Seagrass biofilm communities at a naturally CO_2-rich vent

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Summary

Seagrass meadows are a crucial component of tropical marine reef ecosystems. Seagrass plants are colonized by a multitude of epiphytic organisms that contribute to

broadening the ecological role of seagrasses. To better understand how environmental changes like ocean acidification might affect epiphytic assemblages, the microbial community composition of the epiphytic biofilm of *Enhalus acroides* was investigated at a natural CO_2 vent in Papua New Guinea using molecular fingerprinting and next generation sequencing of 16S and 18S rRNA genes. Both bacterial and eukaryotic epiphytes formed distinct communities at the CO_2-impacted site compared to the control site. This site-related CO_2 effect was also visible in the succession pattern of microbial epiphytes. We further found an increased abundance of bacterial types associated with coral diseases at the CO_2-impacted site (Fusobacteria, *Thalassomonas*) whereas eukaryotes such as certain crustose coralline algae commonly related to healthy reefs were less diverse. These trends in the epiphytic community of *E. acroides* suggest a potential role of seagrasses as vectors of coral pathogens and may support previous predictions of a decrease in reef health and prevalence of diseases under future ocean acidification scenarios.

Keywords: ocean acidification, natural CO_2 vents, seagrass, epiphytes, microbial community composition, coral reef ecology

Introduction

Tropical marine reef ecosystems are hotspots of biodiversity and productivity in an otherwise desert-like marine system. Apart from corals, seagrass meadows are a crucial component of these reef ecosystems. As fish nurseries, nutrient cyclers, organic carbon

producers and sediment stabilizers, seagrass meadows contribute substantially to ecosystem functioning (Orth et al., 2006). Similar to corals (Mouchka et al., 2010), seagrasses are colonized by microorganisms that form epiphytic biofilms on the seagrass leaves (Michael et al., 2008). These biofilms have been shown to affect seagrass physiology as well as their interactions with other reef organisms by e.g. regulating light availability (Sand-Jensen, 1977), influencing the settlement of secondary epibionts and biofouling (Wahl, 1989) or the production of antimicrobial substances (Marhaeni et al., 2011). As such, a seagrass plant and its epiphytic biofilm can be referred to as a seagrass holobiont.

Ocean acidification (OA), defined as a decrease in ocean water pH caused by increased atmospheric CO_2 concentrations, is among the most worrisome threats to coral reef ecosystems (Hoegh-Guldberg et al., 2007). The impacts of OA on corals reach from a decrease of skeletal integrity (Hoegh-Guldberg et al., 2007) to changes in the composition of the microbial biofilm associated with the coral reducing larval settlement and probably coral health (Meron et al., 2011; Webster et al., 2013). Seagrasses, on the other hand, are generally thought to benefit from OA due to the increased availability of CO_2 and bicarbonate for photosynthesis (Koch et al., 2013; Brodie et al., 2014). However, data on how the epiphytic biofilm on seagrass leaves might respond to OA and on the behavior of the seagrass holobiont in future OA scenarios are still sparse.

Several studies have investigated the epiphytic community on seagrass leaves giving detailed information on the composition of bacterial or eukaryotic epiphytes (Uku et al., 2007; Medina-Pons et al., 2009; Hamisi et al., 2013). The effect of ocean acidification on epiphytic communities on seagrass leaves is far less well documented. Previous studies

reported a decrease of calcifying epiphytes such as crustose coralline algae (Martin et al., 2008; Donnarumma et al., 2014) as already seen elsewhere in coral reefs (Fabricius et al., 2011). Donnarumma et al. (2014) also highlighted the decrease in epiphyte diversity with decreasing pH. However, both studies only visually identified epiphytes by using light microscopy and did not address the multitude of cryptic epiphytes detectable only with the increased sensitivity and taxonomic resolution of molecular tools. Our study aims i) to provide a first overview of both bacterial and eukaryotic epiphytes at a molecular level, and ii) to estimate how the epiphytic community on seagrass leaves may change in response to OA. This may thus help increase our understanding of the part the seagrass holobiont may play in the reef ecosystem under future OA scenarios.

