Responses of intertidal bacterial biofilm communities to increasing pCO$_2$ in a naturally acidified system

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Abstract

The effects of ocean acidification on ecosystems remain poorly understood, because it is difficult to simulate the effects of elevated CO_2 levels on entire marine communities in controlled laboratory conditions. Natural systems such as CO_2 seeps that are enriched in CO_2 are being used to help understand the long-term effects of ocean acidification *in situ*. Here, we compared biofilm bacterial community composition on cobbles/boulders and bedrock along a CO_2 gradient in the NW Pacific. Samples sequenced for 16S rRNA showed that different bacterial communities were associated with zones of different seawater pCO_2, and were also distinct between the two rocky habitat types. In both habitats, there was increased bacterial diversity in the biofilm communities in acidified conditions. Differences in pCO_2 were associated with differences in the relative abundance of major bacterial phyla including Cyanobacteria, Bacteroidetes, Proteobacteria, Planctomycetes, and Verrucomicrobia. However, despite the differences in community composition, there is little sign of changes in the bacterial community that would be functionally significant in terms of nutrient cycling and ecosystem structure. As well as direct seawater pH effects, it is possible that increased growth of algae and decreased grazing contributed to the observed shift in bacterial community composition at high CO_2, as documented at several seep systems worldwide. Given the importance of biofilms to coastal ecology, changes in their composition due to globally rising pCO_2 levels requires further investigation to assess the implications for marine ecosystem function. However, the apparent lack of functional shifts in the biofilms despite the pH change may be a reassuring indicator of stability in shallow oceanic biofilm communities in the future.

Keywords: Bacteria, biodiversity, ocean acidification, rocky shore ecology.

Introduction

Bacteria dominate the abundance, diversity and metabolic activities of ocean ecosystems (Azam and Malfatti, 2007). On hard substrates they form attached biofilm communities where they interact in a matrix of extracellular polymeric substances (Decho, 2000; de Carvalho, 2018), which stick the cells to the substrata and protect them against environmental extremes. Marine biofilms rapidly colonise natural and artificial surfaces, and then facilitate the settlement of macroalgae and invertebrates (Lau et al., 2005; Qian et al., 2007). They cycle nutrients and organic matter, and can be a major source of primary productivity in the photic zone, providing an important food resource for higher trophic levels (Thompson et al., 2004; de Carvalho, 2018). Consequently, any changes in biofilm microbial diversity and abundance could have important implications for marine ecosystem structure and function (Weinbauer et al., 2011).

Anthropogenic CO_2 emissions are rapidly changing seawater chemistry, in a process called ocean acidification, and a major research priority is to find out how ecosystems will be affected by these changes (Riebesell and Gattuso, 2015). While marine organisms can tolerate large, rapid changes in carbonate chemistry in coastal regions (Wootton et al., 2008), the long-term effects of decreased mean pH and more frequent episodes of low carbonate saturation and high pCO$_2$ are poorly known. Areas that are naturally exposed to periods of lower pH, lower carbonate saturation and higher pCO$_2$ than those that occurred at pre-industrial levels of atmospheric CO_2 are helping to further our understanding of the ecosystem effects of ocean acidification (Hall-Spencer et al., 2008; Fabricius et al., 2011; Rastrick et al., 2018). This approach has shown that even in coastal ecosystems, where rapid changes in carbonate chemistry are normal, many calcified organisms are adversely affected when the frequency and duration of low carbonate saturation events increase. Concomitant pulses of high levels of dissolved inorganic carbon benefit certain primary producers, causing

shifts in their community composition (Porzio et al., 2011; Connell et al., 2013; Cornwall et al., 2017).

Ocean acidification may directly impact microbial diversity and composition, and indirectly affect microbes through links within food webs, for example with non-bacterial competitors and with grazers (Krause et al., 2012; Sunday et al., 2017). Despite the importance of microbial biofilms, few studies have investigated how ocean acidification might affect them (Liu et al., 2010; Kerfahi et al., 2014). Hypothetically, major biogeochemical processes driven by microbes may not be fundamentally different under future elevated pCO_2 conditions (Joint et al., 2011) since bacterial communities are able to reorganise themselves in response to environmental perturbations (Tolker-Nielsen and Molin, 2000). Aquarium-based experiments have been used to simulate these future conditions, but it is challenging to predict the responses of microbial communities based on these, as they do not incorporate feedbacks and the many indirect effects that might occur as a result of increased pCO_2 in natural systems – for example changes in geochemistry and organism interactions (Das and Mangwani, 2015). For this reason, carbon dioxide seep systems are now being used to investigate the long-term response of microbial communities to ocean acidification (Kerfahi et al., 2014; Morrow et al., 2014; Hassenrück et al., 2015, 2017; O'Brien et al., 2018).

Previous work on biofilm communities at carbon dioxide seeps suggests that their primary productivity will be enhanced by ocean acidification in the photic zone, at least where nutrient levels are sufficient (Lidbury et al., 2012; Johnson et al., 2015) and that there will be broad shifts in their community composition and diversity under increased pCO_2 (Kerfahi et al., 2014; Taylor et al., 2014). These biofilm community responses have also been shown in mesocosm-based studies using experimentally elevated pCO_2 (Witt et al., 2011) with no evidence of loss of biofilm function due to ocean acidification.

Natural biofilm communities on rocky shores are highly variable in terms of community structure and relative abundances, due to abiotic and biotic pressures (Williams et al., 2000), and rocky shore ecology is strongly influenced by shore type e.g. steep cliffs vs boulder fields, or hard vs soft rock substrata (Lewis, 1964). The response of microbial biofilms to the impacts of ocean acidification may differ, depending on habitat type, and may be caused directly by changes in carbonate chemistry, or indirectly due to effects on other organisms (Sunday et al., 2017).

In this study, our aim was to test whether the effects on coastal biofilm bacterial communities of increased seawater pCO_2 in the NW Pacific Ocean are similar to those observed in the Mediterranean Sea (Kerfahi et al., 2014). In Japan, Agostini *et al.* (2018) recently showed that in complex habitats, at increased levels of pCO_2 predominately calcareous organisms were replaced by simplified biofilm-dominated and algal turf-dominated habitats. We used the ocean acidification study system established by Agostini *et al.* (2018) to investigate the bacterial community structure of these biofilms on two types of low rocky shore habitat; cobble/boulder shores, and steep cliff faces. We examined four pCO_2 conditions, from pre-Industrial through to present day and projected future levels, to assess impacts of changing ocean chemistry on bacterial communities. We expected to find major changes in biofilm community composition, given the known sensitivity of bacterial communities to pH (e.g. Lauber et al., 2008; Tripathi et al., 2012), although this type of study has not previously been carried out in the Pacific Ocean.

Materials and methods

Study site

Sampling took place on the South coast of Shikine Island, East of the Izu peninsula, Japan in the North West Pacific Ocean (34° 32'N, 139° 20'E). The Izu archipelago is a chain

of islands approximately 150 km South of Tokyo (Fig. S1) situated at a subtropical-temperate biogeographic boundary due to the influence of the warm North flowing Kuroshio current (Agostini et al., 2018).

Sample collection and DNA extraction

Samples were collected from six sites along a pH gradient ranging from 7.2 to 8.3. At each site, six rock chips were randomly collected along a linear 1 m transect immediately above the low tide line. All samples had the same edaphic parameters, being on boulders (in boulder samples) or cliff faces (in cliff face samples) of the same basaltic lava, on flat faces at vertical westerly orientation and smooth texture. Rock chips were collected in June 2016 using a chisel and placed in individually labelled bags using clean forceps (Fig. S2) and immediately stored at -20˚C. DNA was extracted from each rockchip sample using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The DNA samples were then sent to the Dalhousie University, Canada, for sequencing using an Illumina MiSeq platform.

Seawater chemistry

Geographical co-ordinates of each sample were taken using a GPS. A multi-sensor (U-5000G, Horiba Ltd, Kyoto Japan) was used to measure seawater pH, temperature, salinity and dissolved oxygen. To measure total alkalinity, 100 ml water samples were collected and then filtered at 0.45 μm with cellulose acetate filters (Dismic 045, Advantec Japan). Total alkalinity was measured via titration with HCl at 0.1 mol l^{-1} with a Metrohm titrator (785 DMP titrino) and total alkalinity was calculated by Gran plot from titration point with a pH between 4.0 and 3.0. Carbonate chemistry parameters: partial CO_2 pressure (pCO_2), Aragonite (Ar) and calcite (Ca) saturation state (Ωaragonite, Ωcalcite), and dissolved

149   inorganic carbon (DIC) were calculated using the CO_2SYS software package (Pierrot et al.,

150   2006) using temperature, salinity, pH (NBS scale) and total alkalinity (A_T) with the

151   disassociation constants from Mehrbach et al. (1973), as adjusted by Dickson and Millero

152   (1987), KSO_4 using Dickson (1990), and total borate concentrations from Uppström (1974).

153   The carbonate chemistry for the different sites is presented in Table 1.

154

Sequence analysis

156   The sequenced data generated from Miseq sequencing was processed using the

157   Mothur platform (Schloss et al., 2009). The sequences were aligned using Mothur (default

158   settings: kmer searching with 8mers and the Needleman–Wunsch pairwise alignment

159   method). Next, they were aligned against the EzTaxon-aligned reference (Chun et al., 2007),

160   and further filtered to remove gaps. Sequences were de-noised using the '*pre.cluster*'

161   command in Mothur implemented using a pseudo-single linkage pre-clustering algorithm

162   from Huse *et al.* (2010). Putative chimeric sequences were detected and removed via the

163   Chimera Uchime algorithm contained within Mothur (Edgar et al., 2011) in *de novo* mode,

164   which first splits sequences into groups and then checks each sequence within a group using

165   the more abundant groups as reference. The taxonomic classification was performed using

166   Mothur's version of the RDP Bayesian classifier, using EzTaxon-e database for each

167   sequence at 80% Naïve Bayesian bootstrap cut-off with 1000 iterations. The sequences used

168   in this study have been deposited in the NCBI Sequence Read Archive under accession

169   number SRP172073.

