JPR Advance Access published June 28, 2012

The influence of coastal waters on distributions of heterotrophic protists in the northern East China Sea, and the impact of protist grazing on phytoplankton

KEUN-HYUNG CHOI[1], EUN JIN YANG[2]*, DONGSEON KIM[1], HYUNG-KU KANG[1], JAE HOON NOH[1] AND CHEOL-HO KIM[1]
[1]KOREA OCEAN RESEARCH AND DEVELOPMENT INSTITUTE, ANSAN PO BOX 29, SEOUL 425-600, KOREA AND [2]KOREA POLAR RESEARCH INSTITUTE, INCHEON 406-840, KOREA

*CORRESPONDING AUTHOR: ejyang@kopri.re.kr

Received December 20, 2011; accepted in principle May 16, 2012; accepted for publication May 22, 2012

Corresponding editor: John Dolan

Spatial and temporal variations in the abundance and biomass of heterotrophic protists and of their grazing impact were investigated during five cruises between July 2006 and February 2009 in the continental shelf waters of the northern East China Sea (ECS). Strongly patchy distributions were observed on all cruises, generally with a higher biomass in the western areas affected by the Changjiang River discharge. An opposite pattern was observed in February when the Kuroshio onshore transport is greatest, with a greater biomass in the eastern area. Small heterotrophic dinoflagellates (<20 μm) were most abundant numerically, whereas ciliates contributed the most to the biomass, accounting for 28–58% of the total heterotrophic protist biomass. Small heterotrophic dinoflagellates were more strongly correlated with phytoplankton biomass than were other types of protists. The total protist biomass was often most strongly related to amounts of particulate organic carbon of non-phytoplankton origin, suggesting that their abundance distribution often depended on prey other than phytoplankton, such as heterotrophic bacteria. Heterotrophic protists consumed 30.1–91.5% of Chl *a* production (mean 68.2%), with grazing rates increasing with the phytoplankton biomass. The results suggest that heterotrophic protists were the major consumers of primary production, and that their grazing is one of the most important losses affecting the phytoplankton biomass in the northern ECS.

KEYWORDS: heterotrophic protists; protist herbivory; ciliates; heterotrophic dinoflagellate; Changjiang river; northern East China Sea

INTRODUCTION

Heterotrophic protists, consisting mostly of heterotrophic nanoflagellates (HNF), ciliates and heterotrophic dinoflagellates (HDF), are an important component of the nanozooplankton (<20 μm) and microzooplankton (20–200 μm), and they are important secondary producers in marine ecosystems (Gifford, 1988; Burkill *et al.*, 1993; Levinsen and Nielsen, 2002; Jochem, 2003;

doi:10.1093/plankt/fbs046, available online at www.plankt.oxfordjournals.org.

Landry and Calbet, 2004; Calbet, 2008). Many studies suggest that heterotrophic protists are the dominant herbivorous consumers of phytoplankton in marine environments (Burkill *et al.*, 1987, 1993; Verity *et al.*, 1996), consuming on average over 60% of the daily phytoplankton production on a global basis (Landry and Calbet, 2004). Copepod grazing on phytoplankton, once considered to be the most important component of marine herbivory, appears to be relatively low, accounting for only 10% globally of the primary production consumed daily (Calbet, 2001). Protists, especially large HDFs (lHDFs), are also known to compete with herbivorous meso- and macro-zooplankton for all size classes of phytoplankton (Sherr and Sherr, 2007). Protist grazing, therefore, may affect the rate of organic matter flux from the euphotic zone (Sherr *et al.*, 1992; Calbet and Landry, 2004).

Heterotrophic protist herbivory is also an important mechanism for transferring phytoplankton production to metazoan zooplankton. In oligotrophic waters, where phytoplankton are abundant, but are too small to be directly eaten by mesozooplankton, mesozooplankton production may depend on eating protists (Atkinson, 1996; Roman and Gauzens, 1997). Even in eutrophic waters, heterotrophic protists are frequently preferred over phytoplankton by metazoan zooplankton (Lawrence *et al.*, 2004; Yang *et al.*, 2009, 2010; Saiz and Calbet, 2011). Therefore, it is important to quantify the amount of phytoplankton carbon channeled through heterotrophic protists and the protist production available to the upper trophic levels in marine environments.

The region of interest in this study, the northern East China Sea (ECS) south of Cheju Island off the Korean peninsula, has dynamic hydrographic conditions, with the shallower western shelf water characterized by lower salinity, higher nutrient concentrations and phytoplankton stocks driven by the influx of more productive waters from the coastal area of China. This inflow is known as the East China Sea Current (ECSC) (Yuan and Hsueh, 2010), which incorporates changjiang diluted water (CDW) (Kim *et al.*, 2009). This CDW is defined as a summer plume of $>23^{\circ}$C and <31 salinity (Gong *et al.*, 1996). The deeper eastern section of the ECS has lower nutrient concentrations due to the year-round persistence of the Tsushima Warm Current (TWC) (Kim *et al.*, 2009). The presence of such distinctive water masses across the shelf forms a seasonal thermohaline front, known as the Yangtze-Cheju front, located in the vicinity of 125–126°E (Kim *et al.*, 2009) between the TWC water and the coastal waters of China (Hickox *et al.*, 2000; Park and Chu, 2007; Huang *et al.*, 2010; Yuan and Hsueh, 2010).

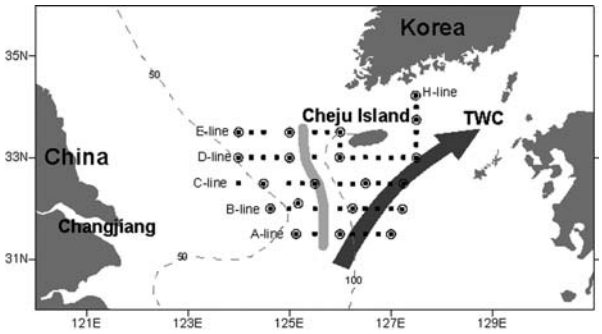

Fig. 1. Sampling stations in the northern East China Sea off Cheju Island south of Korea, with the coastal Changjiang-Cheju thermohaline fronts depicted with a thick gray line. Filled squares indicate hydrocast stations, and open circles are locations at which sampling for parameter measurements was conducted. TWC, Tsushima Warm Current.

The inputs of CDW can significantly affect the distribution of biological and chemical properties (Kim *et al.*, 2009), which could subsequently shape the planktonic food web structure in the region. The coastal water influences on spatial distributions of heterotrophic protist communities and on their herbivory may have significant implications for carbon transfer through the phytoplankton-heterotrophic protist-meso-zooplankton food webs in the region. In the ECS area, studies of heterotrophic protists have focused mainly on the abundance and biomass of ciliates (Chiang *et al.*, 2003; Ota and Taniguchi, 2003), with the study of microzooplankton grazing (Zhang *et al.*, 2006) limited mostly to the central and southern ECS. Heterotrophic protist community distribution and grazing impacts in the northern ECS have not been investigated previously. Therefore, we present an initial undertaking toward improving our understanding of the phytoplankton-heterotrophic protist trophic link in the northern ECS. Our goals were to determine the spatial and seasonal distribution patterns of heterotrophic protists and to determine seasonal variations in grazing impacts of heterotrophic protists on the phytoplankton biomass and production in the northern East China shelf region off Cheju Island, Korea.

METHOD

Field sampling

The study was carried out in the northern ECS off Cheju Island, Korea (31°30′–34°00′N, 124°00′–127°30′E, Fig. 1). A total of five cruises over four seasons were conducted to measure environmental and

biological properties, including suspended solids (SSs), chlorophyll a (Chl *a*), particulate organic carbon and nitrogen (POC, PON, respectively) and the abundance and composition of heterotrophic protists. Cruises were undertaken in the spring (14–24 April 2008), summer (19–25 July 2006 and 18–27 July 2007), autumn (31 October–1 November 2007) and winter (16–22 February 2009). Numbers of stations visited and samples taken on each cruise are shown in Table I.

Water temperature and salinity were measured using a SeaBird9/11 plus conductivity–temperature–depth sensor. Seawater samples for determining concentrations of SS, Chl *a*, POC, PON and heterotrophic protists were collected at various depths, including the subsurface Chl *a* maximum using a rosette sampler with 10-L Niskin bottles mounted on the CTD assembly. Vertical sample profiles of protists were collected during two cruises (19–25 July 2006 and 31 October–1 November 2007), while only near-surface water samples (1 m below the surface) were taken during the other cruises (18–27 July 2007, 14–24 April 2008 and 16–22 February 2009).

