Influence of potential grazers on picocyanobacterial abundance in Lake Biwa revealed with empirical dynamic modeling

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Picocyanobacteria in lakes generally occur as single cells (single-celled picocyanobacteria; SPcy) or colonies (colonial picocyanobacteria; CPcy), and the latter form has been considered an adaption to grazing pressure. In addition to direct effects of grazing, grazers may also have important indirect effects on picocyanobacteria, such as those from nutrient regeneration and trophic cascades. Interactions between picocyanobacteria and their grazers in lakes can thus be complex and difficult to predict. In the present study, we aimed to evaluate the influence of various grazers on SPcy and CPcy in Lake Biwa, Japan. We followed seasonal changes in the abundances of SPcy, CPcy, and their potential grazers biweekly over two years. The data collected were analyzed using empirical dynamic modeling (EDM), a model-free, nonlinear time-series method. We found that heterotrophic nanoflagellates (HNF), rotifers (*Keratella*, *Polyarthra*, and *Trichocerca*), cladocerans, and copepods played important and differing roles in controlling the abundances of SPcy and CPcy. Notably, HNF had an apparent positive influence on SPcy abundance, despite being considered major consumers of SPcy. This result suggested that the enhancement of SPcy growth due to nutrient regeneration by HNF might exceed losses from mortality due to grazing by HNF. EDM also suggested that colony formation by picocyanobacteria may be unidirectional, with SPcy tending to form CPcy. Our findings show that the seasonal dynamics of SPcy and CPcy in Lake Biwa are influenced by a variety of grazers, which may play differing ecological roles in the aquatic food web.

Keywords: empirical dynamic modeling, grazers, heterotrophic nanoflagellates, indirect effects, picocyanobacteria

Introduction

Picocyanobacteria, a diverse group of cyanobacteria defined by cell sizes of less than 2 µm, are numerous and ubiquitous in freshwater and marine ecosystems (Stockner and Antia 1986; Stockner 1988). Despite their small size, these photoautotrophic organisms

contribute largely to phytoplankton biomass and primary production, and play important roles in aquatic ecosystems (Weisse 1993). In freshwaters, single-celled picocyanobacteria (SPcy) dominate in oligotrophic environments and are mainly represented by the genera *Synechococcus* and *Cyanobium* (Fogg 1995; Sanchez-Baracaldo et al. 2005). Colonial picocyanobacteria (CPcy) are also common and often abundant in meso-eutrophic environments (Stockner 1991; Stockner et al. 2000). They consist of colonial species (e.g., *Aphanothece*, *Aphanocapsa*, *Cyanodictyon*) and microcolonies formed by SPcy (Passoni and Callieri 2000; Callieri et al. 2012).

Understanding how the abundances of picocyanobacterial populations are controlled by grazers is essential to elucidating their ecology. Grazing has been considered a key top-down control process affecting SPcy abundance (Horn and Horn 2008). Small protists such as heterotrophic nanoflagellates (HNF) and ciliates are considered the major consumers of SPcy (Stockner and Antia 1986; Šimek et al. 1995 and 1997; Sanders et al. 2000). An uptake of nearly 80% of the carbon produced by SPcy was estimated for HNF and ciliates in an oligotrophic lake (Callieri et al. 2002). Metazoan zooplankton are also important grazers of SPcy. Filter feeders such as planktonic rotifers (e.g., *Keratella*, *Polyarthra*) and cladocerans (e.g., *Daphnia*, *Bosmina*) can feed on pico-sized particles and thus consume SPcy (Gophen and Geller 1984; Weisse 1993; Ronneberger 1998; Callieri et al. 2012). Copepods are also efficient SPcy grazers, even when alternative foods are available (Motwani and Gorokhova 2013). By contrast, CPcy appear to be resistant to grazing (Blomqvist 1996). It has been suggested that colony formation by picocyanobacteria may act as an anti-grazing strategy (Callieri et al. 2012). Grazing experiments have shown that some strains of *Synechococcus* could form microcolonies when co-cultivated with HNF (Callieri et al.

2016). Huber et al. (2017) demonstrated that grazing by *Bosmina* favored the dominance of *Cyanodictyon* over SPcy. However, zooplankton such as *Daphnia* have the ability to ingest particles up to tens of micrometers in size (Ronneberger 1998), and thus small colonies of CPcy may be edible to such large grazers (Van Donk et al. 2011).

Grazers also play other important roles in controlling the abundances of picocyanobacteria based on indirect interactions such as nutrient regeneration and trophic cascades. Heterotrophic protists and metazoan zooplankton can excrete nitrogen and phosphorus and thereby support the growth of phytoplankton (Johannes 1965; Moegenburg and Vanni 1991; Nakano 1994a, b, c). Indeed, Callieri et al. (2004) reported a significant increase in picocyanobacterial photosynthetic efficiency in the presence of *Daphnia* grazing. In addition, predator-prey relationships exist among HNF, ciliates, rotifers, cladocerans, and copepods (Arndt 1993; Sanders et al. 1994; Suzuki et al. 1999; Nakano et al. 2001; Christoffersen and Gonzalez 2003; Brandl 2005), and therefore the abundances of picocyanobacteria may be affected indirectly through trophic cascades of these grazers (Wickham 1995; Sundt-Hansen et al. 2006). Taking the direct effects of grazing into consideration, interactions between picocyanobacteria and grazers in nature can thus be highly complex and difficult to predict.

To elucidate the influence of potential grazers on picocyanobacteria, we carried out a two-year study in Lake Biwa, Japan, focusing on the differing ecological properties of SPcy and CPcy. We collected samples biweekly and followed seasonal changes in abundances of picocyanobacteria (SPcy and CPcy) and their potential grazers (HNF, ciliates, rotifers, cladocerans, and copepods). Finally, we applied empirical dynamic modeling (EDM), which is a model-free, nonlinear time-series analysis method, to the time-series data collected. EDM was developed to specifically

analyze the dynamics of nonlinear systems such as ecosystems, where traditional linear tools are not applicable. Thus, this method is suitable for the analysis of the nonlinear, state-dependent behaviors of picocyanobacteria observed in our study system. The main objectives of the present study are two-fold: 1) identification of potential grazers that affect SPcy and CPcy abundances under natural conditions, and 2) quantification of the overall effects of various grazers on SPcy and CPcy abundances using EDM.

Methods

Sample collection and measurement of environmental variables

Sample collection was conducted biweekly at observation site Ie-1 (35°12'58"N; 135°59'55"E; maximum depth, 73 m) in the north basin of Lake Biwa (Mukherjee et al. 2017) from July 2015 to June 2017. Vertical profiles of water temperature, chlorophyll *a* (Chl-*a*) concentration and photosynthetically active radiation (PAR) throughout the water column were obtained using a CTD profiler (SBE-911 plus; Sea Bird Electronics, Sea-logger, WA, USA). In addition, samples for analysis of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) were collected monthly at depths of 0, 5, 10 and 20 m from August 2015 to July 2016. NH_4-N concentrations were measured using a sensitive fluorometric assay (Holmes et al. 1999). Concentrations of NO_2-N, NO_3-N and DIP were analyzed using an AACS-II autoanalyzer (BRAN+LUEBBE).

Samples of picocyanobacteria, protists and metazoan zooplankton were collected from depths of 0, 5, 10, 15 and 20 m with a 5 L Niskin sampler (General Oceanics, Miami, USA). All samples were collected at around the same time of day (10:00 to 12:00 h). For picocyanobacteria, unfiltered water samples were collected. For

HNF, water samples were prefiltered using a plankton net with a mesh size of 20 μm, and the filtrate was collected. After collection, 100 ml of each water sample was fixed with glutaraldehyde at a final concentration of 1% for enumeration of picocyanobacteria and HNF, and the fixed samples were then stored in the dark at 4 °C. For other zooplankton (ciliates, rotifers, cladocerans, and copepods), 10 L of lake water was concentrated to 100 ml using a plankton net with mesh size 20 μm, then fixed with 5% acid Lugol's solution and stored in the dark.