170

Statistical analysis

172   All samples were standardised by random subsampling to 13,882 reads per sample

173   using the sub.sample command (http://www.mothur.org/wiki/Sub.sample) in Mothur.

174 Richness, diversity indices, and rarefaction values were estimated using Mothur. Variation in

175 the relative abundance of the bacterial phyla among the pH zones was tested using two-way

176 ANOVA in R software package 2.14.2, with habitat type and pH level as fixed factors, after

177 testing for normality. We used Tukey's Honest Significant Difference test for pairwise

178 comparisons. We used the same procedure to test whether OTUs richness and diversity

179 indices differed across different pH levels. OTU-based abundance data were first square root

180 transformed to build the Bray-Curtis distance matrix using the vegdist function in the Vegan

181 package of R (Oksanen et al., 2008). We performed a nonmetric multidimensional scaling

182 (NMDS) plot using the metaMDS function in the vegan package of R. This used the Bray-

183 Curtis distance matrix to assess patterns in bacterial species composition. A permutational

184 multivariate analysis of variance (PERMANOVA) was performed with 999 permutations

185 using the Adonis function in Vegan R package to test if bacterial community composition

186 differed significantly by pH levels and habitat type.

187 The rank order nestedness relationship was also calculated on BINMATNEST

188 (Rodríguez-Gironés and Santamaría, 2006) using default input parameters and null models to

189 test whether the community assemblage in each treatment sample was a subset present in

190 another sample. This approach calculates the p-value for rows and column totals and these

191 were reordered following a packed matrix order from high-to-low nestedness as enumerated

192 by (Dong et al., 2016).

193 Bacterial co-occurrence network analysis was conducted following methods given by

194 (Faust and Raes, 2012; Lima-Mendez *et al.*, 2015). For each group, OTUs that appeared in

195 three or less samples were removed. The relative abundance of OTUs was then used to

196 construct networks using Spearman's correlation and Kullback-Leibler dissimilarity methods.

197 Randomization generated permutation and bootstrap distributions, and *p* values were merged

198 by Brown's method and multiple test correlation with Benjamini-Hochberg. Finally, edges

199   with p_{adj} above 0.05 were discarded.

200   The functional composition of the microbial community was analysed using PICRUSt

201   (Langille et al. 2013) on the OTUs generated from the 16S rRNA data. The PICRUSt

202   program requires a biom-formatted OTU table with OTUs assigned to a Greengenes OTU ID.

203   The 16S OTU table was normalized for copies of the 16S rRNA gene and exported as to

204   *.biom* format for analysis with the software package STAMP (Parks et al 2014). STAMP

205   includes statistical and visualization tools that were used to identify differences in functional

206   potential for the biofilm bacterial communities across different sites along the pH gradient. .

207   Multiple group comparisons of function abundance (KEGG module predictions) were

208   assessed using analysis of variance (ANOVA) followed by *Tukey–Kramer post hoc* tests

209   (confidence interval of 0.95) and reported with corrected *P* values (Storey's FDR multiple

210   test correction approach) in STAMP software (Parks et al 2014).

211

212   **Results**

213   We obtained 583,036 good quality sequences from 36 samples after rarefying to

214   13,882 reads per sample, which were classified into 10,296 operational taxonomic units

215   (OTUs) at 97% similarity level. Most samples showed no sign of reaching an asymptote in

216   OTU richness at the total number of reads available in the rarefaction analysis. This means

217   that more sequences would be required to assess the full taxonomic diversity of bacteria

218   within the biofilms (Fig. S3).

219   Alpha diversity of the bacterial community associated with biofilms across different

220   pH levels was calculated using OTU richness and diversity indices. Bacterial OTU richness

221   was significantly different across all intertidal sites at different pH levels, and highest number

222   of OTUs was observed at sites having the lowest pH (Fig. 1). The OTU richness differed

223   significantly by pH level ($F_{2, 30} = 27.05$, $P < 0.001$) and by the interaction between pH and

224 habitat type ($F_{2, 30}$ = 3.12, P = 0.05). Similarly, Shannon index differed significantly in

225 relation to pH ($F_{2, 30}$ = 15.41, $P < 0.001$) and the interaction between pH and habitat type ($F_{2, 30}$ = 7.01, P = 0.003), with highest diversity found in sites having the lowest pH (Fig. 1).

226 $_{30}$ = 7.01, P = 0.003), with highest diversity found in sites having the lowest pH (Fig. 1).

227 However, the community diversity did not differ between the boulder and cliff-face habitats

228 (OTU richness: $F_{1, 30}$ = 0.83, P = 0.36; and Shannon index: $F_{1, 30}$ = 0.08, P = 0.77).

229 The most dominant bacterial sequences recovered in the present study belonged to

230 *Cyanobacteria* and *Bacteroidetes* representing 36% and 34% of total reads, respectively,

231 followed by *Proteobacteria* with 23% of total reads, and less than 3% for each of the

232 following bacterial phyla: *Planctomycetes*, *Verrucomicrobia*, *Chloroflexi*, *Acidobacteria*,

233 *Actinobacteria*, *Gemmatimonadetes* and *Chlorobi*. 3% of sequences remained unclassified.

234   The relative abundances of phyla in the epilithic bacterial community detected along

235 the pH/CO_2 gradient off Shikine Island showed significant variations across different

236 sampling sites except for *Chloroflexi* and *Chlorobi* (Table S1). The strongest shifts were

237 observed in the most abundant bacterial phyla, with a significant decrease in the relative

238 abundance of *Cyanobacteria* at reduced pH sites. The relative abundance of Bacteroidetes

239 was decreased at medium and low pH, but then increased at very low pH sites. However,

240 Proteobacteria exhibited an increase in relative abundance at low pH then a decrease at

241 extreme low pH sites. The remaining phyla showed either no consistent patterns or no change

242 in abundance across different sites (Fig. 2). The interaction between pH levels and habitat

243 types had a more pronounced effect on bacterial community than only pH level or habitat

244 type. *Cyanobacteria* were influenced by both factors, with lowest abundance was found in

245 boulder zone and sites having lowest pH. Bacteroidetes were significantly affected only by

246 pH with their lowest abundance observed at low pH sites for both boulder and cliff-faced

247 sites. However, *Proteobacteria* were significantly affected only by habitat types showing a

248 decrease in relative abundance at boulder sites. The interaction pH levels and habitat types

249 had a significant effect on most of detected phyla including *Verrucomicrobia*, *Acidobacteria*,

250 *Actinobacteria* and *Gemmatimonadetes*. *Planctomycetes* were significantly affected by pH

251 levels, habitat types and the interaction pH levels*habitat types (Fig. 2, Table S1).

252   More than 25 bacterial orders were detected in our sequences (Fig. 3a).

253 *Sphingobacteriales* were the most abundant order, with a relative abundance of 13.8% of

254 total reads but did not differ at both boulder and cliff-face sites along pH gradient. The

255 relative abundance of almost all detected orders significantly differed in relation to at least

256 one of the studied factors (pH level, habitat type, and interaction pH level*habitat type)

257 (Table S2). The orders *Oscillatoriales*, *Cytophagales*, *Alteromonadales*, *Phycisphaerales*,

258 *Kordiimonadales*, *Chromatiales* and *Balneola* were significantly affected by pH level, habitat

259 type and the interaction of pH level and habitat type. However, *Flavobacteriales* (12.5% of

260 total reads) the second common order significantly differed along pH level and the interaction

261 pH level*habitat type and not with habitat type, with highest abundance found at ambient pH

262 for boulder sites and low pH for cliff-face sites. The relative abundance of *Oscillatoriales* and

263 *Vibrionales* was significantly lower at ambient pH for both boulder and cliff sites.

264 Conversely, *Rhodospirillales* and *Sphingomonadales* were significantly greater at low pH for

265 both habitat types. The remaining orders showed no consistent patterns (Fig. 3a).

266   At the family level, the biofilm bacterial communities at both boulder and cliff-face

267 sites along pH gradient were dominated by *Saprospiraceae* (13.1% of total reads),

268 *Flavobacteriaceae* (12% of total reads), *Prochlorotrichaceae* (7.9% of total reads),

269 *Pleurocapsa* family (7.8% of total reads), *Vibrionaceae* (6.1% of total reads), *Rivulariaceae*

270 (3.9% of total reads), etc. and had significant differences across pH level or/and habitat type

271 except for *Saprospiraceae* (*P*>0.05) the most abundant family (Table S3). Most of the

272 detected families showed no consistent pattern in response to pH and/or habitat type (Fig.

273 3b). For example, *Flavobacteriaceae* were higher at ambient pH in boulder sites and at low

pH in cliff-face sites. *Prochlorotrichaceae* were lower at ambient pH in both boulder and cliff sites. Only habitat type had a significant effect on *Vibrionaceae* and *Rivulariaceae* where *Vibrionaceae* represented a minor component of boulder sites (0.2% of total reads) and major component of cliff sites (14% of total reads), conversely *Rivulariacea*e were greater in boulder sites (6.5% of total reads) and lower in cliff sites (1.5% of total reads). *Pseudoalteromonadaceae* had also lower relative abundance in boulder sites (<0.01% of total reads) compared with cliff sites (7.5% of total reads). *Pseudoalteromonadaceae* were significantly influenced by pH, habitat type and the interaction pH level*Habitat type, with lower abundance observed at lower pH (Fig. 3b).

A non-metric multidimensional scaling (NMDS) using Bray-Curtis distance indicated that both pH levels and habitat type influenced bacterial community composition (Fig. 4). A permutational multivariate analysis of variance (PERMANOVA) results showed that bacterial community composition was not only affected by pH level ($F_{2,29} = 3.54$, $P = 0.001$; Table 2) and habitat type ($F_{1,29} = 8.62$, $P = 0.001$; Table 2), but also by the interaction between pH level and habitat type ($F_{2,29} = 3.65$, $P = 0.001$; Table 2).

Nestedness analysis showed that bacterial communities followed a nested structure ($p < 0.0001$) across different pH levels. We generated a packed matrix order of all samples, in which the nestedness of each sample was categorised from high to low, and the lower ones are nested in the higher ones (Table S4). Samples having the lowest pH in boulder habitat and samples having ambient pH in cliff-face habitat had the lowest rank of nestedness compared to the other sites. Thus, the OTU composition of other sites could be a subset of the bacterial community in the 'Boulder-very low' and 'Cliff-ambient' sites.

