a relatively small number of assemblages. However, due to the heterogeneity of the Rajang River, substantial shifts in OTU diversity were shown, while exhibiting successional changes in community composition downstream. We observed abrupt shifts in terms of richness and diversity as well as bacterial distribution, which was structured according to macro-scale source types. Staley et al. (2015) proposed that variability in microbial communities were less due to the presence/absence but likely due to shifts in relative abundance of OTUs.

R: Agreed and changed.

p. 10 line 342: community composition, overlap between the core microbiome (i.e. free-living and particle-attached portions) of samples were not evident.

R: Agreed and changed.

p. 11 line 346: Change "further supported" with "demonstrated"
R: Agreed and changed.

p. 11 line 351: The short residence time in the Rajang River likely reflected a similar scenario to San Francisco Bay (Reference).
R: Agreed and changed.

p. 11 line 372-378: Delete these sentences. Beta-proteobacteria was already mentioned in the previous paragraph.
R: Agreed and removed.

p. 11 line 380: Were there really Cyanobacterial blooms?
R: Thank you for pointing this out. Cyanobacterial bloom was changed to "the higher abundance of *Cyanobacteria*", which more accurately describes the composition as shown by the abundance in taxa.

p. 12 line 385: Sphingomonas, a purple-sulfur bacteria,
R: Agreed and changed.

p. 12 line 391: indicating its preference for this environment. It's interesting to note that most studies on
R: Agreed and changed to recommended sentence.

p. 12 line 394: In most of these studies, Deinococcus-Thermus was found in low abundance (e.g. 1% in Antarctic marine environments, 1.5% in hypersaline soils; Giudice and Azzaro, 2019; Vera-Gargallo et al., 2019) when compared to the Rajang River.
R: Agreed and changed.

p. 12 line 397: Start new paragraph with: "There was a fundamental shift in bacterial community composition when taking the major taxa into consideration. There was a clear distinction between dry and wet seasons with an overall higher species richness and diversity for the dry season" For the wet season, focus on both the March and September cruises to make a conclusion
R: **Agreed and changed.**

p. 13 line 421-427: Delete these sentences, was already mentioned in Methodology
R: Agreed, the sentences were removed.

p. 13 line 427: There was a continual shift
R: Agreed, changed "is" with "was".

p. 13 line 432: similar to findings by
R: Agreed, changed "akin" to "similar"

p. 13 line 434: likely explaining the reduced relative abundances of some taxa. For example, Chloroflexi has a higher relative abundance upstream while Deinococcus-Thermus shows lower relative abundance downstream.

R: Agreed and changed to recommended sentence.

p. 13 line 438: Delete “salinity gradients”
R: Agreed and removed.

p. 13 line 451: Salinity, DIP () and dissolved oxygen are major environmental drivers of species distribution (References). In this study, marine and brackish peat samples correlated well with salinity.
R: Agreed and changed.

p. 14 line 459-469: Not sure what the authors want to say here. Do they assume there was high or low bacterial productivity?
R: We deduced that even though there was high abundance of associated phyla that may contribute to the production of O_2 (via primary production), the high CO_2 emissions and higher respiratory rate show that there was higher bacterial productivity versus primary production.

p. 14 line 478-480: While the development of unique community structures was strongly influenced by spatial factors, seasonality also played a role. Seasonal variability was also observed between the
R: Agreed and changed to recommended sentence.

p. 14 line 485-490: Again, can the term Cyanobacterial bloom be accurately used?
R: Thank you once again for pointing this out. The sentence was changed to “The greater abundance of Bacteroidetes in March 2017 may be indicative of the community composition adjusting due to the processing of organic material caused by the higher cyanobacterial abundance in the September 2017 cruise. This was similar to a study by Pinhassi et al., (2004), in which the higher abundance of Bacteroidetes follows after an algal bloom.”. This would reduce the assumption of a cyanobacterial bloom. The study quoted (Pinhassi et al. (2004)) was used as an example for probable inference of cyanobacterial bloom but cannot yet be confirmed.

p.15 line 494: “were similar in terms of climate”
R: Agreed, and changed.

p. 15: Start the paragraph with line 515: “The results obtained from this study suggest that the run-off from anthropogenic activities alters the microbial community composition. Anthropogenic disturbances, in particular settlements and logging (secondary forest), led to higher diversity indices (Fig .6). On the contrary, sites surrounded by oil
R: Agreed and changed. The breaking of paragraphs provide better clarity to the overall flow.

p. 16 line 543: The authors refer to “pristine and less pristine environments”. Which sites were classified as pristine, and which were less pristine?
R: Thanks for pointing this out. We have changed this to “anthropogenic perturbations (regions with oil palm plantations and human settlements) led to increased richness but less diversity compared to those that were less affected by anthropogenic perturbations (coastal zone and secondary forest).”

p. 16 line 545: The PICRUST results showed differences between source types

R: Agreed, changed "difference" to "differences"

p. 16 line 550: mixing experiments. This approach will contribute towards a better understanding of the response of microbial communities to anthropogenic perturbations, as well as their role in degrading peat-related run-off from

R: Agreed, and changed to suggested sentence.

We would like thank Ref #2 for the comments and suggestions which helped to improve the manuscript significantly. Our point-by-point responses are posted below, with the reviewer's comments being quoted first and our response (R) below each comment.

The manuscript of Sia et al. describes a study of bacterial communities' distribution in a section of the Rajang River. Overall, the quality and content of the paper is in line with similar publications on lotic bacterial communities, where the community composition is linked to environmental parameters. The strongest point of the study is that is covers multiple time points (different seasons) and several salinity zones. The authors also made an attempt to estimate potential functions of the bacterial communities. I would like to note a detailed and comprehensive Discussion section. However, some revision is necessary. Certain results need to be verified, methods described more in details (please see specific comments). English language could be improved; the manuscript is not free of mistakes and misprints.

Some specific questions and comments:

P 5 L 146 – it is not clear for me how is classification into freshwater and brackish water described in Fig. 1(B). Possibly that is due to the poor quality of the map.

R: Thank you for pointing this out. We removed this sentence "as described in Fig 1. (B)" as Fig. 1(B) is to show the areas with peat only.

P 5 L 150, 152 – Are you sure that those were polycarbonate filters? GF are usually glass fiber filters.

R: Thank you for pointing this out. The correct filter used was Nuclepore™ Track-Etched Polycarbonate Membrane Filter. We have removed the (GF/C) description.

P 5 L 156 – Incorrect reference. Caporaso et al. 2012 describe QIIME pipeline, not Illumina sequencing.

R: Agreed. Changed to Bentley et al. (2008) which describes the first paper that Illumina was based upon.

P 5 L 156 – Could you please add more information on DNA extraction and library preparation procedures, for example, which primers were used for amplification?

R: Thank you for pointing this out. We have included the relevant information in the methods section. It now reads: ….A total of 117 filters were recovered (1 x 3.0 µm was discarded due to contamination) and immediately stored at -20 °C and sent to the Australian Centre for Ecogenomics (ACE), Brisbane for DNA extraction, library preparation and processing utilizing the Illumina (Bentley et al., 2008) platform.

2.2 Illumina Sequencing and Bioinformatics Analyses

Initial upstream processes were carried out by the Australian Centre for Ecogenomics utilizing the ACE mitag pipeline (ACE, 2016). The primers utilized were based on the V3 – V4 hypervariable regions of the 16S rRNA gene.

P 6 L 163 – Reference for Mothur pipeline missing.

R: Thank you for pointing this out. The relevant citation was added (Schloss et al., 2009)

P 6 L 175 –Reference for the GreenGenes database missing.

R: Thank you for pointing this out. The relevant citation was added (DeSantis et al., 2006)

P 7 L 215 – Can you explain why the sequencing depth was so low, especially for some samples? Was it on purpose?

R: Thank you for this question. The minimum sequencing depth was 10,000 reads per sample. After QC and removal of unknown sequences, some samples were left with a very low read count. Given the general lack of data from these systems and to 'lose' as little information /samples as possible, we chose a low read number for the subsampling.

P 7 L 215 – Were the sequences deposited to a public database?

R: Raw sequences have been deposited with the NCBI BioSample database under BioProject ID PRJNA565954.

P 7 L 232 – Are you sure it is "brackish peat" and not "freshwater peat", which seems to me from Fig.2?

R: Yes, thank you for pointing this out. "Brackish peat" was changed to "freshwater peat"

P 8 L 247-249 – This observation is not obvious to me from Fig. 3.

R: Agreed, this portion was removed.

P 8 L 258-259 – was the difference between OTU counts statistically significant?

R: The results shown were plotted based on the calculations from the estimate_richness function in the *phyloseq* package, and hence the observation was more a qualitative observation.

P 10 L 324 – I didn't find any description of the results separately for free-living and particle-attached bacteria, however you discuss them a bit in chapter 4.1 in relation to Supp. Fig. 3. Were the results pooled together for free-living and particle-attached bacteria in Fig. 2-7?

R: Thank you for pointing this out. Yes, for Figures 2 – 7 the results were pooled together for discussion as the difference between free-living and particle-attached bacteria did not exhibit clear distinction and hence was not further elaborated. The following sentence was added in Section 2.2: "Apart from the results and discussion shown for free-living and particle-attached bacteria, the remaining discussion is based on the pooled results of both components"

P 11 L 378-380 – How does the dominance of Proteobacteria indicate its role in nitrogen cycling? Please explain how it is complementary to Cyanobacteria bloom, the message is unclear.

R: The sentence was rephrased as "In a study by Yang et al. (2013), the dominance of Protebacteria influenced the nitrogen cycle via the processes of nitrification and denitrification, in which aeration would increase its abundance and result in higher mortality of cyanobacteria.

P 12 L 394- 397 – "In contrast, most extreme environments show" this sentence sounds strange and needs to be rephrased.

R: Agreed, this sentence was changed to "In most of these studies, Deinococcus-Thermus was found in low abundance (e.g. 1% in Antarctic marine environments, 1.5% in hypersaline soils; Giudice and Azzaro, 2019; Vera-Gargallo et al., 2019) when compared to the Rajang River."