Sample treatment and plankton enumeration

For the enumeration of picocyanobacteria, fixed samples of 1–10 ml were filtered through 0.2 μm polycarbonate membrane filters to retain cells. Duplicate filters were prepared for each sample. An epifluorescence microscope (Olympus BX53, 1000x) was used to enumerate SPcy and CPcy cells under green excitation (530–550 nm). At least 100 cells or 50 fields were counted from each filter. For the enumeration of HNF, fixed samples of 30–50 ml were filtered through 0.8 μm polycarbonate membrane filters. HNF cells on the filter were stained with primulin according to methods in Caron (1983) and enumerated under ultraviolet excitation. Nanoflagellates that exhibited no apparent red chlorophyll fluorescence under green excitation were identified as HNF.

To count metazoan zooplankton, fixed samples were poured into 100 ml cylinders and concentrated through natural sedimentation for at least 48 h. One ml of the concentrated sample was then loaded onto a Sedgewick-Rafter counting chamber (Pyser-SGI Limited, British) and checked under an optical microscope (Olympus BX51, 100x). Each sample was counted twice.

Time series and state space reconstruction (SSR)

Time series can be defined as any set of sequential observations of the system state, and the dynamic behaviors can be delineated as a trajectory of a state over time in a multidimensional state space by plotting time series. Time series taken from ecosystems can be used to trace out trajectories of the system, which provide information on ecosystem dynamics. For example, if one has performed sequential observations on a three-species ecological system, e.g., grasses (primary producer), rabbits (consumer) and foxes (predator), then the dynamics of the three-species system can be reconstructed by plotting time series of grasses, rabbits, and foxes along the x, y, and z axis, respectively, in a three-dimensional state space. The motion of the three-dimensional vectors can be understood as the system behavior.

In a natural ecosystem, however, it is usually impossible to collect time series of all potentially important variables involved in a target system. Fortunately, Takens (1981) offered a theoretical basis to solve this problem: a mathematical theorem, Takens' embedding theorem, demonstrated that a shadow version of the attractor can be reconstructed by a single observed time series. In other words, delineation of trajectories, originally constructed using multivariables, can be possible even if a time series is available only for a single variable (Sauer et al. 1981; Takens 1981). To embed such a single time series, vectors in the putative phase space are formed from time-delayed values of the time series, $\{x(t), x(t-\tau), x(t-2\tau)\ldots, X(t-[E-1]\tau)\}$, where E is the embedding dimension, and τ is the time lag. This procedure, the reconstruction of the original dynamics, is known as State Space Reconstruction (SSR).

Empirical dynamics modeling (EDM)

EDM, a time-series analytical framework rooted in SSR and designed specifically for the analysis of nonlinear dynamics such as ecosystem processes (Sugihara et al. 2012; Ye et al. 2015; Deyle et al. 2016), was applied to our time-series data. Because EDM recovers dynamics directly from time series using SSR, it does not assume any set of equations governing the system, and thus is suitable for analyzing complex systems, for which it is often difficult to make reasonable *a priori* assumptions about their underlying mechanisms. EDM provides tools for various purposes, including the identification of causal factors and quantification of interaction strengths in nonlinear systems where traditional linear statistical tools are not applicable, and has been recently proven effective for analyzing the dynamics of natural complex ecosystems (Ye et al. 2015; Ushio et al. 2018). The analysis workflow in the present study was as follows: first, causal links between picocyanobacteria and other variables (e.g., water temperature, Chl-*a*, or HNF) were identified using convergent cross mapping (CCM; Sugihara et al. 2012); second, when causal links were identified, the interaction strengths between variables were quantified using the multivariate S-map method (Deyle et al. 2016). Detailed descriptions of CCM and the multivariate S-map are available in previous studies (Sugihara et al. 2012; Deyle et al. 2016; Chang et al. 2017; Ushio et al. 2019). Considering the thermal stratification and vertical migration of the plankton community, we used the average of time-series data collected from 0 to 20 m in our analyses. Data were normalized to a zero mean and unit variance prior to EDM analysis. The library size (i.e., the length of time-series data) of most variables was 48, whereas that of CPcy was 38.

First, CCM was applied to detect causal links. Briefly, if two variables are causally related in a dynamic system, they should share the same attractor, making it possible to predict the values of the causal variable by using the reconstructed state space of the effect variable (Sugihara et al. 2012). CCM quantifies how well an effect variable predicts the values of a putative causal variable, and the forecasting accuracy (i.e., cross map skill) and its convergence against the library size are important criteria for determining causality (for more details, see Sugihara et al. 2012). An essential parameter of CCM, the optimal embedding dimension (E), was determined using simplex projection (Sugihara and May 1990). Simplex projection can be used to find the optimal value of E by identifying which E maximizes the forecasting accuracy of a given time series. According to simplex projection, the optimal E for the time series of SPcy and CPcy were 2 and 3, respectively. Another important parameter, time lag (τ) in the lagged coordinate embedding, was set to 1 following a previous study (Chang et al. 2017), which corresponds to 2 weeks in our time series. Due to the time-lagged causal relationship, preliminary CCM was conducted to find the optimal cross-map lag (t_p, time to prediction) for each causal variable. The optimal t_p value which maximized the forecasting accuracy within the range of 0 to −6 (i.e., between now and three months ago) was chosen. Then, CCM was performed to calculate cross map skill and thus identify causal variables that affect SPcy and CPcy. Fisher's z-test and surrogate test were successively applied to determine whether the cross map skill was statistically significant (Chang et al. 2017). Fisher's z-test examines whether the cross map skill obtained using the maximal library length is significantly higher than that obtained using the minimal library length (i.e., convergence). Surrogate test examines whether

the cross map skill is significantly different from the null model expectation generated using surrogate time series.

Second, based on the set of significant causal variables identified through CCM, the multivariate S-map was built to quantify the influence of each variable on SPcy or CPcy, which was approximated using partial derivatives of the causal variables. Time-series data of the effect variable (SPcy or CPcy) and significant causal variables with lag equal to the optimal t_p were used to reconstruct the state space. For example, if the variables Y and Z influence variable X with t_p of −2 and −3, respectively, the state space is reconstructed as follows: $\{X(t), X(t-1), \ldots, X(t-[E-1]), Y(t-1), Z(t-2)\}$, and $X(t+1)$ is predicted using the multivariate S-map. In the multivariate S-map analysis, the nonlinear parameter (θ) that minimizes the forecasting error was chosen according to previous studies (Deyle et al. 2016; Ushio et al. 2018).

Lastly, Spearman's correlation analysis was conducted to infer potential relationships between the abundances of picocyanobacteria and concentrations of nutrients (DIN and DIP). This method was used because the lengths of time series of nutrients (N = 13) were not sufficient for EDM. All analyses described above were carried out using R v3.4.3 (R Development Core Team, 2018). EDM was performed using the "rEDM" package (version 0.7.2, Ye et al. 2015), and the step-by-step tutorial is available at https://ha0ye.github.io/rEDM/index.html.

Results

Seasonal profiles of water temperature, Chl-a, and nutrients

In Lake Biwa, thermal stratification was pronounced from June to October (the stratification period) with a thermocline between 15 and 30 m (Fig. 1a). In August and

September, water temperature in the epilimnion reached as high as 29.8 °C. Water column started mixing in November and circulated totally from February to March (the mixing period). During the mixing period, the average (± standard deviation) water temperature was 8.4 ± 0.5 °C.

The annual mean concentration of Chl-*a* in the euphotic zone (from 0 to 20 m, calculated by PAR, data not shown) was 2.65 ± 1.85 μg L^{-1} (Fig. 1b). Elevated Chl-*a* concentrations (>5 μg L^{-1}) were recorded several times: from April to May 2016, from November 2016 to January 2017 and from May to June 2017. The mean concentration of DIN was 1.64 ± 1.26 μmol L^{-1}. DIN was depleted in the epilimnion during the stratification period but relatively high at other times (Fig. S1a). DIP concentrations were low and remained nearly constant across depths and seasons, with an average of 0.0045 ± 0.0025 μmol L^{-1} (Fig. S1b).

