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

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Abstract

Microbial community composition and diversity in freshwater habitats, especially in lotic environments, are much less studied compared to marine and soil communities. The Rajang River is the main drainage system for central Sarawak in Malaysian Borneo and 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. the 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 the 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

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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 health in 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, River-sea continuum

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1.0 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 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 NH_4^+ 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.

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.,

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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 the high diversity and fast generation time, the first responders to environmental changes (both natural and anthropogenic events such as storms, upwelling and pollutants) are microbial communities (Hunt and Ward, 2015). Liao et al. (2019) show that extensive agricultural land-use in the inter-tidal region of a watershed resulted in the prevalence of bacteria pathogen-like sequences whereas further 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. 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.

Lotic environments are the interface between soil and aquatic environments as terrestrial environments seed microbes into the adjacent water column due to flowing waters (Crump et al., 2012). 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).

Given the rapid development in Sarawak and the hypothesized 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 such as plantations. Linear models are used to examine the relationship between the microbial community structure and their environment. This study aims to investigate (1) the microbial community structure, diversity and probable function across wet and dry seasons in order to (2)

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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

This study was conducted along ~300km of the Rajang river in Sarawak, Malaysia (Figure 1A). This 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, pdf). The coastal plain is dissected into several small distributaries, namely the Igan, Lassa, Paloh and Rajang tributary (Fig. 1(A)). 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 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)).

A total of 59 water samples were collected along salinity-gradients during three (3) cruises (**Fig. 1(A)**), 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)(as described in **Fig. 1(B)**).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. About 250 – 500 mL of water were filtered through 3.0 µm pore size polycarbonate filters GF/C (Cyclopore, 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 polycarbonate (GF/C) filters (Cyclopore, Whatman, Germany). The smaller fraction was referred to as 'free-living' fraction. All filters (117 in total as 1 3.0 µm filter was contaminated and discarded during the filtration process) were immediately stored at -20 °C and sent to the Australian Centre for Ecogenomics (ACE), Brisbane for processing utilizing Illumina (Caporaso et al., 2012) platform.

2.2 Pyrosequencing and Bioinformatics Analyses

Initial upstream processes were carried out by the Australian Centre for Ecogenomics utilizing the ACE mitag pipeline (ACE, 2016). In short, fastq files generated from the Illumina platform were

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processed 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. Subsequent processing steps were then performed utilizing the mothur pipeline. Sequences were aligned against the SILVA alignment (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. The alpha diversity was calculated using the *phyloseq* package R (v.3.5.3). For the analyses of 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 database and not the EzTaxon database used for the phylogeny.

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**). The analyses for nutrients encompassing both inorganic (i.e. Nitrate, NO_3^-, Nitrite, NO_2^-, Ammonium, NH_4^+, Phosphate, PO_4^- and Silicate, SiO_4^{4-}) and organic (dissolved organic nitrate, DON, and dissolved organic phosphate, DOP) fractions were photometrically determined utilizing a SKALAR San^{plus} continuous flow analyser in the State Key Laboratory for 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) while Total Dissolved Nitrogen, TDN, and Total Dissolved Phopshate, 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). An incubation experiment was set up to study the net primary productivity and respiration rate of the Rajang River. Triplicates of samples obtained from Belawai (2°13'47.16"N, 111°12'19.04"E) 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), and similarity analyses (ANOSIM) were executed using PRIMER 7 (Clarke and Gorley, 2015) to determine if for example the various terrestrial source types or different land use determine the structural differences of the bacterial community. By partitioning the community variation, distance-based linear models

