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## Original Article

# Minor impacts of reduced pH on bacterial biofilms on settlement tiles along natural pH gradients at two $CO_2$ seeps in Papua New Guinea

Christiane Hassenrück,[1,*] Halina E. Tegetmeyer,[1,2] Alban Ramette,[1,3] and Katharina E. Fabricius[4]

[1]*Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen, Germany*
[2]*Bielefeld University, Center for Biotechnology – CeBiTec, Universitätsstraße 27, 33615 Bielefeld, Germany*
[3]*Institute of Social and Preventive Medicine, Bern University, Finkenhubelweg 11, 3012 Bern, Switzerland*
[4]*Australian Institute of Marine Science, PMB 3, Townsville, Queensland 4810, Australia*

**Corresponding author: tel: +49 421 2028 838; fax: +49 421 2028 690; e-mail: chassenr@mpi-bremen.de*

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Bacterial biofilms provide cues for the settlement of marine invertebrates such as coral larvae, and are therefore important for the resilience and recovery of coral reefs. This study aimed to better understand how ocean acidification may affect the community composition and diversity of bacterial biofilms on surfaces under naturally reduced pH conditions. Settlement tiles were deployed at coral reefs in Papua New Guinea along pH gradients created by two $CO_2$ seeps. Biofilms on upper and lower tiles surfaces were sampled 5 and 13 months after deployment. Automated Ribosomal Intergenic Spacer Analysis was used to characterize 240 separate bacterial communities, complemented by amplicon sequencing of the bacterial 16S rRNA gene of 16 samples. Bacterial biofilms consisted predominantly of Alpha-, Gamma-, and Deltaproteobacteria, as well as Cyanobacteria, Flavobacteriia, and Cytophagia, whereas taxa that induce settlement of invertebrate larvae only accounted for a small fraction of the community. Bacterial biofilm composition was heterogeneous, with on average only ~25% of operational taxonomic units shared between samples. Among the observed environmental parameters, pH was only weakly related to community composition ($R^2 \sim 1\%$), and was unrelated to community richness and evenness. In contrast, biofilms strongly differed between upper and lower tile surfaces (contrasting in light exposure and grazing intensity). There also appeared to be a strong interaction between bacterial biofilm composition and the macroscopic components of the tile community. Our results suggest that on mature settlement surfaces *in situ*, pH does not have a strong impact on the composition of bacterial biofilms. Other abiotic and biotic factors such as light exposure and interactions with other organisms may be more important in shaping bacterial biofilms on mature surfaces than changes in seawater pH.

**Keywords:** amplicon sequencing, bacterial communities, $CO_2$ vents, community fingerprinting, coral reefs, ocean acidification, natural laboratory, settlement tiles.

## Introduction

The colonization of bare substrata by reef organisms is a crucial process affecting the resilience and recovery of tropical coral reefs. The settlement of coral larvae is especially important to replenish coral cover after reef disturbance (Webster *et al.*, 2004; Witt *et al.*, 2011a) and to prevent a shift from coral to algal dominance (Hughes *et al.*, 2003; Hoegh-Guldberg *et al.*, 2007). Crustose coralline algae (CCA) and their adherent bacterial biofilms play a major role in the mediation of coral larval settlement (Negri *et al.*, 2001; Harrington *et al.*, 2004). Bacterial biofilms can also enhance settlement rates in the absence of CCA (Webster *et al.*, 2004). Certain bacterial strains, e.g. *Pseudoalteromonas* or

*Roseobacter*, produce chemical compounds that trigger the settlement of coral larvae (Negri *et al.*, 2001; Tebben *et al.*, 2011; Sneed *et al.*, 2014; Tebben *et al.*, 2015). Therefore, changes in the community composition of bacterial biofilms, specifically those related to settlement-inducing bacteria, have the potential to affect the rate and success of coral larval settlement (Webster *et al.*, 2011; Sneed *et al.*, 2015).

Ocean acidification (OA), one of the major threats to coral reefs, can alter the composition of bacterial biofilms on various reef substrata (Witt *et al.*, 2011a; Webster *et al.*, 2012, 2013). In incubation experiments, Witt et al. (2011a,b) showed that bacterial biofilms on glass slides differed in community composition after short-term exposure to $CO_2$-enriched seawater, with decreases in the relative abundance of *Roseobacter*. Webster *et al.* (2012) detected an effect of reduced pH treatments on the bacterial community associated with natural reef substrata, including CCA, after 6 weeks of incubation. After further experiments, Webster *et al.* (2013) hypothesized that declines in settlement rates with decreasing pH may be a result of changes in the composition of the bacterial community associated with CCAs.

To go beyond the scope of laboratory experiments, which are limited to observations of organisms outside their natural environment, naturally $CO_2$-rich sites are increasingly being used as analogues for future OA scenarios (Hall-Spencer *et al.*, 2008; Fabricius *et al.*, 2011; Enochs *et al.*, 2015). At these sites, the whole ecosystem is exposed to reduced pH conditions, providing the opportunity to assess the impact of pH changes in a natural environment. First observations from naturally $CO_2$-rich sites supported the conclusion that distinct bacterial biofilm communities develop under reduced pH conditions in temperate rocky shore environments (Lidbury *et al.*, 2012). However, data from naturally $CO_2$-rich sites are still scarce, and have so far been limited to short-term observations. Bacterial biofilms on mature surfaces that have undergone long-term successions, and may thus represent the majority of surfaces available for settlement on coral reefs, are underexplored. Furthermore, potential interactions between bacterial biofilm composition and macro-organisms, such as algae and macro-invertebrates, which are part of such mature surfaces, are not yet well understood.