Seasonal dynamics of picocyanobacteria and potential grazers

The annual average SPcy abundance in the water column above 20 m was 5.64 ± 9.00 × 10^4 cells mL^{-1} during the study period (Fig. 2a). High cell densities (up to 4.50 × 10^5 cells mL^{-1}) were recorded in June and August 2016. Generally, SPcy were highly abundant (>10^5 cells mL^{-1}) throughout the stratification period and were mainly distributed in the epilimnion. During the mixing period, SPcy density decreased to around 10^2 cells mL^{-1} and the cells were almost homogeneously distributed throughout the water column.

The annual average CPcy abundance was 3.22 ± 8.45 × 10^4 cells mL^{-1} (Fig. 2b; calculated using data collected from July 2016 to June 2017, as data from 2015 were incomplete). CPcy density increased dramatically from 10^3 to >3 × 10^5 cells mL^{-1} in the epilimnion at the beginning of July. The majority of CPcy were observed near the

thermocline after 2 weeks and the greatest cell density (up to 4.54×10^5 cells mL^{-1}) was recorded at a depth of 15 m in mid-July 2016. From September onward, CPcy density declined rapidly to $<10^3$ cells mL^{-1} and became undetectable during the mixing period.

HNF were observed throughout the year in the water column, with an average cell density of $7.86 \pm 6.44 \times 10^2$ cells mL^{-1} (Fig. S2a). Generally, HNF were abundant ($>10^3$ cells mL^{-1}) in the epilimnion during stratification periods and remained scarce ($>10^2$ cells mL^{-1}) during mixing periods. By contrast, ciliates were not a major protistan group in Lake Biwa (Fig. S2b). At most times, ciliates were at low abundance (<10 cells L^{-1}) or even below the detection limit, with an annual average cell density of 51.7 ± 153.8 cells L^{-1}. Nevertheless, the genera *Epistylis* and *Codonella* sometimes formed transient blooms in summer or autumn with densities greater than 10^3 cells L^{-1}.

We observed no clear seasonal or vertical trends in the abundances of rotifers, cladocerans, and copepods. These grazers could be abundant in spring, summer or autumn at different depths (Fig. S2c–e). The annual average density of rotifers was 111.6 ± 124.5 individuals L^{-1}. Rotifers in Lake Biwa were highly diverse, so we also recorded seasonal changes in their genus-level composition (Fig. S3). The dominant genera during the study period were *Polyarthra*, *Keratella* and *Trichocerca*, accounting for 55.1%, 11.5%, and 10.8% of total rotifer abundance, respectively. Average densities of cladocerans and copepods were 13.2 ± 15.6 and 58.8 ± 59.1 individuals L^{-1}, respectively. The dominant genera of cladocera were *Daphnia* and sometimes *Bosmina*, whereas copepods were dominated by *Eodiaptomus japonicus*.

Results of EDM and correlation analysis

According to CCM, temperature, HNF, cladocera, copepod, and *Keratella* were identified as causal variables that affected seasonal changes in the abundance of both

SPcy and CPcy, whereas *Polyarthra* affected only SPcy and *Trichocerca* affected only CPcy (Table 1; see details in Fig. S4 and S5). In addition, we also found causal links between SPcy and CPcy. No significant causal link was found between SPcy and other variables such as Chl-*a* concentration, ciliates or non-dominant rotifers (e.g., *Pleosoma*, *Pompholyx*), or between CPcy and those variables (data not shown).

The influences of causal variables on SPcy and CPcy were then quantified using the multivariate S-map (Fig. 3; see Table S1 for optimal parameters of the multivariate S-map). Positive and negative values of interaction strengths can be interpreted as an effect variable tending to increase and decrease, respectively, in response to the increase in a causal variable (Deyle et al. 2016). Although the multivariate S-map method enables the calculation of time-varying interaction strengths, we used time-averaged values of interaction strengths to evaluate the overall effects of causal variables on picocyanobacterial abundance for convenience and simplicity (Table 1; Fig. 4). Therefore, temperature, HNF, and *Polyarthra* had positive effects on SPcy, whereas CPcy, *Keratella*, and cladocera negatively affected SPcy abundance. And copepods had a moderating effect on SPcy. On the other hand, SPcy, HNF, *Trichocerca*, and copepod had positive effects on CPcy abundance, whereas temperature, *Keratella*, and cladocera had negative effects on CPcy.

Lastly, potential relationships among SPcy, CPcy, DIN, and DIP were examined using Spearman's correlation analysis. The results showed that DIN was negatively correlated with SPcy and CPcy ($r_s = -0.668$, $p < 0.001$; $r_s = -0.734$, $p < 0.001$, respectively). DIP was negatively correlated with CPcy ($r_s = -0.528$, $p < 0.001$), but not significantly correlated with SPcy ($r_s = 0.092$, $p = 0.516$).

Discussion

In the present study, we applied EDM for exploring the environmental variables and organisms that are potential drivers of the seasonal dynamics of picocyanobacteria. Interaction strengths estimated using the multivariate S-map revealed how these variables affect picocyanobacterial abundance. In many cases, both positive and negative values were obtained simultaneously, suggesting complex relationships between the causal variable and SPcy or CPcy. The influence by one causal variable on SPcy or CPcy abundance (i.e., the average of interaction strengths) should be regarded as a "net" interaction strength. In other words, the time-averaged interaction strength calculated in the present study indicates whether positive "bottom-up" effects were larger than negative "top-down" effects or not (Deyle et al. 2016).

Effects of environmental variables on picocyanobacteria

The positive influence of temperature on SPcy as revealed through EDM (Fig. 3a) indicates that temperature played an important role in increasing the abundance of picocyanobacteria (Vörös et al. 2009; Jodłowska and Śliwińska 2014). By contrast, CPcy was negatively affected by temperature (Fig. 3b), despite being reported to increase dramatically in the warm summer months (Callieri et al. 2012). This result may be caused by sinking due to the large colony sizes of CPcy (Deng et al. 2016). After forming transient blooms near the water surface, CPcy immediately sank to the metalimnion (15–20 m), where water temperature was relatively low during the stratification period (Fig. 1a and 2b). On the other hand, we did not find a relationship between Chl-*a* concentration and picocyanobacterial abundance. Although picocyanobacteria could be dominant (45% of total Chl-*a*) during the stratification

period, they were not a major group in the phytoplankton community during other parts of the year in Lake Biwa (< 5% of total Chl-*a* in other months; Nagata 1986). Lastly, the negative correlations found between DIN or DIP and picocyanobacterial abundance suggested that low nutrient availability facilitated the dominance of SPcy (Nagata 1986; Schallenberg and Burns 2001; Callieri 2008), and that nutrient limitation could be one of the factors inducing colony formation (Callieri et al. 2012).

Relationships between single cells and colonies

Some strains of SPcy are known to form microcolonies under certain conditions such as ultraviolet radiation or grazing pressure (Jezberová and Komárková 2007; Callieri et al. 2011; Callieri et al. 2016). Conversely, some CPcy genera have single-cell stages in their life histories (Komárková and Šimek 2003) and many genera in the order *Synechococcales* that were originally described as colonial lose their mucilaginous envelopes in cultivation (Komárek et al. 2014). Therefore, transformation between single cells and colonies may occur frequently in lakes. Fortunately, we found significant causal links between SPcy and CPcy, suggesting that they were affected by each other. Furthermore, the multivariate S-map results suggested that SPcy could have enhanced the abundance of CPcy, whereas CPcy decreased that of SPcy (Fig. 4). Therefore, the transformation of the morphology of picocyanobacteria is likely unidirectional in Lake Biwa, with SPcy tending to form CPcy.

