

Inter-taxonomic differences in bacterioplankton community assembly in oligotrophic mountain lakes of east Japan

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ABSTRACT: We examined bacterioplankton community composition (BCC) in 27 oligotrophic freshwater lakes with similar environments, located in the mountainous regions of eastern Japan. We determined BCC by the relative sequence abundance of each taxon using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) targeting the 16S rRNA gene region. Using multiple regression analyses, we investigated the relative importance of environmental and geospatial processes on community assembly of the general bacterioplankton, as well as of the taxonomic groups *Betaproteobacteria* and *Bacteroidetes*. Both processes had significant relationships with community assembly. In particular, environmental processes were relevant, however small the variations among study sites. Community composition of *Betaproteobacteria* was mainly correlated with environmental factors, whereas that of *Bacteroidetes* was primarily associated with geospatial variables, such as the presence of Tsugaru Strait as a geographic barrier. Thus, inter-taxonomic differences are significant in assessing relationships of environmental and geospatial processes with freshwater BCC. Finally, our study provides evidence that biogeographic events are potentially significant for the distribution and diversity of microorganisms, as is the case with macroorganisms. These perspectives should be taken into consideration in order to fully understand bacterial community assembly.

KEY WORDS: Dispersal limitation · Environmental filtering · Geographic barrier · Metacommunity · Microbial biogeography

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INTRODUCTION

Historically, most microbial ecologists have considered species sorting to be essential in predicting geographic patterns of aquatic microorganisms, as it drives community assembly according to local abiotic factors. This approach is based on the prevailing hypothesis that 'everything is everywhere, but the environment selects' (Baas-Becking 1934). Indeed, the relationships of environmental factors with the diversity of aquatic microorganisms have been demonstrated in a number of studies (e.g. Van der Gucht et al. 2007). However, several studies have

highlighted the importance of other processes, such as dispersal (Reche et al. 2005, Yannarell & Triplett 2005).

Recently, species sorting and other processes have been integrated into a framework of microbial community assembly (Lindström & Langenheder 2012) that is based on concepts of microbial biogeography (Martiny et al. 2006) and metacommunity (Logue et al. 2011). Dispersal, which has been labeled a 'geospatial process,' plays a pivotal role in this framework. Thus, a microbial community can be structured by mass effect when the dispersal rate is high, so that inferior competitors are saved from competitive exclu-

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sion by frequent immigration (Mouquet & Loreau 2002). In contrast, if the dispersal rate is low, a microbial community is principally regulated by factors that limit dispersal, such as distance. Furthermore, species sorting as an 'environmental process' can be a predominant force under an intermediate dispersal rate (Lindström & Langenheder 2012). Therefore, environmental and geospatial processes play fundamentally different but complementary roles in shaping aquatic microbial communities (Langenheder & Ragnarsson 2007, Langenheder & Székely 2011, Adams et al. 2014).

However, previous studies examining the relative importance of environmental and geospatial processes have presented remarkably divergent and confusing results (e.g. Van der Gucht et al. 2007, Schiaffino et al. 2011). Thus, several aspects overlooked in previous studies should be addressed in order to draw general conclusions. One of the most essential of these aspects is the difference among microbial taxonomic groups in the expression of geospatial processes. For example, inter-taxonomic differences in long-distance mobility had not been investigated at the macro scale until recently. Indeed, dispersal by attachment to small particles such as dust and aerosol has been considered an important mechanism of bacterial migration to new habitats over long distances (Smith et al. 2011, Yamaguchi et al. 2012, Favet et al. 2013). During dispersal, bacteria are exposed to harsh environmental conditions including extreme desiccation and intense UV irradiation. Hence, there is a need for physiological characteristics that facilitate survival in these conditions (Kellogg & Griffin 2006); these characteristics can potentially differ among taxonomic groups (Zenoff et al. 2006, Dartnell et al. 2010). Taken together, previous studies imply differences in dispersal ability per se among microbial taxonomic groups, but these inter-taxonomic differences have not been extensively studied (Barberán & Casamayor 2010, Székely & Langenheder 2014).