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198 (DistLM) were used to determine the extent of which the bacterial community structure can be
199 explained by environmental variables (Legendre and Anderson, 1999). Normalizing transformations
200 of the environmental variables were carried out prior to execution of DistLM analyses. Hellinger
201 Transformed OTU abundance table was used as the response variable for the variation partition
202 analysis. The authors would like to note that the distLM models are based on only the August 2016
203 and March 2017 cruise as there was a lack of physico-chemical data from the September 2017 cruise
204 due to malfunctioning equipment. Multi-collinearity between variables was tested utilizing the
205 'Draftsman Plot' function in Primer 7 (Clarke and Gorley, 2006; Supplementary Fig. 1). The authors
206 would like to note that the distLM models are based on only the August 2016 and March 2017 cruise
207 as there was a lack of physico-chemical data from the September 2017 cruise due to malfunctioning
208 equipment. However, it is sufficient to draw linkages between the major drivers of microbial
209 communities between seasons as Mar 2017 and September 2017 were considered wet seasons based
210 on the average precipitation (see **Supp. Fig. 1**)
211
212
213 **3.0 Results**
214 **3.1 Clustering of Samples according to ANOSIM Global Test Scores**
215 74,690 high quality bacterial sequences were obtained from a total of 117 samples, with 200 to 2,615
216 sequence reads per sample. The sequences were clustered into 2,087 OTUs at the 97% confidence
217 interval. Instead of displaying bacterial diversity by station, bacterial communities were grouped
218 together according to the R scores obtained from the ANOSIM Global test, with the parameters
219 'cruise', 'source type' and 'land use' showing the highest scores (ANOSIM Global $R = 0.737$, $P < 0.001$, **Table 1**).
220
221
222 **3.2 Shifts in bacterial community structure**
223 The NMDS graph (2D stress score: 0.18, **Fig. 2**), supported ANOSIM results by clustering samples
224 according to (i) source type and land use as well as (ii) cruises.
225
226 The NMDS graph (2D stress score: 0.18, **Fig. 2**) supported ANOSIM results by clustering samples
227 according to (i) source type and land use as well as (ii) cruises. The X axis (MDS1 scores) clearly
228 reflects changes in terms of salinity (river-sea continuum) while the Y axis (MDS2 scores) emulates
229 the different cruises. It is apparent that there were seasonal variations as shown from the lighter shade
230 points, representing the August 2016 samples, compared to those with darker shades representing both
231 March 2017 and September 2017 samples (**Fig. 2**). There are apparent overlaps of samples from
232 mineral soil and brackish peat origin. It can also be observed that there is a gradual shift of samples
233 from mineral soils and freshwater peat towards brackish and then marine samples, with evident
234 transitioning between samples.

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236 **3.3 Bacterial Distribution according to source type and cruise**

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

239

240 **Fig 3** show that the phylum *Deinococcus-Thermus* was abundant in freshwater peat and in mineral
241 soils, albeit at a lesser extent compared to freshwater peat. Taking into consideration seasonality, the
242 relative abundance (%) of *Deinococcus-Thermus* drastically decreased in September 2017. Contrary,
243 the abundance of *Cyanobacteria* was greater within marine as well as brackish peat for the cruises of
244 March 2017 and September 2017 but not for August 2016. For the August 2016 cruise, *Cyanobacteria*
245 were found throughout all source types albeit at lower counts compared to the other cruises. Similar
246 changes in bacterial community were observed during different cruises but at different sections of the
247 river. For the marine and brackish peat portions, the cruises of March 2017 and September 2017 were
248 seen to be more similar to each other as compared to the August 2016 cruise with the anomaly of the
249 *Bacteroidetes* phylum. On the other hand, for the freshwater peat and mineral soils, the cruises of
250 August 2016 and March 2017 had greater resemblance towards each other. Furthermore, there was a
251 distinct split in terms of the bacterial community composition for the four source types across all
252 sampling cruises i.e. marine and brackish peat had similar composition and freshwater peat and
253 mineral soils had similar composition. In terms of a river-sea continuum, the most apparent changes in
254 the community composition were observed during March 2017 which presented an almost step-wise
255 change in bacterial community composition.

256

257 **3.4 Alpha Diversity Indices**

258 Based on the Observed indices (**Fig. 4**), mineral soils generally had the highest counts of unique
259 OTUs. However, during the September 2017 cruise, the freshwater region had the highest values.
260 Based on the Chao1 indices, there was a significant effect of the source type on the observed richness
261 ($p<0.001$), with increasing values from marine to mineral soils. In the March 2017 and September
262 2017 cruise, the Chao1 indices were found to have greater variability as compared to the August 2017
263 cruise. For the September 17 cruise, the values for Chao1 across the brackish peat, freshwater peat as
264 well as mineral soils were all observed to have increased values of Chao1. According to the Shannon
265 indices, the diversity of the microbial communities were significantly different along the different
266 source types ($p<0.001$). In the dry season the Shannon indices were found to be higher than that found
267 in March 17 and September 2017 samples, except for the Brackish peat September 2017 samples. In
268 terms of the Simpson diversity indices, the August 2016 season was found to have the higher values
269 as compared to the March 2017 and September 2017 season.

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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.