Biogeographical distribution of Microbial Communities along the Rajang River-South China Sea Continuum

Edwin Sien Aun **Sia**[1], Zhuoyi Zhu[2], Jing Zhang[2], Wee Cheah[3], Shan Jiang[2], Faddrine Holt Jang[1], Aazani Mujahid[4], Fuh-Kwo Shiah[5], Moritz Müller[1*]

[1]Faculty of Computing, Engineering and Science, Swinburne University of Technology, Sarawak Campus, Jalan Simpang Tiga, 93350, Kuching, Sarawak, Malaysia.

[2]State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Zhongshan N. Road 3663, Shanghai, 200062, China.

[3]Institute of Ocean and Earth Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia.

[4]Department of Aquatic Science, Faculty of Resource, Science and Technology, University Malaysia Sarawak, 93400 Kota Samarahan, Sarawak, Malaysia.

[5]Research Center for Environmental Changes, Academia Sinica, Taipei 11529, Taiwan

Corresponding Author*: Moritz Müller, mmueller@swinburne.edu.my

Abstract

The Rajang River is the main drainage system for central Sarawak in Malaysian Borneo which passes through peat domes whereby peat-rich material is being fed into the system and eventually into the southern South China Sea. Microbial communities found within peat-rich systems are important biogeochemical cyclers in terms of methane and carbon dioxide sequestration. To address the critical lack of knowledge about microbial communities in tropical (peat-draining) rivers, this study represents the first seasonal assessment targeted at establishing a foundational understanding of the microbial communities of the Rajang River-South China Sea continuum. This was carried out utilizing 16S rRNA gene amplicon sequencing via Illumina MiSeq in size-fractionated samples (0.2 and 3.0 μm GF/C filter membranes) covering different biogeographical features/sources from headwaters to coastal waters. The microbial communities found along the Rajang river exhibited taxa common to rivers (i.e. predominance of *β-Proteobacteria*) while estuarine and marine regions exhibited taxa that were common to the aforementioned regions as well (i.e. predominance of *α*- and *γ-Proteobacteria*). This is in agreement with studies from other rivers which observed similar changes along salinity gradients. In terms of particulate versus free-living bacteria, nonmetric multi-dimensional scaling (NMDS) results showed similarly distributed microbial communities with varying separation between seasons. Distinct patterns were observed based on linear models as a result of the changes in salinity along with variation of other biogeochemical parameters. Alpha diversity indices indicated that microbial communities were higher in diversity upstream compared to the marine and estuarine regions whereas anthropogenic perturbations led to increased richness but less diversity. Despite the observed changes in bacterial

community composition and diversity that occur along the Rajang River to sea continuum, the PICRUSt predictions showed minor variations. The results provide essential context for future studies such as further analyses on the ecosystem response to anthropogenic land-use practices and probable development of biomarkers to improve the monitoring of water quality in this region.

Keywords: particle-associated microbes, free-living microbes, 16S rRNA, Rajang river, River-sea continuum

1. Introduction

Biogeochemical transformations are primarily governed by microbial communities (Konopka, 2009), and it is crucial to understand their dynamics in order to predict biosphere modulations in response to a changing climate. Despite the importance of freshwater to society and despite hosting the highest microbial diversity (Besemer et al., 2013), microbial community composition and diversity in freshwater habitats, especially in lotic environments, are much less studied compared to marine and soil communities (Kan, 2018).

Lotic environments are the interface between soil and aquatic environments and aquatic environments as terrestrial environments seed microbes into the adjacent water column due to surface runoff (Crump et al., 2012). Until recently, rivers were thought to be passive channels in the carbon (C) cycling and weathering products until it became clear that rivers regulate for example the transfer of nutrients from land to coastal areas (Smith and Hollibaugh, 1993). Several studies have shown that bacteria are key players in nutrient processing in freshwater systems (Cotner and Biddanda, 2002; Findlay, 2010; Madsen, 2011). Zhang et al. (2018a) stated that the organic matter composition is strongly modified by bacteria as well as its resistance to degradation. Bacteria strongly influence the fluvial organic matter, hence playing a role in carbon cycle (Dittmar et al., 2001) and recent studies in the Rajang river have demonstrated that as indicated by high concentrations of D-form amino acids (Zhu et al., 2019). Moreover, it was demonstrated by Jiang et al. (2019) that dissolved organic nitrogen was mineralized to NH_{4+}, again highlighting the biogeochemical activity and the importance of microbes in the Rajang River. Until now, there has, however, been no study on their diversity yet; a gap that this study aims to fill. Thus, it is essential to understand the dynamics and structure of microbial communities in them to assess their contribution towards biogeochemical fluxes such as carbon and nitrogen (Battin et al., 2008; Raymond et al., 2013), as well as phosphorus cycling (Hall et al., 2013). In addition, the fluxes as well as transformations of organic matter as well as nutrients in aquatic systems are environmentally driven by parameters such as temperature or the availability of nutrients in these ecosystems (Welti et al., 2017). In turn, various gradients (i.e physical, chemical, hydrological or even biological) contribute to the changes in the microbial diversity and distribution living within the lotic environments (Zeglin, 2015).

Next-generation sequencing technologies have enabled a better understanding of the rare or unculturable biosphere which traditional culture methods would not have been able to elucidate (Boughner and Singh, 2016; Cao et al., 2017). Only few studies assessing bacterial community composition have been undertaken in lotic/riverine environments (Fortunato et al., 2012; Ladau et al., 2013; Zwart et al., 2002), with even less focusing on the diversity of surface-attached biofilms in lotic environments, particularly in comparison to biofilm studies in benthic habitats (Zeglin, 2015). Furthermore, bacterial assemblages on suspended particles were shown to differ from free-living

bacterioplankton in a number of studies (Bidle and Fletcher, 1995; Crump et al., 1999) in which the ratios between both fractions are often influenced by the quality of suspended particulate matter (Doxaran et al., 2012). Even less studies attempt to map bacterial community composition in a river-to-sea continuum across multiple seasons and habitats (Fortunato et al., 2012) and it was only recently reported that the most abundant riverine bacterioplankton resemble lake bacteria and can be regarded as 'typical' freshwater bacteria (Lozupone and Knight, 2007; Zwart et al., 2002). Metagenomics studies substantiated the dominance of *Proteobacteria* and *Actinobacteria* whereby *Bacteroidetes, Cyanobacteria, and Verrucomicrobia* were found also found to be abundant in rivers (Cottrell et al., 2005; Kolmakova et al., 2014; Lemke et al., 2009; Newton et al., 2011; Read et al., 2015; Staley et al., 2013). While there are studies related to the freshwater-marine gradients of rivers such as studies by Crump and Hobbie (2005) and Fortunato et al. (2013) and tropical peatlands (Kanokratana et al., 2011; Mishra et al., 2014; Yule et al., 2016; Too et al., 2018), to the author's knowledge, this is the first study which links both freshwater-marine gradients as well as tropical peatlands as a cohesive component (i.e. tropical peat-draining river to coastal ecosystem). Due to their high diversity and fast generation time, microbial communities (Hunt and Ward, 2015) are the first responders to environmental changes (both natural and anthropogenic events such as storms, upwelling and pollutants). Liao et al. (2019) showed that extensive agricultural land-use in the inter-tidal region of a watershed resulted in the prevalence of bacteria pathogen-like sequences whereas Bruland et al. (2008) stated that the assemblages of microbes also vary temporally as a function of oceanographic conditions, river discharge, tidal phase and season.

This study focuses on the Rajang River, which is the longest river in Malaysia and one of the most socio-economically important peat-draining rivers in South East Asia. It transports large amounts of terrestrial material (Müller-Dum et al., 2019), experiences two monsoonal seasons (Sa'adi et al., 2017), and is subjected to anthropogenic disturbances (Gaveau et al., 2016; Miettinen et al., 2016). Thus, it is fundamental to take into consideration both seasonal and anthropogenic influences on the microbial communities of the Rajang River. Given the rapid development in Sarawak and the importance of microbes in several biogeochemical processes in the Rajang river (Jiang et al., 2019; Martin et al., 2018; Müller-Dum et al., 2019; Zhu et al., 2019), it is imperative to study the microbial communities to enable future predictions and management responses. The Rajang river offers the opportunity to study the microbial diversity along a river to sea continuum and at the same time assess influence of natural conditions such as seasons (dry *vs*. wet), different soil types (peat *vs*. mineral soil), as well as anthropogenic disturbances (e.g human settlements and plantations) on microbial succession. This study aims to investigate (1) the microbial community structure, diversity and probable function across wet and dry seasons in order to (2) understand the underlying factors that may influence the spatial and seasonal distribution of the prokaryotic communities and the nutrient dynamics involved in the Rajang River.

2. Methodology

2.1 Study area and sampling strategy

This study was conducted along ~300 km of the Rajang river in Sarawak, Malaysia (**Fig. 1A**). The region has an equatorial climate characterized by constant temperatures, high extensive rainfall and high humidity (Wang et al., 2009, 2005; see also **Supp. Fig. 1**). The Rajang delta system consists of an alluvial valley, an associated coastal plain and a delta plain (Staub and Esterle 1993). The coastal plain is dissected into several small tributaries, namely Igan, Lassa, Paloh and Rajang (**Fig. 1A**). The shoreline experiences tides and seasonally strong waves ranging from 3 – 6 m with intensity increasing from the east to the west. According to Wetlands International (2015), the land surrounding the study sites is characterised by land use change (**Fig 1B)** and a range of anthropogenic activities, such as oil palm and sago plantations (**Fig 1C**), human settlements as well as transportation and sand dredge.

