ENVIRONMENTAL MICROBIOLOGY

Pigmented Nanoflagellates Grazing on *Synechococcus*: Seasonal Variations and Effect of Flagellate Size in the Coastal Ecosystem of Subtropical Western Pacific

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Abstract We investigated seasonal variation of grazing impact of the pigmented nanoflagellates (PNF) with different sizes upon *Synechococcus* in the subtropical western Pacific coastal waters using grazing experiments with fluorescently labeled *Synechococcus* (FLS). For total PNF, conspicuous seasonal variations of ingestion rates on *Synechococcus* were found, and a functional response was observed. To further investigate the impact of different size groups, we separated the PNF into four categories (<3, 3–5, 5–10, and >10 μm). Our results indicated that the smallest PNF (<3 μm PNF) did not ingest FLS and was considered autotrophic. PNF of 3–5 μm in size made up most of the PNF community; however, their ingestion on *Synechococcus* was too low ($0.1–1.9\,Syn\,PNF^{-1}\,h^{-1}$) to support their growth, and they had to depend on other prey or photosynthesis to survive. The ingestion rate of the 3–5 μm group exhibited no significant seasonal variation; by contrast, the ingestion rates of 5–10 and >10 μm PNFs showed significant seasonal variation. During the warm season, 3–5 μm PNF were responsible for the grazing of 12% of *Synechococcus* production, 5–10 μm PNF for 48%, and >10 μm PNF for 2%. Taken together, our results demonstrate that the PNF of 3–10 μm consumed most *Synechococcus* during the warm season and exhibited a significant functional response to the increase in prey concentration.

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Introduction

The factors controlling the fluctuations of *Synechococcus* have drawn much attention ever since their discovery. This single genus of autotrophic cyanobacteria makes up a large part of the picophytoplankton community in oligotrophic oceanic environments worldwide [30, 39, 40]. The genus is responsible for most of the photosynthesis occurring in the North Pacific Ocean [24]. Some studies have investigated the role of viral lysis and protozoan grazing on *Synechococcus*, and the results suggest that the impact of grazing processes is usually more important than viral lysis [6, 26, 32, 42]. Major grazers controlling the biomass and production of *Synechococcus* are ciliates and nanoflagellates, both phagotrophic protozoans [12, 14, 18, 19, 41]. Chen [8] reported that the rate of ingestion *Synechococcus* per ciliate was about two orders of magnitude higher than that per nanoflagellate. However, since the natural abundance of nanoflagellates is much higher than that of ciliates, grazing pressure on *Synechococcus* by the entire nanoflagellate community would be much higher than that by total ciliates. For example, in the North Pacific Ocean, the *Synechococcus* carbon consumed by nanoflagellates was around ten to 1,000 times that of the ciliates [43].

Caron et al. [7] reported that heterotrophic nanoflagellates (HNF) removed as much as 54% of *Synechococcus*

biomass in the surface waters of the Sargasso Sea. Thus, heterotrophic nanoflagellates are likely to be the main player controlling the abundance of *Synechococcus* at least in some marine ecosystems. Pigmented nanoflagellates (PNF) consist of two major groups: autotrophic and mixotrophic nanoflagellates (MNF), with the latter being capable of photosynthesis and particle grazing [34]. In aquatic ecosystems, where PNF are dominant, PNF contribute significantly to bacterivory [8]. For example, Havskum & Riemann [22], using the fluorescently labeled heat-killed bacteria technique, estimated that the feeding of PNF was responsible for up to 86% of the total bacterial grazing in some coastal environments. In some freshwater lakes, PNF have been found to remove more bacteria than either rotifers or ciliates [3]. However, only a few studies [20, 41] have investigated PNF grazing on picophytoplankton (*Synechococcus*). These studies reported PNF to be a major grazer of *Synechococcus* in marine system [20, 33, 41], but their results were of short duration and they used artificial, inert particles as tracers of prey ingestion.

At our study site along the coasts of the subtropical western Pacific, it was noted that the grazing of PNF had a significant impact on *Synechococcus* [41]. Moreover, Safi and Hall [33] reported that PNF had high grazing rates on picophytoplankton-size particles when PNF was dominant. However, these findings are contrary to Hall et al. [20], who observed an apparent preference for the bacteria-sized particles by PNF. The discrepancy may be explained by the observation that the preferred size ratio between predators and their prey is 3:1 for nanoflagellates [21]. That is, PNF with a smaller mean cell volume at 57 μm^3 would prefer to feed on smaller particles than do the HNF that have a larger mean cell volume of 74 μm^3. Nevertheless, if one considers PNF (or HNF) alone, does the size effect remain?

According to a previous study at our study site, Tsai et al. [41] identified PNF as the key grazers of *Synechococcus* populations in our coastal ecological system from May to October and showed that PNF grazed 40% to 86% of *Synechococcus* production. This study asserted that HNF of this size are too small to ingest particles of ~1 μm. The authors also hypothesized that the grazing behavior of all members of the PNF community would be similar. However, considering the wide size range of PNF in the marine system, this hypothesis may be challenged. Indeed, to investigate the energy transfer in the complex marine microbial food web, we need better measurements of the feeding role played by PNF of different sizes.