3.5 Functional Profile of Bacterial Communities

Based on the KEGG pathways (**Fig. 6**), the functional profiles of the microbial communities were predicted for the Aug 2016 and Mar 2017 samples. The metabolic pathways that were selected were based on the active pathways that were exhibited, including the metabolism of Nitrogen, Carbohydrate, Methane and Sulfur metabolism. 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). From **Fig. 6**, it can be seen that the functional gene profiles that were derived from the metagenomic profile were very similar. This was similar to a study by Fortunato and Crump (2015) who observed that the average similarities of the functional gene profiles were 82% from river to ocean. 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. Salinity was the single best predictor variable explaining bacterial community variation (15.27%), followed by Dissolved Inorganic Phosphate at 10.57%. The remaining physico-chemical parameters were dissolved oxygen (9.64%) and Suspended Particulate Matter (6.55%) whereas for the biogeochemical parameters, Silicate (9.27%), Dissolved Organic Phosphate (8.04%), Dissolved Organic Nitrogen (6.37%), Dissolved Organic Carbon (5.27%) and lastly Dissolved Inorganic Nitrogen (4.29%, respectively) made up the remaining variables (all variables $P = 0.001$, except for DIN, $P=0.002$).

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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 as shown in **Fig 7**.

From **Fig. 7**, the distLM model clustered samples from the August 2016 cruise away from the samples of the March 2017 cruise (as seen from the plot points with lighter shades as August 2016 and darker shades as March 2017). Samples originating from the brackish peat as well as marine region (August 2016) irrespective of land use were shown to cluster more strongly towards salinity (as shown from the longer vector from salinity) as well as DIN and DOP, followed by DIP. On the other hand, the brackish peat as well as marine samples from the March 2017 were found to cluster in between DIP and DO. In addition, the samples from August 2016 for freshwater peat and mineral soil -irrespective of land use- clustered towards silicate and DON whereas for March 2017, the samples were shown to cluster towards the SPM vector. Lastly, it was found that samples which are of peat origin were also adjacent to the DOC vector.

4.0 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 allows 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 bacterial community composition

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. 5**) indicate high variation within the system. 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. As shown in **Fig. 3** and **Supp. Fig .5**, the bulk 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, there were abrupt shifts in terms of richness and diversity as well as bacterial distribution which was structured according to macro-scale source types. While there were shifts in the community composition, based on the OTU overlaps, particle association of the samples were not apparent (**Supp. Fig. 3, Supp. Fig. 9**). The similar bacterial community structure in terms of particle association was in line with studies by Noble et al.,

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(1997) and Hollibough et al., (2000) in the Chesapeake Bay (winter season) and San Francisco Bay, respectively. Hollibough et al., (2000) further supported 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 as reported by Müller-Dum et al. (2019) likely reflected similar a similar scenario with the San Francisco Bay.

4.2 Diversity and shifts in bacterial communities along the Rajang river-South China Sea continuum

When comparing with other rivers, the predominance of the *Proteobacteria* phylum, especially within the brackish peat region (**Fig. 3, Supp Fig. 5**) 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%). However, the second-most abundant taxon was the *Proteobacteria* (*β-Proteobacteria*) which 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**). This 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. The predominance of *β-Proteobacteria* in the freshwater region and the predominance of α- and γ-*Proteobacteria* (**Supp. Fig. 4**) 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. Hence, this shows that salinity exhibited a strong influence on the abundances of *Proteobacteria* and *Firmicutes*.

Among the proteobacterial classes, γ-*Proteobacteria* was the most dominant, followed by α-*Proteobacteria*. The high abundance of γ-*Proteobacteria* is in line with Fuchsman et al. (2012) which states that the group is commonly regarded as particle-associated bacteria. When compared across the river-to-sea continuum, the low abundance of β-*Proteobacteria* is in contrast to other literature (Brown et al., 2015; Ghai et al., 2012) whereby the majority of freshwater systems has β-*Proteobacteria* as the most dominant taxa, as the determination takes into account the estuarine as well as the marine regions. The phylum *Proteobacteria* was dominant in all the samples, indicating its role in nitrogen cycling (Yang et al., 2013). The presence of *Protebacteria* in its role in nitrogen cycling is complementary to the *Cyanobacteria* blooms which occur as evidently shown in **Fig. 3**. Furthermore, the higher presence of *Chloroflexi* (Ward et al., 2018) and *Cyanobacteria* (Guida et al.,

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2017) within the marine and brackish peat region indicated its probable role in carbon fixation as reflected by the higher gene counts (carbon fixation pathways in prokaryotes) in the marine and brackish peat regions as compared to the freshwater peat and mineral soil (**Fig .6)**. Furthermore, the presence of the genus *Sphingomonas* indicated the presence of purple-sulfur bacteria which were able to utilize carbon dioxide (carbon fixation pathways in prokaryotes) and oxidation of Hydrogen Sulfide (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 presence of *Deinococcus-Thermus* **(Fig. 3)** was found in freshwater peat environments, indicating its preference for the aforementioned environment. This is interesting to note as 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 contrast, most extreme environments show that *Deinococcus-Thermus* was found in low percentages such as in Antarctic marine environments (1%, Giudice and Azzaro, 2019), 1.5% in hypersaline soils (Vera-Gargallo et al., 2019) as compared to the Rajang River. When taking into consideration the major genera, there is a fundamental shift in bacterial community composition along the continuum (**Fig 3**, **Fig. 4**) together with the bacterial richness and diversity indices, there was a distinct difference 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 is 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). 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 is in line with the River Continuum Concept (Vannote et al., 1980). The presence of peat did not affect the alpha-diversity indices which is reflected in the shift in taxa occurring from freshwater (which includes freshwater peat) towards the saline region (which includes brackish peat). 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.

