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Effects of freshwater input and mariculture (bivalves and macroalgae) on spatial distribution of nanoflagellates in Sungo Bay, China

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ABSTRACT: Sungo Bay in northern China has been used for rearing bivalves and macroalgae for several decades. The bivalve culture areas (B-area) are located in the bay head, and the macroalgae culture areas (M-area) are located outside of the B-area. In field investigations, we compared the nanoflagellates (NF) in the B-area and the M-area during 4 seasons. After the field investigations, enclosure experiments were conducted during summer to study the effects of mariculture (bivalves and macroalgae) on NF. In the warm seasons (summer and autumn), during which there was an obvious freshwater input to the bay (especially during summer), NF abundance was negatively related to salinity and was higher in the B-area than in the M-area. In the enclosure experiments, an increase in NF abundance was observed after Day 4 in the bivalve enclosure, but not in the macroalgae enclosure. Considering that the B-area was the area of lower salinity, and the season of largest freshwater input was also the optimal growth season for bivalves, we suggest that the spatial distribution of NF in the warm seasons in Sungo Bay may be influenced by both freshwater input and mariculture.

KEY WORDS: Nanoflagellates · Mariculture · Freshwater input · Sungo Bay

INTRODUCTION

Nanoflagellates (NF) are a key component of the microbial loop. Phagotrophic NF, which include heterotrophic nanoflagellates (HNF) and mixotrophic NF, are predators of heterotrophic bacteria and photosynthetic picoplankton, and therefore play a pivotal role in transferring prokaryotic and eukaryotic picoplankton production to the classic food web (Sanders et al. 2000, Calbet et al. 2001, Chan et al. 2009, Tsai et al. 2011). From the early formalisation of the microbial loop concept to present-day research, the seasonal and spatial variation of NF and the environmental factors have been widely studied in both freshwater and marine systems because of the significant ecological role of NF in aquatic food webs. Generally, temperature, chlorophyll *a* (chl *a*), nutrient concentrations and prey (bacteria and picophytoplankton) biomass are the major controlling factors in the spatial distribution of NF (Kuuppo 1994, Safi & Hall 1997, Granda & Anadón 2008, Huang et al. 2008). However, there have been few studies on NF in aquaculture areas, which are more readily affected by human activities and more closely associated with our daily lives.

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NF can be captured by bivalves (Dupuy et al. 1999). In contrast, picoplankton are too small to be efficiently retained by most bivalves (Kreeger & Newell 1996). Therefore, NF may represent a trophic link between picoplankton and filter-feeding bivalves (Le Gall et al. 1997, Dupuy et al. 1999). The ingestion and assimilation of bacterial carbon via NF has been demonstrated in mussels (Kreeger & Newell 1996). In some bivalve culture ponds, NF were shown to be an important food source for oysters (Dupuy et al. 2000a,b, 2007). Meanwhile, some bivalves may significantly increase dissolved organic carbon (DOC) concentrations in the surrounding water (McKee et al. 2005).

Carbon fixed by macroalgae during photosynthesis is mainly used to produce structure and storage products. However, excess carbon, accounting for up to 40% of the carbon produced daily during photosynthesis, is released as DOC into the surrounding water (Sieburth 1969, Lucas et al. 1981). Some protists can utilise seaweed-derived polysaccharides and other types of high-molecular-weight polysaccharides (Sherr 1988, Armstrong et al. 2000), whereas bacteria are considered the main consumers of DOC, which is then transferred to higher trophic levels when bacteria are consumed as prey (i.e. by NF). Therefore, seaweeds influence NF through direct or indirect pathways (Armstrong et al. 2000).

Sungo Bay has been used for mariculture since the first successful aquaculture attempts using the seaweed *Laminaria japonica* in 1970. Over the past several decades, mariculture has expanded in this area, and the aquaculture species have changed from the original monoculture of macroalgae to the present polyculture of macroalgae and bivalves (Fang et al. 1996). The bivalves cultivated mainly include *Chlamys farreri* and *Crassostrea gigas*, the retention efficiency of which is reported to drop with decreasing prey size (Dupuy et al. 1999, Zhang et al. 2010). To attain higher production rates and improve aquatic environments, several studies have been conducted in Sungo Bay (Fang et al. 1996, Zhao et al. 1996, Bacher et al. 2003, Mao et al. 2006), and mathematical models have been developed for use in aquaculture assessment and management. However, the microbial loop was not taken into account in these models, despite the microbial loop being important in aquatic systems (Bacher et al. 2003, Nunes et al. 2003, Zhang et al. 2009). The microbial loop may have been ignored in previous studies in Sungo Bay due to a lack of knowledge regarding the *in situ* components of the microbial loop. Moreover, picophytoplankton blooms have frequently appeared in recent years (Kong et al. 2012). NF are regarded as a significant consumer of picoplankton (Christaki et al. 2005, Bręk-Laitinen & Ojala 2011); NF most likely play an important role in the Sungo Bay mariculture ecosystem.

This report presents the first study of the NF in Sungo Bay. The major objective of this study was to evaluate the effects of environmental factors and mariculture on the spatial distribution of NF in the field. The spatial distributions of NF and the relationships between environmental factors and NF distribution were obtained by field surveys. Enclosure experiments *in situ* were conducted to study the effects of mariculture on NF.

