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Changes in microbial communities in coastal sediments along natural CO_2 gradients at a volcanic vent in Papua New Guinea

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Summary

Natural CO_2 venting systems can mimic conditions that resemble intermediate to high pCO_2 levels as predicted for our future oceans. They represent ideal sites to investigate potential long-term effects of ocean acidification on marine life. To test whether microbes are affected by prolonged exposure to pCO_2 levels, we examined the composition and diversity of microbial communities in oxic sandy sediments along a natural CO_2 gradient. Increasing pCO_2 was accompanied by higher bacterial richness and by a strong increase in rare members in both bacterial and archaeal communities. Microbial communities from sites with CO_2 concentrations close to today's conditions had different structures than those of sites with elevated CO_2 levels. We also observed increasing sequence abundance of several organic matter degrading types of *Flavobacteriaceae* and *Rhodobacteraceae*, which paralleled concurrent shifts in benthic cover and enhanced primary productivity. With increasing pCO_2, sequences related to bacterial nitrifying organisms such as *Nitrosococcus* and *Nitrospirales* decreased, and sequences affiliated to the archaeal ammonia-oxidizing *Thaumarchaeota Nitrosopumilus maritimus* increased. Our study suggests that microbial community structure and diversity, and likely key ecosystem functions, may be altered in coastal sediments by long-term CO_2 exposure to levels predicted for the end of the century.

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Introduction

Two hundred years of anthropogenic activities, such as deforestation, agricultural land use and most prominently the use of fossil carbon as energy source, have led to an increase in atmospheric CO_2 concentrations, from long-term stable levels of around 280 µatm $pCO_{2(atm)}$ to presently > 400 μatm (Stocker *et al.*, 2013). The dissolution of atmospheric CO_2 in seawater significantly increases concentrations of dissolved inorganic carbon in the ocean and has reduced mean surface seawater pH by 0.1 units, a process commonly known as ocean acidification (OA). Realistic CO_2 emission scenarios of the Intergovernmental Panel for Climate Change (Stocker *et al.*, 2013) predict atmospheric CO_2 levels of 750 μatm and higher by the end of the century, which will result in a further pH drop of up to 0.4 units (Raven *et al.*, 2005; Andersson *et al.*, 2007). This change would be accompanied by a significant decrease in seawater carbonate ion concentrations and a lower saturation state for calcium carbonate minerals, directly affecting marine calcareous organisms such as coccolithophorids, foraminifera, scleractinian corals and coralline algae (Hall-Spencer *et al.*, 2008; Fabricius *et al.*, 2011; Uthicke *et al.*, 2013). Current OA has already measurable negative impacts on coral ecosystems and this trend will worsen in the future (Pandolfi *et al.*, 2011).

Benthic microbial communities, especially in the oxic layer of the surface of coastal marine sediments, may also be directly influenced by changes in seawater chemistry, potentially leading to different functional responses. Microbial communities play a critical role for ecosystems by supporting the main biogeochemical cycles, such as primary production and remineralization of organic material (Decho, 2000; Thompson *et al.*, 2004; Hewson *et al.*, 2007), by providing protection to larger organisms as biofilms (Mouchka *et al.*, 2010), and/or by influencing the settlement of faunal larvae on benthic substrata

(Webster *et al*., 2004). Despite a growing understanding of the structure of shallow sediment microbiota and of their role in the dynamics of coastal sediment ecosystems (e.g. Böer *et al*., 2009), responses of benthic microbial communities to environmental changes such as OA are still poorly understood.

Recent experimental studies on bacterioplankton and open water microbial assemblages indicated that increased OA might affect microbial processes such as nitrification rates (Beman *et al*., 2011) or activities of certain extracellular enzymes (Piontek *et al*., 2009) and could significantly alter bacterial community structure in the water column (Krause *et al*., 2012). Yet, all existing experiments and studies related to OA effects on microbial communities have, so far, only investigated responses to exposure that lasted days to weeks, and only one study exists on long-term effects on community composition and structure in coastal sediments (Kitidis *et al*., 2011). Community responses to increased pCO_2 observed in short-term experiments obviously do not reflect long-term acclimatization, evolutionary adaptations, complex feedbacks or indirect effects (e.g. changes in local geochemistry, faunal and floral composition), which occur within a natural marine system subjected to decennia or centuries of exposure. To obtain a realistic description of future impacts of chronic OA, ecosystems that are naturally exposed to high *p*CO2 must therefore be studied.

Here, we examined the composition and diversity of bacterial and archaeal communities associated with benthic sediments along a natural pCO_2 gradient, formed by volcanic CO_2 venting. The local conditions provide long-term pCO_2 gradients in the range of acidification projections for the next century, a high purity of the emitted volcanic gas as well as limited confounding effects caused by fluctuations in temperature, salinity or strong currents (Fabricius *et al*., 2011). Although many coastal systems experience natural pH fluctuations due to seasonal or annual changes or to microbial activities such as respiration or nitrification (Harley *et al*., 2006; Joint *et al*., 2010), any general increase in OA due to changes in atmospheric composition will ultimately lift the whole baseline of ecosystems to higher acidification level with peaks above today's maxima. The natural laboratory that we chose here is defined by long-term acidification through CO_2 vents that have been active for at least a century (Fabricius *et al*., 2011). We use this system to characterize potentially adapted microbial communities to OA peak levels expected at the end of this century and beyond (700–1500 μatm pCO_2). Our main hypothesis is that long-term exposure to higher CO_2 significantly affects the diversity of bacterial and archaeal communities. We focused on changes in local (sample) richness (α-diversity) and on community turnover between samples (β-diversity) while also taking into consideration effects on rare community members. Our second hypothesis is that it is possible to identify bacterial or archaeal taxa mostly affected by increased pCO_2 and that such taxa may be indicators of important biogeochemical processes such as the carbon, nitrogen or sulfur cycles reacting sensitively to OA.

Results and discussion

Sampling sites consisted of three geographically separated natural CO_2-seeping areas called Upa-Upasina, Esa'Ala and Dobu, and of adjacent control sites (Table S1, Fig. S1) (Fabricius *et al*., 2011). Because we could not find any significant location-based difference in overall community structure among the three sampling areas, but mostly CO_2 effects, we considered the found CO_2 effects on changes in microbial diversity of general relevance.