Water samples (500 mL) for the determination of Chl *a* were immediately filtered through glass fiber filter paper (Whatman GFF, 25 mm). Size-fractionated Chl *a* was determined on samples passed sequentially through 3 μm polycarbonate membrane filters (47 mm) and glass fiber filters (47 mm). All filtrations were performed under low vacuum pressure (<100 mmHg) or by gravity pressure when using 3-μm filter papers. The Chl *a* extracted in 90% acetone after 24 h was determined with a fluorometer (10-006R, Turner BioSystems, Sunnyvale, CA, USA).

Inorganic nitrate, nitrite and phosphate were analyzed as described in Kim *et al.* (Kim *et al.*, 2009). POC and PON analyses were obtained by filtering seawater through pre-combusted glass fiber filters (WhatmanGF/F, 25 mm). The filters were rinsed in Milli-Q H_2O to remove sea salt, sealed in cleaned Petri dishes and frozen at −20°C onboard. In the laboratory the filters were dried in an oven at 60°C for 2 days. Both POC and PON in the filtered particulate matter were determined using a carbon–nitrogen–sulfur analyzer (CNS EA1110, CE Instruments) after inorganic carbon was eliminated by placing the filtered particulate matter in a desiccator over concentrated HCl for 24 h. The C:Chl *a* ratio was calculated by applying the method of Wienke and Cloern (Wienke and Cloern, 1987) that corrects for the presence of sediment-associated non-phytoplankton carbon.

To determine the abundance of HNF, 100 mL of water was preserved with glutaraldehyde (1%, final concentration), and stored at 4°C < 1 day before staining and filtration. Subsamples of 30–80 mL were filtered through black Nuclepore© filters (pore size, 0.8 μm), stained with proflavin (0.33%) and 4′-6-diamidino-2-phenylindole (DAPI; 5 μg mL^{-1}, final concentration) and mounted onto glass slides that were stored at −20°C until analysis. Samples were examined within 1 month under an epifluorescence microscope (Nikon type 104) at magnifications of ×600 to ×1000. HNF were examined under both UV excitation for DAPI fluorescence and blue excitation for proflavin fluorescence. The autofluorescence of chlorophyll and other pigments was confirmed by switching the filters. At least 50 fields and at least 100 cells were enumerated for each sample. Cell carbon for HNF was derived from measuring cell length and width and calculating cell volume from geometric formulae. At least 100 cells were estimated using an image analyzer for pictures from a camera mounted on the microscope. Cell volume (μm^3) was converted to cell carbon using a factor of 220 fg C μm^{-3} (Bøsheim and Bratbak, 1987).

Ciliate samples were prepared by adding acid Lugol's iodine to 250 mL of seawater (5%, final concentration) and storing them in the dark until analysis. Subsamples of 50–100 mL were concentrated in sedimentation chambers for ≥48 h, and the concentrate was examined with an inverted microscope (Olympus IX 70) at ×100 to ×200. For the ciliates, naked ciliates and loricate ciliates were distinguished. For HDF samples, 250 mL of seawater was preserved with neutralized formalin (2% final concentration) and stored at 4°C in the dark. Subsamples of 50–100 mL were concentrated in a sedimentation chamber for ≥48 h in a refrigerator (4°C); the concentrates were stained with DAPI (5 μg mL^{-1}, final concentration) for 5 min. The whole chamber, or a fraction of it for the smallest and more abundant organisms, was enumerated with an inverted epifluorescence microscope at ×200 to ×400. HDF were classified and recorded as lHDF (>20 μm) and small HDF (sHDF, <20 μm). HDF were distinguished from other flagellates based on cell morphology and structure of the nucleus, especially the unique condensed chromosomes visible by DAPI staining. The samples were analyzed within 1 month of sampling, and sample analysis with the inverted microscope did not exceed 30 min per sample. The abundance of cells in settled samples may have resulted in some underestimation of HDF, especially in the smallest size group (Buck and Garrison, 1988). On the other hand, the formalin preserved HDF, which were stored at 4°C for one month, also may have caused an overestimation of HDF relative to autotrophic dinoflagellate (ADF) abundance because the chlorophyll in any ADF would have likely faded by the time. Ciliate and HDF biovolumes

Table I: Summary of environmental parameters and protozoan density and biomass determined during cruises (geometric mean and 95% confidence limit)

								Density					Biomass			
Cruise	St.	T	S	SS	PON	POC	Chl *a*	Aloricate ciliates	Loricate ciliates	lHDF (cells	sHDF	HNF ($\times 10^3$	Ciliates.B	lHDF.B (μgC	sHDF.B (μgC	HNF.B (μgC
	(*n*)	(°C)		(mg L^{-1})	(μM)	(μM)	(μg L^{-1})	(cells L^{-1})	(cells L^{-1})	L^{-1})	(cells L^{-1})	cells L^{-1})	(μgC L^{-1})	L^{-1})	L^{-1})	L^{-1})
06-Jul	24 (122)	24.5 (24.0–25.0)	30.6 (30.0–31.2)	14.8 (14.4–15.2)	2.6 (2.2–3.0)	19.1 (16.3–22.5)	1.0 (0.8–1.2)	3446 (1274–5618)	133 (85–181)	517 (304–877)	26620 (21219–33395)	1037 (833–1290)	4.2 (3.1–5.8)	0.6 (0.4–1.1)	1.3 (1.0–1.8)	2.2 (1.7–2.8)
07-Jul	31 (165)	25 (24.7–25.4)	32.1 (31.8–32.6)	17.2 (16.9–17.5)	2.1 (1.6–2.6)	12.3 (9.5–16.1)	0.4 (0.3–0.5)	4100 (1030–7170)	166 (58–274)	1857 (1274–2706)	47120 (35357–62795)	2352 (1870–2956)	5.6 (4.5–7.1)	2.6 (1.9–3.6)	2.5 (1.8–3.6)	3.6 (2.8–4.3)
07-Oct	5 (30)	26.4 (25.7–27.1)	34.5 (34.2–34.8)	NA	NA	NA	0.3 (0.1–1.0)	1294 (482–2106)	72 (35–109)	770 (388–1527)	27605 (14986–50850)	1083 (618–1898)	1.9 (0.6–5.8)	1.0 (0.4–2.7)	1.9 (1.3–2.8)	1.7 (1.3–2.3)
08-Apr	21 (122)	13.3 (12.6–14.0)	33.7 (33.5–34.0)	7.9 (5.0–12.4)	3.0 (2.5–3.6)	20.5 (17.2–24.5)	1.2 (0.9–1.7)	10019 (4309–15729)	382 (236–528)	530 (306–920)	46160 (29629–71915)	1344 (907–1990)	5.8 (3.4–10.0)	0.2 (0.1–0.4)	2.2 (1.4–3.3)	2.2 (1.5–3.2)
09-Feb	15 (78)	13.4 (12.0–14.8)	33.9 (33.6–34.3)	23.6 (21.0–26.6)	1.3 (1.1–1.5)	9.5 (7.8–11.5)	0.4 (0.38–0.48)	1504 (680–2328)	75 (28–122)	190 (131–274)	14626 (12151–17605)	298 (252–353)	0.9 (0.5–1.6)	0.2 (0.1–0.4)	0.3 (0.2–0.4)	0.4 (0.36–0.54)

St, number of stations; *n*, total number of samples taken [for heterotrophic protists, vertical samples were collected during two cruises (19–25 July 2006 and 31 October–1 November, 2007), while surface water samples only (1 m below the surface) were taken during the other cruises (18–27 July 2007, 14–24 April 2008 and 16–22 February 2009)]; *T*, temperature; S, salinity; SS, suspended solids, PON, particulate organic nitrogen; POC, particulate organic carbon, lHDF, large heterotrophic dinoflagellates (>20 μm), sHDF, small heterotrophic dinoflagellates (<20 μm), HNF, heterotrophic nanoflagellates. NA, not available.

were estimated from lengths and widths of 50 to 100 cells, according to their geometric shapes (spheres, cones and ellipsoids). The cell volumes (μm^3) of ciliates and HDF were converted to carbon biomass using factors and equations as follows: 0.19 μg C μm^{-3} for naked ciliates (Putt and Stoecker, 1989); carbon (pg) = 44.5 + 0.053 × lorica volume (μm^3) for loricate ciliates (Verity and Langdon, 1984) and carbon (pg) = 0.216 × [volume, μm^3]$^{0.939}$ for HDF (Menden-Deuer and Lessard, 2000).