A total of 59 water samples were collected along salinity-gradients during three (3) cruises (**Fig. 1A**), covering both wet and dry seasons as well as different source types (i.e. mineral or peat soils). Source types sampled were grouped as follows: 1) marine 2) brackish peat 3) freshwater peat and 4) mineral soils. From Sibu towards Kapit (upriver), the riparian zone is mineral soil whereas from Sibu downwards to the coast it consists of peat which was then further divided into freshwater (salinity 0 to ~ 1 PSU) and brackish (salinity 2- 28 PSU). The August 2016 cruise (coloured red) is classified as the dry season based on the lower mean rainfall value as compared to the other two (March 2017 and September 2017), in which the both are classified as the wet season (refer to **Supp. Fig. 1**). The cruise in August 2016 represented the highest sampling frequency in order to obtain complete coverage of representative regions, while the cruises in March and September 2017 were aimed to obtain seasonal representatives for each region. Approximately 250 – 500 mL of water were filtered through 3.0 µm pore size track-etched membranes (Nucleopore™, Whatman, Germany) via vacuum filtration. This was referred to as the 'Particulate-attached' fraction. The filtrate from the 3.0 µm portion was collected in a sterile glass bottle and subsequently filtered through 0.2 µm pore size track-etched membranes (Nucleopore™, Whatman, Germany). The smaller fraction was referred to as 'free-living' fraction. A total of 117 filters were recovered (1 x 3.0 µm was discarded due to contamination) and immediately stored at -20 °C and sent to the Australian Centre for Ecogenomics (ACE), Brisbane for DNA extraction, library preparation and processing utilizing the Illumina platform (Bentley et al., 2008) .

2.2 Illumina Sequencing and Bioinformatics Analyses

Initial upstream processes were carried out by the Australian Centre for Ecogenomics utilizing the ACE mitag pipeline (ACE, 2016). The primers utilized were based on the V3 – V4 hypervariable regions of the 16S rRNA gene. Briefly, fastq files generated from the Illumina platform were quality trimmed with fastqc, primer sequences trimmed with Trimmomatic, and poor quality sequences removed using a sliding window of 4 bases with an average base quality of more than 15. High quality sequences were subsequently processed using the mothur (Schloss et al., 2009) pipeline. Sequences were aligned against the SILVA database (Quast et al., 2013; Yilmaz et al., 2014), 'pre.cluster' command executed for denoising, and chimeric sequences removed using the 'chimera.vsearch' function. Chimera-free 16s rRNA bacterial gene sequences were taxonomically assigned against the EzTaxon database (Kim et al., 2012) using the Naïve Bayesian classifier with a threshold of 80%. The quality-filtered sequences were then clustered into operational taxonomic units (OTUs) at 97% similarity cutoff with singleton OTUs being omitted. In order to reduce bias caused by variations in sample size, high-quality reads were randomly subsampled to 923 reads per sample. Apart from the results and discussion shown for free-living and particle-attached bacteria, the remaining discussion is based on the pooled results of both components. The alpha diversity was calculated using the *estimate_richness* function embedded within the *plot_richness* function found within the *phyloseq* package utilizing R (v.3.5.3). For the analyses of potential functional genes, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, Langille et al., 2013) was utilized. The metagenomics prediction table produced from PICRUSt was utilized to produce pathway abundance profiles using HUMAnN2 (Franzosa et al., 2018). It should be noted that the reconstructed functional genes were based on the GreenGenes (DeSantis et al., 2006) database and not the EzTaxon database used for the phylogeny. Raw sequences have been deposited with the NCBI BioSample database under BioProject ID PRJNA565954.

2.3 Physico-chemical Data and Geochemical Analyses

Monthly precipitation for the period in-between the cruises (August 2016 to September 2017) were obtained from the Tropical Rainfall Measuring Mission website (NASA, 2019) in order to gauge the seasonality (wet or dry; see **Supp. Fig. 1**). In the laboratory, nutrients (Nitrate, NO_3^-, Nitrite, NO_2^-, Ammonium, NH_4^+, Phosphate, PO_4^{3-} and Silicate, SiO_4^{4-}) were photometrically determined utilizing a SKALAR San^{plus} continuous flow analyser in the State Key Laboratory of Estuarine and Coastal Research (SKLEC), Shanghai (details described in (Sia et al., 2019). NH_4^+ and PO_4^{3-} were determined manually following Grasshoff et al., (1999). The total dissolved nitrogen, TDN, and total dissolved phosphate, TDP, were determined indirectly by obtaining the values for NO_3^- and PO_4^{3-} via oxidation with alkaline-persulfate solution (Ebina et al., 1983). The concentrations of dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) are estimated by subtraction of DIN from TDN and PO_4^{3-} from TDP, respectively. Belawai samples (2°13'47.16"N, 111°12'19.04"E) were used in an

incubation experiment to study the net primary productivity and respiration rate of the Rajang River. Technical triplicates were incubated in both light and dark set-ups (refer to **Supp. Table 1** for details).

2.4 Statistical Analyses and distLM model

Ordination visualization, non-metric multidimensional scaling (NMDS, Kruskal-Wallis: Kruskal stress formula: 1; minimum stress: 0.01), similarity analyses (ANOSIM) and coherence plots were executed using PRIMER 7 (Clarke and Gorley, 2015) to determine if the various terrestrial source types or different land use impacted the bacterial community. Permutational multivariate analysis of variance (PERMANOVA) was used based on the Bray-Curtis dissimilarity of Hellinger Transformed resemblance matrix to infer the impact of anthropogenic activities (land use) on the microbial communities. By partitioning the community variation (using a Bray-Curtis dissimilarity matrix resemblance), distance-based linear models (DistLM) were used to determine the extent of which the bacterial community structure can be explained by environmental variables (Legendre and Anderson, 1999). Normalizing transformations of the environmental variables were carried out prior to execution of DistLM analyses using the "Normalise Variables" function in the PRIMER 7 software. Hellinger Transformed OTU abundance table was used as the response variable for the variation partition analysis. The authors would like to note that the distLM models are based on only the August 2016 and March 2017 cruise as there was a lack of physico-chemical data from the September 2017 cruise due to malfunctioning equipment. Multi-collinearity between variables was tested utilizing the 'Draftsman Plot' function in Primer 7 (Clarke and Gorley, 2006; Supp. Fig. 1). However, it is sufficient to draw linkages between the major drivers of microbial communities between seasons as Mar 2017 and September 2017 were considered wet seasons based on the average precipitation (see **Supp. Fig. 1**).

3. Results

3.1 Clustering of Samples according to ANOSIM Global Test Scores

A total of 74,690 high quality bacterial sequences were obtained from a total of 117 samples, with 200 to 2,615 sequence reads per sample. The sequences were clustered into 2,087 OTUs at the 97% confidence interval. Instead of displaying bacterial diversity by station, bacterial communities were grouped together according to the R scores obtained from the ANOSIM Global test, with the parameters 'cruise', 'source type' and 'land use' showing the highest scores (ANOSIM Global $R = 0.737$, $P < 0.001$, **Table 1**). Furthermore, multi-variate analysis showed that the microbial community composition differed among the different land use as well as site nested with land use and source type (**Table 2**).

3.2 Shifts in bacterial community structure

The NMDS graph (2D stress score: 0.18, **Fig. 2**) supported ANOSIM results by clustering samples according to (i) source type and land use as well as (ii) cruises. The X axis (MDS1 scores) clearly reflects changes in terms of salinity (river-sea continuum) while the Y axis (MDS2 scores) emulates the different cruises. It is apparent that there were seasonal variations as shown from the lighter shade points, representing the August 2016 (dry season) samples, compared to those with darker shades representing both March 2017 and September 2017 (wet season) samples (**Fig. 2**). There were clear overlaps of samples from mineral soil and freshwater peat origin. We also observed a gradual shift of samples from mineral soils and freshwater peat towards brackish and then marine samples.

3.3 Bacterial Distribution according to source type and cruise

To further support that the four different source types support distinct bacterial communities, the relative abundance was mapped into a percentage plot (**Fig. 3**).

The core microbial communities along the Rajang River-South China Sea continuum consist of *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Deinococcus-Thermus* and *Cyanobacteria* in varying abundances (**Fig. 3, Supp. Fig. 4**), indicating high variation within the system. The phylum *Deinococcus-Thermus* was abundant in freshwater peat and in mineral soils, albeit at a lesser extent compared to freshwater peat (**Fig. 3)**. Taking into consideration seasonality, the relative abundance (%) of *Deinococcus-Thermus* drastically decreased in September 2017. Contrary, the abundance of *Cyanobacteria* was greater within marine as well as brackish peat for the cruises of March 2017 and September 2017 but not for August 2016. For the August 2016 cruise, *Cyanobacteria* were found throughout all source types albeit at lower counts compared to the other cruises. Similar changes in bacterial community were observed during different cruises but at different sections of the river. For the freshwater peat and mineral soils, the cruises of August 2016 and March 2017 had greater resemblance towards each other. Furthermore, there was a distinct split in terms of the bacterial community composition for the four source types across all sampling cruises i.e. marine and brackish peat had similar composition and freshwater peat and mineral soils had similar composition. In terms

of a river-sea continuum, the most apparent changes in the community composition were observed during March 2017 which presented an almost step-wise change in bacterial community composition.

3.4 Alpha Diversity Indices

Based on the observed indices (**Fig. 4**), mineral soils generally had the highest counts of unique OTUs. However, during the September 2017 cruise, the freshwater region had the highest values. Based on the Chao1 indices, there was a significant effect of the source type on the observed richness ($p<0.001$), with increasing values from marine to mineral soils. In the March 2017 and September 2017 cruise, the Chao1 indices were found to have greater variability as compared to the August 2017 cruise. For the September 17 cruise, we observed increased values of Chao1 across the brackish peat, freshwater peat as well as mineral soils. According to the Shannon indices, the diversity of the microbial communities varied significantly along the different source types ($p<0.001$). In the dry season the Shannon indices were found to be higher than that of March 17 and September 2017 samples, except for the Brackish peat September 2017 samples. In terms of the Simpson diversity indices, the August 2016 season was found to have the higher values as compared to the March 2017 and September 2017 season.

Based on the effects of land use on the diversity indices (**Fig. 5**), the sites which are surrounded by human settlements had higher observed indices (regardless of the cruise), with the exception of the Shannon indices in August 2016. Samples surrounded by secondary forest had the second-highest values with samples from August 2016 repeatedly higher than the other two cruises. There were significant differences ($p<0.001$) between samples from the coastal region with generally lower indices compared to upstream samples (i.e. Human Settlement, Oil Palm and Sago Plantation, Oil Palm Plantation and Secondary Forest).

