

SEASONAL VARIATIONS IN TROPHIC LINKS BETWEEN MICROZOOPLANKTON AND NANOFLAGELLATES IN A COASTAL ECOSYSTEM

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Key words: heterotrophic nanoflagellates, pigmented nanoflagellates, growth rates, bacterial abundance.

ABSTRACT

Using incubation experiments with size-fractionated coastal waters, this study calculated the growth and loss rates of nanoflagellates in a coastal ecosystem of the subtropical western Pacific along a rocky shore in northeastern Taiwan. Samples were taken monthly from September 2014 to August 2015. Seasonal variations in growth rates of heterotrophic nanoflagellates (HNF) and pigmented nanoflagellates (PNF) ranged from 0.17 to 1.13 d⁻¹ and 0.05 to 1.21 d⁻¹, respectively. This study found that grazing had a significant impact on nanoflagellate community (PNF: 0.14 to 0.39 d⁻¹; HNF: 0.12 to 0.52 d⁻¹), accounting for about 15-30% for PNF and 18-60% for HNF growth during the warmer periods. However, during colder period, grazing was not found to have an impact on the mortality of nanoflagellates in this subtropical coastal ecosystem. Furthermore, an interesting phenomenon in this study, composition of nanoflagellates 5-10 μm and > 10 μm size class showed a more pronounced increase in < 20 μm treatments at the end of experiments during the warmer study periods. We suggest that at least two trophic levels within the nanoflagellate community: small nanoflagellates and large nanoflagellates.

I. INTRODUCTION

Nanoflagellates ranging from 2 to 20 μm in size play an important role in the trophic fluxes in the microbial food web, as they are ubiquitous and found to be the most efficient consumers of picoplanktonic cells in aquatic systems (Sanders et al., 1992; Hall et al., 1993; Sherr and Sherr, 2002; Unrein et al., 2013). Thus, they would be expected to play a key role in controlling picoplankton production (Wikner et al., 1990; Sherr

and Sherr, 2002; Tsai et al., 2005; Williams et al., 2008). Nanoflagellates are an important food source for larger protozoans and metazoans, and thus they should function as a link between the microbial compartment and higher trophic levels (Gasol et al., 1995; Nakano et al., 2001).

Two of the most important factors shaping nanoflagellate communities are resource and predation (Jürgens et al., 1996). The relative importance of the two factors to nanoflagellate abundance is believed to depend on their position in the trophic hierarchy and the productivity of the system (Berglund et al., 2005). Some studies have found increase nanoflagellate abundance in areas with bacterial abundance, indicative of prevailing resource control (Sanders et al., 1992; Gasol et al., 1995; Tsai et al., 2008). A weak coupling between bacterial and nanoflagellate abundance, on the other hand, indicates predation control (Wieltschnig et al., 2001). However, while correlative studies can provide an indication of governing factors, they cannot be used to fully understand the complex mechanisms in such an environment. To do this, it is necessary to estimate both nanoflagellate growth and loss rates to determine differences in the two types of nanoflagellate control.

Little is known about the growth and loss rates of nanoflagellates and their seasonal variations in their natural environment (Ferrier-Pagés and Rassoulzadegan, 1994; Weisse, 1997). In previous studies, Nagata (1998) suggested that food supply was a more important factor than temperature in HNF growth in Lake Biwa, while Carrick et al. (1992) reported a linear increase in HNF growth associated with water temperature in Lake Michigan. Furthermore, Weisse (1997) suggested that both bacterial abundance and temperature limited HNF growth in Lake Constance. To the best of our knowledge, no seasonal study has been undertaken to estimate nanoflagellate growth in marine environments. Although one study has reported ciliates to be the most important predators of nanoflagellates, consuming 32-80% of nanoflagellate production in a freshwater environment (Nakano et al., 2001). It is unclear how significant ciliate grazing pressure may be on nanoflagellate communities of other ecosystems. Beside this, some studies have found indirect effects of predation as part of the trophic cascade, based on predation limitation on several trophic levels in nanoflagellate communities (Reckermann and Veldhuis, 1997; Lin et al., 2009). Reckermann and

Veldhuis (1997) found evidence for two trophic levels within nanoflagellates communities: larger nanoflagellates in size 10-20 μm select smaller nanoflagellates (1-5 μm).

