environmental microbiology reports

Environmental Microbiology Reports (2015) **7**(3), 516–525 doi:10.1111/1758-2229.12282

Seagrass biofilm communities at a naturally CO_2-rich vent

Christiane Hassenrück,[1] Laurie C. Hofmann,[2] Kai Bischof[2] and Alban Ramette[1*†]

[1]*HGF MPG Group for Deep-Sea Ecology and Technology, Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, Bremen 28359, Germany.*

[2]*BreMarE, FB 02, University of Bremen, Leobener Str. NW2, Bremen 28359, Germany.*

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 with the control site. This site-related CO_2 effect was also visible in the succession pattern of microbial epiphytes. We further found an increased relative sequence 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.

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 range 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 because of 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 behaviour 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 OA 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 and colleagues (2014) also highlighted a 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 provided by molecular tools. Our study aims (i)

Received 4 September, 2014; accepted 20 February, 2015. *For correspondence. E-mail alban.ramette@ispm.unibe.ch; Tel. +41 316315975; Fax +41 316313648. †Present address: University of Bern, Institute of Social and Preventive Medicine, Finkenhubelweg 11, 3012 Bern, Switzerland.

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; Fig. S1). The sites were previously described as potential sites to study long-term effects of OA on coral reef communities because 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 were collected on total epiphyte cover and carbon and nitrogen content of the seagrass leaves.

Results and discussion

Logger deployments over approximately 44 h at the vent site at Dobu Island (Fig. S1) recorded median pH values of 7.8 in the water column (K. Fabricius, pers. comm.). At the control site, pH values of 8.3 were measured. 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).

Enhalus acroides shoots were collected at approximately 4 m water depth at each of the two sampling sites in May 2013. 18S ribosomal DNA sequences 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 three to five 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 one to four (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 4–5 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 [analysis of variance (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 threefold 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 Kluibenschedl, Harrington, Noonan, and De'ath, 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 ordination plots based on Bray–Curtis dissimilarity coefficients revealed three prominent patterns in the bacterial and eukaryotic community structure (Fig. 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: analysis of similarity (ANOSIM), $R = 0.775$, $P < 0.05$; eukaryotes: $R = 0.692$, $P < 0.05$; Table S1). Only about 30% of the

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; eukaryotes: 18S rRNA gene, 454 sequencing); values constitute mean ± standard error where applicable; for the bacterial sequencing data set Chao1 richness estimates are given in italics with 95% confidence intervals in brackets.

						ARISA		Amplicon sequencing	
		N [%]	C [%]	C : N ratio	Epiphyte cover [%]	Bacteria	Eukaryotes	Bacteria (16S)	Eukaryotes (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
	Second 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
	Third 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
	Second 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
	Third 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