Diversity

α-Diversity. Both observed and estimated bacterial operational taxonomic unit (OTU) richness, as determined by 454 massively parallel tag sequencing (MPTS), increased with increasing pCO_2 levels (linear regression slope coefficients = 324.7, $P = 0.0012$, and 105.6, $P = 0.021$ respectively; Fig. 1, Table S2). No increase in bacterial richness was, however, detected by the community fingerprinting technique automated ribosomal intergenic spacer analysis (ARISA) (Fig. S2, Table S2), which is known to offer lower resolving power as compared with MPTS (Gobet *et al*., 2013). For archaeal communities, a similar, yet weaker trend of increasing richness was found when comparing communities from sites with high pCO_2 with those with medium and control pCO_2 (Mann–Whitney two-tailed test, $U = 0.0$, $P = 0.03$) (Fig. 1, Table S2). Thorough denoising of MPTS data enabled us to include MPTS OTUs originating from the rare biosphere into our ecological analysis. We defined 'rare' OTUs as in (Gobet *et al*., 2012), i.e. OTU occurring either as (i) one sequence in the whole data set (i.e. single sequence OTU absolute; SSO_{abs}), (ii) one sequence in at least one sample (i.e. single sequence OTU relative to a sample; SSO_{rel}), or (iii) two sequences in the whole data set (i.e. double sequence OTU absolute; DSO_{abs}). Based on that definition, 67% and 68% of all bacterial and all archaeal MPTS OTUs, respectively, were considered rare (Table S2). At all those levels of rarity, a significantly lower percentage of rare bacterial and archaeal OTUs was found at control sites when compared with medium or high pCO_2 sites. Although sharing a higher proportion of rare OTUs, medium and high pCO_2 sites could not be significantly distinguished

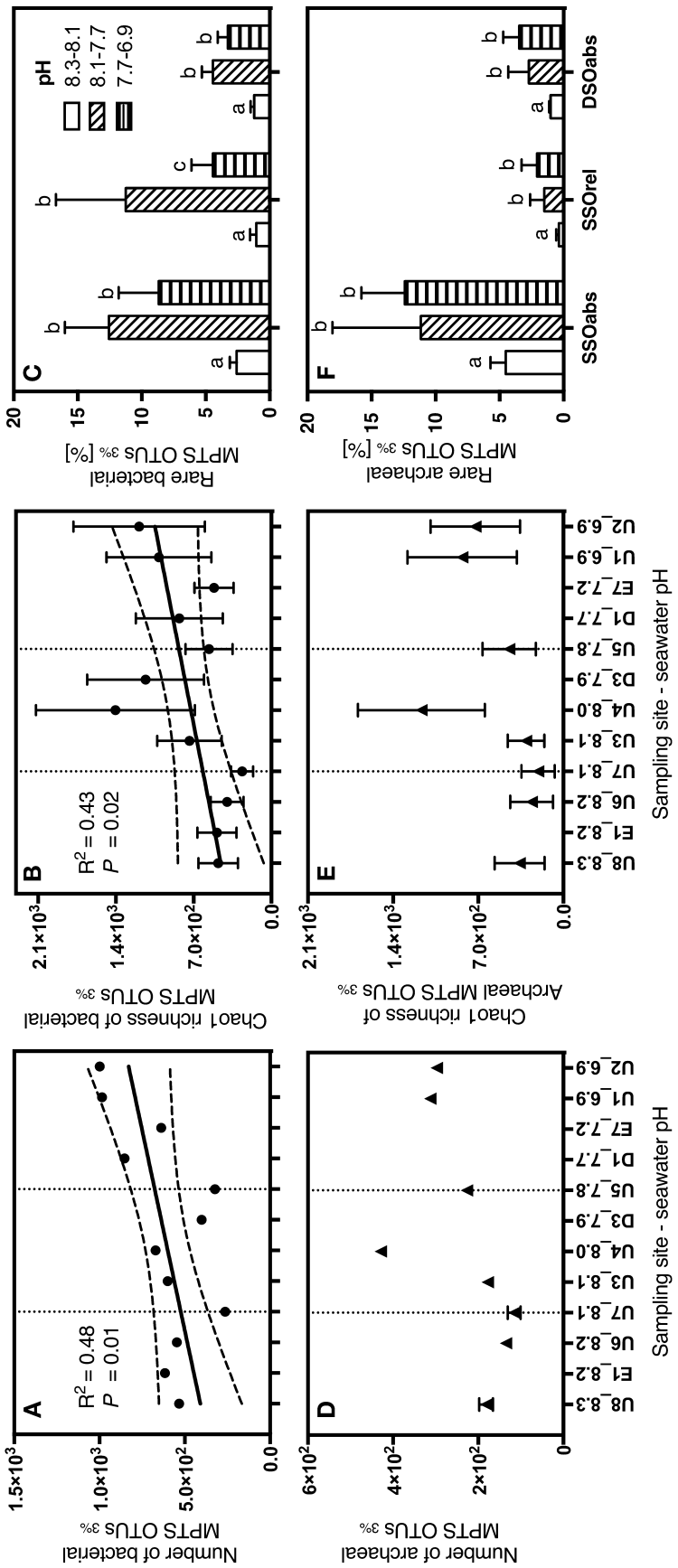

Fig. 1. Observed and predicted (Chao 1 richness estimator) diversity of bacterial (A, B) and archaeal (D, E) communities at all sampling sites, sorted by increasing pCO_2. Richness estimators were calculated by subsampling at the lowest number of sequences in the whole data set ($n = 856$ and 918 for bacteria and archaea respectively). The average percentages per sample of rare bacterial and archaeal MPTS $OTUs_{3\%}$ (as compared with the total number of sequences per sample) at three pCO_2 impact groups (see legend) are shown in C and F respectively. SSO_{abs}, single sequence OTU absolute; SSO_{rel}, single sequence OTU relative; DSO_{abs}, double sequence OTU absolute. See text for more detail.