MATERIALS AND METHODS

Sampling

Sungo Bay, located on the northwest coast of the Yellow Sea, China, is used for raft mariculture of bivalves (*Chlamys farreri* and *Crassostrea gigas*) and macroalgae (*Laminaria japonica* and *Gracilaria lemaneiformis*). Based on our investigation, the culture density of *Chlamys farreri* was about 390 g m^{-3} (wet weight) and *G. lemaneiformis* was about 200 g m^{-3} (wet weight). The cultivation method is longline culture. The organisms are hung into the water column from 0.5 to 7 m. The bivalve culture areas (B-area) are located in the bay head, and the macroalgae culture areas (M-area) are located outside of the B-area (Fig. 1). Samples were collected during 4 trips to Sungo Bay in spring (April 2011), summer (August 2011), autumn (October 2011) and winter (January 2012). Seawater samples were collected using Ruttner bottles (Hydro-Bios). For sites with a water depth of <10 m, water samples were taken at a depth of 0.5 m below the surface. For sites with a depth >10 m, samples were taken at a depth of 10 m. We did not find a significant ($p > 0.05$, paired *t*-test) difference in NF assemblages between 0.5 m and 10 m depths; therefore, average values were used in our results. Water temperature and salinity were measured *in situ* using a YSI Professional Plus meter at the time of sample collection.

Epifluorescence microscopy analysis of NF

Samples (100 ml) used to estimate the abundance of NF were pre-filtered through a nylon mesh (20 µm pore size), then fixed immediately by adding glutaraldehyde (0.5%, final concentration). Subsamples

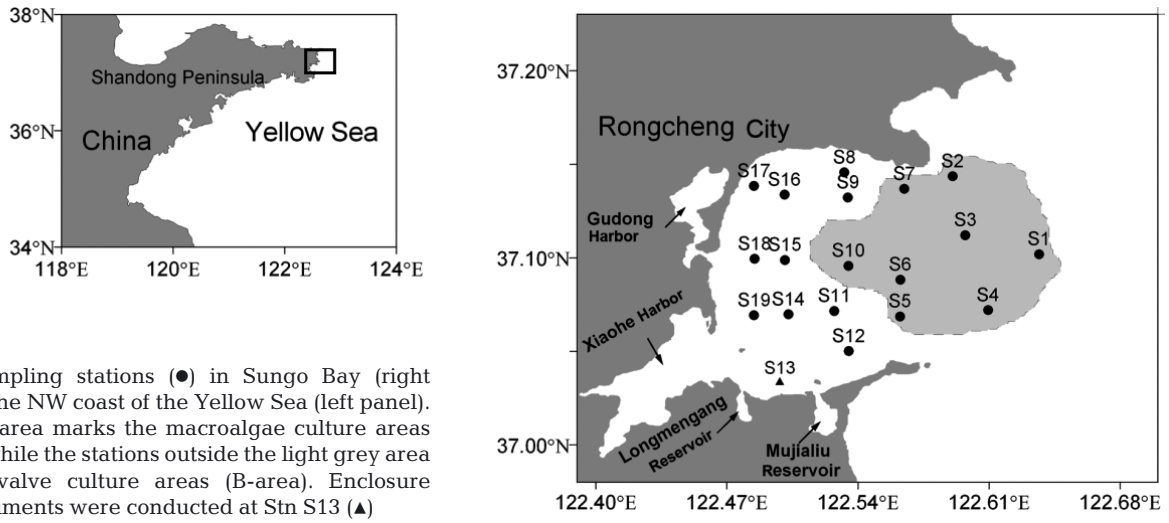

Fig. 1. Sampling stations (●) in Sungo Bay (right panel), on the NW coast of the Yellow Sea (left panel). Light grey area marks the macroalgae culture areas (M-area), while the stations outside the light grey area are the bivalve culture areas (B-area). Enclosure experiments were conducted at Stn S13 (▲)

(20 ml each) were filtered onto polycarbonate black membrane filters (0.22 µm pore size) (Millipore) at low vacuum pressure (<100 mm Hg). After filtration, NF were stained with DAPI for 15 min at a final concentration of 10 µg ml^{-1}. NF were enumerated at 1000× magnification using an epifluorescence microscope (Leica DM 4500B). HNF were identified by their blue fluorescence under UV illumination, and pigmented nanoflagellates (PNF) were distinguished by their orange and red autofluorescence under blue light excitation (Tsai et al. 2010). Length and width were measured using Leica DM 4500 software. To obtain a reliable estimation of abundance, at least 100 NF were counted per sample. In this study, NF were in the size range of 2–10 µm; NF of size range 10–20 µm were not found in any samples.

Flow cytometry analysis of bacteria

Bacteria were enumerated by flow cytometry using a FACS Vantage SE system (Becton Dickinson) equipped with a water-cooled argon laser (488 nm, 1 W, Coherent). Heterotrophic bacteria were stained with SYBR Green I (Molecular Probes) (final dilution: 10^{-4}, v/v) and identified by their green fluorescence (Marie et al. 1997).

Nutrients and chl *a* biomass

The concentrations of dissolved inorganic nitrogen (DIN; sum of NO_3^-, NO_2^- and NH_4^+) and phosphate (PO_4^{3-}) were measured using a Skalar SAN^{plus} autoanalyser, following the methods of Hansen & Koroleff (1999). Seawater used for chl *a* analysis was filtered through GF/F filters (Whatman). Chl *a* was then measured using a Turner Design fluorometer (10-AU-005-CE) after extraction with 90% acetone at –20°C for 24 h in the dark (Parsons et al. 1984). The results were calibrated using a pure chl *a* standard (Sigma).

Enclosure experiments

Three transparent polyethylene bags, each filled with approximately 1000 l of surface seawater, were used to establish enclosures at Stn S13 (Fig. 1) in June 2012. Bags were placed on rafts (with buoys), which provided buoyancy. The bags were suspended, and the seawater in the bags was naturally mixed by the wave and flow, thus the seawater in the bags could be considered to be as homogeneous as the surrounding water. The bags were washed 5 times with natural seawater to eliminate external contamination prior to the experiment. One bag containing merely natural seawater was used as the control. The second bag, into which 13 scallops *Chlamys farreri* (mean wet weight of 28.2 ± 2.4 g $ind.^{-1}$) with their original attaching plates were introduced, was set as the bivalve treatment. The third bag served as the macroalgae treatment, where 200 g (wet weight) of *G. lemaneiformis* was introduced. The biomass of bivalves and macroalgae used in the enclosure experiments was similar to the densities of local aquaculture practices. The bivalves and macroalgae were hung using nylon ropes to prevent them from touching the bottom and walls of the bags. During the experiments, bivalves and macroalgae were cultured in natural seawater

without the artificial food input or nutrient addition. To prevent outside seawater from entering the bags, we set the upper edges of the mouth ~20 cm above the sea surface level. The experiment lasted for 7 d, and sampling was performed at approximately 10:00 h every day. During the experiment, mean temperature and salinity was 20.38 ± 2.15°C and 31.20 ± 0.16 psu, respectively. The NF, bacteria, chl *a* and nutrient measurements were conducted as described in the previous 3 subsections.