Recent research has turned to naturally CO_2-rich systems as models for future OA scenarios (Hall-Spencer et al., 2008; Fabricius et al., 2011; Lidbury et al., 2012; Kerfahi et al., 2014). Unlike laboratory experiments, which are usually restricted to short-term studies, natural sites offer the opportunity to predict OA effects in long-term adapted systems that can be studied in their entirety without the need for experimental manipulation (Hall-Spencer et al., 2008). However, the inherent complexity of natural systems can also confound OA effects and caution is needed in selecting natural CO_2 rich sites for OA research (Vizzini et al., 2013).

Here, the epiphytic biofilm on the leaves of the seagrass *Enhalus acroides* was investigated at a natural CO_2 vent and a control site in Papua New Guinea (PNG; figure S1). The sites were previously described as potential sites to study long-term effects of OA on coral reef communities since the prevailing environmental conditions are assumed to have been stable for up to 100 years (Fabricius et al., 2011). The diversity and

composition of both bacterial and eukaryotic microbial epiphytic communities were assessed using molecular community fingerprinting and next generation sequencing of amplicon libraries. Besides the site-related CO_2 impact, the factor leaf age was included in the analysis to account for different developmental stages of the epiphytic biofilm as well as potential interactions of biofilm development with OA effects. To further characterize the seagrass leaves and their epiphytes, additional data was collected on total epiphyte cover and carbon and nitrogen content of the seagrass leaves.

Results and Discussion

Logger deployments over approx. 44h at the vent site at Dobu Island (Figure S1) recorded median pH values of 7.8 in the water column (Fabricius, pers. communication). At the control site pH values of 8.3 were measured (Hassenrück et al., unpublished). These values were consistent with previous data on the carbonate system at Dobu Island (Fabricius et al., 2011). Apart from the carbonate system, the physicochemical characteristics of the water at the two sampling sites were very similar suggesting that changes in the carbonate system between the vent and the control site were not confounded by any other of the observed parameters (Fabricius et al., 2011).

E. acroides shoots were collected at approximately 4 m water depth at each of the two sampling sites in May 2013. 18S ribosomal DNA sequence confirmed that the seagrass shoots belonged to one species and did not show any pattern by sampling site (data not shown). Each shoot consisted of 3 to 5 leaf pairs that were ranked by their order of budding, i.e. leaf age, with the youngest leaf pair being assigned the first rank. When possible we sampled ranks 1 to 4 (youngest to oldest). On average, *E. acroides* is

expected to produce a new pair of leaves approximately every month (Johnstone, 1979; Brouns and Heijs, 1986; Agawin et al., 2001). The time covered in this study would then amount to four to five months of settlement, although it is possible that growth rates were higher under low pH conditions (Koch et al., 2013). During that time, carbon (C) content of the seagrass leaves decreased with leaf age from approximately 33% to 26% dry weight (ANOVA, $F_{1,38} = 25.986$, $p < 0.001$) and nitrogen (N) content from 2% to almost 1% (Kruskal-Wallis $\chi^2 = 21.262$, df = 3, $p < 0.001$; Table 1). Carbon and nitrogen measurements matched previous measurements of leaves of *E. acroides* (Yamamuro et al., 2004) and were not affected by sampling site suggesting that the substrate type, i.e. the seagrass leaf, was not confounded between sampling sites.

Epiphyte cover increased with leaf age (Table 1). At the vent site, this increase reached only about three fold lower values than under control conditions, most likely due to a lower abundance of pH sensitive organisms such as crustose coralline algae (Corlett and Jones, 2007; Martin et al., 2008, Fabricius et al., unpublished). However, regardless of the trend in epiphyte cover, at the high taxonomic resolution provided by 16S and 18S amplicon sequencing, epiphyte communities seemed to be as diverse at the vent site than at the control site (Table 1).