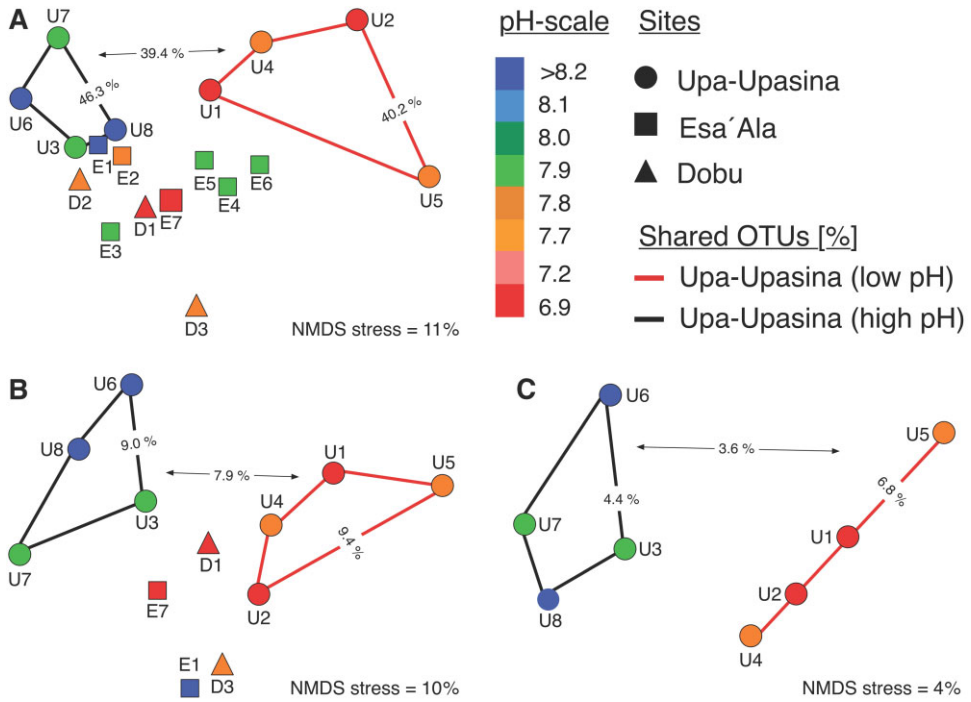

Fig. 2. Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarity matrices of the bacterial ARISA OTU (A), as well as bacterial (B) and archaeal (C) MPTS community tables. Symbol shape indicates site origin, and symbol colour indicates pH exposure. The black and red lines graphically highlight Upa-Upasina samples associated with low and high pH values respectively. Average percentages of numbers of shared OTUs among and between those two groups are indicated to facilitate the interpretation of community shifts.

from each other in terms of community structure (Fig. 1).

At the studied sites, Fabricius and colleagues (2011) observed a shift from coral to algal and seagrass species, and Russell and colleagues (2013) observed an increased productivity and biomass production of certain seagrass species at the CO_2 seep sites as compared with control sites. Despite those changes in the communities of primary producers, sediment organic carbon (OC) and nitrogen (N) concentrations remained at the low values typically found for coral reef environments, and did not correlate with local CO_2 effects (Uthicke *et al*., 2013). Nevertheless, the different types of OC (coral versus algae and seagrass) could affect microbial communities by being more or less accessible for uptake. Additionally, because a benthic cover with fast-growing primary producers is expected to have a much higher growth rate compared with coral species, this shift could have led to an increased turnover of organic matter reaching the sediments, which would then be available as carbon and nutrient source for the reef communities. For complex bacterial communities in oligotrophic arctic sediments, a positive energy-diversity relationship has been described (Bienhold *et al*., 2012). Thus, under the assumption that a higher turnover of carbon sources leads to more carbon-based processes in the system, the increase of bacterial and archaeal richness with pCO_2 in the studied sites could reflect changes in both energy source and availability. A different composition of carbon sources could be an additional factor favouring a richer community. Studies in terrestrial soils further support the theory that a pCO_2-induced plant growth could lead to increased bacterial biomass (Diaz *et al*., 1993; Zak *et al*., 1993) and more diverse communities (Berntson and Bazzaz, 1997).

β-Diversity. In addition to the trend of increasing microbial richness with pCO_2, we also detected a significant shift in community composition (β-diversity) along the eight sites of the well-defined Upa-Upasina pCO_2 gradient using MPTS data: The structure of the microbial communities that were exposed to pCO_2 levels > 700 μatm was significantly different from the structure of communities exposed to lower pCO_2 concentrations [Analysis of Similarity (ANOSIM) $R = 0.67$, $P = 0.02$, and $R = 0.73$, $P = 0.02$, for bacterial and archaeal communities, respectively, based on Bray–Curtis dissimilarities; Fig. 2]. For the bacterial communities, these results were highly congruent with those based on ARISA fingerprints (Fig. 2). Yet, all samples shared between 29% and 57% of OTUs based on ARISA, but only 3–16% of their OTUs based on MPTS. For all control pCO_2 sites (pH 8.3–8.1), the percentage of shared bacterial OTUs was 46% and 9% based on ARISA and MPTS respectively. Similarly, for medium and high pCO_2 sites (pH 8.1–6.9), the percentages of shared OTUs

were 40% and 9% for each technique respectively. When comparing all control sites with medium and high pCO_2 sites, microbial communities shared only 39% of ARISA and 8% of MPTS bacterial OTUs. Archaeal communities shared between 2% and 11% of MPTS OTUs among all sites. Control sites shared 4% of archaeal MPTS OTUs, whereas sites with medium and high pCO_2 shared 7% of archaeal MPTS OTUs. Control and high pCO_2 sites shared only 4% of archaeal MPTS OTUs.

A multivariate analysis with all available environmental data further supported the importance of CO_2 effects on changes in microbial community structure. The best redundancy analysis (RDA) model, which included the factors pCO_2/pH, carbon content and nitrogen content, explained 49% of bacterial community structure variation ($F_{5,6} = 1.2$, $P = 0.018$, based on 1000 permutations), whereas the level of acidification (pH) alone significantly contributed with 9% ($F_{1,10} = 1.04$, $P = 0.030$). The model for archaea explained 67% of community structure variation, whereas the level of acidification alone was marginally significant and explained 18% ($F_{1,6} = 1.3$, $P = 0.060$) of the total community structure variation.

Besides community shifts directly related to environmental parameters, we also assessed possible time-related shifts. For this analysis only, we included bacterial MPTS data from Dobu sites, based on samples taken 1 year after the first sampling (referred to Dobu in 2010 and in 2011 respectively). We found the bacterial community structures of year 2011 to be significantly different from those of 2010 (ANOSIM $R = 0.74$, $P < 0.001$). Nevertheless, when communities from all three sites and both sampling years were analysed together, we found bacterial communities from control sites to be significantly different from those at high pCO_2 sites ($R = 0.34$, $P = 0.017$) (Fig. S3). Communities from medium pCO_2 sites could not be statistically differentiated from those of the control or of the high CO_2 groups when 2010 and 2011 communities were compared.

Overall, these results indicate that high pCO_2 sites not only favour a richer community, in terms of α-diversity, but also select for different dominant microbial types. Although the aforementioned changes in ecosystem structure, such as increased benthic cover and higher nutrient availability, could be responsible for such a restructuring of microbial communities, there could also be pCO_2 effects on specific processes via enzyme kinetics (Piontek *et al*., 2013) or cell homeostasis (Krulwich *et al*., 2011). It has been experimentally shown that a change in seawater chemistry (the stress factor) results in higher abundance and production of complex bacterioplankton communities (Bouvier *et al*., 2012), and similar observations have been made in other studies (Boles *et al*., 2004; Girvan *et al*., 2005). At our study sites, we do not expect such microbial responses to short-term variations in a stress factor as being typical for the analysed communities because the investigated venting areas are supposed to have been stable for several decades (Fabricius *et al*., 2011). Ecologically speaking, the observed increase in microbial species diversity and the larger proportion of rare microbial types at high pCO_2 sites could potentially have a stabilizing effect on local biological processes and ecosystem functioning: Especially, a richer community of rare types might offer a reservoir of new or interchangeable functions (Gobet *et al*., 2012) that might be able to replace those that could be lost or weakened by environmental pressure.