The abundance of the main resource (bacteria) and the predators (ciliates) show large seasonal variations in this study area (Tsai et al., 2008; Chao et al., 2013). To our knowledge, the relative importance of these factors on seasonal variations in nanoflagellates has not been well quantified, neither in oligotrophic nor in eutrophic systems. Here, the aims of the present study were to determine the seasonal variations in growth and grazing rates of nanoflagellates in a coastal ecosystem of the subtropical western Pacific. We expected an increase in the relative importance of predation control in summer, since ciliate are thought to be more abundant in the warm seasons (Chao et al., 2013).

II. MATERIAL AND METHODS

1. Sampling

Samples were collected monthly September 2014 to August 2015 at an established coastal station (25°09.4'N, 121°46.3'E) along a rocky shore in northeastern Taiwan. The environment of this site has been characterized based on data collected from 1999 to 2001 (Tsai et al., 2005). Water temperature there remains constantly above 25°C between June and October, with daytime temperatures generally 0.5-1.5°C higher than those at night (Tsai et al., 2005). Salinity ranges annually from 33.1 to 34.3, the lower value probably reflecting the influence of rainfall runoff. Average monthly nitrate concentrations are highest between November and May, when they may reach 12 μM . Nitrate concentrations decrease to 1 μM between June and October (Tsai et al., 2005). The concentrations of chlorophyll *a* in this study area range from 0.31 mg m^{-3} to 2.41 mg m^{-3} (Tsai et al., 2013).

2. Size-Fractionation Experiments

For each period, 10 L surface seawater sample was collected from 09:00 to 10:00 h in the morning (local time) for short-term (2 days) growth and grazing experiments. Two days was selected because in experiments performed earlier in July initial nanoflagellate growth response could be observed two days after removal of the predators in 20 μm filtered treatments (Fig. 1). Water temperature was measured immediately after the sampling bucket was cast. All samples were brought to the laboratory within 30 min.

Using the differential filtration method (Wright and Coffin, 1984), we estimated the growth and grazing rates of nanoflagellates. Briefly, control treatments (with grazers) were natural unfiltered seawater. For the filtration experiments, a 20- μm pore polycarbonate filter was used to remove the predators of nanoflagellates. The treated samples were transferred into polycarbonate bottles to a volume of 500 mL each and incubated in triplicate in a water bath at *in situ* temperatures.

The net growth rate of nanoflagellate (k , d^{-1}) was calculated for each sample based on microscopic cell counts at the start and the end of the experiment (N_{t_0} and N_t):

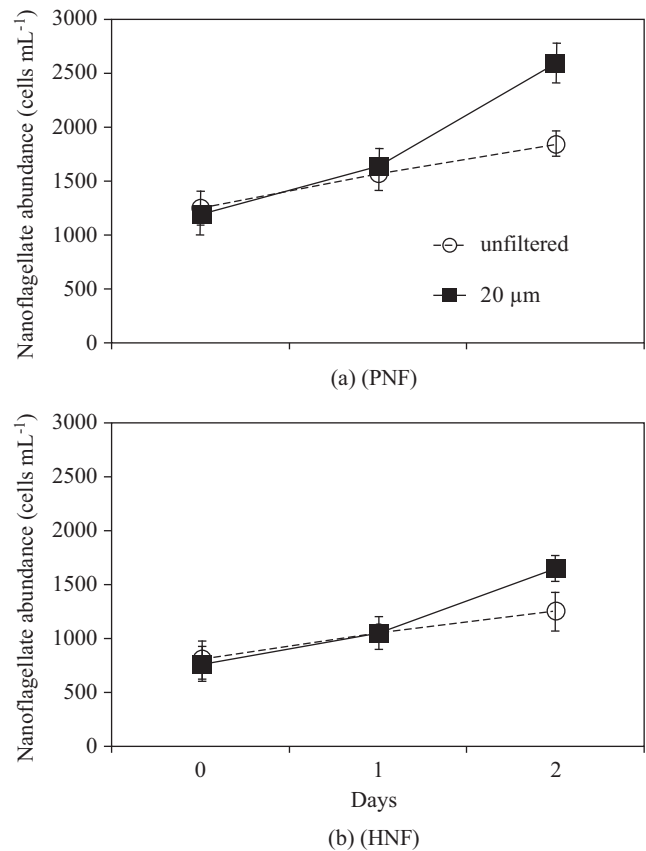


Fig. 1. Pigmented nanoflagellate (PNF) (a) and heterotrophic nanoflagellate abundance (HNF) (b) time-series in July 2015 experiment.

$$k = \ln(N_t / N_{t_0}) / (t - t_0)$$

where t_0 and t are the start and end of the experiment (2 days), respectively. Growth rates (μ , d^{-1}) of nanoflagellate were calculated based on the results from the < 20 μm filtrates. A direct estimate of grazing mortality for nanoflagellates was obtained by calculating the difference in net growth rates between unfiltered and 20 μm filtered treatments.

