



## Bacterioplankton community resilience to ocean acidification: evidence from microbial network analysis

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Ocean acidification (OA), caused by seawater CO<sub>2</sub> uptake, has significant impacts on marine calcifying organisms and phototrophs. However, the response of bacterial communities, who play a crucial role in marine biogeochemical cycling, to OA is still not well understood. Previous studies have shown that the diversity and structure of microbial communities change undeterminably with elevated pCO<sub>2</sub>. Here, novel phylogenetic molecular ecological networks (pMENS) were employed to investigate the interactions of native bacterial communities in response to OA in the Arctic Ocean through a mesocosm experiment. The pMENS results were in line with the null hypothesis that elevated pCO<sub>2</sub>/pH does not affect biogeochemistry processes. The number of nodes within the pMENS and the connectivity of the bacterial communities were similar, despite increased pCO<sub>2</sub> concentrations. Our results indicate that elevated pCO<sub>2</sub> did not significantly affect microbial community structure and succession in the Arctic Ocean, suggesting bacterioplankton community resilience to elevated pCO<sub>2</sub>. The competitive interactions among the native bacterioplankton, as well as the modular community structure, may contribute to this resilience. This pMENS-based investigation of the interactions among microbial community members at different pCO<sub>2</sub> concentrations provides a new insight into our understanding of how OA affects the microbial community.

**Keywords:** Arctic Ocean, community structure, mesocosm experiment, molecular ecological network, ocean acidification.

### Introduction

Since the industrial revolution, the impact of human activity on the global climate has increased greatly as a result of increasing carbon dioxide (CO<sub>2</sub>) emissions from anthropogenic sources. The uptake of anthropogenic carbon dioxide by the ocean has caused a decrease of pH 0.1 units (ocean acidification, OA; IPCC, 2015). Previous studies have demonstrated that some phototrophic communities, like sea grass (Zimmerman *et al.*, 1997; Jiang *et al.*, 2010), diatoms (Riebesell *et al.*, 1993; Baragi *et al.*, 2015; Taucher *et al.*, 2015),

and coccolithophorids (Hiwatari *et al.*, 1995; Lv *et al.*, 2015), have higher photosynthesis rates under a higher partial pressure of carbon dioxide (pCO<sub>2</sub>). However, the response of marine bacterioplankton, a crucial player in marine biogeochemical cycling (Azam, 1998; Jiao *et al.*, 2010), to OA is not well understood at present. Joint *et al.* (2011) proposed that “marine microbes possess the flexibility to accommodate pH change”. Indeed, several mesocosm studies have found that elevated pCO<sub>2</sub> has little effect on bacterial communities in the Arctic Ocean (Allgaier *et al.*, 2008; Tanaka *et al.*, 2008;

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Oliver *et al.*, 2014). The abundance and activity of bacteria in these communities did not differ statistically under three different  $p\text{CO}_2$  treatments (350, 700, and 1085  $\mu\text{atm}$ ; Allgaier *et al.*, 2008). Furthermore, phylogenetic diversity analysis revealed no clear effect of elevated  $p\text{CO}_2$  on a bacterioplankton assemblage in the high Arctic Ocean (Monier *et al.*, 2014). In contrast, a few studies have demonstrated that elevated  $p\text{CO}_2$  has some influence on microbial community composition (Krause *et al.*, 2012; Bowen *et al.*, 2013). Other studies have also shown that the production and growth rates of bacterial isolates were strongly affected by high  $p\text{CO}_2$  and low pH (Takeuchi *et al.*, 1997; Labare *et al.*, 2010; Teira *et al.*, 2012). For example, *Vibrio alginolyticus* growth was suppressed under low  $p\text{CO}_2$  levels (Michael *et al.*, 2010). In contrast, high  $p\text{CO}_2$  stimulated the growth efficiency of one *Flavobacteria* strain (Teira *et al.*, 2012). Moreover, the rate of microbial ammonia oxidation is inhibited by reduced pH in both surface and deep seawater (Huesemann *et al.*, 2002). Conflicting results from the population/ecosystem and species levels indicate that community may play a crucial role in determining the response of microbes to OA. In addition to community structure and the number of species, micro-organism interaction is an important component of diversity (Olesen *et al.*, 2007). For example, ecological networks among different species bridge ecosystem complexity and stability (Montoya *et al.*, 2006). The interaction between plants and pollination enhances the relative resistance of plants to environmental disturbance (Sole and Montoya, 2001; Memmott *et al.*, 2004). Compared with network investigations among macro-organisms (Elton, 1927; Paine, 1980; Bascompte *et al.*, 2003), microbial interactions and ecological networks are understudied and have only been investigated recently with advances in molecular technology (Sherr and Sherr, 2008; Chaffron *et al.*, 2010; Steele *et al.*, 2011). Several microbial network investigations have been conducted on temporal variation in microbial communities and the interactions among bacteria, phage, and protists at the San Pedro Ocean Time-series (SPOT) station off southern California (Chow *et al.*, 2013, 2014). Bacterial interaction has also been proposed as a determinant of phytoplankton bloom dynamics (Tan *et al.*, 2015). Currently, only a few investigations have been performed to illustrate the interaction of microbial communities in response to climate change in soil environments (Zhou *et al.*, 2010; Tu *et al.*, 2015), but no studies have addressed interactions among micro-organisms under elevated  $p\text{CO}_2$  in the marine environment.

A large-scale mesocosm experiment, European Project on Ocean Acidification (EPOCA), was set up in the pelagic coastal area of the Arctic Ocean to assess the impact of OA on the marine ecosystem. Previous EPOCA studies have demonstrated that the phytoplankton community was more susceptible to elevated  $p\text{CO}_2$  than the bacterioplankton community, where pico-phytoplankton and diatoms were stimulated by nutrient addition and elevated  $p\text{CO}_2$ , resulting in increased primary production (Riebesell *et al.*, 2013b). Bacterial activity was subsequently spurred by stimulated primary production and elevated  $p\text{CO}_2$ , leading to a decrease in net production (Engel *et al.*, 2013; Motegi *et al.*, 2013). However, bacterial production and respiration were generally similar among the  $p\text{CO}_2$  mesocosms during the incubation period (Motegi *et al.*, 2013). Nonetheless, bacterioplankton activity was closely related to phytoplankton under both ambient and elevated  $p\text{CO}_2$ , and limited by both nutrient availability and elevated  $p\text{CO}_2$  (Riebesell *et al.*, 2013b). The general diversity and composition of microbial communities has been evaluated by Automated Ribosomal Spacer Analysis (Sperling *et al.*, 2013), T-RFLP, clone libraries (Zhang *et al.*, 2013), and next-generation