Molecular Fingerprinting using Automated Ribosomal Intergenic Spacer Analysis (ARISA)

As a first step to assessing the composition of the epiphytic biofilm of *E. acroides*, the epiphytic community was screened using the molecular fingerprinting technique ARISA (Ramette, 2009; Wolf et al., 2013). ARISA identified 408 bacterial and 321

eukaryotic operational taxonomic units (OTUs). Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity coefficients revealed three prominent patterns in the bacterial and eukaryotic community structure (Figure 1).

First, there was a strong separation of the communities sampled at the vent and the control site, which tended to cluster away from each other (bacteria: ANOSIM, $R = 0.775$, $p < 0.05$; eukaryotes: $R = 0.692$, $p < 0.05$; Table S1). Only about 30% of the bacterial and eukaryotic OTUs were shared between any two samples from the vent and the control site. Redundancy analysis (RDA) further confirmed that both sampling site and leaf age significantly explained part of the variation in the microbial community structure (Table S2). Of the observed parameters, sampling site was the dominant factor responsible for the patterns in epiphytic community structure (bacteria: adjusted R^2 = 27.3%; eukaryotes: adjusted R^2 = 12.4%) with about four times more variation being explained by sampling site than leaf age (Table S2). This pronounced shift in the epiphytic community structure on seagrass leaves between vent and control site further supports previous results, which found a response of bacterial as well as eukaryotic microbes to OA in other habitats (Johnson et al., 2011; Lidbury et al., 2012; Kerfahi et al., 2014).

Second, at each site there appeared to be a successive shift in epiphyte communities from the youngest to older leaves (Table S1). Despite the differences in epiphytic community composition between the vent and the control site, a successional pattern in community composition from younger to older leaves was observed at both sites regardless of CO_2 impact (Figure 1). Since organic matter has been shown to be transferred from the seagrass leaves to the epiphytes (Michael et al., 2008), changes in

carbon and nitrogen content with leaf age as documented here may contribute to the influence of leaf age in shaping epiphyte communities.

Third, apart from the general response to the factors sampling site and leave age, patterns in community structure between samples, i.e. the pairwise similarity between samples, correlated strongly between the bacterial and eukaryotic datasets (Mantel test, $r = 0.64$, $p < 0.05$). The strong correlation seemed unlikely to be caused exclusively by changes in abiotic parameters. A more likely explanation may be that both communities influence and shape each other as previously suggested by (Steele et al., 2011; Sawall et al., 2012).

{insert figure 1}

Amplicon sequencing of epiphytic communities

To taxonomically classify the epiphytic communities on *E. acroides*, eight samples were selected for amplicon sequencing of 16S and 18S rRNA genes for bacterial and eukaryotic communities, respectively (ENA accession PRJEB7181). From each sampling site one sample was chosen for each leaf age. OTU clustering was performed at 97% sequence identity and SILVAngs was used for the taxonomic classification of the OTUs (Quast et al., 2013). A more detailed description of the sequence processing workflow can be found in SI text 1.

Amplicon sequencing of the V4-V6 variable region of the bacterial 16S rRNA gene recovered 2,179 OTUs with about 600 OTUs per sample. Approximately 62% of the OTUs were singletons (47%) or doubletons (15%), which accounted for 8-16% of the total sequence counts per sample. This percentage of rare bacterial types did not

significantly vary between sampling sites (Welch's t-test, t = -0.944, df = 3.817, p > 0.05). The Chao1 index of total OTU richness yielded estimates almost twice as high as the raw counts. There was no significant difference in OTU richness between the sampling sites (Welch's t-test, t = -0.819, df = 2.204, p > 0.05; Table 1). Previous reports on bacterial richness and rare bacterial types using next generation sequencing technology showed inconsistent responses to OA (Kerfahi et al., 2014; Raulf et al., 2015; Hassenrück et al., unpublished), which might be explained by the difference in environments being investigated. As such, the lack of change in bacterial richness and rare bacterial types on seagrass leaves at the vent site should not be generalized beyond the scope of this study.

