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► **To cite this version:**

Storme Zaviar de Scally, Samuel Chaffron, Thulani Peter Makhalanyane. Polar opposites; bacterioplankton susceptibility and mycoplankton resistance to ocean acidification. 2020. hal-02946889

**HAL Id: hal-02946889**

**<https://hal.science/hal-02946889>**

Preprint submitted on 5 Jan 2021

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1 **Polar opposites; bacterioplankton susceptibility and mycoplankton**  
2 **resistance to ocean acidification**

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18 **Running title:** Microbial responses to acidification

19

20 **Keywords:** bacterioplankton, mycoplankton, acidification, Southern Ocean, diversity,  
21 enzymatic activity, carbon, nitrogen, phosphorus

22

23

## 24 **ABSTRACT**

25           Microorganisms form the basis of ocean ecosystems yet the effects of perturbations  
26 such as decreasing pH on microbial community structure, interactions and functionality  
27 remain compared to multicellular organisms. Using an experimental manipulation of  
28 Southern Ocean seawater, we subjected bacterioplankton and mycoplankton to artificial pH  
29 decreases, which are predicted to occur in the future. We show that acidification led to  
30 substantial increases of bacterioplankton diversity, while in contrast it had no effect on  
31 mycoplankton diversity. Our analyses revealed a loss of putative keystone taxa and a  
32 decrease in predicted community interactions as a response to lower pH levels.  
33 Bacterioplankton shifted from generalist to specialist community members, suggesting a  
34 specific stress response to unfavourable conditions. In addition, enzyme activities involved in  
35 nitrogen acquisition were lower at reduced pH levels, suggesting altered organic matter  
36 cycling in a more acidic ocean. Our findings suggest that bacterioplankton and mycoplankton  
37 may respond differentially to future ocean acidification, with potentially negative impacts on  
38 community structure and biogeochemical cycling in the Southern Ocean.

## 39 **IMPORTANCE**

40           Oceans absorb the majority of anthropogenically produced CO<sub>2</sub>, the consequence of  
41 which is ocean acidification, a phenomenon already negatively impacting key marine  
42 organisms. Marine microbial communities form the basis of ocean food webs by generating  
43 nutrients for higher trophic levels, yet the response of these key microbial drivers to  
44 acidification remains unclear. This knowledge deficit is particularly true for understudied  
45 marine ecosystems such as the Southern Ocean. Using a mesocosm approach, we found that  
46 acidification severely impacts microbial community stability, by altering bacterioplankton  
47 community structure, reducing network complexity, and augmenting enzyme activities

48 associated with nitrogen acquisition. This study adds to our understanding of the effects of  
49 ocean acidification on microbial communities, particularly within an environment expected to  
50 be largely effected by future anthropogenically driven climate change.

51

## 52 **INTRODUCTION**

53 The ocean absorbs roughly 30% of anthropogenic carbon dioxide emissions, which  
54 leads to changes in oceanic carbonate chemistry, resulting in both carbonation and  
55 acidification (Orr et al., 2005; Zeebe et al., 2008; Doney et al., 2009; Adler et al., 2011;  
56 Kottmeier et al., 2016). Globally, ocean pH is predicted to decrease by as much as 0.4 and 0.8  
57 units within the next 100 and 300 years, respectively (Caldeira and Wickett, 2003; Orr et al.,  
58 2005), a change which is nearly eight times higher than what has been observed within the  
59 last 25 million years (Pearson and Palmer, 2000). Ocean acidification (OA) can negatively  
60 impact marine macro-organisms, such as calcifiers, adversely affecting their growth and  
61 physiology (Doney et al., 2009; Gazeau et al., 2013; Brandenburg et al., 2019). However, the  
62 extent to which decreasing pH affects diverse bacterioplankton and mycoplankton  
63 communities remains largely unclear.

64 A null hypothesis proposed by Joint and colleagues (2011b) states that due to the  
65 ubiquitous abundance, fast generation time and genetic plasticity of microorganisms,  
66 microorganisms will rapidly adapt to future pH perturbations. However, despite the large  
67 genetic flexibility microorganisms possess, a physiological tipping point, where individual  
68 organisms are no longer able to adapt to perturbations, may be reached (Allison and Martiny,  
69 2008b; Hutchins and Fu, 2017). There is considerable uncertainty and disagreement  
70 regarding the impact of acidification on bacterioplankton and mycoplankton communities,  
71 with reports demonstrating both resistance and susceptibility. Evidence of microbial

72 community resistance to acidification include: unchanged bacterial cell abundance (Grossart  
73 et al., 2006; Allgaier et al., 2008; Krause et al., 2012; Newbold et al., 2012), consistent  
74 composition (Lindh et al., 2013; Roy et al., 2013; Oliver et al., 2014), unaffected or increased  
75 heterotrophic production (Grossart et al., 2006; Arnosti et al., 2011) and increased nitrogen  
76 fixation (Joint et al., 2011a; Rees et al., 2016). Susceptibility has been seen through,  
77 alterations in bacterial and fungal cell numbers (Krause et al., 2013; Endres et al., 2014;  
78 Engel et al., 2014), compositional shifts (Allgaier et al., 2008; Krause et al., 2012; Zhang et  
79 al., 2013) with changes towards pathogenic members (Vega-Thurber et al., 2009; Reich et al.,  
80 2017) and decreased nitrification rates (Beman et al., 2011). Moreover, the increased  
81 expression of proton pump genes identified in a metatranscriptomic study (Bunse et al., 2016)  
82 suggests that acidification may be energetically costly for microbial cells, with possible  
83 negative implications for microbial functionality and biogeochemical cycling. These results  
84 emphasise the need for further investigations into the effects of acidification on complex and  
85 diverse bacterioplankton and mycoplankton assemblages (Hutchins and Fu, 2017).

86         Although the Southern Ocean (SO) covers less than 20% of the global ocean it is a  
87 highly productive ecosystem, facilitating the inter-ocean exchange of nutrients which drives  
88 over 70% of ocean productivity (Mayewski et al., 2009). While the SO has naturally low pH,  
89 it remains vulnerable to anthropogenic induced acidification due to its' low temperature, the  
90 upwelling of CO<sub>2</sub> enriched deep waters, and the fact that approximately 40% of the global  
91 oceanic uptake of CO<sub>2</sub> occurs in the SO (Riebesell and Gattuso, 2015). Several studies have  
92 shown that acidification results in increased resilience in diatoms (Valenzuela et al., 2018)  
93 and functional biodiversity loss (Teixidó et al., 2018). In contrast to other environments  
94 (Newbold et al., 2012; Chen et al., 2013; Huggett et al., 2018; James et al., 2019), the precise  
95 responses of bacterioplankton and mycoplankton remain largely speculative and often

96 unexamined in pristine marine environments. This knowledge is crucial for understanding the  
97 effects of climate change in regions most sensitive to future environmental change.

98 To address this deficit, we assessed the effect of future predicted pH levels (based on  
99 IPCC projections for the years 2100 and 2300, respectively) on both SO bacterioplankton and  
100 mycoplankton diversity, composition, predicted interactions, and functionality using  
101 mesocosm experiments. We predicted that; (1) ocean acidification will decrease both  
102 bacterioplankton and mycoplankton diversity and will significantly alter community  
103 structure; (2) microbial functionality will be both positively and negatively affected by ocean  
104 acidification in terms of carbon and nitrogen/phosphorus decomposition, respectively, and (3)  
105 the total number of covariations and subsequently network stability will decrease with lower  
106 pH levels.