sequencing (Roy *et al.*, 2013), all of which indicated that increased  $p\text{CO}_2$  did not significantly impact the communities. However, because of limited sample numbers and sequencing depth, the above dataset cannot be used to explore the interactions among bacterial groups under elevated  $p\text{CO}_2$ . Thus, we examined the structure and diversity of microbial communities at this site using Illumina sequencing and performed phylogenetic molecular ecological network (pMEN) analysis using the Molecular Ecological Network Analysis Pipeline (MENAP; Deng *et al.*, 2012) for samples collected from nine mesocosms, each having a different  $p\text{CO}_2$  level (175–1085  $\mu\text{atm}$ ), which simulated different acidification scenarios. We tested the null hypothesis that elevated  $p\text{CO}_2$  would have an insignificant or indirect impact on bacterial assemblages as proposed by Joint *et al.* (2011), with a focus on the interactions among bacterioplankton under different  $p\text{CO}_2$  concentrations. Our results indicated an insignificant effect of elevated  $p\text{CO}_2$  on microbial assemblage interactions and connectivity and provide a deep insight into our understanding of bacterioplankton assemblage resistance to climate change.

## Material and methods

### Experimental set-up and sampling

A large-scale mesocosm experiment, supported by EPOCA, was conducted in the Arctic Ocean at Kongsfjorden, Svalbard, Norway (78°56.2'N, 11°54.6'E), from June to July 2010. Experimental set-up details have been described previously (Riebesell *et al.*, 2013a). Briefly, nine mesocosms (45 m<sup>3</sup> volume), each simulating a different  $p\text{CO}_2$  concentration (~175, ~180, ~250, ~340, ~450, ~600, ~675, ~860, and ~1085  $\mu\text{atm}$ ) by bubbled CO<sub>2</sub>, were deployed into the Arctic Ocean. On day 13, nutrients were added to induce phytoplankton bloom development (Schulz *et al.*, 2013).

Sample collection was carried out according to Zhang *et al.* (2013). Briefly, seawater (2 l) collected from nine mesocosms was filtered onto a 0.22  $\mu\text{m}$  pore filter (GTTP, Millipore) to collect microbial cells. The filters were stored at  $-80^\circ\text{C}$  until DNA extraction. In total, we obtained 165 environmental DNA samples from the nine mesocosms over 19 sampling points (not all treatments contain all time points; Supplementary Table S1). Geochemistry and biological parameters were downloaded from the EPOCA website (<http://www.epoca-project.eu>; Nisumaa *et al.*, 2010). The geochemistry parameters included temperature, salinity, pH, oxygen, particle organic carbon (POC), particle organic nitrogen, particle organic phosphorus, NO<sub>3</sub>, Si, PO<sub>4</sub>, and NH<sub>4</sub>; the biological parameters included bacterial production, chlorophyll *a* concentration, bacterial abundance, bacterial respiration, bacterial biomass production, viral abundance, and pico-phytoplankton abundance.

### DNA extraction, Illumina high-throughput sequencing and analysis

DNA was extracted by a freeze-grinding method as described previously (Zhang *et al.*, 2013). The forward (515F, 5'-GTGCCAGCMGCCGCGG-3') and reverse (806R, 5'-GGACTACHVGGGTWTC TAAT-3') primers were used to target the bacterial 16S rDNA gene V4 hyper-variable region (Caporaso *et al.*, 2012). PCR amplification was performed as described by Caporaso *et al.* (2010). The amplicons were then paired-end sequenced using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) and the reads were then analysed through an in-house sequence analysis pipeline (IEG sequence analysis pipeline, <http://zhoulab5.rccc.ou.edu>). Briefly, the sequence length was trimmed (minimum length, 100 bp), Btrim was used to remove sequencing adaptors and low quality regions, and sequences with quality scores <20 were then removed

(Kong, 2011). Thereafter, UCHIME was employed to remove the chimeric sequences (Edgar *et al.*, 2011). The number of sequences from each sample was resampled randomly to 35 000. The sequences were then clustered using the CD-HIT algorithm (Li and Godzik, 2006). The taxonomic assignment was determined using the RDP (Ribosome Database Project) classifier against the RDP 16S rRNA database based on BLAST (Wang *et al.*, 2007).

### pMEN construction and network analysis

Sequence alignment was carried out by the PyNAST alignment method against pre-aligned reference 16S rRNA sequences (Caporaso *et al.*, 2009), followed by approximately maximum-likelihood tree construction using the FastTree method with default settings (Price *et al.*, 2009; Morgan *et al.*, 2010). Comparison of microbial community diversity was performed using the Unifrac method in a phylogenetic context (Lozupone and Knight, 2005, 2007; Lozupone *et al.*, 2006) followed by principal coordinates analysis (PCoA). pMENs analysis was performed based on the relative abundance of all samples through the molecular ecological networks analysis pipeline (<http://ieg2.ou.edu/MENA>; Zhou *et al.*, 2010, 2011; Deng *et al.*, 2012). The whole process and details are given in a previous MENA study (Deng *et al.*, 2012); we briefly introduced the major process in the present study. OTUs that appeared in 13 or fewer samples were removed. The relative abundance of OTUs was log-transformed and missing values were filled with a very small number (0.01) if paired valid values were available. This step ensured a more statistically reliable correlation coefficient between two OTUs. Following data preparation, the relative abundance of each OTU in each sample was used to generate the similarity matrix as the foundation for subsequent steps. Similarity matrices (adjacency matrix) were created for each network based on the pairwise Pearson correlation coefficient across the time-series (one time points lag). To obtain a confident network construction, the threshold of pairwise Pearson correlation coefficient values between OTUs was identified by an RMT-based approach that observed a transition point of nearest-neighbour spacing distribution of eigenvalues from Gaussian to Poisson distribution (Zhou *et al.*, 2010). To compare networks, an identification cut-off of 0.84 (0.85 for 175 and 860  $\mu\text{atm}$  treatments) was used to construct the microbial community networks. Additionally, ecological networks predicted by  $R^2$  ( $R^2 > 0.8$ ) generated based on the random matrix theory (RMT) should be scale-free (Zhou *et al.*, 2010). An identification cut-off of 0.84 for 175 and 860  $\mu\text{atm}$  treatments did not obtain an  $R^2 > 0.8$ , where 0.85 could. To evaluate whether or not the constructed networks were random, we employed a permutation-based null model analysis with 100 permutations and kept the number of nodes and links constant. A Student's *t*-test was performed to evaluate significance differences between random and experimental networks. Once the pMEN was determined, the topological indices were calculated based on the adjacency matrix. Module detection of each network was based on fast greedy modularity optimization (Newman, 2006a). Identification of key module members was based on within-module connectivity ( $Z_i$ ) and among-module connectivity ( $P_i$ ) of each node (Olesen *et al.*, 2007). Eigen-gene, which is a linear combination of genes and eigenvalues (Deng *et al.*, 2012), was detected by singular value decomposition analysis (Alter *et al.*, 2000). Thereafter, trait-based gene significance (GS), which is the correlation of relative abundance of each OTU to a sample trait (e.g.  $p\text{CO}_2$ , temperature), was calculated. Networks were visualized with the NetworkAnalyzer plugin of the Cytoscape 3.1.1 (Assenov *et al.*, 2008). Mantel tests were performed to identify relationships between pMENs and biological and chemical variables (Zhou *et al.*, 2010).