Here, we studied bacterial biofilms on settlement tiles in two tropical coral reef systems, where volcanic $CO_2$ seeps locally reduce seawater pH, thus mimicking conditions expected under OA (Fabricius *et al.*, 2011). The aims of the study were (I) to describe the composition and diversity of the bacterial communities on both the upper and lower sides of mature settlement tiles that were deployed on the reef for 5–13 months, (II) to assess the importance of water pH in shaping bacterial biofilm composition, and (III) to elucidate the relationship between bacterial community composition, abiotic environmental factors, and biotic interactions with other organisms that are part of the successional development on natural reef surfaces. We used a combination of high sample throughput and high resolution molecular techniques to describe bacterial biofilm communities, as well as data on the macroscopic tile community and the carbonate system of the water surrounding the tiles provided in Fabricius *et al.* (2015) for an integrated assessment of bacterial biofilms and the factors shaping their composition and diversity.

## Material and methods

### Sampling

Polyvinyl chloride (PVC) settlement tiles (11.5 cm × 11.5 cm) were deployed along pH gradients created by $CO_2$ seeps at two coral reefs in Papua New Guinea: Upa Upasina on Normanby Island (9.82 S, 150.82 E) and Dobu Island (9.74 S, 150.86 E; Supplementary Figure S1). Sites and tile deployments were described in detail in Fabricius *et al.* (2011). Briefly, 45 tiles per reef were attached horizontally ~2 cm above the reef substrate, in December 2011 (Supplementary Figure S2A and B). Tiles were deployed typically >5 m apart along the reef slopes, at 3 m depth. A subsample of 30 tiles per reef was sampled for the analysis of bacterial biofilms. These 30 tiles covered pH gradients ranging from $pH_T$ (pH total scale) 7.6 to 8.0 at Upa Upasina, and from $pH_T$ 7.4 to 8.0 at Dobu Island. At Upa Upasina, the range of pH values was covered rather evenly, whereas at Dobu Island $pH_T$ values were either lower than 7.8 at the $CO_2$ seeps (20 tiles) or approximately 8.0 or higher at the reference site (10 tiles). Furthermore, the reference site at Dobu Island was farther from the seep site (~2 km) than at Upa Upasina (~500 m; Supplementary Figure S1). Both at 5 and 13 months after deployment, a previously unsampled 2 cm × 2 cm square area was scraped off of the lower and upper sides of each tile with a sterile scalpel immediately after collection, to sample the bacterial biofilm communities on the tiles. Samples were stored in RNAlater (Ambion) in Eppendorf tubes for later DNA extraction. Patches on the settlement tiles with a high density of macro-invertebrates were avoided during the sampling of the biofilm. Data on the carbonate chemistry (pH, $pCO_2$, dissolved inorganic carbon ($C_T$), total alkalinity ($A_T$), calcite and aragonite saturation states Supplementary Table S1) that each tile was exposed to are published in Fabricius *et al.* (2015). Additionally, the percentage cover of the main taxonomic groups on the surfaces of the whole tiles was available as described in Fabricius *et al.* (2015). The taxonomic groups sampled for the upper tile sides were: Crustose coralline algae (CCA), turf algae, green filamentous algae, and brown biofilm; and for the lower tile sides: CCA, turf algae, red algae (*Peyssonnelia*), other macroalgae, cyanobacterial biofilm, foraminifera, bryozoans, sponges, ascidians, bivalves, and polychaetes.

### Molecular analyses

DNA was extracted from 240 biofilm samples using the UltraClean Soil DNA extraction kit (MoBio) following the manufacturer's instructions. For a high sample throughput screening of bacterial diversity and community composition, automated Ribosomal Intergenic Spacer Analysis (ARISA; Fisher and Triplett, 1999) was conducted with all samples as previously described (Ramette, 2009; Hassenrück *et al.*, 2015, 2016). Operational taxonomic units (OTUs) generated by ARISA are referred to as $OTU_{ARISA}$. A table with relative $OTU_{ARISA}$ abundances is available at Pangaea (https://doi.pangaea.de/10.1594/PANGAEA.860795).

Furthermore, a subset of 16 samples from the lower tile side from both sampling times and reefs covering reference and reduced pH conditions was selected for 16S amplicon sequencing to obtain a taxonomic profile of the bacterial biofilm community on the settlement tiles. Paired-end sequences (2 × 300 bp) of the V3–V4 hypervariable region of the bacterial 16S rRNA gene were generated on the Illumina MiSeq platform using the universal bacterial primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (CeBiTec, Bielefeld; Klindworth *et al.*, 2013). Amplicon sequences were quality trimmed, clustered into OTUs, and taxonomically classified using the Silva Ribosomal Database (Quast

*et al.*, 2013) as described previously (Supplementary Table S2; Hassenrück *et al.*, 2016). For the further analysis, singleton OTUs and OTUs that were unclassified on domain level or affiliated with chloroplast and mitochondrial sequences were removed. An overview of the output of the amplicon sequencing is provided in Supplementary Table S3. Further curation of the taxonomic assignment of specific OTUs was done using the online BLAST tool on the NCBI (National Center for Biotechnology Information) website with the 16S ribosomal refseq database (accessed 19.05. 2016). Sequences are available at ENA (European Nucleotide Archive; project accession number PRJEB14127). OTUs generated by amplicon sequencing are referred to as $OTU_{Amplicon}$. A table with $OTU_{Amplicon}$ abundances is available at Pangaea (https://doi.pangaea.de/10.1594/PANGAEA.860795). Bacterial taxon names are further used to designate sequence affiliation to the respective taxon, while abundance of bacterial taxa refers to sequence proportion.

