The ISME Journal (2017) 11, 613–625

www.nature.com/ismej

ORIGINAL ARTICLE

Warming and nutrient enrichment in combination increase stochasticity and beta diversity of bacterioplankton assemblages across freshwater mesocosms

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The current climate warming and eutrophication are known to interactively threaten freshwater biodiversity; however, the interactive effects on lacustrine bacterioplankton diversity remain to be determined. Here, we analyzed the spring bacterioplankton community composition (BCC) in 24 outdoor, flow-through mesocosms (mimicking shallow lake environments) under 3 temperature scenarios and 2 nutrient regimes. Our results revealed that neither long-term warming (8.5 years) nor nutrient enrichment had significant effects on bacterioplankton alpha diversity, whereas long-term enhanced warming (elevated 50% above the IPCC A2 climate scenario) and nutrient enrichment in combination increased bacterioplankton beta diversity. We also found that BCC shifted significantly under enhanced warming and nutrient-enriched conditions towards decreased relative abundances of *Actinobacteria*, *Bacteroidetes* and *Betaproteobacteria*, whereas the percentages of *Cyanobacteria*, total rare phyla and unclassified phyla significantly increased. Null-model tests indicated that deterministic processes played a more important role than stochastic processes in determining BCC. However, the relative importance of stochasticity, primarily ecological drift, was enhanced and contributed to the increased beta diversity of BCC under enhanced warming and nutrient-enriched conditions. Overall, our study suggests that the synergetic effects of warming and nutrient enrichment may result in high variability in the composition of bacterioplankton communities in lacustrine water bodies.

The ISME Journal (2017) **11,** 613–625; doi:10.1038/ismej.2016.159; published online 9 December 2016

Introduction

Biodiversity underpins ecosystem functioning. Determining the mechanisms that generate and maintain biodiversity is therefore important to understand the impacts of future global changes on ecosystems. It has been reported that the Earth has warmed by approximately 0.74 °C since 1850, and by the end of this century, global mean temperatures can increase 3.4 °C with a range of 2.0-5.4 °C under IPCC A2 scenario (Houghton *et al.*, 2001). This increase in mean global temperature gives rise to concern about the fate of biodiversity in various ecosystems in a future warmer climate. Shallow lakes are abundant and ecologically and economically significant in the global landscape (Wetzel, 2001), and freshwater biodiversity is particularly vulnerable to climate warming (Sala *et al.*, 2000; Moss *et al.*, 2011; Jeppesen *et al.*, 2009, 2014) as well as to other anthropogenic changes such as eutrophication (Moss *et al.*, 2011; De Senerpont Domis *et al.*, 2013). Bacterioplankton, an integral component of the planktonic food webs in shallow lake ecosystems, perform important tasks in regenerating nutrients and decomposing organic matter (Hall and Cotner, 2007; Ruiz-González *et al.*, 2015). Currently, studies of warming effects on the lacustrine bacterioplankton community have mainly focused on bacterioplankton growth traits, such as cell densities

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Received 18 February 2016; revised 22 September 2016; accepted 5 October 2016; published online 9 December 2016

(Baulch *et al.*, 2005), abundance (Christoffersen *et al.*, 2006; Markensten *et al.*, 2010), biomass (Hall *et al.*, 2009; Shurin *et al.*, 2012; Özen *et al.*, 2013), metabolism (Hall and Cotner, 2007; Hall *et al.*, 2008) and production (Durán *et al.*, 2016). Most of these studies suggest that warming, when interacting with eutrophication, enhances the growth of lacustrine bacterioplankton (Christoffersen *et al.*, 2006; Shurin *et al.*, 2012; Özen *et al.*, 2013). Nevertheless, to date, little attention has been paid to bacterioplankton diversity patterns and their underlying mechanisms in a changing climate scenario, despite its ecological significance.

The lacustrine bacterioplankton community composition (BCC) is affected by both deterministic and stochastic processes (for example, Anderson *et al.*, 2011; Newton *et al.*, 2011; Logares *et al.*, 2013; He *et al.*, 2014; Ren *et al.*, 2015). Niche-related (deterministic) theory assumes that BCC is shaped by deterministic factors such as environmental stability, habitat heterogeneity, ecosystem productivity and interspecific interactions (for example, Cavender-Bares *et al.*, 2009), whereas shaping of BCC by stochastic processes, including dispersal and ecological drift (Hanson *et al.*, 2012), supposes ecological equivalence among species (for example, Hubbell, 2001). It has been shown that at higher elevations, the lower water temperature and poorer nutrient conditions had stronger deterministic effects in shaping the bacterial community on the river stone surface than at lower elevations (Wang *et al.*, 2012). The effects of stochastic processes on the bacterial community assemblies might, however, be enhanced by warming due to the increased rates of biological activity (for example, colonization, extinction, reproduction and dispersal) created by faster metabolic kinetics in warmer environments (Allen *et al.*, 2002; Brown *et al.*, 2004). Owing to synergistic effects, bacterioplankton assembly processes (deterministic vs stochastic) might be affected even more if the systems are subjected to both warming and nutrient enrichment.

Both deterministic and stochastic processes may contribute to determining the patterns of bacterioplankton diversity under warming and nutrient-enriched conditions. Enhanced warming (EW) may result in changes in the alpha diversity (mean species richness at habitat level) of bacterioplankton communities in accordance with the Red Queen hypothesis, that is, 'the Red Queen runs faster when she is hot' (Carroll, 1917). Evidence suggests that climate warming is generally associated with enhanced primary productivity in shallow lakes with a sufficient nutrient supply (Flanagan *et al.*, 2003; Mooij *et al.*, 2005). This enhanced primary productivity may lead to increased bacterioplankton alpha diversity due to a decrease in niche selection reflecting that by reduced resource competition as 'the larger pie can be divided into more pieces' (Brown, 1981; Fuhrman *et al.*, 2008). The increased environmental disorder and the enhanced stochasticity in community assemblies in a climate warming scenario may increase the site-to-site variations of BCC under similar or even identical conditions (that is, high beta diversity). At certain water temperature and nutrient conditions, shallow water habitats may exhibit multiple stable states under similar or identical nutrient conditions (Scheffer *et al.*, 1993). These between-habitat differences in aquatic ecosystem structures may lead to different BCC. We therefore hypothesize that climate warming and nutrient enrichment (from oligotrophic to eutrophic) may cause changes in bacterioplankton biodiversity patterns.