## 107 **RESULTS**

### 108 **Microbial diversity and community structure in control and acidified mesocosms**

109 Alpha diversity for bacteria and archaea was significantly higher in the low and  
110 medium pH groups in comparison to the control (ANOVA,  $p$ -value  $< 0.001$ ), and both  
111 Shannon diversity (Fig.1a) and OTU richness (Fig.1b) significantly correlated with pH. On  
112 the contrary, fungal alpha diversity was not significantly different between the three  
113 treatment groups (ANOVA,  $p$ -value  $> 0.05$ ), and did not correlate significantly with pH (Fig.  
114 1ab). Bacterial and archaeal community structure was significantly affected by pH treatment  
115 (PERMANOVA,  $p$ -value  $< 0.05$ , betadisper  $p$ -value  $> 0.05$ ), and clustered according to  
116 treatment type (Fig. 2a). In contrast to bacteria and archaea, fungal community structure was  
117 not significantly influenced by pH treatment (PERMANOVA,  $p$ -value  $< 0.05$ , betadisper  $p$ -  
118 value  $< 0.05$ ), as no distinct clusters were seen according to treatment group (Fig. 2b). Thus,  
119 bacterioplankton and mycoplankton community composition clearly display differential

120 responses to acidification. Whereas several bacterioplankton genera either increased or  
121 decreased in abundance under acidified conditions, mycoplankton composition remained  
122 stable, with the exception of Agariomyctes. Specific genera and species differences are  
123 further described in Supporting Information (Fig. S3).

#### 124 **Functional community responses to ocean acidification**

125 Extracellular enzymatic activities were detected in all samples, where the activities of  
126 carbon and phosphorus acquiring enzymes did not vary significantly with pH treatment (Fig.  
127 3). In contrast, nitrogen acquiring enzymes NAG and LAP significantly decreased at lower  
128 pH. Enzymes involved in carbon acquisition, namely BG and BX, showed no significant  
129 increase in response to pH treatment (ANOVA,  $p$ -value  $> 0.05$ ). AP, involved in phosphorus  
130 acquisition, showed no significant increase or decrease in activity in response to the pH  
131 treatment (ANOVA,  $p$ -value  $> 0.05$ ). NAG activity decreased in the low group across  
132 sampling days, although this was not significant from the control group (ANOVA,  $p$ -value  $>$   
133  $0.05$ ). Notably, LAP was shown to be significantly lower in the medium and low pH  
134 treatment groups relative to the control at day 21 of the mesocosm experiment (ANOVA,  $p$ -  
135 value  $< 0.05$ ). Activities were correlated to pH levels using both linear regression (Fig. 3) and  
136 Spearman's rho. Both results indicated that only NAG was significantly positively associated  
137 with pH ( $r = 0.42$ ,  $p$ -value  $< 0.05$ ; Fig. 3a), whereby NAG activity decreased at lower pH  
138 levels. LAP activity, although significantly different between treatment and control groups,  
139 could not be predicted by pH, likely due to the lower activity in the medium pH group than  
140 the low pH group.

#### 141 **Community structures and system level changes associated to ocean acidification**

142 Table S2 shows topological features of bacterial and archaeal community networks.  
143 All networks exhibited scale-free characteristics, typical of biological association networks,

144 were small world, modular, and significantly different from generated random networks (see  
145 Supporting information for details).

146 Network structure, complexity and community composition differed markedly  
147 between the three treatment groups (Table S2, Fig. 4). The medium network was the largest  
148 followed by the low and control networks, showing that acidification resulted in larger  
149 networks. Interestingly, although the medium network displayed the highest diversity and  
150 connectivity, the low network was the least complex (Table S2). The medium network  
151 contained the greatest number of edges, the largest average degree (i.e. average number of  
152 edges per node in the network) and the shortest harmonic geodesic distance (i.e. distance  
153 between all nodes in the network).

154 The number of modules in the medium and low networks were greater than the  
155 control network (Table S2). Modules were largest and most connected in the medium  
156 network, followed by the control and the low network, reflecting patterns observed from  
157 overall network structure. Intriguingly, the percentage of positive associations increased in  
158 response to acidification, where the low network had 77.82% positive associations, the  
159 medium network contained 77.25% and the control network only 74.70%, highlighting that  
160 reduced pH levels resulted in a slight increase in positive associations as opposed to co-  
161 exclusions (Fig. 4, Table S2). The overall taxonomic composition of the largest modules at  
162 both phylum and class level did not differ markedly between the three treatment groups,  
163 although some modules exhibited a dominance of different taxonomic groups (Fig. 4).  
164 Alphaproteobacteria, Bacteroidetes and Gammaproteobacteria were the dominant taxa in  
165 almost all modules and were also the dominant taxa in the overall bacterioplankton  
166 community. Actinobacteria occurred more frequently in the medium and low networks, and  
167 Euryarchaeota was found exclusively in the control network (Fig. S2 and Table S1).  
168 Unassigned sequences constituted a relatively high proportion in several modules, and were



169 highest in the low network, suggesting that cryptic unknown species may increase in  
170 abundance in response to acidification in the SO. Furthermore, no modules consisted of a  
171 single class or phylum, showing a low degree of phylogenetic assortativity within the  
172 association networks, which suggest a potential high capacity for biotic interactions in SO  
173 microbial communities.

174 Network topological metrics can be computed for each node/OTU by assessing the  
175 within-module connectivity ( $Z_i$ ) as well as the between-module connectivity ( $P_i$ ), which can  
176 indicate the potential ecological role of each OTU. These parameters can then be used to  
177 classify each OTU into four network-based categories; peripherals, connectors, module hubs  
178 and network hubs (Fig. 5a). Ecologically, peripherals may be considered as specialists, while  
179 connectors and module hubs may represent generalists and network hubs could be classified  
180 as super generalists. Additionally, the last three can be regarded as potential keystone taxa, on  
181 the sole basis of their high connectivity, since the overall network structure would be  
182 significantly altered if they were removed.

183 The majority of OTUs in all three networks were classified as peripherals, with most  
184 edges occurring within their own modules (Fig. 5a). Several peripheral OTUs had a  $P_i$  value  
185 of 0, indicating that these nodes were connected solely to other OTUs within the same  
186 module (74% of OTUs in the control network, 70% in the medium network and 84% in the  
187 low). No network hubs (super generalists) were detected in any network. Interestingly, the  
188 relative abundance of several peripheral taxa changed in response to acidification (Fig. 5b,  
189 see Supplemental Information for details).

190 The number and taxonomic affiliation of module hubs and connectors detected (Table S3)  
191 differed noticeably between the three networks. Five module hubs were detected in the  
192 control network, five in the medium network and only three in the low network (Fig. 4, 5a).

193 All control hubs consisted of phylum Bacteroidetes OTUs and unassigned sequences, with  
194 the orders *Flavobacteriales* and *Saprospirales*. Medium hubs belonged to classes  
195 Flavobacteriia, Gammaproteobacteria and Alphaproteobacteria. Low hubs consisted of  
196 Alphaproteobacteria, Flavobacteriia and unassigned bacteria. The relative abundance of  
197 module hubs ranged from 0.01% to 0.74%. The control network contained five connectors,  
198 the medium network contained three and the low network had only one. Control and medium  
199 connectors consisted of Alphaproteobacteria (order *Rhodobacterales* and *Rickettsiales*,  
200 respectively) and Flavobacteriia (order *Flavobacteriales*), whereas the low connector  
201 belonged to Bacteroidetes (order *Cytophagales*). Connector relative abundance ranged from  
202 0.005% to 3.24%, with all but one OTU with an abundance of less than 1%, underlying the  
203 disconnection between species centrality and relative abundance. As hubs and connectors can  
204 be considered potential keystone taxa, Alphaproteobacteria and Flavobacteriia represented  
205 prominent putative keystone taxa within all networks, although differences between networks  
206 can be detected at finer taxonomic resolution (Table S3).

## 207 **DISCUSSION**

### 208 **Diversity does not equal stability**

209 Microbial community stability amidst perturbations is thought to be promoted by  
210 increased diversity, due to the link between diversity and ecosystem health and  
211 multifunctionality (Girvan et al., 2005; Delgado-Baquerizo et al., 2016; Shade, 2017).  
212 Similarly, loss of community diversity following environmental disturbance is often linked to  
213 community susceptibility. However, it has recently been suggested that assumptions of  
214 increased diversity leading to increased stability are false, at least for some ecosystems, as the  
215 role of diversity is dependent on the environment or ecosystem and disturbance itself (Shade,  
216 2017). Community members are not necessarily interacting mutualistically in diverse

217 compositions, and taxonomic diversity does not absolutely equate to functional diversity  
218 (Louca et al., 2016). In this study, we found that bacterioplankton alpha diversity is  
219 significantly higher in acidified compared to ambient mesocosms, in contrast to what has  
220 been hypothesised. Simultaneously, bacterioplankton community structure was significantly  
221 altered in response to CO<sub>2</sub>, with the relative abundances of several key community members  
222 significantly changing. We suggest that increased bacterioplankton diversity is an indication  
223 of community susceptibility, or “scramble” rather than stability in the Southern Ocean. The  
224 high diversity identified for bacterioplankton communities highlight that polar marine  
225 regions, and the SO specifically, contain high levels of unexplored diversity (Dickinson et al.,  
226 2016).