### Statistical analysis

To visualize patterns in bacterial community composition, non-metric multidimensional scaling (NMDS) plots were calculated based on the Bray–Curtis dissimilarity matrix of relative $OTU_{ARISA}$ abundances. Analysis of similarity (ANOSIM) was conducted to test for differences in community composition between reefs, sampling times, tile sides, and pH categories. pH categories were defined by $pH_T$ values of >7.95 (reference), 7.95–7.80 (intermediate), and <7.80 (low). At Dobu Island, no samples were available for the intermediate pH category. Pairwise comparisons were corrected for multiple testing (Benjamini and Hochberg, 1995). Furthermore, the contribution of pH values to explaining variation in bacterial community composition based on ARISA, while accounting for other parameters (reef, tile side, and sampling time), was tested using redundancy analysis (RDA) and variation partitioning. Water column parameters that were highly correlated with pH, i.e. dissolved organic carbon concentration ($C_T$), $pCO_2$, calcite and aragonite saturation state, were excluded from the RDA models. Total alkalinity ($A_T$) did not strongly correlate with pH but did not improve the RDA models. Prior to the RDA the bacterial community table was corrected for compositionality effects of proportional data using a centred log ratio (clr) transformation (Fernandes *et al.*, 2014). The significance of the individual parameters of the RDA models was assessed using restricted permutation tests. Bacterial community richness (based on $OTU_{ARISA}$ number) and evenness (based on the inverse Simpson index; see Oksanen *et al.*, 2015) were tested with the same models.

Patterns in bacterial community composition between ARISA and sequencing data of the same samples were compared using Mantel tests based on Bray–Curtis and Jaccard dissimilarity matrices.

To investigate interactions between abiotic and biotic factors influencing the composition of the bacterial biofilm, the directional relationships between water pH, bacterial community composition, and the macroscopic community on the settlement tiles were evaluated using path analysis (Legendre and Legendre, 1998; Bienhold *et al.*, 2012; Sawall *et al.*, 2012). Path analysis allows a mathematical assessment of the likelihood of ecological scenarios, i.e. path models, based on *a priori* hypotheses about direct and indirect causal dependencies between blocks of variables (Legendre and Legendre, 1998). Here, path models were constructed separately for upper and lower tile sides and the two sampling times using z-transformed pH values, clr-transformed bacterial $OTU_{ARISA}$ data, and clr-transformed percentage tile cover. To generate the *a priori* hypotheses about the original path models, the effects of water pH, reef and tile cover on bacterial community composition were tested in separate RDAs per tile side and sampling time (Supplementary Table S4). The original path models included the following effects between variables: water pH on tile cover, water pH on bacterial communities, tile cover on bacterial communities (and vice versa). To account for effects of unknown parameters that may differ between reefs, alternative models including an effect of reef on bacterial communities and on tile cover were calculated. In summary, the models included water pH and reef as exogenous variables and bacterial community composition and tile cover as endogenous variables. The correlation between the variables was assessed using the RV coefficient (Robert and Escoufier, 1976). Unexplained variation of endogenous variables was represented by the coefficient of non-determination (Legendre and Legendre, 1998). The goodness-of-fit of the path models was evaluated with the Chi-square test, as well as the Akaike and Baysian Information Criterion (AIC and BIC, respectively). The best models were selected based on a non-significant Chi-square test and the minimum AIC.

All statistical analyses were conducted in R (R Core Team, 2015) using the packages vegan (Oksanen *et al.*, 2015), FactoMineR (Husson *et al.*, 2016), and sem (Fox *et al.*, 2015). Significance was evaluated at a significance level of 0.05.

## Results

### Bacterial community fingerprinting

The biofilm material collected from 4 $cm^2$ of the settlement tiles varied considerably in amount, shape and composition (Supplementary Figure S2C–F). Some samples only contained very little material, whereas others were filled with approximately 0.5 ml of brown, red or green algal material with occasional traces of calcareous substances. The macroscopic community on the whole tiles was dominated by crustose coralline algae, especially at reference pH conditions, the red algae *Peyssonnelia* sp., and turf algae (Supplementary Figure S3).

ARISA of the bacterial communities on the settlement tiles detected a total of 451 $OTU_{ARISA}$ with an average of 143 $OTU_{ARISA}$ per sample (25th percentile: 127, 75th percentile: 163). We did not detect an effect of pH, tile side or sampling time on OTU number or evenness. However, the bacterial communities were slightly more diverse at Dobu Island with $147.6 \pm 2.5$ $OTU_{ARISA}$ (mean ± standard error) and an inverse Simpson of $48.1 \pm 1.4$, compared to $139.7 \pm 2.6$ and $33.7 \pm 1.3$ at Upa Upasina, respectively (Table 1).

NMDS showed a highly heterogeneous composition of the bacterial communities with on average only 25% shared $OTU_{ARISA}$ between any two samples (Figure 1). In the first two NMDS dimensions the strongest visible patterns in bacterial community composition were between upper and lower tile sides. A weak pattern by pH category was mainly recognizable in the NMDS dimensions two and three (Figure 1). ANOSIM confirmed that at a given sampling time and tile side, the bacterial communities were different between pH categories (Supplementary Table S5). However, the separation of the bacterial communities between pH categories was weak, especially at Upa Upasina, with

ANOSIM R values of less than 0.5. The differences in bacterial community composition between reference and low pH categories at Dobu Island were more pronounced with an ANOSIM R between 0.51 and 0.88, except for samples from the lower tile side after 13 months deployment (Supplementary Table S5).