Identification of key microbial types affected by CO_2 effects

General compositional changes. From a total of 39 535 bacterial sequences in our main data set, we taxonomically identified 4892 (out of 7443) bacterial OTUs, among which 1.5% occurred in at least 50% of all sites and two of the three sampling areas. From these 1.5%, 27% had a significant positive and 21% a significant negative linear relationship with increasing pCO_2, corresponding to 41% and 18% of bacterial sequences in the data set respectively. From a total of 21 021 archaeal sequences, we taxonomically identified 2749 (out of 3367) archaeal OTUs, among which 1.9% occurred in at least 50% of all sites in the Upa-Upasina sampling area. From these 1.9%, 15% had a significant positive and 2% a significant negative linear relationship with increasing pCO_2, corresponding to 25% and 37% of archaeal sequences in the data set respectively.

To further identify the microbial types that contributed the most to the observed changes in microbial richness and community composition at high pCO_2 sites, we performed a detailed taxonomic analysis of the 16S rRNA gene libraries: From all taxonomically identified bacterial MPTS OTUs, taxonomic information could be assigned to 98.5% on the phylum, 96.7% on the class level and 80.9% on the family level. From all taxonomically identified archaeal OTUs, 96.5% could be taxonomically assigned to the phylum level, 91.3% to the class level and 48.6% to the family level. At all sites, the communities were dominated by the bacterial classes *Alphaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria, Flavobacteria*, *Acidobacteria* and *Planctomycetacia* (Fig. S4). The archaeal communities were dominated at all sites by the *Crenarchaeota* classes *Marine Group I*, *Thermoprotei* and *pSL12* as well as the *Euryarchaeota* classes *Thermoplasmata*, *Methanomicrobia* and *Halobacteria* (Fig. S5).

Among the bacterial types (here considering various levels of taxonomic resolution) that only occurred at control, medium or high pCO_2 sites, none could be

conspicuously associated to a known microbial function (Table S3). One explanation could be that a pCO_2 increase of approximately 1000 μatm is not extreme enough to favour the development of completely different or endemic microbial communities when compared with local background (control) conditions, but rather leads to gradual shifts among the coexisting communities. Supporting this hypothesis, we found significant linear increases in relative sequence abundances with decreasing pH for a large number of bacterial and archaeal sequences when the *P* values of the partial multiple regression models were set to < 0.1.

At broad taxonomic resolution levels, we found significant linear increases in relative sequence abundance with decreasing pH for the bacterial phyla *Bacteroidetes* ($R^2 = 0.47$, $P = 0.014$) and *Proteobacteria* ($R^2 = 0.35$, $P = 0.042$), as well for the dominant archaeal *Crenarchaeota* class *Thermoprotei* ($R^2 = 0.43$, $P = 0.078$) (Table S4). An overall significant decrease of sequence abundances with decreasing pH was found for the bacterial phyla *Nitrospirae* ($R^2 = 0.38$, $P = 0.032$) and *Actinobacteria* ($R^2 = 0.28$, $P = 0.079$) as well as for the *Crenarchaeota* class *Marine Group I* ($R^2 = 0.49$, $P = 0.052$; Fig. 3) (Table S5).

Taxa potentially associated with the nitrogen cycle. Marine Group I are suspected to be among the most abundant marine microorganisms (DeLong, 1992) and made up to 37% of all archaeal sequences in our data set. Nevertheless, one prominent OTU, whose representative sequence was identified as *Nitrosopumilus maritimus* [99% nucleotide (nt) identity], made up to 13% of all archaeal sequences and increased in relative sequence abundance with seawater acidification ($R^2 = 0.51$, $P = 0.046$; Fig. 3; Table S5). As a member of the newly defined phylum *Thaumarchaeota*, which consists of a major group of ammonia-oxidizing archaea (AOA), previously assigned to the diverse *Marine Group I* (Könneke *et al*., 2005), *N. maritimus* is known for its high affinity to oxygen and for growing chemoautotrophically on ammonia with inorganic carbon as sole carbon source (Walker *et al*., 2010). Concomitantly, the only sequences of ammonia-oxidizing bacteria (AOB) identified in our data significantly decreased with increasing seawater acidification and were identified as *Gammaproteobacteria Nitrosococcus* sp. ($R^2 = 0.32$, $P = 0.057$; Fig. 3; Table S4), representing 1% of all bacterial sequences.

It is known that many AOA, in contrast to AOB, seem to be adapted to thrive even under oligotrophic ammonia concentrations, with *N. maritimus* even showing the highest substrate affinity to ammonia among all microorganisms known to date (Martens-Habbena *et al*., 2009). Another very distinctive property of many *Thaumarchaeota*, which is of special relevance to OA, is their ability to perform nitrification at neutral or lower pH: Lehtovirta-Morley and colleagues (2011) even reported an acidophilic nitrifying *Thaumarchaeota*, isolated from soil, with an optimum pH < 5. Despite the ratios of ammonia oxidation to nitrite being in the range of known AOB, it has therefore been suggested that AOA might use a different nitrification mechanism (Martens-Habbena *et al*., 2009; Walker *et al*., 2010; Stahl and de la Torre, 2012).

Although most strains of nitrifying bacteria are known to be pH sensitive with an optimal growth range between pH 7 and pH 8 (Tarre *et al*., 2004), there is evidence for the existence of acid-tolerant AOB communities (De Boer and Kowalchuk, 2001; Gieseke *et al*., 2006). Acid tolerance does not seem to be connected to any uncommon AOB taxa, but clearly requires physiological adaptations, such as ammonium ion transporters (Gieseke *et al*., 2006) because AOB require NH_3 for their ammonia monooxygenase. Because NH_3 is decreasing 10-fold with any 1-unit reduction in pH through protonation to NH_4^+, non-acid-tolerant communities may become substrate limited in acidified seawater (Stahl and de la Torre, 2012). It is not yet clear how common acid-tolerant AOB communities are in marine coastal sediments. Our observation of a decrease in AOB sequences therefore tends to support a previous study that reported a decrease in bacterial ammonia oxidation rates as a common response to acidification in marine environments (Beman *et al*., 2011), while keeping in mind that changes in sequence relative abundance do not necessarily translate directly to changes in process rates.