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420 **4.3 Factors determining bacterial community composition**

421 While there is difficulty in assessing microbial communities in lotic environments due to the
422 heterogeneity of the physicochemical parameters that lotic environments are subjected to (Zeglin,
423 2015), the major drivers of microbial communities should still be assessed. While only two cruises
424 (August 2016 and March 2017) were used due to the lack of physico-chemical data for the September
425 2017 cruise, it is sufficient to draw linkages between the major drivers of microbial communities
426 between seasons as March 2017 and September 2017 were both considered wet seasons based on the
427 average precipitation (see **Supp. Fig. 1**). As shown in **Fig. 2**, it can be observed that there is a
428 continual shift in microbial communities, suggesting mixing of the microbial communities from the
429 headwaters to the coast (Fortunato et al., 2012) which has also been observed along the Upper
430 Mississippi River (Staley et al., 2015) and along the Danube River (Savio et al., 2015). Based on the
431 linear model (**Fig. 7**), salinity is an important factor in driving the shift in microbial communities
432 (**Table 2**), akin to findings by Herlemann et al. (2011) along a 200 km salinity gradient in the Baltic
433 Sea**.** The dispersal of taxa of microbial communities from fresh to marine waters faces a strong barrier
434 due to salinity (Fortunato and Crump, 2015), likely explaining the reduced relative abundances of
435 *Chloroflexi* upstream and in turn the reduced *Deinococcus-Thermus* downstream (**Fig. 3**). Such
436 dispersals are further influenced by transitional waters such as estuaries and plumes whereby the
437 microbial communities are exposed to rapidly changing physico-chemical conditions such as salinity
438 gradients, nutrients, temperature as well as sporadic anthropogenic inputs (Crump et al., 2004). While
439 the distribution of the core microbial communities are indicative of the river-sea continuum, it is
440 noteworthy that several phyla were distinctly associated with specific source types. The distinct shift
441 in bacterial taxa for example from Freshwater to Brackish waters (and lack thereof between
442 freshwater peat and brackish peat; **Fig. 3**) indicates that peat did not have a significant effect on the
443 distribution of bacterial taxa. This is further supported by the fact that DOC (as a proxy for organic
444 matter of peat origin) only accounts for 5.27% of the community variation (**Table 2**). A study on
445 blackwater rivers in the Orinoco Basin, Venezuela (Castillo et al., 2004) showed that increased DOC
446 resulted in higher bacterial production, however, the change in bacterial production is not a reflection
447 of its influence on the community composition. This was supported based on a simple respiration
448 experiment conducted in Aug 2016 (**Supp. Table 1**) whereby the respiration rate (0.44 ± 016 g DO L^{-1}
449 d^{-1}) was higher than that of the primary production rate (0.39 ± 0.08 g DO L^{-1} d^{-1}).

450

451 According to Peter et al. (2011) and Wilhelm et al. (2015)salinity, DIP (biogeochemical parameter)
452 and Dissolved Oxygen (physical parameter) had major impacts on the distribution of species. This is
453 neatly supported by the distribution of samples on the distLM fitted dbRDA graph (**Fig. 7**) whereby
454 the affinity for each of the samples correlates to the physical environment (e.g. the samples which
455 group along the salinity vector were the samples which correlate with the marine as well as brackish