In this context, the aims of the present study are (1) to investigate whether PNF of different sizes has a differential impact on the removal of *Synechococcus* in the coastal western Pacific Ocean and (2) to determine whether there are seasonal differences in the PNF grazing on *Synechococcus*. To carry out our study, we categorized PNF into different

sizes and used the fluorescently labeled *Synechococcus* (FLS) technique to estimate their ingestion rates for the first 6 months (January–July 2005). We also used FLS to study seasonal variations of the grazing on *Synechococcus* by PNF that were not categorized by size for another 10 months (August 2005–October 2006). The seasonality of ingestion rates of protozoans may be influenced by different environmental and biological factors such as temperature, initial prey concentration, and light [2, 5, 15]; thus, we also attempted to identify which factors are more important in regulating the grazing behavior of PNF.

Materials and Methods

Study Site and Abundance Determinations

Samples were collected at a coastal station (25°09.4' N, 121°46.3' E) off a rocky shore in northeastern Taiwan (Fig. 1). Based on background information collected from 1999 to 2001 in Tsai et al. [42], a seasonal oscillation in temperature was observed with higher temperatures (>25°C) occurring in June to October, a time when nutrient concentrations were low (NO$_3$<1 μM l^{-1}). Annual salinity ranged from 33 to 34 with occasional drops below 33, probably caused by rain fall. To estimate the contribution of PNF to the consumption of *Synechococcus*, we collected samples of seawater once per month from 2005 to 2006. On each sampling day, seawater was collected from 09:00 to 10:00 in the morning (local time). We also collected samples from 20:00 to 22:00 at night (local time) from September 2005 to October 2006. Water temperature was measured immediately after the bucket cast. All samples were brought to the laboratory within 30 min after collection. In the period between January 2005 and October 2006, reported values of temperature and abundances of PNF and *Synechococcus* were averages of day and night measurements (Fig. 2).

Samples taken to measure *Synechococcus* and nanoplankton were fixed immediately after collection by adding glutaraldehyde to a final concentration of 1% (v/v). We filtered subsamples of *Synechococcus* (2 to 4 ml) and nanoflagellates (20 ml) at low pressure (<100 mm Hg) onto black Nuclepore filters (0.2 and 0.8 μm for *Synechococcus* and nanoflagellates, respectively). A Millipore filter was used as a pad placed beneath the Nuclepore filter to ensure uniform distribution of cells. Cells left on filter membranes were stained with 4'6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg ml^{-1} [31], and examined under epifluorescence microscopy at ×1,000 (Nikon Optiphot-2). PNF were defined as cells that contained chloroplasts with red autofluorescence under blue excitation light. Autotrophic picoplankton (cyanobacteria *Synechococcus*) were collected on a Nuclepore filter without DAPI staining and

Figure 1 Map illustrating the location of study site
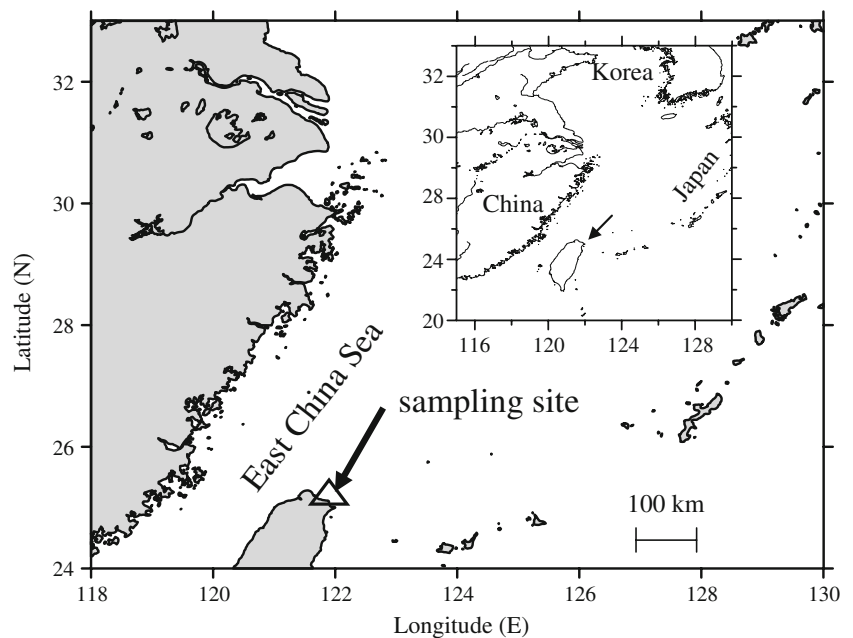
identified by their orange autofluorescence. To ensure reliability of the estimates, we counted the *Synechococcus* and PNF cells using 10 and 50 fields of view (average of 200 and 250 cells), respectively.
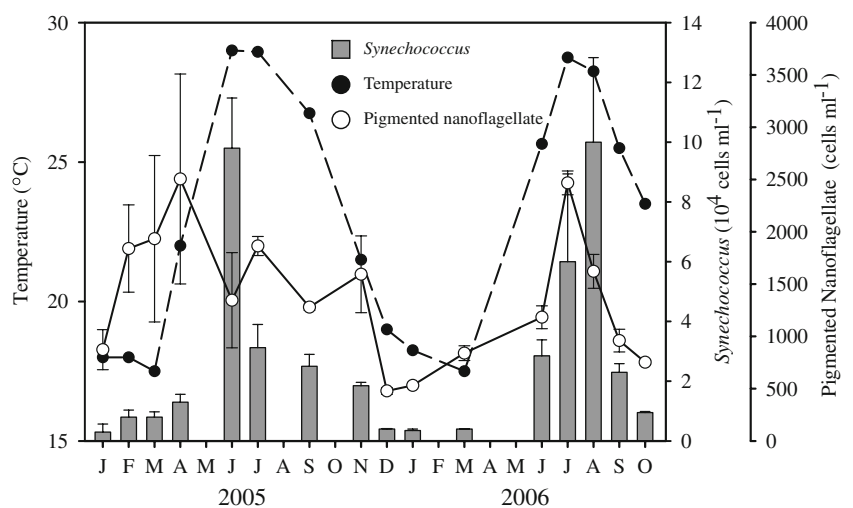
Grazing Experiments