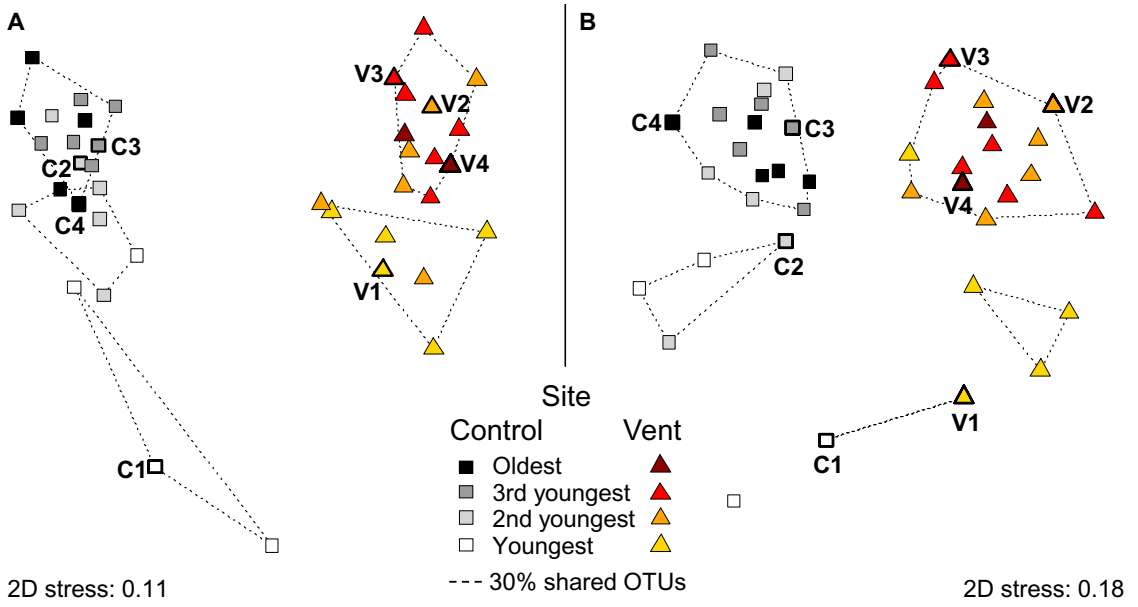

Fig. 1. Non-metric multidimensional scaling (NDMS) plot based on the Bray–Curtis dissimilarity matrix for bacteria (A) and on the Jaccard dissimilarity matrix for eukaryotes (B) on leaves of *E. acroides*; both bacterial and eukaryotic communities were assessed using ARISA; dashed hulls representing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

bacterial and eukaryotic OTUs were shared between any two samples from the vent and the control site. Redundancy analysis 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 (Fig. 1). Because 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 data sets (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 and colleagues (2011) and Sawall and colleagues (2012).

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 Text S1.

Amplicon sequencing of the V4–V6 variable region of the bacterial 16S rRNA gene recovered 2179 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), 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 3928 OTUs. 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 and colleagues (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 (Fig. 2A). The high percentage of *Gammaproteobacteria* 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 in 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 *Leptolyngbya*, this response has been documented before in a temperate system (Russell *et al*., 2013), whereas *Lyngbya* is expected to react more to changes in temperature and nutrient availability than to OA (Paerl and Huisman, 2009).