To reveal changes in the biodiversity patterns of lacustrine bacterioplankton communities under warmer and nutrient-enriched conditions and to investigate the underlying mechanisms, including the relative contribution of and interplay between deterministic and stochastic processes, we investigated spring BCC in 24 outdoor mesocosms in Central Jutland, Denmark. These mesocosms simulate shallow lake systems under two nutrient regimes (oligotrophic and eutrophic) and three temperature scenarios, one control, one heated according to IPCC climate scenario A2 and one heated according to A2 +50% (increase of 50%). Our bacterioplankton samples were collected in mesocosms that had run for 8.5 years. Using high-throughput sequencing, we analyzed the composition and the alpha and beta diversity of the spring bacterioplankton communities in the mesocosms. Additionally, null model analyses were performed to determine the potential mechanisms driving the shifts in BCC and the changes in alpha and beta diversity. We found that enhanced climate warming (elevated 50% above the IPCC A2 climate scenario), particularly in concert with nutrient enrichment, caused shifts in aquatic BCC and increased stochasticity and beta diversity of the BCC in the experimental mesocosms.

Materials and methods

Mesocosm experiment

The mesocosms used in our experiment are located in a lowland valley in Central Jutland, Denmark (56° 14′N, 9°31′E). They are part of a long-term experiment that has run continuously since August 2003 (Liboriussen *et al.*, 2005). This experiment represents the world's longest-running lake mesocosm experiment to study the impacts of climate change. The experimental set-up included 24 fully mixed outdoor mesocosms (diameter: 1.9 m, water depth: 1 m, lake sediment: 0.2 m). Each mesocosm is a flow-through system in which a timer-controlled magnetic valve adds groundwater and an overflow pipe drains off excess surface water (Liboriussen *et al.*, 2005). The retention time of the mesocosms is approximately 2.5 months, mimicking natural freshwater shallow lakes in the region. This experiment combines three temperature scenarios with two nutrient

levels in four replicates. The temperatures in the 24 mesocosms, 8 unheated (control), 8 heated relative to IPCC climate scenario A2 for the period 2071–2100 (warming, W), and 8 heated according to A2+50% increase (enhanced warming, EW) were automatically controlled by an electrically powered heating system relative to the ambient temperature in the unheated mesocosms (Liboriussen *et al.*, 2005). In this experiment, IPCC climate scenario A2 and A2 +50% are based on a regional downscaling (average over five 25 × 25 km grid cells) to the experiment area (adjusted taking seasonal variation into account by monthly adjustment of the heating). The modeled temperature difference for the A2 scenario is generally higher in August to January (max: 4.4 °C in September) than during the rest of the year (min: 2.5 °C in June). The Prudence ensemble climate models were used as the basis (Christensen and Christensen, 2003) for the downscaling. Half of the 24 mesocosms were nutrient enriched by adding Ca $(NO_3)_2$ and Na_2HPO_4 solutions weekly, maintaining a constant loading of 538 mg N and 54 mg P per mesocosm each week (27.1 mg N m^{-2} d^{-1} and 2.7 mg P m^{-2} d^{-1}). The other half of the 24 mesocosms remained unenriched and received only natural nutrient input from the groundwater (total nitrogen: 51 ~ 71 μg N l^{-1} and total phosphorus: 2 ~ 20 μg P l^{-1}) (Liboriussen *et al.*, 2005). Initially, planktivorous fish (male *Gasterosteus aculeatus*) were stocked in natural densities consistent with the nutrient treatment (Liboriussen *et al.*, 2005); one at low and 12 at high concentrations. Since the year 2006, fish were allowed to breed in the high nutrient mesocosms by substituting some males with females. In total, we had six different treatments – control, W and EW under ambient nutrient conditions and NP, W and NP and EW and NP under nutrient-enriched conditions. NP indicates nitrogen and phosphorus enrichment. The distribution and coverage of macrophytes were made uniform before initiating heating of the mesocosms in late August 2003 (Liboriussen *et al.*, 2005). Most low nutrient mesocosms exhibited clear water conditions with macrophytes (*Elodea canadensis* and *Potamogeton crispus*) throughout the study, whereas nutrient enriched mesocosms typically had no or sparse submerged vegetation and were either turbid, dominated by phytoplankton or clear, dominated by filamentous algae (Nielsen *et al.*, 2013). Further information on the background, design details and operating characteristics of the mesocosms can be found in Liboriussen *et al.* (2005).

Sampling and chemical analyses

We collected 8-l pooled water samples from 3 uniformly distributed sites in each of the 24 mesocosms using a 1-m long tube water sampler integrating the whole water column on two sampling occasions (on 27 March and 2 April 2012, respectively). The samples from the two occasions were taken as time replicates. Approximately 400-500 ml of the collected water was used for bacterial analysis and filtered through 0.2-μm Isopore filters (Millipore, Billerica, MA, USA). The filters were stored at − 20 °C until further analysis.

The water temperature and dissolved oxygen levels of the mesocosms were measured continuously and recorded every 30 min. Gross primary production and ecosystem respiration of the mesocosms were calculated from high-frequency data (every 30 min in all mesocosms) on oxygen and temperature according to Nielsen *et al.* (2013). To estimate the proportion of the mesocosms occupied by plants (total macrophyte and filamentous algae), percentage cover and plant height were assessed. Plant abundance was estimated as per cent volume inhabited of the water column. Water total nitrogen (TN) and total phosphorus (TP) were analyzed according to the standard methods (Søndergaard *et al.*, 1990).

DNA extraction, amplification, MiSeq sequencing and data processing

DNA was extracted by the standard phenol-chloroform method (Wu *et al.*, 2007) and purified using a PowerClean DNA Clean-Up Kit (Mo Bio Laboratories, Carlsbad, CA, USA). The V4 hypervariable regions of bacterial 16S rRNA genes were amplified with the primers F515 (5′-GTGCC AGCMGCCGCGG-3′) and R806 (3′-TAATCTW TGGGVHCATCAG-5′) (Li *et al.*, 2014). For pooling multiple samples in one run of Illumina sequencing, a unique 12-mer tag for each DNA sample was added to the 5′ end of both primers. Three replicates of each sample were PCR-amplified in a 25 μl reaction, which contained 2.5 μl 10 × PCR Accuprime buffer II, 0.5 U of AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 0.2 μM each primer, and 10 ng of genomic DNA. Cycling conditions were as follows: 94 °C for 1 min followed by 25 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 25 s, extension at 68 °C for 45 s, and a final extension at 68 °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), 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 MiSeq platform.