Grazing rates of PNF on *Synechococcus* were estimated based on their ingestion of fluorescently labeled *Synechococcus*. *Synechococcus* (CH0129) was previously isolated from a surface water sample of northeastern Taiwan, and grown in f/2 media. FLS was stained with 5-(4,6-dichlorotriazin-2-yl) aminofluorescein according to the method described by Sherr and Sherr [38]. The mean size of the FLS was about 1 μm, similar to that of *Synechococcus* in situ. We distinguished the natural

Synechococcus from FLS by the bright green color from FLS under blue excitation light using epifluorescence microscopy.

In grazing experiments, which were done in triplicate, we filtered surface water samples though a 20 μm nylon mesh into 500 ml polycarbonate bottles and then added the tracer particles (FLS) to a concentration of 20% to 30% of the total counts of *Synechococcus* in the experiment bottle [1, 35] (Table 1). Bottles were incubated in a water bath at in situ temperature and light intensities. At 0, 15, 30, 45, and 60 min after the FLS was added, 20 ml subsamples were preserved with glutaraldehyde (to 1% v/v final concentration). We counted PNF, *Synechococcus*, and FLS ingested by PNF under an epifluorescence microscope at ×1,000 magnification. The nanoflagellate uptake rate on FLS was calculated by linear regression from the change of

Figure 2 Seasonal variation of *Synechococcus* abundance (10^4 cells ml^{-1}; *shaded square*), surface water temperature (°C; *shaded circle*), and pigmented nanoflagellate (cells ml^{-1}; *unshaded circle*) in 2005 and 2006. These values represent daily averages (*error bars*=±1 standard deviation)

Table 1 Initial concentrations (cells ml^{-1}) of *Synechococcus*, FLS, and PNF in the experimental bottles of grazing experiments

	Date	E.T.[a]	*Synechococcus* (10^4ml^{-1})	FLS added (ml^{-1})	FLS:syn ratio (%)	PNF (ml^{-1})
2005	January	Day	0.3	781	28	873
	February	Day	0.8	2,342	28	1,840
	March	Day	0.8	2,774	35	1,934
	April	Day	1.3	4,160	32	2,505
	June	Day	9.8	21,570	22	1,346
	July	Day	2.8	6,232	22	1,773
		Night	4.4	8,326	19	1,955
	September	Day	2.1	7,140	34	1,309
		Night	2.9	9,280	32	1,251
	November	Day	2	4,524	23	1,227
		Night	1.7	4,525	26	1,961
	December	Day	0.4	1,450	38	446
		Night	0.4	916	26	511
2006	January	Day	0.3	1,028	35	523
		Night	0.4	1,110	28	540
	March	Day	0.4	986	28	769
		Night	0.4	1,092	31	910
	June	Day	2.3	3,501	15	1,292
		Night	3.4	4,767	14	1,074
	July	Day	3	4,485	15	2,354
		Night	9	11,525	13	2,582
	August	Day	7	16,908	24	1,784
		Night	13	33,027	26	1,460
	September	Day	2	6,524	33	1,069
		Night	2.6	9,449	37	851
	October	Day	1	3,476	35	775
		Night	0.9	3,035	33	730

FLS fluorescently labeled *Synechococcus*, *PNF* pigmented nanoflagellate

[a] Experiment time: day time, 09:00 to 10:00; night time, 20:00 to 22:00

average number of FLS PNF^{-1} over time [37, 41]. Normally, when we calculated the FLS PNF^{-1} over time, we used every time point to make a linear regression. In some cases, the uptake of FLS saturated after 30 min and we used the first three time points (0, 15, and 30 min) to calculate the uptake rate on FLS by linear regression. The ingestion rate (*Syn* PNF^{-1} h^{-1}) was then estimated from the ratio between concentration of FLS and abundance of *Synechococcus* in the incubation bottle (Table 1). To compare loss of *Synechococcus* with its production, we estimated consumption rates (*Syn* ml^{-1} h^{-1}) by multiplying ingestion rates on *Synechococcus* and total PNF per milliliter. To estimate the ingestion rate of PNF belonging to different size classes on *Synechococcus*, we divided PNF into four size categories: <3, 3–5, 5–10, and >10 μm, and recorded their FLS content separately from January to July 2005.