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

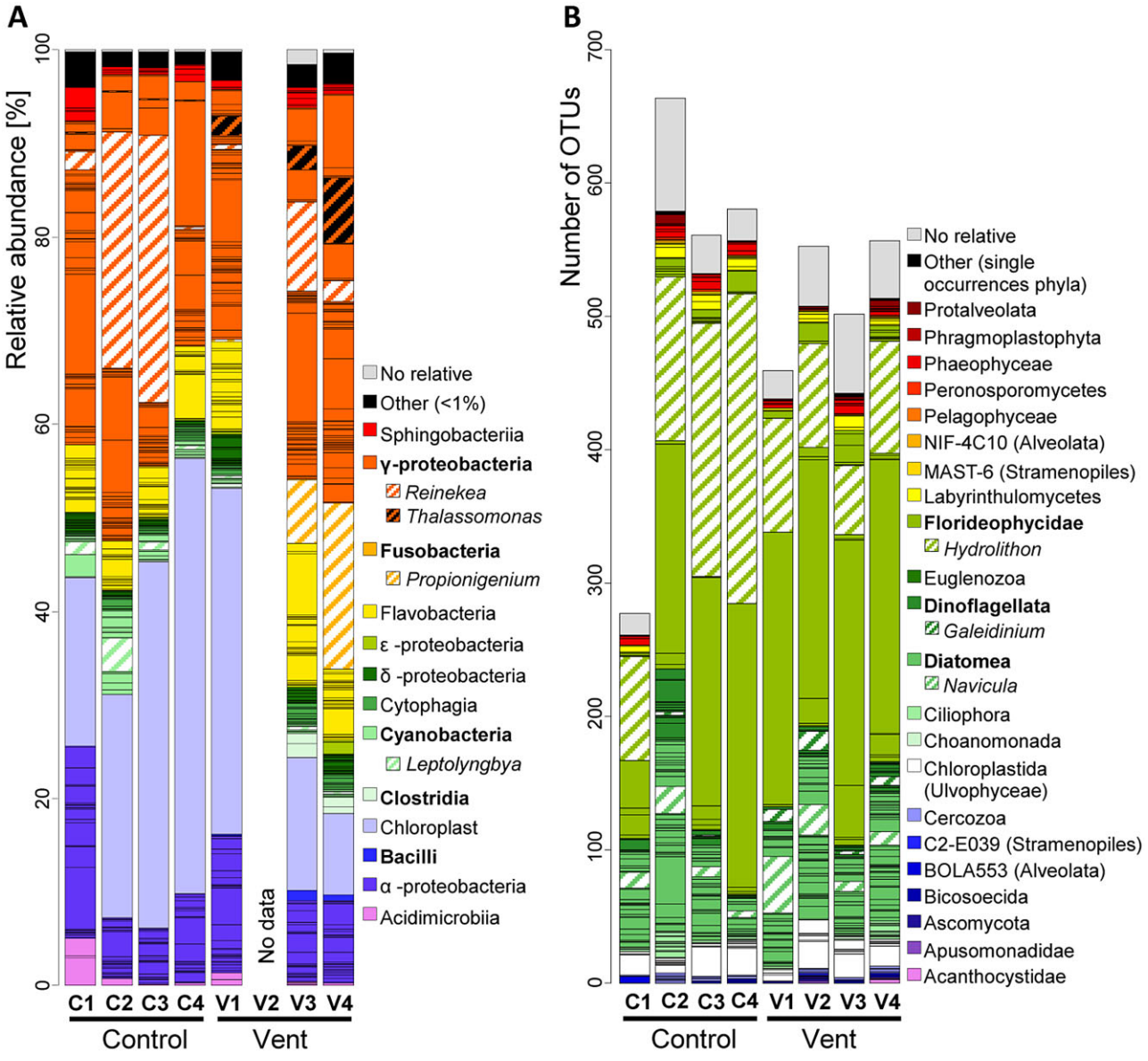

Fig. 2. Taxonomic composition of the epiphytic biofilm on leaves of *E. acroides*: (A) bacterial community based on the relative abundance of OTUs (16S rRNA gene sequences, 454 sequencing); (B) eukaryotic community based on the presence/absence of OTUs (18S rRNA gene sequences, Illumina sequencing). Bars are coloured 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 (Tables S3 and S5). Samples are ordered by leaf age (left: youngest, right: oldest) within sampling site.

implicated in coral diseases (Vega Thurber *et al*., 2009; Sweet *et al*., 2013).

Alphaproteobacteria, *Gammaproteobacteria* 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 data set, 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 and colleagues (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 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 two 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 with 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 with the control site, although so far, no direct evidence exists for that matter (Fabricius *et al*., 2011). H_2S was detected in the sediment (A. Fink, pers. comm.) 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). This general trend of an increase in disease-associated bacterial OTUs at the vent site has also been observed at PNG in corals (Morrow *et al*., 2014).

Taxonomic composition of eukaryotic epiphytes

Eukaryotic OTUs were 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, Fig. 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, whereas 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 with 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 on their comparative competitiveness (Koch *et al*., 2013).

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.

Acknowledgements

We thank the scientists and crew of the cruise to Papua New Guinea for their support during the sampling, especially Katharina Fabricius (AIMS, Australia), Dirk de Beer and Artur Fink (both MPI for Marine Microbiology, Bremen) for their continued advice. The research was funded by the German Federal Ministry of Education and Research (BMBF) in the framework of the BIOACID II project, the Max Planck Society and the University of Bremen.

References

Agawin, N.S.R., Duarte, C.M., Fortes, M.D., Uri, J.S., and Vermaat, J.E. (2001) Temporal changes in the abundance, leaf growth and photosynthesis of three co-occurring Philippine seagrasses. *J Exp Mar Bio Ecol* **260:** 217–239.

Bourne, D.G., van der Zee, M.J.J., Botté, E.S., and Sato, Y. (2013) Sulfur-oxidizing bacterial populations within cyanobacterial dominated coral disease lesions. *Environ Microbiol Rep* **5:** 518–524.

Brodie, J., Williamson, C.J., Smale, D.A., Kamenos, N.A., Mieszkowska, N., Santos, R., *et al*. (2014) The future of the northeast Atlantic benthic flora in a high CO2 world. *Ecol Evol* **4:** 2787–2798.

Brouns, J.J.W.M., and Heijs, F.M.L. (1986) Production and biomass of the seagrass *Enhalus acoroides* (L.f.) Royle and its epiphytes. *Aquat Bot* **25:** 21–45.

Celdrán, D., Espinosa, E., Sánchez-Amat, A., and Marín, A. (2012) Effects of epibiotic bacteria on leaf growth and epiphytes of the seagrass *Posidonia oceanica*. *Mar Ecol Prog Ser* **456:** 21–27.