Fig. 5 and Fig. 6 illustrated the interactions between taxa across different pH levels. Associations of bacterial taxa at very low pH sites outnumbered low and ambient pH sites, no matter the rock type. Positive edges were higher than negative edges, except for low pH cliff

and ambient boulder sites where the number of positive edges was equal with negative edges (Fig. 5). Furthermore, non-random edges distribution with regard to phylum was observed. We found that both intra-phylum and inter-phylum interaction edges of Bacteroidetes were the highest in all samples (Fig. 6).

The functional classification based on KEGG module predictions (Fig. S4) revealed that biofilm microbial communities show some a significant shift in most of the functions in Boulder and Cliff sites across different pH levels. Analysis of PICRUSt-predicted functional profiles for methane metabolic pathways suggest a decrease in gene abundance at lower pH for both boulder and cliff sites (Fig. 7a). Sulfur metabolism showed the opposite pattern with greater gene abundance at lower pH (Fig. 7b). The analysis of nitrogen metabolic pathway suggested no significant difference in gene abundance at both habitat types across different pH level (ANOVA, Tukey–Kramer, $P < 0.05$; Fig. 7c). Genes related to energy metabolic pathways increased at lower pH at both boulder and cliff sites (Fig. 7d). Carbon fixation potential in Prokaryotes showed an increase then a decrease in gene abundance related to carbon fixation in Prokaryotes at boulder sites along pH gradient. These genes decreased at medium pH then increased at low pH at cliff sites (Fig. 7e). The opposite pattern was observed for genes related to carbon fixation pathway in photosynthetic organisms (Fig. 7f).

Discussion

We found that bacterial community composition in biofilms on two types of rocky shore habitat was affected at CO_2 seeps (Heat map Fig). Pre-industrial, present day and projected future levels of seawater pCO_2 each had their own discrete communities of biofilm bacteria, raising the possibility that the transition from 300-400 ppm CO_2 in ocean seawater over the past 200 years has already affected biofilm communities worldwide. Biofilm growing at even higher than present-day levels of CO_2 increased in bacterial biodiversity.

14

324   This is very similar to the biofilm shifts recorded from present-day to high pCO$_2$ conditions

325   in the Mediterranean Sea (Lidbury et al., 2012; Kerfahi et al., 2014) and in mesocosm

326   conditions (Witt et al., 2011) and suggests a general response of biofilms to increasing CO$_2$.

327   Responses to ocean acidification conditions varied between bacterial taxa, some of

328   these increasing whilst others decreased in relative abundance resulting in changes in species

329   richness and diversity (Fig. 1, Fig. 2 and Fig. 3). These findings mirror those of Taylor *et al.*

330   (2014) at CO$_2$ seeps in Italy, who found that shifts in bacterial biofilm communities involved

331   a similar set of bacterial phyla to those recorded in our study (Fig. 2). Marine

332   bacterioplankton communities also respond to changes in seawater CO$_2$ with changes in

333   community composition, yet the functions of those communities remain resilient to

334   acidification (Lindh et al., 2013; Lin et al., 2018), as predicted by Joint *et al.* (2011).

335   Holobiont communities can also be affected by ocean acidification conditions. Meron

336   *et al.* (2012) reported shifts in coral bacterial community composition along a pH gradient of

337   8.1-7.3 caused by CO$_2$ seeps in Italy but found that many taxa were resilient. However,

338   Webster *et al.* (2016) carried out an eight week-long mesocosm experiment and found that

339   coral-associated bacterial taxa were tolerant of elevated pCO$_2$ (including *Proteobacteria*,

340   *Bacteroidetes*, *Fusobacteria*, *Verrucomicrobia*, *Chloroflexi* and *Planctomycetes*) with no

341   community shifts in these taxa between pH 8.1 (pCO$_2$ 479–499 µatm) and pH 7.9 (pCO$_2$

342   738–835 µatm) although they acknowledged that a longer-term experiment might have

343   revealed an effect. In contrast, shifts in hard substratum biofilm microbial community

344   structure were recorded by Webster et al. (2013) who simulated the effects of ocean

345   acidification by bubbling CO$_2$ into flow-through aquaria and recorded the appearance of new

346   taxa within bacterial biofilm communities as the pH fell. This is similar to the responses we

347   found in relation to pH treatments on Japanese rocky shores, suggesting that perhaps

348   holobiont communities are under tighter control exerted by the host, compared to rocky

349   biofilm communities.

350   It is interesting that there are no major shifts in relative abundance functional groups,

351   for example N fixers and photosynthesizers. Both taxonomic and functional analysis showed

352   that major groups of bacteria (at family and genus levels) remained abundant at the higher

353   pCO2 levels suggesting that the primary productivity and nutrient cycling of the system did

354   not fundamentally changed, and that most essential biogeochemical functions were still

355   present (Fig. 2) such as the nitrogen fixing activities *Cyanobacteria* and *Chloroflexi.*. It is

356   also interesting to note that chemotrophs such as *Halothiobacillus*, *Guyparkeria*,

357   *Thiobacillus*, *Sulfuritortus*, *Nitrosomonas*, *Gallionella* and *Ferriphaeselus* are absent even at

358   the lowest pH treatments. This negative result simultaneously suggests that there may be little

359   change in biogeochemical cycling as a result of such coastal biofilms, and confirms that the

360   CO_2-rich vents are not contaminated with S-containing gases and are indeed pure CO_2 as was

361   originally proposed based on gas and seawater chemistry measurements.

362   However, our conclusions on functional attributes of the biofilms are based only on

363   taxonomic composition and relative abundances. It would be interesting to study the

364   metagenome of these systems as a range of microbial functional processes are thought to be

365   sensitive to ocean pH shifts (Das and Mangwani, 2015).

366   Network analysis revealed that sites having very low pH were more connected than

367   low and ambient pH sites (Fig. 5 and Fig. 6). Network analysis which is based on statistically

368   significant tests of correlation helps to illustrate interactions between taxa, and between

369   particular taxa and particular gene functions (Barberán *et al.*, 2012; Faust and Raes, 2012;

370   Mendes *et al.*, 2014). In networks based on correlation between bacterial OTUs, co-presence

371   associations were stronger than mutual exclusion, implying that most interactions were

372   positive at all studied pH levels, a pattern which is consistent with Lima-Mendez *et al.*,

373   (2015). However, the nearly equal proportions of positive and negative edges demonstrate

374   that taxon-taxon interactions may be regulated by environmental factors. An intact species-

375   rich system would be expected to have greater network complexity due to stable and

376   predictable interactions (Wagg *et al.*, 2014). Our results showed that the sites with very low

377   seawater pH had more complex networks and higher diversity, which indicates that these

378   biofilm communities experienced greater environmental heterogeneity (Lin *et al.*, 2018).

379   *Bacteroidetes* exhibited the most intra-phylum and inter-phylum associations, suggesting that

380   *Bacteroidetes* play crucial roles in the biofilm communities we sampled.

381   Overall, we found that bacterial biofilms had considerable resilience to ocean water

382   acidification, retaining a similar high diversity and functional structure, and stronger network

383   complexity. This contrasts with the simplification of the taxonomic and functional diversity

384   system of larger organisms brought about by decreasing pH in these same coastal systems.

385   Agostini et al. (2018) showed that at the acidified sites on both of the habitat types we

386   studied, biofilm and turf algae predominated. The normal succession that occurs on rocky

387   shores had been truncated with simpler and less diverse macrobenthic communities, in line

388   with the findings of Kroeker et al. (2013) and Brown *et al.* (2018).

389   There are likely to be multiple mechanisms that drive changes in biofilm bacterial

390   composition along gradients of increasingly acidified conditions. These may include the

391   direct effects of low pH, or low carbonate, or high pCO$_2$ as well as the indirect effects that

392   occur due to changes in habitat and/or organism interactions. We consider it likely that the

393   bacterial community shifts we observed along pH gradients were party driven by changes in

394   the broader ecology of the rocky shore system food webs. At our high pCO$_2$ sites there was a

395   marked reduction in calcified grazers such as littorinid snails, limpets and chitons and an

396   increase in eukaryotic primary producers, such as diatoms (Agostini et al., 2018) all of which

397   would be expected to affect the thickness, extent and composition of biofilm communities.

398   To date, there had been no other studies on the effects of ocean acidification on

399   biofilms in Asia. Our comparison of marine biofilms along a natural pH gradient in Japan

400   supports the view that while there may be pervasive effects of rising pCO$_2$ levels on marine

401   bacterial biofilm communities, the functional effects may not be major. This warrants further

402   investigation, given that acidification may disrupt biofilm settlement cues for commercially

403   important shellfish, and may affect grazing habitat for a range of marine animals.

404   Our observations contrast with mounting evidence that increasing levels of seawater

405   pCO$_2$ simplifies marine habitats, maintaining them in an early successional state (Kroeker et

406   al., 2013; Brown et al., 2018). Agostini et al. (2018) showed that biodiverse rocky shore

407   communities (including calcareous macroflora and fauna) were gradually replaced by

408   simpler, less biodiverse communities that are dominated by non-calcified algae and biofilms

409   in areas affected by periods of low carbonate saturation and high dissolved inorganic carbon.

410   Here we showed that at the microbial level bacterial diversity and biofilm connectedness

411   increased, and while communities shifted with unknown effects on biofilm function. Whilst

412   the biofilms will still be fixing and cycling carbon and nutrients, it is possible that a range of

413   other functions (e.g. provision of settlement cues) may be affected. Given the importance of

414   biofilms to coastal ecology, changes in their composition due to globally rising pCO$_2$ levels

415   requires further investigation to assess the implications for marine ecosystem function.

416

References

Agostini, S., Harvey, B. P., Wada, S., Kon, K., Milazzo, M., Inaba, K., et al. (2018). Ocean acidification drives community shifts towards simplified non-calcified habitats in a subtropical-temperate transition zone. *Sci. Rep.* 8, 11354. doi:10.1038/s41598-018-29251-7.