Statistical analysis

Differences in physical (temperature), chemical (DIN and PO_4^{3-}) and biological values (chl *a*, bacteria, and NF) between the B-area and M-area were assessed using a *t*-test. One-way ANOVA and least significant difference (LSD) test for multiple comparisons were performed to evaluate the differences in NF or bacterial abundance among different seasons. Pearson correlation analysis was employed to analyse the relationships between all variables in the field survey. To reveal differences among different enclosures, all data from the enclosure experiments were analysed with repeated-measures ANOVA. All analyses were carried out using the statistical program SPSS 16.0, with a significance level of 5%.

RESULTS

Spatial variation of environmental factors and NF abundance over 4 seasons

The distribution of salinity was relatively homogeneous in winter and spring in Sungo Bay, and salinity was <31 psu in spring. The salinity gradient was large in summer but small in autumn (Fig. 2A–D). However, a temperature gradient existed throughout the year. In spring and summer, temperature decreased from the inner bay to outer bay, while in autumn and winter, it increased from the inner to the outer bay (Fig. 2E–H). The gradient distribution of the chl *a* biomass appeared in the warm seasons and increased from the bay mouth to the bay head (Fig. 2J,K). In winter, chl *a* biomass was high in the bay mouth (Fig. 2L), while in spring high chl *a* biomass was observed in both the bay mouth and the bay head (Fig. 2I).

Total nanoflagellate (TNF) abundance ranged from 0.32×10^3 to 171.62×10^3 cells ml^{-1} during the study period. A gradient distribution of TNF appeared in the warm seasons but was relatively homogeneous in the cold seasons (winter and spring) (Fig. 3A–D). PNF contributed 41.50, 65.18, 65.96 and 43.02% of TNF abundance in spring, summer, autumn and winter, respectively. PNF and HNF distributions were similar to the distribution of TNF (Fig. 3E–L).

Differences between B-area and M-area

Water temperature was significantly different between the B-area and the M-area over the 4 seasons ($p < 0.05$, *t*-test) (Table 1). No significant differences were observed regarding the other measured environmental factors (i.e. salinity, chl *a*, DIN and PO_4^{3-}) between the 2 areas in the cold seasons ($p > 0.05$, *t*-test) (Table 1). However, in the warm seasons, salinity was significantly lower in the B-area than in the M-area; the concentrations of nutrients (except for DIN in autumn) and chl *a* biomass were significantly higher in the B-area than in the M-area ($p < 0.05$, *t*-test) (Table 1). Bacterial abundance remained higher in the B-area than in the M-area throughout the year ($p < 0.05$, *t*-test) (Table 1).

According to the presence or absence of pigments, NF were divided into HNF and PNF. The NF community was dominated by PNF in the warm seasons and HNF in the cold seasons (Fig. 4). The abundances of TNF, PNF and HNF were significantly higher in the B-area than in the M-area ($p < 0.05$, *t*-test) in the warm seasons, but were similar between the 2 areas during the cold seasons ($p > 0.05$, *t*-test) (Fig. 4). Size ranges of NF of 2–5 µm and 5–10 µm were distinguished. NF of both size classes were more abundant in the B-area than in the M-area during the warm seasons (Fig. 5). Both the abundance of NF (TNF, HNF, PNF, 2–5 µm NF and 5–10 µm NF; Figs. 4 & 5) and bacteria (Table 1) showed a significant ($p < 0.05$) seasonal variation, with the lowest value in winter and highest value in summer.

Variations in NF, nutrients, chl *a* and bacteria in experimental enclosures

DIN concentrations were not significantly different among the 3 enclosures (repeated-measures ANOVA, $p > 0.05$) (Fig. 6A). Compared with other enclosures, the concentration of PO_4^{3-} was significant higher in the bivalve treatment (repeated-measures ANOVA, $p < 0.01$). However, no significant differences (repeated-measures ANOVA, $p > 0.05$) were detected between the macroalgae treatment and the control