Although AOA seem to dominate ammonia-depleted regions of the ocean, there are many environments, such as coastal ecosystems, where they are outcompeted by substrate competition with AOB and phytoplankton for ammonia (Martens-Habbena *et al*., 2009; Stahl and de la Torre, 2012). Although we could not find any significant change in sequence abundances of major nitrogen-fixing bacteria or archaea in the sediment, Hutchins and colleagues (2009) reported evidence for elevated nitrogen fixation rates in the water column, based on a fertilizing effect of CO_2 as well as the range expansion of temperature sensitive nitrogen-fixing organisms, in a warmer ocean. Potentially higher availability of ammonium, from nitrogen fixation in the water column, which was not measured in this study, might offer an advantage to AOB, whereas AOA, which generally seem better adapted to oligotrophic ammonium levels (Stahl and de la Torre, 2012), could profit from lower pH levels and from the depletion of AOB in an acidified future ocean. In a short-term incubation experiment with coastal arctic sediment, Tait and colleagues (2014) could show that 2 weeks of increased *p*CO2 exposure at 760 μatm leads to a decreased transcription of bacterial ammonia-oxidizing genes (*amoA*), whereas archaeal *amoA* transcript

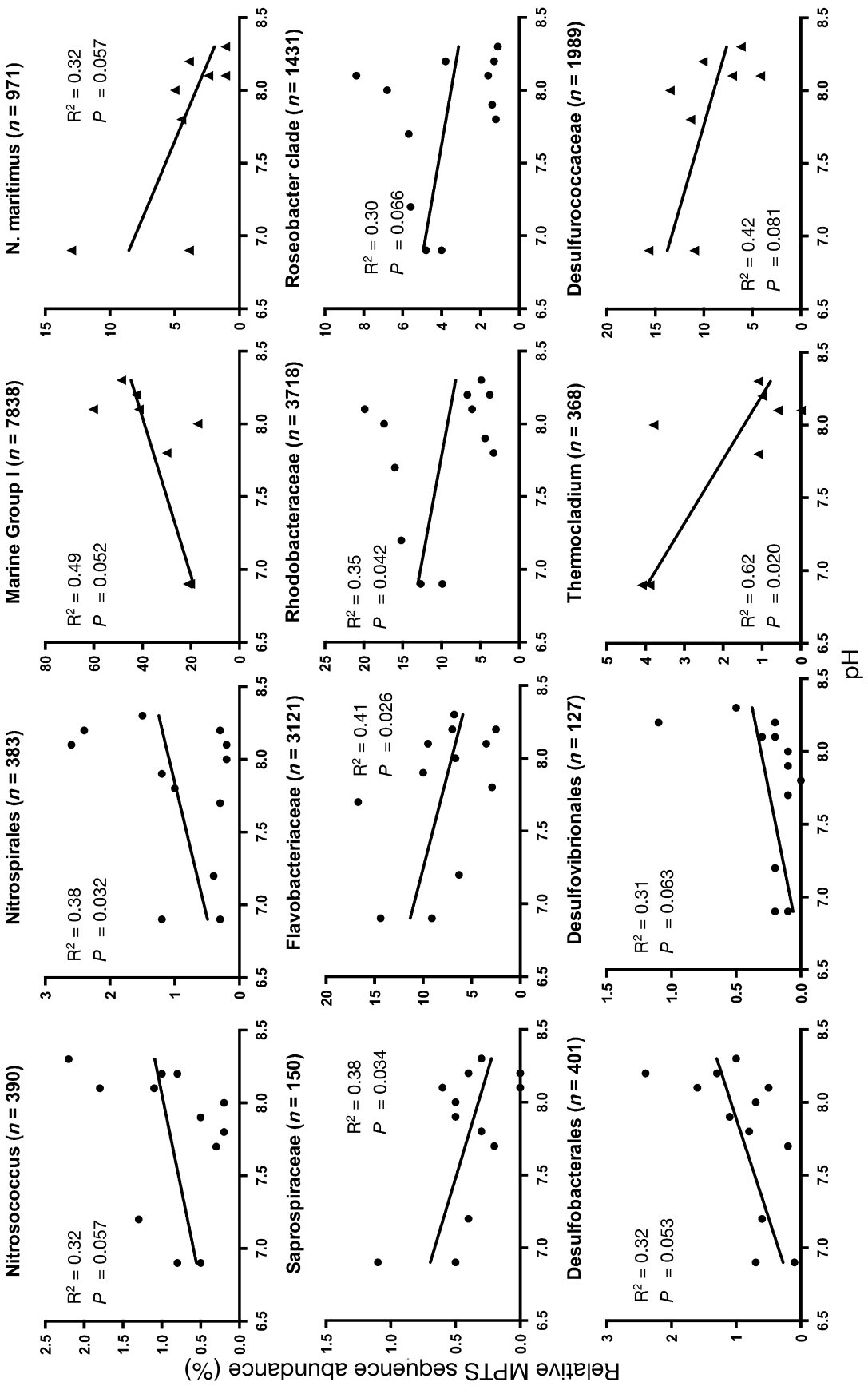

Fig. 3. Significant linear relationships of relative MPTS sequence abundances (y-axes) with pH (x-axes) of selected bacterial (circles) and archaeal (triangles) taxa. The taxonomic group and the total number of sequences in the data set (*n*) are displayed on top of each graph. For the statistical analysis, the *P* values of the partial multiple regression models were set to < 0.1.

numbers seemed not to be affected. At such acidification levels, Gazeau and colleagues (2014) could not detect any effect on the simultaneously measured sediment nitrogen fluxes within the limited time frame of this experiment, suggesting that AOA could, up to a certain point, compensate for putatively lower AOB activity. Such an activity shift from AOB to AOA could also explain the finding by Kitidis and colleagues (2011), who detected no effect on ammonia oxidation in coastal sediments with a history of long-term exposure to increased CO_2 from vents off the island of Ischia in the Mediterranean Sea.

Although in our data set, the increased occurrence of *N. maritimus* sequences with acidification was accompanied by a decrease in AOB sequences, it is interesting to note that sequences of all identified *Nitrospirales*, which made up 49% of all nitrite-oxidizing bacteria (NOB) identified in our data set, decreased with lower pH values ($R^2 = 0.38$, $P = 0.032$; Fig. 3; Table S4). This could suggest that ammonia oxidation rates might in fact be lower in communities that have an increased proportion in AOA, and this hypothesis should be tested in future work. Nevertheless, the quantitative aspect of the trends reported in our study should be taken with caution as it is the case with any polymerase chain reaction (PCR)-based methods, which do not represent true changes in organism abundances.