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peat region. Samples influenced by dissolved oxygen (**Fig. 7**) are from the estuarine region which showed an almost anoxic zone (refer to **Supp. Fig. 7**). The low availability of oxygen is mirrored in higher counts (samples belonging to the brackish peat category showed highest counts regardless of phyla as well as season; **Supp. Fig. 5**). Higher counts (particularly *Chloroflexi* and *Cyanobacteria*) do, however, not reflect higher primary production within this zone. While zones of coastal estuaries are usually deemed to have higher primary productivity, it can be inferred that the depletion in oxygen and higher pCO_2 emissions (Müller-Dum et al., 2019) within the brackish peat region of the August 2016 campaign was a result of high bacterial productivity. This can be further supported by the high suspended particulate matter (SPM) as a proxy of turbidity of the brackish peat (**Supp. Fig. 7**) which may have resulted in the reduced primary productivity, which in turn can explain the lower dissolved oxygen values. As aforementioned earlier, the respiration 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} d^{-1}). This was similar to a study in the Scheldt River whereby the higher bacterial production occurred in the turbidity maxima together with the depletion of oxygen (Goosen et al., 1995). However, the relative abundance of bacterial OTUs were higher in the estuary as well as marine region, reflecting that while the microbial communities are structured by salinity, the abundance is more a reflection of the nutrients available, especially in estuaries which exhibit circulation patterns which can result in localised nutrient-rich conditions (They et al., 2019). This was supported by the higher relative abundance of oxidative phosphorylation genes as well as nitrogen metabolism within the brackish peat and further supported by Jiang et al. (2019) demonstrated through incubations studies whereby N transformations in the Rajang River estuary mixing zone was higher than in the Rajang River and coastal region.

While the development of unique community structures is strongly influenced by spatial factors, an influence of seasonality could also be observed with samples from March 2017 being distinctly different from the other two cruises (August 2016 and September 2017; **Supp. Fig. 3**). Seasonal variability was also observed between the source types, particle association and down to the genus level (**Fig. 2**, **Supp. Fig. 3** and **Supp. Fig. 6**). 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 following an algal bloom (Pinhassi et al., 2004). In the September 2017 season, it is probable that the time sampled was still during an algal bloom, as indicated by the higher abundance of *Cyanobacteria*. Moreover, the shifts in community composition from Aug 2016

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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 seasons, 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 is hypothesized that the September 2017 might be more optimal for picoplankton proliferation (**Supp. Fig. 8**). 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**).

4.4 Possible pathogenic bacteria and/or anthropogenic influence and land-use change

According to Reza et al. (2018) the taxa *Flavobacterium* is a potential fish pathogen which 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 is the sixth most abundant class (**Supp. Fig. 5**). 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. Furthermore, 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. 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). 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. However, the results obtained from this study evidently 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 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. 10** 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

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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.

5.0 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 led to increased richness but less diversity in the less pristine environments compared to those that were more pristine. Even though there were observed changes in bacterial community composition and diversity that occur along the Rajang River to sea continuum, the PICRUST predictions showed minor variations. 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 in order to further gauge the response of the microbial communities towards anthropogenic perturbations as well as the role of microbial communities in degrading peat-related run-off from the surrounding riparian regions.

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859 **Tables**

860

861 **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

862

863 **Table 2:** Proportion of combined community variation based on marginal DistLM test that is
864 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
	Dissolved oxygen	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
	DON	4.2218	0.001	6.37

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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.

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 based on a linear model (DistLM) and plotted against the bacterial community composition.

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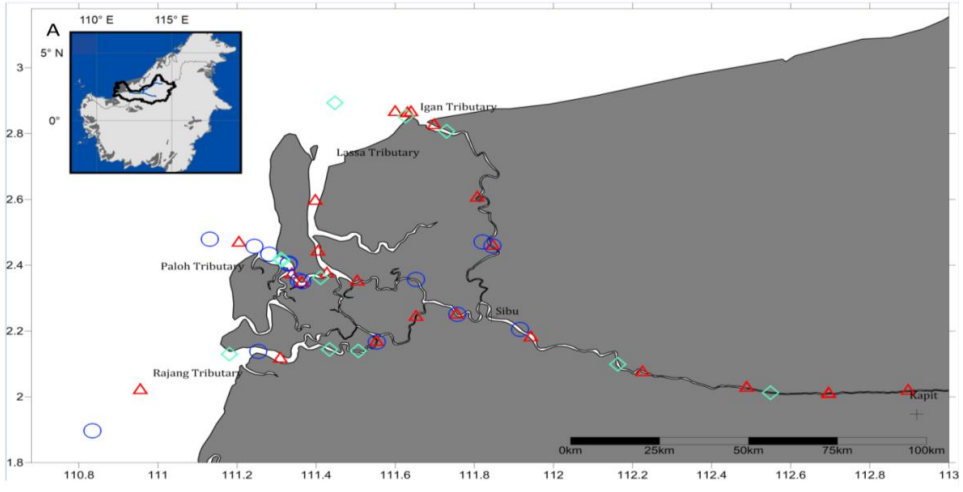
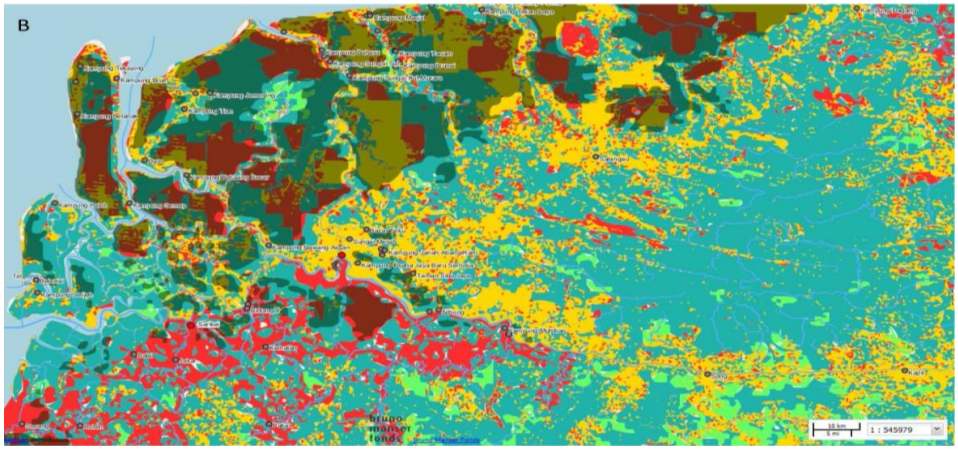
893 **Figures**