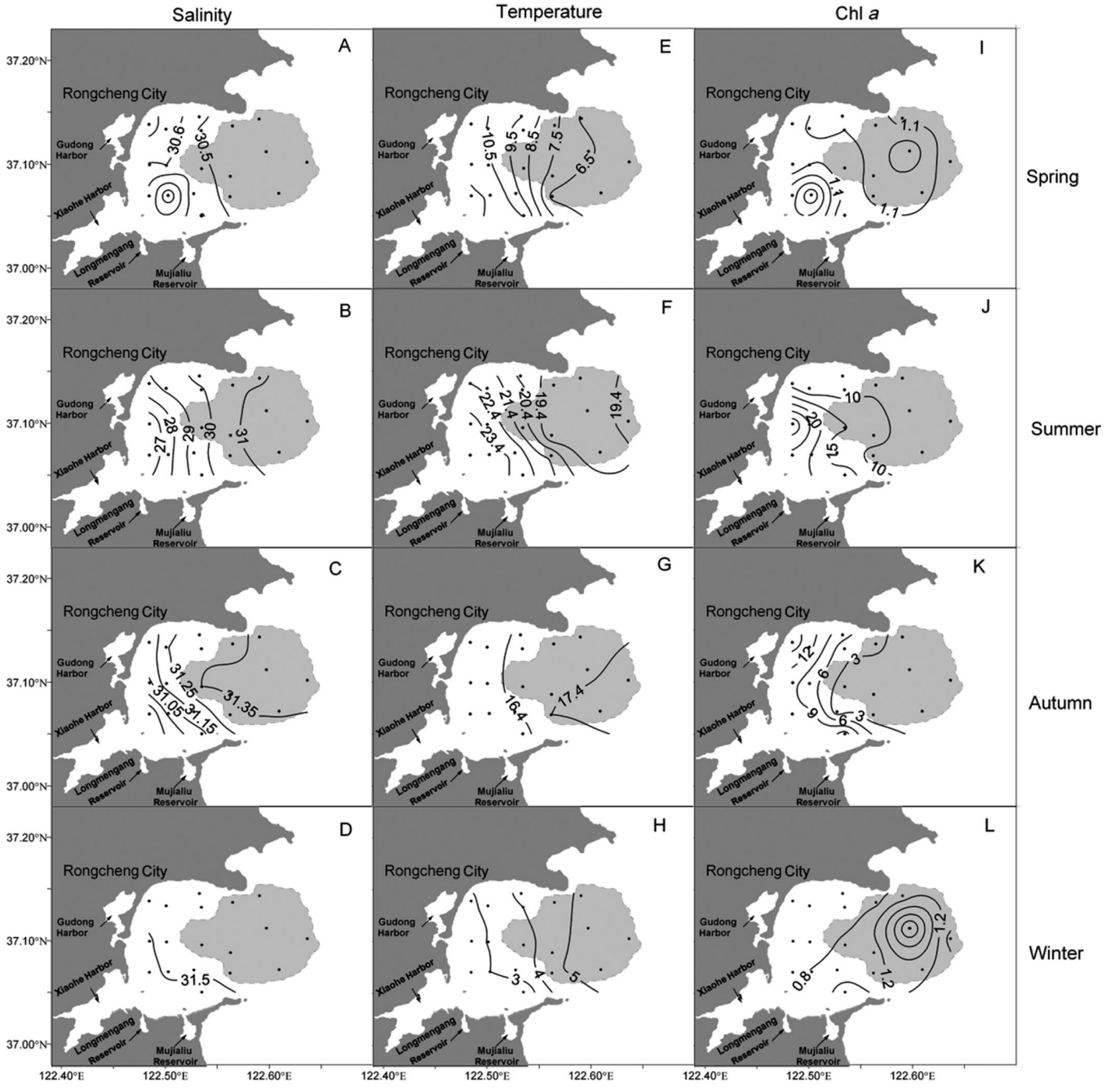

Fig. 2. Spatial variations in (A–D) salinity (psu), (E–H) temperature (°C) and (I–L) chl *a* biomass (µg l^{-1}) over 4 seasons. Sampling stations, M-area and B-area are marked as in Fig. 1

(Fig. 6B). The differences in chl *a* biomass were significant among the 3 enclosures (repeated-measures ANOVA, $p < 0.01$). Chl *a* biomass was significantly higher in the bivalve treatment than in the control, and was significantly lower in the macroalgae treatment than in the control (repeated-measures ANOVA, $p < 0.05$) (Fig. 7A).

Bacterial abundance was significantly higher in the bivalve treatment than in the other enclosures (repeated-measures ANOVA, $p < 0.01$) and increased steadily with time to 4.68 times the initial level. Though bacterial abundance was higher in the macroalgae treatment than in the control after Day 2, the differences between the macroalgae treatment and the control were not significant (repeated-measures ANOVA, $p > 0.05$) (Fig. 7B).

No significant differences in NF abundance (including TNF, HNF, PNF, 2–5 µm NF and 5–10 µm