Corlett, H., and Jones, B. (2007) Epiphyte communities on *Thalassia testudinum* from Grand Cayman, British West Indies: their composition, structure, and contribution to lagoonal sediments. *Sediment Geol* **194:** 245–262.

Dishaw, L.J., Flores-Torres, J., Lax, S., Gemayel, K., Leigh, B., Melillo, D., *et al*. (2014) The gut of geographically disparate *Ciona intestinalis* harbors a core microbiota. *PLoS ONE* **9:** e93386.

Donnarumma, L., Lombardi, C., Cocito, S., and Gambi, M.C. (2014) Settlement pattern of *Posidonia oceanica* epibionts along a gradient of ocean acidification: an approach with mimics. *Mediterr Mar Sci* **15:** 498–509.

Fabricius, K.E., Langdon, C., Uthicke, S., Humphrey, C., Noonan, S., De'ath, G., *et al*. (2011) Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. *Nat Clim Chang* **1:** 165–169.

Frias-Lopez, J., Zerkle, A.L., Bonheyo, G.T., and Fouke, B.W. (2002) Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. *Appl Environ Microbiol* **68:** 2214–2228.

Frias-Lopez, J., Klaus, J.S., Bonheyo, G.T., and Fouke, B.W. (2004) Bacterial community associated with black band disease in corals. *Appl Environ Microbiol* **70:** 5955–5962.

Garcia, G.D., Gregoracci, G.B., Santos, E.D.O., Meirelles, P.M., Silva, G.G.Z., Edwards, R., *et al*. (2013) Metagenomic analysis of healthy and white plague-affected *Mussismilia braziliensis* corals. *Microb Ecol* **65:** 1076–1086.

Gradoville, M.R., White, A.E., Bo, D., Church, M.J., and Letelier, R.M. (2014) Diversity trumps acidification: lack of evidence for carbon dioxide enhancement of *Trichodesmium* community nitrogen or carbon fixation at Station ALOHA. *Limnol Oceanogr* **59:** 645–659.

Graue, J., Engelen, B., and Cypionka, H. (2012) Degradation of cyanobacterial biomass in anoxic tidal-flat sediments: a microcosm study of metabolic processes and community changes. *ISME J* **6:** 660–669.

Hall-Spencer, J.M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S.M., *et al*. (2008) Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature* **454:** 96–99.

Hamisi, M., Díez, B., Lyimo, T., Ininbergs, K., and Bergman, B. (2013) Epiphytic cyanobacteria of the seagrass *Cymodocea rotundata*: diversity, diel nifH expression and nitrogenase activity. *Environ Microbiol Rep* **5:** 367–376.

Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., Gomez, E., *et al*. (2007) Coral reefs under rapid climate change and ocean acidification. *Science* **318:** 1737–1742.

Hutchins, D.A., Fu, F.-X., Zhang, Y., Warner, M.E., Feng, Y., Portune, K., *et al*. (2007) CO2 control of *Trichodesmium* N2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry. *Limnol Oceanogr* **52:** 1293–1304.

Johnson, V.R., Brownlee, C., Rickaby, R.E.M., Graziano, M., Milazzo, M., and Hall-Spencer, J.M. (2011) Responses of marine benthic microalgae to elevated CO2. *Mar Biol* **160:** 1813–1824.

Johnstone, I.M. (1979) Papua New Guinea seagrasses and aspects of the biology and growth of *Enhalus acoroides* (L.f.) Royle. *Aquat Bot* **7:** 197–208.

Kerfahi, D., Hall-Spencer, J.M., Tripathi, B.M., Milazzo, M., Lee, J., and Adams, J.M. (2014) Shallow water marine sediment bacterial community shifts along a natural CO2 gradient in the Mediterranean sea off Vulcano, Italy. *Microb Ecol* **67:** 819–828.

Koch, M., Bowes, G., Ross, C., and Zhang, X.-H. (2013) Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Glob Chang Biol* **19:** 103–132.

Li, S., Sun, L., Wu, H., Hu, Z., Liu, W., Li, Y., and Wen, X. (2012) The intestinal microbial diversity in mud crab (*Scylla paramamosain*) as determined by PCR-DGGE and clone library analysis. *J Appl Microbiol* **113:** 1341–1351.