The method of ANOVA was used for testing the significance of seasonal variation of the abundance of *Synechococcus* and PNF, and ingestion rate and consumption rate of PNF. The simple linear regression analyses between grazing rate of PNF and prey abundance or water temperature were performed to determine the controlling factor of grazing rate of PNF. Next, we used multivariate regression analysis to find out which factor (prey abundance or water temperature) was a major controlling factor of the ingestion rates of four different sizes of PNF. Specifically, we normalized variables to unit mean and variance so that the temperature and *Synechococcus* abundance are directly comparable; we then calculated the beta weights for the prey abundance and water temperature and determined the relative contribution of the two factors [27]. All analyses were conducted using SPSS software.

The size composition of PNF was calculated as the percentage of each size classes in the whole PNF community (Fig. 4a). In addition, the size composition of FLS-containing PNF was calculated by dividing the

number of FLS-containing PNF in each size class by the number of FLS-containing PNF (Fig. 4b). To avoid underestimation of the percentage of mixotrophic organisms in the PNF community, we calculated the percentage of PNF that ingested the FLS during our grazing experiments according to Poisson EZ method [4].

Results

Water Temperature, PNF, and *Synechococcus* Abundance

The water temperature exhibited a regular seasonal pattern with high values in summer and low values in winter, and the *Synechococcus* abundance closely tracked this seasonality (Fig. 2). The monthly water temperatures varied from 17°C (March) to 29°C (July; Fig. 2). The monthly *Synechococcus* abundance ranged from 0.3×10^4 to 10×10^4 cells ml^{-1} and reflected the seasonal temperature changes ($r^2 = 0.64$, $p < 0.05$) with the highest values (about 10×10^4 cells ml^{-1}) in June 2005 and August 2006 and the lowest values (0.3×10^4 cells ml^{-1}) in January 2005 and 2006 (Fig. 2). PNF abundance, however, did not follow temperature ($r^2 = 0.19$, $p > 0.05$) and exhibited different seasonal variations in these 2 years. The highest (about 2,500 cells ml^{-1}) values were found in April 2005 and July 2006, about 1 or 2 months before the highest density of *Synechococcus* (June 2005; Fig. 2), and the low values were found in December 2005 and January 2006 (about 500 cells ml^{-1}).

Grazing Experiments

Using FLS to measure ingestion, we provided the first estimates of seasonal impacts of PNF grazing upon *Synechococcus* in the coastal waters of the western subtropical Pacific Ocean. In our study (Table 1), no significant difference was found between the day and night experiments (paired t test, $p = 0.77$) so that the average values were presented (Fig. 3b). The magnitudes and trends of PNF ingestion rate were different in 2005 and 2006 (Fig. 3a). In 2005, ingestion rates increased from January to July, leveled off between July and September, and increased again in November. In 2006, an obvious seasonal variation was observed. The highest ingestion rates appeared in summer (0.83 *Syn* PNF^{-1} h^{-1}, August 2006), and the lowest rate was in October (0.08 *Syn* PNF^{-1} h^{-1}). Moreover, the consumption rate (i.e. the total *Synechococcus* ingested by PNF) followed a pattern similar to that of the ingestion rate of PNF, with the highest rate occurring in August 2006 (1.1×10^3 *Syn* ml^{-1} h^{-1}) and the lowest in December 2005 (0.045×10^3 *Syn* ml^{-1} h^{-1}; Fig. 3a).