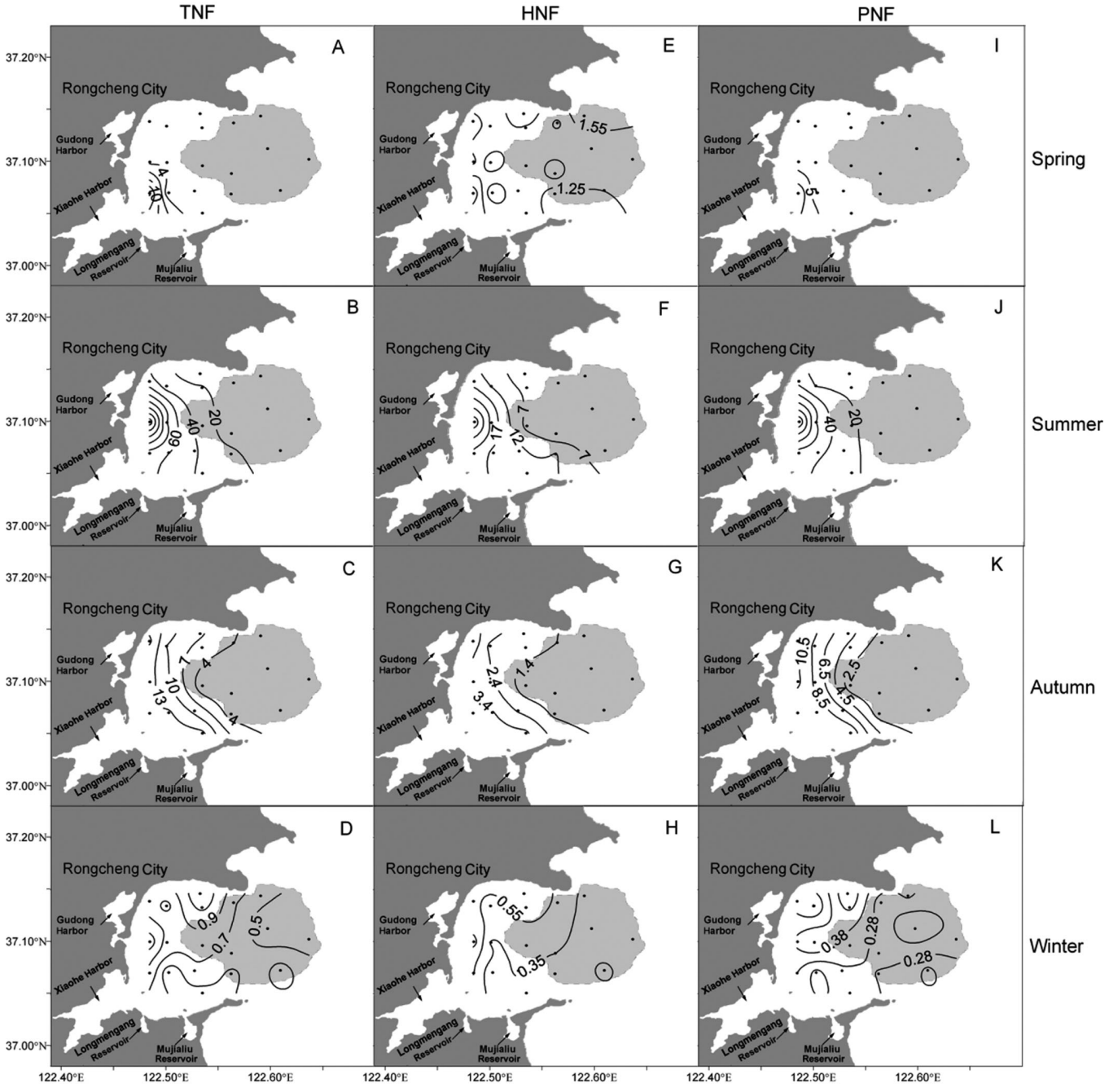

Fig. 3. Spatial variations in (A–D) total nanoflagellate (TNF) abundance, (E–H) heterotrophic nanoflagellate (HNF) abundance and (I–L) pigmented nanoflagellate (PNF) abundance (all: $\times 10^3$ cells ml^{-1}) over 4 seasons. Sampling stations, M-area and B-area are marked as in Fig. 1

NF) were detected among the 3 enclosures (repeated-measures ANOVA, $p > 0.05$). However, the effect of time on NF abundances was significant (Day × Treatment, $p > 0.05$). The abundance of TNF, HNF, PNF and 2–5 µm NF were clearly higher in the bivalve treatment than in the other 2 enclosures during the last 3 d. However, no obvious differences in the 5–10 µm NF were found among the 3 enclosures even during the last 3 d, indicating that the increased abundance of NF in the bivalve treatment was the result of an increased abundance of 2–5 µm NF. No obvious differences were found with respect to the NF (TNF, HNF, PNF, 5–10 µm NF and 2–5 µm NF) abundance between the control and the macroalgae treatment in the last 3 d (Figs. 8 & 9).

Table 1. Comparison of environmental factors and bacterial abundance (mean ± SD) in the bivalve (B-area) and macroalgae culture areas (M-area). *Significant difference (Diff.; $p < 0.05$, *t*-test) between the 2 areas. DIN: dissolved inorganic nitrogen; ns: not significant

	Spring			Summer			Autumn			Winter		
	M-area	B-area	Diff.	M-area	B-area	Diff.	M-area	B-area	Diff.	M-area	B-area	Diff.
Temperature (°C)	7.40 ± 1.56	10.58 ± 1.02	*	19.49 ± 1.06	22.73 ± 1.30	*	17.25 ± 0.26	15.91 ± 0.49	*	4.89 ± 0.66	2.95 ± 0.79	*
Salinity (psu)	30.46 ± 0.06	30.56 ± 0.15		30.87 ± 0.63	28.31 ± 1.55	*	31.36 ± 0.02	31.06 ± 0.23	*	31.55 ± 0.01	31.50 ± 0.08	
Chl *a* (μg l^{-1})	1.24 ± 0.31	1.30 ± 0.73		9.23 ± 3.31	18.17 ± 11.23	*	1.38 ± 1.21	10.20 ± 5.29	*	1.17 ± 0.72	0.71 ± 0.30	
DIN (μM)	19.29 ± 7.95	13.4 ± 6.21		10.05 ± 4.84	20.98 ± 12.29	*	50.84 ± 41.96	26.48 ± 31.35	ns	22.92 ± 7.73	18.40 ± 6.95	
PO_4^{3-} (μM)	0.26 ± 0.19	0.36 ± 0.21		0.10 ± 0.04	0.14 ± 0.06	*	0.03 ± 0.02	0.08 ± 0.05	*	0.05 ± 0.06	0.05 ± 0.01	
Bacteria (×10^6 cells ml^{-1})	1.28 ± 0.37	1.83 ± 0.15	*	2.45 ± 0.90	5.17 ± 1.28	*	0.82 ± 0.32	3.30 ± 1.47	*	0.27 ± 0.05	0.33 ± 0.06	*

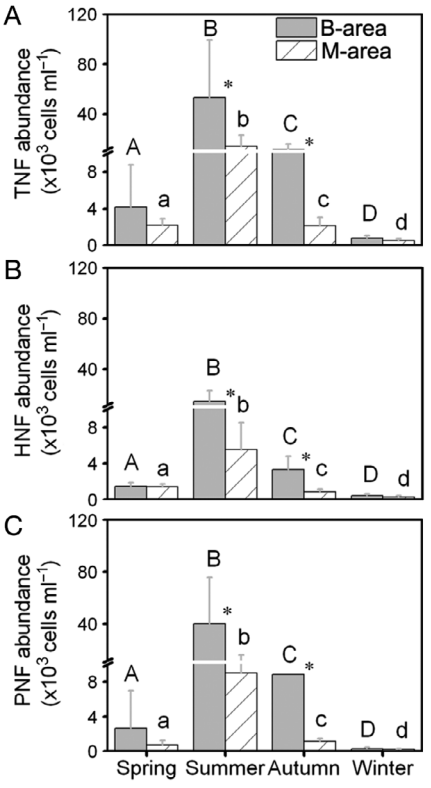

Fig. 4. Mean (+SD) abundance of (A) total nanoflagellate (TNF), (B) heterotrophic nanoflagellate (HNF) and (C) pigmented nanoflagellate (PNF) in the B-area and M-area (see Fig. 1). *Significant difference ($p < 0.05$, *t*-test) between the 2 areas in the same season. Different uppercase and lowercase letters indicate significant ($p < 0.05$) differences among 4 seasons for B-area and M-area, respectively