Another important aspect is the presence of geographic barriers (Lomolino et al. 2010). To date, the role of geographic barriers in community assembly has rarely been investigated (Schauer et al. 2010), owing to the popular assumption that microorganisms have extremely high dispersal ability (Smith et al. 2011, Yamaguchi et al. 2012, Favet et al. 2013). Indeed, various identical nucleotide sequences have often been detected in comparable environments worldwide (Hahn 2003, Martiny et al. 2006). However, Whitaker et al. (2003) discovered local endemism in hyper-thermophilic archaea isolated from

hot springs in 3 different continents. This finding suggests that for some bacterial groups, geographic barriers are a significant factor limiting dispersal. Two recent studies attempted to explicitly test the significance of geographic barriers for bacterioplankton community assembly in freshwater environments (Drakare & Liess 2010, Schiaffino et al. 2011). Schiaffino et al. (2011) found that microbial community structures between lakes significantly diverged at an inter-continental scale in the presence of a geographic barrier, but Drakare & Liess (2010) did not detect any such phenomenon at a regional scale. These contradictory findings highlight the need to examine geographic barriers as a determinant of bacterial community assembly.

In this study, we evaluated the relationship of environmental and geospatial processes with bacterioplankton community assembly, using data from 27 lakes that we have previously investigated (Fujii et al. 2012). We focus on oligotrophic lakes with similar environments, because spatially structured taxonomic groups may be masked by the apparent importance of environmental processes when the variation in such processes is large. We also focus on *Beta-proteobacteria* and *Bacteroidetes*, both of which are predominant in freshwater environments worldwide (Zwart et al. 2002, Newton et al. 2011). These taxonomic groups exhibit many differences in ecological and physiological characteristics that determine environmental adaptability (Wu et al. 2006, Percent et al. 2008) and substrate availability (Burkert et al. 2003, Eiler & Bertilsson 2004, Kolmonen et al. 2004). Some lineages of *Betaproteobacteria* have been grouped into cosmopolitan clades in freshwater lakes, because nucleotide sequences from this group have been detected globally (Hahn 2003, Newton et al. 2011). In contrast, there are few freshwater cosmopolitan clades in *Bacteroidetes*, with the exception of the SOL cluster in family *Saprospiraceae* (Schauer et al. 2005). Such differences in geographical patterns may reflect differences in dispersal ability and environmental tolerance.

Here, we hypothesize that the relationship of environmental and geospatial processes with bacterial community assembly varies among taxonomic groups. The specific goals of this study were (1) to determine the relative importance of geospatial variables on bacterioplankton community assembly within a narrow range of environmental variables, (2) to examine the differences in the significance of geospatial variables among taxonomic groups, and (3) to evaluate the association of geographic barriers with bacterioplankton community composition (BCC).

MATERIALS AND METHODS

Study sites

We investigated 41 freshwater lakes in the mountainous region of eastern Japan, located between latitudes 36° 3' and 43° 41' N, with altitudes ranging from 419 to 2700 m (Fujii et al. 2012). Samples from each lake were collected once in either July or August of 2005 and 2006. Of these 41 lakes, we chose 27 oligotrophic ones (Fig. 1) where the total phosphorus (TP) concentration was less than $10 \mu\text{g P l}^{-1}$, and which were similar in other aspects, including pH, electric conductivity (EC), dissolved organic carbon (DOC), and chlorophyll *a* (chl *a*) concentration (Fujii et al. 2012). These lakes are located at Honshu Island and Hokkaido Island, which are the main islands in Japan and are located approx. 20 km apart on opposite sides of Tsugaru Strait (Fig. 1). Tsugaru Strait is a known key geographic barrier restricting the distribution of flora and fauna in eastern Japan (Dobson 1994). The geographic distance between lakes ranges from 40 m to 997 km.

