



# Bacterioplankton Metacommunity Processes across Thermal Gradients: Weaker Species Sorting but Stronger Niche Segregation in Summer than in Winter in a Subtropical Bay

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**ABSTRACT** Thermal effluents from nuclear power plants greatly change the environmental and ecological conditions of the receiving marine water body, but knowledge about their impact on microbial ecology is limited. Here we used high-throughput sequencing of the 16S rRNA gene to examine marine bacterioplankton metacommunity assembly across thermal gradients in two representative seasons (i.e., winter and summer) in a subtropical bay located on the northern coast of the South China Sea. We found high heterogeneity in bacterioplankton community compositions (BCCs) across thermal gradients and between seasons. The spatially structured temperature gradient created by thermal effluents was the key determinant of BCCs, but its influence differed by season. Using a metacommunity approach, we found that in the thermal discharge area, i.e., where water is frequently exchanged with surrounding seawater and thermal effluent water, the BCC spatial patterns were shaped by species sorting rather than by mass effects from surrounding seawater or by dilution of thermal effluent water by surrounding seawater. However, this effect of species sorting was weaker in summer than in winter seawater. In both seasons, the bacterioplankton community structure was predominately determined by niche sharing; however, the relative importance of niche segregation was enhanced in summer seawater. Our findings suggest that for the seasonal differences in metacommunity processes, the BCCs of subtropical summer seawater were more sensitive to temperature and were more difficult to predict than those of winter seawater in the face of different intensities of thermal impacts.

**IMPORTANCE** Understanding the mechanisms of bacterial community assembly across environmental gradients is one of the major goals of marine microbial ecology. Thermal effluents from two nuclear power plants have been present in the subtropical Daya Bay for more than 20 years and have generated a comparatively stable and long thermal gradient (a temperature increase from 0 to 10°C). The environmental patches across thermal gradients are heterogeneous and very strongly interconnected on a microbial scale; thus, this is a useful model for the study of the metacommunity processes (i.e., patch dynamics, species sorting, mass effects, and neutral processes) that underlie marine bacterioplankton assembly. The significance of our research is to reveal how environmental conditions and dispersal-related processes interact to influence bacterioplankton metacommunity processes and their seasonal differences across thermal gradients. Our results may advance the understanding of marine microbial ecology under future conditions of global warming.

**KEYWORDS** marine ecosystem, metacommunity processes, microbial ecology, subtropical bay, thermal impacts

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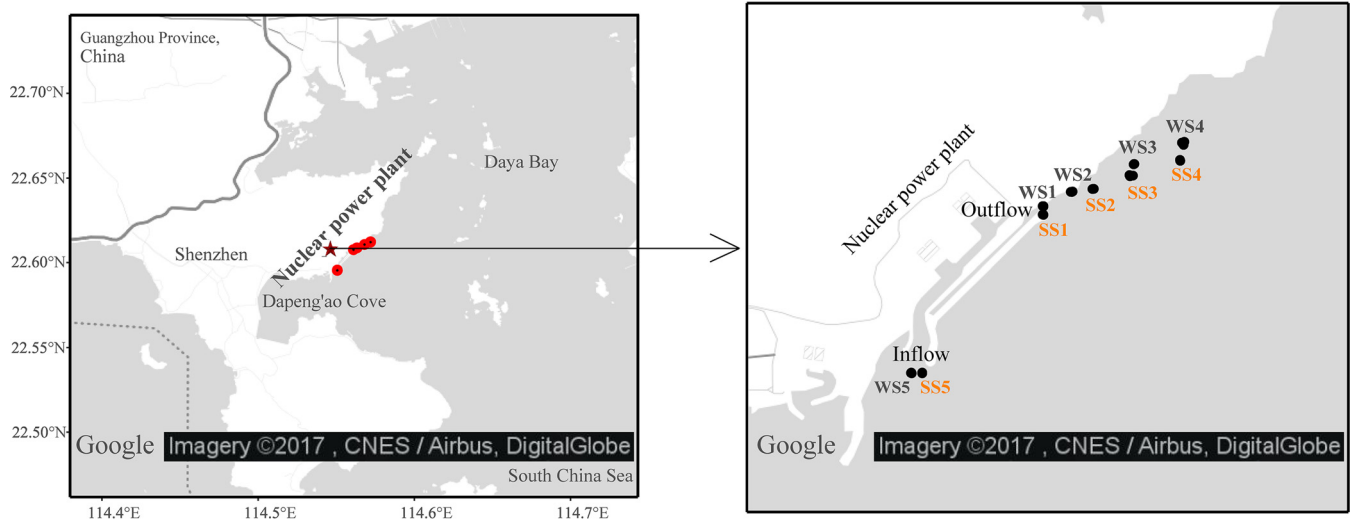
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The increasing number of marine nuclear power plants has given rise to concerns about the undesirable thermal effects of power plant cooling systems on marine environments and ecology (1–3). Bacterioplankton play important roles in the ecological processes of marine ecosystems (4, 5). Despite their ecological importance, the response of bacterioplankton communities to thermal effects is not as well understood as those of phytoplankton (1, 6–9), protozoa (10), zooplankton (11), and fish (12). Current studies about thermal effects on marine bacterioplankton communities have focused mainly on production (13), respiration (14), and the growth rate (15). Studies suggest that thermal effluents have profound impacts on bacterioplankton metabolism abilities and that the effects are dependent on the season (13–15). Bacterioplankton community compositions (BCCs) subject to thermal effects, however, have rarely been examined by use of high-throughput sequencing, except for a recent study showing that thermal effluents from a coal power plant (water temperature, 15.0 to 18.6°C) had significant effects on marine spring BCCs (16). The habitats with more thermal effluents had lower relative abundances of *Alphaproteobacteria* and *Gammaproteobacteria* but higher relative abundances of *Cyanobacteria* (16). Our knowledge about the thermal impact of nuclear power plants on BCCs is thus inadequate. We know little about the seasonal differences in BCC responses to thermal effluents and the underlying ecological mechanisms.

Exploring the mechanisms of bacterioplankton assembly is one of the major goals of marine microbial ecology (17–19). Generally, aquatic bacterioplankton community structure is shaped by environmental or dispersal-related processes (see, e.g., references 20 to 22). Metacommunity theory incorporates the interplay between environmental conditions and dispersal-related processes into four main categories: patch dynamics, species sorting, mass effects, and neutral processes (23). Dispersal-related processes include mass effects and dispersal limitation (20, 23). Very high dispersal rates can result in mass effects, with taxa existing in less suitable habitats due to continuous supply (23, 24), whereas very low dispersal rates can result in dispersal limitation, leading to purely spatial biogeography patterns (20). Neutral processes imply a lack of differences in bacterial fitness and niche, so that community structure depends on demographic stochasticity (23, 24). Species sorting in metacommunity concepts is similar to niche separation and sharing (25, 26), emphasizing niche roles above and beyond spatial dynamics (18, 23).