Raw reads of the 16S rRNA gene sequences were processed using the software package Mothur (V. 1.30.0, http://www.mothur.org, 2013) according to the MiSeq standard operating procedure (Kozich *et al.*, 2013). In brief, the raw reads were combined, denoised, trimmed, quality-filtered and aligned to the SILVA v119 databases (Quast *et al.*, 2013) using Mothur. After initial processing, the quality sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level. Each of the OTU representative sequences was classified using

the freshwater bacterial database (Newton *et al.*, 2011) at the recommended bootstrap threshold of 80% (Wang *et al.*, 2007). Unclassified representative sequences were further classified using the SILVA v119 database at 80% bootstrap threshold (Wang *et al.*, 2007). After quality filtration, we obtained a total of 1 562 698 sequences from the 48 samples, with 13 436 to 62 813 sequences per sample. To avoid possible sequencing bias, all singletons and OTUs occurring in only one sample were excluded from the OTU table. To correct for the difference in sequencing depth, the minimum number of sequences in the whole sample (that is, 10 255 sequences per sample) was randomly sub-sampled to calculate appropriate metrics.

Diversity estimation and null model test

Alpha diversity (here, the number of $OTUs_{0.03}$, Kunin *et al.*, 2010) was determined using the vegan package (Oksanen *et al.*, 2013) in the R statistical environment (R Development Core Team, 2008). Beta diversity (BCC dissimilarities within treatments on each sampling occasion) was examined using two common dissimilarity metrics: Jaccard's and Bray-Curtis's dissimilarity. Jaccard's dissimilarity is an abundance-unweighted incidence-based metric, while Bray-Curtis takes differences in species abundances into account. Both Jaccard's and Bray-Curtis's dissimilarities were calculated using the vegdist command in the vegan package (Oksanen *et al.*, 2013) in R. To identify the exact factors (for example, warming, nutrient enrichment or their interaction effects) driving the changes in bacterioplankton diversity, the diversity index was grouped in three different ways. First, mesocosms under the same warming scenarios were allocated to the same group that included two different nutrient regimes. Second, mesocosms under the same nutrient regime were categorized into one group that included three different warming scenarios. Finally, mesocosms under different warming scenarios and nutrient regimes were categorized into different groups.

Beta diversity can provide insight into community assembly mechanisms (Chase and Myers, 2011). Three community assembly processes can affect beta diversity: purely deterministic, purely stochastic, or interactions between deterministic and stochastic processes (Chase *et al.*, 2011). Since beta diversity is the ratio between gamma and alpha diversity, to exclude the effect of alpha and gamma diversity on the variations in beta diversity and to uncover potential mechanisms underlying the bacterioplankton community assemblage, a null-model analysis was conducted (Zhou *et al.*, 2014). In the null model analysis, the community data matrix was randomized using an Independent Swap algorithm (Gotelli, 2000) with the randomizeMatrix command in the R picante package. The number of OTUs per mesocosm and the number of mesocosms occupied by each OTU were each fixed at a constant value (Gotelli, 2000). This randomized process was replicated 999 times to obtain 999 null communities of each observed community. In the null model analysis, incidence-based Jaccard's dissimilarities were adopted in a permutational analysis of multivariate dispersions (Zhou *et al.*, 2014) in the R vegan package to test the differences between the observed number of shared species (OTUs) between two observed communities and the expected number of shared species between two average null-expected communities. In addition, in order to determine the relative importance of deterministic and stochastic processes in shaping the bacterioplankton community assembly, the beta diversity of the observed communities and the mean 999 null communities was calculated as the BCC incidence-based Jaccard's dissimilarities within different treatments, and the standardized effect size (SES) was estimated as the differences in beta diversity between the observed communities and the mean value of the 999 null communities divided by the standardized deviation of the beta diversity in the 999 null communities. If SES is closer to zero, it suggests that the relative importance of stochastic processes increases. Standardized effect size was also grouped in three different ways as for alpha and beta diversity.

Statistical methods

Because samples collected from the same mesocosm on two sampling occasions were not independent of each other, we performed repeated-measures approaches to test the significant effects of different treatments on the parameters involved in this study. To assess the significant differences of environmental characteristics, OTU richness, and the relative abundance of phylum and of lineages and clades under different treatments, we conducted repeated-measures analysis of variance tests (ANOVA) and repeated-measures Friedman's tests (when the normality test was violated), which were both followed by *post hoc* comparisons. Repeated-measures ANOVA were performed in the stats package in R, while the subsequent repeated-measures *post hoc* comparisons were conducted using a linear mixed-effects model in the R multcomp package (Hothorn *et al.*, 2008). Repeated-measures Friedman's tests and the subsequent *post hoc* analyses were performed in the R agricolae package (De Mendiburu, 2014).

To assess the effects of the different treatments on the changes in BCC, permutational multivariate analysis of variance (PERMANOVA) was performed using the R vegan package. Since both beta diversity and SES were not independent parameters, we conducted permutational analysis of variance (PERANOVA) to test the effects of different treatments on the changes in both beta diversity and SES in the R RVAideMemoire package (Hervé, 2015). Neither PERMANOVA nor PERANOVA include repeated-measures modules, we therefore did repeated measures by using strata to block the sampling time (Andrew and Lilleskov, 2009).

The relative abundances of the main lineages or clades of bacterioplankton communities under different climate warming scenarios and nutrient conditions were depicted in a heat map with the pheatmap package (Kolde, 2013) in R. The incidence-based Jaccard's dissimilarity and Bray-Curtis dissimilarity of BCC were visualized using non-metric multidimensional scaling in the R vegan package. The relationships between BCC and environmental factors were determined by canonical correlation analysis with an automatic stepwise model using permutation tests in the R vegan package. The models were validated by analysis of variance.

Availability of supporting data

The sequence data were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) under the accession numbers SRX1016058-SRX1016105. Mothur sequence processing commands and R data analysis codes are available in Github (https://github.com/wisdom503/Phylogeny-and-bioinformatics-analysis-of-aquatic-bacterial-16S-rDNA).

Results

Shift in BCC at warming and nutrient enrichment

We found that BCC was significantly impacted by warming scenarios (PERMANOVA, incidence-based Jaccard's dissimilarity: $F = 1.724$, $P = 0.002$, Figure 1a; Bray-Curtis's dissimilarity: $F = 2.081$, $P = 0.001$, Figure 1b; Table 1) and nutrient levels (incidence-based Jaccard's dissimilarity: $F = 3.305$, $P = 0.001$, Figure 1a; Bray-Curtis's dissimilarity: $F = 4.807$, $P = 0.001$, Figure 1b; Table 1). However, the main effects of both warming and nutrient conditions could not sufficiently explain the distributions of BCC in the different mesocosms as warming and nutrient conditions exhibited a significant interaction (incidence-based Jaccard's dissimilarity: $F = 1.765$, $P = 0.001$, Figure 1a; Bray-Curtis's dissimilarity: $F = 2.089$, $P = 0.001$, Figure 1b; Table 1).