**Table 1.** Contribution and significance of observed environmental factors to explaining the variation in bacterial richness ($OTU_{ARISA}$ number), evenness (inverse Simpson index) and community composition on the settlement tiles, based on redundancy analysis and variation partitioning.

| Source of variation | Adjusted $R^2$ | df | *F* | P |
|---|---|---|---|---|
| Richness | | | | |
| Complete model | 0.030 | 4,191 | 2.494 | 0.041 |
| Reef | 0.021 | 1,191 | 5.057 | 0.030 |
| pH | −0.004 | 1,191 | 0.180 | 0.678 |
| Tile side | 0.009 | 1,146 | 2.391 | 0.132 |
| Sampling time | −0.002 | 1,146 | 0.663 | 0.427 |
| Evenness | | | | |
| Complete model | 0.181 | 4,191 | 11.750 | 0.001 |
| Reef | 0.131 | 1,191 | 31.813 | 0.001 |
| pH | 0.002 | 1,191 | 1.517 | 0.236 |
| Tile side | 0.000 | 1,146 | 0.985 | 0.306 |
| Sampling time | −0.006 | 1,146 | 0.009 | 0.925 |
| Community composition | | | | |
| Complete model | 0.088 | 4,191 | 5.703 | 0.001 |
| Reef | 0.012 | 1,191 | 3.598 | 0.001 |
| pH | 0.010 | 1,191 | 3.193 | 0.001 |
| Tile side | 0.051 | 1,146 | 11.576 | 0.001 |
| Sampling time | 0.013 | 1,146 | 3.489 | 0.001 |

Significance was assessed using restricted permutations. $R^2$: explained variation, df: numerator and denominator degrees of freedom, *F*: test statistic, P: *p*-value.

Furthermore, differences in bacterial community composition between reefs were detected among samples from the same pH category with ANOSIM R values ranging from 0.38 to 0.75. These differences were in some cases more pronounced than differences in bacterial community composition between pH categories at the same reef (Supplementary Table S5).

Redundancy analysis showed that the observed environmental parameters (pH, reef, tile side, and sampling time) explained only a small, yet significant, fraction of the variation in bacterial community composition ($R^2 = 8.8\%$; Table 1). Among these parameters, tile side was the most important one explaining 5% of the variation, compared to less than 1.5% attributed to the other observed parameters, including pH (Table 1).

## Path analysis

We evaluated the interactions between abiotic and biotic factors influencing the composition of the bacterial biofilms by constructing directional path models to assess the relationships between water pH, bacterial community composition (ARISA data), and the macroscopic community (tile cover) on the settlement tiles. On the lower tile side, the best model showed significant path coefficients from water pH to tile cover and from tile cover to bacterial community composition at both sampling times (Figure 2). The direct path from water pH to bacterial community composition was not statistically significant as expected based on RDA (Supplementary Table S4), and was discarded in the final path model. Reversing the path from tile cover to bacterial community composition or including reef as exogenous variable did not improve the path models for the lower tile side.

On the upper tile side, the directional relationships between water pH, tile cover and bacterial community composition were more difficult to assess. The fit of the best model was poorer

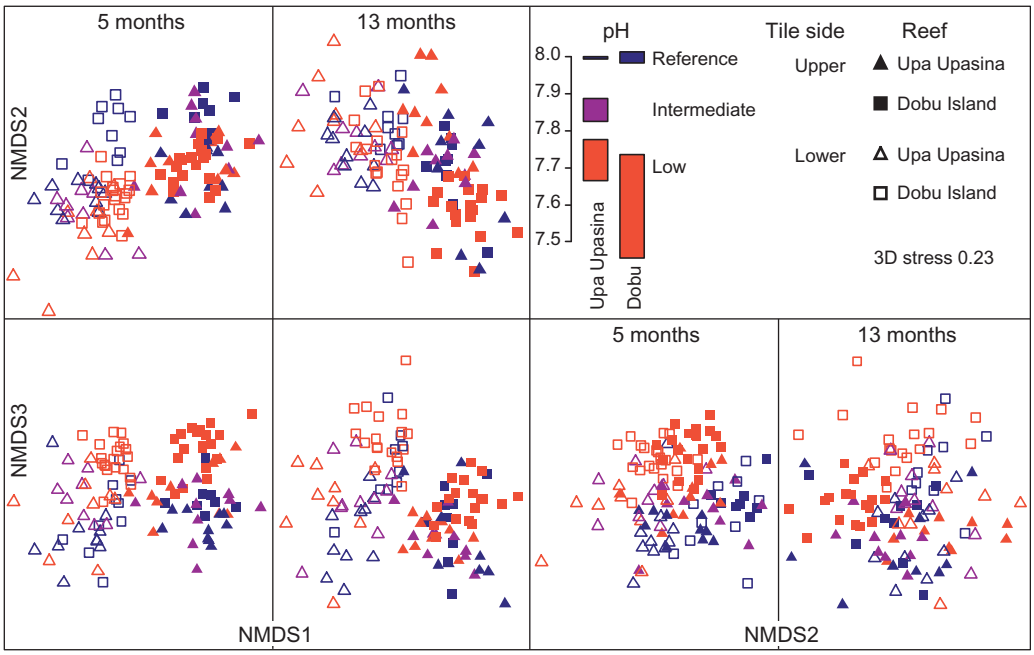


**Figure 1.** Non-metric multidimensional scaling (NMDS) plot of the bacterial communities on the upper and lower surfaces of the settlement tiles after 5 and 13 months, based on Bray–Curtis dissimilarity of the ARISA data set. Average percentage of shared $OTU_{ARISA}$: 25%. The range of pH values covered per pH category at each reef is shown as bars along a pH scale.

compared to the models for the lower tile side, especially after 13 months of deployment, with lower path coefficients and higher coefficients of non-determination (Figure 2). Unlike on the lower tile sides, it seemed that on the upper tile side water pH was directly influencing bacterial community composition, which then had an effect on tile cover. However, after 13 months of deployment path models including instead paths from water pH to tile cover and from tile cover to bacterial community composition were equally as likely as the one displayed in Figure 2. Furthermore, including reef in the path models improved the model fit according to the BIC, although the AIC increased (Figure 2).