Heterotrophic protist herbivory experiments

Specific phytoplankton growth rates and protist grazing rates on phytoplankton were estimated using a dilution method measuring the total Chl *a* concentration (Landry and Hassett, 1982). All equipment was cleaned with 10% HCl and thoroughly rinsed with copious distilled water. Surface seawater was prescreened into a 20 L carboy by gentle reverse filtration through a 200-μm mesh immersed in the seawater to remove copepods. Particle-free seawater was prepared with gravity filtration through a Pall Acropak 0.8/0.2 μm 500 filter capsule pre-washed with 10% trace-metal grade HCl followed by Milli-Q and seawater rinses. The experimental water was then diluted with 0.2-μm-filtered seawater to obtain duplicate bottles with proportions of 100%, 75, 55 and 20% of the sample water. Dilution series were set up in eight 1.3 L polycarbonate bottles, which were amended with a nutrient mixture (10 μM $NaNO_3$ and 1 μM Na_2HPO_4) to ensure that nutrients would not become limiting during incubations. In July 2006, two additional undiluted bottles were prepared without nutrient added to assess the natural growth of the phytoplankton. As we found no nutrient limitation for the phytoplankton growth rate (see results below), control bottles for whole water with no nutrient amended were not set up on the following cruises. All paired experimental bottles were screened to collection-depth light levels with neutral density screening and incubated for 24 h on deck in seawater-cooled incubators. Subsamples were collected from replicate bottles at 0 and 24 h to determine the Chl *a* concentration. For all experiments, a linear regression model was used to plot the best−fit relationship between the phytoplankton net growth rate and the dilution level (Landry and Hassett, 1982). Instantaneous phytoplankton growth with nutrient enrichment (k) and grazing rate (g) is defined as the y-intercept and the negative slope of the relationship, respectively. In July 2006, we compared the net phytoplankton growth rate (μ_o) in the undiluted bottle without added nutrients and the net growth rate (μ) in the undiluted bottle with added nutrients, because the addition of nutrient in the bottle would provide an overestimation of phytoplankton growth rates. Instantaneous phytoplankton growth rates (k') in July 2006 were derived from the net phytoplankton growth rate (μ_o) in the undiluted bottles without added nutrients, with the addition of the grazing rate (g) from dilution experiments ($k = \mu_o + g$).

Heterotrophic protistan grazing impacts on the Chl *a* standing stock (%SS) and Chl *a* production (% P) were calculated according to Verity *et al.*, (Verity *et al.*, 1993):

$$\%\text{SS grazed} = (1 - \exp^{-g}) \times 100$$
$$\%\text{P grazed} = 100 \times (1 - \exp(-g))/(1 - \exp(-k)).$$

The phytoplankton carbon consumption (G) by heterotrophic protists was calculated using

$$G = g \times C_m$$
$$C_m = C_0[\exp(k - g)t - 1]/(k - g)t,$$

where C_m is the mean concentration of the phytoplankton carbon biomass during the experiment and C_o is the initial concentration of the phytoplankton carbon biomass. The intercept may not always be positive, which indicates the possible natural mortality from parasites or viruses that can make the instantaneous phytoplankton growth rate naturally negative.

Data analysis

Regression analyses were conducted to examine relationships among the biological properties. The errors of the regressions were examined for violation of the assumptions of equal variance and normal distribution of errors. The statistical analyses were performed with R, an object-oriented open-source program (R Development Core Team, 2006).

RESULTS

Variation among cruises

The concentrations of both POC and Chl *a* were greatest among the cruises in July 2006 and April 2008 (Table I). The heterotrophic protist biomass followed the temporal pattern of numerical abundance, with February 2009 values, except for lHDF, being lower than those of the other cruises. HNF were numerically most abundant on all cruises, distantly followed by sHDF. Ciliates accounted for 28−58% of the total heterotrophic protist biomass, followed by HNF (Table I). lHDF showed a much lower biomass in April 2008 compared with the other protist groups.

Spatial distribution patterns

In July 2006, the surface water temperature generally declined toward the eastern part of the shelf (Fig. 2), but no clear spatial pattern was observed in July 2007. Cross-shelf variation was more evident in February 2009 and April 2008 with warmer waters to the north and east and colder waters to the south and west (Figs 4 and 5). Salinity showed an increasing cross-shelf pattern toward the eastern part on all cruises (Figs 2–5). In July 2006, there was a plume of low salinity surface waters on the western part, with salinity as low as 27 (Fig. 2; Table I). A much weaker coastal water effect was observed in summer 2007, reflecting the large inter-annual variation in coastal water input in the region. Coastal water and CDW input to the region was observed in both February 2009 and April 2008, with the temperature steadily increasing to the east. In February 2009, the salinity at all stations was >32, the highest of all cruises.

Nutrients also displayed frontal patterns, with sharp changes over the study area (Figs 2–5). Nitrate distribution was opposite to that of phosphate in July 2007 (Fig. 3), but on the other cruises they were positively correlated. SS concentration showed quite varied spatial patterns among the cruises. It was higher in the eastern part in July 2006 (Fig. 2) and high throughout the study area in July 2007, with strongly elevated levels in the western sector in both April 2008 and February 2009. POC concentrations were always greater in the western parts on all cruises, as was Chl *a*, except in February 2009 when the pattern was reversed (Fig. 5). In July 2006, the distributions of both POC and Chl *a* strongly reflected the coastal water plume impinging on the western side, with high concentrations of POC (>30 p.p.m.) and Chl *a* present in the low salinity water (Fig. 2). POC in July 2006 appeared to be mostly particles containing Chl *a*, but the distribution on other cruises was apparently much influenced by non-phytoplankton SSs (e.g. February 2009, Fig. 5). Chl *a* concentration was greatest in waters with high nutrients, except in February 2009 when Chl *a* and nutrients had opposite patterns (Fig. 5).

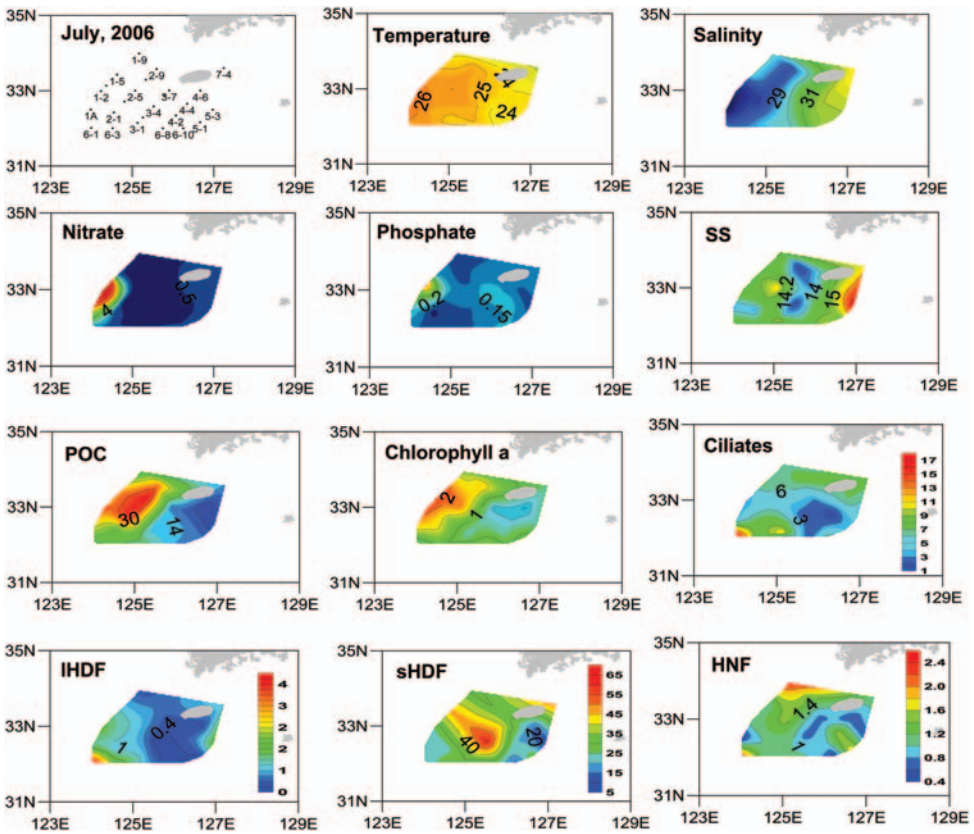

Fig. 2. Surface distribution of physical and biological properties in July 2006 in the northern East China. Temperature (°C), Nitrate and phosphate (μmol kg^{-1}), SS, suspended solids(mg L^{-1}); POC, particulate organic carbon (μM), Chlorophyll a (μg L^{-1}), ciliates (cell × 10^3 L^{-1}); lHDF, large heterotrophic dinoflagellates (cell × 10^3 L^{-1}); sHDF, small heterotrophic dinoflagellates (cell × 10^3 L^{-1}); HNF, heterotrophic nanoflagellates (cell × 10^6 L^{-1}).