### 227 **Mycoplankton resistance to acidification**

228 In contrast to our initial hypothesis, we did not observe a significant effect of ocean  
229 acidification on mycoplankton diversity. Instead, fungal community diversity and structure  
230 was stable, with the exception of Agaricomycetes, which increased in relative abundances at  
231 lower pH levels (Table S3). Agaricomycetes is a class of Basidiomycota that contains several  
232 yeast species (Jones, 2011; Jones et al., 2015), suggesting an increase in abundance of  
233 adapted fungal morphologies in response to acidification. However, the increase of  
234 Agaricomycetes phylotypes did not significantly affect overall fungal diversity or community  
235 structure. We argue that mycoplankton may contain a higher genetic plasticity and degree of  
236 physiological adaptation than bacterioplankton and are therefore resistant to future  
237 acidification. Culture dependant studies have shown that both terrestrial and marine fungi can  
238 rapidly adapt to a variety of environmental conditions, such as hot and cold temperatures and  
239 anoxia (Richards et al., 2012; Tsuji et al., 2013; Rédou et al., 2015). Additionally, fungi have  
240 larger pH tolerance ranges and optima for growth than bacteria (Rousk et al., 2010).  
241 Moreover, fungi possess chitin-rich cell walls (Taylor and Cunliffe, 2016), which may

242 provide enhanced resistance. Source habitat significantly influences mycoplankton  
243 community structure (Panzer et al., 2015), further suggesting that SO fungal communities  
244 may be adapted to the pelagic environment and therefore less effected by pH variations. The  
245 lack of a significant mycoplankton community response in SO waters to pH levels predicted  
246 in the next 300 years, suggests the resistance of marine fungi to acidification in these  
247 systems.

#### 248 **Altered organic matter cycling in a future acidic ocean**

249       Microbially produced extracellular enzymes break down complex organic substrates,  
250 mediating heterotrophic carbon and nutrient recycling in marine environments (Arnosti,  
251 2014). Enzymes measured for nutrient acquisition showed activity in all samples, indicating  
252 active bacterioplankton and/or mycoplankton heterotrophic communities within surface SO  
253 waters, since members of both assemblages are capable of producing each enzyme (Jones,  
254 2011; Arnosti, 2014; Hoarfrost and Arnosti, 2017). The observed results of exoenzyme  
255 activities were congruent with our original hypothesis. Carbon and phosphorus degrading  
256 enzymes showed no substantial shifts in activity in response to pH treatment, suggesting  
257 constant, and not elevated carbon and phosphorus utilisation. Nitrogen acquiring enzymes,  
258 LAP and NAG, however, were negatively associated with pH, implying altered heterotrophic  
259 nitrogen recycling in a future more acidic ocean.

260       LAP and NAG activity responses contrasted to previous studies, which have found  
261 LAP activity to increase (Piontek et al., 2010; Endres et al., 2014) or remain unchanged  
262 (Yamada et al., 2008; Burrell et al., 2016). The decreased LAP and NAG activities suggest  
263 that nitrogen acquisition by bacterioplankton may be negatively impacted by pH, possibly  
264 due to increased physiological stress placed on microbial cells in a more acidic ocean. Bunse  
265 *et al.* (2016) found that bacterioplankton respond to a pH decrease of 0.2 units, applied over a

266 period of only nine days, by increasing the expression of pH homeostasis genes. The  
267 exposure to lower extracellular pH likely increased physiological stress, resulting in  
268 intensified energy expenditure on cell maintenance, at the expense of bacterial growth (Bunse  
269 et al., 2016). Therefore, it is likely that the microbial communities producing LAP and NAG  
270 were negatively impacted by acidification. Reduced pH may have caused a loss of  
271 community members responsible for producing the nitrogen acquiring enzymes (Allison and  
272 Martiny, 2008a), ultimately decreasing cellular growth and fitness. Ultimately, microbial  
273 communities are essential producers of enzymes responsible for recycling organic matter,  
274 especially within nutrient deficient regions such as the SO. Although further research is  
275 necessary, alterations in bacterioplankton communities and their functions within the  
276 oligotrophic SO suggest negative future effects on ocean nutrient cycling.

#### 277 **Bacterioplankton community network shifts as a response to acidification**

278 Network topology, size and structure shifted markedly in response to reduced pH,  
279 with prominent differences between the control, medium and low bacterioplankton networks,  
280 congruent with our third hypothesis. Altered network structure and the loss of potential  
281 keystone taxa at lower pH levels suggest that marine microbial communities will be impacted  
282 by future acidification. Modules within networks may represent specific ecological niches  
283 (Olesen et al., 2007; Dupont and Olesen, 2012), and high interconnectedness between  
284 modules may indicate communication or co-operation between niches. The control group,  
285 although containing a large number of modules, did not contain a high number of connections  
286 between modules, and several between module connections were negative associations (Fig.  
287 4). The large number of modules observed in the control group suggests that SO  
288 bacterioplankton communities form distinct niches and that both intermodular and  
289 intramodular predicted interactions may represent a balance between competition and  
290 mutualism. For example, microorganisms compete for nutrients and exhibit different growth

291 constraints (Steele et al., 2011; Eiler et al., 2012; Brum et al., 2016) and also communicate  
292 via mechanisms such as quorum sensing (Hmelo, 2017). This study supports the notion that  
293 SO bacterioplankton communities are highly complex with a multitude of putative biotic  
294 interactions.

295         The higher network connectivity and complexity in the medium pH group suggests  
296 that bacterioplankton responded to a pH decrease of 0.4 units, associated with an increase in  
297 network stability (Fig. 4). Indeed, pH has been shown to alter community network structure  
298 (Barberan et al., 2012), resulting in larger and more complex phylogenetic and functional  
299 networks in response to elevated soil CO<sub>2</sub> levels (Zhou et al., 2010; Zhou et al., 2011).  
300 Furthermore, network stability is thought to be promoted by increased modularity, as  
301 disturbances may spread more slowly through a modular than a non-modular network  
302 (Olesen et al., 2007). Module 4 contained the highest number of intra-module connections,  
303 where the majority of co-occurrences were positive (Fig. 4). The high intra-module  
304 connectivity is likely indicative of cooperation or mutualism between phylogenetically distant  
305 species within a specific niche. In contrast, several intermodular connections were negative,  
306 suggesting that increased physiological stress may result in more competitive interactions  
307 between niches at lower pH levels within the SO. This competition may be due to increased  
308 physiological stress on weaker community members, leading to the development of  
309 specialists with more efficient mechanisms for using the available nutrients. However, more  
310 research is needed to determine the cause of competition observed. Ultimately, future pH  
311 decreases of 0.4 units may promote increased bacterioplankton community interactions and  
312 network stability.

313         In contrast, the low network was the least complex, with reduced intermodular  
314 connectivity. This may suggest that microbial niches or guilds decreased the amount of  
315 interactions compared to the control and medium networks. This decreased cooperation may

316 be due to the increased physiological stress placed on bacterioplankton communities at pH  
317 levels expected in the next 300 years. Acidification can promote direct physiological changes  
318 of microbial communities, whereby the increased energetic cost associated with cellular  
319 maintenance may lead to reduced microbial growth and fitness (Gilbert et al., 2008; Bunse et  
320 al., 2016). Decreased cellular fitness may result in altered functionality (Bunse et al., 2016)  
321 eventually perturbing community interactions. The increased stress induced by a pH decrease  
322 of 0.8 units likely resulted in the substantial alterations of the network structure, with  
323 potentially deleterious effects on bacterioplankton community structure in a future acidified  
324 ocean scenario.