3.5 Functional Profile of Bacterial Communities

Based on the potential KEGG pathways (**Fig. 6**), the functional profiles of the microbial communities were predicted for the Aug 2016 and Mar 2017 samples. The main functions found were oxidative phosphorylation (20.09%), carbon fixation pathways in prokaryotes (19.00%) and methane metabolism (18.36%), respectively. This was then followed by nitrogen metabolism (11.50%), carbon fixation in photosynthetic organisms (7.67%), inorganic ion transport and metabolism (5.68%). The remaining functional groups were photosynthesis, sulphur metabolism, inositol phosphate metabolism, phosphotransferase system (PTS), carbohydrate metabolism, phosphonate and phosphinate metabolism and lastly mineral absorption (4.92%, 4.31%, 2.96%, 2.34%, 1.83%, 1.11% and 0.23%, respectively). Clear differences were observed between source types and seasons and potential KEGG pathways displayed similar composition among samples originating from either (i) marine and brackish peat, or (ii) freshwater peat and mineral soil. In terms of gene abundances, the March 2017 samples (wet season) were found to have higher gene abundances with the highest counts in brackish peat followed by marine

samples. However, marine samples in August 2016 displayed slightly higher gene counts compared to the brackish peat.

3.6 Distance-based Linear Model of bacterial communities and environmental parameters

Marginal DistLM was performed in order to gauge the extent of physicochemical parameters or environmental variables accounting for a compelling proportion of variation in the bacterial communities. Significant vectors of environmental variables (R_2>0.3892, P <0.001) were calculated based on a linear model (DistLM) and plotted against the bacterial community composition (**Fig 7**). Salinity was the single best predictor variable explaining bacterial community variation (15.27%), followed by DIP (10.57%). The remaining physico-chemical parameters were dissolved oxygen (DO, 9.64%) and suspended particulate matter (SPM, 6.55%) whereas for the biogeochemical parameters, Silicate (9.27%), DOP (8.04%), DON (6.37%), dissolved organic carbon (DOC, 5.27%) and dissolved inorganic nitrogen (DIN, 4.29%) respectively made up the remaining variables (all variables P = 0.001, except for DIN, P=0.002).

The distLM model clustered samples from the August 2016 cruise separately from the March 2017 samples. Brackish peat, as well as marine samples from August 2016, correlated more strongly with salinity, irrespective of land use. On the contrary, the March 2017 samples were found to cluster separately with DO. In addition, the August 2016 mineral soil samples correlated with silicate.

4. Discussion

This study presents seasonal and spatial distribution of particulate-attached and free-living bacteria in the longest river in Malaysia in an attempt to map the bacterial community composition of the water column across several habitats with relation to the riparian zones and anthropogenic activities in a river-to-sea continuum. Our dataset develops a comparison of the microbial community across two dimensions: spatial biogeography from headwaters to the coastal zone as well as through time (seasonally). The rich supporting dataset also allows us to assess underlying nutrient dynamics influencing the microbial communities.

4.1 General diversity of core bacterial communities along the Rajang river-South China Sea continuum in comparison with global systems

The majority of bacterial taxa were restricted to a relatively small number of assemblages. Dominant phyla typically found in Malaysian peat swamps such as *Proteobacteria* (Kanokratana et al., 2011; Too et al., 2018; Tripathi et al., 2016) are found throughout the Rajang river whereas *Acidobacteria* is not a major phylum in the Rajang river. However, due to the heterogeneity of the Rajang River, substantial shifts in OTU diversity were shown, while exhibiting successional changes in community composition downstream. We observed abrupt shifts in terms of richness and diversity as well as bacterial distribution, which were structured according to macro-scale source types. Staley et al. (2015) proposed that variability in microbial communities were less due to the presence/absence but likely due to shifts in relative abundance of OTUs. While there were shifts in the community composition, overlap between the core microbiome (i.e. free-living and particle-attached portions) of samples were evident (**Supp. Fig. 2, Supp. Fig. 8**). The similar bacterial community structure in terms of particle association was in line with studies by Noble et al. (1997) and Hollibough et al. (2000) in the Chesapeake Bay (winter season) and San Francisco Bay, respectively. Hollibough et al. (2000) demonstrated that the difference or similarity of the particle association of bacterial community was due to the origin as well as composition of the particles, particularly in marine snow or estuarine particles. In the aforementioned study, there was limited metabolic divergence and similar communities between the estuarine turbidity maxima and the river samples. Due to the short residence time, the rapid exchange of organisms likely reduced the divergence of phylogenetic composition. The short residence time in the Rajang River likely reflected a similar scenario to San Francisco Bay (Müller-Dum et al. 2019). When comparing with other rivers, the predominance of the *Proteobacteria* phylum, especially within the brackish peat region (**Fig. 3, Supp. Fig. 4**) was similar to a recent study on the Pearl River Delta (Chen et al., 2019). In another study by Doherty et al. (2017) on the mainstem of the Amazon River (a blackwater influenced river, similar to the Rajang River), *Actinobacteria* were much more abundant (25.8%) compared to the Rajang River (11.95%).

4.2 Factors determining bacterial community composition

4.2.1 Spatial and environmental drivers

As shown in **Fig. 2**, it can be observed that there was a continual shift in microbial communities, suggesting mixing of the microbial communities from the headwaters to the coast (Fortunato et al., 2012) which has also been observed along the Upper Mississippi River (Staley et al., 2015) and along the Danube River (Savio et al., 2015). The decrease in richness and evenness was similar to a study conducted by Savio et al. (2015) in which the bacterial evenness and richness declined downriver, which was in line with the River Continuum Concept (Vannote et al., 1980). The presence of peat did not affect the alpha-diversity indices which was reflected in the shift in taxa occurring from freshwater (which includes freshwater peat) towards the saline region (which includes brackish peat).

Salinity, DIP and DO are major environmental drivers of species distribution (Peter et al. 2011; Wilhelm et al., 2015). In this study, marine and brackish peat samples correlated well with salinity. This was neatly supported by the distribution of samples on the distLM fitted dbRDA graph (**Fig. 7**) whereby the affinity for each of the samples correlates to the physical environment (e.g. the samples which group along the salinity vector were the samples which correlate with the marine as well as brackish peat region. The predominance of *β-Proteobacteria* in the freshwater region and the predominance of α- and γ-*Proteobacteria* (**Supp. Fig. 3**) in the estuarine region is typical as the main group in seawaters (Nogales et al., 2011) and similar to findings by Silveira et al. (2011) on the bacterioplankton community along the river-to-ocean continuum from the Parnaioca River towards the Atlantic Ocean. This shows that salinity exhibited a strong influence on the abundances of *Proteobacteria* and *Firmicutes*. Furthermore, based on the linear model (**Fig. 7**), salinity was an important factor in driving the shift in microbial communities (**Table 3**), similar to findings by Herlemann et al. (2011) along a 200 km salinity gradient in the Baltic Sea**.** The dispersal of taxa of microbial communities from fresh to marine waters faces a strong barrier due to salinity (Fortunato and Crump, 2015), likely explaining the reduced relative abundances of some taxa (**Fig. 3**). For example, *Chloroflexi* has a higher relative abundance upstream while *Deinococcus-Thermus* shows lower relative abundance downstream. Such dispersals are further influenced by transitional waters such as estuaries and plumes whereby the microbial communities are exposed to rapidly changing physic-chemical conditions such as nutrients, temperature as well as sporadic anthropogenic inputs (Crump et al., 2004).

While the distribution of the core microbial communities are indicative of the river-sea continuum, it is noteworthy that several phyla were distinctly associated with specific source types. The distinct shift in bacterial taxa for example from Freshwater to Brackish waters (and lack thereof between freshwater peat and brackish peat; **Fig. 3**) indicates that peat did not have a significant effect on the distribution of bacterial taxa. This was further supported by the fact that DOC (as a proxy for organic matter of peat origin) only accounts for 5.27% of the community variation (**Table 3**). A study on blackwater rivers in

990 the Orinoco Basin, Venezuela (Castillo et al., 2004) showed that increased DOC resulted in higher
991 bacterial production, however, the change in bacterial production was not a reflection of its influence
992 on the community composition. This was supported based on a simple respiration experiment conducted
993 in Aug 2016 (**Supp. Table 1**) whereby the respiration rate (0.44 ± 016 g DO L^{-1} d^{-1}) was higher than
994 that of the primary production rate (0.39 ± 0.08 g DO L^{-1} d^{-1}).
995
996 Samples influenced by DO (**Fig. 7**) are from the estuarine region which showed an almost anoxic zone
997 (refer to **Supp. Fig. 6**). The low availability of oxygen was mirrored in higher counts (samples
998 belonging to the brackish peat category showed highest counts regardless of phyla as well as season;
999 **Supp. Fig. 4**). However, higher counts (particularly the phylum *Chloroflexi* and *Cyanobacteria* which
1000 are normally associated with production of oxygen via primary productivity) do not reflect higher
1001 primary production within this zone. Zones of coastal estuaries are usually deemed to have higher
1002 primary productivity, however, it can be inferred that the depletion in oxygen and higher pCO_2
1003 emissions (Müller-Dum et al., 2019) within the brackish peat region of the August 2016 campaign was
1004 a result of high bacterial productivity. This can be further supported by the high SPM as a proxy of
1005 turbidity of the brackish peat (**Supp. Fig. 6**) which may have resulted in the reduced primary
1006 productivity, which in turn can explain the lower DO values. As aforementioned earlier, the respiration
1007 rate (0.44 ± 016 g DO L^{-1} d^{-1}) was higher than that of the primary production rate (0.39 ± 0.08 DO L^{-1}
1008 d^{-1}). This was similar to a study in the Scheldt River whereby the higher bacterial production occurred
1009 in the turbidity maxima together with the depletion of oxygen (Goosen et al., 1995).
1010
1011 **4.2.1.1 Functional potential of major taxa according to source types**
1012 In the Rajang River, the relative abundance of bacterial OTUs were higher in the estuary as well as
1013 marine region, reflecting that while the microbial communities are structured by salinity, the abundance
1014 was more a reflection of the nutrients available, especially in estuaries which exhibit circulation patterns
1015 which can result in localised nutrient-rich conditions (They et al., 2019). This was further supported by
1016 the higher relative abundance of oxidative phosphorylation genes as well as nitrogen metabolism within
1017 the brackish peat and further supported by Jiang et al. (2019) demonstrated through incubations studies
1018 whereby N transformations in the Rajang River estuary mixing zone was higher than in the Rajang
1019 River and coastal region. In a study done by Yang et al., (2013), the dominance of *Proteobacteria*
1020 influenced the nitrogen cycle via the processes of nitrification and denitrification, in which aeration
1021 would increase its abundance and result in higher mortality of *Cyanobacteria*. Hence, lower
1022 *Proteobacteria* abundance resulted in the higher abundance of *Cyanobacteria* which occur as evidently
1023 shown in **Fig. 3**. Furthermore, the higher presence of *Chloroflexi* (Ward et al., 2018) and *Cyanobacteria*
1024 (Guida et al., 2017) within the marine and brackish peat region indicated its probable role in carbon
1025 fixation as reflected by the higher gene counts (carbon fixation pathways in prokaryotes) in the marine
1026 and brackish peat regions as compared to the freshwater peat and mineral soil (**Fig .6)**. Furthermore,