Amplicon sequencing of the V4 variable region of the eukaryotic 18S rRNA gene recovered 3,928 OTU. OTU number per sample ranged from 277 (C1) to 664 (C2; Table 1). Similar to the bacterial OTU richness, there was no significant trend in the OTU number between sampling sites (Welch's t-test, t = 0.034, df = 3.454, p > 0.05). This result was consistent with that of Lidbury et al. (2012) who did not detect a response of eukaryotic microbial richness on settlement tiles to OA using a molecular fingerprinting technique. However, the scarcity of OA studies on eukaryotic microbes applying next generation sequencing technology does not allow for a more comprehensive discussion on how eukaryotic epiphyte richness may respond to OA.

Taxonomic composition of bacterial epiphytes

Most of the bacterial sequences belonged to the phylum Proteobacteria (51%), with Gammaproteobacteria (38%) and Alphaproteobacteria (11%) constituting the majority.

The next most abundant phyla were Cyanobacteria (30%, chloroplast sequences 27%), Bacteroidetes (12%, Flavobacteria: 8%) and Fusobacteria (4%), which were especially abundant on older leaves at the vent site (Figure 2A). The high percentage of Gamma- and Alphaproteobacteria was consistent with previous observations on bacterial epiphytes of tropical seagrasses (Weidner et al., 2000; Uku et al., 2007). The high percentage of chloroplast sequences may be explained by the origin of the samples, which were taken in the photic zone from a chloroplast-containing substratum that was also colonized by algae. We identified several taxa that may potentially be influenced by sampling site and/or age of the seagrass leaves (Table S3). Notice that taxa that seemed to be predominantly affected by leaf age are not further discussed here, because the main objective of our study was to describe potential OA effects on epiphytic microbes.

Cyanobacteria appeared to have a higher relative abundance at the control site than at the CO_2-impacted vent site. Predictions of OA effects on free living cyanobacteria are controversial and range from no effect on metabolic rates (Gradoville et al., 2014) to an increase in carbon and nitrogen fixation (Hutchins et al., 2007; Lomas et al., 2012). In microbial biofilms, OA seemed to decrease cyanobacterial abundance and diversity (Witt et al., 2011; Russell et al., 2013). In complex assemblages, cyanobacteria are supposed to benefit less from OA than other photosynthetic organisms such as chlorophytes, and may thus be outcompeted by them (Low-Décarie et al., 2014). In agreement with this hypothesis, cyanobacteria seemed to decrease in relative abundance with decreasing pH in this study: e.g. the two nitrogen fixing genera *Leptolyngbya* and *Lyngbya*, which are known epiphytes of seagrasses (Uku et al., 2007; Hamisi et al., 2013), were more abundant at the control site, the latter even being unique to the control site. In the case of

253 *Leptolyngbya*, this response has been documented before in a temperate system (Russell
254 et al., 2013), whereas *Lyngbya* is expected to react more to changes in temperature and
255 nutrient availability than to OA (Paerl and Huisman, 2009).

256 Contrarily to cyanobacteria, Deltaproteobacteria, Bacilli, Fusobacteria and Clostridia
257 seemed to increase in relative abundance at the vent site. Within the Deltaproteobacteria
258 this increase was mostly due to an increase in the relative abundance of OTUs of the
259 order Bdellovibrionales at the vent site as also observed by Raulf et al. (2015) in
260 sediments from PNG. The responses of Bacilli and Fusobacteria were mostly due to an
261 increase in the relative abundance of only one OTU belonging to the genus *Paenibacillus*
262 and to the family Leptotrichiaceae, respectively. For *Paenibacillus*, this response has
263 previously been observed in sediments under elevated pCO_2 (Kerfahi et al., 2014). The
264 fusobacterial OTU was among the most abundant OTUs in the dataset (3.5% of all
265 sequences) and was further identified as a relative of *Propionigenium* sp. with a sequence
266 identity of 93% to the latter (NCBI accession number KC918186). Fusobacteria are a
267 group of strictly anaerobic bacteria, which have been associated with tidal flat sediments,
268 where they contribute to organic matter degradation (Graue et al., 2012), and are present
269 in the gut microflora of marine invertebrates (Li et al., 2012; Dishaw et al., 2014;
270 Rungrassamee et al., 2014) and coral biofilm (Morrow et al., 2012). There is evidence
271 that Fusobacteria associated with corals increase in abundance under OA (Vega Thurber
272 et al., 2009), which might support our results, although the exceptionally high sequence
273 abundance of Fusobacteria at the vent site was restricted to the two oldest leaves.
274 Noticeably, Fusobacteria as well as Clostridia have further been implicated in coral
275 diseases (Vega Thurber et al., 2009; Sweet et al., 2013).