The interaction effect of warming and nutrients was also evident at coarse taxonomic levels (Figure 2, Supplementary Figure S1). We found that compared with ambient mesocosms (control), the relative abundances of *Actinobacteria* (repeated-measures *post hoc* Tukey test: $F = 627.5$, $P = 0.025$), *Bacteroidetes* ($F = 75.93$, $P = 0.073$) and *Betaproteobacteria* ($F = 888$, $P = 0.021$) significantly decreased in the EW and NP mesocosms (Figure 2), while the relative abundances of *Cyanobacteria* ($F = 1223$, $P = 0.018$), total rare phyla (proportion $< 1.5\%$; $F = 3143$, $P = 0.011$) and unclassified phyla ($F = 206$, $P = 0.043$) significantly increased in the EW and NP mesocosms (Figure 2). In addition, the relative abundances of the dominant clades including acI-A ($F = 1527$, $P = 0.016$), Lhab-A1 ($F = 64.58$, $P = 0.079$) and Lhab-A2 ($F = 167.8$, $P = 0.049$) all significantly decreased in the EW and NP mesocosms, compared with ambient mesocosms (Supplementary Figure S1).

When relating BCC variations to environmental factors (Table 2), we found that the BCC in the heated and nutrient-enriched mesocosms (that is, W and NP and EW and NP) was significantly positively correlated with gross primary production (permutation test: $P = 0.005$; Supplementary Figure S2), whereas the BCC in all nutrient-unenriched mesocosms and most nutrient-enriched but unheated mesocosms (that is, control, W, EW and NP) was significantly positively correlated with plant coverage (permutation test: $P = 0.01$; Supplementary Figure S2).

Variations in bacterial diversity and null model analyses

We found that neither warming nor nutrients had significant effects on OTU richness in the different

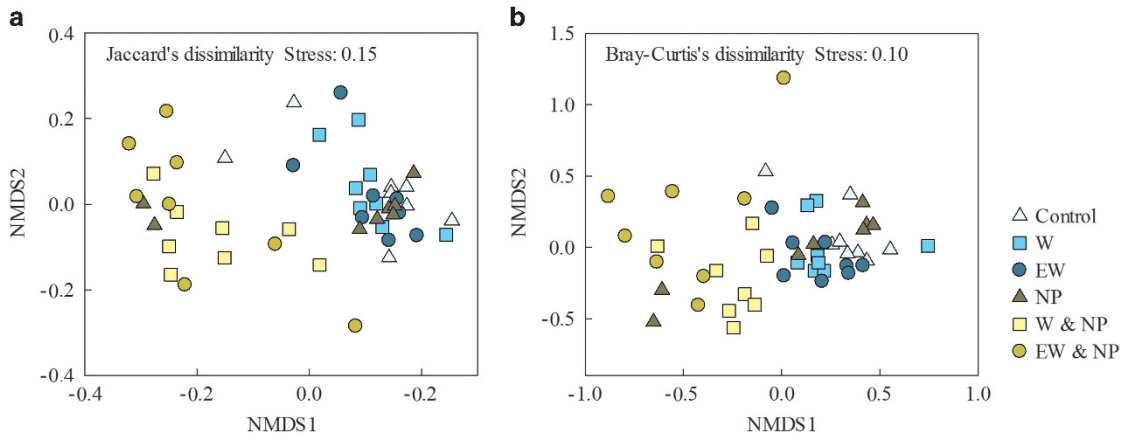

Figure 1 Non-metric multidimensional scaling (NMDS) plots derived from the incidence-based Jaccard's (**a**) and Bray-Curtis's (**b**) dissimilarities of BCC, with symbols color and shaped by warming and nutrient treatments. Control, ambient temperature and nutrition conditions; W, warming; EW, enhanced warming; NP, nitrogen and phosphorus enrichment.

Table 1 Permutational multivariate analysis of variance (PERMANOVA) based on incidence-based Jaccard's dissimilarity and Bray-Curtis's dissimilarity in bacterioplankton community composition (BCC) in experimental mesocosms characterized by different warming and nutrient conditions

Index	*incidence-based Jaccard's dissimilarity*				*Bray-Curtis's dissimilarity*			
	d.f.	R^2	*F model*	*P*	*d.f.*	R^2	*F model*	*P*
Warming (W)	2	0.066	1.724	0.002**	2	0.075	2.081	0.001**
Nutrient (NP)	1	0.063	3.305	0.001**	1	0.087	4.807	0.001**
W:NP	2	0.068	1.765	0.001**	2	0.075	2.089	0.001**
Residuals	42	0.803			42	0.761		

*$P<0.05$; **$P<0.01$.
Significance tests were performed using *F*-tests based on sequential sums of squares from permutations of the raw data.

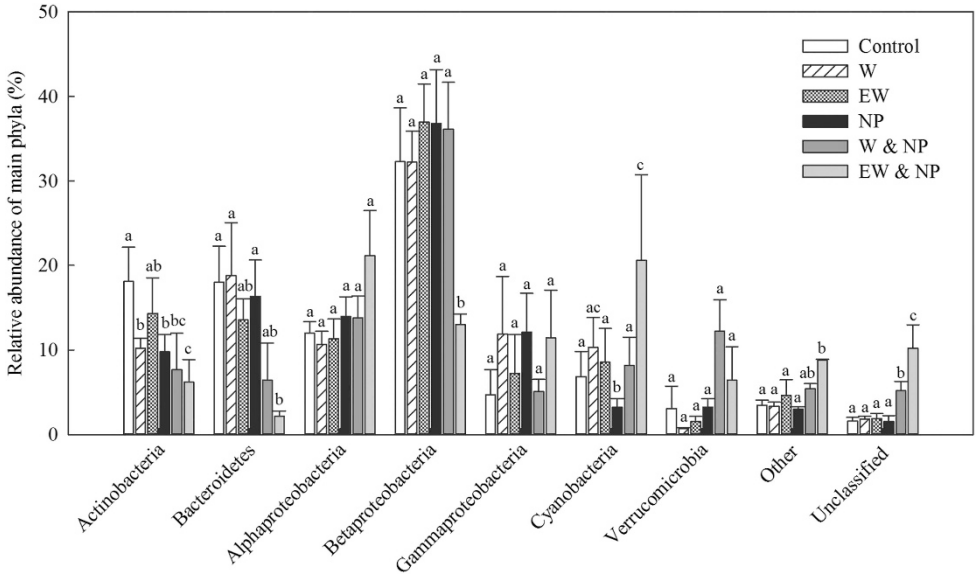

Figure 2 Relative abundances of the dominant bacterial taxa under different warming and nutrient conditions. All data are presented as mean ± s.e.m. Significant ($P<0.05$) differences among the different treatments are indicated by letters above the bars. Control = ambient temperature and nutritional conditions; W, warming; EW, enhanced warming; NP, nitrogen and phosphorus enrichment.