3. Bacterial, *Synechococcus* spp. Nanoflagellate and Ciliate Abundance Counts

Picoplankton (Bacteria, *Synechococcus* spp.) and nanoflagellates were counted using an epifluorescence microscope (Nikon Optiphot-2) (1000 \times). Subsamples of 1-2 mL or 20 mL were filtered onto 0.2 μm or 0.8 μm black Nuclepore filters for picoplankton and nanoflagellates, respectively. Samples were stained with DAPI at a final concentration of 1 $\mu\text{g mL}^{-1}$ (Porter and Feig, 1980) to count bacteria and heterotrophic nanoflagellates (HNFs). Pigmented nanoflagellates (PNF) and HNF were detected and counted based on the absence or presence of chlorophyll autofluorescence using a separate filter set optimized for chlorophyll or DAPI under a 1000 \times epifluorescence microscope (Nikon-Optiphot-2). Bacteria and HNF were identified by

Table 1. Monthly variation of PNF and HNF net growth rate in unfiltered and 20 μm treatments. PNF and HNF loss rate by ciliates.

Month/Year	PNF net growth rate (d^{-1})		HNF net growth rate (d^{-1})		Ciliate grazing on PNF	Ciliate grazing on HNF
	Unfiltered	20 μm	Unfiltered	20 μm		
Sep-14	0.94 \pm 0.08	1.08 \pm 0.13	0.82 \pm 0.08	1.00 \pm 0.06	0.14 \pm 0.12	0.17 \pm 0.06
Oct-14	0.64 \pm 0.05	0.66 \pm 0.06	0.79 \pm 0.07	0.91 \pm 0.08	nd	0.12 \pm 0.07
Nov-14	0.14 \pm 0.02	0.16 \pm 0.04	0.20 \pm 0.05	0.27 \pm 0.08	nd	nd
Dec-14	0.05 \pm 0.01	0.05 \pm 0.02	0.11 \pm 0.06	0.18 \pm 0.04	nd	nd
Jan-15	0.15 \pm 0.04	0.28 \pm 0.05	0.11 \pm 0.04	0.17 \pm 0.06	0.14 \pm 0.05	nd
Feb-15	0.24 \pm 0.05	0.27 \pm 0.07	0.26 \pm 0.07	0.39 \pm 0.09	nd	nd
Mar-15	0.52 \pm 0.10	0.49 \pm 0.06	0.58 \pm 0.12	0.58 \pm 0.08	nd	nd
Apr-15	0.54 \pm 0.09	0.54 \pm 0.06	0.35 \pm 0.11	0.36 \pm 0.06	nd	nd
May-15	0.80 \pm 0.06	1.02 \pm 0.08	0.54 \pm 0.10	0.73 \pm 0.09	0.22 \pm 0.08	0.20 \pm 0.09
Jun-15	0.78 \pm 0.09	1.17 \pm 0.13	0.30 \pm 0.08	0.82 \pm 0.15	0.39 \pm 0.11	0.52 \pm 0.12
Jul-15	1.03 \pm 0.04	1.21 \pm 0.06	0.93 \pm 0.05	1.13 \pm 0.06	0.18 \pm 0.05	0.20 \pm 0.06
Aug-15	0.86 \pm 0.05	1.16 \pm 0.04	0.84 \pm 0.07	1.01 \pm 0.05	0.30 \pm 0.05	0.17 \pm 0.05

nd: not determined.