Alphaproteo-, Gammaproteo- and Flavobacteria did not show a response to sampling site on class level. However, at a higher level of taxonomic resolution, several taxa appeared to be affected by sampling site (Table S3). Among the most abundant OTUs in the dataset, those potentially influenced by sampling site belonged to the Gammaproteobacteria, i.e. *Thalassomonas* (1.6%) and *Marinomonas* (3.8%), which were more abundant at the vent site, and *Reinekea* (7.2%) and *Melitea* (2.3%), which were more abundant at control site. Sequence comparison of the OTU belonging to *Thalassomonas* showed a high sequence identity (99%) to the sequence retrieved by Webster et al. (2013; NCBI accession number JQ178640), which was associated with the crustose coralline algae *Hydrolithon* at low pH. It was further closely related (96% sequence identity) to *Thalassomonas loyana* (NCBI accession number NR043066), the causative agent of white plague-like disease in corals (Thompson et al., 2006), suggesting a potentially pathogenic role. The OTU of *Marinomonas* was related to *Marinomonas poseidonica* (99% sequence identity, NCBI accession number NR074719), which has been reported to be beneficial to seagrass (Celdrán et al., 2012) and may contribute to increased growth rates at the vent site. *Reinekea* is a genus that might play an important role in the degradation of organic matter after phytoplankton blooms (Teeling et al., 2012). Its reduced abundance at the vent site may be caused by the decreased availability of degradable material presumably due to the lower percentage of epiphyte cover. However, it also belongs to the order Oceanospirillales, which are common in coral biofilms and expected to decrease in abundance in diseased corals (Mouchka et al., 2010). Hardly anything is known about the genus *Melitea* and, although it has been

mentioned before in OA research, its response to elevated pCO_2 still remains largely unknown (Meron et al., 2011).

The direction of potential changes (i.e. the increase or decrease) in relative OTU abundance from control to vent site or vice versa appeared to be related to total OTU abundance. Whereas approximately equal numbers of abundant OTUs (defined by more than 1% total sequence abundance) increased towards either the vent or control site, more OTUs of intermediate abundance level (defined by more than 2 sequence occurrences but less than 1% total sequence abundance) tended to increase towards the vent site than towards the control site (Table S4). Although not seen in the rare bacterial types as previously discussed, this trend might be comparable to the increase in rare types with decreasing pH observed in marine sediments at PNG (Raulf et al., 2015).

Among these increasing OTUs, sulfur oxidizers were overrepresented, some of which – but not all – were unique to the vent site. This suggests that a higher concentration of sulfur compounds that can be metabolized by bacteria might be present in the water column at the vent compared to the control site, although so far no direct evidence exists for that matter (Fabricius et al., 2011). H_2S was detected in the sediment (Artur Fink, pers. communication) and gas, but H_2S levels in the water column did not exceed values typically observed for seawater (Fabricius et al., 2011). On the other hand, sulfur-oxidizing bacteria might also constitute a contamination from the sediment and might not even be active on the seagrass leaves. Furthermore, apart from their biogeochemical function, sulfur-oxidizing bacteria have also been associated with coral diseases (Frias-Lopez et al., 2002, 2004; Bourne et al., 2013). Their increased relative abundance may therefore not only be attributable to sulfide seepage. Other OTUs of

intermediate abundance, which increased at the vent site, belonged to genera such as *Shewanella* and *Vibrio*, which again have been related to coral diseases (Mouchka et al., 2010; Meron et al., 2011; Garcia et al., 2013; Sweet et al., 2013). The general trend of an increase in disease-associated bacterial OTUs at the vent site has also been observed in corals in PNG (Morrow et al., 2014).