Thermal effluents from nuclear power plants generate thermal gradients in the discharge area (2, 8, 27). The environmental patches across thermal gradients are heterogeneous and very strongly interconnected on a microbial scale (2, 8); thus, this is a useful model with which to study the environmental and dispersal-related processes that underlie marine bacterioplankton assembly (18). Bacterioplankton are easily dispersed through both flowing thermal effluent water and water flows and tides from the surrounding seawater. High dispersal rates in the thermal effluent area may induce mass effects with strong source-sink relations among heterogeneous habitats (23). Across thermal gradients, mass effects probably swamp the species-sorting roles of thermal effects and result in greater difficulty in predicting bacterioplankton communities on the basis of local environmental characteristics (23). The BCC patterns across thermal gradients may be determined by mass effects from surrounding seawater or spatial factors due to the continuous dilution effect of surrounding seawater on thermal effluent water. In addition, due to seasonal differences in hydrologic and environmental conditions, bacterioplankton metacommunity processes across thermal gradients may differ by season. However, thus far, research on the potential ecological mechanisms of bacterioplankton assembly subject to thermal effects is scarce, and we know little about the way in which the environmental conditions and dispersal-related processes interact to influence bacterioplankton community structure and their seasonal differences in response to thermal effects.

To determine the spatial distribution of bacterioplankton communities across thermal gradients, their seasonal differences, and the underlying ecological mechanisms, we used high-throughput sequencing of the 16S rRNA gene to study marine BCCs

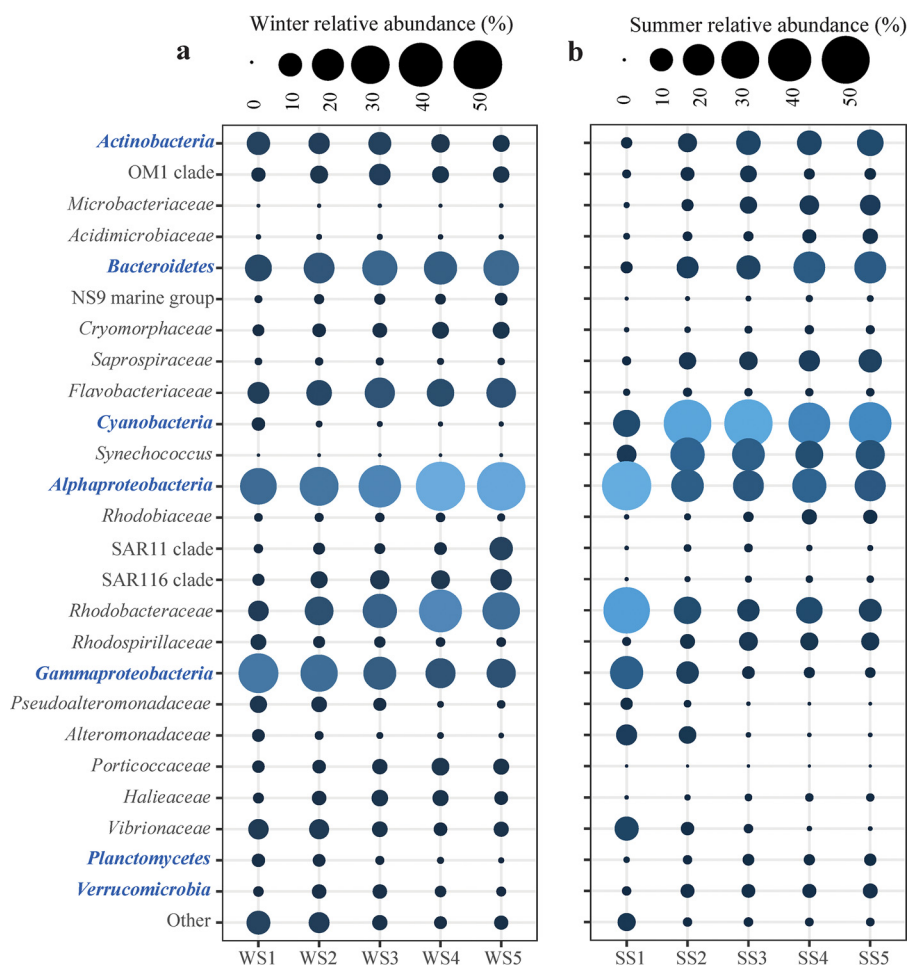


**FIG 1** Sampling sites across the thermal gradients in subtropical Daya Bay on the northern coast of the South China Sea. Five sites were set for sampling in each season. Four sites were located along the thermal effluent (WS1, WS2, WS3, and WS4 for the winter and SS1, SS2, SS3, and SS4 for the summer), and the remaining site (WS5 for the winter and SS5 for the summer) was set as a control and was located in the inflow area of the cooling system upstream of the thermal effluents. The maps were generated based on an open-access Google satellite map using the ggmap package (<https://github.com/dkahle/ggmap>) in the R statistical computing environment (<https://www.R-project.org>).

across thermal gradients in two representative seasons (i.e., winter and summer) in a subtropical bay (Daya Bay) located on the northern coast of the South China Sea (Fig. 1). In the area studied, thermal effluents from the cooling systems of two nuclear power stations have been present for more than 20 years and have generated a comparatively stable and long thermal gradient (a temperature increase from 0 to 10°C [2, 8]). There are strong seasonal differences in both hydrologic and environmental conditions in the area studied. For instance, compared to summer, winter is usually characterized by hydrologic conditions of a higher tide level and higher current velocity but lower wind speed (6, 28, 29) and has the environmental traits of lower temperatures but higher concentrations of dissolved oxygen (DO) (2, 3). We used a metacommunity approach (17, 18, 22) as a logical framework for studying the impact of the environmental conditions and dispersal-related processes underlying bacterioplankton assembly (30, 31). The potential species-sorting processes (e.g., niche separation and sharing) were further examined by a co-occurrence network analysis of bacterioplankton communities (32, 33). We hypothesized that (i) in the thermal discharge area, i.e., where water is frequently exchanged with surrounding seawater and thermal effluent water, the thermal effluents may have nonsignificant species-sorting effects on marine bacterioplankton assembly; (ii) the BCC pattern across the thermal gradient may depend on spatial factors because of the continuous dilution effect of surrounding seawater on thermal effluent water; and (iii) due to seasonal differences in hydrologic and environmental conditions, bacterioplankton metacommunity structure and processes under thermal impacts may differ by season.