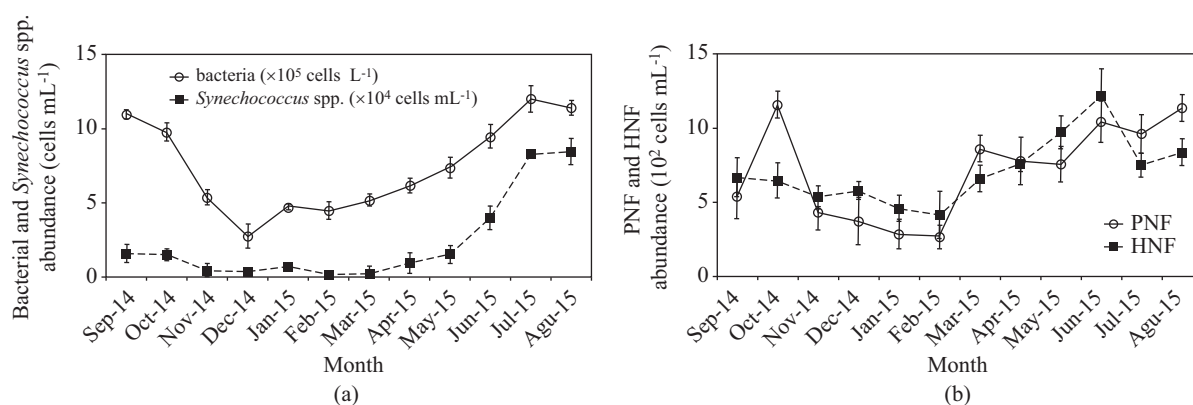


Fig. 2. Monthly variations of bacterial and *Synechococcus* spp. (a) and nanoflagellate abundance (b) during the study period.

their blue fluorescence under UV illumination. PNF and *Synechococcus* spp. were identified by their orange and red autofluorescence under blue excitation light. To obtain reliable estimates of abundance, at least 50 nanoflagellates, 400 *Synechococcus* spp. and 800 bacteria were counted per sample.

All cells were sized by eyepiece micrometer. For nanoflagellates, linear dimensions (length and width) of at least 50 cells per sampling event were measured. Nanoflagellate cells were grouped in three size categories according to cell length: cells from 2 to 5 μm , from 5 to 10 μm and cells > 10 μm .

For ciliates, 500 mL water samples from the surface were fixed with neutralized formaldehyde (2% final concentration) (Stoecker et al., 1989) and preserved at 4°C until analysis. To obtain a reliable ciliate abundance count, a 500 mL water sample was concentrated into a 100 mL subsample with a 20 μm mesh size net, after which the subsamples (100 mL) were settled in an Utermöhl chamber (Utermöhl). The entire area of the Utermöhl chamber

was examined at 200 \times or 400 \times using an inverted microscope (Nikon-TMD 300).

4. Statistical Analysis

As the distribution of variables did not meet normality, a non-parametric Mann-Whitney test was applied in order to search for median differences (Sigma Stat version 3.5). In this study, the non-parametric Mann-Whitney test was used to determine significant differences between the net growth rates of nanoflagellates in unfiltered and 20 μm filtered treatments. When significant differences were observed, a direct estimate of grazing mortality for nanoflagellates was obtained by calculating the difference in net growth rates between unfiltered and 20 μm filtered treatments. Seasonal variances in nanoflagellate growth and grazing rates were compared using one-way analysis of variance (one-way ANOVA). When significant differences were observed, a *post-hoc* Tukey's comparison test was also performed.

Potential relationships between variables were tested by linear Person correlations and multiple regressions. STATISTICA 7.0 software was used to perform all statistical operations. A probability value of < 0.05 was considered significant.

III. RESULTS

1. Abundance of Prokaryote and Nanoflagellates

During the study period, surface water temperatures showed strong seasonality with maximum values recorded during the summer period (29.5°C in August 2015) and minimum values during winter (16.5°C in February 2015). Bacterial and *Synechococcus* spp. abundances ranged from 3.2×10^5 to 11.8×10^5 cells mL^{-1} and 0.2×10^4 to 8.3×10^4 cells mL^{-1} , respectively (Fig. 2(a)). Furthermore, PNF and HNF abundance were also found to have definite seasonal peaks during the warmer months, concomitantly with the higher abundance of bacteria and *Synechococcus* spp. (Fig. 2(b)).