the presence of the genus *Sphingomonas*, a purple-sulphur bacteria which were able to utilize carbon dioxide (carbon fixation pathways in prokaryotes) and oxidation of hydrogen sulphide (sulphur metabolism) (Pfennig, 1975) (**Fig. 6**). In the case of *Firmicutes*, the higher abundance of *Firmicutes* in the brackish region was reflective of the overall production as opposed to selective growth of the particular source type, as *Firmicutes* were found throughout all four source types. The highest level of *Deinococcus-Thermus* **(Fig. 3)** was found in freshwater peat environments, indicating its preference for this environment. This was interesting to note that most studies on bacterial community composition show that the phylum *Deinococcus-Thermus* occurs in a higher abundance in extreme environments such as in hot springs (Zhang et al., 2018b) or in studies that are analogous for Mars (Joseph et al., 2019). In most of these studies, *Deinococcus-Thermus* was found in low abundance (e.g. 1% in Antarctic marine environments, 1.5% in hypersaline soils; Giudice and Azzaro, 2019; Vera-Gargallo et al., 2019) when compared to the Rajang River.

4.2.2 Seasonality as a driver of microbial community compositition

While the development of unique community structures was strongly influenced by spatial factors, seasonality also played a role. When taking into consideration the major genera, there was a fundamental shift in bacterial community composition along the continuum (**Fig 3**, **Fig. 4**). The second-most abundant taxon, *Proteobacteria* (*β-Proteobacteria*) peaked during seasons of high discharge. The same pattern of peaking during high discharge can be observed in the Rajang River with considerably higher relative abundance in the wet season (Fig. 3) which could be a result of the intense rainfall that led to the large input of freshwater (Silveira et al., 2011), and ultimately resulting in a "trickling" over microbial pattern from the freshwater to the brackish region. Furthermore, there was a distinct difference in terms of bacterial richness and diversity indices between the dry season (August 2016) and both wet seasons, with September 2017 having higher observed indices while the March 2017, while being a wet season as well had lower or variable observed indices. This difference in the two wet seasons could be the due to the different stages of phytoplankton bloom as mentioned earlier whereby the September 2017 was during an algal bloom while the March 2017 was after an algal bloom event. This was reflected in the Simpson index as well as the indices for September 2017 being lower than those of the August 2016 or March 2017 samples. Similarly, Zhou et al. (2018) demonstrated that the Simpson Indices for bacteria increased after the onset of an algal bloom (Brackish peat, September 2017) whereas the Shannon indices was at the lowest (Brackish peat, March 2017) (when assuming that the region in which phytoplankton blooms occur was the brackish peat region). Overall, there was greater diversity (based on Shannon Indices) in the dry season (August 2016) than the wet seasons (March and September 2017) whereas there were greater OTUs in the wet season (Observed index).

Seasonal variability was also observed between the source types, particle association and down to the genus level (**Fig. 2**, **Supp. Fig. 2** and **Supp. Fig. 5**). Based on the precipitation as an indicator of the

seasonality, a probable "transitioning" phase was observed in the dry season (August 2016) with the microbial communities being more alike with the March 2017 samples (**Fig. 8**) when comparing both wet seasons (March 2017 and September 2017). Within the phylum rank **(Fig. 3)**, the presence of *Cyanobacteria* during the March and September 2017 cruises indicates the influence of seasonality. However, while March 2017 and September 2017 were both considered to be wet seasons based on the precipitation, in terms of the relative abundance, there are considerable differences between the two cruises. The greater abundance of *Bacteroidetes* in March 2017 may be indicative of the community composition adjusting due to the processing of organic material caused by the higher cyanobacterial abundance in the September 2017 cruise. This was similar to a study by Pinhassi et al., (2004), in which the higher abundance of *Bacteroidetes* follows after an algal bloom. Moreover, the shifts in community composition from August 2016 to March 2017 and from March 2017 to September 2017 are indicative of the influence of seasonality. While March 2017 and September 2017 were similar in terms of climate, September 2017 had higher precipitation during that month, which led to higher run-off from the riparian region as compared with the March 2017 wet season. This could have led to the increase in cyanobacteria, which was also reflected increase of picoplankton size class during the wet season where it was hypothesized that the September 2017 might be more optimal for picoplankton proliferation (**Supp. Fig. 7**). Furthermore, in comparison, August 2016 and March 2017 were similar in terms of the proportion of the relative abundance of the community composition (**Fig. 3**).

4.2.3 Land-use change and anthropogenic drivers

There has been little to no literature regarding the changes in microbial community composition as a result of land-use changes that occur within this region, particularly throughout the catchment area of the Rajang River. The results obtained from this study suggest that the run-off from anthropogenic activities alters the microbial community composition. The *Cytophaga-Flavobacterium-Bacteroidetes* group, or rather known as the CFB group, are commonly associated with humans (Weller et al., 2000), reflecting anthropogenic influences on the samples, especially within the brackish areas which has several human settlements and plantations. This was shown in the coherence plots in **Supp. Fig. 10** and **Supp. Fig. 11** whereby the CFB group in the *Bacteroidetes* phylum were more pronounced in areas with influence of oil palm plantations. Lee-Cruz et al. (2013) demonstrated that conversions of oil palm plantations from tropical forests are much more severe as compared to logged over forests in terms of bacterial community composition whereby logged over forests was shown to exhibit some resilience and resistance (to a certain extent). This was shown in the clustering of bacterial taxa adjacent to the oil palm plantation regardless of the source type (**Supp. Fig. 12**) in which the vector of *Flavobacteriia* fall under the samples of oil palm plantation in the brackish peat and to a certain extent, the vector of *Bacteroidia* along the oil palm plantation samples in the freshwater peat. Furthermore, among the identified possible pathogenic bacteria, according to Reza et al. (2018), the taxa *Flavobacterium* is a potential fish pathogen and is commonly found in freshwater habitats (Lee and Eom, 2017) as well as

coastal pelagic zones (Eilers et al., 2001). In the Rajang river, it was the sixth most abundant class (**Supp. Fig. 4**). This is cause for concern as it was found to be high in the coastal regions as well as brackish regions where fisheries and fishing activities are concentrated.

Anthropogenic disturbances, in particular, settlements and logging (secondary forest) led to higher diversity indices (**Fig .6**). On the contrary, sites surrounded by oil palm plantations displayed the lowest diversity indices, supporting results by Mishra et al. (2014) who found similar results in peatlands. Furthermore, the OTU overlapping of major anthropogenic activities (i.e. settlements and oil palm plantations) in **Supp. Fig. 9** reflected the possibility of higher abundance of generalists as compared to sensitive species (Jordaan et al., 2019) as microbial communities generally adapt to permanent stress events such as increased concentrations of inorganic or organic nutrients. In another study conducted by Fernandes et al. (2014), anthropogenically-influenced mangroves had 2x higher the amount of *γ-Proteobacteria* compared to pristine mangroves. This was similar to the March 2017 cruise along the Rajang River, whereby *γ-Proteobacteria* was the predominant class in the marine and brackish peat region along with the significant increase in *Bacteroidetes* as aforementioned, which can be associated to anthropogenic activities. On the other hand, during the dry season, the diversity of the "less-disturbed" region was higher than the disturbed regions. However, it should be noted that the coastal zone generally has the lowest richness and diversity amongst the other regions regardless of the presence or absence of anthropogenic activities. Hence, the extent of salinity intrusion may also result in the loss of diversity and richness of the microbial communities (Shen et al., 2018) in the Rajang River.

5. Conclusion

This study represents the first assessment of the microbial communities of the Rajang River, the longest river in Malaysia, expanding our knowledge of microbial ecology in tropical regions. The predominant taxa are *Proteobacteria* (50.29%), followed by *Firmicutes* (22.35%) and *Actinobacteria* (11.95%). The microbial communities were found to change according to the source type whereby distinct patterns were observed as a result of the changes in salinity along with variation of other biogeochemical parameters. Alpha diversity indices indicate that the microbial diversity was higher upstream as compared to the marine and estuarine regions whereas anthropogenic perturbations (regions with oil palm plantations and human settlements) led to increased richness but less diversity compared to those that were less affected by anthropogenic perturbations (coastal zone and secondary forest). The PICRUSt results showed differences between source types. Areas surrounded by oil palm plantations showed the lowest diversity and other signs of anthropogenic impacts included the presence of CFB-groups as well as probable algal blooms. In order to further gauge and substantiate the functional and metabolic capacity of the microbial communities within each specific source type, metaproteomics as well as metabolomics should be carried out along with mixing experiments. This approach will contribute towards a better understanding of the response of microbial communities to anthropogenic perturbations, as well as their role in degrading peat-related run-off from the surrounding riparian regions.