## RESULTS

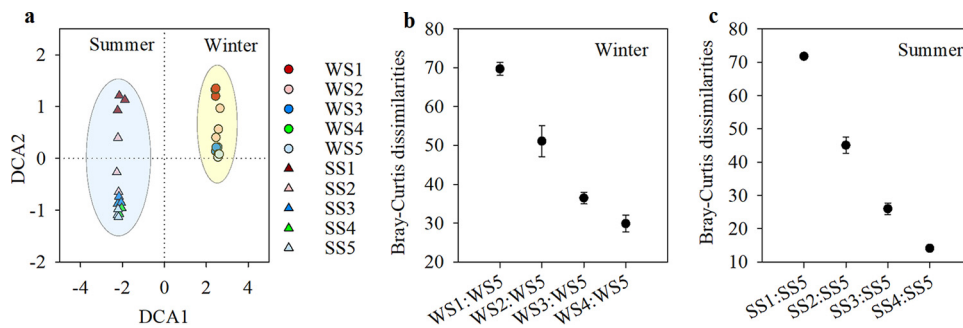
**Environmental characteristics.** In each season studied, the thermal effluents from the two nuclear power plants had profound influences on the water temperature (see Fig. S1 and S2 in the supplemental material). In the winter, the water temperature increased from 21°C to 31°C, and in the summer, the temperature increased from 31°C to 39°C. In both seasons, the water temperature decreased significantly along our sampling sites (from site 1 to site 5) ( $P, <0.05$  by one-way analysis of variance [ANOVA] in both cases) (Fig. S1 and S2), whereas the DO content of the water increased significantly ( $P, <0.05$  by one-way ANOVA in both cases) (Fig. S1 and S2). In both seasons studied, the total organic carbon (TOC),  $\text{NH}_4^+\text{-N}$ ,  $\text{PO}_4^{3-}\text{-P}$ , and  $\text{SiO}_3^{2-}$  contents



**FIG 2** Differences in the spatial distributions of the relative abundances of bacterioplankton clades across the thermal gradients in winter (a) and summer (b) seawater. The phyla and subphyla are shown in blue boldface. Following the abundant phyla and subphyla (*Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria*), their abundant families are listed.

had no obvious differences across the thermal gradient ( $P, >0.05$  by one-way ANOVA in all cases) (Fig. S1 and S2). Between the two seasons studied, the environmental characteristics temperature, pH, and  $\text{PO}_4^{3-}\text{-P}$  and  $\text{NO}_3^-\text{-N}$  concentrations were significantly higher in summer than in winter seawater ( $P, <0.05$  by the  $t$  test in all cases) (Fig. S1 and S2), whereas DO and  $\text{SiO}_3^{2-}$  concentrations were significantly lower in summer than in winter seawater ( $P, <0.05$  by the  $t$  test in both cases) (Fig. S1 and S2).

**Shifts in community structure.** The main bacterioplankton in seawater in both summer and winter were *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Planctomycetes*, and *Verrucomicrobia*. The relative abundances of these bacterioplankton groups were significantly different for the two seasons ( $P, <0.05$  by the  $t$  test in all cases) (Fig. 2), and for each season, the relative abundances were significantly different across the thermal gradient ( $P, <0.05$  by one-way ANOVA in all cases) (Fig. 2). In the winter, the thermal impact increased the relative abundances of *Actinobacteria*, *Cyanobacteria*, *Gammaproteobacteria*, *Planctomycetes*, and other rare phyla (relative abundance of total sequences,  $<1\%$ ) and decreased the relative abundances of *Bacteroidetes* and *Alphaproteobacteria* (Fig. 2a). However, in the summer, the sites with more effluents had higher relative abundances of *Alphaproteobacteria* and *Gammaproteobacteria* and lower relative abundances of *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* (Fig. 2b). The relative abundance of *Cyanobacteria* (primarily *Synechococcus*) was significantly higher in sea-



**FIG 3** (a) Detrended correspondence analysis (DCA) of bacterioplankton community structures in winter and summer seawater. (b and c) BCC dissimilarities between sites along the thermal effluent (winter: WS1, WS2, WS3, and WS4; summer: SS1, SS2, SS3, and SS4) and control sites (winter, WS5; summer, SS5) located in the inflow area in winter (b) and summer (c) seawater.

water in the summer than in the winter (Fig. 2a and b), and the relative abundance increased as the thermal effluents intensified but decreased when the water temperature was approximately 39°C (Fig. 2a and b).

At the level of operational taxonomic units (OTUs), our results showed that winter and summer had significantly different BCCs across the thermal gradients, as revealed by detrended correspondence analysis (DCA) and permutational multivariate analysis of variance using distance matrices (PERMANOVA) ( $F = 39.687$ ;  $P < 0.01$ ) (Fig. 3a; see also Table S1 in the supplemental material). In each season, significant differences in community structure were detected among the five sampling sites by PERMANOVA ( $F = 5.919$ ,  $P < 0.01$  for winter;  $F = 9.284$ ,  $P < 0.01$  for summer) (Table S1), and the sites with more thermal effluents had BCCs that differed more dramatically from those of the control site (Fig. 3b and c). Pairwise comparisons by PERMANOVA showed that in the winter, the BCCs were significantly different between any two thermal effluent sites ( $P, < 0.05$  in all cases), except for the comparisons of WS2 and WS3 and the comparisons of WS3 and WS4 (Table S1). However, in the summer, the BCC differences in all pairwise comparisons of the thermal effluent sites (from site 1 to site 4) were significant ( $P, < 0.05$  in all cases) (Table S1).