## Taxonomic composition of bacterial communities

The bacterial community on the lower tile side of the settlement tiles was dominated by Alphaproteobacteria (28%), Gammaproteobacteria (22%), Cyanobacteria (8.5%), Flavobacteriia (7.0%), Deltaproteobacteria (6.6%), and Cytophagia (4.7%; Figure 3). At class-level resolution the bacterial community was similar across all sequenced samples, although at the $CO_2$ seep at Dobu Island, a higher prevalence of Epsilonproteobacteria and Proteobacteria Incertae Sedis was detected (Figure 3). Among these two bacterial classes most sequences were related to the genera *Sulfurovum* and *Sulfurimonas*, and *Candidatus* Thiobios, respectively. Additionally, the gammaproteobacterial family Thiotrichaceae displayed a higher sequence proportion at the $CO_2$ seep at Dobu Island. Furthermore, a large proportion of the bacterial community could not be classified at a high taxonomic resolution, with more than 50% of the sequences unclassified at genus level.

Generally, patterns in bacterial community composition were comparable between ARISA and sequencing data (Bray–Curtis dissimilarity: Mantel test, $r = 0.504$, $p = 0.001$; Jaccard dissimilarity: Mantel test, $r = 0.461$, $p = 0.001$). At $OTU_{Amplicon}$ resolution the composition of the bacterial community was as heterogeneous as in the ARISA data set with a similarly low number of shared OTUs of approximately 20% shared $OTU_{Amplicon}$.

Bacterial taxa mediating the settlement of invertebrate larvae, such as bacterial strains of the genera *Alteromonas*, *Vibrio*, *Pseudoalteromonas*, *Erythrobacter*, *Acinetobacter*, *Photobacterium*, *Shewanella*, *Bacillus*, *Pseudomonas*, and *Colwellia*, were of particular interest (Table 2). $OTU_{Amplicon}$ closely related to bacterial species comprising potentially settlement-inducing strains generally only accounted for a small proportion of the sequences per sample with approximately 1% or less. The exceptions were tiles from the reference site at Dobu Island, where they constituted 6–14% of the sequences after 13 months of deployment (Table 2). Among these putative settlement-inducing genera, *Alteromonas*,

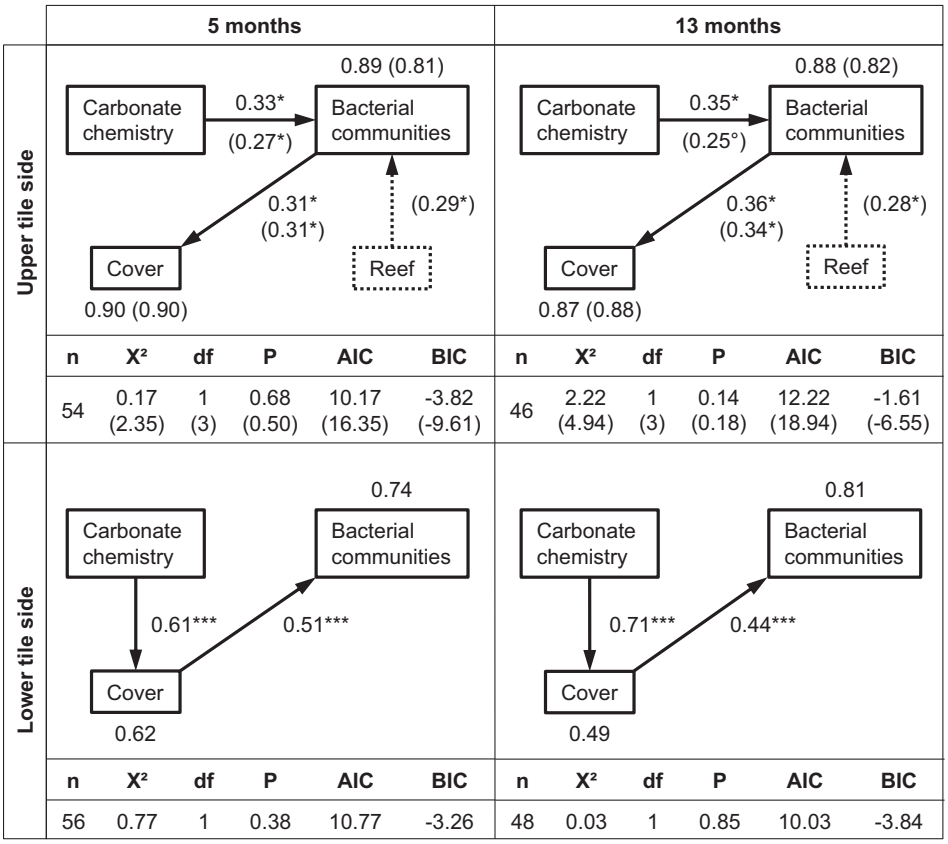


**Figure 2.** Final models of the path analyses to test for directional relationships between water pH, bacterial community composition, and the macroscopic tile communities (represented by percentage tile cover) on the settlement tiles for each tile side and sampling time. Values associated with arrows: path coefficients, values associated with factors: coefficient of non-determination (unexplained variation). n: number of observations, $X^2$: goodness-of-fit test statistic, df: degrees of freedom, P: *p*-value of goodness-of-fit test, AIC: Akaike Information Criterion, BIC: Baysian Information Criterion. Significance of path coefficients: $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), $p < 0.1$ (°). For the upper tile side, alternative models were calculated including "Reef" as exogenous variable (dotted arrows and values in parentheses).