Effects of protists on picocyanobacteria

HNF have been regarded as key grazers of SPcy that contribute strongly to the latter's mortality losses (Nagata 1988; Callieri et al. 2002), and thus HNF are expected to have negative effects on SPcy abundance. However, the opposite result was obtained from

EDM, with HNF increasing SPcy abundance rather than reducing them (Fig. 4). A possible explanation for this is the enhancement of SPcy growth due to nutrient regeneration by HNF exceeding mortality losses due to grazing by HNF. This is consistent with the results in previous studies (Ferrier-Pages and Rassoulzadegan 1994; Selph et al. 2003), though it is impossible to quantify the amount of nutrients excreted respectively by HNF or other grazers from nutrient samples we collected.

The other possible explanation is the presence of trophic cascade, as HNF fall within the food size ranges of a variety of predators and are vulnerable to predation in aquatic environments (Pace et al. 1998; Nakano et al. 2001). We investigated the influence of putative predators on HNF using EDM, and found that *Polyarthra* and cladocera showed negative influences on HNF abundance (Table S2), suggesting the presence of top-down controls on HNF by these predators (Pourriot 1977; Stemberger and Gilbert 1985; Jürgens et al. 1996). So it is likely that grazing pressure of HNF on picocyanobacteria can be suppressed by predation of *Polyarthra* and cladocerans. Furthermore, HNF were enumerated at the community level in the present study, and grazing on SPcy by HNF is species-specific (Callieri et al. 2012). Therefore, the HNF species that prey on SPcy could be minor in our samples, which could result in the moderate influence of SPcy on HNF (Table S2).

On the other hand, the positive effect on CPcy abundance by HNF (Fig. 4) suggested that HNF play an important role in stimulating colony formation by picocyanobacteria, possibly through grazing on single cells, as previously reported (Callieri et al. 2016). Indeed, microcolony-forming bacteria generally cannot be consumed by HNF due to their large size (Hahn et al. 2000), and we found an apparent

365 negative effect of CPcy on HNF (Table S2), which suggests that HNF are unlikely to
366 graze on CPcy.
367 Ciliates are also important grazers of picocyanobacteria (Šimek et al. 1995 and
368 1997), but EDM did not show a significant causal link between their abundance and that
369 of SPcy or CPcy. Ciliates may not be involved in controlling picocyanobacterial
370 abundance, due to their low abundance in the north basin of Lake Biwa (Yoshida et al.
371 2001b).

372 ***Effects of metazoan zooplankton on picocyanobacteria***

373 The relationships between metazoan zooplankton and picocyanobacteria can be more
374 complex because they are both potential grazers of picocyanobacteria and predators of
375 HNF and small zooplankton. For example, rotifers feed significantly on nanoflagellates
376 and small ciliates (Arndt 1993), whereas cladocerans prey upon a wide range of food
377 particle sizes (1–50 μm) that includes small protists (Gophen and Geller 1984; Stockner
378 and Porter 1988; Sanders et al. 1994). Copepods use a variety of hunting and feeding
379 techniques, enabling them to prey on diverse planktonic animals (Suzuki et al. 1999;
380 Brandl 2005). The influence of rotifers, cladocerans, and copepods on SPcy or CPcy
381 can thus be interpreted as the synergistic effects of grazing, nutrient regeneration, and
382 trophic cascades. In addition, we also conducted EDM analyses to investigate whether
383 and how these grazers could be affected by other variables (Table S2). However,
384 potential causal variables of metazoan zooplankton, such as abundances of bacteria,
385 phytoplankton and their predators, are not available. The reconstructed dynamics of
386 metazoan zooplankton might not be reasonably resolved, and thus the interaction
387 strengths calculated by the multivariate S-map might be counter-intuitive and difficult
388 to explain. Therefore, detailed discussion on influences of causal variables on metazoan

zooplankton can be speculative and should be avoided as possible.

Rotifers that have been previously reported to prey on picocyanobacteria are *Keratella* and *Polyarthra* (Callieri et al. 2012), both of which were dominant genera in Lake Biwa (Yoshida et al. 2001b). *Keratella* showed apparent top-down control on picocyanobacterial abundance (Fig. 4), indicating that they may be effective grazers of both SPcy and CPcy (Pourriot 1977; Callieri et al. 2012; Table S2, positive influence of CPcy on *Keratella*). The positive effect of *Polyarthra* on SPcy (Fig. 4) suggested that *Polyarthra* may enhance SPcy abundance through preying on HNF, as discussed previously. The bacterivorous rotifer *Trichocerca* enhanced the abundance of CPcy (Fig. 4), and the negative influence of CPcy on *Trichocerca* may suggest that CPcy were not grazed by *Trichocerca* (Table S2). However, it is unclear whether grazing pressure from *Trichocerca* plays a role in inducing colony formation, as they had no effect on SPcy (Table 1). Overall, rotifers have seldom been investigated as grazers of picocyanobacteria, and therefore further research is needed to clarify the food chain between rotifers and picocyanobacteria.

Cladocerans are well-known grazers of SPcy (Callieri et al. 2012), and they had an apparent negative influence on SPcy abundance (Fig. 4). Similar to the rotifer *Keratella*, cladocerans (mainly *Daphnia*) induced the decrease in CPcy abundance (Fig. 4), suggesting that they graze on CPcy. Although the CPcy found during the present study generally had large colony sizes (up to several hundred μm), microcolonies ranging from several to tens of micrometers can be eaten by cladocerans (Ronneberger 1998; Table S2, positive influence of CPcy on cladocera).

The effect of copepods on SPcy abundance was nearly moderate, despite their ability to ingest SPcy effectively (Fig. 4; Motwani and Gorokhova 2013; Table S2,

positive influence of SPcy on copepod). A possible reason for this finding is that the negative effects of grazing were offset by positive indirect effects, especially through trophic cascades, as copepods represent the highest trophic level among grazers of picocyanobacteria. A trophic cascade involving copepods may also be a major contributor to their positive influence on CPcy (Fig. 4). Copepods increased the abundance of CPcy, possibly by preying on microzooplankton such as *Keratella* (Yoshida et al. 2001a) that have negative effects on CPcy abundance.

Conclusions

Increasing picocyanobacterial abundance along with climate change in future have been indicated in oceans (Flombaum et al. 2013). In lakes, growing blooms of picocyanobacteria also have been reported in recent years, and some species of picocyanobacteria can produce harmful toxins and secondary metabolites thus causing problems to public health (Jakubowska and Szeląg-Wasielewska 2015; Jasser and Callieri 2016; Śliwińska-Wilczewska et al. 2018). Despite the increasing impacts of picocyanobacteria on aquatic ecosystems, however, ecology of them remain largely unclear. So far, few studies have been reported discussing the comprehensive impacts of grazers on picocyanobacteria.

In the present study, we found that HNF, *Keratella*, *Polyarthra*, *Trichocerca*, cladocerans and copepods had important impacts on SPcy and CPcy, and played various roles in controlling their abundances (Fig. 4). Notably, we found that HNF might stimulate the growth of SPcy through indirect positive effects such as nutrient regeneration in excess of direct negative effects such as grazing, which is a novel result. We also found that single cells of picocyanobacteria tended to form colonies, possibly due to the positive effects of HNF, *Trichocerca* and copepods on CPcy. Our findings

clearly show that natural seasonal dynamics of picocyanobacteria in Lake Biwa are influenced by a variety of grazers, and that the influences of grazers in complex natural food webs are often counter-intuitive. Furthermore, because SPcy and CPcy are influenced by different grazers, they may thus play differing ecological roles in the aquatic food web. It should be noticed that we did not conduct any *in situ* or laboratory experiments to validate the results of the present study. Further research, especially *in situ* manipulative experiments, is needed to elucidate the detailed interspecific interactions among picocyanobacteria and their grazers.