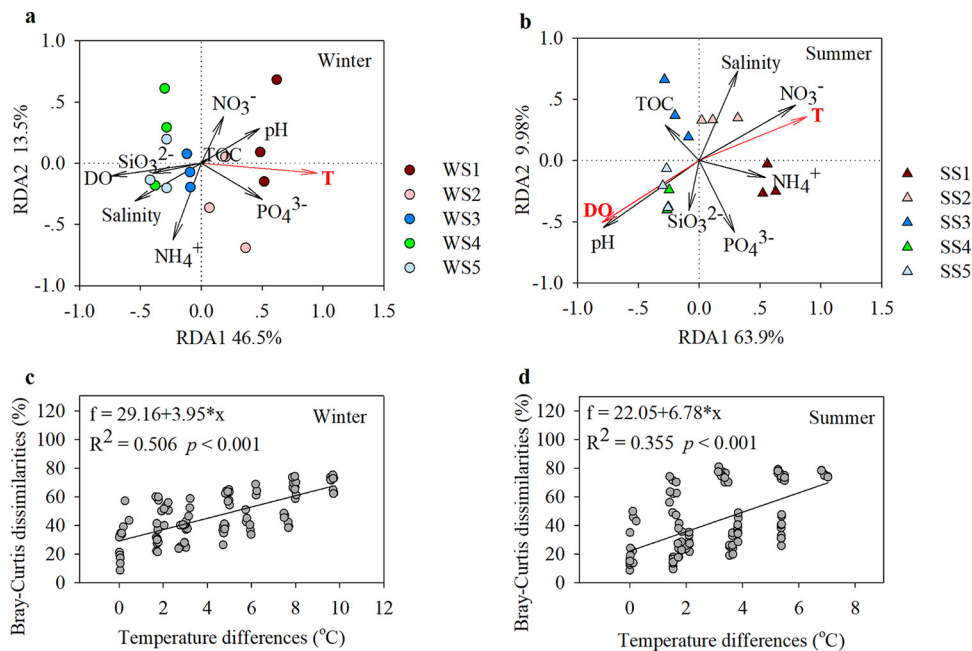
**Relating community structure to environmental factors and geographic distances.** Variance partitioning analysis (VPA) for each season revealed that the subset of pure environmental variables explained a larger portion of the BCC variation in winter (32.7%) than in summer (13.9%) seawater (Table 1). The geographic distances between sampling sites also had an effect on bacterioplankton community structure and, by themselves, explained 7.8% of winter community structure and 2.3% of summer community structure (Table 1). The VPA results were further tested by redundancy analysis (RDA)-based partial permutation tests, and the results showed that in both seasons, bacterioplankton community structures across the thermal gradient were

**TABLE 1** Variance partitioning analysis for BCCs<sup>a</sup>

Factor(s)	% of variation in BCC <sup>b</sup>	
	Winter	Summer
Pure E	32.7	13.9
Pure G	7.8	2.3
Mixed effect	25.5	22.2
Residuals	34.0	61.6

<sup>a</sup>E, environmental variables; G, geographic distance between sampling sites; mixed effect, mixed effects of geographic distance and environmental variables; residuals, unexplained components. The PCNM (principal coordinates of neighborhood matrix) method was performed to transform geographic distances between any pair of sites within S1, S2, S3, and S4 into 7 principal coordinates (from pcnm1 to pcnm7) in each season.

<sup>b</sup>The best subset of environmental parameters (winter: temperature; summer: temperature and dissolved oxygen) and geographic distance (winter: pcnm1 and pcnm4; summer: pcnm1 and pcnm7) in the VPA was chosen by the BIOENV procedure.

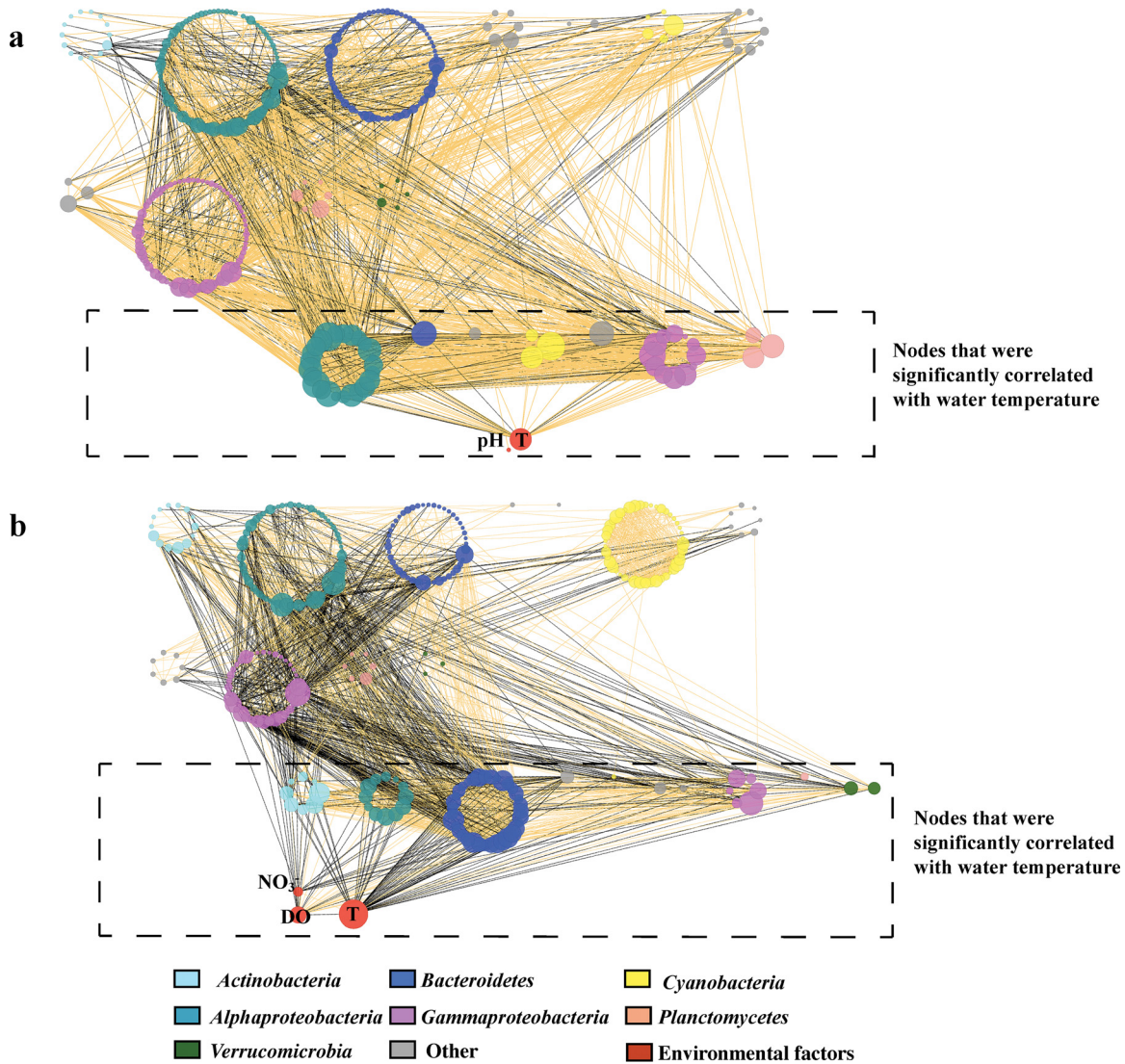


**FIG 4** (a and b) Redundancy analysis (RDA) of bacterioplankton community compositions (BCCs) and the environmental factors examined in winter (a) and summer (b) seawater. The environmental factors that correlated significantly with BCCs are shown in red. (c and d) Correlations between Bray-Curtis dissimilarities in BCC and temperature differences, determined by pairwise comparisons of sampling sites, in winter (c) and summer (d) seawater.