Sample collection

Water temperature, pH, and EC were measured at the deepest point in each lake (Fujii et al. 2012). At

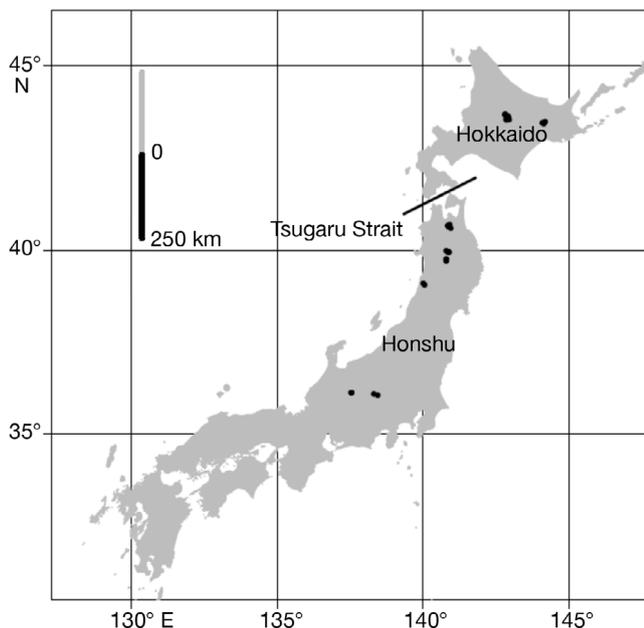


Fig. 1. Map of the 27 lakes investigated (filled circles) in Honshu and Hokkaido in eastern Japan, which are separated by Tsugaru Strait, a geographic barrier. For some of the adjacent lakes, the circles overlap each other

the same location in the lake, water samples were collected a maximum of 3 times at different depths in isothermal lakes, or at 3 depths above the thermocline in stratified lakes. Duplicate samples were collected at each depth, and pooled. One portion of these samples was used to measure chl *a*, DOC, and TP, while the other part was filtered, using a sterile $0.22 \mu\text{m}$ pore size Sterivex-GP filter (Millipore), to collect planktonic microorganisms. Post-filtration, filters were immediately immersed in RNAlater (Ambion) and stored at 4°C until DNA extraction.

BCC analysis

A subset of a dataset in our previous publication (Fujii et al. 2012) was used as BCC data for this study. Briefly, the dataset was generated using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), followed by gel imaging and sequencing. Unique band positions were considered operational taxonomic units (OTUs), since, in theory, such a band position should contain identical sequences derived from the same taxon. To quantitatively evaluate BCC, the relative intensity of each band was used to estimate the relative abundance of each OTU, which, in turn, is a measure of the dominance of the taxonomic group. We also sequenced DGGE bands to define the phylogenetic affiliation of each OTU using the RDP (ribosomal database project) Classifier (Wang et al. 2007), with a bootstrapping classification confidence threshold of 80%. In cases where more than one sequence was observed in the same band position, the phylogenetic affiliation of the most intensive band was selected to represent the OTU. Nucleotide sequences were previously deposited in DDBJ/EMBL/GenBank (Fujii et al. 2012).

Statistical analyses

To examine the relative importance of environmental and geospatial variables on BCC, 2 hypotheses were tested: to what extent (1) physico-chemical variables (temperature, pH, EC, DOC, and chl *a*) and geographic distance between lakes are correlated with the dissimilarity of bacterioplankton communities, and (2) physico-chemical variables, geographic barriers (i.e. the Tsugaru Strait), and spatial coordinates (latitude and longitude) determine the composition of bacterioplankton communities. Among the physico-chemical variables, temperature, pH, and EC as a measure of salinity have strong influence on

various bacterial activities, including enzymatic activity, ATP synthesis, plasmic membrane stability, and osmoregulation. In contrast, DOC is a measure of organic carbon subsidized from surrounding terrestrial regions, and chl *a* is an index of primary production, both of which represent carbon available for bacterial growth. To avoid multiple collinearity and loss of degree of freedom, other variables were not included.