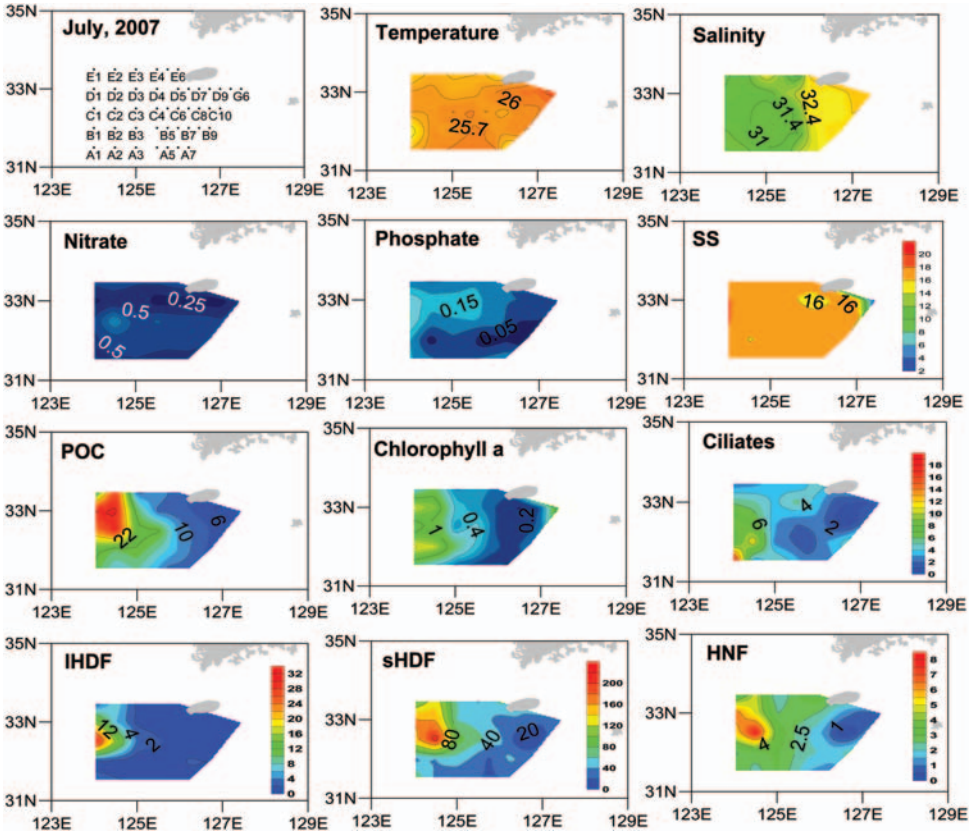

Fig. 3. Same as in Fig. 2, but for July 2007.

The spatial patterns of heterotrophic protists are hard to generalize. None of the protists was obviously associated with Chl *a* or POC distributions in July 2006 (Fig. 2). Ciliates and lHDF showed a positive abundance relationship, while the highest concentration of sHDF was observed together with low abundance of both ciliates and lHDF (Fig. 2). On the July 2007 cruise all protists were positively associated with the distributions of both Chl *a* and POC (Fig. 3), but the peak ciliate density was shifted to the south. In April 2008, both sHDF and HNF were generally correlated with the distributions of Chl *a* and POC, while both ciliates and lHDF were at low concentration in the south-western part with highest concentrations along the thermohaline front between colder and less saline coastal water and warmer more saline oceanic water (Fig. 4). In February 2009, all protist groups seemed to be associated positively with Chl *a* and negatively with POC (Fig. 5).

Vertical distribution patterns

To examine the vertical structure across the shelf, cross-sectional distributions were drawn for line B (Fig. 1). The water column was highly stratified in July of both 2006 and 2007, with intrusion of saline water along the bottom in the eastern part (Figs 6 and 7). Consequently, a nutricline was observed, above high-nutrient concentrations in the intermediate subsurface waters, especially in the central part (Fig. 6). Stratification disappeared in February and April, and nutrients were vertically homogeneous (Figs 8 and 9). Patchy nutrient pools, especially of phosphate, were observed over the western part of the shelf. On all cruises, water mass fronts, seen in the distributions of temperature and salinity, were present either as salt-wedges (July of 2006 and 2007) or as vertical (April 2008 and February 2009) transitions between colder, less saline, freshwater-influenced coastal water to the west and warmer more saline TWC water to the east.

High concentrations of SSs were observed in the bottom waters in the central region on July cruises (Figs 6 and 7). More bottom sediments were entrained into the water column during April 2008 and February 2009 when it was more mixed (Figs 8 and 9), contributing much of the POC content. On July cruises, when the water column was stratified, high POC concentrations in the surface reflected the distribution patterns of

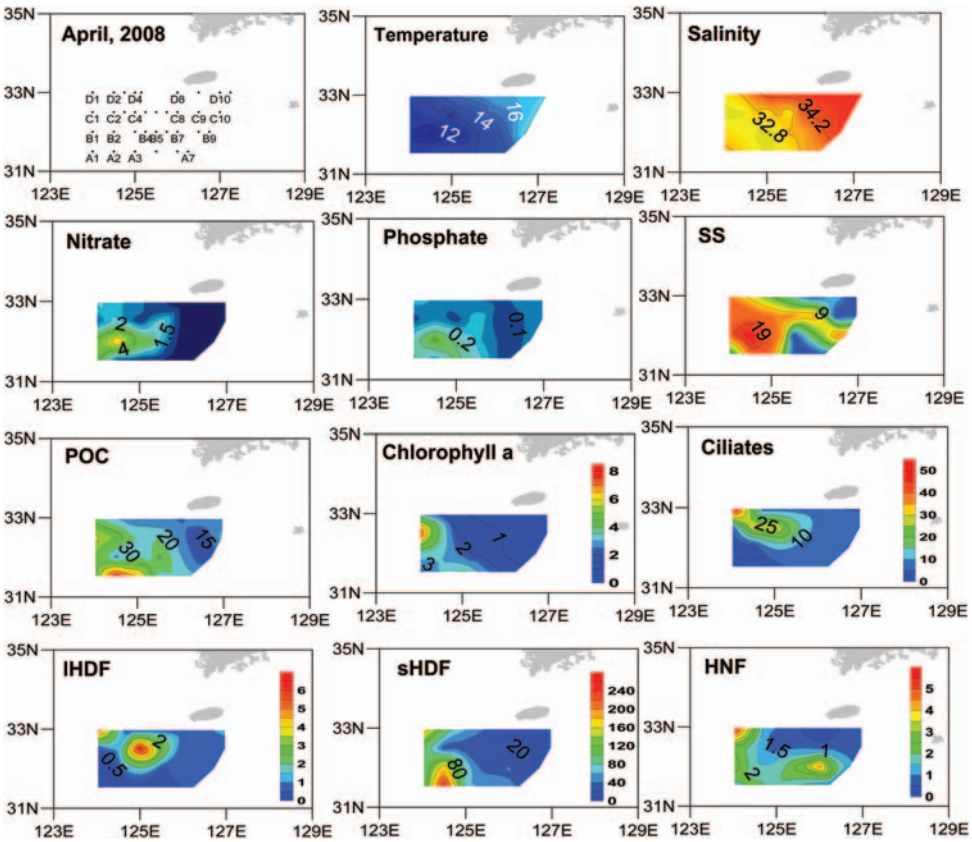

Fig. 4. Same as in Fig. 2, but for April 2008.

phytoplankton carbon. Chl *a* concentration generally showed subsurface maxima, except in April 2008 when it decreased progressively with depth. In July 2006, the patchy high concentrations of Chl *a* were found in waters of lowest SS concentration. Two Chl *a* maxima were observed in July 2007, a shallower one (~5–15 m) to the west and a deeper one (~25–35 m) to the east. In April 2008, Chl *a* concentration was similar to that of July 2007, despite greater inorganic nutrient availability in surface waters. The distributions of protist numbers differed from that of Chl *a*. None of the cross-sectional distributions of protists on line B appeared to be associated with either Chl *a* or POC distributions. Ciliates, lHDF and HNF all had their greatest concentrations restricted to the surface waters in the west with sharp declines with depth. In contrast, sHDF had a subsurface maximum in the east (Fig. 6).