2. Nanoflagellate Growth and Grazing Mortality

Table 1 summarizes monthly variations in net growth rate of nanoflagellates (HNF and PNF) in different treatments (unfiltered and $< 20 \mu\text{m}$ treatments). Grazing was assumed to be negligible in the $< 20 \mu\text{m}$ treatments, thus, HNF and PNF growth rates in the $< 20 \mu\text{m}$ treatments ranged from 0.17 to 1.13 d^{-1} and 0.05 to 1.21 d^{-1} , respectively (Table 1, Fig. 3(a)). Nanoflagellate grazing rates were calculated as the difference in the net growth rates between unfiltered and $< 20 \mu\text{m}$ fractions. No significant differences in net growth rates of nanoflagellates between unfiltered and $< 20 \mu\text{m}$ treatments were observed during the colder season (November-April; $< 25^{\circ}\text{C}$), except in January (Mann-Whitney test, $p > 0.05$) (Table 1). During that same colder period, grazing was also not found to have an impact on the mortality of nanoflagellates (Fig. 3(b)). In the warmer season (May-October; $> 25^{\circ}\text{C}$), however, grazing had a significant impact on nanoflagellate community (PNF: 0.14 to 0.39 d^{-1} ; HNF: 0.12 to 0.52 d^{-1}) (Table 1, Fig. 3(b)), accounting for about 15-30% for PNF and 18-60% for HNF growth (Fig. 3(c)).

3. Effect of Bacterial Abundance and Temperature on Nanoflagellate Growth Rates

In this study, nanoflagellates were largely dependent on prey supply and temperature, as HNF growth rate increased significantly with increases in bacterial abundance ($R^2 = 0.49$, $n = 12$) and temperature ($R^2 = 0.80$, $n = 12$) (Table 2). A multiple regression analysis was carried out to determine the relative importance of the main correlates for HNF growth rate. The model for which the R^2 was obtained clearly highlights the importance of temperature for HNF growth rate (HNF = $-0.94 + 0.06$ Temperature + 0.03 bacterial abundance, $R^2 = 0.81$, $n = 12$) (Table 2). Furthermore, we also found that both bacterial abundance and temperature had highly significant ($p < 0.05$) relationships to seasonal variations in PNF growth rates in this coastal ecosystem (Table 2).

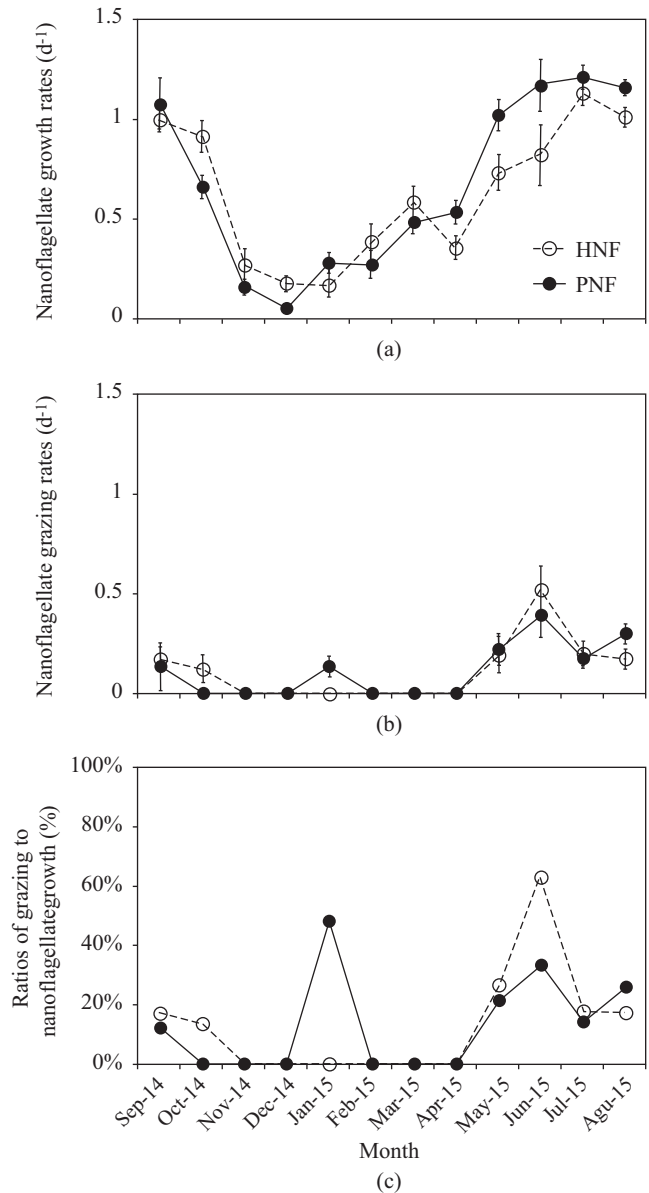


Fig. 3. Monthly variations of nanoflagellate growth rates (a), grazing rates (b) and the ratios of grazing to nanoflagellate growth (c) during the study period.