First, we used multiple regressions on distance matrices (MRM; Tuomisto et al. 2003) to measure how dissimilarity matrices among *Bacteria*, *Betaproteobacteria*, and *Bacteroidetes* correlated with environmental and spatial matrices. Dissimilarity matrices were calculated using the Bray-Curtis index, while environmental matrices were constructed from differences in physico-chemical variables. Spatial matrices simply consisted of geographic distances between lakes. A matrix of differences in sampling year was also included as a covariate to accommodate possible temporal changes. Matrices were transformed into standard normal distributions with mean 0 and standard deviation 1 to avoid differences in units. In MRM, multiple linear regressions of a response matrix are applied to explanatory matrices, where, as with ordinary multiple regressions, the coefficient of one explanatory variable is estimated by adjusting others (Lichstein 2007). Analyses were performed in the R language and environment for statistical computing (R Development Core Team 2013), using the package *ecodist* (Goslee & Urban 2007). Statistical significance of estimated coefficients was tested using permutation analysis.

Second, we performed permutational multivariate analysis of variance using distance matrices (PERMANOVA; Anderson 2001) to investigate association of environmental and geospatial variables with community composition of *Bacteria*, *Betaproteobacteria*, and *Bacteroidetes*. PERMANOVA fits linear models and partitions the sums of squares of multivariate data among sources of variation. Although PERMANOVA is analogous to parametric multivariate analysis of variance (MANOVA), it is an alternative to both MANOVA and ordination in describing how variation is attributed to different variables, and is robust to non-normality (Anderson 2001). In PERMANOVA models, matrices of the relative abundance of *Bacteria*, *Betaproteobacteria*, and *Bacteroidetes* were taken to be response variables. Physico-chemical

variables, region (Honshu and Hokkaido), spatial coordinates, and sampling year were used as explanatory variables. Significance was tested by permutation with pseudo-*F* ratios. Calculations were performed using the R package *vegan* (Oksanen et al. 2013).

RESULTS

Based on DGGE, 50 bacterial OTUs were present in samples obtained from 27 oligotrophic lakes. A total of 104 different bands were successfully sequenced, allowing phylogenetic affiliation to be assigned to each OTU using the RDP. Among the 50 bacterial OTUs, *Betaproteobacteria* were the most dominant with 20 OTUs, followed by *Bacteroidetes*, *Actinobacteria*, *Alphaproteobacteria*, and *Cyanobacteria* with 15, 2, 2 and 1 OTUs, respectively. The remaining 10 OTUs could not be classified.

MRM detected differences in the significance of environmental and spatial variables to the dissimilarity index of different taxonomic groups. The dissimilarity matrix for *Bacteria* and *Bacteroidetes* was significantly correlated with geographic distance and DOC (Table 1). In contrast, EC was the only significant predictor for communities of *Betaproteobacteria* (Table 1). Sampling year was significant for *Bacteroidetes* only. Temperature, pH, and chl *a* did not significantly affect BCC (Table 1). In terms of relative importance, the coefficient for DOC was highest among all variables for *Bacteria* and *Bacteroidetes* (Table 1). Geographic distance was third and second in *Bacteria* and *Bacteroidetes*, respectively (Table 1). For *Betaproteobacteria*, the contribution of EC was largest (Table 1).

PERMANOVA also demonstrated the varying relationship of environmental and geospatial variables

Table 1. Coefficients estimated from multiple regressions of dissimilarity matrices for *Bacteria*, *Betaproteobacteria*, and *Bacteroidetes*, with geographic distance (distance), temperature, pH, electric conductivity (EC), dissolved organic carbon (DOC), chlorophyll *a* (chl *a*), and sampling year (year). Significant *p*-values ($p < 0.05$) are highlighted in **bold**