Biomass relationship between phytoplankton and heterotrophic protists

To compare the ratio of the heterotrophic protist biomass to the phytoplankton biomass, we used a phytoplankton-C:Chl *a* ratio of 90. Total heterotrophic protist biomass was nearly equal to that of phytoplankton in waters of a low phytoplankton biomass, such that heterotrophic protists represented a significant reservoir of biogenic carbon (Fig. 10). However, the heterotrophic protist biomass relative to that of phytoplankton declined strongly with an increasing phytoplankton biomass and became insignificant, <2% of the phytoplankton biomass when the phytoplankton biomass was >200 μg C L^{-1}.

The relationship of Chl *a* to the heterotrophic protist biomass was examined with linear regressions. No positive relationship was observed, except for July 2007 when the protist biomass was log transformed (slope = 0.71, $R^2 = 0.29$, $P < 0.001$). Partial regression analyses were also performed between Chl *a* and the heterotrophic protist biomass, in which the residuals of the regression between Chl *a* and salinity were used as an independent variable. Protists were strongly and positively correlated with changes in the concentration of their phytoplankton food in both summer 2007 (slope = 0.43, $R^2 = 0.29$, $P < 0.001$) and spring 2008 (slope = 0.56, $R^2 = 0.25$, $P = 0.02$) (Fig. 11).

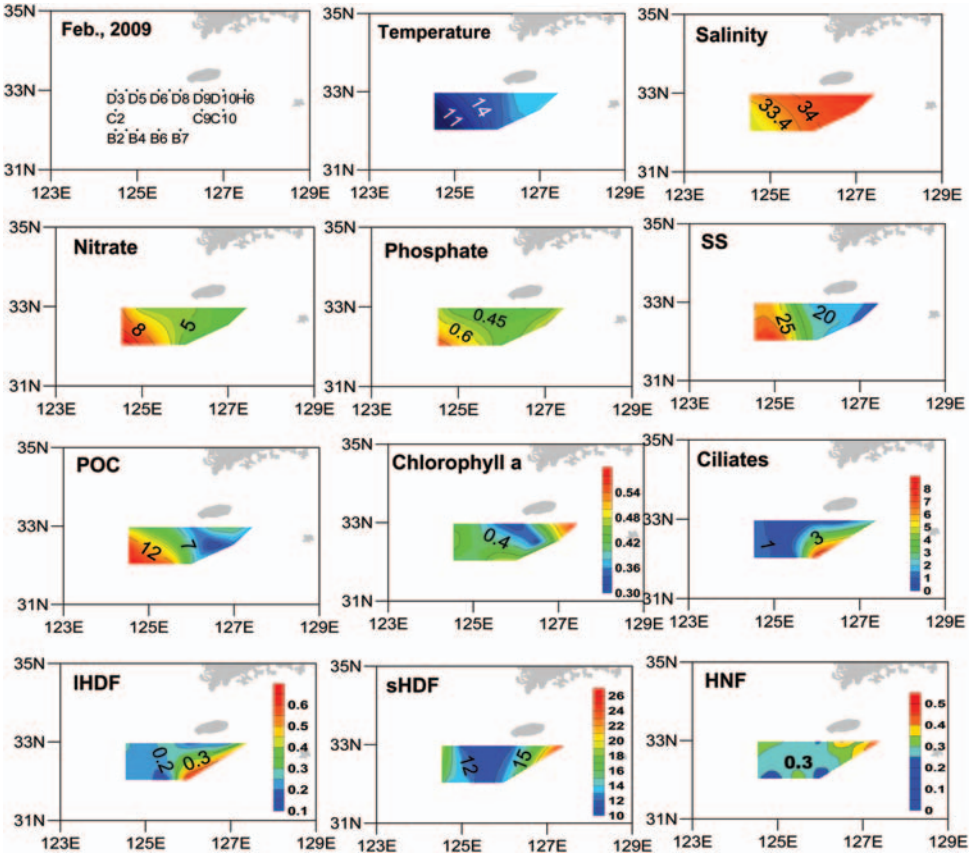

Fig. 5. Same as in Fig. 2, but for February 2009.

Phytoplankton growth and the heterotrophic protist grazing rate

The net phytoplankton increase measured in July 2006 in whole water differed only slightly with and without nutrient amendment (paired *t*-test, $P > 0.05$, Table II). Consequently, phytoplankton specific growth rates (k), as determined with nutrient-amended dilution experiments, were little different from those (k') determined in whole water with no nutrients added. The specific growth rate ranged between 0.25 day^{-1} and 87 day^{-1}, with an overall mean of 0.76 day^{-1} (Table II). Grazing rates (g) ranged from 0.11 to 1.09 day^{-1}, with an average of 0.45 day^{-1}. Both k and g were positively and linearly related with Chl *a* concentrations of up to 2.5 μg L^{-1} ($R^2 = 0.27$, $P < 0.001$ for the growth rate, and $R^2 = 0.24$, $P = 0.01$ for the grazing rate); however, the growth and grazing rates declined sharply at higher Chl *a* concentrations (Fig. 12). Low k and g values at the highest Chl *a* concentrations reflected the presence at some stations of large centric diatoms that protists cannot ingest (e.g. Strom and Welschmeyer, 1991). The phytoplankton growth rate in July 2007 (mean 0.83 day^{-1}) was significantly higher than the February rate of 0.42 day^{-1} [one-way analysis of variance (ANOVA), $P = 0.02$]. The July 2006 growth rate (0.61 day^{-1}) was not significantly different from the February rate. July 2006 protist grazing rates of 0.38 and 0.55 day^{-1} were significantly greater than the February rate of 0.18 day^{-1} ($P < 0.006$).

Heterotrophic protists were found to graze 10.4–66.4% of Chl *a* standing stocks daily, and 30.0–91.5% (mean of 68.8%) of Chl *a* production (Table II). The grazing impact increased linearly with the Chl *a* concentration and generally increased with the heterotrophic protist biomass (Fig. 8). The average February grazing impact (48.9%) on the Chl *a* production was lower than that in July (67.6 and 79.6% for 2006 and 2007, respectively). The estimated daily ration of the protists was 940% of body carbon per day (Fig. 8).

DISCUSSION

Heterotrophic protists were more abundant when the Chiangjiang outflow impinged on the study area, giving their distribution pattern an inverse relationship