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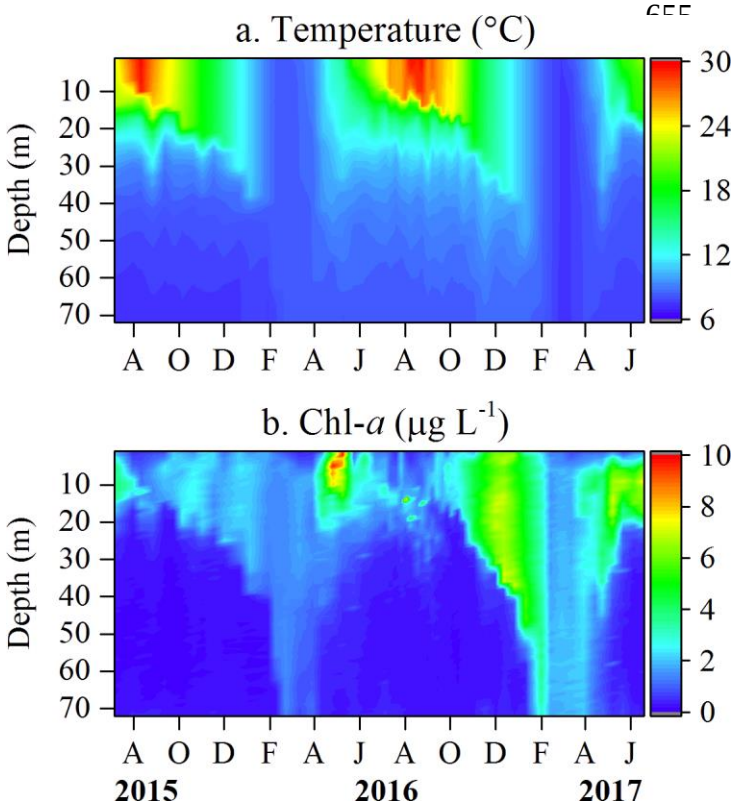
Table 1. Significant causal variables affecting the abundances of single-celled picocyanobacteria (SPcy) and colonial picocyanobacteria (CPcy) identified by CCM, and according time-averaged interaction strengths calculated by the multivariate S-map.

Effect variable	Causal variable	t_p	ρ_{max}	$\Delta\rho$	P_z	P_s	Time-averaged interaction strength
SPcy	Temperature	0	0.75	0.42	0.000	0.048	0.164
	CPcy	−2	0.57	0.46	0.000	0.004	−0.467
	HNF	−1	0.58	0.31	0.007	0.006	0.324
	Keratella	−4	0.42	0.44	0.001	0.024	−0.213
	Polyarthra	−5	0.51	0.47	0.000	0.003	0.133
	Trichocerca	0	0.26	0.13	0.342*	0.081*	
	Cladocera	0	0.62	0.54	0.000	0.001	−0.300
	Copepod	−3	0.54	0.41	0.001	0.001	0.008
CPcy	Temperature	−2	0.91	0.59	0.000	0.001	−0.299
	SPcy	−2	0.82	0.57	0.000	0.001	0.125
	HNF	−3	0.81	0.63	0.000	0.001	0.212
	Keratella	−4	0.49	0.47	0.000	0.011	−0.059
	Polyarthra	0	0.12	0.16	0.267*	0.309*	
	Trichocerca	−1	0.47	0.30	0.019	0.004	0.262
	Cladocera	−1	0.29	0.29	0.040	0.044	−0.081
	Copepod	−6	0.55	0.45	0.000	0.002	0.139

t_p: cross-map lag; ρ_{max}: ρ at maximal library size; $\Delta\rho$: ρ at maximum library size minus ρ at minimum library size; P_z: *P* value of Fishfer's z test; P_s: *P* value of surrogate test; *: not statistically significant at the 0.05 level.

653 Figure 1. Seasonal changes in vertical profiles of (a) water temperature and (b)
654 chlorophyll-*a* concentration.



662 Figure 2. Seasonal changes in vertical abundances of (a) single-celled picocyanobacteria
663 (SPcy) and (b) colonial picocyanobacteria (CPcy).
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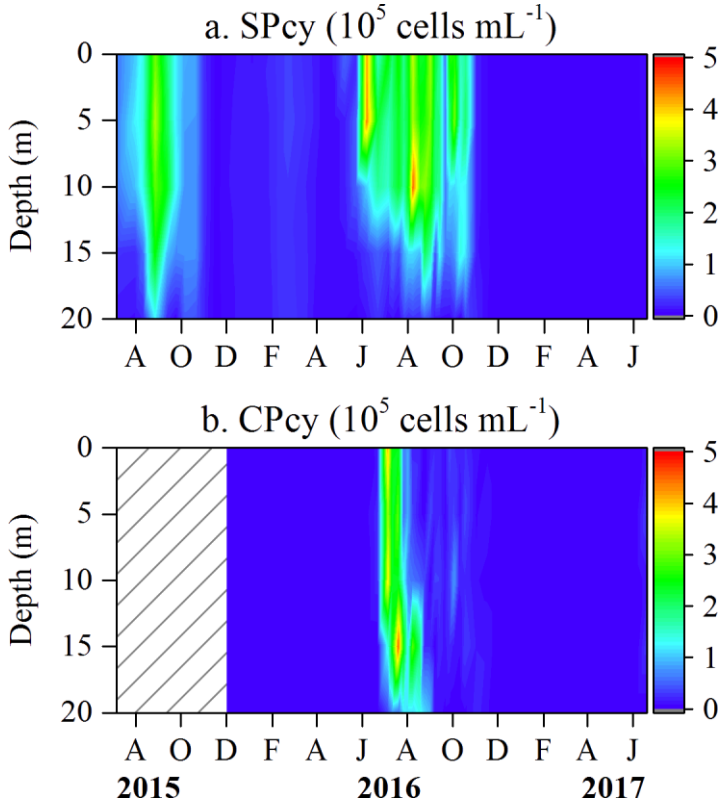
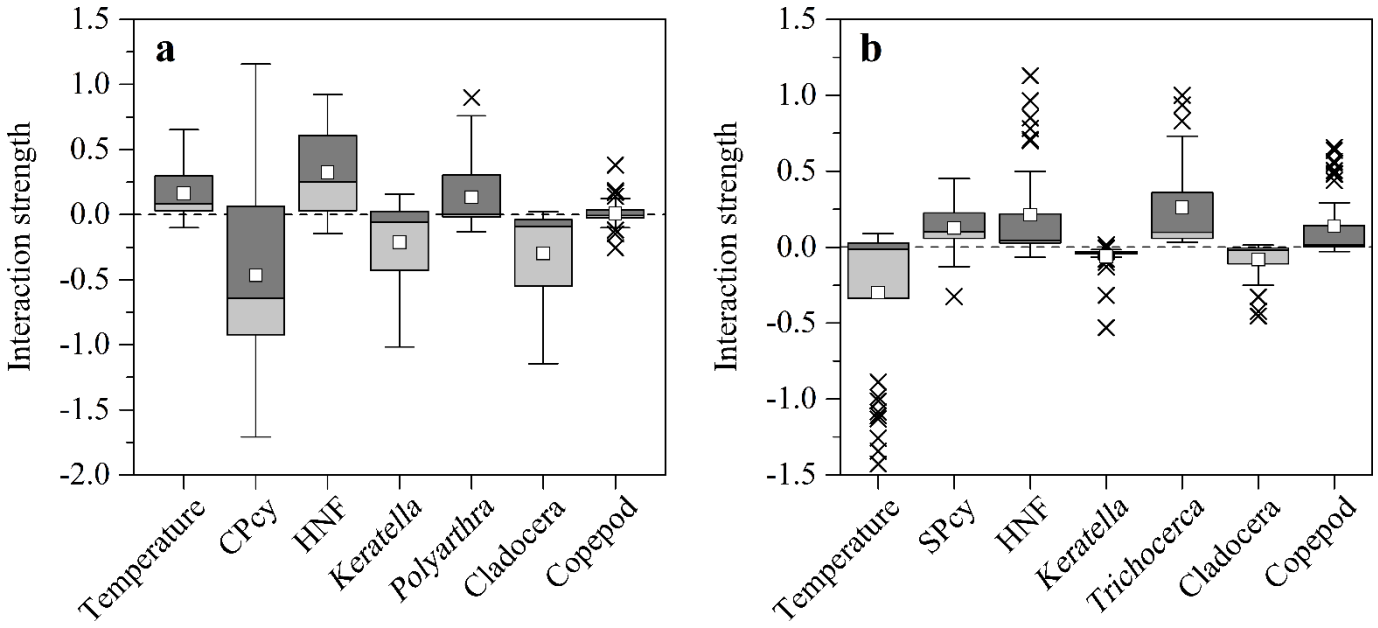