### 325 **Keystone bacterioplankton in the SO and responses to acidification**

326 Several potential keystone taxa were identified in all three networks, defined by their  
327 integral topological role in maintaining network structure and stability (Deng et al., 2012).  
328 The number of connectors decreased in the medium network, suggesting a switch between  
329 single microorganisms responsible for connecting niches to several community members  
330 linking different modules. The number of module hubs and connectors decreased  
331 dramatically in the low network. Loss of keystone members in a network decreases network  
332 stability and can lead to a collapse of network structure due to their high level of connectivity  
333 (Dunne et al., 2002; Olesen et al., 2007). The observed decrease in keystone nodes in the low  
334 network therefore correlates to the less stable network structure. Moreover, the reduced  
335 frequency of module hubs and connectors yet again suggests a lower frequency of  
336 community interactions or relationships in response to extreme levels of acidification.

337 The constructed networks are based on co-abundances and do not necessarily  
338 represent true biological interactions (Shi et al., 2016; Röttgers and Faust, 2019). However,  
339 co-occurrence networks have been instrumental for investigating the structure of microbial

340 assemblages across biomes (de Menezes et al., 2015; Murdock and Juniper, 2019), in the  
341 detection of true viral host interactions (Chow et al., 2014; Soffer et al., 2015), the association  
342 of phylotype shifts to specific environmental parameters and functions (Valverde et al., 2015;  
343 Van Goethem et al., 2017), as well as in the detection of metabolic dependencies resulting in  
344 the culture of previously uncultivable microorganisms (Duran-Pinedo et al., 2011).  
345 Furthermore, networks provide valuable insight into complex community structures and  
346 stability, particularly in the face of perturbations, and give information on community  
347 organisation not portrayed by diversity metrics alone (Zhou et al., 2010). Thus, our results  
348 provide a framework for complex community predicted interactions in response to  
349 acidification (Fig. 6) where individual species interactions may be experimentally tested in  
350 future studies.

351 In summary, we have shown that ocean acidification significantly alters bacterioplankton,  
352 but not mycoplankton, communities. Exoenzyme activities involved in nitrogen acquisition  
353 were substantially reduced at lower pH levels, suggesting a diminished availability of  
354 nutrients in surface waters and amended nutrient recycling. Additionally, bacterioplankton  
355 network structure and keystone taxa were significantly impacted by increased CO<sub>2</sub>. We  
356 propose that future acidification may have adverse consequences on microbial communities  
357 and biogeochemical cycling, particularly within regions most sensitive to future climatic  
358 changes.

## 359 **MATERIALS AND METHODS**

### 360 **Sampling and mesocosm experiment**

361 Seawater was collected during the Marion Island Relief Cruise aboard the RV SA  
362 Agulhas II from April 2016 to May 2016. Approximately 500 L of seawater was collected  
363 from surface waters (5 m) using the underway system at a single site (46°00.065' S,



364 38°00.37' E). The water was distributed into nine mesocosms (50 L volume) with the sterile  
365 drums filled to near capacity to keep the headspace small (volume 5 - 10ml) in order to  
366 minimize the exchange of CO<sub>2</sub>. The mesocosms consisted of three biological replicates for  
367 each pH treatment group, where treatments are defined as follows: a control (i.e. pH 8.0)  
368 which is the natural pH of the environment, a medium pH, which mimics predicted pH  
369 decreases within the next 100 years (0.4 units, i.e. pH 7.6), as well as a low pH treatment  
370 which exceeds the predicted decrease in pH within the next millennium (i.e. pH 7.2). All  
371 values were allowed to deviate by  $\pm 0.05$  pH units in order to account for natural variability  
372 within the SO environment (Kapsenberg et al., 2015). The mesocosms were exposed to light  
373 on a 12 hr day/night cycle, where light was allowed to penetrate the top of the mesocosms in  
374 order to mimic the natural light conditions at 5 m. Mixing of mesocosms was performed via  
375 consistent O<sub>2</sub> bubbling (HAILEA Air Compressor, Guangdong, China), as well as manual  
376 stirring throughout the experiment. Each treated mesocosm contained a pH probe (MODEL  
377 PH-301C, SAGA, Tainan City, Taiwan, pH accuracy  $\pm 0.1\%$  + 2 digits) which consistently  
378 recorded the seawater pH. Physico-chemical variables within each mesocosm such as  
379 temperature, salinity, conductivity and pH, were also manually recorded twice a day  
380 throughout the duration of the experiment using a HANNA probe (MODEL HI 98195,  
381 Padova, Italy, pH accuracy  $\pm 0.02$  units, temperature accuracy  $\pm 0.15^\circ\text{C}$ , conductivity and  
382 salinity accuracy  $\pm 1\%$  of reading). Changes in pH were induced via CO<sub>2</sub> bubbling and the  
383 experiment was run for a total of 21 days, during which pH changes were induced on the first  
384 day (day 0) of the experiment. Both O<sub>2</sub> and CO<sub>2</sub> bubbling were introduced into mesocosms  
385 via sterile plastic tubing, where the amount of gas was controlled using a regulator. Samples  
386 were collected every 7 days at noon, including at the start (day 0) of the experiment. Physico-  
387 chemical variables such as temperature, salinity, and nutrients were recorded throughout the  
388 experiment (see Fig S1).

## 389 **Enzymatic activities**

390 Seawater collected from each sampling point in the mesocosm experiment was  
391 assayed for the activity of 5 enzymes, including  $\beta$ -glucosidase (BG),  $\beta$ -xylosidase (BX),  $\beta$ -N-  
392 acetyl-glucosaminidase (NAG), alkaline phosphatase (AP), and leucine aminopeptidase  
393 (LAP). The potential activities of these enzymes were tested using fluorescent assays, and  
394 enzyme activities were performed and calculated as described previously (Sinsabaugh, 1994;  
395 Papanikolaou et al., 2010; Hoarfrost and Arnosti, 2017). Briefly, 200  $\mu$ L of seawater was  
396 added to 96-well black flat-bottom microplates (Greiner Bio One, Frickenhausen, Germany),  
397 where four replicate wells were used per sample. A concentration of between 1 mM to 1.5  
398 mM of each enzyme substrate was then added to the 96 well plates. Plates were incubated for  
399 2 hours at 4°C in the dark. Fluorescence was measured from the black 96 well plates using a  
400 Spectramax® Paradigm Multi-Mode Microplate Reader (Molecular Devices, USA) with an  
401 emission wavelength of 450 nm and an excitation wavelength of 360 nm.

## 402 **Molecular analysis**

403 Seawater (2 L) collected from each sampling point in the mesocosm experiment was  
404 filtered through 0.2  $\mu$ M pore cellulose acetate membrane filter (Sartorius Stedim Biotech,  
405 Göttingen, Germany). Nucleic acid was subsequently extracted from each of the filtered  
406 samples using the PowerWater DNA Isolation Kit (MoBio, Carlsbad, USA), according to the  
407 manufacturer's instructions. DNA was sent to MR DNA ([www.mrdnalab.com](http://www.mrdnalab.com), Shallowater,  
408 USA) for paired-end sequencing (2 x 300bp) of the 16S rRNA gene and the internal  
409 transcribed spacer (ITS) 1 rDNA variable region on the Illumina MiSeq platform (Illumina,  
410 San Diego, CA, USA), as detailed previously (Phoma et al., 2018; Vargas-Gastélum et al.,  
411 2019). The marine specific primer pair 515F-Y and 926R (Walters et al., 2016) was used to  
412 PCR amplify hypervariable regions V4 and V5 of the bacterial and archaeal 16S rRNA gene.

413 ITS region 1 was targeted and PCR amplified using the primer pair ITS1F (Gardes and  
414 Bruns, 1993) and ITS2 (White et al., 1990). All samples were barcoded on the forward  
415 primer to allow for multiplex sequencing.

416 Sequences were analysed using QIIME v 1.8.0 (Caporaso et al., 2010). Sequences  
417 were demultiplexed according to barcode identity and were quality filtered according to  
418 QIIME default parameters, with exceptions for the ITS dataset. For the ITS dataset, the p  
419 score applied was altered to 0.45 to ensure that all reads >200bp were included in the dataset,  
420 provided that they met the other default quality parameters (i.e. q 19, r 3, etc.). Chimeras  
421 were detected both *de novo* and against the Greengenes reference library (DeSantis et al.,  
422 2006) for 16S rRNA genes and the UNITE reference database for the ITS region (Kõljalg et  
423 al., 2013) using usearch61 (Edgar et al., 2011). Operational taxonomic units (OTUs) were  
424 clustered at a similarity threshold of 97% using the uclust method and the Greengenes and  
425 UNITE reference database for 16S rRNA gene and ITS region datasets, respectively.