mesocosms (two-way repeated-measures ANOVA: warming: $F=0.027$, $P=0.973$; nutrients: $F=1.775$, $P=0.410$, Figure 3). However, based on both incidence-based Jaccard's (Figure 4a) and Bray-Curtis's dissimilarity (Figure 4b), warming and nutrient enrichment demonstrated interaction effects on beta diversity. We observed a significant difference in bacterioplankton beta diversity between different warming scenarios in the two different nutrient regimes (PERANOVA, incidence-based Jaccard's dissimilarity: $F=182.51$, $P=0.009$, Figure 4a (i); Bray-Curtis's dissimilarity: $F=25.456$, $P=0.03$, Figure 4b(i)) and, compared with other treatments, beta diversity significantly increased in the EW scenarios ($P<0.05$ in all cases; Figures 4a(i) and b (i)). In the two nutrient regimes (each including three different warming scenarios), a significantly higher beta diversity was found in the nutrient-enriched mesocosms (incidence-based Jaccard's dissimilarity: $F=135.04$, $P=0.058$, Figure 4a(ii); Bray-Curtis's dissimilarity: $F=266.64$, $P=0.037$, Figure 4b(ii)). There was also a significant difference in beta diversity among the different warming and nutrient treatments (incidence-based Jaccard's dissimilarity: $F=8.508$, $P=0.017$, Figure 4a(iii); Bray-Curtis's dissimilarity: $F=6.811$, $P=0.024$, Figure 4b(iii)). However, compared with other treatments, beta diversity significantly increased in the EW and NP mesocosms ($P<0.05$ in all cases; Figures 4a(iii) and b(iii)).

Null model analysis revealed that warming and nutrient enrichment also had interactive effects on the relative importance of deterministic and stochastic processes in shaping the bacterioplankton community assembly, assessed via the SES. We observed that among the different warming scenarios (that each included two different nutrient regimes), a significantly higher SES was detected at higher temperatures ($P<0.05$ in all cases; Figure 5a). In the two nutrient regimes (each including three different warming scenarios), a significantly higher

Table 2 Summary of the environmental characteristics in the mesocosms under different climate and nutrient conditions

Index	*T (°C)*	*DO (mg l^{-1})*	*GPP(mg O_2 l^{-1} day^{-1})*	*ER (mg O_2 l^{-1} day^{-1})*	*NPP (mg O_2 l^{-1} day^{-1})*	*PVI (%)*	*TN (mg l^{-1})*	*TP (mg l^{-1})*
Control	7.67 ± 0.65^{a}	13.11 ± 0.25^{a}	3.17 ± 0.30^{a}	2.68 ± 0.21^{a}	0.488 ± 0.137^{a}	6.85 ± 2.59^{ab}	0.160 ± 0.072^{a}	0.014 ± 0.003^{a}
W	10.24 ± 0.62^{ac}	13.69 ± 0.39^{ab}	3.38 ± 0.20^{a}	2.79 ± 0.18^{ab}	0.595 ± 0.0764^{ab}	8.74 ± 2.36^{ab}	0.027 ± 0.005^{a}	0.008 ± 0.001^{a}
EW	11.52 ± 0.61^{c}	13.05 ± 0.40^{a}	3.49 ± 0.19^{a}	2.90 ± 0.18^{ab}	0.582 ± 0.092^{ab}	10.56 ± 1.55^{b}	0.037 ± 0.010^{a}	0.009 ± 0.001^{a}
NP	8.11 ± 0.67^{a}	12.51 ± 0.55^{a}	4.51 ± 0.34^{ab}	4.09 ± 0.45^{bc}	0.412 ± 0.176^{a}	2.77 ± 1.63^{ab}	4.600 ± 0.277^{bc}	0.085 ± 0.037^{ab}
W and NP	10.47 ± 0.65^{ac}	16.42 ± 0.87^{c}	5.21 ± 0.44^{b}	3.94 ± 0.35^{c}	1.263 ± 0.261^{b}	0^{a}	5.737 ± 0.744^{b}	0.248 ± 0.046^{c}
EW and NP	11.42 ± 0.62^{a}	15.66 ± 0.46^{bc}	5.34 ± 0.37^{b}	4.63 ± 0.35^{c}	0.707 ± 0.201^{ab}	2.5 ± 1.64^{a}	3.831 ± 0.530^{c}	0.129 ± 0.034^{bc}

DO, dissolved oxygen; GPP, gross primary production; ER, ecosystem respiration; NPP, net primary production; PVI, percent volume inhabited; TN, total nitrogen; TP, total phosphorus. Repeated-measures ANOVA, Friedman's tests (when the normality test was violated), and *post hoc* comparisons were performed to test the differences among the different treatments. Significant ($P<0.05$) differences among the different treatments are indicated by letters. Control, ambient temperature and nutritional conditions; W, warming; EW, enhanced warming; NP, nitrogen and phosphorus enrichment.

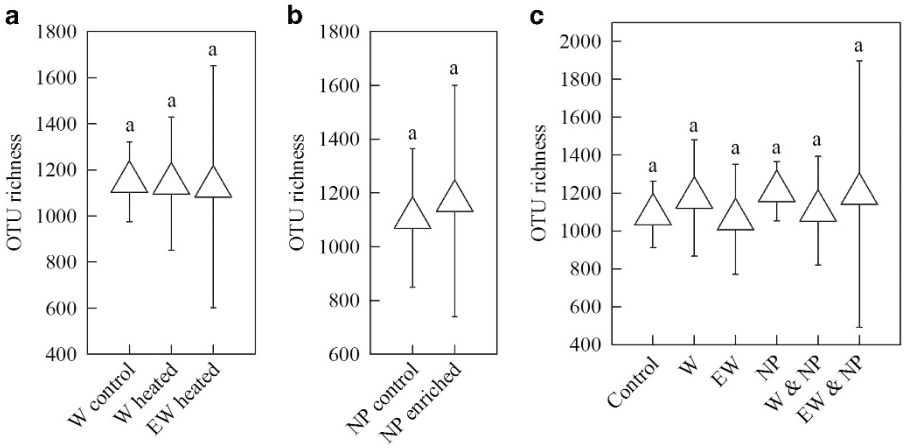

Figure 3 Bacterioplankton operational taxonomic unit (OTU) richness. (**a**) OTU richness under different warming scenarios. OTUs in the same warming scenario were allocated to the same group; (**b**) OTU richness under different nutrient conditions. OTUs under similar nutrient conditions were allocated to the same group; (**c**) OTU richness under each warming scenario and nutrient condition. All data are presented as mean ± s.e.m. Significant ($P<0.05$) differences among the different treatments are indicated by letters above the bars. Control = ambient temperature and nutritional conditions; W = warming; EW = enhanced warming; NP = nitrogen and phosphorus enrichment.