Azam, F., and Malfatti, F. (2007). Microbial structuring of marine ecosystems. *Nat. Rev. Microbiol.* 5, 782. doi:10.1038/nrmicro1747.

Barberán A, Bates S T, Casamayor E O, Fierer N. 2012. Using network analysis to explore co-occurrence patterns in soil microbial communities. *The ISME journal.* **6**: 343.

Brown, N. E. M., Milazzo, M., Rastrick, S. P. S., Hall-Spencer, J. M., Therriault, T. W., and Harley, C. D. G. (2018). Natural acidification changes the timing and rate of succession, alters community structure, and increases homogeneity in marine biofouling communities. *Glob. Change Biol.* 24, e112–e127. doi:10.1111/gcb.13856.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K., et al. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* 57, 2259–2261. doi:10.1099/ijs.0.64915-0.

Connell, S. D., Kroeker, K. J., Fabricius, K. E., Kline, D. I., and Russell, B. D. (2013). The other ocean acidification problem: CO_2 as a resource among competitors for ecosystem dominance. *Philos. Trans. R. Soc. B Biol. Sci.* 368, 20120442. doi:10.1098/rstb.2012.0442.

Cornwall, C. E., Revill, A. T., Hall-Spencer, J. M., Milazzo, M., Raven, J. A., and Hurd, C. L. (2017). Inorganic carbon physiology underpins macroalgal responses to elevated CO_2. *Sci. Rep.* 7, 46297. doi:10.1038/srep46297.

Das, S., and Mangwani, N. (2015). Ocean acidification and marine microorganisms: responses and consequences. *Oceanologia* 57, 349–361. doi:10.1016/j.oceano.2015.07.003.

de Carvalho, C. C. (2018). Marine biofilms: A successful microbial strategy with economic implications. *Front. Mar. Sci.* 5, 126. doi:10.3389/fmars.2018.00126.

Decho, A. W. (2000). Microbial biofilms in intertidal systems: an overview. *Cont. Shelf Res.* 20, 1257–1273. doi:10.1016/S0278-4343(00)00022-4.

Dickson, A. G. (1990). Thermodynamics of the dissociation of boric acid in potassium chloride solutions from 273.15 to 318.15 K. *J. Chem. Eng. Data* 35, 253–257. doi:10.1021/je00061a009.

Dickson, A. G., and Millero, F. J. (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Res. Part Oceanogr. Res. Pap.* 34, 1733–1743. doi:10.1016/0198-0149(87)90021-5.

Dong, K., Tripathi, B., Moroenyane, I., Kim, W., Li, N., Chu, H., et al. (2016). Soil fungal community development in a high Arctic glacier foreland follows a directional replacement model, with a mid-successional diversity maximum. *Sci. Rep.* 6, 26360.

doi:10.1038/srep26360.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi:10.1093/bioinformatics/btr381.

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. Change* 1, 165–169. doi:10.1038/nclimate1122.

Faust K, Raes J. 2012. Microbial interactions: From networks to models. *Nature Reviews Microbiology.* **10**: 538-550.

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. doi:10.1038/nature07051.

Hassenrück, C., Hofmann, L. C., Bischof, K., and Ramette, A. (2015). Seagrass biofilm communities at a naturally CO_2-rich vent. *Environ. Microbiol. Rep.* 7, 516–525. doi:10.1111/1758-2229.12282.

Hassenrück, C., Tegetmeyer, H. E., Ramette, A., Fabricius, K. E., and Handling editor: Woodson CB (2017). Minor impacts of reduced pH on bacterial biofilms on settlement tiles along natural pH gradients at two CO_2 seeps in Papua New Guinea. *ICES J. Mar. Sci.* 74, 978–987. doi:10.1093/icesjms/fsw204.

Huse, S. M., Welch, D. M., Morrison, H. G., and Sogin, M. L. (2010). Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environ. Microbiol.* 12, 1889–1898. doi:10.1111/j.1462-2920.2010.02193.x.

Johnson, R. V., Brownlee, C., Milazzo, M., and Hall-Spencer, M. J. (2015). Marine microphytobenthic assemblage shift along a natural shallow-water CO_2 gradient subjected to multiple environmental stressors. *J. Mar. Sci. Eng.* 3. doi:10.3390/jmse3041425.

Joint, I., Doney, S. C., and Karl, D. M. (2011). Will ocean acidification affect marine microbes? *ISME J.* 5, 1. doi:10.1038/ismej.2010.79.

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 CO_2 gradient in the Mediterranean Sea off Vulcano, Italy. *Microb. Ecol.* 67, 819–828. doi:10.1007/s00248-014-0368-7.

Krause, E., Wichels, A., Giménez, L., Lunau, M., Schilhabel, M. B., and Gerdts, G. (2012). Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach. *PLoS One* 7, e47035. doi:10.1371/journal.pone.0047035.

Kroeker, K. J., Gambi, M. C., and Micheli, F. (2013). Community dynamics and ecosystem simplification in a high-CO_2 ocean. *Proc. Natl. Acad. Sci. U. S. A.* 110, 12721–12726. doi:10.1073/pnas.1216464110.

Lau, S. C., Thiyagarajan, V., Cheung, S. C., and Qian, P.-Y. (2005). Roles of bacterial

community composition in biofilms as a mediator for larval settlement of three marine invertebrates. *Aquat. Microb. Ecol.* 38, 41–51. doi:10.3354/ame038041.

Lauber, C. L., Strickland, M. S., Bradford, M. A., and Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol. Biochem.* 40, 2407–2415. doi:10.1016/j.soilbio.2008.05.021.

Lewis, J. R. (1964). *The Ecology of Rocky Shores*. London, UK: English Universities Press.

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. doi:10.1016/j.marpolbul.2012.02.011.

Lima-Mendez G, Faust K, Henry N, Decelle J, Colin S, Carcillo F, Chaffron S, Ignacio-Espinosa J C, Roux S, Vincent F. 2015. Determinants of community structure in the global plankton interactome. *Science.* **348**: 1262073.

Lin, X., Huang, R., Li, Y., Li, F., Wu, Y., Hutchins, D. A., et al. (2018). Interactive network configuration maintains bacterioplankton community structure under elevated CO_2 in a eutrophic coastal mesocosm experiment. *Biogeosciences* 15, 551–565. doi:10.5194/bg-15-551-2018.

Lindh, M. V., Riemann, L., Baltar, F., Romero-Oliva, C., Salomon, P. S., Granéli, E., et al. (2013). Consequences of increased temperature and acidification on bacterioplankton community composition during a mesocosm spring bloom in the Baltic Sea. *Environ. Microbiol. Rep.* 5, 252–262. doi:10.1111/1758-2229.12009.

Liu, J., Weinbauer, M. G., Maier, C., Dai, M., and Gattuso, J. P. (2010). Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. *Aquat. Microb. Ecol.* 61, 291–305. doi:10.3354/ame01446.

Mehrbach, C., Culberson, C. H., Hawley, J. E., and Pytkowicz, R. M. (1973). Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* 18, 897–907. doi:10.4319/lo.1973.18.6.0897.

Mendes L W, Kuramae E E, Navarrete A A, Van Veen J A, Tsai S M. 2014. Taxonomical and functional microbial community selection in soybean rhizosphere. *The ISME journal.* **8**: 1577-1587.

Meron, D., Rodolfo-Metalpa, R., Cunning, R., Baker, A. C., Fine, M., and Banin, E. (2012). Changes in coral microbial communities in response to a natural pH gradient. *ISME J.* 6, 1775. doi:10.1038/ismej.2012.19.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9, 386. doi:10.1186/1471-2105-9-386.

Morrow, K. M., Bourne, D. G., Humphrey, C., Botté, E. S., Laffy, P., Zaneveld, J., et al. (2014). Natural volcanic CO_2 seeps reveal future trajectories for host–microbial associations in corals and sponges. *Isme J.* 9, 894. doi:10.1038/ismej.2014.188.

O'Brien, P. A., Smith, H. A., Fallon, S., Fabricius, K., Willis, B. L., Morrow, K. M., et al. (2018). Elevated CO_2 has little influence on the bacterial communities associated with the pH-tolerant coral, massive *Porites* spp. *Front. Microbiol.* 9, 2621. doi:10.3389/fmicb.2018.02621.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G. L., Solymos, P., et al. (2008). The vegan package. *Community Ecol. Packag.-Forge R-Proj. Orgprojectsvegan*.

Pierrot, D., Lewis, E., and Wallace, D. W. R. (2006). *MS Excel Program Developed for CO_2 System Calculations, ORNL/CDIAC-105*. Oak Ridge, Tennessee.

Porzio, L., Buia, M. C., and Hall-Spencer, J. M. (2011). Effects of ocean acidification on macroalgal communities. *J. Exp. Mar. Biol. Ecol.* 400, 278–287. doi:10.1016/j.jembe.2011.02.011.

Qian, P.-Y., Lau, S. C., Dahms, H.-U., Dobretsov, S., and Harder, T. (2007). Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling and aquaculture. *Mar. Biotechnol.* 9, 399–410. doi:10.1007/s10126-007-9001-9.

Rastrick, S. S. P., Graham, H., Azetsu-Scott, K., Calosi, P., Chierici, M., Fransson, A., et al. (2018). Using natural analogues to investigate the effects of climate change and ocean acidification on Northern ecosystems. *ICES J. Mar. Sci.*, fsy128–fsy128. doi:10.1093/icesjms/fsy128.

Riebesell, U., and Gattuso, J.-P. (2015). Lessons learned from ocean acidification research. *Nat. Clim. Change* 5, 12–14. doi:10.1038/nclimate2456.

Rodríguez-Gironés, M. A., and Santamaría, L. (2006). A new algorithm to calculate the nestedness temperature of presence–absence matrices. *J. Biogeogr.* 33, 924–935. doi:10.1111/j.1365-2699.2006.01444.x.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi:10.1128/AEM.01541-09.