### Nucleotide sequence accession number

Sequences obtained in this study were uploaded to the MG-RAST database under the ID number 4626065.3.

### Results

A previous study sequenced samples from this same experimental site, collected across nine time-points, using the Illumina HiSeq2000 platform (Roy *et al.*, 2013); however, network analysis studies and our preliminary tests suggested that this sampling frequency does not result in accurate and reliable network analysis. Therefore, we expanded this sample set to include 19 time-points for each of nine  $p\text{CO}_2$  treatments, nine more time-points than Roy *et al.* (2013; Supplementary Table S1). These DNA samples were also used to investigate the bacterioplankton community by T-RFLP in a previous study (Zhang *et al.*, 2013). Results from different sequencing platforms or different runs on the same platform can affect the estimation of microbial diversity (Zhou *et al.*, 2013), so all samples were sequenced together on the Illumina MiSeq platform. The microbial community structure revealed in our study (discussed below) did not show obvious differences from those of Roy *et al.* (2013). In total,  $\sim 8\,900\,000$  paired-end reads were generated after quality control in this study, with an average length of  $\sim 250$  bp (Supplementary Table S1). To increase the reliability of comparison among samples, the sequences were resampled with a threshold of 35 000.

### Bacterial community composition and diversity

Similar bacterial community composition and structure were observed in the nine  $p\text{CO}_2$  treatments during the entire incubation period (Table 1, Figure 1, and Supplementary Figure S2), which is consistent with previous results (Roy *et al.*, 2013; Sperling *et al.*, 2013; Zhang *et al.*, 2013). The *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Cyanobacteria*/chloroplast were predominant in all nine mesocosms. The relative abundance of these microorganisms varied over the incubation period. For example, the *Proteobacteria* dominated at the beginning of the incubation period and then decreased in relative abundance; while the *Bacteroidetes* exhibited a relatively high abundance at the end of the incubation, which could be a response to the phytoplankton bloom (Figure 1). Meanwhile, the rarefaction curves for all samples revealed variations in richness and diversity among samples (Supplementary Figure S1). The bacterioplankton community diversity, including Chao index and PD, as well as richness, decreased during the incubation period in all  $p\text{CO}_2$  treatments (Supplementary Figures S2 and S3). The weighted PCoA results revealed that the bacterial community structures were altered by nutrient addition, although there was no clear pattern among different  $p\text{CO}_2$  treatments (Supplementary Figure S4).

### Microbial community interactions

The increased number of sequences obtained by high-throughput sequencing technology afforded us the unprecedented opportunity to investigate the interaction and connectivity within these microbial communities using network analysis. pMEN analysis is a novel RMT-based framework for studying microbial interaction, which has been used in long-term  $\text{CO}_2$  experiments (Zhou *et al.*, 2010, 2011; Tu *et al.*, 2015). In the pMEN analysis, a node in pMEN indicates an OTU, while a link indicates a correlation between two connected nodes. The connectivity, also called node degree, indicates the connection strength of a node; therefore,

**Table 1.** Percentage of major phylum and class bacterioplankton community OTUs in nine  $p\text{CO}_2$  mesocosms.

Mesocosm	M3		M7		M2		M4		M8		M1		M6		M5		M9			
	175	Percent <sup>b</sup> s.d.	180	Percent <sup>b</sup> s.d.	250	Percent <sup>b</sup> s.d.	340	Percent <sup>b</sup> s.d.	425	Percent <sup>b</sup> s.d.	600	Percent <sup>b</sup> s.d.	675	Percent <sup>b</sup> s.d.	860	Percent <sup>b</sup> s.d.	1085	Percent <sup>b</sup> s.d.	Elevated	
Proteobacteria	52.46 <sup>b</sup>	8.02	56.45	9.13	53.05	7.84	49.88	6.87	49.09	5.05	52.99	9.35	52.20	8.18	52.26	8.54	52.19	7.81	54.46	51.67
Alphaproteobacteria (class)	71.36 <sup>c</sup>	14.10	71.62	12.46	68.83	13.70	68.06	9.54	71.33	11.32	68.84	8.89	71.88	7.97	71.54	9.56	75.32	9.18	52.36	42.83
Betaproteobacteria (class)	6.09	3.88	4.99	2.41	6.18	4.24	5.74	2.48	6.31	3.87	7.88	3.36	5.04	2.53	7.69	3.70	5.66	3.26	4.99	4.98
Gammaproteobacteria (class)	21.79	10.12	22.55	10.80	24.20	10.33	25.32	8.40	21.71	8.29	22.47	6.68	22.52	6.84	19.86	7.22	18.55	6.47	18.16	10.32
Bacteroidetes	32.99	12.30	25.14	12.85	30.28	13.07	32.53	10.99	31.61	14.39	30.69	13.35	29.06	11.08	30.00	12.25	29.44	10.67	29.06	30.52
Flavobacteria (class)	90.30	5.49	90.31	6.44	84.44	10.24	90.85	4.96	89.59	4.91	90.85	3.36	87.26	7.75	86.24	9.88	86.17	11.86	90.30	87.92
Sphingobacteria (class)	1.18	0.66	2.40	3.23	2.08	2.21	1.68	1.59	2.07	2.96	1.85	1.78	2.25	2.34	2.87	5.93	1.47	0.80	1.79	2.38
Actinobacteria	5.12	3.09	7.03	4.27	6.86	6.13	6.40	3.98	5.91	3.47	6.28	3.18	7.17	4.73	7.03	4.42	6.62	5.25	6.08	6.61
Firmicutes	0.33	0.33	0.44	0.40	0.38	0.38	0.24	0.19	0.47	0.35	0.30	0.31	0.40	0.51	0.67	0.84	0.41	0.30	0.38	0.41
Cyanobacteria/chloroplast	7.68	11.27	5.79	9.15	4.57	6.17	7.10	10.09	7.39	11.19	6.01	8.34	7.92	11.02	5.45	5.60	8.26	10.90	6.73	10.21
Others	2.43	1.55	6.20	7.36	5.81	6.40	5.19	6.35	10.01	6.83	4.73	6.02	4.64	6.40	6.51	7.54	4.36	4.64	4.31	5.89

<sup>a</sup>Ambient indicates ambient  $p\text{CO}_2$  cosmosms contained M3 and M7, while elevated indicates the other 7 mesocosms.