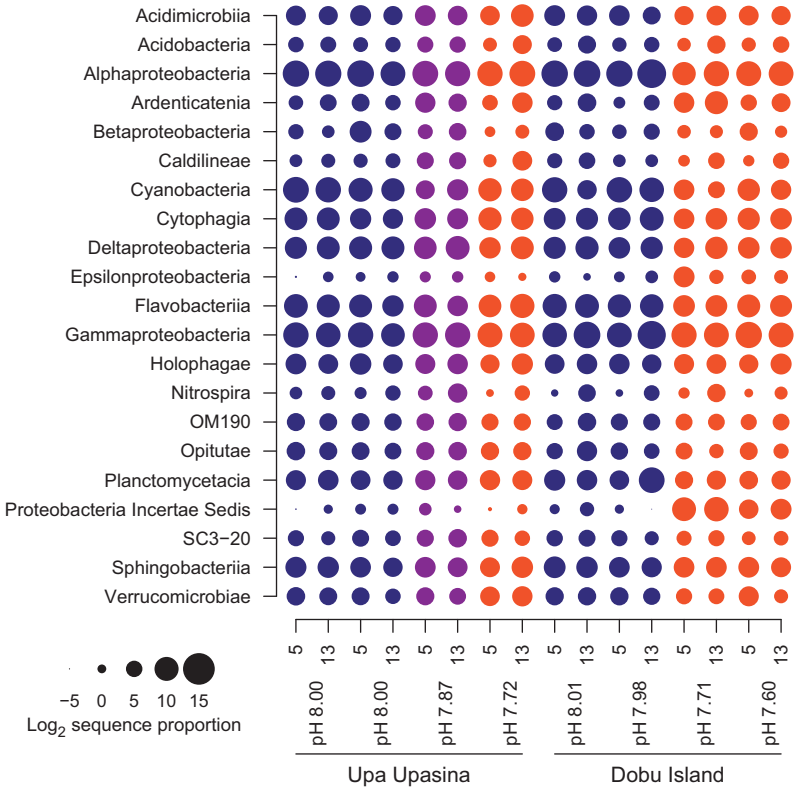


**Figure 3.** Taxonomic composition of the bacterial biofilm on the lower tile sides. The dotplot shows clr-transformed sequence proportions of dominant bacterial classes with a relative sequence abundance of at least 1% in at least one sample. The difference in the sizes of the dots illustrates $\log_2$-fold changes in sequence proportion. Sequences affiliated with the taxa shown in the dotplot constitute more than 90% of the sequences per sample. Order of plotting by reef (Upa Upasina, Dobu Island), decreasing pH [pH categories: reference (dark blue), intermediate (purple), low (orange)] and sampling time (5 months, 13 months).

*Pseudoalteromonas* and *Vibrio* were most abundant on the settlement tiles (Table 2).

## Discussion

In this study, we documented the composition of bacterial biofilms on settlement tiles deployed along natural pH gradients at two coral reefs in Papua New Guinea. The most striking feature of the bacterial biofilm communities was their highly heterogeneous composition. This high degree of heterogeneity complicated the detection of patterns in bacterial community composition. The amount of variation in community composition explained by the observed environmental parameters, pH, reef, tile side and sampling time, was below average for studies on microbial community composition (Hanson *et al.*, 2012). However, our model only included few environmental parameters and it is likely that other biotic or abiotic factors may contribute to further explain patterns in the composition of these bacterial communities.

Of the observed environmental parameters, tile side was the most important factor in explaining patterns in community composition. Tile side can act as proxy for various environmental parameters, predominantly light exposure and grazing pressure, but also current velocities or proximity to the substrate, to which the settlement tiles were attached. Light exposure has been documented to have a major influence on community development in settlement experiments before (Lidbury *et al.*, 2012; Sawall *et al.*, 2012). Here, light exposure of the upper tile surfaces was similar between sites, as all tiles were affixed at a depth of 3 m, and seawater concentrations of nutrients and particulate matter did not differ significantly between the sites (Smith *et al.*, accepted). The increased light availability on the upper tile side would favour the growth of photosynthetic algae and cyanobacteria, potentially resulting in a different trophic structure and therefore a different community composition (Ylla *et al.*, 2009; Sawall *et al.*, 2012). Furthermore, numerous scrape marks indicated frequent fish grazing on the exposed upper tile side (Fabricius *et al.*, 2015).