Taxonomic composition of eukaryotic epiphytes

The richness of eukaryotic OTUs was dominated by Florideophycidae, which mostly consisted of crustose coralline algae (Corallinophycidae, 2282 OTUs) and Rhodymeniophycidae (235 OTUs), followed by diatoms (695 OTUs), Ulvophyceae (171 OTUs) and dinoflagellates (145 OTUs, figure 2B). This composition conforms with the findings of microscopy-based work on tropical seagrasses which also reported a prevalence of crustose coralline algae (Corlett and Jones, 2007; Martin et al., 2008).

Potential changes in OTU richness were related to genera of the taxa Corallinophycidae, Dinoflagellata and Diatomea (Table S5). Corallinophycidae were slightly less diverse at the vent site, especially on the older leaves where they only retained about 65% of their OTUs. As calcifying organisms, crustose coralline algae are likely to suffer from OA (Martin et al., 2008; Fabricius et al., 2011; Donnarumma et al., 2014). However, some genera appear to be more vulnerable to elevated pCO_2 than others. Here, *Hydrolithon* the most diverse genus of crustose coralline algae on the leaves of *E. acroides* lost about two thirds of its OTUs, and *Lithophyllum*, which disappeared completely at the vent site, seemed especially susceptible to acidified conditions. Severe declines in *Hydrolithon* have also been observed on settlement tiles in PNG (Fabricius et

al., unpublished). The calcite deposits of *Hydrolithon* and *Lithophyllum* contain a high percentage of magnesium, while e.g. *Spongites* which was the only crustose coralline algae unique to the vent site, deposits calcite with little magnesium content – a form that is less susceptible to reduced pH than high-Mg calcite (Smith et al., 2012). These differences in calcite composition may contribute to the resilience of crustose coralline algae under OA (Ries, 2011; Ragazzola et al., 2013).

The genus *Galeidinium* (Dinoflagellata) was more diverse at the vent compared to the control site. However, the impacts of OA on dinoflagellates, in general, and *Galeidinium*, in particular, are not very well studied, so that potential implications of an increased diversity of *Galeidinium* under elevated pCO_2 cannot yet be predicted.

Diatoms showed a variable response to sampling site with *Navicula* and *Grammatophora* being more diverse at the vent and *Cyclophora* and *Cylindrotheca* at the control site. These changes in the diversity of diatoms largely concurred with previous findings, which predicted an increase in the genera *Grammatophora* and *Navicula* under OA with a coinciding decrease of *Cyclophora* and *Cylindrotheca* (Johnson et al., 2011; Singh and Singh, 2014), which was also the case here. Although photosynthetic organisms in general are expected to benefit from OA, species-specific responses depend on the respective ability of each organism to utilize inorganic carbon during photosynthesis and their comparative competitiveness (Koch et al., 2013).

{insert figure 2}

Conclusion: does epiphyte composition change due to OA?