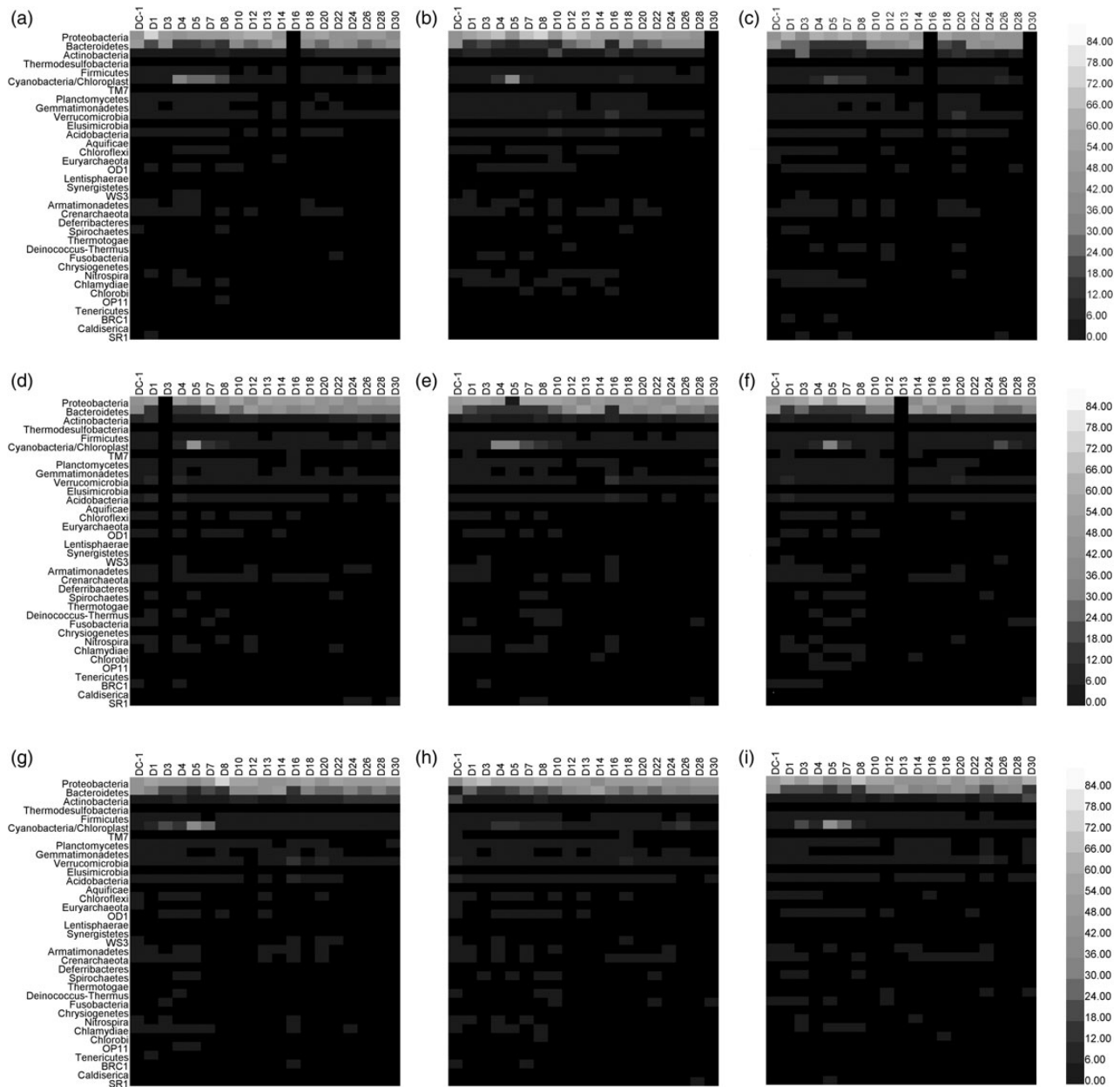
<sup>b</sup>Average phylum OTU percentage of total OTUs with standard deviation.

<sup>c</sup>Average class OTU percentage of total phylum OTUs with standard deviation.

higher average connectivity means a more complex network. Geodesic distance means the shortest path between two nodes, while a smaller average geodesic distance means the nodes in a network are closer. The average clustering coefficient means the extent of a module in a network, where the clustering coefficient describes how well a node is connected with its neighbours. The network can be separated into communities or modules (Newman, 2006b). The modularity value indicates how well a network can be divided into modules. In our study, elevated  $p\text{CO}_2$  generally had no consistent influence on the connectivity of micro-organisms observed via pMENs analysis (Table 1). For example, the size (number of total nodes) of pMENs under 175 and 180  $\mu\text{atm } p\text{CO}_2$  were smaller than for higher-level treatments (250, 425, 600, and 675  $\mu\text{atm}$ ), while they were similar to those from the 860 and 1085  $\mu\text{atm}$  mesocosms. Additionally, the total number of links and average connectivity were similar among the nine  $p\text{CO}_2$  treatments. Average geodesic distance and average clustering coefficients revealed a discrepancy change along the  $p\text{CO}_2$  gradient. The modularity of nine pMENs, which contained at least four modules each, suggested that the bacterial communities in these mesocosms were highly complex (Olesen et al., 2007). Strong negative correlations were observed in all nine pMENs, while positive correlations primarily occurred in submodules (Figure 2). These results suggested potential competition among bacterial lineages in these ecosystems.

Connectivity analysis among or within the modules showed that different microbial clades played different roles in the pMENs (Figure 3). From an ecological perspective, the peripherals may represent specialists, whereas module hubs and connectors may be more generalists and network hubs may be super-generalists (Olesen et al., 2007; Deng et al., 2012). The relatively higher abundance of *Alphaproteobacteria* and *Flavobacteria* suggest that they play crucial roles in the pMENs. The number of connectors was greater under 250, 600, and 1085  $\mu\text{atm } p\text{CO}_2$ , which suggested a greater complexity in these microbial communities (Supplementary Figure S5). Larger numbers of OTUs belonging to *Alphaproteobacteria* and *Flavobacteria* were observed under 250 and 600  $p\text{CO}_2$ . Network module hubs are another important factor in network topology. Compared with connectors, there were fewer module hubs, but they were dominated by *Gammaproteobacteria* (Supplementary Table S4). Unexpectedly, no network connectors or module hubs were detected under 175 and 860  $\mu\text{atm}$ . Consistent with previous studies, no network hub was detected in any pMEN (Zhou et al., 2010, 2011; Deng et al., 2012). These results indicated that *Alphaproteobacteria*, *Gammaproteobacteria*, and *Flavobacteria* play an important role in maintaining the stability of microbial ecosystems in these mesocosms.