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Tables

Table 1: ANOSIM Global Test scores based on various parameters

Parameters tested, 999 permutations, random sampling	**ANOSIM Global Test, R**	**P value**
Cruise (Wet/Dry season)	0.439	0.001
Source Type	0.422	0.001
Land use	0.182	0.001
Particle Association	0.037	0.001
Source Type, Land use	0.415	0.001
Cruise, Source Type, Particle Association,	0.708	0.001
Cruise, Source Type, Land use	0.737	0.001

Table 2: Results of permutational multivariate analysis of variance (PERMANOVA)

Parameters tested, 9999 permutations, permutation of residuals under a reduced model	***df***	***F***	***P***
Land Use	7	1.54	0.0016
Site (nested with land use and particle attached)	33	2.27	0.0001
Site (nested with source type and land use)	13	2.60	0.0001

df represents degrees of freedom.

Table 3: Proportion of combined community variation based on marginal DistLM test that is explained by each predictor variable using two cruises (August and March 2017)

Category	**Variable**	**Pseudo-F**	***P*-value**	**Proportion explained (%)**
Physico-chemical parameters	Salinity	9.6128	0.001	13.42
	DO	6.6151	0.001	9.64
	SPM	4.3486	0.001	6.55
Biogeochemical parameters	DIP	4.2218	0.001	10.57
	Silicate	9.269	0.001	9.27
	DOP	5.4246	0.001	8.04
	DOC	3.4495	0.001	5.27
	DON	4.2218	0.001	6.37

Figure Captions

Fig. 1: Location of Rajang River within Sarawak, Malaysia (inset). (A) shows the stations sampled during three (3) different cruises; August 2016 (red triangles), March 2017 (blue circles) and September 2017 (cyan diamonds). (B) GIS data from 2010 (Sarawak Geoportal, 2018) indicating various forest types. Red colour represents non-forest areas (2010), yellow represents non-forest areas (2013), light green represents primary forests, teal represents secondary forests whereas dark green represents potential peat swamp forests.(C) Digitized NREB map obtained from Wetlands International, (2015). The map shows the plantation cover as determined from Landsat showing licensed oil palm and sago plantations (licensed).

Fig. 2*:* Non-metric Multi-dimensional Scaling (NMDS) graph of samples according to cruise, source type as well as land use.

Fig. 3: Relative abundance (%) of dominant bacterial (at phylum level, top 10) along the various source types (Marine, Brackish Peat, Freshwater Peat, Mineral Soils) across 3 cruises/seasons

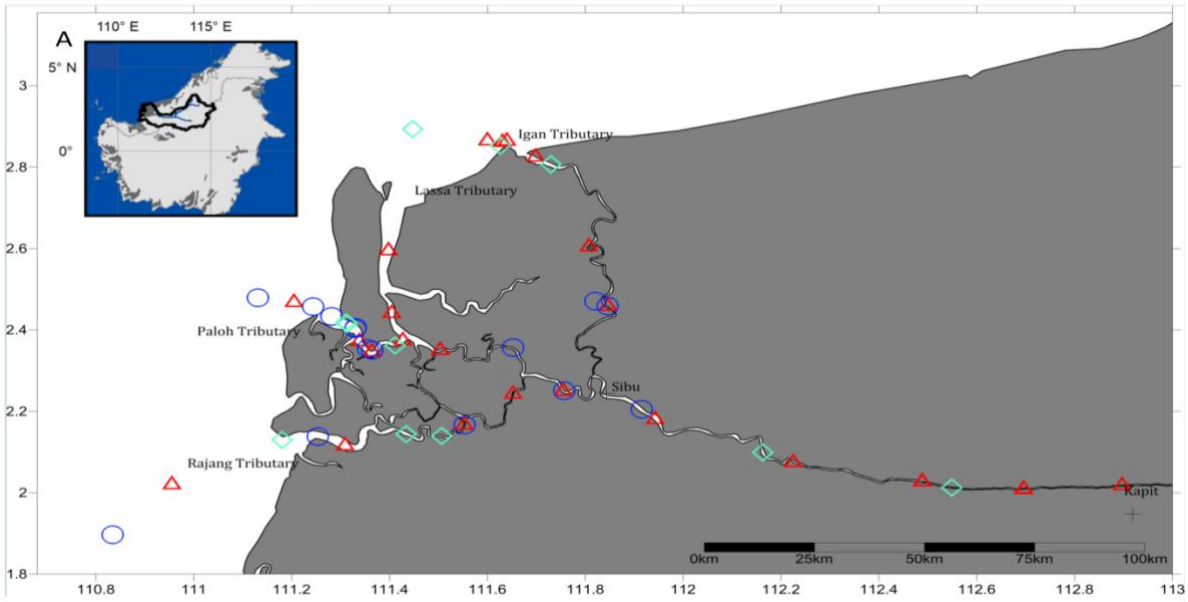
Fig. 4: The calculated α-diversity indices (Observed, Chao1, Shannon, Simpson and Inverse Simpson) of the four different source type along the salinity gradient.

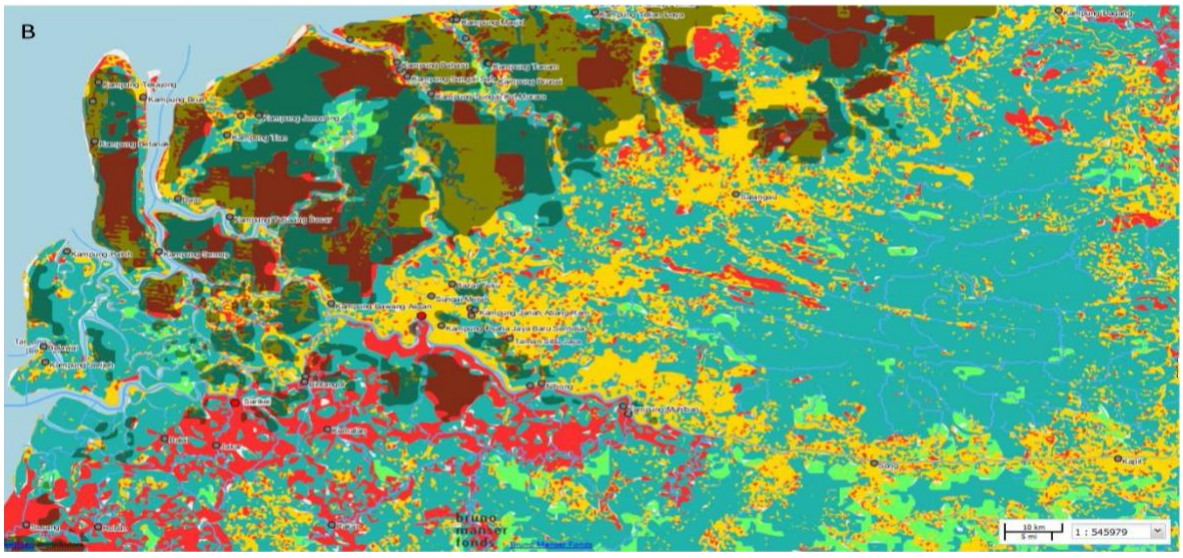
Fig. 5: The calculated α-diversity indices (Observed, Chao1, Shannon, Simpson and Inverse Simpson) of the Land Use types (Coastal Zone, Coastal Zone with Plantation (OP) influence) Coastal Zone with Plantation (Sago and Oil Palm influence), Human Settlement, Oil Palm and Sago mixed Plantation, Oil Palm Plantation and Secondary Forest)

Fig. 6: The relative abundance of predicted functional profiles in the four source types across two seasons based on KEGG Pathways

Fig. 7*:* Distance-based Redundancy Analysis (dbRDA) plot of cruise, source type and land use on a linear model (DistLM) of normalised predictor variables