Taxa potentially related to the sulfur cycle. It is not expected that seawater acidification directly affects sulfur or sulfate reduction in sediments (Koschorreck, 2008), but among the sulfate-reducing bacteria, we found sequences of two obligate anaerobic deltaproteobacterial groups, the acetate-oxidizing *Desulfobacterales* and the non-acetate-oxidizing *Desulfovibrionales* with a significant negative linear relationship with increasing seawater acidification ($R^2 = 0.32$, $P = 0.053$; and $R^2 = 0.31$, $P = 0.063$ respectively; Fig. 3; Table S4). Together, they represent 14% of all bacterial sequences in the data set that could be identified as potential sulfate reducers. In contrast to bacterial sulfate reducers, we found a linear increase with acidification for sequences of the sulfate-reducing *Crenarchaeota* family *Thermocladium* ($R^2 = 0.62$, $P = 0.020$; Fig. 3; Table S5), which represented 22% of all putative sulfate-reducing archaea in our data set as well as the sulfur-reducing *Crenarchaeota* order *Desulfurococcaceae* ($R^2 = 0.42$, $P = 0.081$; Fig. 3; Table S5). Their OTUs were distantly related to *Thermocladium modestius* (74% nt identity), which has been shown to grow optimally at pH 4.0 and *Ignicoccus pacificus* (74% nt identity) that can grow between pH 4.5–7.0 respectively (Itoh *et al*., 1998; Huber *et al*., 2000).

Taxa potentially related to extreme conditions. *Thermocladium* species and *Desulfurococcaceae* species seem to be limited to extreme temperatures from 60 to 100°C (Madigan *et al*., 1997; Huber *et al*., 2000). Besides *Thermocladium*, we found sequences of other putatively extremophilic microorganisms with positive linear relationships to decreasing pH, such as sequences of the *Euryarchaeota* class of *Thermoplasmatales*, which contains three thermophilic and extremely acidophilic genera, including the most acidophilic of all known organisms with a favoured growth range at pH 0.5–4.0 (Cowan, 2000). We found bacterial sequences associated with the genus *Rhodothermus*, which is currently assigned to the *Rhodothermaceae* of the phylum *Bacteroidetes*, to significantly increase in abundance with increasing CO_2 effect ($R^2 = 0.30$, $P = 0.065$; Table S4). Species described so far grow at a pH range of 6.0–8.0 and temperatures range of 55–80°C (Marteinsson *et al*., 2010). Also, an increase in sequences of the order *Flavobacteriaceae*, which is known to have very limited tolerance to elevated temperatures above 50°C and which is one of the most dominant bacterial groups found at our high pCO_2 sites, questions the occurrence of an active extremophilic community in the sampled sediments. Because the seeping is of volcanic origin, one explanation could be that extremophilic members actually grow in the deeper hot subsurface and are transported to the surface through CO_2 seepages. As such, they might be considered as contaminants of the surface sedimentary communities.

Taxa related to the carbon cycle. Among the dominant bacterial phyla, *Bacteroidetes* (10% of all bacterial sequences), we found sequences associated with the order *Saprospiraceae*, which significantly increased with increasing pCO_2 ($R^2 = 0.38$, $P = 0.034$; Fig. 3; Table S4). *Saprospiraceae* are well known as major heterotrophic consumers of plant biomass and are common in marine littoral sand and coastal zones in various locations worldwide (Delk and Dekker, 1972; Saw *et al*., 2012). Sequences of the dominant family *Flavobacteriaceae* (8% of all bacterial sequences) significantly increased with seawater acidification ($R^2 = 0.41$, $P = 0.026$; Fig. 3; Table S4), with the most dominant OTUs being closely related to *Aquimarina* species, especially one prominent OTU ($R^2 = 0.53$, $P = 0.007$; Table S4), which made up to 14% of all *Flavobacteriaceae* sequences and was closely related to *Aquimarina mytili* (95% nt identity). This species was recently isolated and described to grow optimally at pH 7.0 and 25–30°C (Park *et al*., 2012). Another prominent *Flavobacteriaceae* OTU ($R^2 = 0.33$, $P = 0.051$, representing 26% of all *Flavobacteriaceae*; Table S4) was found to be closely related to *Actibacter sediminis* (96% nt identity) with an optimum growth at at pH 6.0 and 37°C (Kim *et al*., 2008). Generally, most members of the *Flavobacteriaceae* are all widely distributed in oxic marine environments and are aerobic chemoheterotrophs.

Typically, they abundantly occur in sediments when sufficient oxygen is available, for instance within surface mixed layers with oxygenating influence by bioturbation or oceanic currents (Bowman, 2006). Similar to *Saprospiraceae*, *Flavobacteriaceae* play a prominent role in the degradation of complex polymeric substrates in marine environments and are often associated with the occurrence of large amounts of plant detrital biomass (Bowman, 2006; Klippel *et al*., 2011). The higher cover of algae and seagrass at the high pCO_2 sites might therefore be an even more relevant reason for the observed increase in both *Flavobacteriaceae* and *Saprospiraceae* sequences.

Another major group of bacteria that significantly increased in sequence abundance with pCO_2 was the *Alphaproteobacteria* order *Rhodobacteraceae* ($R^2 = 0.35$, $P = 0.042$, representing 9% of all bacterial sequences; Fig. 3; Table S4). These are known to include acidophilic types, with a growth optimum at pH 6.0 (Madigan *et al*., 1997). But among the *Rhodobacteraceae* bacteria, the group of the so-called *Roseobacter* clade seemed to most prominently profit from low pH conditions (linear increase $R^2 = 0.30$, $P = 0.066$; Fig. 3; Table S4), making up to 39% of all *Rhodobacteraceae* sequences. Two very prominent OTUs with positive linear relationships to decreasing pH were closely related to *Phaeobacter daeponensis* (100% nt identity), which was isolated from a tidal flat in the Yellow Sea, Korea, and to *Phaeobacter gallaeciensis* (92% nt identity), which was isolated from the German Wadden Sea. Both species have been reported to grow optimally at seawater pH < 7.0–8.0 and have a chemoheterotrophic and obligate aerobic metabolism (Martens *et al*., 2006; Yoon *et al*., 2007). Generally, bacteria of this group have been isolated from habitats all over the world and are known to be one of the most abundant groups in marine environments (Martens *et al*., 2006).

Many *Flavobacteriaceae*, *Saprospiraceae* and *Roseobacter* types are often found associated with marine algae, seagrasses and coastal biofilms (Buchan *et al*., 2005). Interestingly, Teira and colleagues (2012) observed no direct effect on cell numbers or biomass of *Rhodobacteraceae* strain MED165 and *Flavobacteriaceae* strain MED217 when experimentally treated with increased pCO_2 alone. This supports our hypothesis that those bacterial groups could profit from a pCO_2-induced increase in algal and seagrass biomass instead of being directly influenced by the seawater chemistry. CO_2 effects of increased seawater acidification on *Alphaproteobacteria* and *Rhodobacteraceae* as well as *Bacteroidetes* and *Flavobacteriaceae* have been reported before: At Upa-Upasina, also one of our study sites, Morrow and colleagues (2014) observed that increasing CO_2 in the seawater was accompanied by an increase in relative MPTS sequence abundance of *Flavobacteria* associated with the hard coral *Porites cylindrica*. Also Vega Thurber and colleagues (2009) experimentally showed that numbers of *Flavobacteriaceae* on coral surfaces increased under low pH conditions, whereas Meron and colleagues (2010) reported a positive effect of acidified seawater on the occurrence of *Rhodobacteraceae* on coral tissue. Nevertheless, because many *Flavobacteriaceae* and *Rhodobacteraceae* are also associated with coral diseases (Cooney *et al*., 2002; Meron *et al*., 2010), the reason for an increase in abundances could, in those cases, be related to the corals themselves, which may be stressed by acidification and therefore more prone to infections.