894 

895 

896 **Fig. 1**

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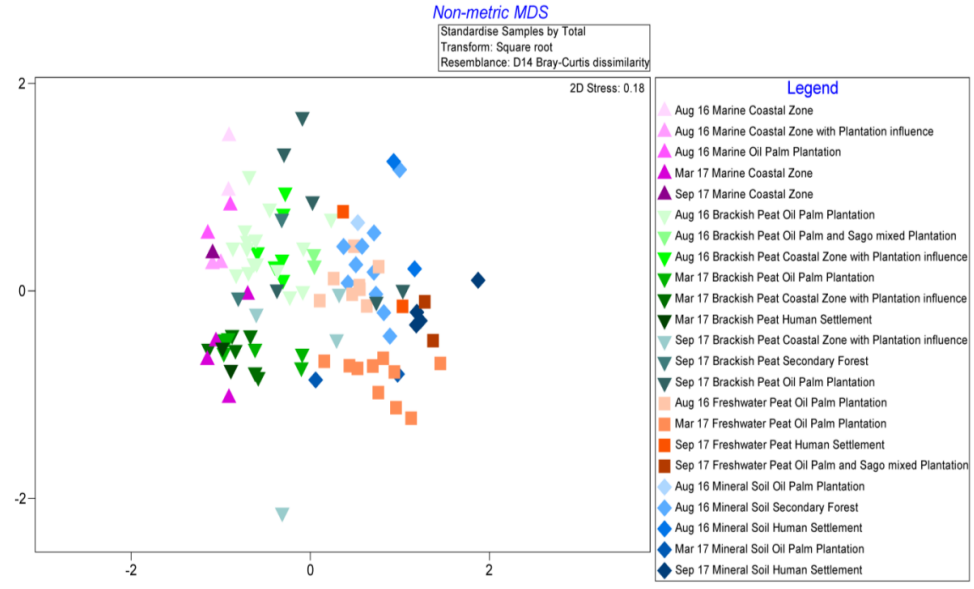