significantly explained by purely environmental factors ( $F = 7.192$ ,  $P < 0.001$  for winter;  $F = 9.495$ ,  $P < 0.001$  for summer) rather than by geographic distances only ( $F = 1.024$ ,  $P > 0.05$  for winter;  $F = 1.152$ ,  $P > 0.05$  for summer) (Table 1). A larger portion of community structure could not be explained by the selected environmental factors and geographic distances in summer (61.6%) than in winter (34.0%) seawater (Table 1). In addition, strong mixed effects of the selected environmental factors and geographic distances (25.5% for winter; 22.2% for summer) were detected (Table 1).

We found that in each season, the seawater temperature was a key environmental factor in determining bacterioplankton community structure across the thermal gradient (Fig. 4a and b) and that in any pairwise comparison of sampling sites, the Bray-Curtis dissimilarities in BCC correlated positively with temperature differences ( $R^2 = 0.506$ ,  $P < 0.001$  for winter;  $R^2 = 0.355$ ,  $P < 0.001$  for summer [Fig. 4c and d]). However, the regression coefficient for the correlation between the BCC dissimilarities and the temperature differences was significantly higher for seawater in summer than in winter ( $P < 0.01$ ) (Fig. 4c and d).

**Shifts in bacterioplankton network structure.** OTU co-occurrence patterns and their relationships with environmental factors were investigated by co-occurrence network analysis (Fig. 5). A network of 332 nodes and 2,183 links was obtained for winter BCCs (Fig. 5a), and the network was fragmented into 8 components (Table 2). The summer BCCs resulted in a network of 314 nodes and 2,226 links that was fragmented into 4 components (Fig. 5b; Table 2). The observed networks of both the winter and summer bacterioplankton communities were significantly different from the corresponding random networks in the topological properties of average shortest path length, clustering coefficient, and topological coefficient ( $P, < 0.05$  in all cases). The observed-versus-random network clustering coefficient ratio (log response ratios, 0.99 for the winter network and 0.94 for the summer network) showed that the connectivity of the two networks fitted the power law model well and that the two networks were scale free. In addition, the observed clustering coefficients and characteristic path lengths were both greater than those of the corresponding random networks with the



**FIG 5** Co-occurrence network structures in winter (a) and summer (b) seawater. The networks are visualized with group attribute layouts based on phylum or subphylum. Nodes that were significantly correlated with water temperature are shown in dashed boxes. The colors of the nodes indicate different phylum or subphylum OTUs, as shown in the key at the bottom. An orange line indicates a positive interaction between two OTUs (nodes), while a black line indicates a negative interaction.

same network nodes and edges (Table 2), indicating that the networks of both the winter and summer BCCs showed small-world characteristics. In both seasons, the bacterioplankton communities tended to be copresent more than would be expected by chance; however, fewer copresence links and more exclusion links were observed in the co-occurrence network for the summer than in that for the winter (Table 2). The keystone species OTUs (depicted as nodes with a high degree in the network) differed greatly between the winter and the summer networks (see Fig. S3 in the supplemental material). The top 50 keystone OTUs belonged mainly to the phyla *Alphaproteobacteria* and *Gammaproteobacteria* in the winter network and were affiliated with the phyla *Bacteroidetes* and *Alphaproteobacteria* in the summer network (Fig. S3).

For both the summer and winter networks, temperature was among the most connected nodes. The subnetwork built around temperature showed that more OTUs correlated significantly with the temperature in seawater in the summer than in the winter (Fig. 5; see also Fig. S4 in the supplemental material). In the winter subnetwork, temperature tended to have positive correlations with OTUs (37 positive links; 46 total links), including the classified clades belonging to the phyla *Alphaproteobacteria* (12

**TABLE 2** Major topological properties of the empirical bacterioplankton co-occurrence networks in seawater in winter and in summer and their associated random networks

Parameter	Winter		Summer	
	Observed	Random	Observed	Random
No. of original OTUs	500		500	
No. of nodes	332	332	314	314
No. of edges	2,183	2,183	2,226	2,226
Avg no. of neighbors	13.15	13.15	14.18	14.18
Clustering coefficient	0.407	0.041	0.384	0.044
Betweenness centrality	0.0087	0.0047	0.016	0.0047
Closeness centrality	0.308	0.394	0.254	0.407
Network diam	11	4	14	4
Network radius	1	3	1	3
Network centralization	0.155	0.039	0.183	0.038
Network density	0.04	0.04	0.045	0.045
Characteristic path length	3.86	2.539	4.784	2.461
Network heterogeneity	1.122	0.271	0.952	0.264
Connected components	8	1	4	1
Log response ratio	<b>0.99</b>		<b>0.94</b>	
Copresence links	<b>1,696</b>		<b>1,429</b>	
Exclusion links	<b>487</b>		<b>797</b>	
Exclusion links/total links	<b>0.22</b>		<b>0.36</b>	

positive links; 20 total links), *Gammaproteobacteria* (9 positive links; 11 total links), and *Planctomycetes* (3 positive links; 3 total links) (Fig. S4). However, in the summer subnetwork, temperature tended to have negative correlations with OTUs (61 negative links; 68 total links), including the classified clades belonging to the phyla *Actinobacteria* (8 negative links; 8 total links), *Bacteroidetes* (26 negative links; 27 total links), *Alphaproteobacteria* (8 negative links; 11 total links), and *Gammaproteobacteria* (4 negative links; 6 total links) (Fig. S4). In the subnetworks that included only temperature-related and unrelated nodes, fewer copresence links and more exclusion links were observed in seawater in the summer than in the winter (Fig. 5; see also Table S2 in the supplemental material). By removing the OTUs that had significant correlations with temperature from the networks, subnetworks were generated (see Fig. S5 in the supplemental material), and these showed fewer copresence links and more exclusion links in seawater in the summer than in the winter (Fig. S5 and Table S2).