426 16S rRNA gene OTUs were assigned a taxonomy using the Greengenes (DeSantis et  
427 al., 2006) reference database. Taxonomy was assigned to ITS region OTUs using a  
428 combination of the UNITE database in QIIME and the Warcup Fungal ITS training set in the  
429 RDP classifier (Wang et al., 2007; Kõljalg et al., 2013; Deshpande et al., 2016). For the RDP  
430 classifier, taxonomic classifications were kept at the default confidence threshold of equal to  
431 or greater than 80% for classification to the root only, after which the default confidence  
432 threshold was decreased to 50% for all subsequent assignments. Following taxonomy  
433 assignment, singletons were removed from the datasets and both datasets were rarefied to the  
434 lowest number of sequences per sample (53,267 for 16S rRNA gene and 10,370 for ITS  
435 region), using a single rarefaction. All sequences are available on the SRA database under the  
436 following accession number: SRP126760

## 437 **Network analysis**

438 Ecological association networks were constructed using Random Matrix Theory  
439 (RMT) through the Molecular Ecological Network Analysis (MENA) pipeline  
440 (<http://ieg2.ou.edu/MENA/>) (Deng et al., 2012). Individual networks were constructed for the  
441 three treatment groups (i.e. one low, medium, control) where all days were combined,  
442 excluding day zero (i.e. nine biological replicates per network, see Supporting Information  
443 for details). All networks were visualised in Cytoscape v 3.4.0 (Shannon et al., 2003).

## 444 **Statistical analysis**

445 All statistical analyses were performed using R statistical software version 3.2.2. (R Core  
446 Team, 2013). Alpha diversity was calculated using the *vegan* package (Oksanen et al., 2011).  
447 Statistically significant differences in diversity measures between each treatment group were  
448 determined via the *aov* function (analysis of variance, ANOVA) in *vegan* and the post-hoc  
449 TukeyHSD test. Microbial community structure was visualised by non-metric  
450 multidimensional scaling (nMDS) ordination plots with the *metamds* function of *vegan* using  
451 Bray-Curtis dissimilarity and visualised using *ggplot2*. Significant differences in microbial  
452 community structure were determined via PERMANOVA (permutational analysis of  
453 variance) using the *adonis* and *betadisper* functions in *vegan*. The *aov* and *tukeyHSD*  
454 functions of *vegan* were used to determine significant differences between treatment groups  
455 for each of the enzyme activities. Correlations between taxon abundances, enzyme activities  
456 and pH were calculated using Spearman's rho, via the *rcorr* function of the *Hmisc* package.  
457 Relationships between pH and enzyme activities, bacterial, archaeal and fungal diversity and  
458 taxon abundance were modelled using linear regressions (*lm* function).

459

460 **ACKNOWLEDGEMENTS**

461 We are grateful to the National Research Foundation (NRF) (Grant ID 100052 SZdS) the  
462 South African National Antarctic Programme (SANAP 110717), and the University of  
463 Pretoria for funding. TPM also wishes to acknowledge the Fulbright Visiting Scholar  
464 Program for providing sabbatical funding. We wish to acknowledge Mr. A. van der Walt and  
465 Mr. M. Clayton for useful discussions on the content of the manuscript.

466

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693

694 **Figures legends**

695 **Fig. 1:** Relationships between bacterial and fungal diversity and experimentally induced  
696 seawater pH changes. Shannon diversity or H' index (a) and richness or observed species (b)  
697 are indicated in the top and bottom panels, respectively. Solid lines represent the fitted  
698 regression model. The percentage of variance explained by the model or  $R^2$ , as well as the  
699 significance level of the model, is indicated in the bottom left corner of each sub-figure.  
700 Fungal diversity relationships with pH were non-significant ( $p$ -value > 0.5), and therefore no  
701 regression line is shown.

702 **Fig. 2:** Non-metric multi-dimensional scaling (nMDS) ordination plots of bacterioplankton  
703 and mycoplankton communities constructed using Bray-Curtis dissimilarity. The left (a) and  
704 right (b) panels represent the 16S rRNA gene and ITS 1 region datasets, respectively. Points  
705 represent samples and are coloured and shaped according to treatment group and sampling  
706 day, respectively. Stress values are indicated at the bottom of each sub-figure.

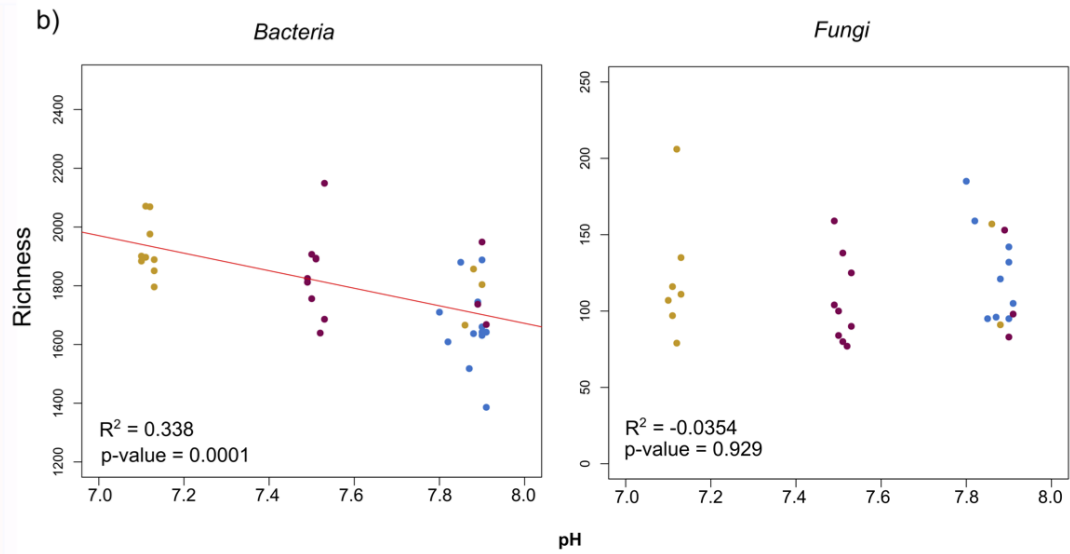
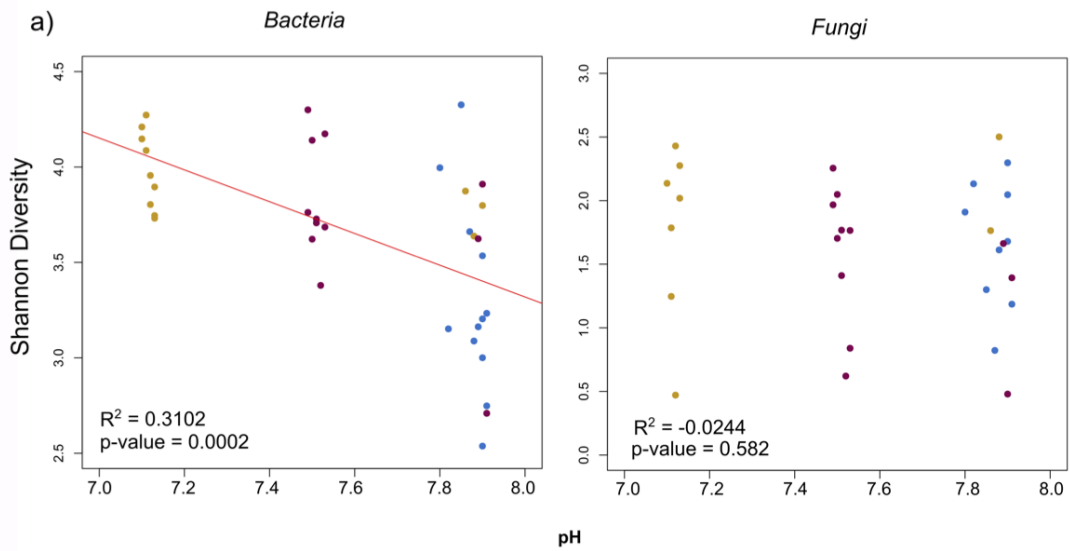
707 **Fig. 3:** Relationship between extracellular enzymatic activities and experimentally induced  
708 acidification. The solid red line represents the fitted regression model. The percentage of  
709 variance explained by the model or  $R^2$ , as well as the significance level of the model, is  
710 indicated in the left corner of each sub-figure. No fitted regression line is shown for all  
711 exoenzyme activities which were not significantly correlated with pH ( $p$ -value > 0.5). Points  
712 are coloured by the three treatment groups where orange, purple and blue indicate low,  
713 medium and control, respectively.