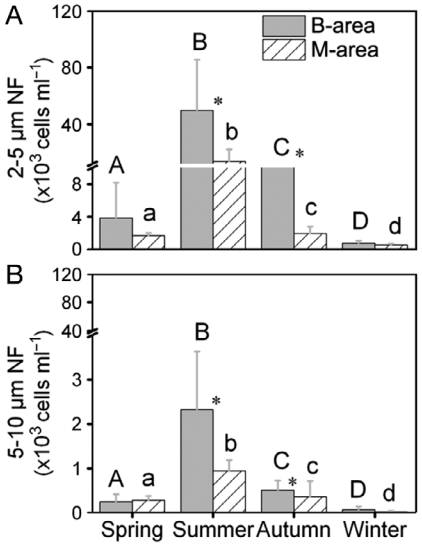

Fig. 5. Mean (+SD) abundance of (A) 2–5 μm nanoflagellates (NF) and (B) 5–10 μm NF in the B-area and M-area (see Fig. 1). *Significant difference ($p < 0.05$, *t*-test) between the 2 areas in the same season. Different uppercase and lowercase letters indicate significant ($p < 0.05$) differences among 4 seasons for B-area and M-area, respectively

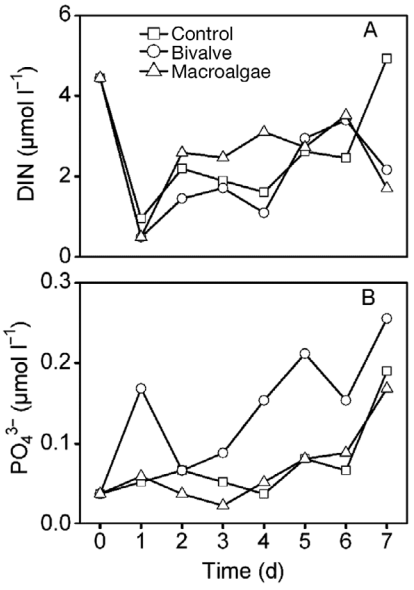

Fig. 6. Variations in concentrations of (A) dissolved inorganic nitrogen (DIN) and (B) PO_4^{3-} in the enclosure experiments over time

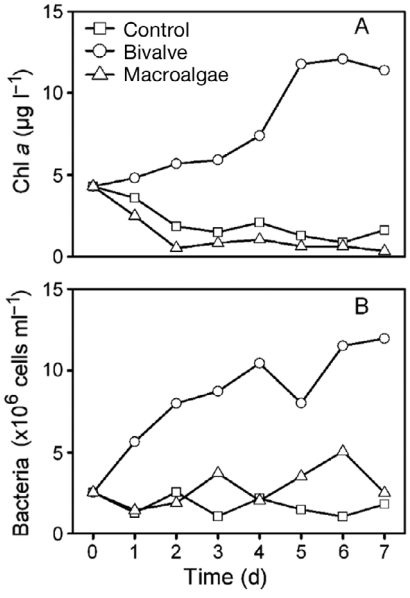

Fig. 7. Variations in (A) chl *a* biomass and (B) bacterial abundance in the enclosure experiments over time

DISCUSSION

In this study, a field survey was carried out to reveal relationships between NF and environmental factors; enclosure experiments *in situ* were conducted to study the effects of mariculture on NF. The aim was to assess the possible effects of environmental factors and mariculture on the spatial distribution of NF in Sungo Bay.

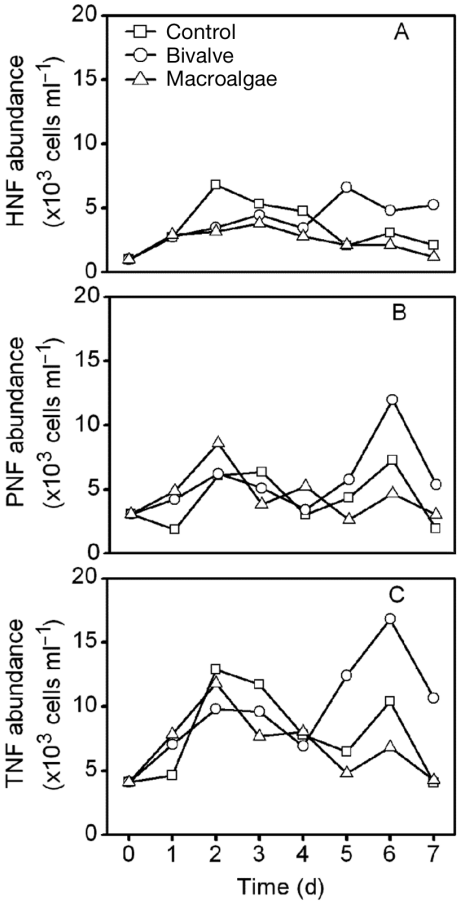

Fig. 8. Variations in abundance of (A) heterotrophic nanoflagellates (HNF), (B) pigmented nanoflagellates (PNF) and (C) total nanoflagellates (TNF) in the enclosure experiments over time

Comparison with other bays in East Asia

During the study period, NF abundance in Sungo Bay varied from 0.32×10^3 to 171.62×10^3 cells ml^{-1} (Fig. 3A–D), which was within the typical range for NF in planktonic ecosystems (10^2–10^5 cells ml^{-1}) (Lee & Patterson 2002, Huang et al. 2008). However, NF were more abundant in Sungo Bay than in the open waters of the Yellow Sea, except in winter, when the water temperature was lower in Sungo Bay than in the open water (Lin 2012). Interestingly, although Sungo Bay is a phosphorus (P)-deficient system (Sun et al. 2007), NF abundance levels in Sungo Bay appeared to be higher than in other eutrophic bays in East Asia, except for Funka Bay, which was sampled during a spring pre-bloom period for phytoplankton (Table 2) (Lee et al. 2001, Choi et al. 2003, Kamiyama 2004, Yang et al. 2008). The higher abundance of NF in Sungo Bay may relate to both the specific environmental conditions and mariculture in the bay.