## DISCUSSION

The thermal effluents from two nuclear power plants in Daya Bay have been present for more than 20 years and have generated comparatively stable and long thermal gradients (a temperature increase from 0 to 10°C) in the discharge areas. The environmental patches across thermal gradients are heterogeneous and very strongly interconnected on a microbial scale; thus, this is a useful model for the study of the metacommunity structure and processes of bacterioplankton. Our results showed that the bacterioplankton community structure was highly heterogeneous across the thermal gradients and between winter and summer. Using a metacommunity approach, we found that the spatial BCC patterns in the thermal discharge area were significantly explained by purely environmental factors rather than by purely spatial factors, suggesting that species sorting had a strong effect on bacterioplankton assembly. However, this species-sorting effect was weaker in summer than in winter seawater. Co-occurrence network analysis revealed that bacterioplankton communities coexisted more (copresence links) than expected by chance in both seasons; however, the exclusion links/total links ratio was higher in summer seawater than in winter seawater.

**Thermal effluents had significant impact on bacterioplankton community structure; however, the effect differed by season.** High heterogeneity of bacterioplankton community structure was detected across the thermal gradients and between seasons, even at coarse levels of taxonomic resolution, reflecting the different environmental optima of bacterioplankton taxa across the thermal gradients and between



seasons (34–37) or differences in the intensity of water warming (16). In findings similar to our winter results, decreased relative abundances of *Alphaproteobacteria* and *Gammaproteobacteria* in warmer waters were also observed by Xiong et al. (16) and Von Scheibner et al. (38) in investigations of water temperatures from about 20°C to 30°C. In addition, we showed that high temperatures of <38°C promoted the relative abundance of *Cyanobacteria* (primarily *Synechococcus*), supporting previous observations that temperature was an important factor influencing *Synechococcus* distribution and was positively correlated with the abundance of *Synechococcus* when the temperature was in an adaptive range (39, 40). In summer seawater, we found lower percentages of *Actinobacteria* at sites with more thermal effluents, which was probably related to the obviously increased relative abundance of *Cyanobacteria*. This finding is consistent with previous reports showing negative relationships between the relative abundances of *Cyanobacteria* and *Actinobacteria* in aquatic ecosystems (19, 41). Furthermore, *Bacteroidetes* have been found to be likely to attach to marine eukaryotic algae or animals (42, 43). The decreased abundances of eukaryotic algae and zooplankton under thermal impacts in our study area (2, 3, 8) might therefore be one of the reasons for the lower relative abundances of *Bacteroidetes* at sites with higher levels of effluents in summer seawater.

Among all the environmental factors examined, the spatially structured temperature created by thermal effluents played the strongest role in determining bacterioplankton metacommunity structure across thermal gradients in both seasons studied. Differences in traits cause different bacterial populations to have different sensitivities to temperature during their growth processes (44–47); thus, temperature is one of the most important factors in shaping bacterioplankton community structure (19, 35, 48). This finding was further confirmed by the positive linear correlations between BCC dissimilarities and temperature differences in any pairwise comparison of sampling sites. A higher regression coefficient was also observed for summer seawater than for winter seawater, indicating that summer bacterioplankton communities showed higher turnover in response to temperature and thus were more sensitive to thermal impacts than winter bacterioplankton communities. This finding might be related to the fact that to keep up with the increased rates of biological activity (for example, colonization, extinction, reproduction, and dispersal) created by faster metabolic kinetics in warmer environments (i.e., summer versus winter coastal ecosystems), aquatic organisms must maintain higher turnover at higher temperatures (11, 49).

In a network analysis, we found that temperature had more positive correlations with OTUs in winter seawater and showed more negative correlations with OTUs in summer seawater. In Daya Bay, the surface water temperature is >25°C during most of the year, reaching a minimum of 14°C in January and a maximum of 32.8°C in July (50). Because of the adaptation of local bacterioplankton communities to the ambient temperature (51), most bacterioplankton may have an optimum temperature near the average water temperature of Daya Bay. In the winter, the background sea temperature in the bay was 21°C, and increased water temperatures caused by thermal effluents might promote the growth of most bacterioplankton taxa. In the summer, the ambient seawater temperature was 31°C, which might be higher than the optimum temperature of many bacterioplankton taxa; thus, the increased water temperatures caused by thermal effluents might stress and inhibit the growth of more bacterioplankton taxa (52).

**BCC spatial patterns were predominantly shaped by species sorting, but this effect was weaker in summer than in winter seawater.** We found significant heterogeneity in the BCCs across the thermal gradients in both seasons, suggesting that in the thermal discharge area, i.e., where water is frequently exchanged with the surrounding seawater (current velocity, 0.05 to 0.10 m/s [28, 29]), the high dispersal rates of bacterioplankton communities between specific habitats and the surrounding seawater did not induce mass effects with BCC homogenization across thermal gradients. The VPA revealed that in each season, the BCC variations across the thermal gradient were significantly explained by purely environmental factors rather than by

geographic distances only, suggesting that species sorting played a more important role than the spatially dependent dilution role of surrounding seawater on the thermal effluent water in generating BCC spatial patterns across thermal gradients. Previous studies suggest that the strong effect of species sorting is most likely due to the extremely high population growth rates of bacteria (21, 30). Studies of currents that focused on the dynamics of bacterioplankton community structure have confirmed that BCCs can rapidly track changes in the environment (53–55). Therefore, the persistence of newly arrived bacterioplankton populations that migrated via flowing water was more likely to be determined by species sorting of heterogeneous patches than by mass effects (56). This result was in accordance with recent studies that revealed that bacterial spatial distributions were more closely linked to local environmental characteristics than to purely spatial factors (30, 57).

Our results showed that across thermal gradients, purely environmental factors explained a smaller portion of bacterioplankton community structure in summer than in winter seawater, suggesting weaker species sorting of bacterioplankton metacommunity processes in summer than in winter seawater. A larger portion of the BCC variation could not be explained by the selected environmental factors and geographic distances for summer than for winter seawater, suggesting that bacterioplankton assembly might be more stochastic and difficult to predict on the basis of the environmental and spatial factors examined in summer than in winter seawater. This result was further confirmed by linear correlations of the BCC Bray-Curtis dissimilarities with temperature differences between any pair of sampling sites, which show a lower correlation coefficient for summer correlations than for winter correlations. Previous studies showed that the exponential increase in the metabolic rate as the temperature increases affects nearly all biological processes (11, 49). Thus, in the two seasons studied, high temperatures in summer seawater may simply increase the stochasticity of the colonization and extinction of bacteria across thermal gradients. Moreover, the higher level of disorder in biological and environmental conditions created by faster metabolic kinetics and irregular molecular movement in summer seawater may also contribute to the enhanced stochasticity of the bacterioplankton assembly (11, 19, 58) and make bacterioplankton assembly more difficult to predict on the basis of environmental and geographic factors in summer than in winter seawater. Our results also revealed strong mixed effects of temperature and geographic distance in both seasons, emphasizing that marine bacterioplankton assembly was determined by spatially structured environmental gradients (16, 59).