Size compositions of PNF varied through time and so did the ingestion pattern of different size groups (Fig. 4). At
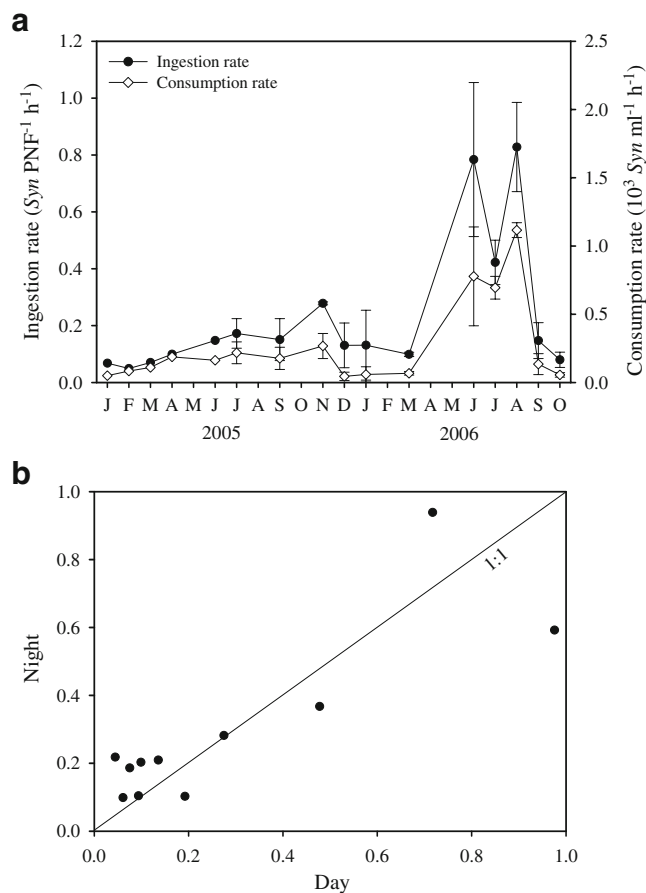
Figure 3 a Time series of ingestion rate (*Syn* PNF^{-1} h^{-1}) and consumption rate (10^3 *Syn* ml^{-1} h^{-1}) of PNF. *Error bars* = ±1 standard deviation and **b** scatter plot illustrating the relationship between day and night ingestion rates of PNF. The diagonal line indicates the 1:1 relationship

our study site, there was a high abundance of 3–5 μm PNF over the entire study period (ranging from of 45% to 70% and with an average of 62%; Fig. 4a). The smallest PNF (cells < 3 μm) was composed of the unidentified unicellular organisms and contributed to between 7% and 30% of the total PNF (Fig. 4a). No PNF within this size range was found to ingest FLS in our incubation experiments. The 3–5 μm fraction made up most of the FLS-containing PNF (Fig. 4b). PNF > 10 μm appeared during the summer period, and their contribution to the grazing on FLS clearly increased during this time (Fig. 4b). Based on the Poisson EZ method [4], we estimated the percentage of the active grazer in PNF as only 1.3% to 3.8% (mean 2.35%).

As for grazing effect, the ingestion rates of 3–5 μm PNF remained low (0.1–1.9 *Syn* PNF^{-1} h^{-1}) from January to July (Fig. 5a). In June, there was a marked fluctuation in abundance of PNF of sizes between 5–10 and >10 μm. At that time, the ingestion rates associated with these two size categories reached their maximum (14 and 12 *Syn* PNF^{-1} h^{-1},
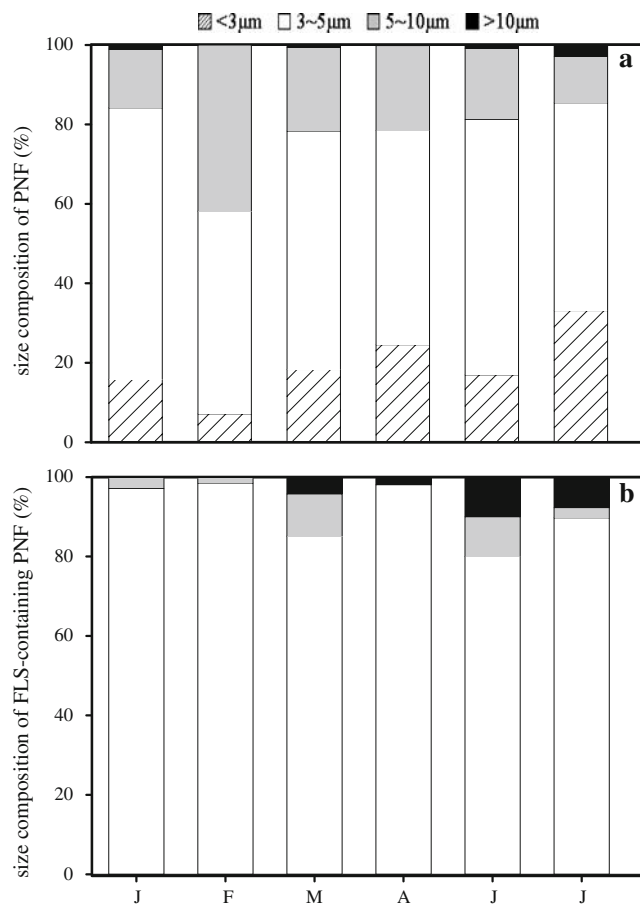
Figure 4 **a** The percentage of PNF belonging to four different size categories (<3, 3–5, 5–10, and >10 μm) and **b** the percentage of total FLS-containing PNF in the four categories from January to July 2005
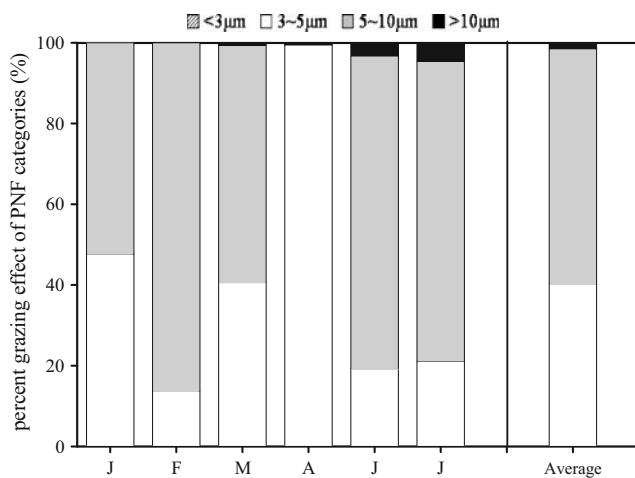
Discussion