Fig. 2

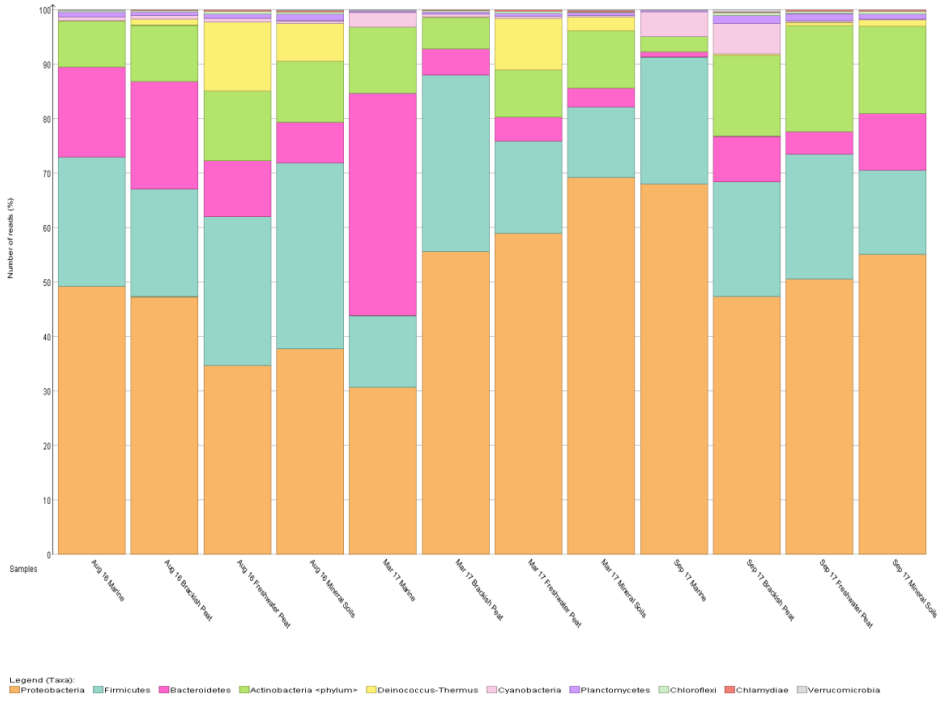

Fig. 3

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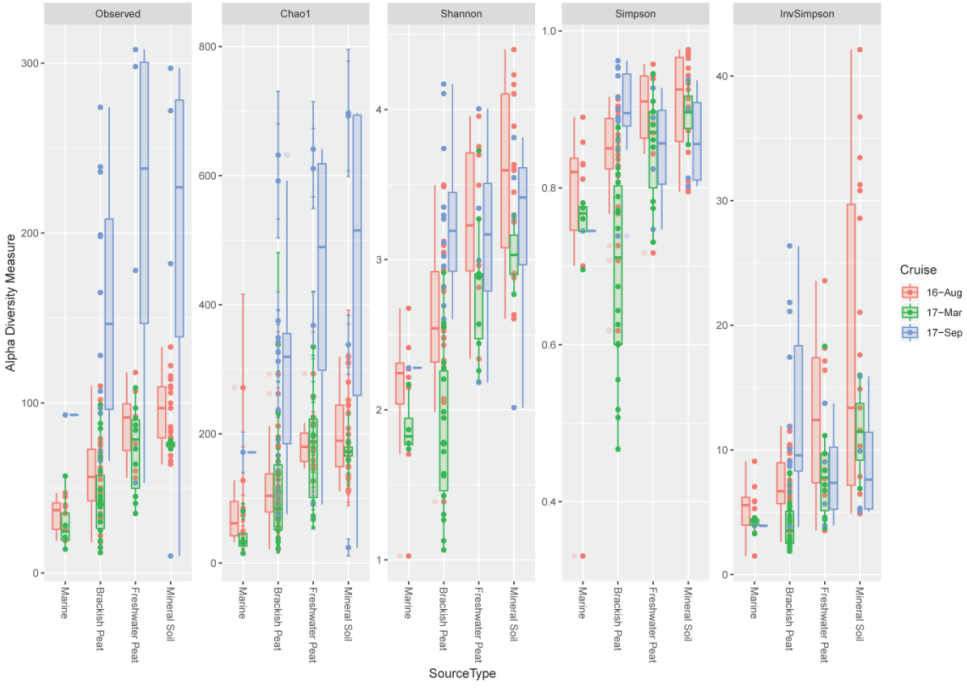

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905 **Fig. 4**

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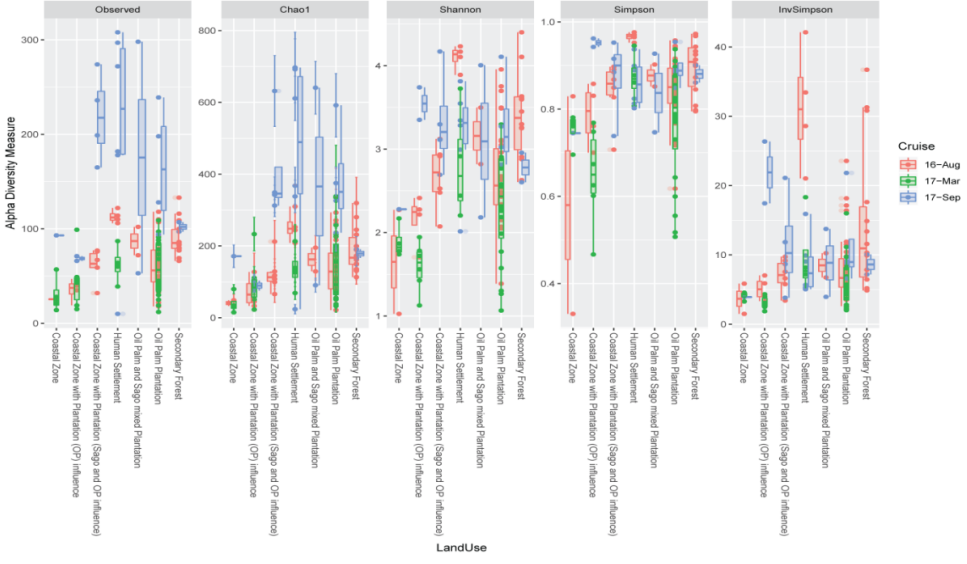