Figure 3. The effects of causal variables on picocyanobacterial abundance determined using the multivariate S-map. The boxplots show the interaction strengths of causal variables on the abundances of (a) SPcy and (b) CPcy. The bottom and top of each box show the lower (25%) and upper (75%) quartiles, respectively; the band and square within each box represent the median and the mean, respectively; whiskers indicate the minimum and maximum; and crosses represent outliers. Note that original time series were standardized, and thus the interaction strengths from different variables can be compared directly to discuss the relative importance of each variable.



675 Figure 4. The effects of grazers on picocyanobacterial abundance, and the relationship
676 between SPcy and CPcy. Numbers beside the arrows represent time-averaged values of
677 interaction strengths.

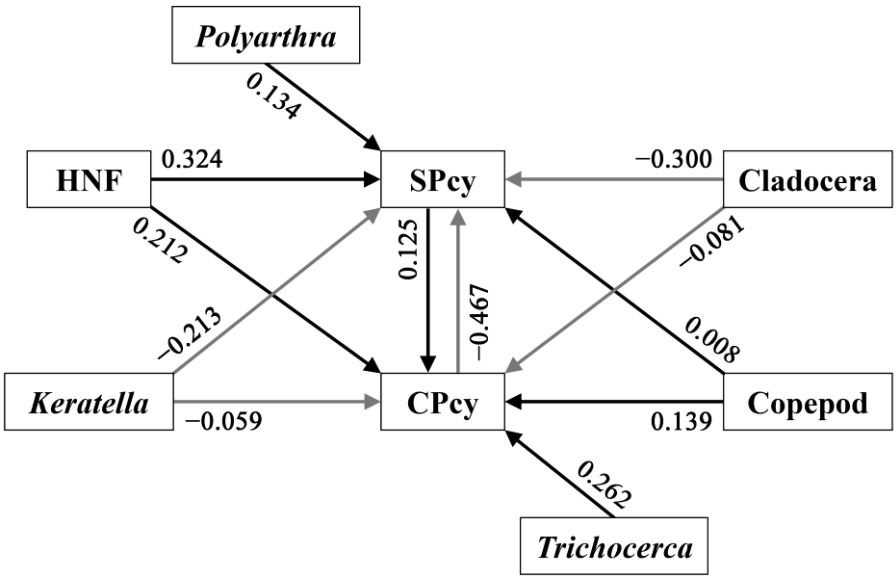


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Table S1. Optimal parameters of the multivariate S-map.

Variable to predict	θ	Number of predictions	ρ	MAE	RMSE	*P* value
SPcy	3.75	36	0.90	0.28	0.46	0.000
CPcy	1.45	35	0.65	0.43	0.86	0.000

θ: the nonlinear parameter; ρ: the forecasting accuracy; MAE: mean absolute error; RMSE: root mean square error; *P* value: *P* value that ρ is significantly greater than zero using Fishfer's z-transformation.

686 Table S2. Significant causal variables affecting the abundances of HNF and metazoan
687 zooplankton identified by CCM, and according time-averaged interaction strengths
688 calculated by the multivariate S-map.

Effect variable	Causal variable	t_p	ρ_{max}	$\Delta\rho$	P_z	P_s	Time-averaged interaction strength
HNF	SPcy	0	0.63	0.30	0.007	0.045	−0.002
	CPcy	−5	0.55	0.53	0.000	0.013	−0.119
	Polyarthra	−3	0.27	0.30	0.033	0.046	−0.297
	Cladocera	−1	0.51	0.53	0.000	0.004	−0.058
Keratella	CPcy	−6	0.48	0.42	0.001	0.017	0.483
	HNF	0	0.30	0.26	0.047	0.036	0.072
	Polyarthra	−5	0.27	0.32	0.023	0.046	−0.142
	Cladocera	−2	0.26	0.32	0.023	0.027	0.024
Polyarthra	HNF	−3	0.40	0.36	0.007	0.030	−0.035
	Cladocera	−5	0.77	0.60	0.000	0.001	0.331
Trichocerca	CPcy	0	0.51	0.49	0.000	0.009	−0.091
	HNF	−5	0.51	0.52	0.000	0.028	0.417
	Cladocera	−5	0.63	0.59	0.000	0.001	−0.006
	Copepod	−2	0.56	0.58	0.000	0.045	0.083
	Polyarthra	0	0.30	0.33	0.017	0.042	0.436
Cladocera	CPcy	−6	0.53	0.51	0.000	0.017	0.170
	HNF	−2	0.39	0.34	0.011	0.049	0.273
	Polyarthra	−6	0.55	0.37	0.002	0.006	0.400
	Trichocerca	−1	0.46	0.25	0.046	0.004	−0.154
Copepod	SPcy	0	0.56	0.39	0.001	0.003	0.321
	Cladocera	0	0.34	0.36	0.009	0.009	0.062
	Trichocerca	−2	0.44	0.36	0.006	0.003	−0.317

689 t_p: cross-map lag; ρ_{max}: ρ at maximal library size; Δρ: ρ at maximum library size minus
690 ρ at minimum library size; P_z: *P* value of Fishfer's z test; P_s: *P* value of surrogate test.

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693 Figure S1. Seasonal changes in vertical distributions of (a) dissolved inorganic nitrogen
694 (DIN) and (b) dissolved inorganic phosphorus (DIP) concentrations.
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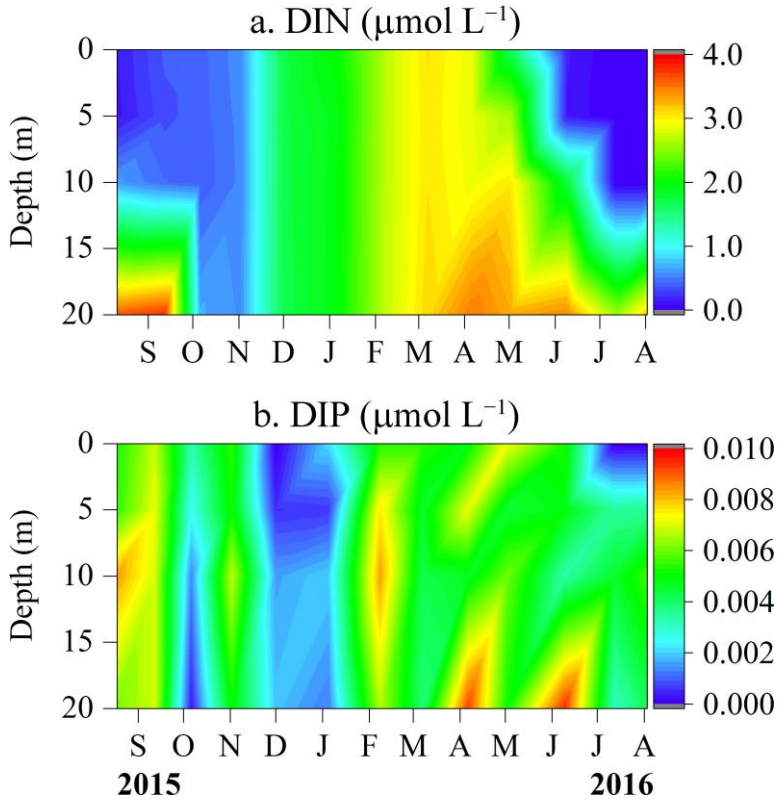


Figure S2. Seasonal changes in vertical abundances of picocyanobacteria grazers: (a) HNF, (b) ciliate, (c) rotifer, (d) cladocera, and (e) copepod.