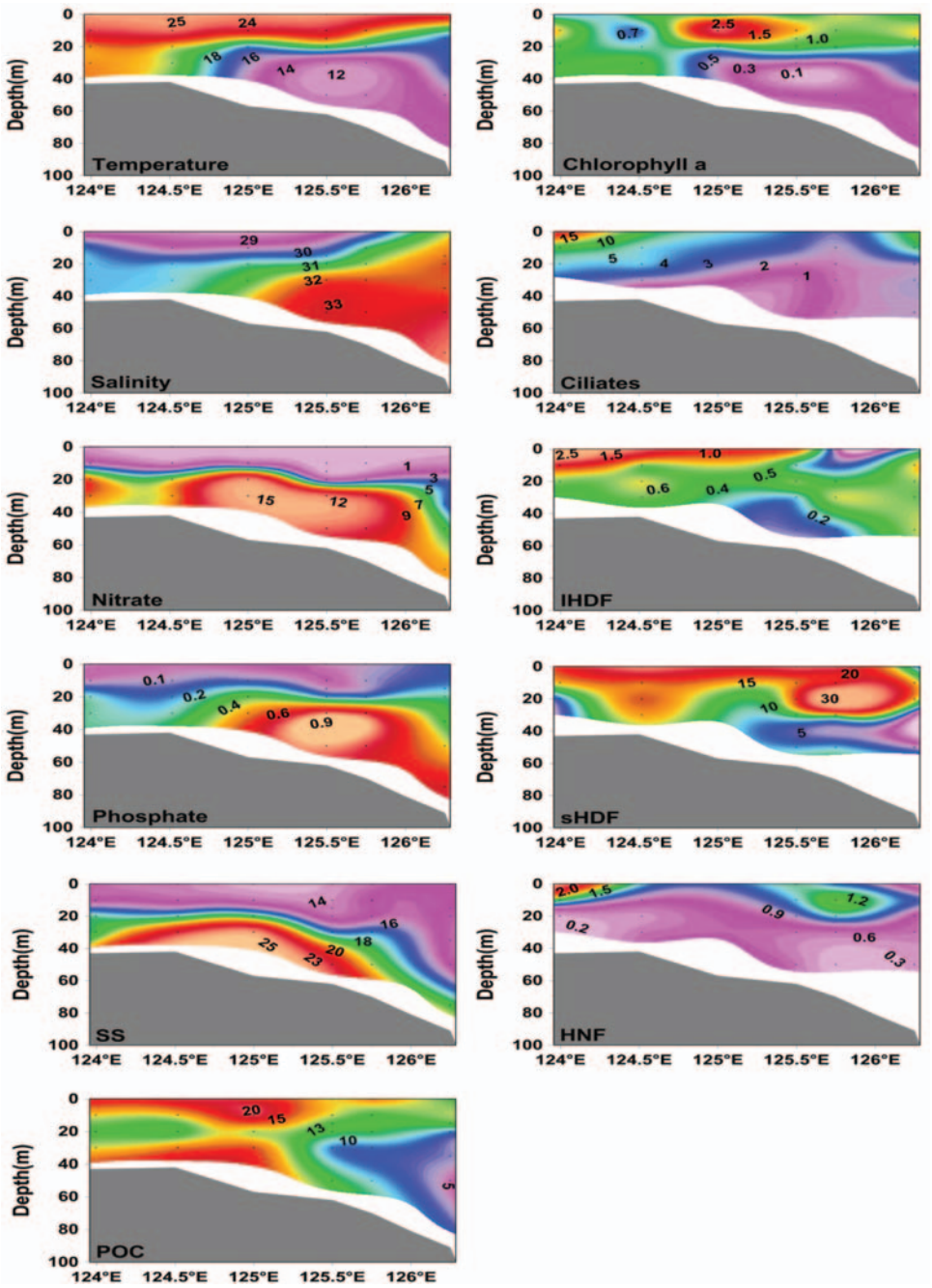

Fig. 6. Vertical distribution of physical and biological properties in July 2006 along a transect in line B (Fig. 1). Temperature (°C), nitrate and phosphate (μmol kg^{-1}), SS, suspended solids (mg L^{-1}), POC, particulate organic carbon (μM), Chlorophyll a (μg L^{-1}), ciliates (cell × 10^3 L^{-1}); lHDF, large heterotrophic dinoflagellates (cell × 10^3 L^{-1}); sHDF, small heterotrophic dinoflagellates (cell × 10^3 L^{-1}); HNF, heterotrophic nanoflagellates (cell × $10^6 L^{-1}$).

with salinity. Similar patterns have been documented by McManus and Fuhrman (McManus and Fuhrman, 1990), Jochem (Jochem, 2003) and Breed *et al.* (Breed *et al.*, 2004). The extent of the inflow of the Changjiang River water is primarily determined by the seasonal wind direction (Chang and Isobe, 2005). Chainag *et al.* (Chainag *et al.*, 2003) reported a positive relationship between ciliate density and the Changjiang discharge. Cross-shelf variations affected by coastal water intrusion were also observed in the POC:PON ratios in the water column, which generally tended to decrease from the western shelf toward

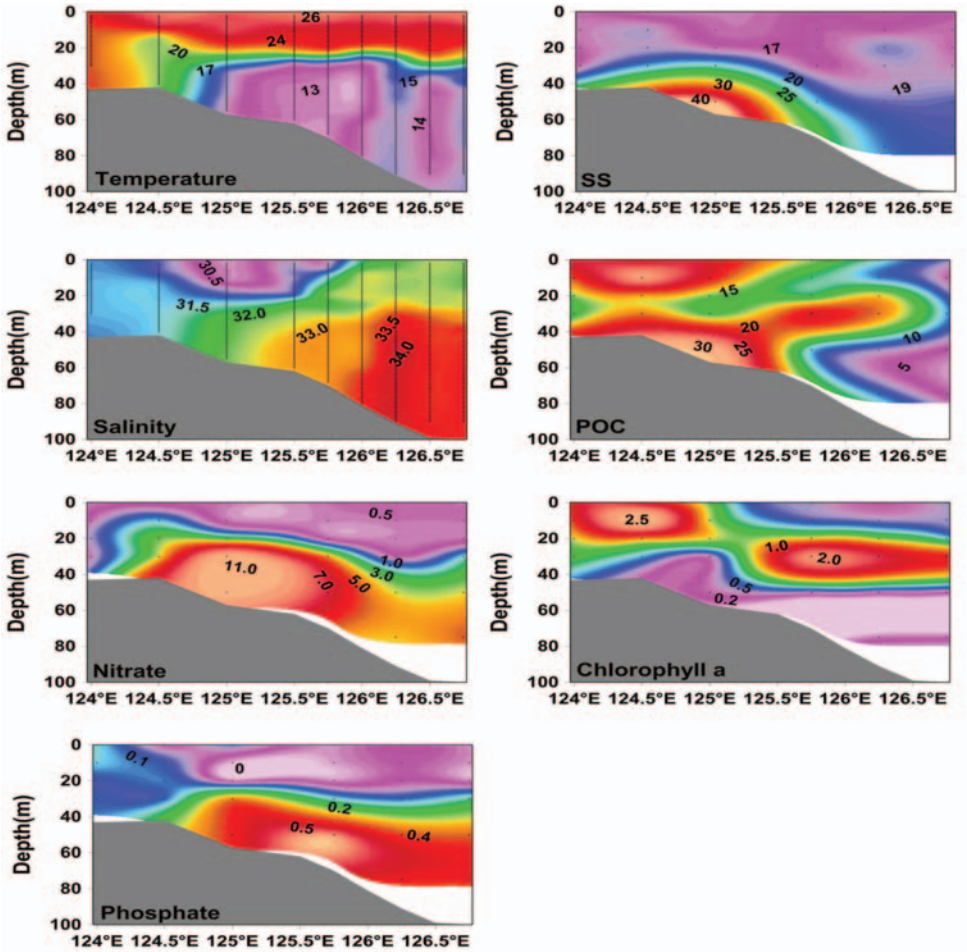

Fig. 7. Same as in Fig. 6, but for July 2007.

the eastern part (Choi *et al.* submitted). In summer, CDW dominates the surface waters of the region, as shown by the depressed surface salinity (Fig. 2), driven from the west by southerly winds. In autumn, the northerly wind pushes the Changjiang River plume to the south, causing it to extend southward along the coast. That leads to the cessation of the coastal upwelling in the autumn along the Chinese coast (Chuang *et al.*, 1993). Freshwater discharge diminishes in winter from the large volumes in summer that flush monsoonal rainfall seaward. As in autumn, the prevailing northerly wind in winter pushes the CDW plume to the south. Furthermore, the onshore transport of the Kuroshio water, the TWC, is strongest in winter (Guo *et al.*, 2006). We observed an exception to the cross-shelf biomass trend in February, when the Chl *a* concentration and the abundance of protists were greater on the eastern part of the shelf (Fig. 5). Phytoplankton may have benefited from warm temperatures that, in combination with higher nutrient levels due to vertical mixing and reduced amounts of SS, may have stimulated their growth.

Our data show that sHDF (<20 μm) were of numerical importance, about an order of magnitude more abundant than ciliates, which were the third most abundant protists (Fig. 2; Table I). The relative abundance ratios among heterotrophic protists may be driven in part by the size and biomass structure of prey available to them. In low-production ecosystems, where prokaryotic cells and picoautotrophs are the dominant primary producers, small flagellates are numerically dominant and comprise the major proportion of small grazers (Landry *et al.*, 1984; Roman *et al.*, 1995; Calbet, 2008). Conversely, heterotrophic and mixotrophic dinoflagellates are often the dominant grazers in more productive ecosystems (Calbet, 2008). Although the contribution of picophytoplankton to the total Chl *a* seemed unrelated to the total Chl *a* concentration (Table II), sHDF appeared to be more related to the total Chl *a* distribution than were other heterotrophic protists (Fig. 2).