Table 2. Comparison of nanoflagellate abundances in Sungo Bay with those reported in other bays. HNF: heterotrophic nanoflagellates, PNF: pigmented nanoflagellates

Study area	Location	Study period	Nanofla-gellates	Abundance ($\times 10^3$ cells ml^{-1})	Reference
Gyeonggi Bay	Middle northern region of Yellow Sea, Korea	Jan 1997–Dec 1999	HNF	1.94	Yang et al. (2008)
Masan Bay	Southern coast of Korea	Aug 1999	HNF	1.04 ± 0.72	Choi et al. (2003)
Funka Bay	SW of Hokkaido, Japan	Mar 1999	HNF	2.86	Lee et al. (2001)
Hiroshima Bay	Seto Inland Sea, Japan	Jun–Jul 1995	HNF	0.52–11.06	Kamiyama (2004)
Sungo Bay	NW coast of Yellow Sea, China	Apr 2011–Jan 2012	HNF	3.77 ± 6.00	This study
			PNF	8.73 ± 19.46	This study

Temperature limitation in winter

Water temperature characterised the seasonal pattern of NF by affecting physiological processes as well as food density (Weisse 1991, 1997, Hansen & Christoffersen 1995, Zhao et al. 2003). When the abundance of bacteria approaches a threshold of 3×10^5 cells ml^{-1}, the effective grazing of NF is limited (Berninger et al. 1991). In winter, both in the B-area ($3.28 \times 10^5 \pm 0.63 \times 10^5$ cells ml^{-1}) and M-area ($2.67 \times 10^5 \pm 0.46 \times 10^5$ cells ml^{-1}), bacterial abundance was near the threshold density of effective grazing for NF, and thus, NF in both areas likely faced a shortage of food. Therefore, we speculate that the lack of a significant difference in NF abundance between the B-area and M-area in winter is probably due to the low temperature (<5°C average), which limits the growth of bacteria (Tsai et al. 2005).

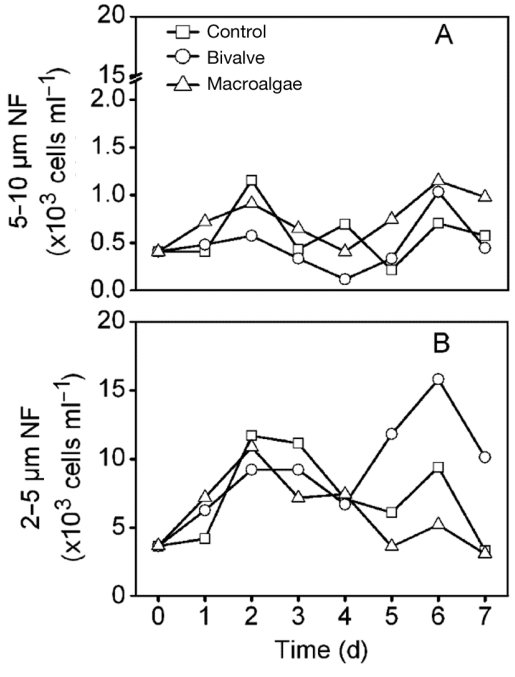

Fig. 9. Variations in abundance of (A) 5–10 µm nanoflagellates (NF) and (B) 2–5 µm NF in the enclosure experiments over time

Effects of freshwater input on distribution of NF

Together with water temperature, salinity is also used as an indicator of different water masses (with different nutrient levels) when analysing the spatial distribution of NF (Sherr et al. 1988, Andersson et al. 1994, Vázquez-Domínguez et al. 2012, Lin et al. 2014). However, Sungo Bay is a shallow bay with an average depth of 7.5 m (Zhang et al. 2009). Surface water temperature in the shallow system is mainly controlled by air temperature and solar heat gain rather than water mass interactions. Therefore, we used salinity as the only indicator of freshwater discharge in this study. Freshwater influenced Sungo Bay mainly in the warm seasons (Fig. 2A–D). Salinity in the spring was <31 psu (Fig. 2A), most likely due to several rain events prior to sampling.

Sungo Bay is a P-deficient and nitrogen-eutrophic system (Sun et al. 2007), where the growth of osmotrophic organisms (bacteria and autotrophic PNF) may depend on P concentration rather than DIN. Both in summer and autumn, Sungo Bay experienced a freshwater influx (Fig. 2B,C), and the abundance of both NF and bacteria were negatively related to salinity (Table 3). Freshwater may bring a certain amount of terrestrial nutrients, which likely resulted in higher PO_4^{3-} concentrations in the B-area (Table 1). It has been reported that higher nutrient supplies might result in a higher abundance of NF (Safi & Hall 1997, Huang et al. 2008, Tsai et al. 2010). Therefore, freshwater input in the warm seasons may have caused higher TNF abundance in the B-area than in the M-area (Fig. 4).