The Mantel test was performed to investigate the correlation between environmental variables and micro-organism interactions. The OTU significance (GS) in the pMENs were calculated, which were defined as the square of the Pearson correlation coefficient ( $r^2$ ) of OTU abundance profile with environmental traits (Deng et al., 2012), and did not significantly correlate with either biological or chemical parameters in almost all of the pMENs, except for the 860  $\mu\text{atm}$  treatment (Supplementary Table S2). Furthermore, rare individual variables were significantly correlated with the network topological properties. However, the 250 and 340  $\mu\text{atm}$  pMENs were significantly correlated with particle organic phosphorus and  $\text{NO}_2$  concentration, respectively. The 675 and 860  $\mu\text{atm}$  pMENs were significantly correlated with incubation day and pH, and bacterial abundance, viral abundance, and particle organic nitrogen, respectively. These significant correlations indicated connectivity



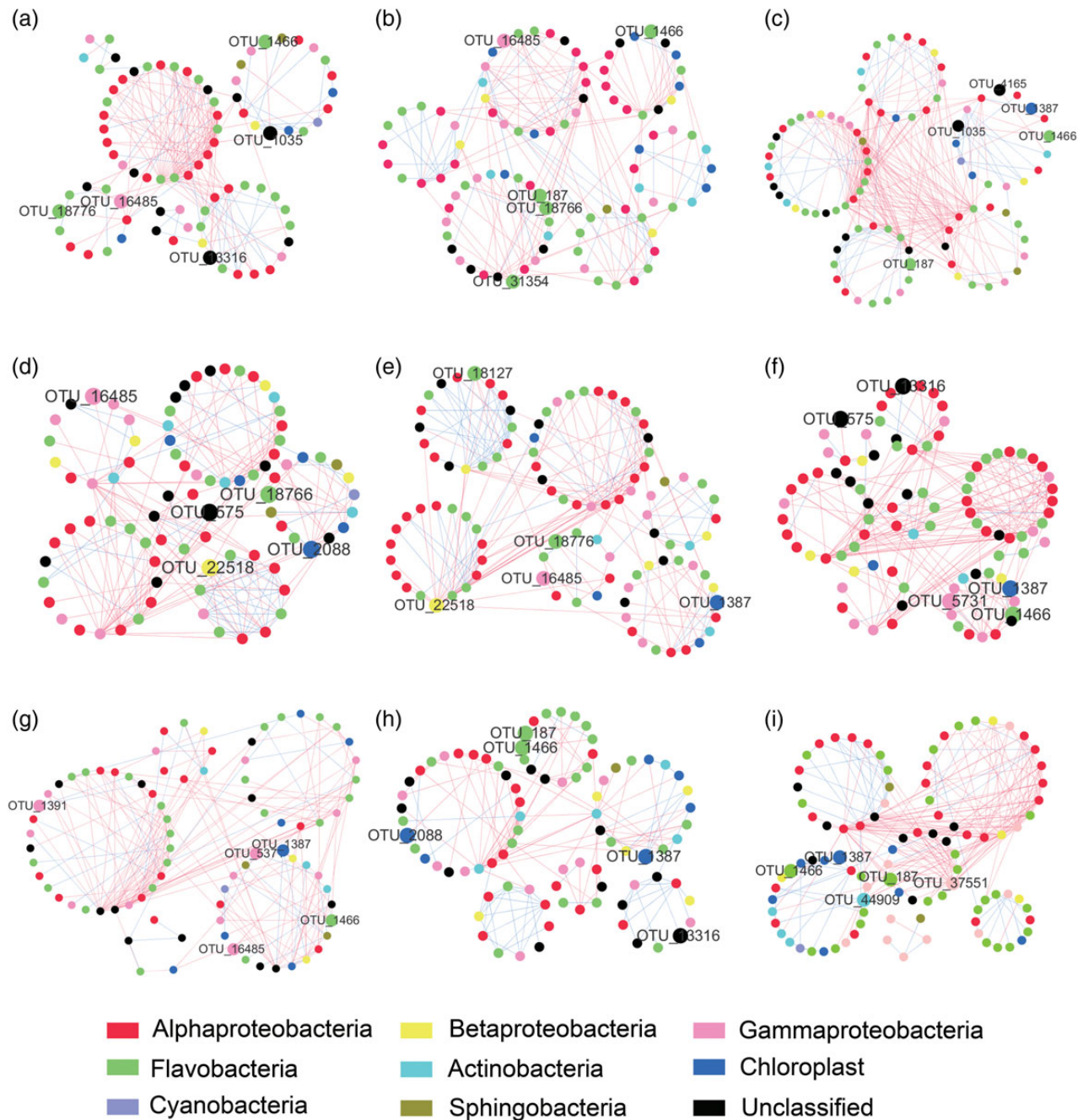
**Figure 1.** Heatmap of sample compositions based on the proportion of each classes. Black represents the non-detected class; colour from dark to light indicates the proportion from low to high. The  $p\text{CO}_2$  concentrations ( $\mu\text{atm}$ ) from A to I are 175, 180, 250, 340, 425, 600, 675, 860, and 1085. x-axis (D and a number) indicate the incubation day from  $-1$  (DC-1) to day 30.

among these micro-organisms under  $860 \mu\text{atm } p\text{CO}_2$  because both were significantly associated with changes in biological and geochemical variables. However, there was no clear trend in these correlations between topology properties and environmental variables along the  $p\text{CO}_2$  gradient, which suggested that elevated  $p\text{CO}_2$  had little effect on the global interaction properties. Mantel analysis was also used to investigate the correlation between environmental variables and pMEN microbial composition. Similarly, the Mantel test results between pMEN topology and environmental variables revealed that few OTUs were significantly correlated with environmental variables (Supplementary Table S3). *Cyanobacteria*/chloroplast were significantly correlated with environmental properties under the 180 and  $1085 \mu\text{atm } p\text{CO}_2$  treatments. *Betaproteobacteria* and *Gammaoproteobacteria* were significant in the  $675 \mu\text{atm } p\text{CO}_2$

pMEN. These results indicated that *Proteobacteria* were more sensitive to changes in marine variables, especially under high  $p\text{CO}_2$ . They also indicated that no specific bacteria was significantly correlated with changes in either environmental variables or  $p\text{CO}_2$  concentrations.