Sunday, J. M., Fabricius, K. E., Kroeker, K. J., Anderson, K. M., E, B. N., Barry, J. P., et al. (2017). Ocean acidification can mediate biodiversity shifts by changing biogenic habitat. *Nat. Clim. Change* 7, 81–85. doi:10.1038/nclimate3161.

Taylor, J. D., Ellis, R., Milazzo, M., Hall-Spencer, J. M., and Cunliffe, M. (2014). Intertidal epilithic bacteria diversity changes along a naturally occurring carbon dioxide and pH gradient. *FEMS Microbiol. Ecol.* 89, 670–678. doi:10.1111/1574-6941.12368.

Thompson, R., Norton, T., and Hawkins, S. (2004). Physical stress and biological control regulate the producer–consumer balance in intertidal biofilms. *Ecology* 85, 1372–1382. doi:10.1890/03-0279.

Tolker-Nielsen, T., and Molin, S. (2000). Spatial organization of microbial biofilm communities. *Microb. Ecol.* 40, 75–84. doi:10.1007/s002480000057.

Tripathi, B. M., Kim, M., Singh, D., Lee-Cruz, L., Lai-Hoe, A., Ainuddin, A., et al. (2012).

Tropical soil bacterial communities in Malaysia: pH dominates in the equatorial tropics too. *Microb. Ecol.* 64, 474–484. doi:10.1007/s00248-012-0028-8.

Uppström, L. R. (1974). The boron/chlorinity ratio of deep-sea water from the Pacific Ocean. *Deep Sea Res. Oceanogr. Abstr.* 21, 161–162. doi:10.1016/0011-7471(74)90074-6.

Wagg C, Bender S F, Widmer F, van der Heijden M G. 2014. Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proceedings of the National Academy of Sciences.* **111**: 5266-5270.

Webster, N., Negri, A., Botté, E., Laffy, P., Flores, F., Noonan, S., et al. (2016). Host-associated coral reef microbes respond to the cumulative pressures of ocean warming and ocean acidification. *Sci. Rep.* 6, 19324. doi:10.1038/srep19324.

Webster, N., Negri, A., Flores, F., Humphrey, C., Soo, R., Botte, E., et al. (2013). Near-future ocean acidification causes differences in microbial associations within diverse coral reef taxa. *Environ. Microbiol. Rep.* 5, 243–251. doi:10.1111/1758-2229.12006.

Weinbauer, M. G., Mari, X., and Gattuso, J.-P. (2011). "Effect of ocean acidification on the diversity and activity of heterotrophic marine microorganisms," in *Ocean Acidification* (Oxford, UK: Oxford University Press), 83–98.

Williams, G. A., Davies, M. S., and Nagarkar, S. (2000). Primary succession on a seasonal tropical rocky shore: the relative roles of spatial heterogeneity and herbivory. *Mar. Ecol. Prog. Ser.* 203, 81–94. doi:10.3354/meps203081.

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. doi:10.1111/j.1462-2920.2011.02571.x.

Wootton, J. T., Pfister, C. A., and Forester, J. D. (2008). Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. *Proc. Natl. Acad. Sci. U. S. A.* 105, 18848–18853. doi:10.1073/pnas.0810079105.

Yachi, S., and Loreau, M. (1999). Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis. *Proc. Natl. Acad. Sci. U. S. A.* 96, 1463–1468. doi:10.1073/pnas.96.4.1463.

Table legends

Table 1. Sea water chemistry across different sampling sites along pH gradient.

Table 2. Results of multivariate PERMANOVA showing significant differences in biofilm bacterial community composition between different sites along pH gradient.

Figure legends

Fig. 1. Biofilm bacterial alpha-diversity (mean ± SE) at different seawater pH levels in Shikine Island, Japan. Variation of OTUs richness in **(a)** boulder sites and **(b)** cliff sites. And variation of Shannon index **(c)** boulder sites and **(d)** cliff sites.

Fig. 2. Relative abundance of biofilm bacterial phyla detected along a pH gradient in Shikine Island, Japan.

Fig. 3. Relative abundance of biofilm bacterial taxa detected along a pH gradient in Shikine Island, Japan at **(a)** order level and **(b)** family level.

Fig. 4. NMDS ordination of total bacterial community composition among **(a)** different pH levels and **(b)** habitat types, based on Bray-Curtis distance.

Fig. 5. Frequency of co-occurrence patterns of sampled sites along a pH gradient in Shikine Island, Japan.

Fig. 6. Number of inter-Phylum and intra-Phylum co-presences and mutual exclusions of sampled sites (A: very low cliff site; B: low cliff site; C: ambient cliff site; D: very low boulder site; E: low boulder site; F: ambient boulder site).

Fig. 7. Box plot representing the relative abundance of the (a) Methane metabolism, (b) Sulphur metabolism, (c) Nitrogen metabolism, (d) Energy metabolism, (e) Carbon fixation in Prokaryotes and (f) Carbon fixation in photosynthetic organisms. The analysis was based on KEGG module

predictions using 16S data with the software PICRUSt. Results from multiple group comparisons in function abundance (ANOVA, Tukey–Kramer, $P < 0.05$) is reported as corrected P-value (Storey's FDR multiple test correction approach). The median value is shown as a line within the box and the mean value as a star. Error bars represent standard deviations.

Supplementary Online Material

Table S1. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial phyla across different sites.

Table S2. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial orders across different sites.

Table S3. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial families across different sites.

Table S4. Samples of "Boulder-Very low" and "Cliff-Ambient" sites formed the lowest nest while the other samples are basically a subset of these.

Fig. S1. Location of sample sites off Shikine Island, Japan.

Fig. S2. Rock biofilm sampling along a pH gradient off Shikine Island, Japan.

Fig. S3. Rarefaction curves comparing rock biofilm bacterial communities along a seawater pH gradient off Shikine Island, Japan.

Fig. S4. Predicted differences in biofilm bacterial functional categories (PICRUSt) across boulder and cliff sites off Shikine Island, Japan.