Figures





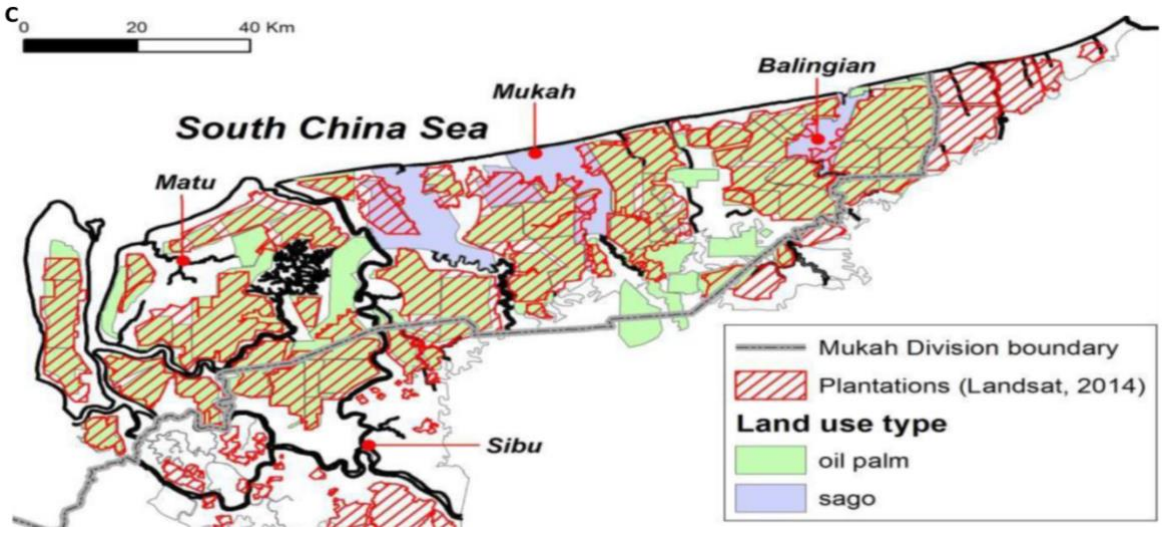


Fig. 1

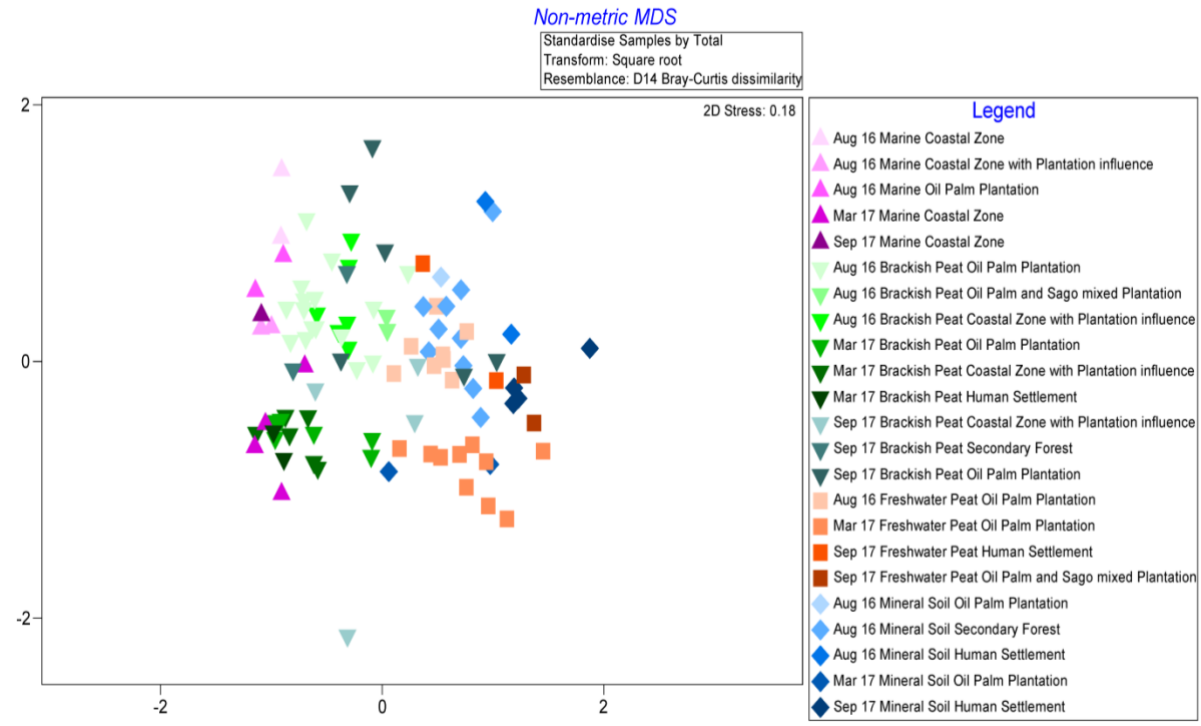

Non-metric MDS
Standardise Samples by Total
Transform: Square root
Resemblance: D14 Bray-Curtis dissimilarity
2D Stress: 0.18
Legend
Aug 16 Marine Coastal Zone
Aug 16 Marine Coastal Zone with Plantation influence
Aug 16 Marine Oil Palm Plantation
Mar 17 Marine Coastal Zone
Sep 17 Marine Coastal Zone
Aug 16 Brackish Peat Oil Palm Plantation
Aug 16 Brackish Peat Oil Palm and Sago mixed Plantation
Aug 16 Brackish Peat Coastal Zone with Plantation influence
Mar 17 Brackish Peat Oil Palm Plantation
Mar 17 Brackish Peat Coastal Zone with Plantation influence
Mar 17 Brackish Peat Human Settlement
Sep 17 Brackish Peat Coastal Zone with Plantation influence
Sep 17 Brackish Peat Secondary Forest
Sep 17 Brackish Peat Oil Palm Plantation
Aug 16 Freshwater Peat Oil Palm Plantation
Mar 17 Freshwater Peat Oil Palm Plantation
Sep 17 Freshwater Peat Human Settlement
Sep 17 Freshwater Peat Oil Palm and Sago mixed Plantation
Aug 16 Mineral Soil Oil Palm Plantation
Aug 16 Mineral Soil Secondary Forest
Aug 16 Mineral Soil Human Settlement
Mar 17 Mineral Soil Oil Palm Plantation
Sep 17 Mineral Soil Human Settlement

Fig. 2

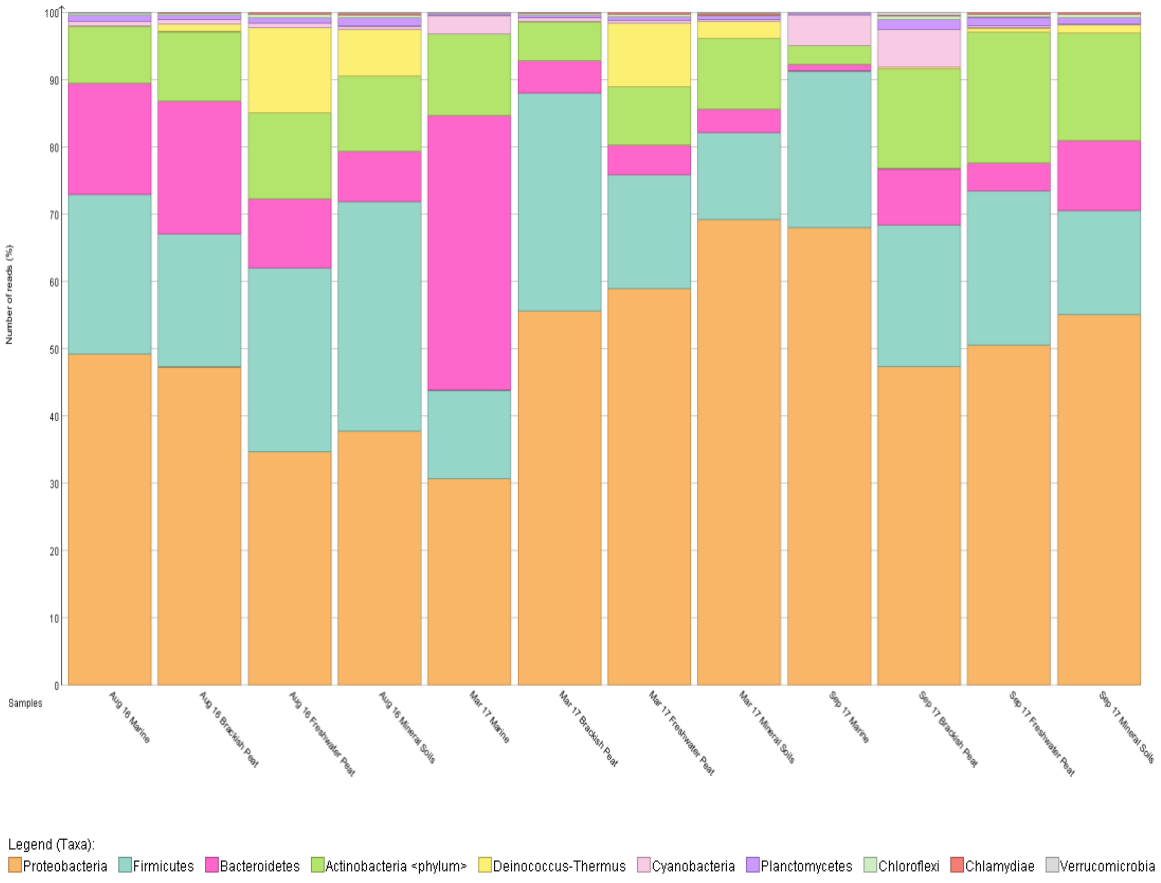
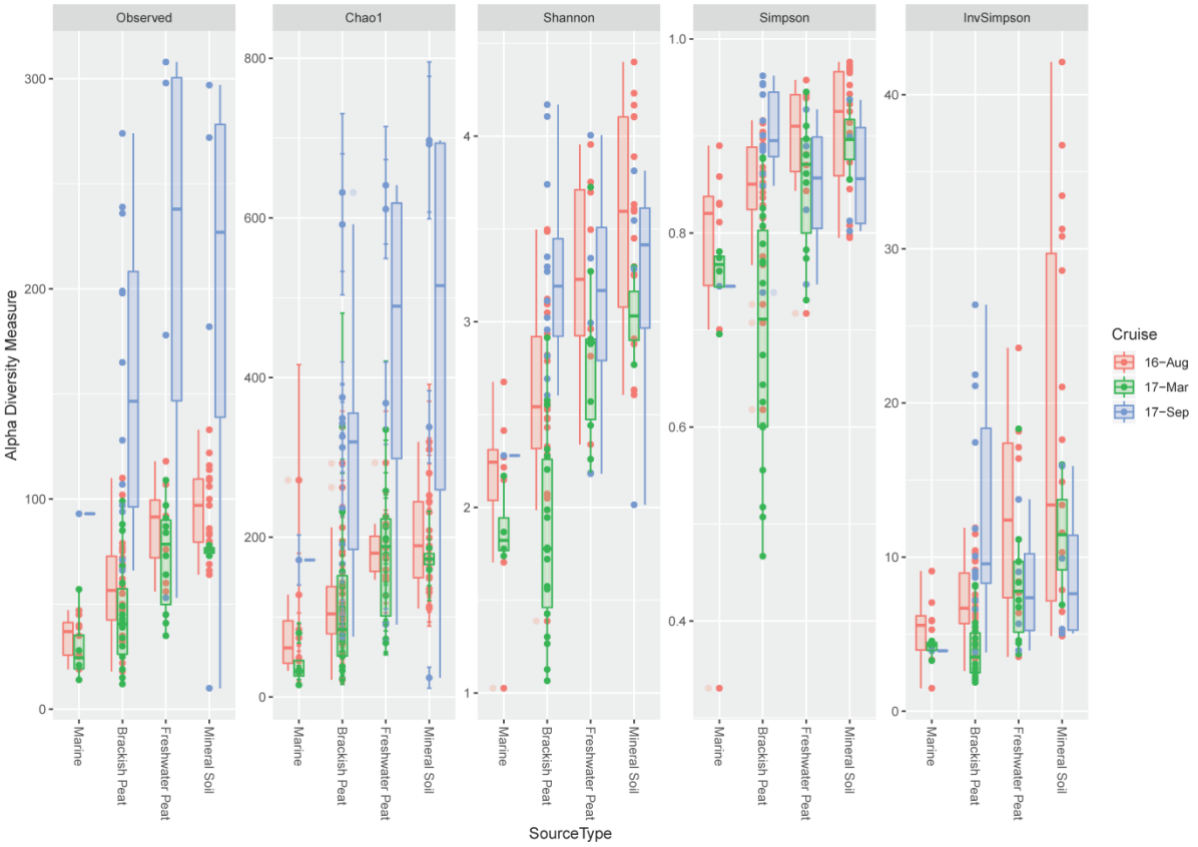