## Discussion

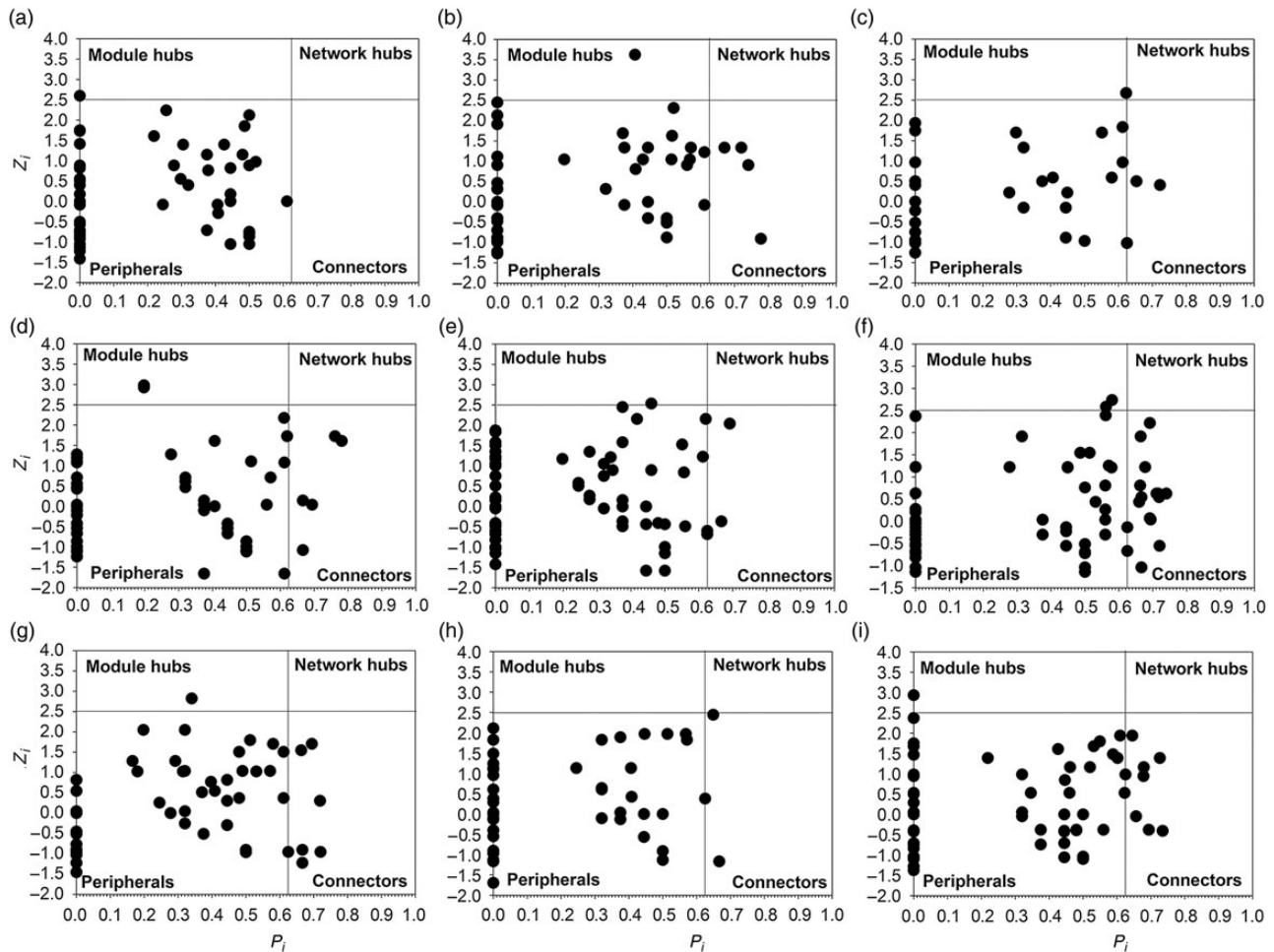
Understanding the response of bacterioplankton communities to elevated  $p\text{CO}_2$  is important for evaluating climate change effects on the ocean ecosystem. However, how the marine bacteria respond to elevated  $p\text{CO}_2$  and decreased pH is not well understood and remains controversial (Liu *et al.*, 2010; Joint *et al.*, 2011). The EPOCA mesocosm experiments have provided a great opportunity to answer this critical question at the community-level (Riebesell *et al.*, 2013b). The present study surveyed bacterioplankton communities by MiSeq



**Figure 2.** Bacterioplankton network interactions under 175 (a), 180 (b), 250 (c), 340 (d), 425 (e), 600 (f), 675 (g), 860 (h), and 1085 (i)  $\mu\text{atm } p\text{CO}_2$ . Five to seven modules were formed under different  $p\text{CO}_2$  concentrations. Each node represents an out, which signified a species. Node colours indicate different phyla. Each line connects two OTUs. A blue line indicates a positive interaction between two individual nodes suggesting a mutualism or cooperation, while a red line indicates a negative interaction suggesting predation or competition. Top five highest-abundance OTUs are indicated by bigger dots and marked with OTU identification numbers. The cycle composed of several nodes is a module in the pMEN, which is more correlated in a module than between other modules. Only the module containing more than five OTUs are displayed.

sequencing of 16S rRNA gene amplicons and RMT-based ecological network analysis. Previous studies have demonstrated that phytoplankton respond significantly to elevated  $p\text{CO}_2$  (Riebesell *et al.*, 2013a, b; Schulz *et al.*, 2013). Moreover, primary production and bacterial protein production are simultaneously stimulated by elevated  $p\text{CO}_2$  (Engel *et al.*, 2013; Motegi *et al.*, 2013; Piontek *et al.*, 2013), which generally results in net production decreases by the end of incubation experiments (Engel *et al.*, 2013). Our results indicated

that the phylogenetic diversity and structure of bacterioplankton communities were generally similar among different  $p\text{CO}_2$  treatments, which is consistent with previous EPOCA bacterial community investigations (Roy *et al.*, 2013; Sperling *et al.*, 2013). Furthermore, the pMEN analysis suggested that the bacterioplankton community did not respond significantly to elevated  $p\text{CO}_2$  at the community level, which means that our study agreed with the null hypothesis that changes in  $p\text{CO}_2$  concentration have little



**Figure 3.** pMEN submodules under nine different  $p\text{CO}_2$  treatments. Each dot represents an OTU from nine different  $p\text{CO}_2$  treatments (a–i indicate  $p\text{CO}_2$  concentration from 175 to 1085  $\mu\text{atm}$ ). The  $Z$ – $P$  plot showing OTU distribution based on their module-based topological role according to within-module ( $Z$ ) and among-module ( $P$ ) connectivity. The nodes of  $Z_i > 2.5$  and  $P_i < 0.625$  are indicated as the module hubs that were more closely connected within the module, while the nodes of  $Z_i < 2.5$  and  $P_i > 0.625$  are the connectors that were more closely connected to nodes in other modules. Peripherals of  $Z_i < 2.5$  and  $P_i < 0.625$  are considered specialists in each module, while the network hubs of  $Z_i > 2.5$  and  $P_i > 0.625$  are super-generalists.

influence on the affected microbial communities (Joint *et al.*, 2011) and the bacterioplankton community had a certain resilience to the  $p\text{CO}_2$  perturbations. The present study provides novel insights into how bacterioplankton communities respond to ongoing  $p\text{CO}_2$  increases.