We detected a highly diverse bacterial and eukaryotic community on the leaves of *E. acroides*. Although OTU richness seemed unaffected, our results overall suggest a pronounced and interconnected shift in bacterial and eukaryotic community composition of the epiphytic biofilm of *E. acroides* with changes in the carbonate system of the surrounding water. Besides organisms well-known to respond to elevated pCO_2, this shift may also include taxa that have not been identified in OA research before. In some cases, a potential response to elevated pCO_2 was only visible at a very high level of taxonomic resolution. We further detected an increased prevalence of microbial sequence types associated with coral diseases at the vent site under elevated pCO_2 conditions. This agrees with the hypothesis that coral reefs experiencing elevated pCO_2 levels will be more susceptible to diseases than reefs not yet exposed to OA (Hoegh-Guldberg et al., 2007). It further highlights the potential of seagrasses as vectors of coral pathogens (Sweet et al., 2013) and stresses the point that seagrasses should be viewed as a holobiont when making predictions about OA effects and ecological consequences in coral reefs. Given the high diversity of the epiphytic community on seagrass leaves, an accurate assessment of the interaction of seagrasses with other components of reef ecosystems will also require further knowledge of their epiphytic community composition.

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Table 1: Carbon (C) and nitrogen (N) content in percentage dry weight, C:N ratio and epiphyte cover of the leaves of *E. acroides*, the number of bacterial and eukaryotic OTUs obtained through ARISA and amplicon sequencing (Bacteria: 16S rRNA gene, Illumina sequencing; Eukarya: 18S rRNA gene, 454 sequencing); values constitute mean +/- standard error where applicable; for the bacterial sequencing dataset Chao1 richness estimates are given in italics with 95% confidence intervals in brackets.

Figure 1: Non-metric multidimensional scaling (NDMS) plot based on the Bray-Curtis dissimilarity matrix for bacteria (A) and on the Jaccard dissimilarity matrix for eukaryotes (B) on leaves of *E. acroides*; both bacterial and eukaryotic communities were assessed using ARISA; dashed hulls representing a minimum of 30% shared OTUs between samples within the hull; labeled points: samples selected for 16S/18S amplicon sequencing.

Figure 2: Taxonomic composition of the epiphytic biofilm on leaves of *E. acroides*, A: bacterial community based on the relative abundance of OTUs (16S rRNA gene sequences, 454 sequencing); B: eukaryotic community based on the presence/absence of OTUs (18S rRNA gene sequences, Illumina); bars colored by bacterial class or eukaryotic phylum, separated by genus; hatched areas: examples of genera potentially influenced by site and/or leaf age; bold: bacterial classes or eukaryotic phyla potentially

influenced by sampling site (Table S3 and S5); samples ordered by leaf age (left: youngest, right: oldest) within sampling site.

Supporting information

SI text 1: Sequence processing workflow.

Table S1: Analysis of similarity (ANOSIM) results to test the influence of sampling site and leaf age on the similarity of bacterial and eukaryotic communities based on ARISA; upper panel: ANOSIM R based on Bray-Curtis dissimilarity coefficient, lower panel: ANOSIM R based on Jaccard dissimilarity coefficient; 1-4 representing leaf ages from youngest to oldest; [a] only 2 samples for oldest leaves from the vent site; false discovery rate (fdr)-adjusted p-values < 0.1 (°), < 0.05 (*), < 0.01 (**), < 0.001 (***), ANOSIM R of non-significant results are not reported.

Table S2: RDA results to identify factors significantly explaining the variation in bacterial and eukaryotic communities based on ARISA; results shown for RDA based on hellinger-transformed relative abundances and presence/absence (binary) data.

Table S3: Bacterial taxa potentially affected by sampling site based on relative OTU abundance; information is given on the abundance of the respective taxa (high: > 1% total abundance, rare: singletons and doubletons), the number of taxa of the next lower taxonomic level (subtaxa), taxa that cumulatively contributed to 70% of the dissimilarity between sampling sites (SIMPER analysis based on Bray-Curtis dissimilarity

coefficient), factors potentially affecting abundance based on an uncorrected significance threshold of 0.05 and 0.1 (effect), evaluation of the response of potentially impacted taxa (response to sampling site and/or leaf age, minor response), direction of potential change in abundance and whether taxa were exclusive to either sampling site.

Table S4: Number of bacterial (upper panel) and eukaryotic (lower panel) taxa potentially affected by sampling site and the direction of that effect (increase in abundance/diversity); in brackets: number of taxa unique to that site.