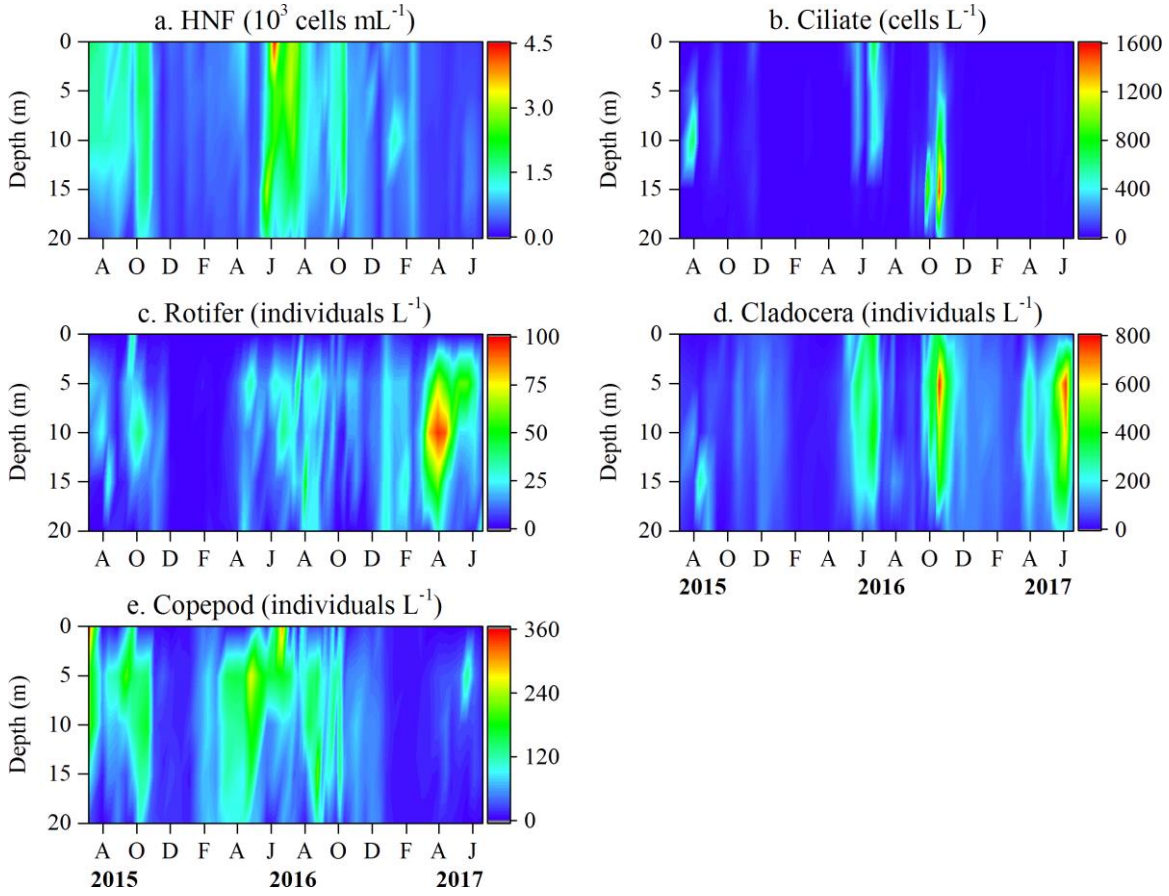


Figure S3. Seasonal changes in the genus-level composition of rotifers.

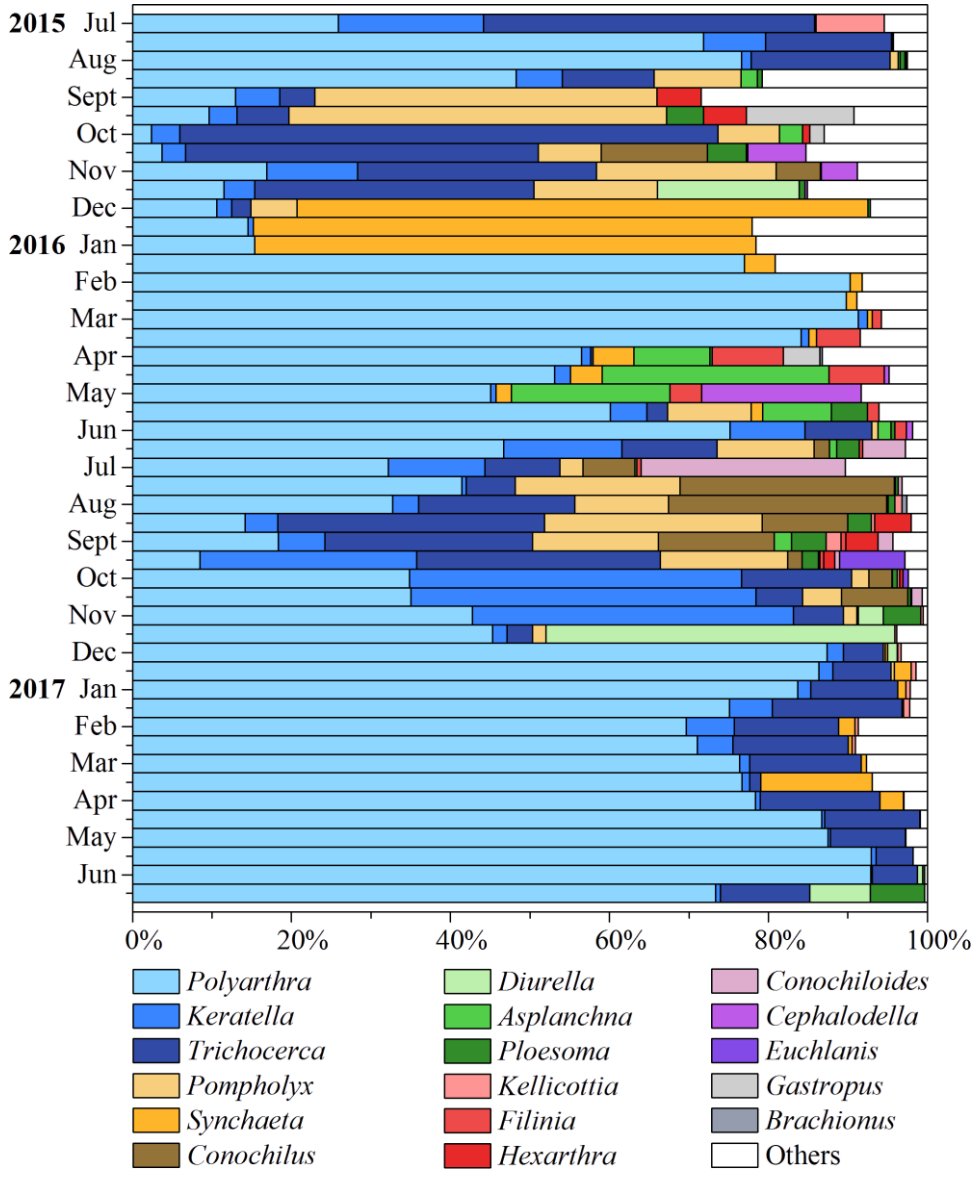
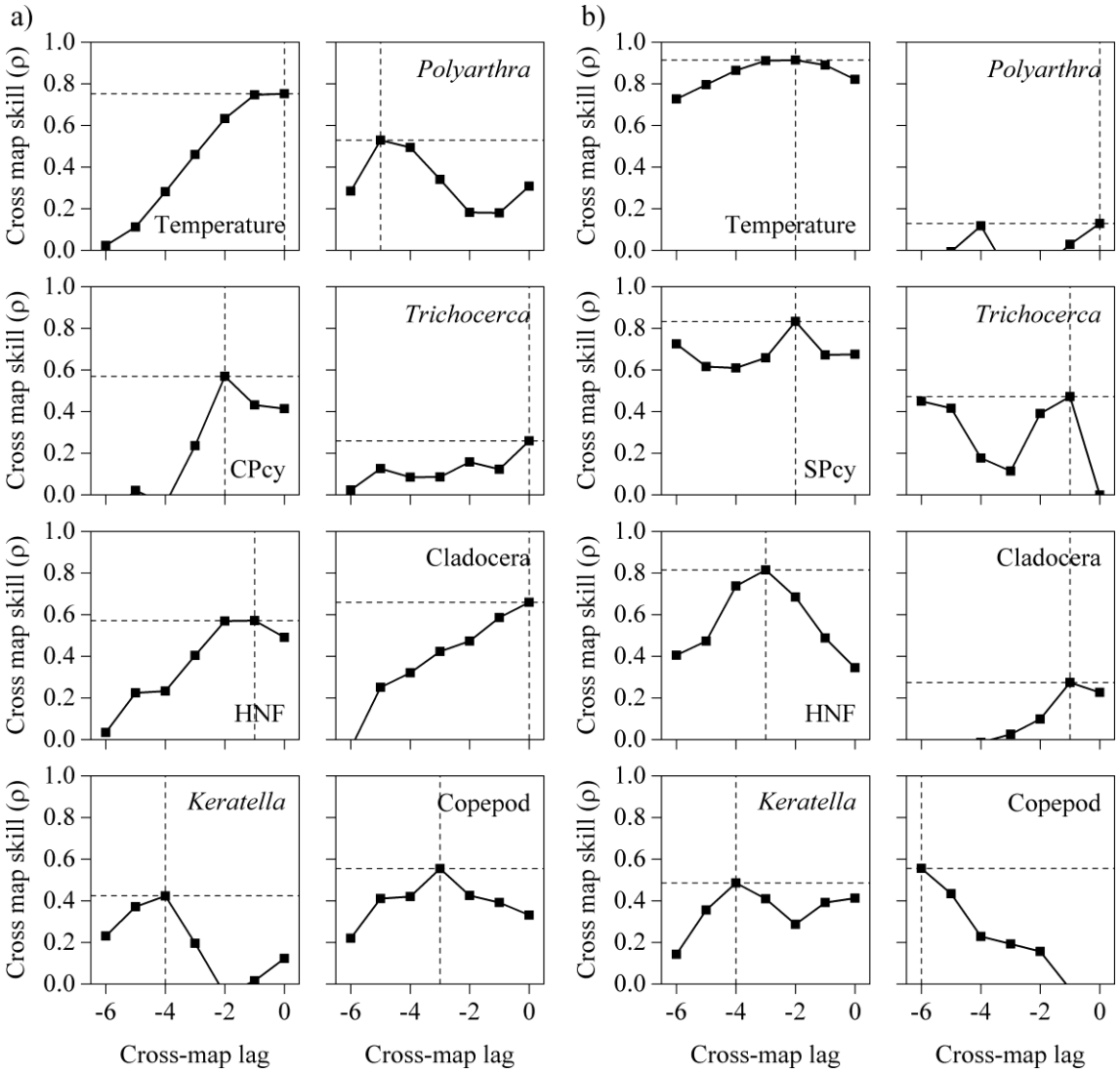