Generally, high diversity is thought to allow bacterial communities to resist disturbance, this is known as the “insurance effect” (Boles *et al.*, 2004). Joint *et al.* (2011) suggested that high diversity is one of the mechanisms employed by bacterioplankton resist to elevated  $p\text{CO}_2$  and decreased pH. Besides, micro-organisms do not live in isolation but form complex ecological interaction webs, which are also closely related to community stability (Faust and Raes, 2012). Changes global interactions (e.g. connectivity and modularity) among bacterioplankton communities along the  $p\text{CO}_2$  gradient were unclear in the present study (Table 2 and Figure 2). These results suggested that the community interaction webs were relatively stable, despite the  $p\text{CO}_2$  changes. In an ecological network, a negative correlation suggests a competitive relationship between species (Faust and Raes, 2012). The competition between species should yield less variation in total abundance

or biomass, known as compensatory dynamics (Gonzalez and Loreau, 2009). A community with more competitors would be more stable under environmental fluctuations through maintaining relatively low covariations in community densities (Ives and Hughes, 2002). Klug *et al.* (2000) conducted a microcosm experiment and suggested strong compensatory dynamics when plankton were directly affected by pH perturbations. Furthermore, the competition might increase both the amplitude and asynchrony of population fluctuations generated by the asynchrony in species responses to the environment, which is the basis for functional compensation between species and potentially stabilizes aggregate community or ecosystem properties (Loreau, 2010). Ecosystem modelling indicates that asynchrony in species intrinsic rates of natural increase could stabilize the community (Fowler *et al.*, 2012). Therefore, the dominant competitive relationships observed in our study (Figure 2) might sustain population diversity and enhance ecosystem resilience to perturbations.

Additionally, the high modularity observed in our study (Table 2) might result in a more complex ecosystem that would be more stable than dispersal ecosystems. Because of the tighter

**Table 2.** Topological properties of the microbial communities' phylogenetic molecular networks under nine  $p\text{CO}_2$  concentrations and their rewired random networks.

$p\text{CO}_2$ ( $\mu\text{atm}$ )	Experimental networks								Random networks		
	Cut-off	Total nodes	Total links	$R^2$ of power-law	Avg. connectivity	Avg. geodesic distance	Avg. clustering coefficient	Modularity	Avg. geodesic distance	Avg. clustering coefficient	Avg. modularity
175	0.85	86	187	0.843	4.349	4.088 <sup>a</sup>	0.224 <sup>a</sup>	0.452 <sup>a</sup>	2.941 ± 0.136	0.133 ± 0.021	0.384 ± 0.010
180	0.84	99	185	0.878	3.737	3.572 <sup>a</sup>	0.177 <sup>a</sup>	0.558 <sup>a</sup>	3.153 ± 0.178	0.076 ± 0.018	0.454 ± 0.011
250	0.84	108	315	0.836	5.833	2.281 <sup>a</sup>	0.218 <sup>a</sup>	0.330 <sup>a</sup>	2.629 ± 0.146	0.180 ± 0.017	0.303 ± 0.008
340	0.84	92	186	0.850	4.043	3.062 <sup>a</sup>	0.213 <sup>a</sup>	0.561 <sup>a</sup>	3.642 ± 0.153	0.157 ± 0.023	0.442 ± 0.011
425	0.84	116	226	0.850	3.897	2.773 <sup>a</sup>	0.243 <sup>a</sup>	0.592 <sup>a</sup>	3.164 ± 0.189	0.068 ± 0.014	0.451 ± 0.010
600	0.84	113	219	0.887	3.876	2.488 <sup>a</sup>	0.079 <sup>a</sup>	0.453 <sup>a</sup>	2.909 ± 0.227	0.068 ± 0.013	0.442 ± 0.009
675	0.84	100	215	0.801	4.300	2.692 <sup>a</sup>	0.178 <sup>a</sup>	0.465 <sup>a</sup>	2.974 ± 0.167	0.082 ± 0.017	0.411 ± 0.010
860	0.85	95	154	0.870	3.242	3.435 <sup>a</sup>	0.148 <sup>a</sup>	0.638 <sup>a</sup>	3.275 ± 0.256	0.047 ± 0.015	0.513 ± 0.015
1085	0.84	99	245	0.831	4.949	2.965 <sup>a</sup>	0.293 <sup>a</sup>	0.461 <sup>a</sup>	2.838 ± 0.114	0.141 ± 0.019	0.357 ± 0.010

<sup>a</sup>Significant difference between experimental network and random network by the  $t$ -test ( $p < 0.001$ ).

interactions between nodes within the modules, the disturbances that influenced these nodes were unlikely to spread to other modules. For example, the OTU\_1466 in the 175  $\mu\text{atm}$  pMEN only connected with nodes within that module, hence the abundance variation of OTU\_1466 must affect more than two nodes to spread this variation to other modules (Figure 2). The extinction of a module hub might cause the module to fragment, having little or no cascading impact on other modules. In contrast, the extinction of connectors might cause the network to fragment but with minor impacts on the interactions within the isolated modules (Olesen *et al.*, 2007). Therefore, compared with a non-modular structure, a modular structure should dampen the rapid spread of disturbance in a community (Olesen *et al.*, 2007). Since each module in a pMEN indicates an ecological niche (Chaffron *et al.*, 2010), our observation that negative relationships dominated between rather than within the modules suggested that the variations within a niche did not affect community stability. Therefore, competitive relationships and the modular structure of bacterioplankton communities provided resistance to variations in  $p\text{CO}_2$  in the present mesocosm experiment. However, previous studies have shown that interactions among soil bacterial communities are influenced by elevated atmospheric  $\text{CO}_2$  over a longer time-scale (i.e. annually; Zhou *et al.*, 2010, 2011; Tu *et al.*, 2015). Whether or not marine bacterioplankton also respond to long-term  $p\text{CO}_2$  changes is worthy of investigation.