Table S5: Eukaryotic taxa potentially affected by sampling site based on the presence/absence of OTUs; information is given on the number of taxa of the next lower taxonomic level (subtaxa), factors potentially affecting OTU number (diversity) based on an uncorrected significance threshold of 0.15, evaluation of the response of potentially impacted taxa (response to sampling site and/or leaf age, minor response), direction of potential change in diversity and whether taxa were exclusive to either sampling site.

Figure S1: Sampling area in Papua New Guinea showing the two sampling sites, which were approximately 2 km apart (control site: S9.752, E150.854, vent site: S9.737, E150.869).

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Table 1: Carbon (C) and nitrogen (N) content in percentage dry weight, C:N ratio and epiphyte cover of the leaves of *E. acroides*, the number of bacterial and eukaryotic OTUs obtained through ARISA (SI text 1) and amplicon sequencing (Bacteria: 16S rRNA gene, Eukarya: 18S rRNA gene, SI text 1); values constitute mean +/- standard error where applicable; for the bacterial sequencing dataset Chao1 richness estimates are given in italics with lower confidence interval/upper confidence interval in brackets.

						ARISA		Amplicon sequencing	
		N [%]	C [%]	C:N ratio	Epiphyte cover [%]	Bacteria	Eukarya	Bacteria (16S)	Eukarya (18S)
Control	All ages	1.42 ± 0.08	28.87 ± 0.8	20.98 ± 0.71	18.08 ± 2.45	96.52 ± 3.27	86.14 ± 2.56	507.5 ± 31.45 *917.31 ± 49.13*	520.75 ± 84.25
	Youngest	1.98 ± 0.18	34.63 ± 2.21	17.89 ± 2.5	3.25 ± 3.25	111.25 ± 6.02	72.5 ± 3.8	585 *913.76 (822.52/1040.05)*	277
	2nd youngest	1.54 ± 0.1	29.68 ± 0.73	19.56 ± 0.88	14.3 ± 5.5	96.83 ± 7.49	86.67 ± 4.97	532 *991.53(863.52/1168.96)*	664
	3rd youngest	1.2 ± 0.04	28.07 ± 0.8	23.47 ± 0.42	22.33 ± 2.72	94.83 ± 2.4	93.17 ± 3.36	462 *984.34 (827.90/1207.66)*	561
	Oldest	1.19 ± 0.04	25.83 ± 0.89	21.87 ± 1.31	22.54 ± 3.56	86.4 ± 6.31	88 ± 4.69	451 *779.63 (680.69 /921.19)*	581
Vent	All ages	1.41 ± 0.12	26.93 ± 1.8	19.96 ± 0.79	6.61 ± 1.6	135.95 ± 2.82	89.68 ± 2.71	619.67 ± 48.22 *1044.09 ± 187.94*	517.75 ± 23.24
	Youngest	2.05 ± 0.05	32.02 ± 0.42	15.68 ± 0.42	2.5 ± 1.5	139.6 ± 7.64	97.4 ± 7.06	594 *933.61 (839.61/1063.58)*	459
	2nd youngest	1.25 ± 0.21	24.87 ± 3.83	20.15 ± 0.74	8.7 ± 2.2	133.5 ± 5.94	87.33 ± 4.36	NA	553
	3rd youngest	1.07 ± 0.18	24.26 ± 3.91	22.65 ± 1.3	8.55 ± 3.84	133.83 ± 2.7	84.33 ± 3.19	552 *788.18 (718.60 /886.80)*	502
	Oldest	1.29 ± 0.04	28.36 ± 0.1	22.04 ± 0.76	3.75 ± 0.75	140.5 ± 7.5	93.5 ± 7.5	713 *1410.49 (1240.43/1635.38)*	557
Total		1.41 ± 0.07	27.92 ± 0.97	20.48 ± 0.53	12.81 ± 1.77	408	329	2179 *3811.43 ± 142.83*	3928