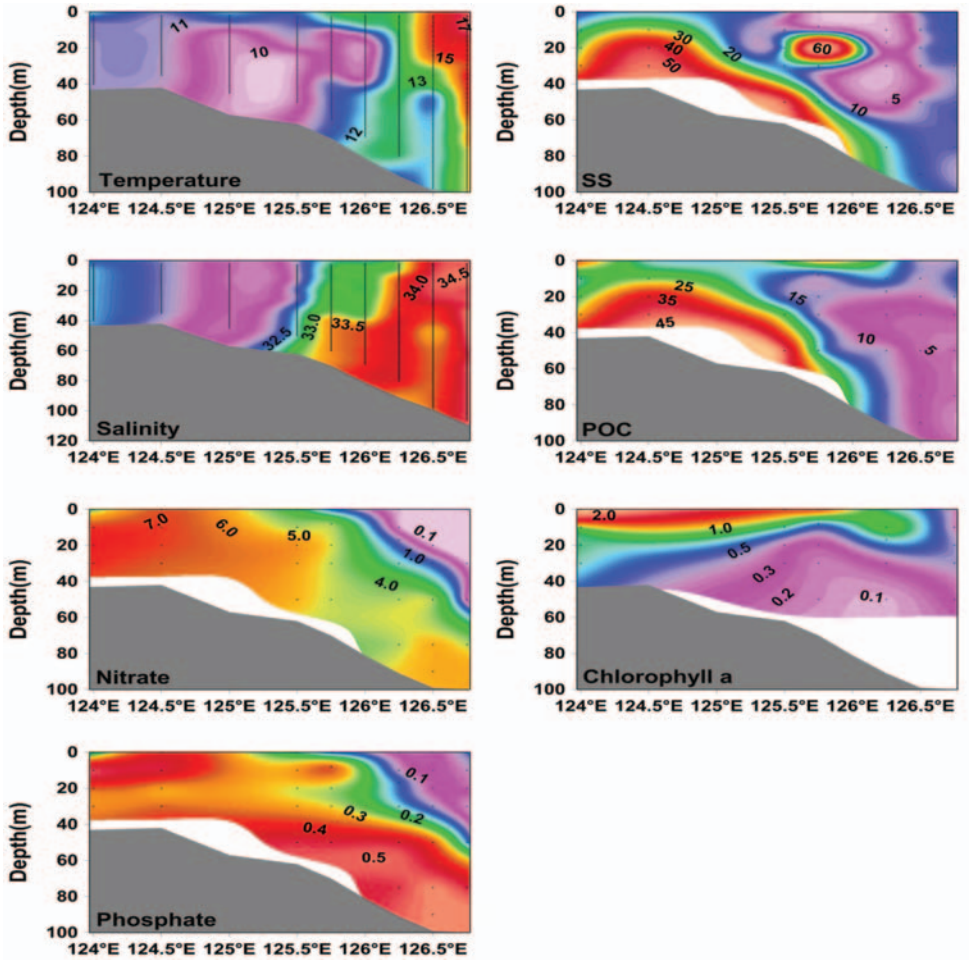

Fig. 8. Same as in Fig. 6, but for April 2008.

sHDFs appear to be an important prey of small metazoan zooplankton in this region, especially of small copepods, whereas ciliates may be a preferred prey for larger copepods. Abundant copepods in the study area, particularly *Paracalanus* spp. and *Oithona* spp., were small (0.2−1 mm), contributing 50% to the mesozooplankton biomass. Medium size (1−2 mm) copepods (e.g. *Calanus sinicus* and *Cosmocalanus darwinii*) contributed 35% (Kang, 2008; Choi *et al.*, 2011). Wu *et al.* (Wu *et al.*, 2010) showed in the northern ECS, where *Paracalanus* spp. are dominant in abundance, that ciliates and sHDF provide the majority of mesozooplankton carbon ingestion, even when the phytoplankton biomass is greater than that of both heterotroph groups. Suzuki *et al.* (Suzuki *et al.*, 1999) similarly demonstrated that *Paracalanus* spp. feeds on HDF and ciliates, with sHDF providing more than twice as much food as ciliates. On the other hand in studies by Yang *et al.* (Yang *et al.*, 2009), medium size (1−2 mm) copepods (e.g. *Calanus sinicus*) exhibited strong selectivity for heterotrophic protozoa in the 20- to 50-μm and 50- to 100-μm size categories, while auto- and heterotrophic protozoa <10 μm were not grazed. Ciliates were most numerous and the biomass dominants in April 2008, and the abundance ratio of the ciliates to sHDF was greater in April and February, when the water temperature was lower than on the July cruises (Figs 4 and 5). The large and numerically dominant calanoid copepod, *Calanus sinicus*, is more abundant in the cold season in the ECS (Kang, 2008), implying that it may take advantage of the abundant occurrence of ciliates in April. Huo *et al.* (Huo *et al.*, 2008) reported that *Calanus sinicus* ingested ciliates preferentially over other components of the microplankton in the Yellow Sea. The numerical importance of sHDF suggests their importance not only as major grazers of phytoplankton, but also as a major link to the

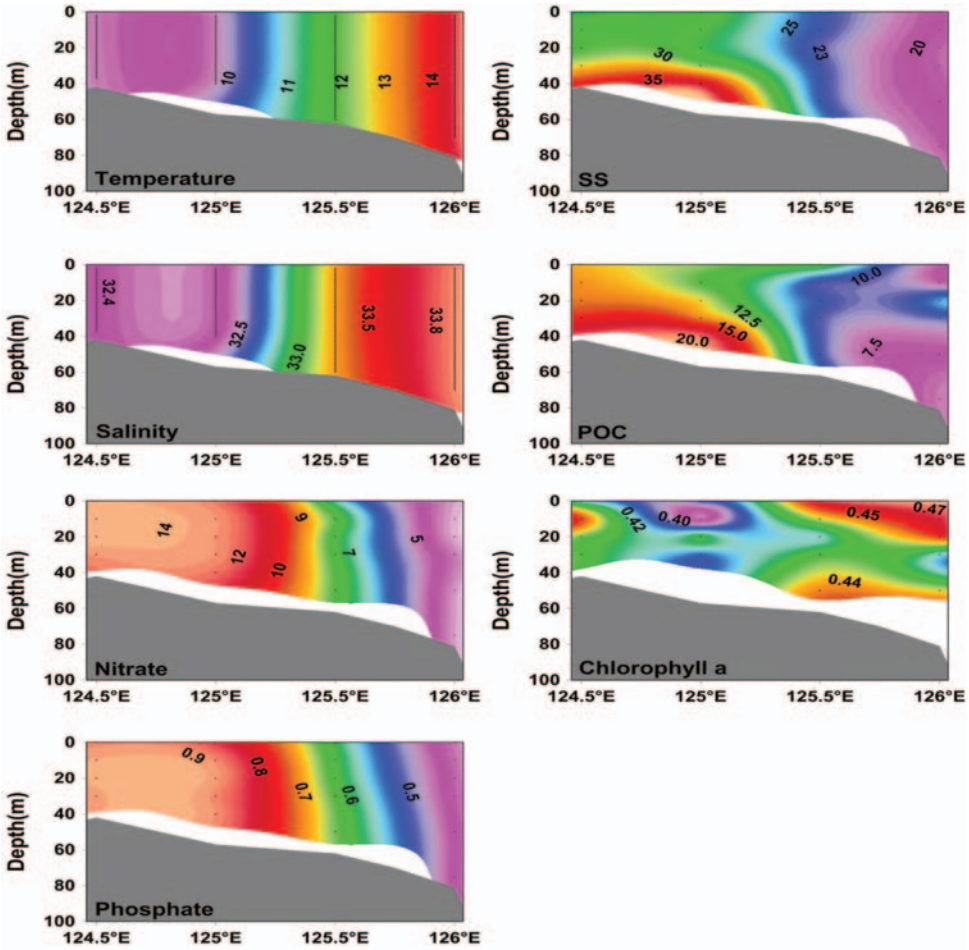

Fig. 9. Same as in Fig. 6, but for February 2009.

mesozooplankton food chain in the region. The role of sHDF in transferring phytoplankton C to larger predators in the region deserves further study.

Although the numerical importance of sHDF suggests their importance as grazers of phytoplankton in the study area, other prey such as heterotrophic bacteria could be an important factor in determining the spatial and seasonal variation of protist distributions. HNF mostly consume bacteria (Sanders *et al.*, 1992), and Chiang *et al.* (Chiang *et al.*, 2003) reported that the peak of ciliate abundance observed in summer was driven not only by phytoplankton but also by increased availability of bacteria in the ECS. Although HDFs are known to be capable of feeding on prey items of their own size because HDF have complex feeding behaviors and mechanisms that enable them to feed on larger prey (Hansen, 1991; Jakobsen and Hansen, 1997), they are also major grazers of marine heterotrophic bacteria (Jeong *et al.*, 2008). The bacterial biomass was measured at four stations during our October 2007 cruise (Lee *et al.*, 2012a) averaging 4.0 ± 1.3 mg C m^{-2}, assuming 10 fg $cell^{-1}$(Kawasaki *et al.*, 2011), which accounted only for 8% of the total microplankton biomass. The strong correlation between protists and POC, but not between protists and Chl *a* in July 2007, together with the seeming lack of a spatial relationship of protists with Chl *a* in February 2009 suggests that protists may often depend primarily on prey (e.g. heterotrophic bacteria) other than phytoplankton or that sHDF were grazing on other sHDF or other microzooplankton.