Lidbury, I., Johnson, V., Hall-Spencer, J.M., Munn, C.B., and Cunliffe, M. (2012) Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent ecosystem. *Mar Pollut Bull* **64:** 1063–1066.

Lomas, M., Hopkinson, B., Losh, J., Ryan, D., Shi, D., Xu, Y., and Morel, F. (2012) Effect of ocean acidification on cyanobacteria in the subtropical North Atlantic. *Aquat Microb Ecol* **66:** 211–222.

Low-Décarie, E., Fussmann, G.F., and Bell, G. (2014) Aquatic primary production in a high-CO2 world. *Trends Ecol Evol* **29:** 223–232.

Marhaeni, B., Radjasa, O.K., Khoeri, M.M., Sabdono, A., Bengen, D.G., and Sudoyo, H. (2011) Antifouling activity of bacterial symbionts of seagrasses against marine biofilm-forming bacteria. *J Environ Prot (Irvine,. Calif).* **02:** 1245–1249.

Martin, S., Rodolfo-Metalpa, R., Ransome, E., Rowley, S., Buia, M.-C., Gattuso, J.-P., and Hall-Spencer, J. (2008) Effects of naturally acidified seawater on seagrass calcareous epibionts. *Biol Lett* **4:** 689–692.

Medina-Pons, F.J., Terrados, J., López-López, A., Yarza, P., and Rosselló-Móra, R. (2009) Evaluation of the 18S rRNA clone library approach to study the diversity of the macroeukaryotic leaf-epiphytic community of the seagrass *Posidonia oceanica* (L.) Delile. *Mar Biol* **156:** 1963–1976.

Meron, D., Atias, E., Iasur Kruh, L., Elifantz, H., Minz, D., Fine, M., and Banin, E. (2011) The impact of reduced pH on the microbial community of the coral *Acropora eurystoma*. *ISME J* **5:** 51–60.

Michael, T.S., Shin, H.W., Hanna, R., and Spafford, D.C. (2008) A review of epiphyte community development: surface interactions and settlement on seagrass. *J Environ Biol* **29:** 629–638.

Morrow, K.M., Moss, A.G., Chadwick, N.E., and Liles, M.R. (2012) Bacterial associates of two Caribbean coral species reveal species-specific distribution and geographic variability. *Appl Environ Microbiol* **78:** 6438–6449.

Morrow, K.M., Bourne, D.G., Humphrey, C., Botté, E.S., Laffy, P., Zaneveld, J., *et al*. (2014) Natural volcanic CO2 seeps reveal future trajectories for host-microbial associations in corals and sponges. *ISME J.* **2:** 1–15.

Mouchka, M.E., Hewson, I., and Harvell, C.D. (2010) Coral-associated bacterial assemblages: current knowledge and the potential for climate-driven impacts. *Integr Comp Biol* **50:** 662–674.

Orth, R.J., Carruthers, T.J.B., Dennison, W.C., Duarte, C.M., Fourqurean, J.W., Heck, K.L., Jr, *et al*. (2006) A global crisis for seagrass ecosystems. *Bioscience* **56:** 987–996.

Paerl, H.W., and Huisman, J. (2009) Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. *Environ Microbiol Rep* **1:** 27–37.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al*. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41:** D590–D596.

Ragazzola, F., Foster, L.C., Form, A.U., Büscher, J., Hansteen, T.H., and Fietzke, J. (2013) Phenotypic plasticity of coralline algae in a high CO2 world. *Ecol Evol* **3:** 3436–3446.

Ramette, A. (2009) Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl Environ Microbiol* **75:** 2495–2505.

Raulf, F.F., Fabricius, K.E., Uthicke, S., de Beer, D., Abed, R.M.M., and Ramette, A. (2015) Changes in microbial communities in coastal sediments along natural CO2 gradients at a volcanic vent in Papua New Guinea. *Environ Microbiol* doi:10.1111/1462-2920.12729.

Ries, J.B. (2011) Skeletal mineralogy in a high-CO2 world. *J Exp Mar Bio Ecol* **403:** 54–64.

Rungrassamee, W., Klanchui, A., Maibunkaew, S., Chaiyapechara, S., Jiravanichpaisal, P., and Karoonuthaisiri, N. (2014) Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*). *PLoS ONE* **9:** e91853.