We studied the mechanisms behind seasonal variations in PNF grazing on *Synechococcus* in subtropical western Pacific coastal waters. In a previous study, Chen [9] reported that the entire ciliate community removed ~3% of *Synechococcus* production per day in spite of high ingestion rate being observed for individual ciliates. Furthermore, Tsai et al. [41] found that HNF was practically incapable of grazing *Synechococcus*, and demonstrated the important role played by the PNF in the regulation of *Synechococcus*. However, we found that not all PNF contributed to mixotrophy (Fig. 4b). Our results indicate that the smallest PNF (<3 μm PNF) was possibly autotrophic as they did not ingest any fluorescently labeled *Synechococcus* (Fig. 4b). This finding is consistent with a previous study [44] that observed no ingestion of fluorescence-labeled bacteria by PNF<3 μm in size. For larger size groups (3–5, 5–10, and >10 μm), we did find
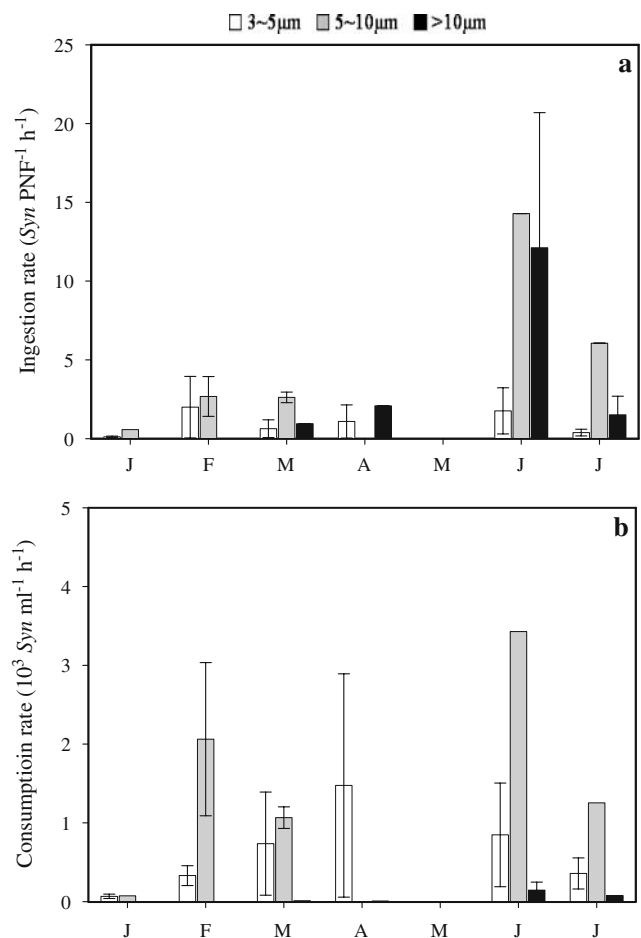
Figure 5 **a** The ingestion rates (*Syn* PNF^{-1} h^{-1}) and **b** consumption rates (10^3 *Syn* ml^{-1} h^{-1}) of PNF in three different size categories (3–5, 5–10, and >10 μm) from January to July 2005. *Error bars*=±1 standard deviation

respectively; Fig. 5a). Moreover, the ingestion rate of PNF 5–10 μm and PNF>10 μm was two- to sixfold higher, respectively, in June and July than in January to April. Multiplying the ingestion rate by the number of each size PNF per milliliter generated the consumption rate for each size of PNF on *Synechococcus*. Except for January and April, PNF 5–10 μm had a greater consumption rate (range, 1.25×10^3 to 3.43×10^3 *Syn* ml^{-1} h^{-1}) than did other PNF size categories (range, 3–5 μm:0.36×10^3 to 0.85×10^3 *Syn* ml^{-1} h^{-1} and >10 μm:0.08×10^3 to 0.15×10^3 *Syn* ml^{-1} h^{-1}; Fig. 5b). The largest size PNF (>10 μm) made up only about 3% of the total PNF abundance (Fig. 4a); therefore, its consumption of *Synechococcus* was generally low (<0.5×10^3 *Syn* ml^{-1} h^{-1}; Fig. 5b). With the exception of April, PNF 5–10 μm were responsible for 52% to 86% of the grazing on *Synechococcus* (Fig. 6), and PNF>10 μm accounted for relatively low percentage (ranged from 1% to 5%; Fig. 6). For the whole study period (January to July 2005), 3–5 μm PNF and 5–10 μm PNF were responsible for, on average, 40% and 58% of the grazing, respectively (Fig. 6).