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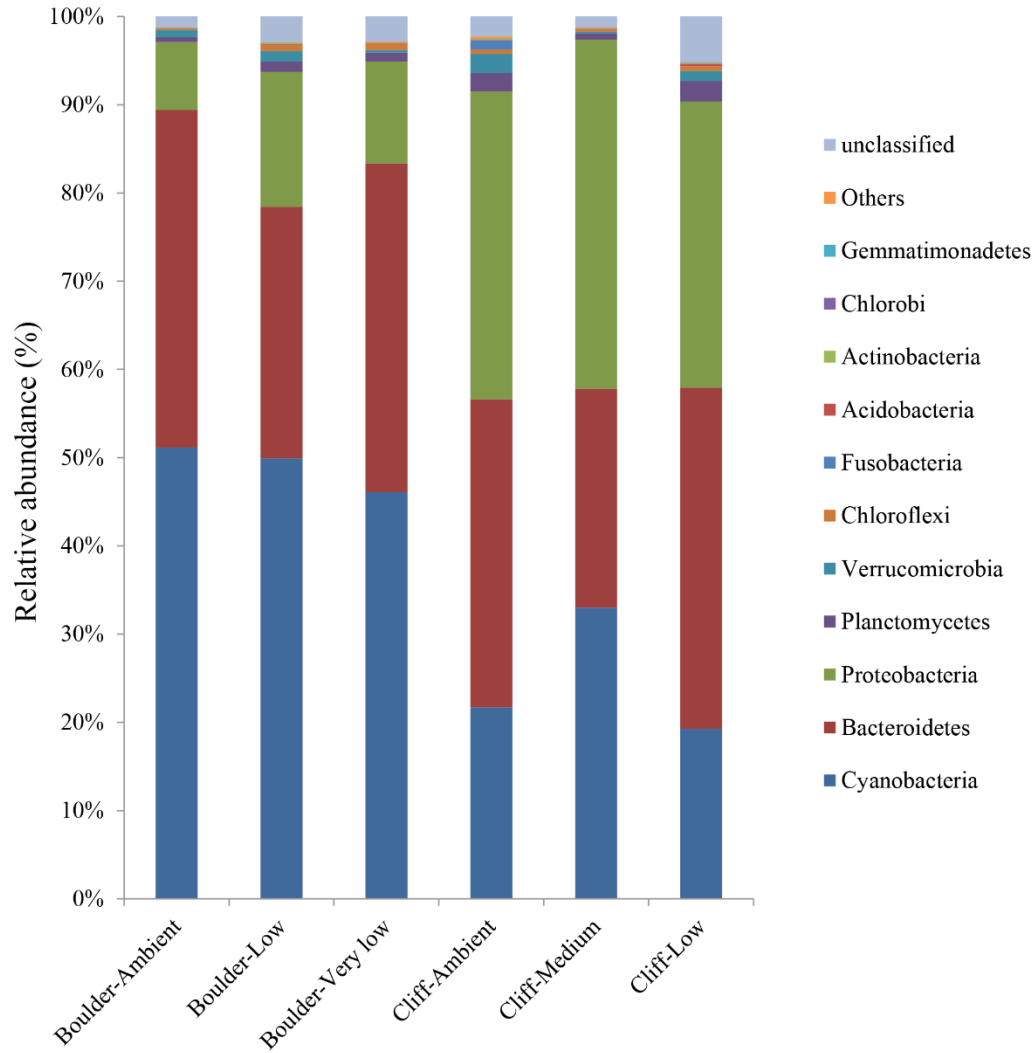
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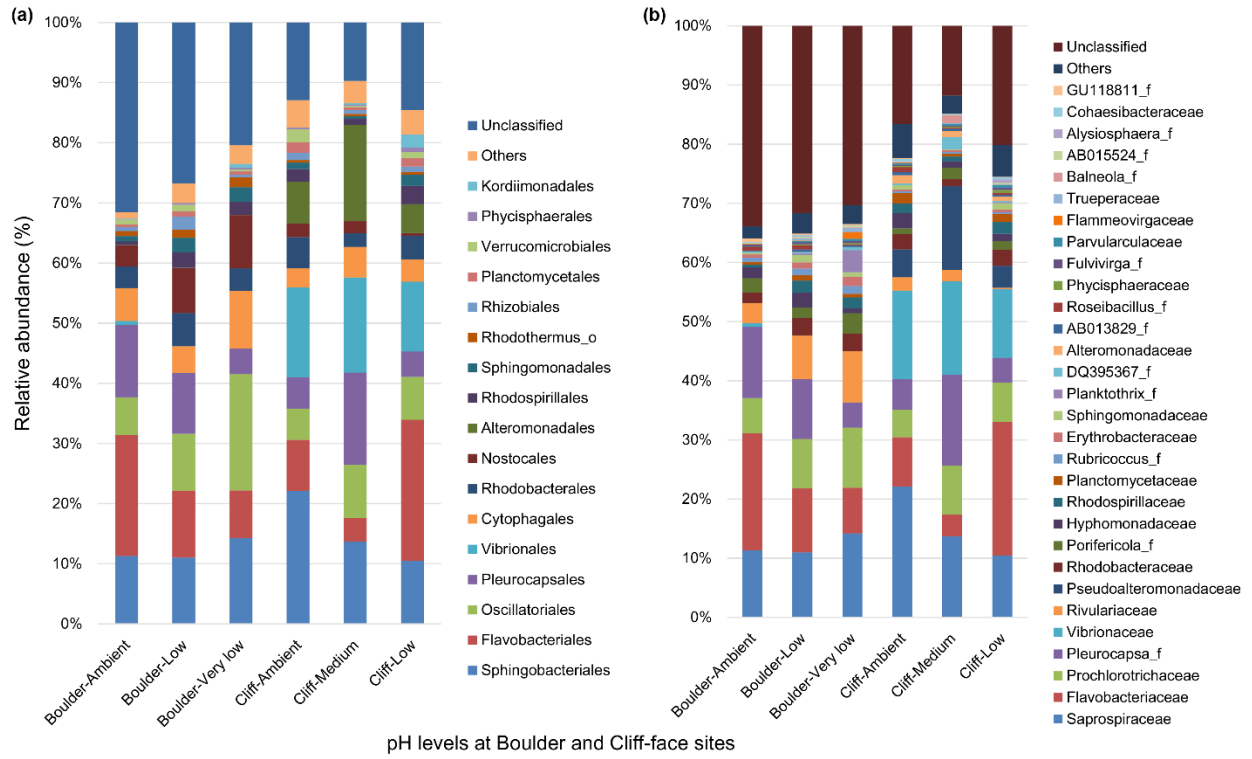
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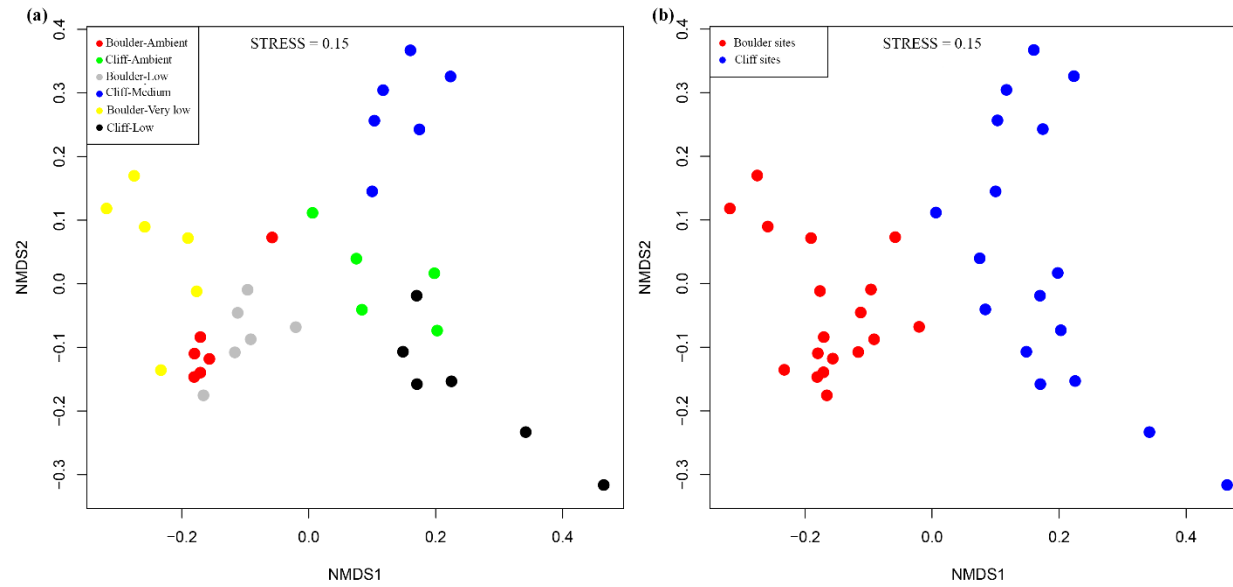
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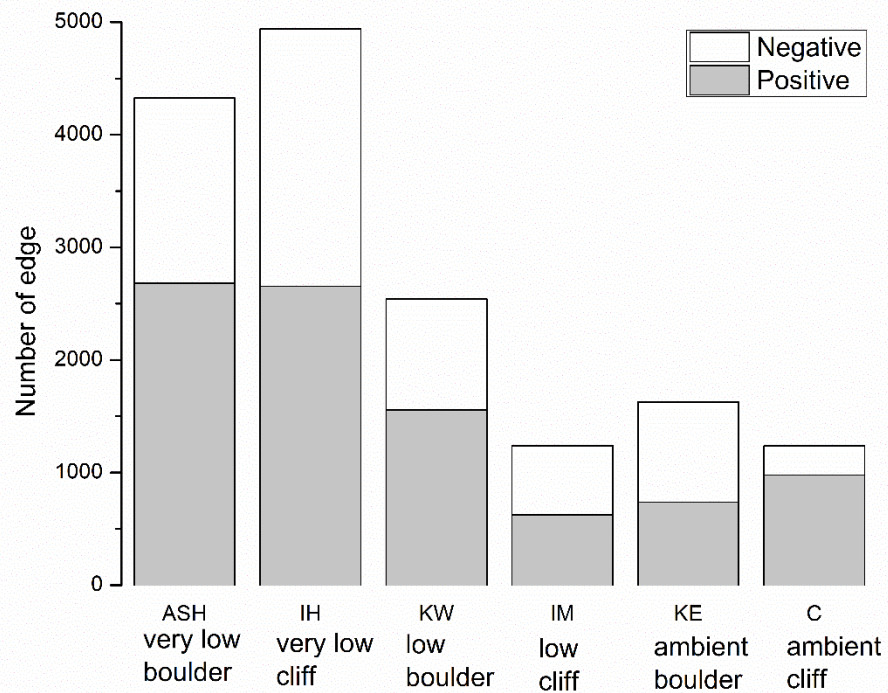
Fig. 5. Frequency of co-occurrence patterns of sampled stations.
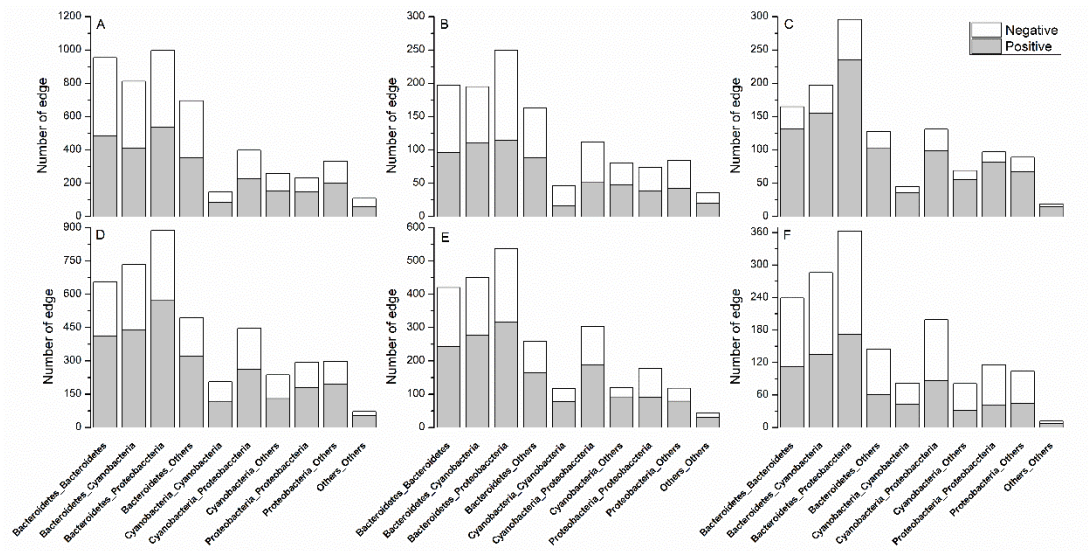
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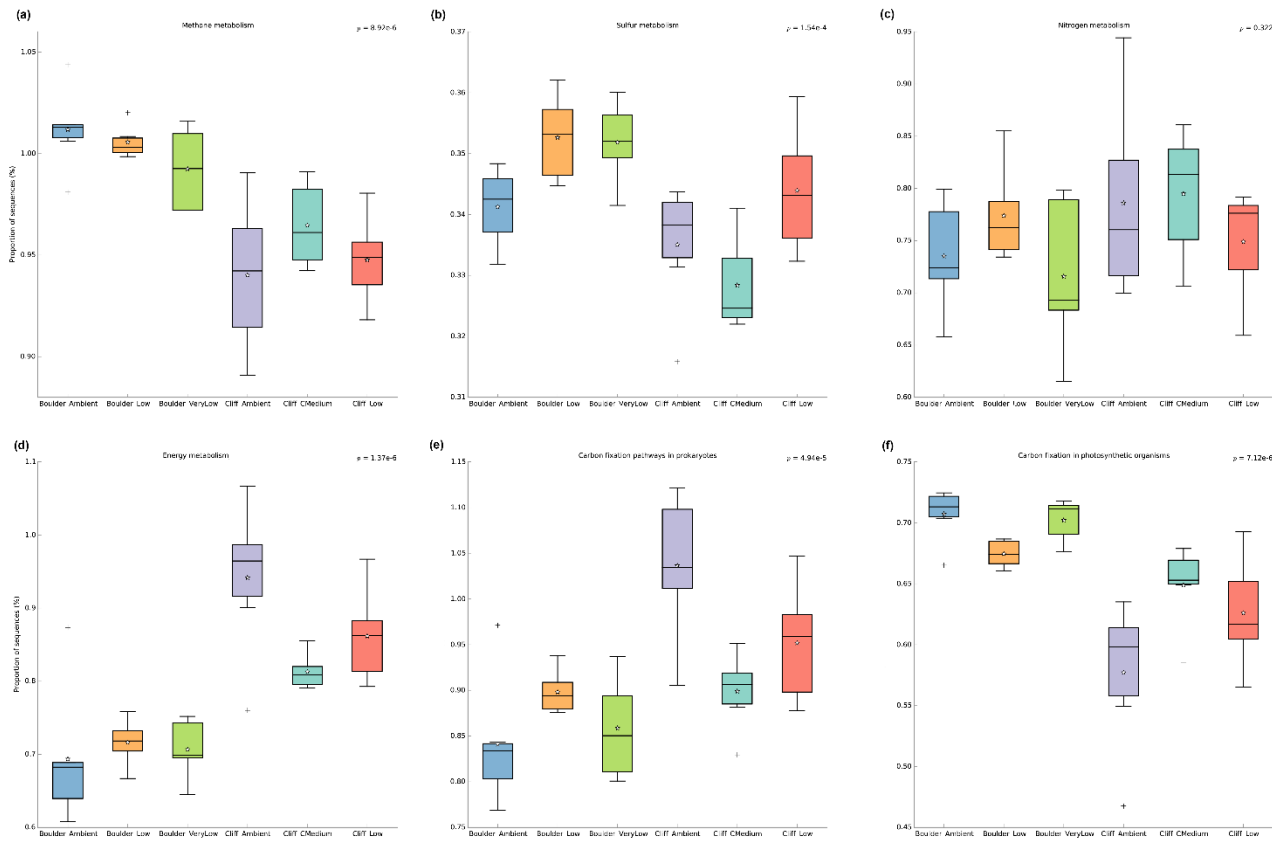
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Table 1. Sea water chemistry across different sampling sites along pH gradient.