However, the correlation between salinity and nutrients was not significant (Table 3), which was likely due to the patchy distribution of bivalves in the

Table 3. Correlations between total nanoflagellate (TNF) abundance, bacterial abundance and salinity and other factors in Sungo Bay in the 4 seasons. DIN: dissolved inorganic nitrogen. Correlation is significant (in **bold**) at *the 0.05 level (2-tailed) or **the 0.01 level (2-tailed)

		Bacteria	Chl *a*	PO_4^{3-}	DIN	Salinity
Spring	TNF	0.405	**0.611****	0.224	−0.169	0.125
	Bacteria	–	0.186	−0.057	−0.153	**0.483***
	Salinity	**0.483***	0.086	−0.013	−0.295	–
Summer	TNF	**0.645****	**0.681****	−0.061	0.382	**−0.784****
	Bacteria	–	**0.729****	0.032	0.445	**−0.829****
	Salinity	**−0.829****	**−0.859****	−0.005	−0.424	–
Autumn	TNF	**0.967****	**0.929****	0.351	−0.270	**−0.893****
	Bacteria	–	**0.940****	0.371	−0.228	**−0.944****
	Salinity	**−0.944****	**−0.805****	−0.290	0.169	–
Winter	TNF	−0.106	0.110	−0.073	−0.142	0.038
	Bacteria	–	−0.148	−0.216	−0.234	−0.216
	Salinity	−0.216	−0.001	0.038	0.300	–

bay. Nutrients released by the bivalves might lower the correlation between salinity and nutrient concentrations. The correlations between bacteria and NF abundances and nutrient concentrations were also not significant (Table 3), likely because the bivalves' grazing may have had effects on the abundance of bacteria and NF. In sum, the spatial variations of the NF in Sungo Bay could not be only attributed to the influence of freshwater discharge.

Effects of nutrient release and grazing of bivalves on NF

In fact, the nutrient release by bivalves plays an important role in aquaculture areas (Carlsson et al. 2012, Cranford et al. 2012) and has been used to explain the predominance of bacteria, autotrophic flagellates and picoeukaryotes in areas of intense bivalve farming (Dupuy et al. 2000a). Bacterial growth is known to be stimulated by phosphate addition when ambient phosphate concentration drops <0.1 µM (Morris & Lewis 1992, Carlsson & Caron 2001). With bacterivores and autotrophic PNF being the predominant assemblages in the 2–5 µm NF (Unrein et al. 2007, Jürgens et al. 2008, Tsai et al. 2011), nutrient excretion (mainly P) by bivalves may increase the abundance of 2–5 µm NF either directly by increasing the abundance of autotrophic NF or indirectly by increasing the prey of bacterivorous NF. The abundance of 2–5 µm NF contributed ~90% of the TNF abundance (data not shown) in our study and bivalve cultivation substantially increased the concentration of PO_4^{3-} (Fig. 6B). Thus, we suggest that the release of P by bivalves may have caused the elevated abundance of TNF observed during the last 3 d of our enclosure experiment.

Based on microscopic observations, ~7 µm NF dominated in 5–10 µm NF, and >3 µm NF dominated in 2–5 µm NF in this study. The size ratio between protist predators and their optimal prey is suggested to be 3:1 (Hansen et al. 1994). Therefore, the main predator of 2–5 µm NF may be a protist >10 µm in size, and the main predator of 5–10 µm NF may be a protist >20 µm.

Bivalves cannot efficiently filter picoplankton (Kach & Ward 2008), and their retention efficiency decreases with decreasing particle size (Charles et al. 1999). Further, small flagellates (<5 µm) are poorly retained compared to protists with a size >5 µm (Dupuy et al. 2000b). In Sungo Bay, the retention rate of scallops *Chlamys farreri* was 8% on 2 µm particles and 45% on 8 µm particles, and the retention rate dropped with decreasing particle size (Zhang et al. 2010). Therefore, the feeding selectivity of the bivalves probably relieved the grazing pressure of <5 µm NF. This may explain why the abundance of 2–5 µm NF increased in the bivalve enclosure experiment, while 5–10 µm NF remained relatively stable over time in the same enclosure (Fig. 9).

Though no significant differences in NF abundance were found among the 3 enclosures (repeated-measures ANOVA, $p > 0.05$), NF abundance in the bivalve treatment increased during the last 3 d. The enclosure experiments were maintained for only 7 d, but our results are similar to a 1 yr long experiment conducted by Dupuy et al. (2000a) in an oyster farming pond, where <5 µm NF developed at a more intense rate than in the control pond (without oysters). Therefore, it would be reasonable to expect that NF abundance might remain higher in the bivalve enclosure than other enclosures for a longer period of time.

The optimal temperature for *C. farreri* is between 19 and 22°C, and the scallop stops growing at around 4°C (Yang et al. 1999, Zhang et al. 2004). Though the enclosure experiments were conducted only in summer, it would be reasonable to expect that the feeding and P-release of the bivalves would be significantly greater in summer and autumn (15.5–23.8°C) than in spring and winter (2.2–10.6°C).

Effects of macroalgae on NF

Bacteria and macroalgae competed with phytoplankton for nutrients (Sfriso & Pavoni 1994, Pedersen & Borum 1996, Passow et al. 2007, Thingstad et al. 2007, Thingstad & Cuevas 2010). The introduction of macroalgae enhanced the competition for P, and this was confirmed by the decrease in chl *a* biomass in the macroalgae enclosure (Fig. 7A). The competition for limited P among different taxonomic groups may limit the use of DIN. Though the DOC released by macroalgae is available for bacterial growth (Hatcher et al. 1977, Pregnall 1983), the P deficiency may limit the increase in bacterial abundance even in the presence of excess DOC. The P deficiency and the insignificant increase of bacteria may explain the lack of a significant difference in NF abundance between the macroalgae treatment and the control. Overall, at least in summer, NF abundance should be higher in the B-area than in the M-area if the impacts of mariculture are considered individually.

In conclusion, both freshwater input and mariculture of bivalves and magroalgae are likely important factors influencing the spatial distribution of NF in warm seasons in Sungo Bay.

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