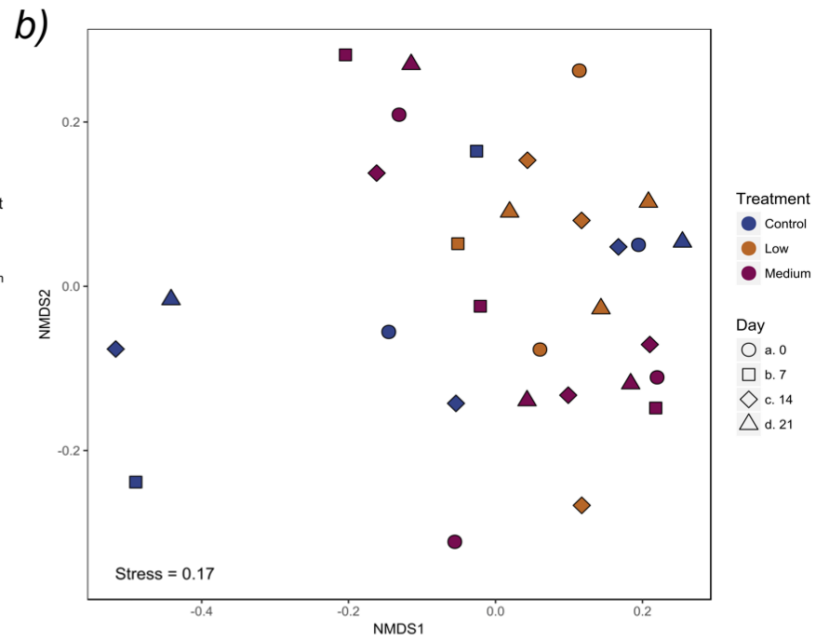
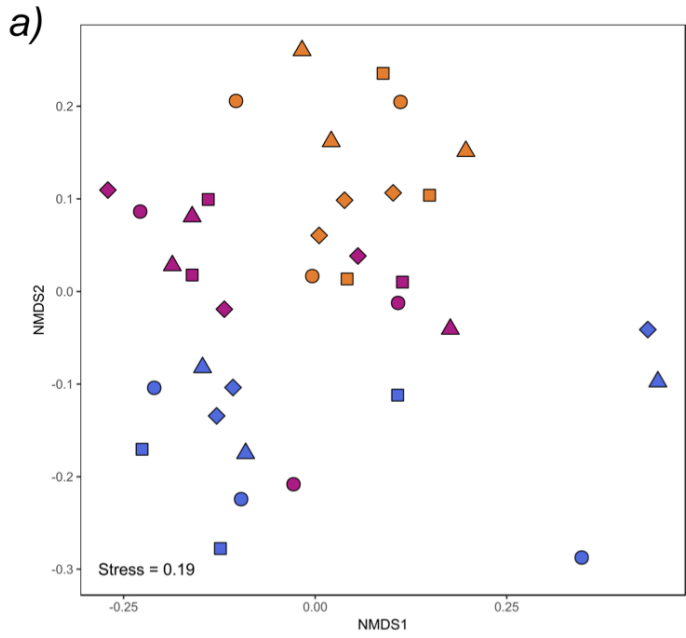
714 **Fig. 4:** Ecological bacterioplankton association networks for the control, medium and low pH  
715 treatment groups. Nodes correspond to OTUs, where node size is plotted according to node  
716 degree, or connectedness. Node colours correspond to phyla and classes. Module taxa  
717 compositions are indicated with numbered donut charts, where numbers within a specific

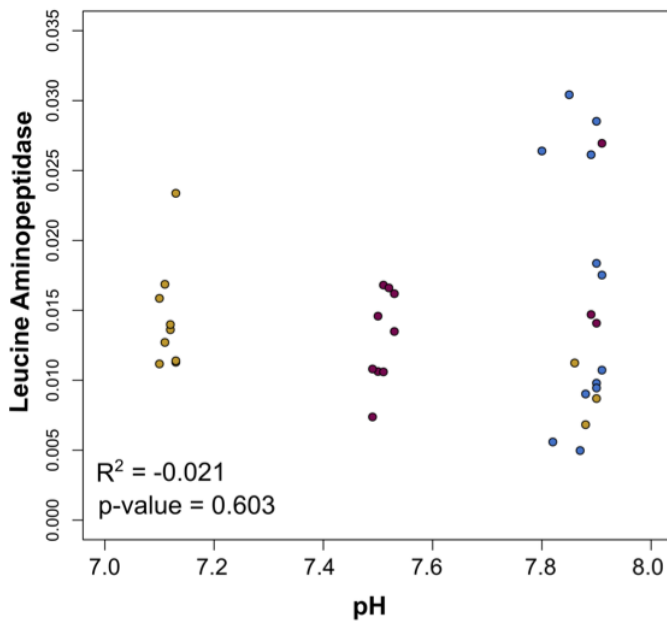
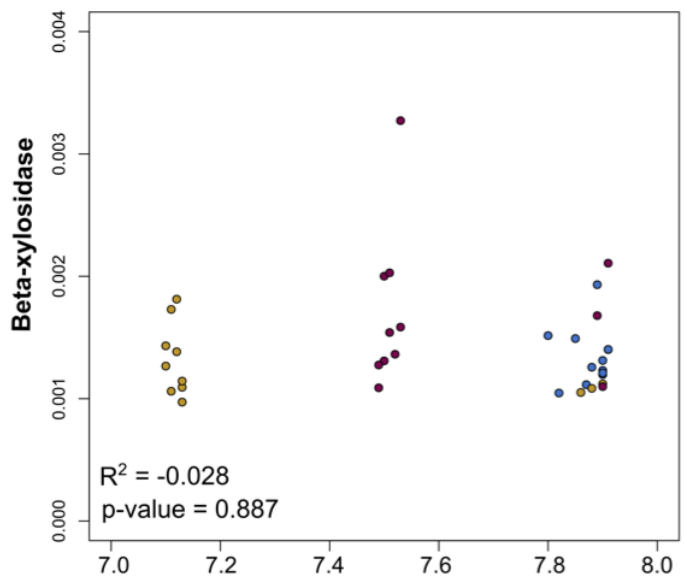
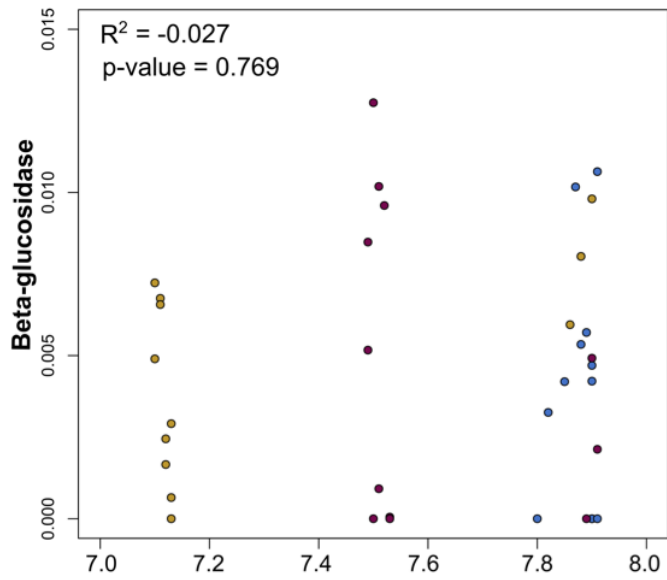
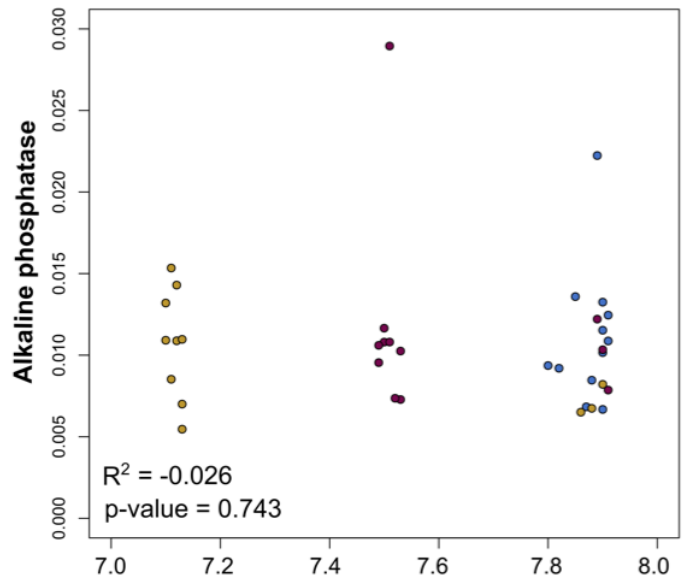
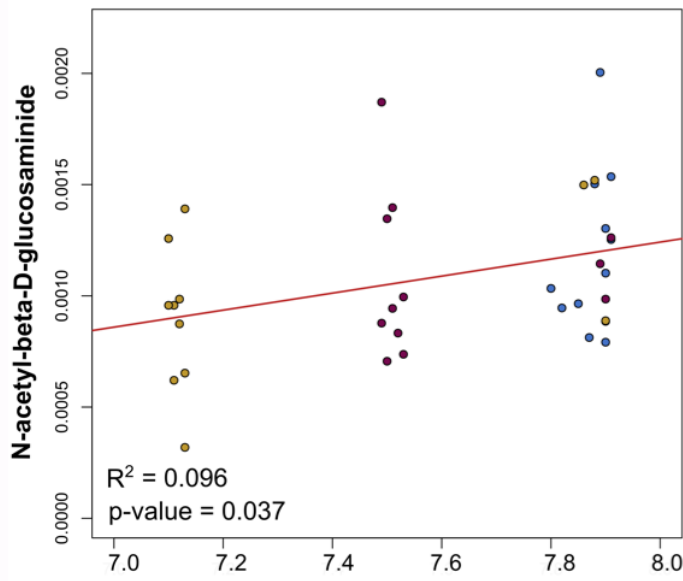
718 chart correspond to a particular module. Associations are defined by Spearman's' rho  
719 correlation coefficient and are deemed significant by random matrix theory (RMT). Positive  
720 co-occurrences are shown with a blue line whereas negative co-exclusions are indicated with  
721 a red line. Black boxes indicate within module keystones (module hubs) and between module  
722 keystones (connectors), respectively.