In an ecological network, the module hubs and connectors are considered keystone species, which have a crucial role in sustaining community stability (Paine, 1969). We found a relatively larger proportion of *Gammaproteobacteria* and *Flavobacteria* in pMEN module hubs and connectors in our study (Supplementary Figure S5 and Table S4). The relative abundance of *Gammaproteobacteria* and *Flavobacteria* did not change significantly among the  $p\text{CO}_2$  treatments (Roy *et al.*, 2013; Supplementary Figure S6) and they were not significantly correlated with geochemical variables under elevated  $p\text{CO}_2$ . Therefore, these stable keystone species likely provided stability to the entire bacterial community. Monier *et al.* (2014) found the relative abundance of two groups of *Gammaproteobacteria* (*Alteromonadales* and *Oceanospirillales*) responded significantly to variations in  $p\text{CO}_2$  in the north Arctic Ocean, but they did not respond significantly to decreased pH in terms of general phylogenetic structure. In our experiment, these potentially sensitive groups were not the major components and only a few OTUs belonging to these

two *Gammaproteobacteria* groups were found in nine pMENs. Furthermore, we did not observe a clear trend in the interaction between the *Gammaproteobacteria* and their closest neighbours with elevated  $p\text{CO}_2$  (Supplementary Figures S7 and S8).

Despite their general structure, bacterioplankton communities were insensitive to elevated  $p\text{CO}_2$  in the EPOCA experiments, the low abundance species had the potential to respond to elevated  $p\text{CO}_2$ . Roy *et al.* (2013) found that some rare species, like *Methylotenera*, *Fluviicola* and *Colwellia*, were significantly correlated with changes in  $p\text{CO}_2$ . However, only a few of these bacterioplankton appeared within the nine pMENs in our analysis. For example, *Colwellia* appeared in the high  $p\text{CO}_2$  treatment at the end of the incubation period, but was almost non-existent before that (Roy *et al.*, 2013; Supplementary Figure S9). Because variations in keystone species have a greater impact on ecosystem stability and resilience than rare species (Paine, 1969), we used the major components of the bacterioplankton community to construct the networks.

Elevated  $p\text{CO}_2$  had increased the POC and dissolved organic carbon (DOC) production by the end of the incubation period; therefore, bacterial protein activity and production were also stimulated (Motegi *et al.*, 2013; Piontek *et al.*, 2013). However, bacterial production, respiration, and growth efficiency were similar over the incubation period among the different  $p\text{CO}_2$  treatments, which suggested that elevated  $p\text{CO}_2$  had little impact on bacterial metabolism at the community level (Motegi *et al.*, 2013). Previous meta-analyses have found no evidence that bacterial community growth efficiency is stimulated by elevated  $p\text{CO}_2$  (Liu *et al.*, 2010; Motegi *et al.*, 2013). We did not find any significant correlation between network topology and bacterial production (except for the 675  $\mu\text{atm}$  treatment) and picophytoplankton abundance change. These results suggested that primary production stimulation had little influence on these keystone components. In fact, the variations in DOC and POC production during the incubation were greater those among the different  $p\text{CO}_2$  levels (Engel *et al.*, 2013). This is perhaps partially explained by the insignificant network response to  $p\text{CO}_2$  among different mesocosms. However, another possible reason is different bacterial assemblage living strategies, i.e. free-living vs. particle-attached. Allgaier *et al.* (2008) reported that the free-living fraction of a bacterioplankton community was significantly correlated with elevated  $p\text{CO}_2$  in a temperate fjord in Norway. However, the dynamics of the bacterioplankton community particle-attached fraction, compared with the free-



living fraction, had a strong relationship with phytoplankton bloom, and exhibited a higher richness at the end of the incubation period in the EPOCA experiments (Sperling *et al.*, 2013). The richness of the free-living fraction, however, decreased at the same time (Sperling *et al.*, 2013). Thus, we would expect that the networks based upon entire bacterioplankton communities in the present study could not reflect the response of particle-attached bacteria to  $p\text{CO}_2$  changes. Additionally, the lower bacterial abundance during this period combined with increased viral lysis in the higher  $p\text{CO}_2$  mesocosm suggested top-down control on bacterial production (Brussaard *et al.*, 2013), which might also not have reflected pMENS and suggested the importance of top-down control during this experiment. Therefore, the network analysis involved in both bacterioplankton and virioplankton, even zooplankton, would provide a more comprehensive explanation of the future effect of elevated  $p\text{CO}_2$  in the ocean.

A few studies have found that decreased pH significantly influences specific bacterial groups in various oceans (Krause *et al.*, 2012; Maas *et al.*, 2013). However, their results were not consistent with ours; this might have been because of the difference between pH manipulations by HCl and  $\text{CO}_2$ . Manipulation with HCl, which modifies the total alkalinity (TA) and pH with constant dissolved inorganic carbon (DIC), is not the most accurate approach to mimic future carbonate chemistry (Hurd *et al.*, 2009; Krause *et al.*, 2012). In contrast,  $\text{CO}_2$  addition induces a pH decrease and DIC increase when TA is constant (Hurd *et al.*, 2009). Even the carbonate chemistry parameters were similar between both manipulation methods when  $p\text{CO}_2 < 700$  ppm (Hurd *et al.*, 2009). However, when pH was modified to 7.5 by HCl, the  $[\text{HCO}_3^-]$  value was 22% lower compared with the pH value obtained by  $p\text{CO}_2$  manipulation (Hurd *et al.*, 2009). The previous study has demonstrated that this difference in DIC affects some algal groups, e.g. those lacking a carbon-concentrating mechanism exhibited lower growth rate in pH 7.5 by HCl manipulation compared with  $p\text{CO}_2$  manipulation (Hurd *et al.*, 2009). Yet, the impact of this difference on the bacterioplankton is still unclear. Because mixotrophic micro-organisms are widespread carbon in the world's oceans (Hugler and Sievert, 2010), methodological differences may have affected the response of bacteria to changes in pH. Thus,  $p\text{CO}_2$  manipulation is the most appropriate method available to mimic future changes in oceanic carbonate chemistry.

## Conclusion

The hypothesis that “marine microbes possess the flexibility to accommodate pH change” is primarily based on the observation that microbial populations confront large variations in pH, both short-term and seasonally, in marine environments (Joint *et al.*, 2011). This hypothesis is supported by recent studies at the microbial community level (Allgaier *et al.*, 2008; Newbold *et al.*, 2012; Roy *et al.*, 2013; Sperling *et al.*, 2013; Zhang *et al.*, 2013). In combination with the present study, these data suggest that a larger population size and higher diversity within a microbial community contribute to the resistance of communities to high  $p\text{CO}_2$ . Additionally, our network analysis provides further evidence that complex interactions (e.g. interspecies competition and modularity) among individual microbial species in a natural population help microbes resist and recover from pH and  $p\text{CO}_2$  disturbances. This could explain the contradictory observations between OA effects on single species (e.g. pure culture) and natural marine microbe populations.

## Supplementary data

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

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