Variable	<i>Bacteria</i>		<i>Betaproteobacteria</i>		<i>Bacteroidetes</i>	
	Coefficient	<i>p</i>	Coefficient	<i>p</i>	Coefficient	<i>p</i>
Distance	0.198	0.021	0.141	0.078	0.191	0.024
Temperature	0.128	0.196	0.079	0.418	0.145	0.165
pH	0.100	0.330	0.066	0.518	0.032	0.754
EC	0.244	0.036	0.389	0.005	-0.060	0.694
DOC	0.250	0.019	0.174	0.098	0.376	0.007
Chl <i>a</i>	0.092	0.407	0.095	0.390	-0.038	0.766
Year	-0.002	0.941	-0.052	0.102	0.084	0.029

with the composition of bacterioplankton communities, in general agreement with results from MRM. In *Bacteria*, region, longitude, temperature, pH, EC, and DOC were significantly correlated with community composition (Table 2). In contrast, *Betaproteobacteria* and *Bacteroidetes* communities were significantly affected only by EC and region, respectively (Table 2). Sampling year did not have any significant correlation. EC was the most important variable to explain the community composition of both *Bacteria* and *Betaproteobacteria*, based on coefficients of determination. The community composition of *Bacteroidetes* could be explained to the largest extent by region (Table 2).

DISCUSSION

Based on MRM and PERMANOVA, both environmental and geospatial processes were significant for BCC in general. This suggests that both play essential roles in bacterioplankton community assembly in oligotrophic mountain lakes in eastern Japan. However, geospatial processes may be less important, as indicated by higher coefficient estimates for environmental processes, where BCC of *Bacteria* was significantly explained by EC, temperature, DOC, and pH. Previous studies arrived at different conclusions about the significance of environmental and geospatial processes at the continental scale. For example, Schiaffino et al. (2011) reported that both processes were essentially equally important to BCC in oligo- and eutrophic lakes in Antarctica and Patagonia (>2100 km). In contrast, Van der Gucht et al. (2007)

demonstrated the significance of environmental filtering in a large number of meso- and eutrophic lakes in Europe (>2500 km), with marginal contribution from dispersal. Thus, environmental and geospatial processes appear to vary in significance, depending on environmental conditions, spatial configurations, and historical formation of study sites. A possible explanation might be differences in trophic state. In meso- and eutrophic lakes, contributions of geospatial processes might be masked due to environmental heterogeneity, such as a wide range in pH and organic matter content.

Our results also highlight the contrasting roles of environmental and geospatial variables on *Betaproteobacteria* and *Bacteroidetes*. In *Betaproteobacteria*, salinity had a strong correlation with BCC, in agreement with Wu et al. (2006). Further, species sorting may be the predominant force, since spatial variables were not significant. In contrast, spatial variables were consistently significant in *Bacteroidetes*. Thus, dispersal limitation may primarily govern the distribution of this group. Differences between groups indicate that the importance of environmental and geospatial processes should be examined for each group. However, the significance of both processes on the overall bacterial community may reflect the dominance of *Betaproteobacteria* and *Bacteroidetes*. Our results are markedly different from those of previous studies (Barberán & Casamayor 2010, Székely & Langenheder 2014). In those studies, environmental processes were found to be of greater significance to both taxonomic groups, whereas we found *Bacteroidetes* to be structured mainly by geospatial processes. The difference may be due to the range of environmental conditions examined.

We focused exclusively on oligotrophic lakes with little environmental variation, while previous studies addressed bacterial systems with large environmental fluctuations, where communities of different compositions may form from the same taxonomic groups.