Figure S4. Time-delayed convergent cross mapping (CCM). (a) SPcy and (b) CPcy cross-mapping causal variables. Crossed dash lines indicate the optimal cross-map lag (t_p), which maximizes cross map skill (ρ).



710 Figure S5. CCM at the optimal cross-map lag. (a) SPcy and (b) CPcy cross-mapping
711 causal variables
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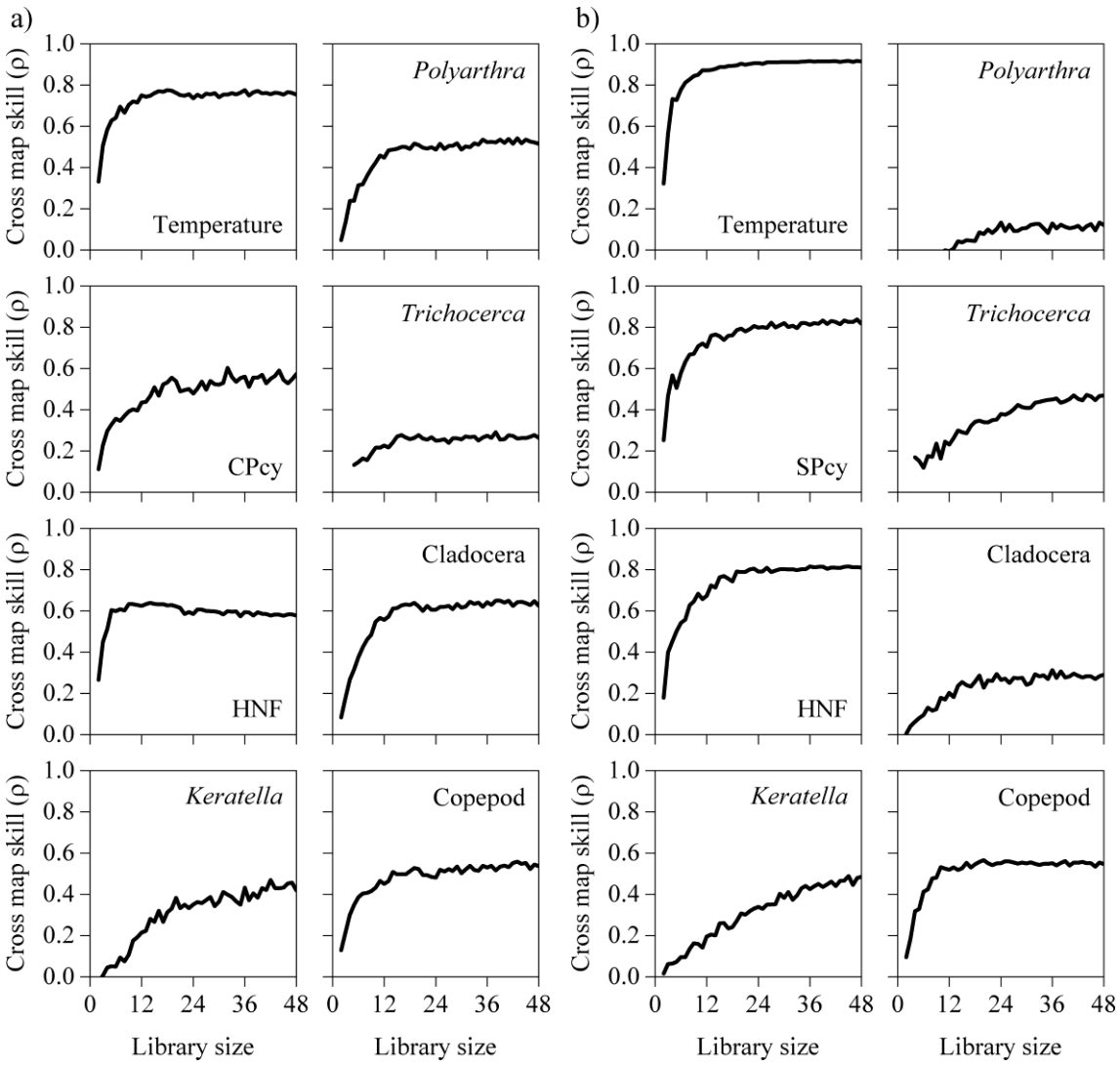

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