SES was found in the nutrient-enriched mesocosms ($F = 198.9$, $P = 0.048$; Figure 5b). In addition, SES under different warming and nutrient treatments was found to increase significantly in the EW and NP mesocosms ($P<0.05$ in all cases; Figure 5c). Null model analysis also showed that irrespective of BCC grouping (according to warming, nutrient enrichment, and different warming and nutrient treatments), the observed bacterioplankton communities all differed significantly from the null expectation, which included only stochastic community assembly processes (permutational analysis of multivariate dispersion: $P<0.001$ in all cases).

Discussion

In this study, we used high-throughput MiSeq sequencing to examine spring bacterioplankton biodiversity patterns and their underlying mechanisms (that is, deterministic vs stochastic processes) in early spring in 24 freshwater mesocosms. These mesocosms have been subjected to unique long-term (8.5 years) treatments (nutrient enrichment crossed with warming), allowing the investigation of the interactive impacts of simulated climate warming and nutrient enrichment on BCC in systems that were free of initial transients state that characterizes many experimental systems (Stewart *et al.*, 2013). High-throughput Miseq sequencing is a powerful technique to explore bacterioplankton community diversity (Zhou *et al.*, 2011, 2015) despite a strong technical stochastic effect, for example, PCR amplification, sequencing errors and chimeric sequences (for example, Schloss *et al.*, 2011; Zhou *et al.*, 2011, 2015). In order to correct possible sequencing bias, we removed less frequently encountered OTUs

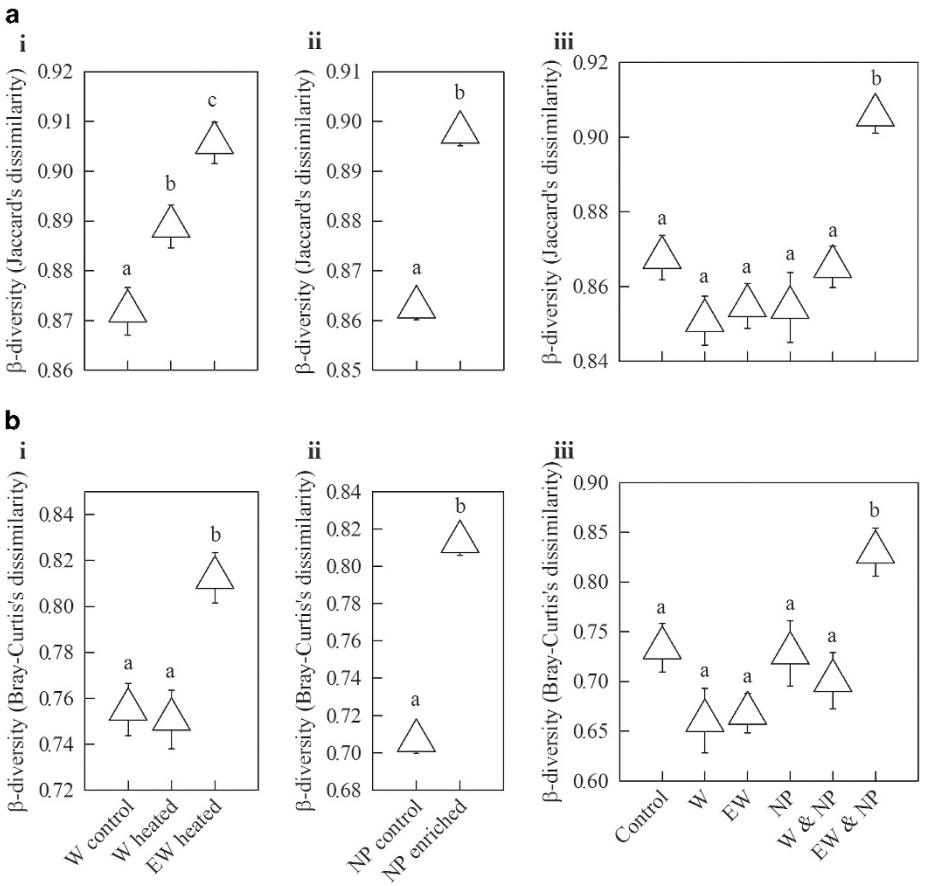

Figure 4 Bacterioplankton beta diversity, as measured by BCC incidence-based Jaccard's (**a**: i, ii and iii) and Bray-Curtis's (**b**: i, ii and iii) dissimilarity. i: beta diversity under different warming scenarios. BCC in the same warming scenario was allocated to the same group; ii: beta diversity under different nutrient conditions. BCC under similar nutrient conditions was allocated to the same group; iii: beta diversity under each warming scenario and nutrient condition. All data are presented as mean ± s.e.m. Significant ($P<0.05$) differences among treatments are indicated by letters above the bars. Control, ambient temperature and nutrition conditions; W, warming; EW, enhanced warming; NP, nitrogen and phosphorus enrichment.

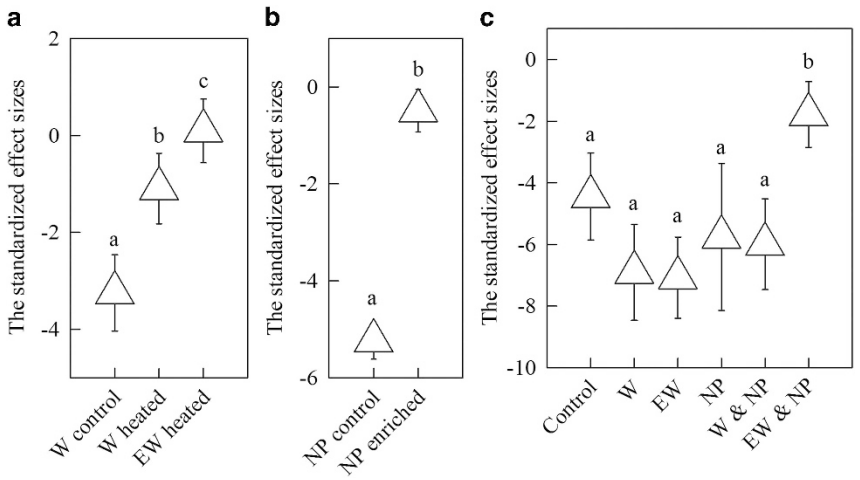

Figure 5 The standardized effect sizes (SES) based on null model analysis. (**a**) SES under different warming scenarios. BCC in the same climate scenario was allocated to the same group; (**b**) SES under different nutrient conditions. BCC at similar nutrient conditions was allocated to the same group; (**c**) SES under each warming scenario and nutrient condition. All data are presented as mean ± s.e.m. Significant ($P<0.05$) differences among the different treatments are indicated by letters above the bars. Control, ambient temperature and nutrition conditions; W, warming; EW, enhanced warming; NP, nitrogen and phosphorus enrichment.