Russell, B.D., Connell, S.D., Findlay, H.S., Tait, K., Widdicombe, S., and Mieszkowska, N. (2013) Ocean acidification and rising temperatures may increase biofilm primary productivity but decrease grazer consumption. *Philos Trans R Soc Lond B Biol Sci* **368**(1627):20120438. doi: 10.1098/rstb.2012.0438

Sand-Jensen, K. (1977) Effect of epiphytes on eelgrass photosynthesis. *Aquat Bot* **3:** 55–63.

Sawall, Y., Richter, C., and Ramette, A. (2012) Effects of eutrophication, seasonality and macrofouling on the

diversity of bacterial biofilms in equatorial coral reefs. *PLoS ONE* **7:** e39951.
Singh, S.P., and Singh, P. (2014) Effect of CO2 concentration on algal growth: a review. *Renew Sustain Energy Rev* **38:** 172–179.
Smith, A.M., Sutherland, J.E., Kregting, L., Farr, T.J., and Winter, D.J. (2012) Phylomineralogy of the coralline red algae: correlation of skeletal mineralogy with molecular phylogeny. *Phytochemistry* **81:** 97–108.
Steele, J.A., Countway, P.D., Xia, L., Vigil, P.D., Beman, J.M., Kim, D.Y., *et al*. (2011) Marine bacterial, archaeal and protistan association networks reveal ecological linkages. *ISME J* **5:** 1414–1425.
Sweet, M.J., Bythell, J.C., and Nugues, M.M. (2013) Algae as reservoirs for coral pathogens. *PLoS ONE* **8:** e69717.
Teeling, H., Fuchs, B.M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C.M., *et al*. (2012) Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336:** 608–611.
Thompson, F.L., Barash, Y., Sawabe, T., Sharon, G., Swings, J., and Rosenberg, E. (2006) *Thalassomonas loyana* sp. nov., a causative agent of the white plague-like disease of corals on the Eilat coral reef. *Int J Syst Evol Microbiol* **56:** 365–368.
Uku, J., Björk, M., Bergman, B., and Díez, B. (2007) Characterization and comparison of prokaryotic epiphytes associated with three East African seagrasses. *J Phycol* **43:** 768–779.
Vega Thurber, R., Willner-Hall, D., Rodriguez-Mueller, B., Desnues, C., Edwards, R.A., Angly, F., *et al*. (2009) Metagenomic analysis of stressed coral holobionts. *Environ Microbiol* **11:** 2148–2163.
Vizzini, S., Di Leonardo, R., Costa, V., Tramati, C.D., Luzzu, F., and Mazzola, A. (2013) Trace element bias in the use of CO2-vents as analogues for low-pH environments: implications for contamination levels in acidified oceans. *Estuar Coast Shelf Sci* **134:** 19–30.
Wahl, M. (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Mar Ecol Prog Ser* **58:** 175–189.
Webster, N.S., Uthicke, S., Botté, E.S., Flores, F., and Negri, A.P. (2013) Ocean acidification reduces induction of coral settlement by crustose coralline algae. *Glob Chang Biol* **19:** 303–315.
Weidner, S., Arnold, W., Stackebrandt, E., and Pu, A. (2000) Phylogenetic analysis of bacterial communities associated with leaves of the seagrass *Halophila stipulacea* by a culture-independent small-subunit rRNA gene approach. *Microb Ecol* **39:** 22–31.
Witt, V., Wild, C., Anthony, K.R.N., Diaz-Pulido, G., and Uthicke, S. (2011) Effects of ocean acidification on microbial community composition of, and oxygen fluxes through, biofilms from the Great Barrier Reef. *Environ Microbiol* **13:** 2976–2989.
Wolf, C., Frickenhaus, S., Kilias, E.S., Peeken, I., and Metfies, K. (2013) Regional variability in eukaryotic protist communities in the Amundsen Sea. *Antarct Sci* **11:** 1–11.
Yamamuro, M., Umezawa, Y., and Koike, I. (2004) Internal variations in nutrient concentrations and the C and N stable isotope ratios in leaves of the seagrass *Enhalus acoroides*. *Aquat Bot* **79:** 95–102.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. 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).
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.
Text S1. Sequence processing workflow.