4. Increased Percentage Composition of Nanoflagellates

During the warmer seasons ($> 25^{\circ}\text{C}$, May-September), as a whole, nanoflagellates $2-5 \mu\text{m}$ in size, were responsible for $> 90\%$ of nanoflagellate community. However, after 2 days incubated time, nanoflagellate in $< 20 \mu\text{m}$ treatment showed marked composition variations compared to the unfiltered treatment (Fig. 4). An interesting phenomenon in this study, composition of nanoflagellates $5-10 \mu\text{m}$ and $> 10 \mu\text{m}$ size class showed a more pronounced increase in $< 20 \mu\text{m}$ treatments at the end of experiments during the warmer study periods (Fig. 4(a)). However, there was no significant change in composition of nanoflagellates in unfiltered treatments (Fig. 4(b)).

Table 2. Effect of bacterial abundance and temperature on nanoflagellate growth rate during the study period, using linear Pearson correlations and multiple regressions test. R^2 (%), percentage of variation explained.

Independent variable	R^2 (%)	p
HNF		
Bacterial abundance	48.9	< 0.05
Temperature	79.5	< 0.01
Bacterial abundance \times Temperature	81.2	< 0.01
PNF		
Bacterial abundance	55.7	< 0.05
Temperature	86.9	< 0.01
Bacterial abundance \times Temperature	93.4	< 0.01

VI. DISCUSSION

In this study, monthly short-term experiments were conducted to determine growth and grazing rates of nanoflagellates in a coastal ecosystem over a one-year period. The results of these laboratory grazing experiments under defined conditions makes possible a clearer interpretation of estimates of the grazing potential of microzooplankton. The present study found that grazing removed between 15% and 60% of nanoflagellate production.

1. Growth Rates of Nanoflagellates

The selective filtration method allowed us to estimate the growth rates of nanoflagellate community. These growth rates were similar to those reported in other environmental systems. HNF growth rates obtained by various studies on different environments have ranged from -0.34 to 2.57 d^{-1} (Nagata, 1988; Weisse, 1991; Carrick et al., 1992; Chrzanowski and Šimek, 1993; Berglund et al., 2005). This wide range appears to mainly reflect seasonal variation, with the highest growth rates generally found in summer. We found HNF to be 6.6 times the growth rate in July than in January (Table 1). While differences in the functional biology of the HNF communities may have contributed to the variation in growth rates (Boenigk and Arndt, 2002), to date, most of the variations in HNF growth rates in natural systems have been attributed to bacterial abundance and temperature (Landry et al., 1984; Weisse, 1991; Weisse and Scheffel-Möser, 1991; Wieltchnig et al., 2001). In our study, making use of pooled data, HNF growth rate also increased significantly with increases in bacterial abundance and temperature (Table 2). One of our previous studies of the same site showed HNF of $2\text{-}3 \mu\text{m}$ in size dominated the HNF community, making up 69 to 89% of the total HNF abundance (average 79%), while 44% of the total measured bacterivory was attributed to the HNF community (Tsai et al., 2011). Thus, HNFs represent the most probable link between bacteria and higher trophic levels. Our results showed that HNF growth was primarily limited by resource in the coastal waters of the subtropical western Pacific. It is unknown why temperature had a linear effect on HNF growth feeding rates (Peters, 1994). Similarly, Carrick et al. (1992) found a positive linear relationship between HNF growth and water temperature in Lake Michigan. In addition, several studies that