across the entire quality-filtered sample set. Two data sets were generated by discarding OTUs occurring in less than two samples and less than three samples, respectively (Zhou *et al.*, 2011). Furthermore, to correct for the difference in sequencing depth, we performed two normalized methods, one is rarefying and the other is a variance stabilizing transformation (McMurdie and Holmes, 2014). In total, we used four different methods (two different cutoffs of OTUs multiply by two normalized methods) to denoise the technical stochastic effects (Supplementary Data S1). The main results of the four methods were consistent (Figures 1 and 3–5, and Supplementary Figures S3–S11). The results revealed that neither warming nor nutrient enrichment had significant effects on bacterioplankton alpha diversity (Figure 3, Supplementary Figures S4 and S9); however, the combined effects of EW and nutrient enrichment induced a significant shift in BCC (Figure 1 and Supplementary Figure S3). Bacterioplankton beta diversity (Figure 4 and Supplementary Figures S5–S7) and the relative importance of deterministic and stochastic processes (Figure 5, Supplementary Figures S8) also changed significantly by the synergetic influences of EW and nutrient enrichment.

BCC is sensitive to slight warming with nutrient enrichment

Because different bacterial populations have different temperature optima, temperature is considered a major driving factor in determining the lacustrine bacterioplankton community, including their size structure (Daufresne *et al.*, 2009), metabolism (Hall and Cotner, 2007; Hall *et al.*, 2008), biomass (Hall *et al.*, 2009) and community composition (Adams *et al.*, 2010; Ren *et al.*, 2013). For example, Hall *et al.* (2009) found that lacustrine bacterioplankton communities incubated at different temperatures (4, 14 and 24 °C) had different nutrient use efficiency. However, in several other studies where only small increases in temperature were tested, water temperature had no significant effects on the growth rate (Ducklow *et al.*, 1999), abundance (Christoffersen *et al.*, 2006), biomass (Özen *et al.*, 2013) or community composition (Scheibner *et al.*, 2014) of aquatic bacteria. However, small increases in water temperature significantly modified the effect of nutrients on both bacterioplankton abundance (Christoffersen *et al.*, 2006) and biomass (Özen *et al.*, 2013). Similarly, we found that slight warming did not by itself significantly change BCC in the mesocosms, but that the BCC shifted when warming acted in concert with nutrient enrichment. At phylum level, non-significant differences were observed under the independent effects of warming; however, the EW scenarios combined with nutrient enrichment (that is, EW and NP) clearly decreased the relative abundance of *Actinobacteria*, *Bacteroidetes* and *Betaproteobacteria*, and increased the percentages of *Cyanobacteria*, total rare phyla and unclassified phyla. This finding is inconsistent with those in a previous study by Scheibner *et al.* (2014), showing that warming (6 °C increase in Baltic Sea water temperature) increased the relative abundance of *Betaproteobacteria* and *Flavobacteria* and reduced the abundance of *Alphaproteobacteria* and *Gammaproteobacteria*. The conflicting results perhaps reflect different optima of bacterioplankton taxa between freshwater and marine habitats (Newton *et al.*, 2011) or differences in warming intensity or timing. However, in accordance with our results, previous investigations worldwide have demonstrated warming-induced increases of *Cyanobacteria* abundance and their proportion in total phytoplankton biomass in eutrophic shallow lakes (for example, Jeppesen *et al.*, 2009; Markensten *et al.*, 2010). Previous studies have shown that the relative abundance of *Cyanobacteria* increase with a decrease of that of *Actinobacteria* in freshwater reservoirs (for example, Ghai *et al.*, 2014) and cyanobacterial cultures contained lower proportions of *Limnohabitans* bacteria compared to other phytoplankton cultures (Šimek *et al.*, 2011). We also found lower percentages of *Actinobacteria* (mainly acI-A) and *Limnohabitans* (mainly Lhab-A1 and Lhab-A2) in EW and nutrient-enriched mesocosms, which may be related to the increased relative amount of *Cyanobacteria*. In addition, the high relative abundance of PnecB in all experimental mesocosms (receiving groundwater and rainwater only) may suggest a potential coupling between PnecB and phytoplankton, as implicated in other studies (for example, Horner-Devine *et al.*, 2003; Wu and Hahn, 2006).

Neither warming nor nutrient enrichment had significant effects on bacterioplankton alpha diversity

Earlier studies have shown that bacterioplankton diversity varies along a primary productivity gradient and that the richness of different bacterial groups may respond differently to changes in primary productivity (Horner-Devine *et al.*, 2003). We found that the primary productivity in the heated and nutrient-enriched mesocosms was significantly higher than in the control mesocosms (Table 2), as demonstrated in other international studies (Flanagan *et al.*, 2003; Mooij *et al.*, 2005) as well as in the mesocosms used in our experiment (Nielsen *et al.*, 2013). However, we observed no significant differences in average bacterial alpha diversity between the different warming and nutrient treatments based on OTU richness (Figure 3 and Supplementary Figure S4) and Faith's phylogenetic diversity (Faith, 1992) (Supplementary Data S1 and Supplementary Figure S9). This may be attributed to several factors: first, the limited range and the lack of a distinct gradient of primary productivity in this study may have prevented us from detecting the threshold underlying the diversity primary

productivity relationship. Second, the high BCC heterogeneity in mesocosms within the same nutrient and temperature treatment may also obscure differences in diversity between different primary productivity levels. Third, the high dispersal rate of bacteria and other random processes involved in shaping the bacterial community assemblage in the 24 mesocosms in such a limited space may obscure us from detecting changes in alpha diversity. This suggestion is supported by our null-model test of phylogenetic diversity, in which increased stochasticity in determining the bacterial phylogenetic assemblage was observed at increased temperature and nutrient loading, where values of the SES of phylogenetic diversity were closer to zero under heated and nutrient-enriched conditions (Supplementary Data S1 and Supplementary Figure S10).