We detected only a minor effect of water pH on bacterial biofilm composition. Although the pH effect was statistically significant, its biological relevance is questionable since pH only explained 1% of community variation. These results were supported by the weak separation of bacterial communities between pH categories, with ANOSIM R values either not statistically significant or below values that are generally considered to show a strong separation of bacterial communities (Shade *et al.*, 2007; Zinger *et al.*, 2011). At Dobu Island, the distinction between reference and low pH communities was stronger than at Upa Upasina. However, it is possible that at Dobu Island the pH effect is enhanced by the larger geographic distance between reference and $CO_2$ seep sites of approximately 2 km compared to 500 m at Upa Upasina. Furthermore, at similar pH values, there were differences between bacterial biofilm communities between the two reefs, suggesting a regional variability in bacterial community

**Table 2.** Relative sequence abundance [%] of putative settlement-inducing bacterial $OTU_{Amplicon}$ on the lower sides of the eight settlement tiles selected for sequencing, after 5 and 13 months of deployment at a range of pH conditions.

| | Upa Upasina | | | | Dobu Island | | | |
|---|---|---|---|---|---|---|---|---|
| | $pH_T$ | | | | $pH_T$ | | | |
| | 8.00 | 8.00 | 7.87 | 7.72 | 8.01 | 7.98 | 7.71 | 7.60 |
| **5 months** | | | | | | | | |
| *Alteromonas* | 0.04 | 0.15 | 0.02 | 0.03 | 0.39 | 0.23 | 0.06 | 0.33 |
| *Vibrio* | 0.15 | 0.20 | 0.12 | 1.21 | 0.91 | 0.75 | 0.58 | 0.43 |
| *Pseudoalteromonas* | 0.02 | 0.06 | 0.02 | 0.15 | 0.42 | 0.72 | 0.15 | 0.04 |
| *Erythrobacter* | 0.24 | 0.13 | 0.20 | 0.31 | 0.27 | 0.19 | 0.20 | 1.51 |
| *Acinetobacter* | <0.01 | 0.01 | 0.02 | 0.31 | <0.01 | 0.03 | 0.01 | 0.01 |
| *Photobacterium* | 0.03 | 0.06 | 0.03 | 0.05 | 0.05 | 0.02 | 0.05 | 0.04 |
| *Shewanella* | 0 | <0.01 | <0.01 | 0.02 | 0.01 | 0.20 | 0.01 | 0 |
| *Bacillus* | 0.01 | 0.01 | <0.01 | 0.01 | 0.01 | 0.04 | 0.01 | <0.01 |
| *Pseudomonas* | 0 | 0.01 | <0.01 | <0.01 | 0 | 0 | 0 | 0 |
| *Colwellia* | 0 | <0.01 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total [%] | 0.49 | 0.64 | 0.42 | 2.08 | 2.07 | 2.17 | 1.06 | 2.37 |
| **13 months** | | | | | | | | |
| *Alteromonas* | 0.16 | 0.27 | 0.24 | 0.27 | 2.20 | 6.11 | 0.33 | 0.37 |
| *Vibrio* | 0.30 | 0.18 | 0.27 | 0.40 | 1.71 | 1.73 | 0.83 | 0.44 |
| *Pseudoalteromonas* | 0.05 | 0.06 | 0.22 | 0.16 | 1.55 | 5.56 | 0.30 | 0.27 |
| *Erythrobacter* | 0.21 | 0.06 | 0.04 | 0.07 | 0.26 | 0.26 | 0.03 | 0.20 |
| *Acinetobacter* | <0.01 | 0.04 | 0.01 | 1.48 | 0.02 | 0.10 | <0.01 | <0.01 |
| *Photobacterium* | 0.02 | 0.02 | 0.04 | 0.02 | 0.03 | 0.02 | 0.07 | 0.02 |
| *Shewanella* | <0.01 | 0.02 | 0.01 | <0.01 | 0 | <0.01 | 0 | <0.01 |
| *Bacillus* | 0.01 | 0.02 | 0.01 | 0.01 | 0.04 | 0.05 | 0.02 | 0.01 |
| *Pseudomonas* | 0 | <0.01 | 0 | 0 | 0.01 | 0 | 0 | 0 |
| *Colwellia* | 0 | 0 | 0 | 0 | 0 | <0.01 | 0.01 | 0 |
| Total [%] | 0.76 | 0.66 | 0.83 | 2.40 | 5.83 | 13.84 | 1.59 | 1.32 |

The representative sequences of the $OTU_{Amplicon}$ exhibited a high sequence similarity to bacterial species identified as potentially settlement-inducing for various invertebrate larvae in Huggett *et al.* (2006), Tebben *et al.* (2011), Tran and Hadfield (2011), Sneed *et al.* (2014), and Sharp *et al.* (2015).

composition that may complicate a generalization of findings regarding pH effects on bacterial biofilm communities. Such differences in community composition between reefs may also explain the slightly higher richness and evenness of the bacterial biofilm at Dobu Island.

The weak pH effect on bacterial community composition that we detected was in agreement with OA studies on planktonic bacteria, which reported only limited effects of reduced pH on bacterial community composition (Newbold *et al.*, 2012; Lindh *et al.*, 2013; Oliver *et al.*, 2014). In contrast, our results did not agree with the majority of previous observations on OA effects on bacterial biofilm development and composition including those from naturally $CO_2$-rich systems, which pointed to a rather strong effect of pH on bacterial community development and composition (Witt *et al.*, 2011a; Lidbury *et al.*, 2012; Webster *et al.*, 2012). However, the duration of previous experiments was much shorter than the period of deployment here. Therefore, the relative importance of the environmental parameters governing community dynamics may change after the first months of settlement.