Number of reads (%)
Samples
Aug 16 Marine
Aug 16 Brackish Peat
Aug 16 Freshwater Peat
Aug 16 Mineral Soils
Mar 17 Marine
Mar 17 Brackish Peat
Mar 17 Freshwater Peat
Mar 17 Mineral Soils
Sep 17 Marine
Sep 17 Brackish Peat
Sep 17 Freshwater Peat
Sep 17 Mineral Soils
Legend (Taxa):
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria <phylum>
Deinococcus-Thermus
Cyanobacteria
Planctomycetes
Chloroflexi
Chlamydiae
Verrucomicrobia

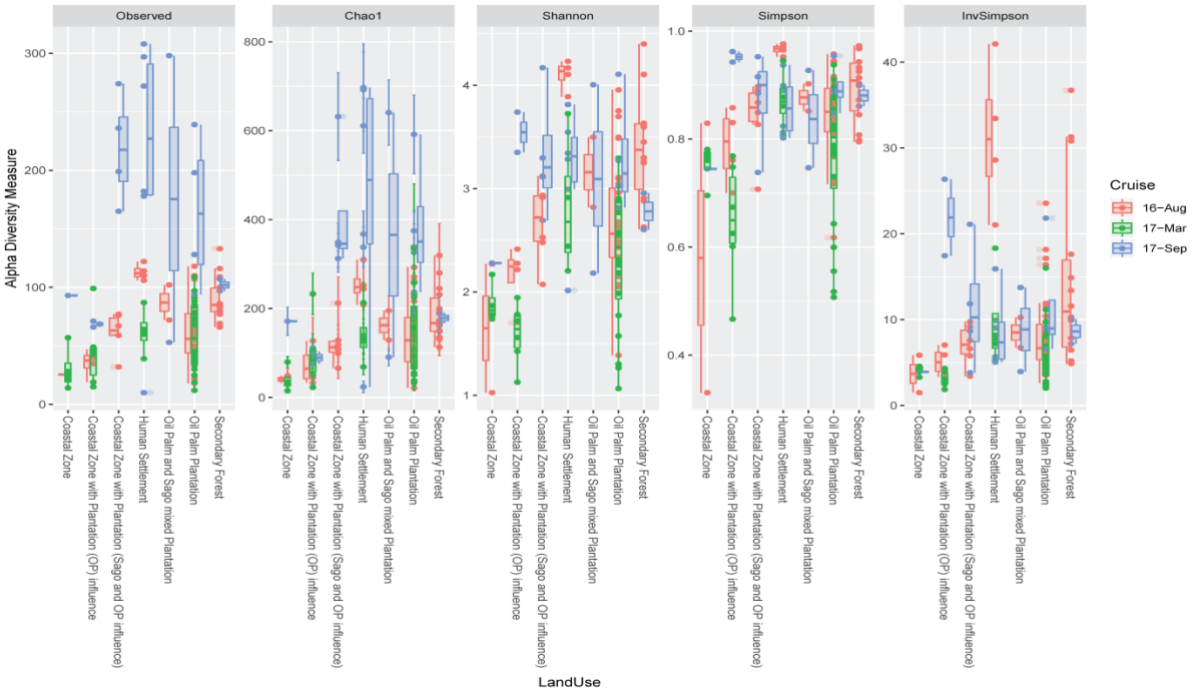
Fig. 3



1506

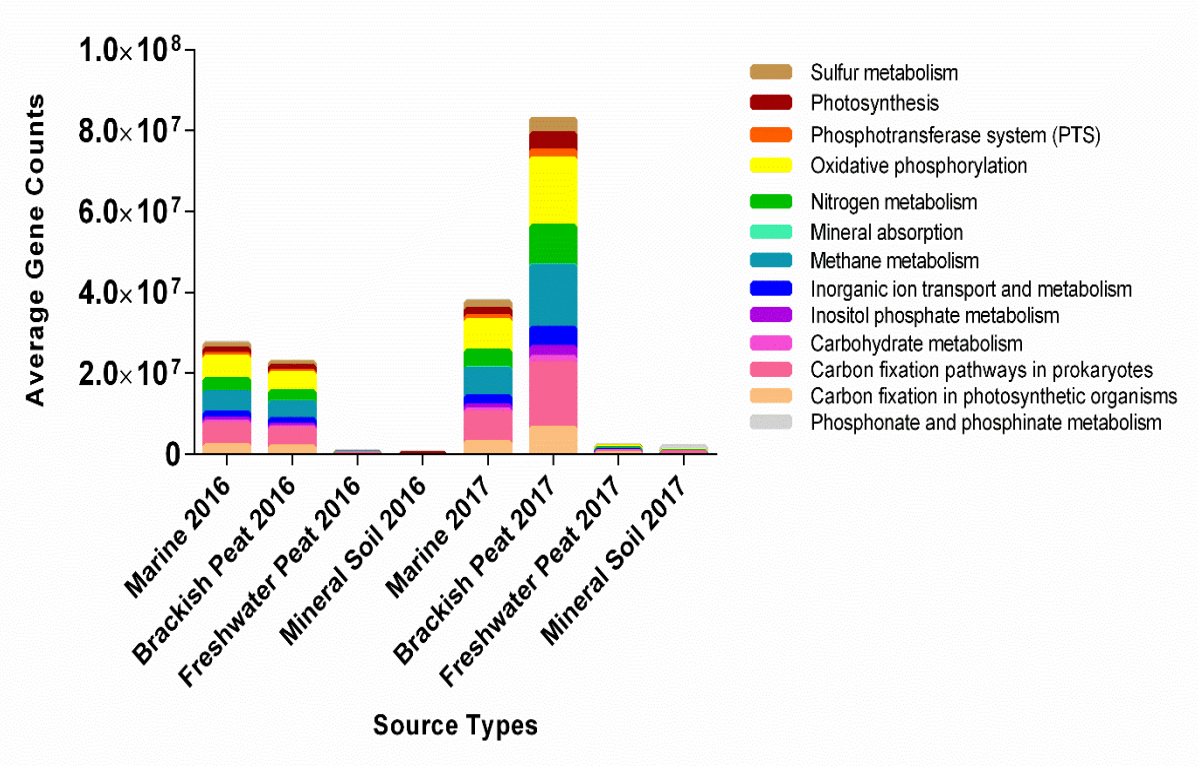
1507 **Fig. 4**

1508

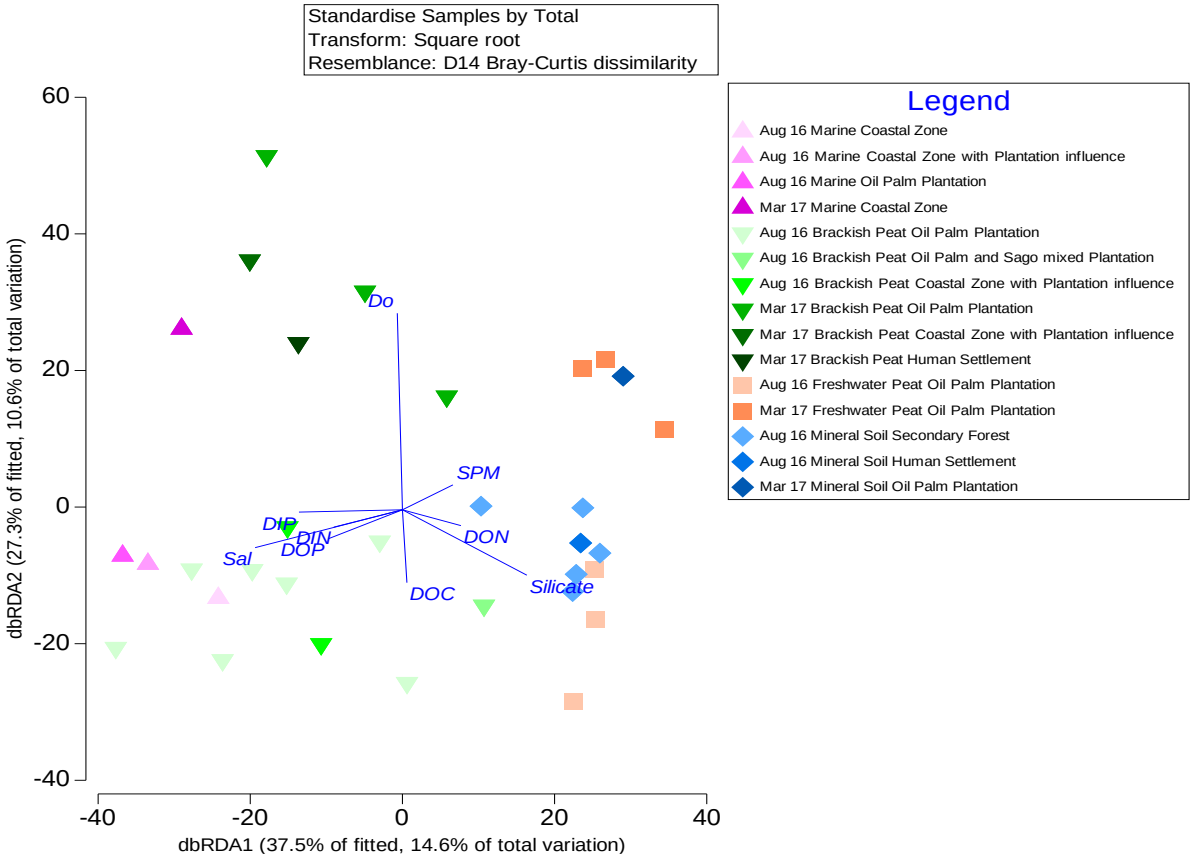


1509

1510 **Fig. 5**



1511 **Fig. 6**



1512

1513 **Fig. 7**