High stochasticity and beta diversity of BCC caused by the interaction effects of EW and nutrient enrichment

Previous studies have found loss of beta diversity among benthic animals (Donohue *et al.*, 2009) and fish (Menezes *et al.*, 2015) in eutrophic lakes, but interaction effects of nutrients and warming are less well studied. In this study, we observed that long-term warming and nutrient-enrichment had interaction effects that significantly increased the beta diversity of bacterioplankton communities. Such synergistic effects of warming and nutrient enrichment also caused high variability in BCC, even among replicate mesocosms under the same conditions. In our null model test, we found that the observed bacterioplankton community differed significantly from the null expectation, suggesting that deterministic processes play a more important role than stochastic processes in determining the community assembly. However, the relative importance of stochastic processes increased in the heated and nutrient-enriched mesocosms, where the values of SES grouped in three different ways all were much higher than those in the unheated and nutrient ambient mesocosms, and all were very close to zero as well. Another null-model test of bacterial beta diversity also revealed that under warming and nutrient-enriched conditions, bacterioplankton communities deviated much less from the null expectation, which included only stochastic community assembly processes (Supplementary Data S1 and Supplementary Figure S11). Thus, both model tests revealed that the enhanced relative importance of stochastic processes (or in other words the decreased relative importance of deterministic processes) in structuring the BCC contributed to the increased beta diversity in mesocosms under heated and nutrient-enriched conditions (Figures 4 and 5, Supplementary Figures S5–S8 and S11).

It is generally accepted that four processes, selection, drift, speciation and dispersal, are involved in controlling BCC. Selection is deterministic and drift is stochastic, whereas speciation and dispersal may contribute to both (Chase *et al.*, 2011). In our study, the 24 mesocosms were randomly placed within a 20×20 m open area in a lowland valley (Liboriussen *et al.*, 2005). Because bacterioplankton are highly abundant and can disperse through air, dust and rain (Östman *et al.*, 2010; Lindström and Östman, 2011), we assume that BCCs in each of the 24 mesocosms share the regional species pool as these outdoor systems were open to the air and received similar groundwater daily. Dispersal might therefore not be a limiting factor for the community assembly in this narrow open area. Moreover, the retention time of our experimental mesocosms is approximately 2.5 months, which is too short to allow sufficient evolution and speciation of bacterial species based on 16S rRNA genes (Ochman *et al.*, 1999). Bacterial communities on the surface of biofilms and in sediments are likely to have higher persistence; however, they may have very weak effects on planktonic bacteria in the water columns of long-term stable ecosystems due to habitat-specific bacterial community compositions (for example, Ye *et al.*, 2009; He *et al.*, 2014). Therefore, the observed BCC changes were most likely driven by environmental selection (a deterministic process) and ecological drift (a stochastic process). It is not surprising that the BCC was driven by environmental selection. At a certain temperature and nutrient loading level, shallow lakes can exhibit alternative states under similar conditions, implying the occurrence of between-habitat differences in aquatic ecosystem structure and function (Scheffer *et al.*, 1993). We also observed large variations in macrophyte coverage, respiration and primary production, even among replicate mesocosms (Table 2). Such differences will act as environmental selection and will change the BCC across the mesocosms within or between different treatments. High beta diversity of bacteria has previously been observed at times of high productivity and environmental heterogeneity in the metacommunity, partially reflecting environmental selection (Langenheder *et al.*, 2012).

In addition to deterministic processes, stochastic processes (that is, ecological drift) also played an important role in determining the bacterial community assembly and contributed to the increased beta diversity in mesocosms under heated and nutrient-enriched conditions. Several mechanisms may contribute to the enhanced role of stochasticity in the warmer and nutrient-enriched mesocosms. First, temperature has a direct effect on the kinetics of metabolism. The exponential increase in metabolic rate as temperature increases affects nearly all biological processes (Allen *et al.*, 2002; Brown *et al.*, 2004). Thus, the rising temperature may simply increase the stochasticity of the colonization and extinction of bacteria, resulting in ecological drift among the mesocosms. Moreover, the increased disorder in ecological conditions created by faster metabolic kinetics in warmer and nutrient-enriched

environments may also increase the stochasticity of the bacterioplankton community assembly, such as random colonization or extinction and unpredictable perturbations, alluding to the past changes in environmental conditions (Feuchtmayr *et al*., 2009) and biological structures (Christoffersen *et al*., 2006; Özen *et al*., 2013). Second, nutrient enrichment might enhance the stochasticity in structuring BCC as nutrient enrichment may accelerate the growth of some rare bacterial species and thus amplify the initial differences in bacterial composition (Zhou *et al*., 2014). The interaction between warming and nutrient enrichment was clearly demonstrated in our experiment and led to high stochasticity and beta diversity. Warming strengthens a significant effect of nutrient enrichment on the assembly processes of bacterioplankton communities, and nutrient enrichment exacerbated the impact of warming on regulating the relative importance of deterministic and stochastic processes. Third, our system is a flow-through system with a retention time of ca. 2.5 months. The stochasticity in bacterial immigration and emigration by the flow-through water might also be enhanced by the interaction effects of warming and nutrient enrichment.

Conclusions

Our results revealed that warming and nutrient enrichment in combination increase stochasticity and beta diversity of spring bacterioplankton communities in freshwater mesocosms mimicking shallow lake environments. This study is the first attempt to investigate bacterioplankton biodiversity patterns in a future warmer climate and with changing nutrient conditions in freshwater ecosystems. We propose that bacterial community may turn more variable across freshwater bodies when warming acts in concert with nutrient enrichment.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We are very grateful to Lissa Skov Hansen for her excellent work with the chemical analyses and Anne Mette Poulsen for English language assistance. We also acknowledge Te Cao, Haijun Wang, Femke van Beersum, Yuanjiao Lv, Lijun Zhou, Huabing Li, Peng Xing, Jin Zeng, Biying Zhao and Yujin Wang for their assistance with the experiment and data analysis. We especially thank Ryan J. Newton for access to the freshwater bacterial database. This work was supported by the National Science Foundation (31225004, U1202231, 31500376), the MARS project (Managing Aquatic ecosystems and water resources under multiple stress) funded under the Seventh EU Framework Programme, Theme 6 (Environment including Climate Change), Contract No. 603378 (http://www.mars-project.eu), 'CLEAR' (a Villum Kann Rasmussen Centre of Excellence project), CRES, CIRCE, and projects of the Chinese Academy of Sciences (QYZDJ-SSW-DQC030, KZZD-EW-TZ-08).

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Supplementary Information accompanies this paper on *The ISME Journal* website (http://www.nature.com/ismej)