Habitat type	pH zone	Salinity (psu)	Temp (°C)	A_T (μmol kg^{-1})	DIC (μmol kg^{-1})	pH$_{NBS}$	pCO$_2$ (matm)	HCO$_3^-$ (μmol kg^{-1})	CO$_3^{2-}$ (μmol kg^{-1})	Ω_{Ca}	Ω_{Ar}
Boulder	Ambient	34.0 ± 0.1	20.5 ± 0.7	2241.7 ± 12.5	1930.3 ± 20.6	8.28 ± 0.03	290.6 ± 25.1	1702.7 ± 33.1	218.3 ± 13.4	5.26 ± 0.32	3.42 ± 0.21
Boulder	Low	34.0 ± 0.2	20.4 ± 0.7	2201.4 ± 57.0	2141.5 ± 23.9	7.70 ± 0.07	1320.6 ± 225.4	2029.7 ± 27.9	69.4 ± 11.3	1.67 ± 0.27	1.09 ± 0.18
Boulder	Very low	34.1 ± 0.1	20.5 ± 0.5	2162.7 ± 31.7	2253.7 ± 37.6	7.24 ± 0.10	4003.9 ± 877	2100.2 ± 16.0	25.2 ± 6.5	0.61 ± 0.16	0.40 ± 0.10
Cliff	Ambient	34.0 ± 0.0	19.4 ± 0.5	2237.4 ± 1.0	1938.9 ± 36.3	8.27 ± 0.06	298.6 ± 51	1719.6 ± 58.1	209.4 ± 23.5	5.04 ± 0.57	3.27 ± 0.37
Cliff	Medium	34.0 ± 0.1	17.5 ± 1.1	2249.7 ± 2.1	2028.1 ± 35.9	8.15 ± 0.07	419.2 ± 82.3	1853.0 ± 55.1	160.5 ± 22.4	3.86 ± 0.54	2.49 ± 0.35
Cliff	Low	34.0 ± 0.0	18.5 ± 0.7	2281.7 ± 31.3	2186.8 ± 82.4	7.81 ± 0.22	1181.5 ± 672.4	2054.4 ± 103.6	92.3 ± 42.1	2.22 ± 1.01	1.44 ± 0.66

Supplementary Online Material

Responses of intertidal bacterial biofilm communities to increasing $p\mathrm{CO_2}$ in a naturally acidified system

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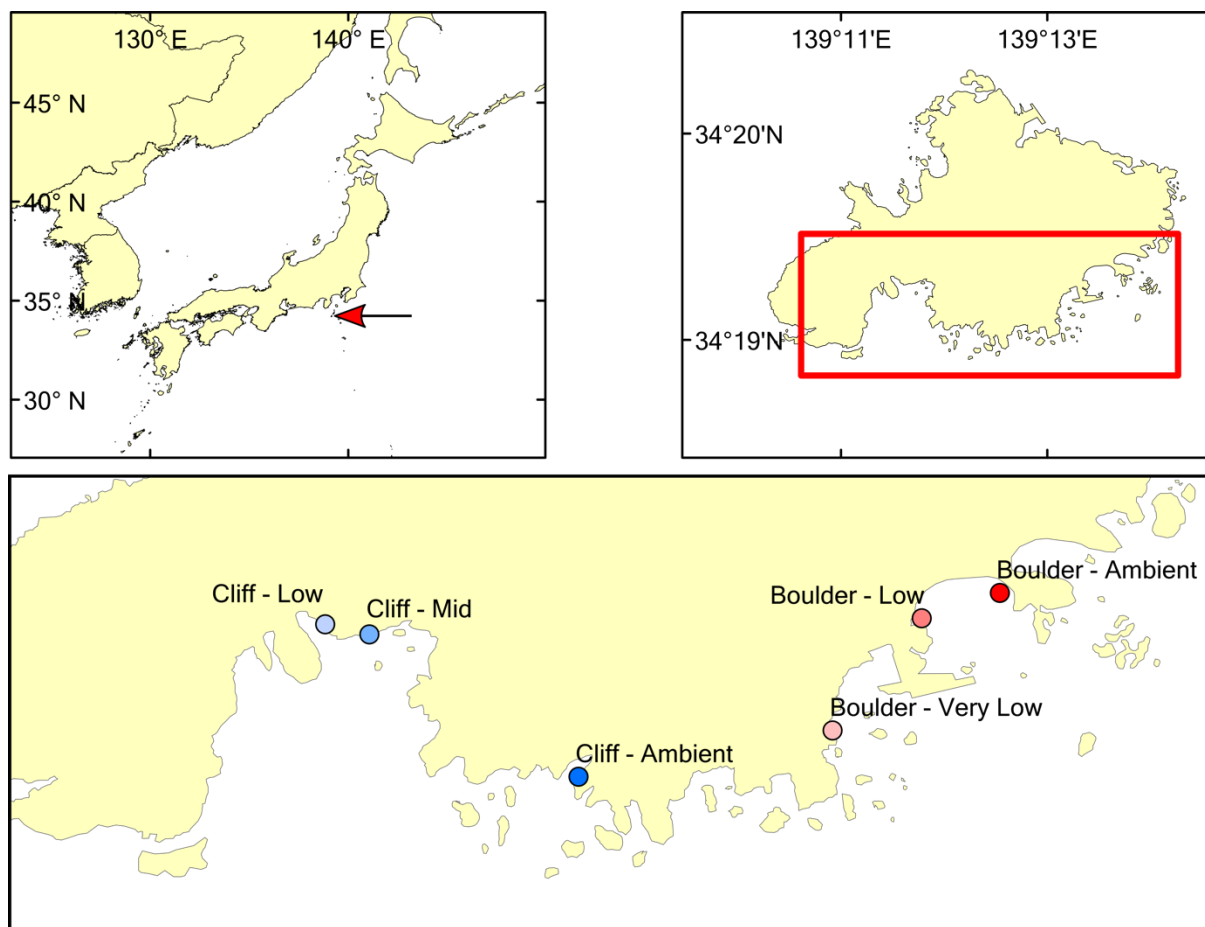
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Table S1. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial phyla across different sites.

		df	Mean square	F model	P value
Cyanobacteria	**Habitat type**	**1**	**5279**	**67.76**	**<0.001**
	pH	2	234	3	0.064
	Habitat type * pH	2	133	1.7	0.19
Bacteroidetes	Habitat type	1	28.8	0.31	0.58
	pH	**2**	**451**	**4.85**	**0.01**
	Habitat type * pH	2	26.4	0.28	0.75
Proteobacteria	**Habitat type**	**1**	**5229**	**38.33**	**<0.001**
	pH	2	134	0.98	0.38
	Habitat type * pH	2	29	0.21	0.8
Planctomycetes	**Habitat type**	**1**	**5.81**	**12.48**	**0.001**
	pH	**2**	**1.52**	**3.27**	**0.05**
	Habitat type * pH	**2**	**3.67**	**7.9**	**0.001**
Verrucomicrobia	Habitat type	1	1.5	1.58	0.21
	pH	2	2.49	2.68	0.08
	Habitat type * pH	**2**	**4.38**	**4.61**	**0.01**
Chloroflexi	Habitat type	1	0.08	0.45	0.5
	pH	2	0.38	2.09	0.14
	Habitat type * pH	2	0.44	2.43	0.1
Fusobacteria	Habitat type	1	1.04	0.96	0.33
	pH	2	1.11	1.02	0.37
	Habitat type * pH	2	1.06	0.97	0.38
Acidobacteria	Habitat type	1	0.03	2.93	0.09
	pH	2	0.02	1.72	0.19
	Habitat type * pH	**2**	**0.05**	**3.71**	**0.03**
Actinobacteria	Habitat type	1	0.0004	0.07	0.79
	pH	2	0.009	1.47	0.24
	Habitat type * pH	**2**	**0.02**	**4.48**	**0.01**
Chlorobi	Habitat type	1	0.00005	0.02	0.8
	pH	2	0.006	3.02	0.06
	Habitat type * pH	2	0.001	0.52	0.59
Gemmatimonadetes	Habitat type	1	0.001	2.58	0.11
	pH	2	0.0009	1.7	0.19
	Habitat type * pH	**2**	**0.002**	**4.01**	**0.02**