In biofilms developing on glass slides and exposed to acidified seawater from the Great Barrier Reef, some *Flavobacteriaceae* were reported to increase in relative abundances with decreasing pH, whereas, in contrast to our results, members of the *Roseobacter* clade showed a decreasing trend with rising pCO_2 (Witt *et al*., 2011). In addition, Krause and colleagues (2012) experimentally showed that the community composition of microbial batch cultures, originating from the North Sea water column, significantly changed under the influence of small reductions in seawater pH and was accompanied by pH-dependent dissimilarities mostly caused by changes in *Flavobacteriaceae* and by a higher proportion of *Rhodobacteraceae* at ambient seawater pCO_2.

For some bacterial and archaeal taxa, which generally showed significant linear relationships with decreasing pH, we found sequence occurrences of subgroups, which could better be described by quadratic relationships, which suggests modal distributions rather than linear changes along the pH gradient. Nevertheless, with the exception of sequences related to unidentified *Marine Group I* OTUs, quadratic relationships of sequence occurrences with pH were scarce in our data set and seemed to represent rather complex relationships, for which it might be too speculative to discuss functional ecological patterns apart from possible niche differentiations. Nevertheless, we provide the results based on quadratic relationships and those related to microbial taxa without obvious ecological functions in the Supporting Information section (Text S1 and Tables S6 and S7).

Conclusions

Our results strongly support the hypothesis that the nitrogen cycle might be directly affected by rising oceanic pCO_2, with possible disadvantages for AOB and advantages for better adapted AOA. At our study sites, CO_2-induced seawater acidification leads to a gradual increase in the cover of benthic primary producers, namely algae and seagrasses. The expected increase in biomass turno-

ver may explain the observed increase in organic matter degrading bacterial groups from the *Flavobacteriaceae*, *Roseobacter* clade or *Saprospiraceae*. Besides these changes in taxonomic groups, we clearly evidenced an increase in bacterial and archaeal richness and an increase in rare OTUs along the acidification gradient. Even after factoring out the temporal shift in communities between two sampling years, we still observed a strong structuring effect of increased pCO_2 on bacterial and archaeal communities. Therefore, our study clearly reveals the sensitivity of complex natural bacterial and archaeal communities to seawater acidification, as expected for our future oceans, and identified the microbial components that are most likely to be affected.

Experimental procedures

Samples and sites

The three natural CO_2-seeping areas, called Upa-Upasina, Esa'Ala and Dobu, are located around the D'Entrecasteaux Islands in the Milne Bay Province of Papua New Guinea (Fig. S1) and were first described by Fabricius and colleagues (2011). The gas composition at the studied sites has been measured by Fabricius and colleagues (2011). At all three venting areas, the gas consists of over 99% CO_2, with traces of O_2, N_2 and CH_4, whereas no C_2H_2 or N_2O could be detected. The gas emitted at Upa-Upasina and Esa'Ala is H_2S-free, whereas the gas at Dobu contains about 163 ppm H_2S. Arsenic and 10 heavy metals (V, Cr, Co, Ni, Cu, Ga, Mo, Cd, Pb and U) were measured in the seawater above the benthos at all control and seep areas by Uthicke and colleagues (2013). They found no significant difference between seep and non-seep sites, and all concentrations were in ranges expected for pristine seawater. All seeps investigated in this study are assumed to have existed for at least seven decades (Fabricius *et al*., 2011).

Our study was based on 18 samples taken in 2010 and 6 samples taken in 2011, of the upper three centimetres of the oxic zone of sandy sediment at a water depth of 1–4 m (Table S1). All samples were immediately frozen and stored at −20°C until analysis. Control sites consisted of five sediment-sampling sites in 2010 and two in 2011, which were located at proximity of the seeps with very similar environmental settings, but not directly affected by CO_2 emissions. Their seawater pH levels ranged from 8.3 to 8.1 (NBS scale), corresponding to typical ambient pCO_2 concentrations of 360–400 μatm. The 13 sediment-sampling sites from 2010 with increased seawater acidification levels were sampled to represent a gradient, ranging from pH 8.0 to pH 6.9 (pCO_2 concentrations of 620–1500 μatm). The most continuous pH gradient was found at the site Upa-Upasina, from which eight samples were taken in 2010, including two control areas at opposite sites from the venting area along the coast. In 2011, two additional samples were taken from a CO_2-seeping site near Dobu with an average pH of 7.7, as well as two samples from a close-by venting area with an average pH of 6.8, and two from the Dobu control site (pH 8.2). Although the samples from 2010 represent the main data set for this study, the six samples from 2011 were used to get an estimate of community shift over time. The pH values in the overlaying water and in the upper sediment layer were measured during the field expeditions in 2010 and 2011, when the samples for this study were taken.

In terms of benthic cover, Fabricius and colleagues (2011) observed that with decreasing seawater pH, structurally complex coral communities gradually shift to assemblages dominated by slow-growing, long-lived and structurally simple *Porites*. Despite this change in community composition, coral cover remained stable in the range of pH 8.1–7.8, whereas no reef development was found at sites with > 1000 μatm pCO_2. For plant and algal cover, a loss of coralline species with decreasing pH was accompanied by an increase in seagrass and non-calcareous macroalgae (Fabricius *et al*., 2011; Russell *et al*., 2013). In surface sediments, concentrations of OC, nitrogen and siliceous spicules did not change along the gradient, whereas inorganic carbon, for instance in form of the remains of calcareous species, was only found in relevant amounts at sites with pCO_2 < 1000 μatm (Uthicke *et al*., 2013).

DNA extraction and microbial community fingerprinting

Total community DNA from each sample was isolated from 1 g well-mixed sediment using UltraClean Soil DNA Isolation kits (MoBio Laboratories, Carlsbad, CA, USA). Two aliquots with a final volume of 50 µl of Tris-EDTA buffer were stored at −20°C until analysis. DNA concentrations were determined spectrophotometrically using a NanoQuant infinite M2000 (Tecan Group, Männedorf, Switzerland). The DNA of all 18 sediment samples from 2010 was analysed via the molecular fingerprinting technique ARISA (Fisher and Triplett, 1999) using a triplicate PCR approach. For each PCR, the amount of DNA was adjusted to 25 ng. PCR amplification, analysis of amplified fragments via capillary electrophoresis and subsequent binning into OTUs were done as described previously (Ramette, 2009). An OTU was considered present in a sample only if it appeared in at least two out of the three PCR replicates for that sample.