We also detected a weak effect of sampling time on bacterial community composition. The time period between the start of the experiment and the first sampling spanned 5 months, which was most likely sufficient for the formation of a fully established bacterial biofilm community, especially in warm tropical waters (Witt *et al.*, 2011b; Sawall *et al.*, 2012). Any further changes in community composition may represent the temporal variability of already established bacterial communities in response to changes in the environment. Furthermore, due to the long duration of the experiment, the settlement tiles continued to be colonized by macro-organisms, such as algae and macro-invertebrates, whose presence and succession patterns may influence the development of the bacterial biofilm. It is known that different reef organisms host distinct bacterial communities (Barott *et al.*, 2011; Morrow *et al.*, 2012; Sneed *et al.*, 2015), suggesting that there may be strong biotic interactions between biofilm bacteria and other members of the tile community. These biotic interactions may result in the formation of microenvironments, with small-scale environmental conditions unrelated to water column chemistry, and may be more important than large-scale abiotic factors in shaping bacterial community composition. Indeed, the high heterogeneity of the bacterial biofilm may be caused by close associations with other organisms on the settlement tiles. Reef organisms also metabolically modulate the seawater chemistry in their vicinity, taking up $CO_2$ through photosynthesis and releasing metabolites (Kleypas *et al.* 2011). The boundary layers, in which the biofilms develop, may therefore experience very different pH conditions to those measured within the water column.

The further exploration of the relationships between bacterial community composition and the macroscopic tile community using path models strongly supported the importance of such biotic interactions. On the sheltered lower side of the settlement tiles, the same directional relationships were observed at both

sampling times, suggesting that these interactions were already well established after 5 months of deployment. While the macroscopic community on the lower tile sides seemed to be influenced by pH, direct pH effects on bacterial community composition were unlikely, but may rather be mediated by changes in the macroscopic tile community. On the exposed upper tile side, direct pH effects on bacterial community composition, which in turn influences the macroscopic tile community, were statistically more likely. However, these path models still contained a large proportion of unexplained variation. Furthermore, although the relationship between bacterial community composition and the macroscopic tile community was well supported, the analysis of these interactions may have been biased. Due to the need to preserve the biofilms as quickly as possible, data on the cover of macro-organisms within the 4 $cm^2$ squares were not obtained, and the available data on total macro-organism cover may have underestimated their role. Especially on the heterogeneous lower sides, the 4 $cm^2$ square that was sampled for the analysis of the bacterial biofilm may not have been representative for the whole tile community. Therefore the correlation between the bacterial community composition and the macroscopic tile community only constitutes an approximation. Still, we were able to gain a better insight into the ecological dynamics on the settlement tiles and the role of pH in such dynamics.

Whereas community fingerprinting and path analysis facilitated a robust analysis of general patterns in bacterial community composition and interactions that were based on a large number of samples, amplicon sequencing provided more detailed information on the taxonomic composition of the bacterial biofilm on the lower tile sides. Because the sheltered lower tile sides were ecologically of greater interest, with a higher heterogeneity of macroscopic communities and a higher expected settlement rate of invertebrate larvae compared to the upper tile sides (Maida *et al.*, 1994), only samples from the lower tile sides were selected for sequencing. Generally, the bacterial biofilm composition was consistent with previous observations from settlement tile experiments and natural substrates from coral reefs (Kriwy and Uthicke, 2011; Webster *et al.*, 2011; Witt *et al.*, 2011a; Sneed *et al.*, 2015). Many bacterial sequences could not be reliably classified at genus level, indicating that a large proportion of the bacterial community may consist of so far uncharacterized bacteria. Putative settlement-inducing $OTU_{Amplicon}$ only accounted for a small fraction of the sequences from most biofilm samples. Assuming that settlement of invertebrate larvae is dependent on the amount of settlement-inducing bacteria (Tebben *et al.*, 2011), the role of the bacterial biofilm in the mediation of larval settlement may be minor. We further detected an increased prevalence of certain sulfur-oxidizing taxa exclusively at the $CO_2$ seep at Dobu Island, such as the family Thiotrichaceaea, and the genera *Sulfurimonas*, *Sulfurovum*, and *Candidatus* Thiobios, which are typically found in sulfur-rich environments including hydrothermal vents (Rinke *et al.*, 2006; Meyer and Kuever, 2007; Meier *et al.*, 2016). Unlike the $CO_2$ seep at Upa Upasina, the seep at Dobu Island emitted small amounts of hydrogen sulphide and further contained microbial mats on the sediment in the immediate vicinity (<0.5 m) of some of the $CO_2$ bubble streams (Fabricius *et al.*, 2011; Hassenrück *et al.*, 2016). Therefore, additionally to geographic distance between reference and seep sites, the presence of sulfide may constitute a further reason for the rather strong differences in bacterial community composition along the pH gradient at Dobu Island compared to Upa Upasina. Furthermore, it may also explain the difference in bacterial community composition detected between the two reefs at similar pH. Such differences are most likely not attributable to pH, and suggest that in areas with extremely low pH values ($pH_T < 7.6$) the pH gradient at Dobu Island may be confounded by changes in other environmental factors.

In conclusion, our results do not support strong effects of reduced pH conditions, such as those expected under future OA, on bacterial biofilms on 5 and 13 months old surfaces in tropical coral reefs. Other abiotic and biotic factors such as light exposure or the presence of macro-organisms on the settlement tiles may be more important in shaping bacterial communities. However, we have only begun elucidating the factors that determine the development and composition of bacterial biofilms. Although we did not detect strong pH effects on bacterial biofilms at the time of sampling in this study, this does not preclude that other stages in the development of bacterial biofilms during colonization processes are not influenced by pH. Sampling of earlier successional stages, as well as a more detailed analysis of the interactions between the members of the tile community, including coral recruits, should be employed for a more comprehensive understanding of pH effects on bacterial colonization processes on coral reefs, and potential repercussions on coral settlement.

## Supplementary data

Supplementary material is available at the ICESJMS online version of the manuscript.

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