Table S2. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial orders across different sites.

		df	Mean square	F value	P value
Sphingobacteriales	Habitat type	1	95.67	1.49	0.23
	pH	2	75.79	1.18	0.32
	Habitat type * pH	2	159.8	2.49	0.09
Flavobacteriales	Habitat type	1	11.9	0.31	0.57
	pH	**2**	**229**	**6.07**	**0.006**
	Habitat type * pH	**2**	**634.8**	**16.8**	**<0.001**
Oscillatoriales	**Habitat type**	**1**	**191.7**	**9.89**	**0.003**
	pH	**2**	**169.3**	**8.74**	**0.001**
	Habitat type * pH	**2**	**127.9**	**6.60**	**0.004**
Pleurocapsales	Habitat type	1	2.56	0.07	0.78
	pH	**2**	**217.5**	**6.19**	**0.005**
	Habitat type * pH	**2**	**109.4**	**3.11**	**0.05**
Vibrionales	**Habitat type**	**1**	**1734.4**	**10.25**	**0.003**
	pH	2	16.7	0.09	0.90
	Habitat type * pH	2	13.7	0.08	0.92
Cytophagales	**Habitat type**	**1**	**56.83**	**14.03**	**<0.001**
	pH	**2**	**18.16**	**4.48**	**0.01**
	Habitat type * pH	**2**	**32.84**	**8.10**	**0.001**
Rhodobacterales	Habitat type	1	2.10	0.61	0.43
	pH	2	1.11	0.32	0.72
	Habitat type * pH	**2**	**19.01**	**5.52**	**0.009**
Nostocales	**Habitat type**	**1**	**228.1**	**15.25**	**<0.001**
	pH	2	13.61	0.91	0.41
	Habitat type * pH	2	38.75	2.59	0.09
Alteromonadales	**Habitat type**	**1**	**760.9**	**32.70**	**<0.001**
	pH	**2**	**106.3**	**4.56**	**0.01**
	Habitat type * pH	**2**	**107**	**4.59**	**0.01**
Rhodospirillales	Habitat type	1	0.65	0.34	0.56
	pH	2	4.84	2.58	0.09
	Habitat type * pH	**2**	**8.12**	**4.32**	**0.02**
Rhodothermus_o	**Habitat type**	**1**	**6.38**	**32.18**	**<0.001**
	pH	2	0.59	2.98	0.06
	Habitat type * pH	2	0.44	2.22	0.12
Rhizobiales	Habitat type	1	0.25	0.96	0.33
	pH	**2**	**1.64**	**6.17**	**0.005**
	Habitat type * pH	**2**	**4.12**	**15.54**	**<0.001**

Planctomycetales	**Habitat type**	**1**	**2.80**	**9.29**	**0.004**
	pH	2	0.54	1.81	0.18
	Habitat type * pH	**2**	**2.27**	**7.53**	**0.002**
Verrucomicrobiales	Habitat type	1	1.45	1.51	0.22
	pH	2	2.67	2.79	0.077
	Habitat type * pH	**2**	**8.31**	**4.34**	**0.022**
Phycisphaerales	**Habitat type**	**1**	**0.25**	**5.86**	**0.02**
	pH	**2**	**0.56**	**12.91**	**<0.001**
	Habitat type * pH	**2**	**0.16**	**3.72**	**0.03**
Kordiimonadales	**Habitat type**	**1**	**3.02**	**4.21**	**0.04**
	pH	**2**	**6.74**	**9.41**	**<0.001**
	Habitat type * pH	**2**	**2.31**	**3.22**	**0.05**
Parvularculales	Habitat type	1	0.08	2.61	0.11
	pH	**2**	**0.20**	**6.22**	**0.005**
	Habitat type * pH	2	0.004	0.13	0.87
Balneola_o	**Habitat type**	**1**	**1.64**	**3.78**	**0.06**
	pH	**2**	**1.59**	**3.66**	**0.03**
	Habitat type * pH	**2**	**1.63**	**3.75**	**0.03**
Chromatiales	**Habitat type**	**1**	**0.23**	**32**	**<0.001**
	pH	**2**	**0.05**	**6.96**	**0.003**
	Habitat type * pH	**2**	**0.10**	**13.68**	**<0.001**
Myxococcales	Habitat type	1	0.002	0.52	0.47
	pH	**2**	**0.03**	**7.74**	**0.001**
	Habitat type * pH	2	0.01	2.71	0.08

Table S3. Results from two-way ANOVA showing the variation of the relative abundance of biofilm bacterial families across different sites.

		df	Mean square	F value	P value
Saprospiraceae	Habitat type	1	95.77	1.45	0.23
	pH	2	76.24	1.18	0.31
	Habitat type * pH	2	160	2.49	0.09
Flavobacteriaceae	Habitat type	1	13.8	0.37	0.54
	pH	**2**	**220.3**	**5.94**	**0.006**
	Habitat type * pH	**2**	**600.6**	**16.22**	**<0.001**
Pleurocapsa family	Habitat type	1	2.53	0.07	0.79
	pH	**2**	**217.3**	**6.19**	**0.005**
	Habitat type * pH	**2**	**109.4**	**3.11**	**0.05**
Vibrionaceae	**Habitat type**	**1**	**1734.4**	**10.25**	**0.003**
	pH	2	16.7	0.09	0.90
	Habitat type * pH	2	13.7	0.08	0.92
Rivulariaceae	**Habitat type**	**1**	**225.5**	**15.24**	**<0.001**
	pH	2	12.13	0.82	0.45
	Habitat type * pH	2	40.89	2.76	0.07
Pseudoalteromonadaceae	**Habitat type**	**1**	**508.4**	**22.64**	**<0.001**
	pH	**2**	**99.8**	**4.44**	**0.02**
	Habitat type * pH	**2**	**99.7**	**4.44**	**0.02**
Rhodobacteraceae	Habitat type	1	1.49	1.41	0.24
	pH	2	1.94	1.84	0.17
	Habitat type * pH	**2**	**5.21**	**4.94**	**0.01**
Porifericola family	**Habitat type**	**1**	**10.50**	**11.31**	**0.002**
	pH	2	2.01	2.17	0.13
	Habitat type * pH	**2**	**4.26**	**4.59**	**0.01**
Hyphomonadaceae	Habitat type	1	0.05	0.04	0.83
	pH	**2**	**4.24**	**3.75**	**0.03**
	Habitat type * pH	**2**	**4.42**	**3.91**	**0.03**
Planctomycetaceae	**Habitat type**	**1**	**2.80**	**9.29**	**0.004**
	pH	2	0.54	1.081	0.18
	Habitat type * pH	**2**	**2.27**	**7.53**	**0.002**
Rubricoccus family	**Habitat type**	**1**	**5.11**	**44.70**	**<0.001**
	pH	2	0.22	1.96	0.15
	Habitat type * pH	**2**	**0.36**	**3.14**	**0.05**
Erythrobacteraceae	**Habitat type**	**1**	**4**	**16.64**	**<0.001**
	pH	**2**	**0.94**	**3.94**	**0.03**
	Habitat type * pH	2	0.62	2.58	0.9

Sphingomonadaceae	Habitat type	1	0.28	1.41	0.24
	pH	**2**	**0.73**	**3.67**	**0.03**
	Habitat type * pH	**2**	**2.23**	**11.12**	**<0.001**
Planktothrix family	**Habitat type**	**1**	**14.42**	**15.06**	**<0.001**
	pH	**2**	**14.04**	**14.66**	**<0.001**
	Habitat type * pH	**2**	**12.01**	**12.54**	**<0.001**
Alteromonadaceae	**Habitat type**	**1**	**9.72**	**16.23**	**<0.001**
	pH	2	0.36	0.61	0.54
	Habitat type * pH	2	0.45	0.75	0.47
Phycisphaeraceae	**Habitat type**	**1**	**0.12**	**4**	**0.05**
	pH	**2**	**0.20**	**6.72**	**0.003**
	Habitat type * pH	2	0.03	1.30	0.28
Flammeovirgaceae	**Habitat type**	**1**	**1.29**	**6.75**	**0.01**
	pH	**2**	**1.09**	**5.68**	**0.008**
	Habitat type * pH	**2**	**1.22**	**6.40**	**0.004**
Alysiosphaera family	**Habitat type**	**1**	**0.32**	**6.45**	**0.01**
	pH	**2**	**0.28**	**5.61**	**0.008**
	Habitat type * pH	**2**	**0.39**	**7.9**	**0.001**
Cohaesibacteraceae	**Habitat type**	**1**	**0.15**	**8.2**	**0.007**
	pH	2	0.01	0.92	0.40
	Habitat type * pH	**2**	**0.14**	**7.37**	**0.002**

1 **Table S4.** Samples of "Boulder-Very low" and "Cliff-Ambient" sites formed the lowest nest while the
2 other samples are basically a subset of these.

Group	Rank order of nestedness
Boulder-Very low 1	16
Boulder-Very low 2	15
Boulder-Very low 3	3
Boulder-Very low 4	5
Boulder-Very low 5	23
Boulder-Very low 6	13
Cliff-Ambient 1	14
Cliff-Ambient 2	17
Cliff-Ambient 3	1
Cliff-Ambient 4	2
Cliff-Ambient 5	6
Cliff-Low 1	12
Cliff-Low 2	31
Cliff-Low 3	33
Cliff-Low 4	10
Cliff-Low 5	18
Cliff-Low 6	4
Cliff-Medium 1	25
Cliff-Medium 2	11
Cliff-Medium 3	7
Cliff-Medium 4	35
Cliff-Medium 5	32
Cliff-Medium 6	22
Boulder-Ambient 1	30
Boulder-Ambient 2	34
Boulder-Ambient 3	19
Boulder-Ambient 4	8

Boulder-Ambient 5	27
Boulder-Ambient 6	24
Boulder-Low 1	20
Boulder-Low 2	29
Boulder-Low 3	9
Boulder-Low 4	21
Boulder-Low 5	28
Boulder-Low 6	26

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