MPTS

The combination of the molecular techniques ARISA and MPTS, used in this study, has been successfully applied before (Bienhold *et al*., 2012; Gobet *et al*., 2013; Jacob *et al*., 2013), because it allows an extensive assessment of overall α- and β-diversity, while leaving the opportunity of targeting specific samples via MPTS for a deeper diversity analysis and for taxonomic identification of key microbial indicators. Samples for which complete and comparable environmental parameters were available were chosen for MPTS. The sequencing was done via the Roche 454 FLX+ system (Roche, Basel, Switzerland) at the Research and Testing Laboratory (Lubbock, Texas, USA). For bacterial communities, DNA of 12 samples from 2010 and all 6 samples from 2011 were amplified with primers targeting the bacterial V1-V3 region (Gray28F 5′-TTTGATCNTGGCTCAG-3′). For the archaeal community, eight samples from 2010 from site Upa-Upasina were amplified with primers targeting the archaeal V6 region (Arch349F 5′-GYGCASCAGKCGMG AAW-3′). Sequences were deposited with the European

Nucleotide Archive under study accession number PRJEB7705 and sample accession numbers ERS580491-ERS580498 for archaeal samples U1 to U8, respectively, and sample accession numbers ERS580473- ERS580490 for bacterial samples U1-U8, E1, E7, D1, D3, CST, CSB, BST, BSB, ST and SB respectively.

Denoising (i.e. pyrosequencing noise removal), trimming, error correction (chimera and PCR errors) of the MPTS sequences were implemented with the *mothur* pipeline (version 1.23.1; Schloss *et al*., 2009). For alignment and taxonomic assignment of the bacterial and archaeal sequences, SILVA reference databases (both, version 115) provided with the *mothur* pipeline were used. MPTS OTUs were defined at the 3% nt difference level. OTU richness was estimated with the Chao1 richness estimator, which takes into consideration the number of rare sequences while correcting for the estimated undiscovered species being present in a sample (Chao, 1984). Chao1 richness was only calculated after re-sampling every sample to the lowest number of sequences found in any sample. The inverse Simpson's index (Simpson, 1949) was used to measure the evenness of the diversity in each sample as the index is maximized when all OTUs are equally represented. For all bacterial and archaeal OTUs significantly changing with the pCO_2 gradient, we performed a systematic search for described microbial nucleotide sequences via the BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST; standard nucleotide data base for bacterial and archaeal 16S rRNA genes; Altschul *et al*., 1990) using the best representative sequences for each OTU, followed by filtering of the results for sequences matching with > 90% nt identity, > 98% query coverage and E-value < 10^{-6}.

Statistical analysis

The multivariate statistical analyses of all molecular data from ARISA and MPTS, along with the measured environmental parameters, were performed with the R statistical language (version 2.15.0; R Core Team, 2013, URL: http://www.R-project.org), using the R packages 'MASS' (Venables and Ripley, 2002) and 'vegan' (Oksanen *et al*., 2013) to perform non-metric multidimensional scaling, one-way ANOSIM, stepwise model selection, canonical RDA and associated permutation tests (reviewed in Ramette, 2007). For the single OTU analysis, changes in the sequence abundance of an OTU were considered to be significant when the *P* values of the partial multiple regression model (1000 permutations) were < 0.1, after false discovery rate correction (Benjamini and Hochberg, 1995). To quantify the specific pCO_2 effects on community variation while disentangling the effects of other measured parameters, we performed RDA with models including the environmental parameters pCO_2/pH, total carbon content, nitrogen content, as well as geographic distances between the sampling sites (Table S1, partly published by Uthicke *et al*., 2013). Scripts of the MULTICOLA software (version 1.5, available at URL: http://www.mpi-bremen.de/Software_2.html; Gobet *et al*., 2010) were used to assess the effects of rare MPTS OTUs on the reported ecological interpretations. For some statistical analysis, sites were grouped into the three categories (control, medium and high pCO_2), corresponding to the pH ranges of 8.3–8.1, 8.1–7.7 and 7.7–6.9 respectively. Statistical tests to compare models of linear and quadratic relationships of relative MPTS sequence abundances with environmental parameters were performed as described in Makarenkov and Legendre (2002). Significant linear relationships of taxa and OTUs were only considered if there was no significantly better quadratic model found.

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Supporting information

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

Fig. S1. Map of sampling area (map from Google 2014), with the seeping sites Upa-Upasina (A), Esa'Ala (B) and Dobu (C). The coordinates for each sample can be found in Table S1.

Fig. S2. Numbers of bacterial ARISA OTU per seawater pH category, i.e. control (pH 8.3–8.1), medium (pH 8.1–7.7) and high (pH 7.7–6.9) pCO_2 sites.

Fig. S3. Non-metric multidimensional scaling ordination of Bray–Curtis dissimilarity matrices based on bacterial community data (MPTS $OTU_{3\%}$) from samples of 2010 and 2011 (dotted line). The size of the symbol dots is a (non-linear) representation of differences in pCO_2 exposure at the respective sites. Coloured shapes highlight the three CO_2 impact groups 'control' (grey), 'medium pCO_2' (blue) and 'high pCO_2' (red).

Fig. S4. Dominant bacterial phyla (A) and classes (B) at the studied sites. The taxonomic assignments are based on MPTS sequences using the SILVA 16S rRNA reference database. At the top the site names with the respective seawater pH are indicated.

Fig. S5. Dominant archaeal phyla (A) and classes (B) at the studied sites. The taxonomic assignment is based on MPTS sequences using the SILVA 16S rRNA reference database. At the top the site names with the respective seawater pH are indicated.

Table S1. Overview of all 24 samples with time of sampling, sampling location, sampling depth, type of molecular analysis and measurements of environmental parameters.

Table S2. Summary of bacterial and archaeal sample diversity, based on ARISA and MPTS and abundance of rare bacterial and archaeal MPTS OTUs.

Table S3. Number of bacterial and archaeal OTUs and taxa, characterized by MPTS sequences which either uniquely occur at samples from the categories 'high', 'medium' and 'control pCO_2' or show linear or quadratic relationships with pH.

Table S4. Bacterial taxa and MPTS OTUs, which were found to significantly increase in terms of relative sequence abundances with increasing seawater pCO_2.

Table S5. Archaeal taxa and MPTS OTUs, which were found to significantly increase in terms of relative sequence abundances with increasing seawater pCO_2.

Table S6. Bacterial taxa and MPTS OTUs, which were found to have a significant quadratic relationship with increasing pCO_2.

Table S7. Archaeal taxa and MPTS OTUs, which were found to have a significant quadratic relationship with increasing pCO_2.

Text S1. Taxa with no obvious ecological functions, Quadratic relationships of microbial MPTS sequence occurrence with pH.