907

908 **Fig. 5**

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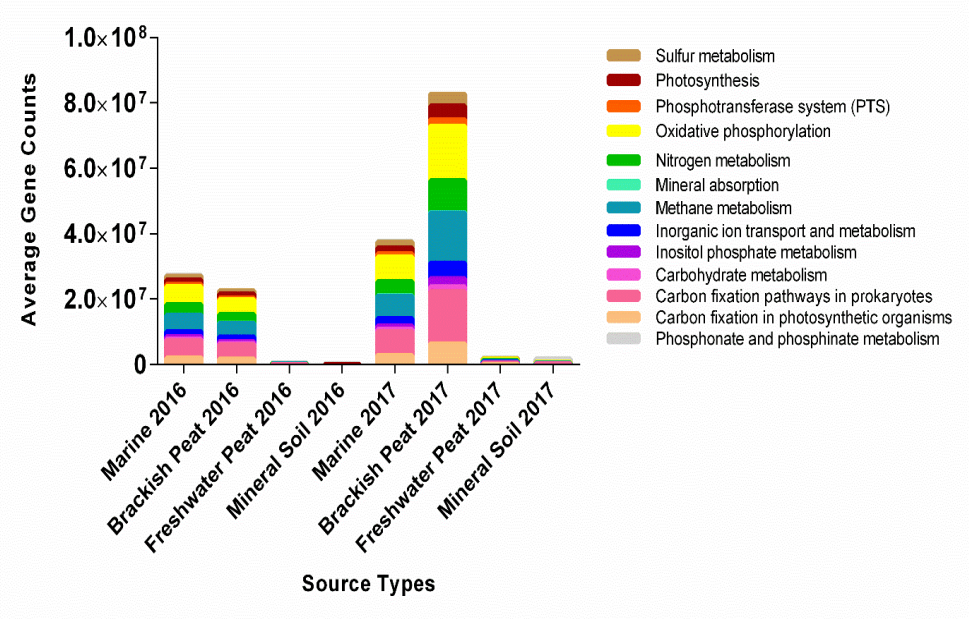
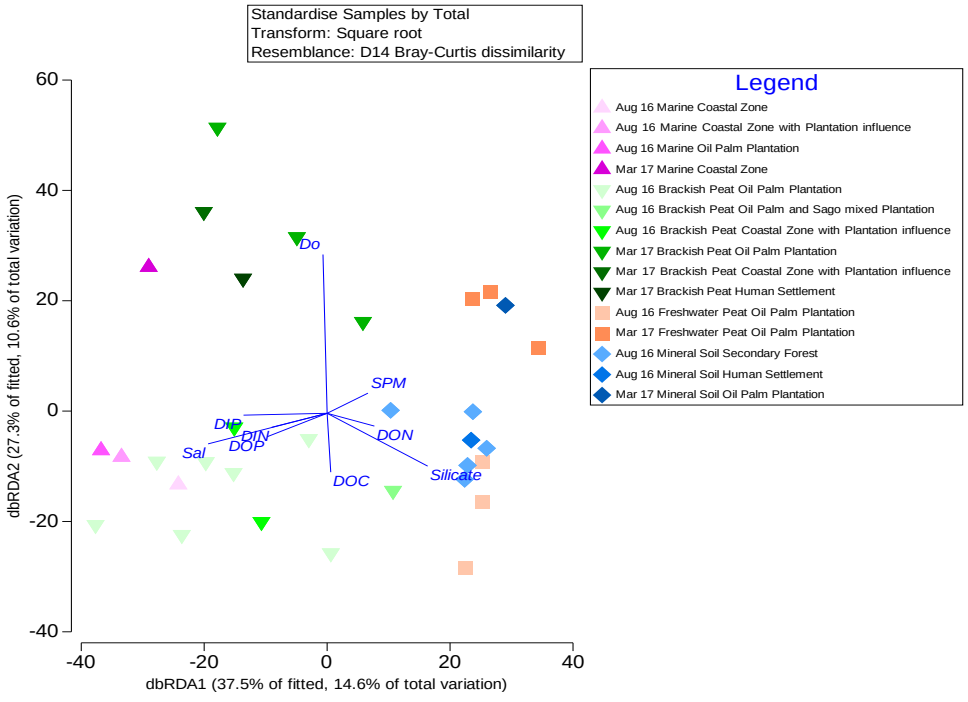


909 **Fig. 6**



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911 **Fig. 7**