723 **Fig. 5:** Plot of the within-module connectivity ( $Z_i$ ) and between-module connectivity ( $P_i$ ) of  
724 each node or OTU in the three ecological networks and taxon relative abundances.  
725 Topological roles of OTUs are indicated either as peripherals ( $Z_i \leq 2.5, P_i \leq 0.62$ ), connectors  
726 ( $Z_i \leq 2.5, P_i > 0.62$ ), module hubs ( $Z_i > 2.5, P_i \leq 0.62$ ) and network hubs ( $Z_i > 2.5, P_i >$   
727  $0.62$ ). The abundance of peripherals, connectors and module hubs between the three  
728 treatment groups is indicated in (b), where the control group is the centremost donut ring, the  
729 medium group the middle ring and the low group the outer donut ring.

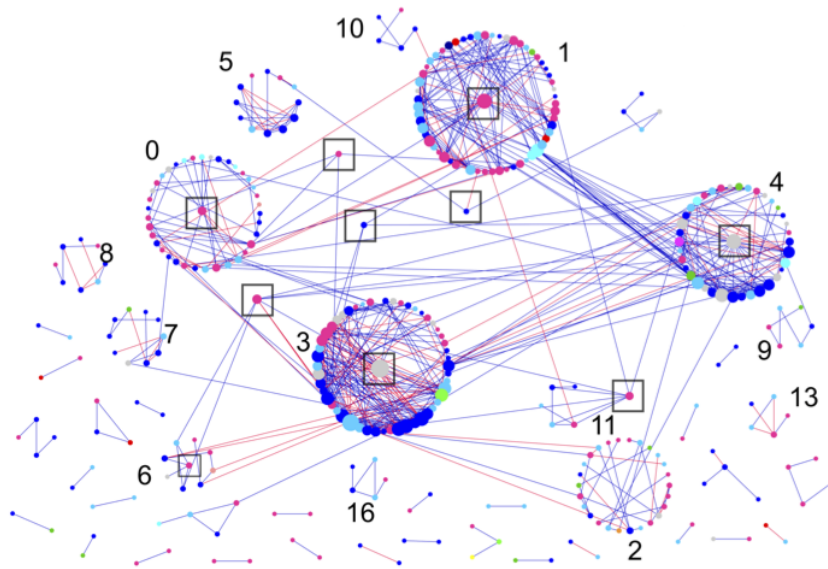
730 **Fig. 6:** Conceptual diagram depicting ocean acidification effects on SO surface microbial  
731 community structure and functionality. The left and right panel indicate current and future pH  
732 levels, respectively. Ovals represent microbial communities, where the lightest colour is  
733 indicative of bacteria, the middle colour archaea and the darkest colour fungi. Headings  
734 indicate different processes; the first panel shows the process of  $\text{CO}_2$  exchange with the  
735 atmosphere and the associated consequences on ocean  $\text{H}^+$  concentration, pH and a bacterial  
736 cell (bacterial cell = yellow oval; proton pump = red star); the second panel indicates current  
737 and future microbial community interactions; the last panel shows functional processes  
738 utilised to degrade high molecular weight DOM, the subsequent products, and the usage of  
739 the resultant products by microbial communities and the higher trophic system. Abbreviations  
740 are as follows: HMW = High molecular weight; LMW = Low molecular weight; C = carbon;  
741 N = nitrogen;  $\text{H}^+$  = hydrogen ions; BG = beta-glucosidase; BX = beta-xylosidase; NAG = N-  
742 acetyl-glucosaminidase; LAP = leucine aminopeptidase.