Figure 6 The percentage grazing effect (%) of PNF in three different size categories (3–5, 5–10, and >10 μm) from January to July 2005

that they occupied a major portion (60% to 92%) in PNF, and were able to ingest FLS (Fig. 4a). It has been reported that around 2% to 38% of PNF are mixotrophic in the Georges Bank and 9% to 49% in the Bay of Aarhus, respectively [22, 35]. A higher proportion of mixotrophs (53%) has also been reported for surface waters of the Sargasso Sea [1]. Based on these results, it seems that the phagotrophic PNF exerts a significant grazing effect on bacteria. In our study, the portion of *Synechococcus*-feeding MNF (active grazers feeding on FLS) varied between 1.3% and 3.8% of total PNF as estimated by the Poisson EZ method [4]. This range was much lower than values reported in other studies [1, 20, 33]. Both Safi and Hall [33] and Hall et al. [20] used the picophytoplankton size beads in their measurements, and observed 57% and >50% of PNF were mixotrophic, respectively. In addition, Arenovski et al. [1] found 50% of PNF containing ingested FLS. Although these studies did not use Poisson EZ method to correct their estimates of *Synechococcus*-feeding MNF, these estimates still represent higher values.

The prey type may cause the difference in percentages of *Synechococcus*-feeding MNF. For example, fluorescently labeled beads cannot be digested but FLS can. It is possible that the digestion of FLS was very fast and caused underestimation of active grazer on FLS [4]. However, the percentage of active grazers did not significantly increase when fluorescently labeled beads (diameter 1 μm) were used as food particles (Chan, unpublished result). Gonzãlez et al. [16] reported that HNF in eutrophic systems contained higher percentages of active grazers than does HNF in oligotrophic system. The low percentages of *Synechococcus*-feeding MNF at our study site may represent a characteristic of the particular ecosystem.

Grazing Impact of PNF

The impact of community consumption of PNF on *Synechococcus* biomass varied from 14% to 53% per day (mean 29%) in this study, which is consistent with previous studies [6, 11, 25, 33, 35]. Generally, the grazing pattern of PNF was mainly affected by temperature and the availability of food. In our study, we found that the ingestion rates of total PNF were linearly correlated with water temperatures and the abundance of *Synechococcus* ($r^2 = 0.49$ and 0.94 respectively, $p < 0.05$; Table 2). However, based on comparisons of beta weight (t_1 and t_2) in multivariate regression analysis [27], we found that the *Synechococcus* abundance was a stronger factor in controlling the ingestion rates of PNF than temperature (Table 2).

The ingestion rate responding to prey availability can be described as a functional response [10, 11, 23, 29]. Several studies suggested that the ingestion rates of HNF increase linearly with the increase in picoplankton concentrations [7, 11, 13], which has been characterized as type I functional response. Most studies showed that the ingestion rates of HNF on bacteria or *Synechococcus* represented a type I functional response, although Grover [17] used heterotrophic microflagellate feeding experiments in laboratory cultures and showed a clear saturating type II functional response for two species of diatom. In our study, ingestion by PNF was not the saturated type but rather linear (functional response I; Table 2). This is the first study to report in situ evidence that PNF functionally responds to abundance of *Synechococcus*.

Ingestion rates measured at our study site were somewhat higher than published values. To some extent, our results should be closer to the true ingestion rate in natural environments because stained *Synechococcus* instead of latex beads were used as food particles. There is evidence to indicate that nanoflagellates ingest *Synechococcus* more actively [28, 36]. However, a minor overestimation may exist in our results. Since the *Synechococcus* that we used to produce FLS were grown in nutrient-sufficient conditions, this kind of high-quality food may induce higher ingestion rates [36]. Also, the addition of FLS inevitably increased food concentrations for flagellates in our experiments [28], which may cause overestimation according to the functional response defined by our multiple regression analysis (ingestion rate=0.009 temperature+0.039 *Syn*−0.058). In our experiments, the addition of FLS was carefully controlled to about 20% of the natural abundance of *Synechococcus*, which translates to an overestimation between 5% and 15% when the temperature range is set from 20°C to 25°C and the range of *Synechococcus* abundance is set from 10^4 to 10^5 cells ml^{-1}.