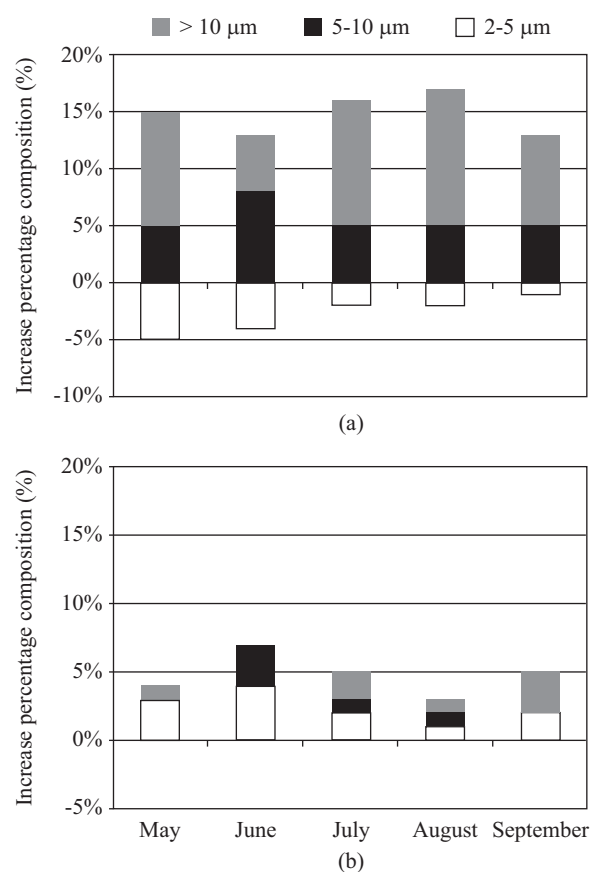


Fig. 4. Increase percentage composition in terms of abundance of three groups of nanoflagellate populations after 2 days incubation time in $< 20 \mu\text{m}$ treatments (a) and unfiltered treatments (b), respectively.

investigated the effects of temperature on the transfer of carbon between bacteria and protists have shown that temperature has a positive effect on bacterial grazing rates (Rose and Caron, 2007; Tsai et al., 2008; Vaqué et al., 2009; Lara et al., 2013). Thus, we suspected that temperature as a major environmental forcing factor for HNF growth rate (Table 2).

2. Predation Limitation

This study found that impact of grazing on nanoflagellates

accounted for about 15-30% and 18-60% of PNF and HNF growth rates, respectively (Fig. 3(c)). We believe that the predation was not the only control of nanoflagellate abundance in this study. Microplankton grazing and viral lysis can also be responsible for nanoflagellate mortality, producing changes in the dynamics and structure of protist communities (Brussaard et al., 2004; Massana et al., 2007; Saura et al., 2011; Weinbauer et al., 2015). However, this study did not analyze the effect of viruses on the HNF community. It is known that viruses may act as predators on eukaryotic populations and thereby decrease the net growth rates (Brussaard, 2004). In this study, nanoflagellate mortality due to viruses should be the same in both unfiltered and $< 20 \mu\text{m}$ treatments since viruses are small enough to pass into the $< 20 \mu\text{m}$ treated water samples. Based on these reasons, we suggest that HNFs primarily were limited by resource, although a simultaneous predation limitation was measured.

Furthermore, it has been established that consumers can exert powerful control on the population dynamics of other organisms, both through direct predation and trophic cascading. Some studies have indicated a tightly structured predator-prey coupling within the nanoflagellate assemblage, with the predator on a higher trophic level affecting more than one lower trophic level (Sherr et al., 1992; Calbet et al., 2001). Reckermann and Veldhuis (22) also found evidence for at least four trophic levels within the microbial food web: picophytoplankton, small HNFs, and large HNFs and ciliates. In the present study, we found that the percentage composition of nanoflagellate $> 10 \mu\text{m}$ in size increased largely at the end of the incubations in $< 20 \mu\text{m}$ treatments during the warmer seasons (Fig. 4(a)). This finding suggests that the absence of ciliates allowed the development of larger nanoflagellates ($> 10 \mu\text{m}$), which in turn consumed smaller nanoflagellates (2-5 μm) directly. Our observations confirm those of an early study reported that HNFs in the 2-5 μm size-fractions are most probably consumed by 5-20 μm HNF (Calbet and Landry, 2001). In this situation, we suggest that there was control of small nanoflagellates by larger nanoflagellates ($> 10 \mu\text{m}$), and this could explain in part why the ciliate grazing was not control nanoflagellate growth.

In conclusion, bacterial abundance and temperature were the variables mostly associated with seasonal variations in growth rates of HNF and PNF. Nanoflagellate carbon flux through the microbial loop showed strong seasonal oscillations. During the warmer part of the year (May to October), a part of carbon of nanoflagellates was channeled through the microbial loop by ciliate grazing, suggesting that it could be an important link between bacteria and higher trophic levels. Furthermore, there was a trophic cascade effect within nanoflagellate community during the warmer seasons. On the other hand, during the colder part of the year (November to April), nanoflagellate carbon flux was absent in the microbial loop.

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