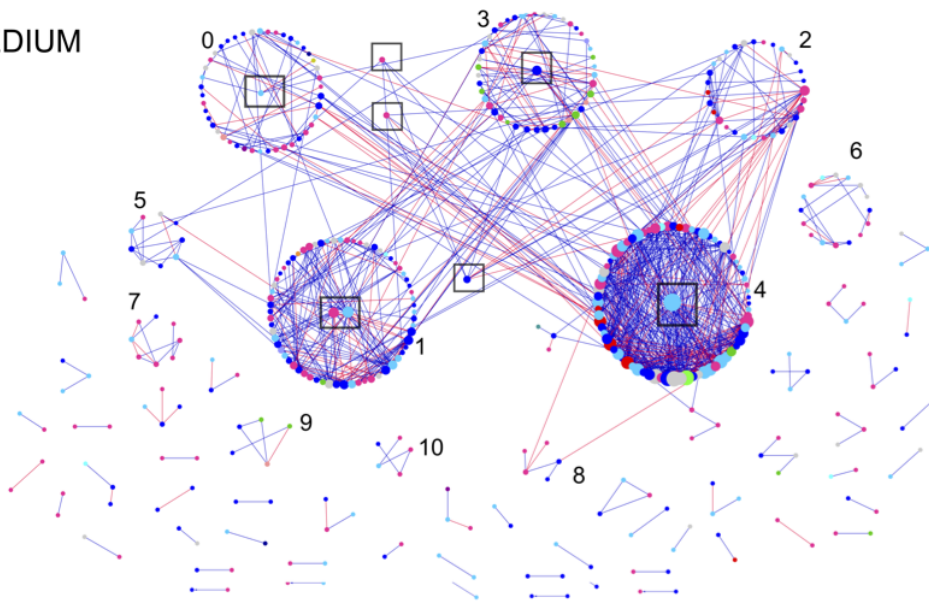


# CONTROL

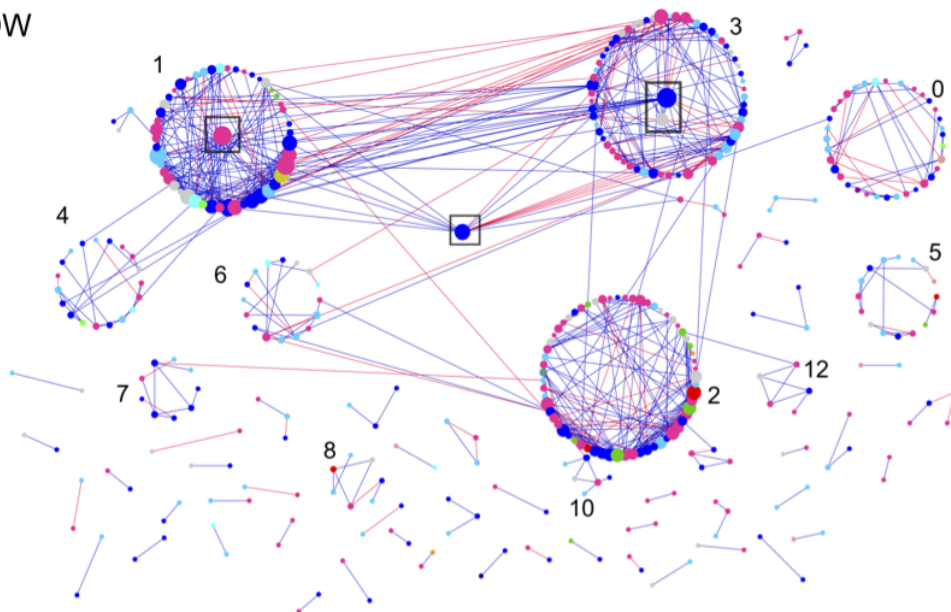


- Actinobacteria
- Bacteroidetes
- Chlorobi
- Cyanobacteria
- GN02
- NKB19
- Planctomycetes
- Proteobacteria
- Alphaproteobacteria
- Betaproteobacteria
- Deltaproteobacteria
- Gammaproteobacteria
- SBR 1093
- Verrucomicrobia
- Unassigned
- Crenarchaeota
- Euryarchaeota

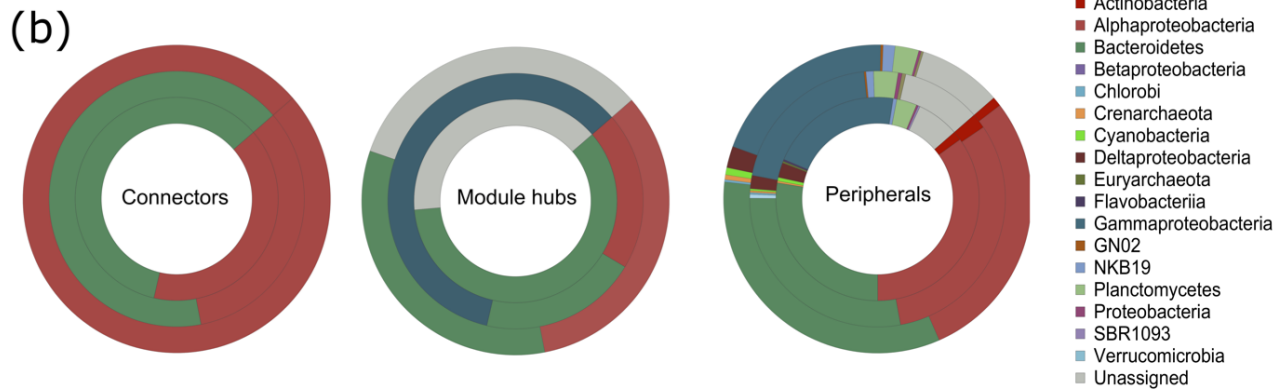
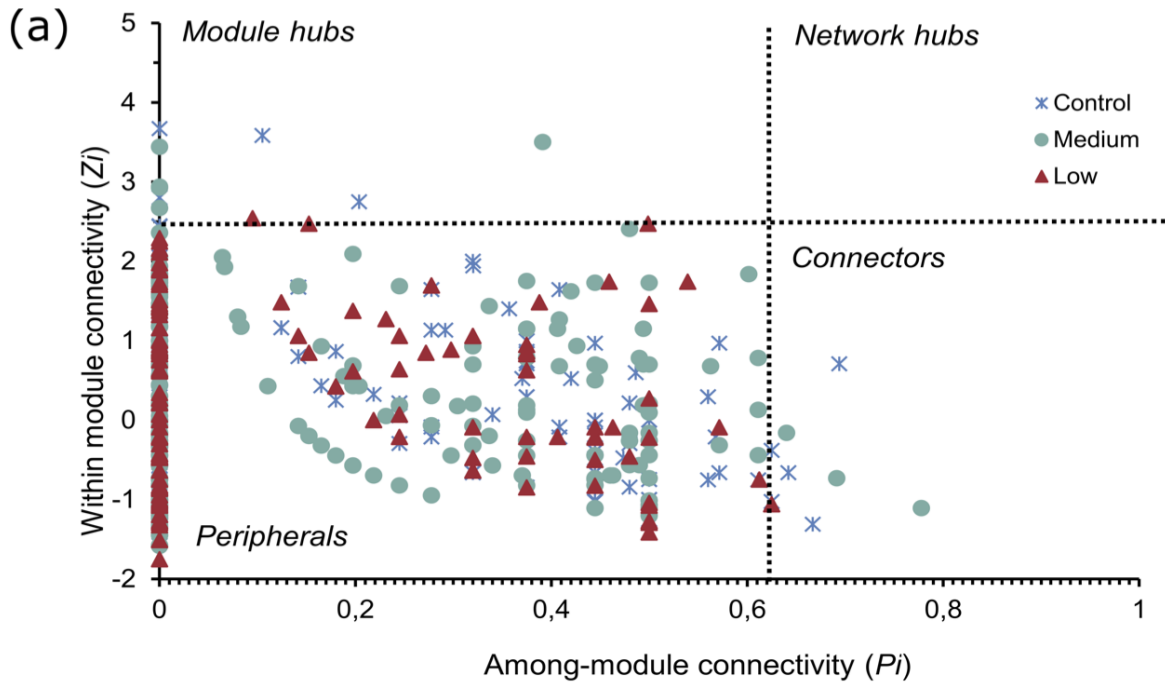
# MEDIUM



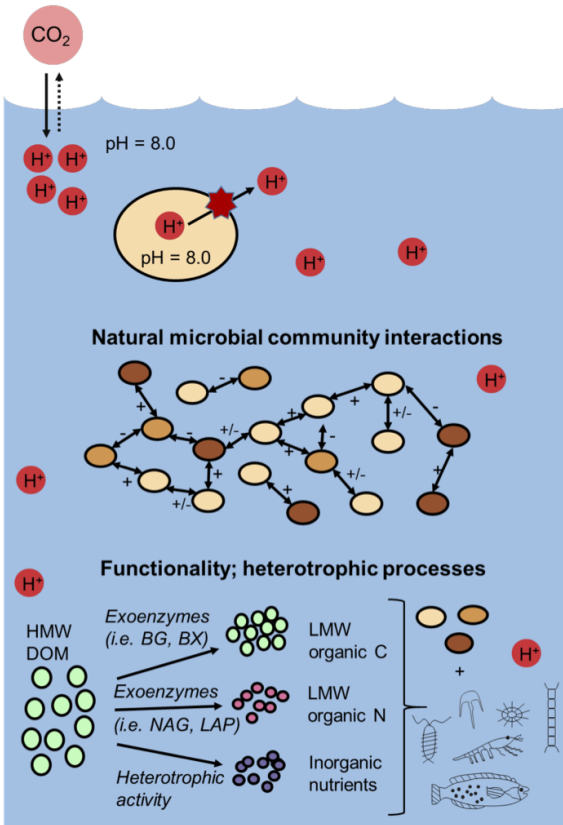
# LOW







**Current Southern Ocean microbial communities**



**Future Southern Ocean microbial communities**