At our study site, nutrient concentration was low (NO$_3$<1 μM l^{-1}) between June and October [42]. Phagotrophic behavior may be necessary for these PNF to obtain

Table 2 Correlations and beta weights of multivariate regressions between predator (all PNF community, <3, 3–5, 5–10, and >10 μm) and surface water temperature (°C) and *Synechococcus* abundance (10^4 cells ml^{-1})

Ingestion rate (*Syn* PNF^{-1} h^{-1})	Temperature				*Synechococcus*			
	r^2	p	t_1[a]	n	r^2	p	t_2[b]	n
Total PNF	0.24	<0.01	0.233[c]	27	0.34	<0.001	0.767[c]	27
3–5 μm PNF	0.01	>0.05		15	0.02	>0.05		15
5–10 μm PNF	0.59	>0.05		15	0.93	<0.01		15
>10 μm PNF	0.93	<0.05	0.139[d]	15	0.97	<0.001	0.861[d]	15

PNF pigmented nanoflagellate

[a] $t_1 = (b_1/(b_1+b_2))$

[b] $t_2 = (b_2/(b_1+b_2))$

[c] After multiple regression, beta=0.146(b_1), p=0.533 (temperature); beta=0.48(b_2), p=0.049 (*Syn*), R^2 =0.35

[d] After multiple regression, beta=0.182(b_1), p=0.26 (temperature); beta=1.123(b_2), p=0.003 (*Syn*), R^2 =0.98

essential nutrient for growth. Mixotrophs with this growth strategy would be expected to respond favorably to increasing prey abundance.

Differences in Grazing Patterns Between the PNF Size Classes

Among different size groups of PNF (3–5, 5–10, and >10 μm), we found that about 90% of the FLS-containing PNF at our study site was attributed to 3–5 μm PNF (Fig. 4b). However, their per capita ingestion rate remained low throughout the study period (0.1–1.9 *Syn* PNF^{-1} h^{-1}; Fig. 5a). Considering that 3–5 μm PNF had a high abundance but showed the lowest ingestion rate on *Synechococcus* in warmer months, we speculate that PNF of this size class may rely on other prey or autotrophy for their supply of nutrients to remain abundant and maintain their dominance in the PNF community (Fig. 4a). One possible alternative food source is bacteria, as Unrein et al. [44] found that the <5 μm PNF made up most bacterivory nanoflagellates (ranging from 19% to 61%) throughout the year in an oligotrophic coastal system. However, we did not estimate PNF bacterivory in this study and further studies are needed.

We examined the grazing effects of 5–10 and >10 μm PNF on *Synechococcus* and found that their grazing patterns had a noticeable seasonality, higher during the warmer months (Fig. 5a). The ingestion rate of PNF 5–10 μm was significantly correlated with the abundance of *Synechococcus* (r^2=0.93, p<0.01) but not temperature, while the ingestion rate of PNF>10 μm was significantly correlated with the abundance of *Synechococcus* and water temperature (Table 2). However, using multivariate regression to calculate beta weights of *Synechococcus* abundance and temperature factors, we found that *Synechococcus* abundance (t_2=0.861) played a greater role in influencing

the PNF ingestion rates than water temperature (t_1=0.139; Table 2). These results suggest that *Synechococcus* is an important energy source for 5–10 and >10 μm PNF in the coastal waters of western subtropical Pacific Ocean.

Christaki et al. [11] assembled the ingestion rates of HNF in various environments and showed that the ingestion rates range from 0.008 to 0.5 *Syn* flagellate^{-1} h^{-1}. To our knowledge, no studies have estimated the grazing rates on picophytoplankton (*Synechococcus*) by the PNF. Some studies used beads (1 μm) similar to *Synechococcus* in size to measure the ingestion rates of PNF, and obtained 0.02 and 0.04 beads PNF^{-1} h^{-1} in the coastal area and open ocean, respectively [20, 33]. Compared with these studies, the ingestion rates at our study site were relatively high, with 5–10 μm PNF ingesting as much as 14 *Syn* PNF^{-1} h^{-1} (Fig. 5a). This discrepancy suggests the importance in subcategorizing PNF grazers according to size in order to estimate the grazing parameters in microbial food webs.

Our study provided a better understanding of trophic structures and the energy flow within the microbial loop. To show this, we compared the growth rates of *Synechococcus* in 2005 (Tsai, unpublished data) with the grazing rates (>3 μm PNF) of our study. The growth rate of *Synechococcus* was higher in the warmer months, amounting to 4,900 *Syn* ml^{-1} h^{-1}. Comparing the production rate of *Synechococcus* with the grazing rates of different PNF size categories, we found that 3–5 μm PNF were responsible for ingesting 12% of *Synechococcus* production, 5–10 μm PNF for 48% and >10 μm PNF for 2%. By contrast, another study at the same site indicated that ciliates accounted for the removal of only about 3% of *Synechococcus* production [9]. Thus, about 60% of *Synechococcus* production loss was attributable to PNF in size of 3–10 μm. If we correct for the overestimation of ingestion rates of each category PNF, it would still be about 58% of *Synechococcus* production.

While recent studies have demonstrated the importance of PNF as phagotrophs in marine ecosystems [22, 35, 41, 44], there are limited data on the grazing impact of mixotrophic populations upon picophytoplankton [33, 35, 41]. Our study demonstrated that the PNF of different sizes showed significant variations in their grazing patterns on *Synechococcus* in the subtropical western Pacific.

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