**Niche sharing dominated the processes of species sorting, but the relative importance of niche segregation was enhanced in summer seawater.** Co-occurrence networks allow a deeper analysis of the ecological processes structuring microbial communities, such as neutral processes and species sorting (e.g., niche sharing and segregation) (32, 33). Our results showed that the observed co-occurrence networks of both the winter and summer bacterioplankton communities were remarkably different from the corresponding random networks, suggesting that species sorting plays a more important role than neutral processes in shaping marine bacterioplankton assembly. This finding was in agreement with our VPA and other evidence in recent studies (19, 33). Our study also revealed that bacterioplankton communities coexisted more (copresence links) than expected by chance in both seasons. Copresence links could be caused by niche sharing because of ecological interactions, such as facilitative or mutual attraction, while exclusion links could result from niche segregation due to competition interactions (32, 60). In the thermal discharge seawater we studied, co-occurrence bacteria tended to show more niche sharing than niche segregation in both winter and summer seawater, suggesting that although co-occurrence taxa were more likely to share nutritional resources and thus compete more, they tended to co-occur frequently, probably because they also shared other traits (e.g., cross-feeding, coaggregation, or cocolonization) that allowed them to survive together.

The summer seawater temperature was higher than the optimum temperature of many bacterioplankton taxa, and the increased temperatures caused by thermal efflu-

ents might inhibit the growth of many heat-sensitive taxa (52). Therefore, the roles of niche segregation between heat-sensitive and -insensitive OTUs might be enhanced in summer seawater (52). This inference was further confirmed by the co-occurrence network analysis, which showed a higher ratio of negative correlations between the relative abundances of the temperature-related and non-temperature-related OTUs in the summer network than in the winter network. This finding probably reveals one of the reasons for the higher sensitivity of bacterioplankton communities to temperature in summer seawater than in winter seawater. Moreover, in the subnetworks excluding temperature-related OTUs, a higher ratio of negative correlations between the relative abundances of OTUs was also observed for summer seawater than for winter seawater, suggesting that in addition to temperature, other factors (e.g., resources) might also have had a species-sorting effect on bacterioplankton assembly and resulted in niche segregation between co-occurrence OTUs in summer seawater. In the bay we studied, where there is no shortage of nutrients, since it has been a typical aquaculture area for years (50), bacterioplankton growth may not be constrained by resources in the winter. However, in the summer, with further increases in the water temperature and higher metabolic rates of bacterioplankton communities, resource consumption and demand probably both continued to increase and likely resulted in the insufficiency of certain resources for the growth of some bacterioplankton in higher-temperature habitats (58). Therefore, the roles of niche segregation between resource-sensitive and -insensitive OTUs might be enhanced in summer seawater (61). With the increased roles of niche segregation, co-occurrence bacterioplankton OTUs in summer seawater tended to have fewer copresence links and more exclusion links than those in winter seawater.

**Conclusion.** Our results suggest that in the thermal effluent area, i.e., where water is exchanged very frequently with the surrounding seawater and thermal effluent water, the spatial patterns of marine BCCs were significantly shaped by species sorting rather than by mass effects or by the dilution of thermal effluent water by the surrounding seawater. However, this species-sorting effect was weaker in summer than in winter seawater. In addition, bacterioplankton metacommunity structure was predominately shaped by niche sharing in both seasons, but the niche segregation roles were enhanced in summer community assembly. Finally, we propose that with the enhanced niche segregation between heat-sensitive and -insensitive taxa and the increased stochastic assembly processes in summer seawater, bacterioplankton communities were more sensitive to thermal impacts and were more difficult to predict in summer than in winter seawater in the face of different intensities of thermal impacts.

## MATERIALS AND METHODS

**Study area.** Our study area, Daya Bay, is located in the northern South China Sea (22.5°N to 22.9°N, 114.5°E to 114.9°E) between Shenzhen and Huizhou in Guangdong Province near Hong Kong (Fig. 1). The region of Daya Bay is characterized by a subtropical climate. The area of Daya Bay is 650 km<sup>2</sup> at flood tide, and its depth is between 6 and 15 m. The surface water salinity ranges from 22 to 33, and the temperature ranges from 15°C to 32°C (2, 8, 27). The bay is dominated by an irregular semidiurnal tide with a narrow tidal range. The mean wind velocity is 4.4 m/s in winter and 4.8 m/s in summer (8, 27). The two nuclear power stations are located in Dapeng Cove, which is an important maricultural area (27). The 1,800-MW Daya Bay Nuclear Power Station (DNPS) has operated since 1994 and discharges heated water at a rate of  $2.9 \times 10^7$  m<sup>3</sup>/year (3). Another nuclear power station, the Lingao Nuclear Power Station (LNPS), which is located near the DNPS, has been in operation since 2002. In the cooling system, cooling water is drawn from intake points in a coastal water body. After going through the cooling systems, heated water is released back into the same water body at a unified outlet site away from the intake points. The velocity of thermal effluents at the outlet site is about 2.0 m/s (3, 28, 29). By the influence of currents, the velocity of effluents is slowed down, with a mean velocity of about 0.08 m/s in the discharge area (3, 28, 29). Thermal effluents elevate the temperature of the receiving water body and significantly affect the ecosystems in Daya Bay (2, 27).

**Water sampling and chemical determination.** In our study area, Daya Bay, the warmest months in the year of sampling (i.e., 2017) included June, July, August, and September, with an average high temperature of 31.0°C; the coldest months were January, February, and December, with an average high temperature of 21.0°C. Samples were collected on 7 January and 28 July 2017, which were typical winter and summer days in the subtropical bay with water temperatures of 21.0°C and 31.0°C, respectively. For this study, we set five sampling sites for each season. Four sites were located along the direction of the thermal effluent (winter: WS1, WS2, WS3, and WS4; summer: SS1, SS2, SS3, and SS4), and the remaining site (winter, WS5; summer, SS5) was set as a control and was located in the inflow area of the cooling