BCC of *Bacteria* and *Bacteroidetes* was significantly different between Honshu and Hokkaido, in contrast to previous studies which reported that regional differences were caused by environmental changes correlated with geographic distance, rather than by geospatial processes per se (Yanarella & Triplett 2005, Van der Gucht et al. 2007, Schiaffino et al. 2011). Our

Table 2. Coefficients of determination calculated from permutational multivariate analyses of variance using community compositions of *Bacteria*, *Betaproteobacteria*, and *Bacteroidetes* for region (Honshu and Hokkaido), latitude, longitude, temperature, pH, electric conductivity (EC), dissolved organic carbon (DOC), chlorophyll *a* (chl *a*), and sampling year (year). Significant p-values ($p < 0.05$) are highlighted in **bold**

Variable	<i>Bacteria</i>		<i>Betaproteobacteria</i>		<i>Bacteroidetes</i>	
	Coefficient	p	Coefficient	p	Coefficient	p
Region	0.058	0.025	0.035	0.322	0.079	0.011
Latitude	0.026	0.582	0.019	0.755	0.058	0.093
Longitude	0.055	0.035	0.057	0.072	0.052	0.145
Temperature	0.056	0.025	0.056	0.060	0.047	0.206
pH	0.051	0.047	0.043	0.198	0.044	0.275
EC	0.133	0.001	0.187	0.001	0.053	0.123
DOC	0.053	0.044	0.043	0.165	0.051	0.150
Chl <i>a</i>	0.029	0.498	0.036	0.320	0.016	0.897
Year	0.035	0.282	0.020	0.741	0.043	0.260
Residuals	0.504		0.504		0.559	

findings are probably not artifacts observed in previous studies, because we focused only on oligotrophic lakes to exclude extreme environmental variation, and considered sampling year in multiple regressions. Thus, our data may provide evidence for a role of geographic barriers in the assembly of specific taxonomic groups. Generally, bacteria can disperse to other habitats via attachment to biotic and abiotic objects such as birds, aerosols, and lithospheric particles (Hervàs et al. 2009, Grossart et al. 2010). By investigating airborne bacteria, Hervàs et al. (2009) and Smith et al. (2013) found that dispersal ability was different among taxonomic groups, and observed that some *Betaproteobacteria* can be dispersed over large distances, unlike *Bacteroidetes*. Therefore, *Betaproteobacteria* may be less affected by geographic barriers. However, auto-correlations of environmental variables and other unknown environmental parameters might generally bias the analysis of spatial processes. This could plausibly explain contradictory reports on the occurrence and role of dispersal limitation.

On a different note, we should comment on the reliability of PCR-DGGE in examining BCC. Limited detectability of only 1% of the total population has been reported for this method (Muyzer et al. 1993). In addition, a single DGGE band occasionally contains sequences of multiple bacterial taxa, even at the same band position. In such a case, major bacterial taxa may mask minor ones. These limitations imply that our approach may examine only dominant bacterial species (Bent & Forney 2008). Thus, higher-resolution techniques such as next generation sequencing may be potentially more effective. However, the purpose of this study was to test the hypothesis that the relative importance of environmental and geospatial processes on bacterial community assembly varied according to taxonomic groups. For this purpose, PCR-DGGE was sufficient and appropriate, because it compares BCC in lakes at the same resolution.

In summary, this study showed that environmental and geospatial processes play complementary roles in the assembly of the overall bacterioplankton community, via niche partitioning and dispersal limitation. We demonstrated that the importance of environmental and geospatial processes differs among taxonomic groups. Finally, we discovered that biogeographic events are potentially associated with the distribution of some bacterial groups. These results highlight 2 key points toward understanding the assembly of bacterioplankton communities. (1) The possibility of ecological trade-off among taxonomic groups. In this study, *Betaproteobacteria*, which may

disperse easily, was restricted to a narrow range of environmental conditions, whereas *Bacteroidetes*, which may have lower dispersal ability, was more environmentally adaptable. This result indicates that important processes may be masked if taxonomic groups with distinct traits are not specified during analysis. (2) The ecological role of geographic barriers. In this study, the distribution and diversity of *Bacteroidetes* were uniquely determined by a geographic barrier, even at regional scale. Therefore, it is also possible that BCC will not be sufficiently defined if geographic barriers are overlooked. These perspectives should potentially contribute not only toward understanding microbial ecology, but also toward practical issues, such as management of airborne pathogens that disperse from their original habitat.

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