system upstream of the thermal effluents (Fig. 1). In winter and summer, the site layouts were inconsistent due to the differences in hydrologic conditions (e.g., higher tide level and higher current velocity in winter than in summer seawater [28, 29]). At each sampling site, we obtained three replicates. The maps of the sampling sites were generated based on an open-access Google satellite map using the ggmap package (<https://github.com/dkahle/ggmap>) in the R statistical computing environment (<https://www.R-project.org>) (Fig. 1). A 5-liter surface seawater sample was collected at each replicate site and was filtered through 0.2- $\mu\text{m}$ -pore-size Isopore filters (Millipore, Billerica, MA, USA). Before the experiments, the filters were unsealed and UV sterilized in a clean bench. The filters were stored at  $-70^{\circ}\text{C}$  until further analyses of the bacterioplankton communities. The water temperature, dissolved oxygen, pH, and salinity were measured in the field. Approximately 500 ml of surface water was collected for the nutrient analysis. Ammonium nitrogen ( $\text{NH}_4^+$ ), nitrate nitrogen ( $\text{NO}_3^-$ ), soluble reactive phosphate ( $\text{PO}_4^{3-}$ ), and silicate ( $\text{SiO}_3^{2-}$ ) levels (all measured in micromoles per liter) were determined using a UV-visible spectrophotometer (UV2450; Shimadzu, Tokyo, Japan) according to marine monitoring specifications (62). Total organic carbon (TOC) was measured using a TOC analyzer (TOC-V<sub>CPH</sub>; Shimadzu, Tokyo, Japan) according to a standard procedure (62).

**DNA extraction, amplification, sequencing, and data processing.** Genomic DNA was extracted from the biomass collected on the filters using a PowerWater DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) and was purified using a PowerClean DNA clean-up kit (MoBio Laboratories, Carlsbad, CA, USA). DNA was quantified, and its quality was determined, using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The V3 and V4 hypervariable regions of bacterial 16S rRNA genes were amplified with primers F341 (5'-CCTACGGGAGGAGCAGCAG-3') and R806 (3'-GGACTACHVGG GTWTCTAAT-5'). To pool multiple samples in one Illumina sequencing run, a unique 12-mer tag was added to the 5' end of each primer for each DNA sample. Three replicates of each sample were PCR amplified in a 50- $\mu\text{l}$  reaction mixture, which contained 25  $\mu\text{l}$  2 $\times$  PCR Premix *Taq*, 10 mM each primer, 60 ng of genomic DNA, and 20  $\mu\text{l}$  of nuclease-free water. Cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were visualized on 1% agarose gels, and the positive amplicons were quantified using the PicoGreen dsDNA assay kit (Invitrogen Corporation, Carlsbad, CA, USA) and were equally combined and purified with Zymo's Genomic DNA Clean & Concentrator kit (Zymo Research Corporation, Irvine, CA, USA). Finally, amplicons were sequenced using the Illumina HiSeq 2500 platform.

Raw reads of the 16S rRNA gene sequences were processed using the mothur software package (version 1.30.0, 2013) (<http://www.mothur.org>) according to the MiSeq standard operating procedure (63). In brief, the raw reads were combined, denoised, trimmed, quality-filtered, and aligned to the SILVA databases (64) using mothur. After initial processing, the high-quality sequences were clustered into OTUs at a 97% similarity level. Each of the representative OTU sequences was classified using the SILVA databases at the recommended bootstrap threshold of 80% (65). To minimize bias caused by sequencing depth, all singletons and OTUs occurring in only one sample were excluded from the OTU table. To correct for differences in sequencing depth, the minimum number of sequences in the whole sample (i.e., 40,851 sequences per sample) was randomly subsampled for analyses of the following metrics described below.

**Network analysis.** In each season studied, we selected the 500 most abundant OTUs, which accounted for 88.2% of the total winter sequences and 83.9% of the total summer sequences, for the performance of co-occurrence network analyses. We removed OTUs present in less than one-third of the samples, because they could cause artifactual associations (32). By using the Cytoscape plugin CONET (<http://psbweb05.psb.ugent.be/conet/>), significantly positive and negative interactions were identified based on an ensemble approach that combined five different measures: Bray-Curtis and Kullback-Leibler dissimilarities, Pearson and Spearman correlations, and mutual information (32). These five similarity measures were computed to cover a wide range of relationships (e.g., linear or nonlinear), to address noise and outliers, and to reduce the impact of choosing a single measure (32). For each measure, we requested the 1,000 top positive and negative edges in the "threshold-setting menu" (32). *P* values were further computed from method- and edge-specific permutations and bootstrap score distributions with 1,000 iterations each. Measure-specific *P* values were merged using Brown's method and were corrected for multiple testing by the Benjamini-Hochberg method. The unstable edges with scores outside the 0.95 range of their bootstrap distribution were removed. Co-occurrence networks were further visualized by Cytoscape, version 3.5.1, with a group attribute layout based on the phylum or subphylum (66). The subnetworks of nodes (OTUs) that had nonsignificant correlations with the seawater temperature were also generated. The topology of the resulting undirected network was investigated using the implemented tool network analyzer of Cytoscape (67) and was compared to an Erdős-Rényi random network of similar size, which was calculated by the implemented tool Network Randomizer, version 1.1.3.

**Statistical analyses.** To assess the significant differences in the environmental characteristics and the relative abundances of phyla and of lineages and clades across the thermal gradients and between seasons, we conducted one-way ANOVA followed by *post hoc* comparisons and Student *t* tests using the stats package in R. A detrended correspondence analysis (DCA) and permutational multivariate analysis of variance using distance matrices (PERMANOVA) were performed to test the significant differences in the BCCs across the thermal gradient and between seasons using the vegan package in R. Using the lmpPerm package in R, analysis of covariance (ANCOVA) with permutation tests was used to test the differences between the summer and winter regression lines formed between BCC dissimilarities and temperature differences for any pair of sampling sites.

The PCNM (principal coordinates of neighborhood matrix) method was used to transform geographic distances between any pair of sites within S1, S2, S3, and S4 into rectangular data that were suitable for constrained ordination or regression. A partial redundancy analysis (RDA)-based variance partitioning analysis (VPA) was carried out to partition the variations in BCCs across sites S1, S2, S3, and S4 into purely environmental, purely geographic distance-related, mixed geographic distance- and environment-related, and unexplained components (30, 31). The best subset of environmental parameters and geographic distance in the VPA was chosen by the BIOENV procedure. The VPA results were further tested by RDA-based partial permutation tests. To further identify the key environmental factors that significantly explained the BCC pattern across the thermal gradient, RDA was performed. The models in RDA were validated by analysis of variance. PCNM, VPA, BIOENV procedure, RDA, and RDA-based partial permutation tests were all carried out in the vegan package of R. The top 50 keystone OTUs and the OTUs that had significant links to the seawater temperature were visualized by heat maps using the phyloseq package in R.

**Accession number(s).** The sequence data were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (<https://www.ncbi.nlm.nih.gov/sra>) under accession number [SRP158638](https://www.ncbi.nlm.nih.gov/sra/SRP158638).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02088-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 1.4 MB.

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