The proportion of heterotrophic protists relative to total protists (autotrophs + heterotrophs) declined with the increasing autotrophic biomass (Fig. 10). This may be due to mesozooplankton grazing pressure on heterotrophic protists. Such a relationship can arise from enhanced phytoplankton growth under reduced grazing pressure by heterotrophicprotists (Nejstgaard *et al.*, 1997; Schnetzer and Caron, 2005; Jang *et al.*, 2010; Wu *et al.*, 2010), or the presence of an alternative food source in the waters of low

phytoplankton biomass. Alternatively, such an inverse relationship can be induced by high turnover rates of phytoplankton resulting from tight coupling between phytoplankton and heterotrophs, which is made most likely by more efficient grazing processes (Wassmann, 1990; Baines *et al.*, 1994; Banse, 1995; Gasol *et al.*, 1997; Chen and Liu, 2010). However, that possibility seems unlikely, as indicated by grazing rates generally increasing with the phytoplankton biomass (Fig. 11), although an increase in the phytoplankton biomass generally involves an increase in large phytoplankton (>20 μm), which would make large phytoplankton less prone to ingestion by protists. Lastly, the negative tendency may have occurred purely by physical processes (e.g. horizontal transport related to the coastal water plume).

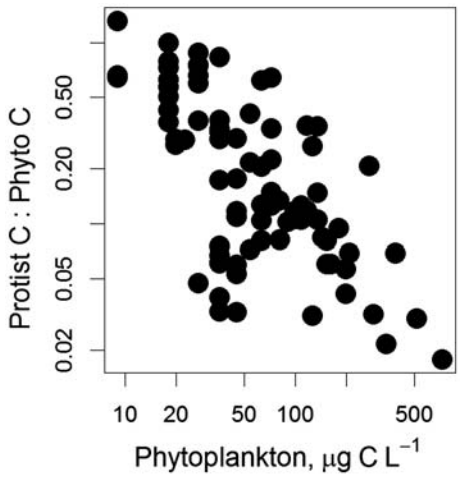

Fig. 10. The ratio of heterotrophic protist biomass to phytoplankton biomass. Phytoplankton biomass was estimated using a C:Chl *a* ratio of 90 in the region.

We employed dilution experiments to estimate protist grazing rates. Both specific growth rates and grazing rates estimated from the dilution experiments were in good agreement with reported values in the literature. Specific phytoplankton growth rate results (*k*: 0.25 day^{-1} to 1.87 day^{-1} with an overall mean of 0.72 day^{-1}) averaged 38% higher than the values of 0.29–0.86 day^{-1}, with a mean of 0.54 day^{-1} for the phytoplankton growth rate as determined by dilution experiments performed across a broader area of the Eastern China Sea during a more limited period (Zhang *et al.*, 2006). The difference could be due in part to the nutrient added to the incubation bottles in our studies to control for nutrient regeneration due to protist grazing. In July 2006, the average growth rate of phytoplankton without nutrient addition. μ_o was statistically indistinguishable from that with nutrients. μ (paired *t*-test, $P > 0.05$). However, we cannot rule out the possibility of overestimation in the phytoplankton growth rate during July and October 2007 when we observed a low nutrient concentration (Table II). Therefore, it is more likely that the difference was from the different temporal scales over which the Zhang *et al.* dilution experiments were performed in the ECS, rather than from the lack of stimulation of phytoplankton growth by nutrient amendments. Assuming the summer primary production and the Chl *a* concentration of ~700 mg C m^{-2} day^{-1} (unpublished data) and 0.7 mg m^{-3} (Table I), respectively,

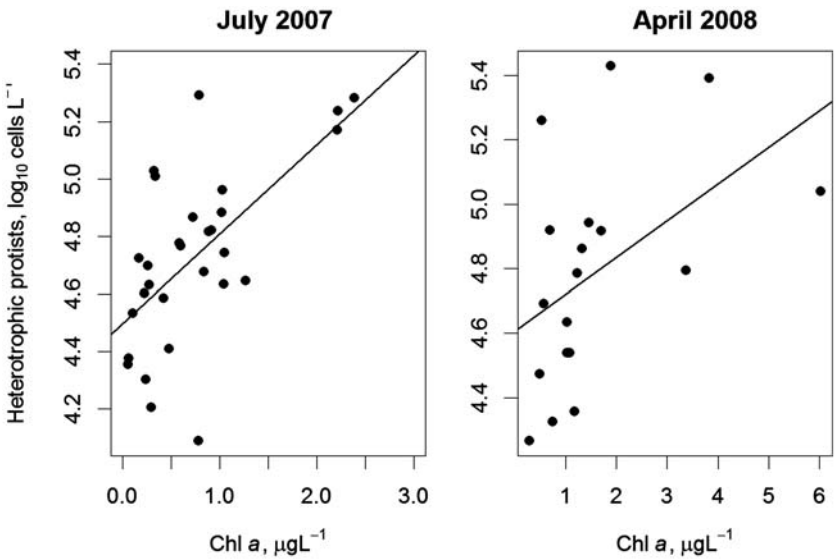

Fig. 11. Chl *a* versus total heterotrophic protist abundance in which the effect of salinity was treated as constant. The regression results for July 2007: slope = 0.31, R^2= 0.38, P< 0.001; and slope = 0.11, R^2= 0.17, P= 0.04 for April 2008.

Table II: Summary of grazing experiments for each station of each cruise in the northern East China Sea

Date	Station	PO_4 (μM)	NO_3 (μM)	Chl *a* (μg L^{-1})	Pico-Chl *a* (%)	μ_o (day^{-1})	μ (day^{-1})	*k* (day^{-1})	*k′* (day^{-1})	*g* (day^{-1})	*R*	Chl-SS (%)	Chl-P (%)
06-Jul	2–1	0.03	0.96	1.99	72.5	0.37	0.41	1.34	1.4	1.04	0.9	64.7	87.6
06-Jul	6–1	0.1	1.31	1.53	85	0.01	0.01	0.35	0.3	0.31	0.92	26.7	90.3
06-Jul	1–9	0.06	0.35	0.74	—	0.25	0.29	0.58	0.5	0.3	0.81	25.9	59.1
06-Jul	2–9	0.06	0.77	3.16	45.1	0.54	0.54	0.88	0.9	0.34	0.73	28.8	49.3
06-Jul	4–6	0.09	0.89	0.8	84.1	0.22	0.21	0.53	0.6	0.37	0.87	30.9	75.1
06-Jul	5–3	0.08	1.02	0.65	—	0.22	0.23	0.46	0.5	0.26	0.85	22.9	62
07-Jul	B1	0.17	0.42	1.56	14.1			1.87		0.73	0.76	51.8	61.3
07-Jul	B2	0.03	0.58	1.18	—			1.52		1.09	0.93	66.4	84.9
07-Jul	B5	0.01	0.57	0.29	65.4			0.41		0.33	0.88	28.1	83.5
07-Jul	B9	0.01	0.53	0.28	67.7			0.42		0.37	0.95	30.9	89.4
07-Jul	D1	0.25	0.17	3.6	7			0.68		0.36	0.99	30.2	62.2
07-Jul	D3	0.18	0.25	0.7	42.5			1.7		0.65	0.83	47.8	58.5
07-Jul	D5	0.1	0.1	0.16	77.8			0.7		0.59	0.95	44.6	88.5
07-Jul	D9	0.05	0.1	0.1	66.7			0.62		0.53	0.95	41.1	89
07-Jul	G6	0.07	0.57	0.14	—			0.81		0.71	0.93	50.8	91.5
07-Oct		0.13	0.16	0.49	83.1			0.7		0.56	0.97	42.9	85.2
07-Oct		0.08	0.11	0.84	73.1			1.28		0.85	0.89	57.3	79.3
09-Feb	B2	0.9	13.84	0.4	24.1			0.3		0.16	0.83	14.8	57
09-Feb	B4	0.86	12.59	0.32	12.8			0.41		0.29	0.85	25.2	65
09-Feb	B7	0.43	4.22	0.4	39.1			0.25		0.11	0.83	10.4	47
09-Feb	D3	0.57	8.05	0.48	7.6			0.33		0.15	0.88	13.9	51.5
09-Feb	D5	0.43	5.04	0.4	11.4			0.6		0.31	0.86	26.7	59
09-Feb	D6	0.41	5	0.35	21.2			0.44		0.17	0.83	15.6	44.9
09-Feb	D8	0.44	5.42	0.3	16.9			0.9		0.19	0.8	17.3	30.1

μ_o, net growth in whole water without nutrient added; μ, net growth rate with nutrient; *k*, instantaneous growth rate; *k′*, instantaneous growth rate estimated without nutrient ($\mu_o + g$); *g*, grazing rate; Chl-SS, percent of Chl-*a* standing stock grazed daily by microzooplankton with nutrient added; Chl-P, percent of Chl-*a* production grazed daily by microzooplankton. See the "METHOD" section.

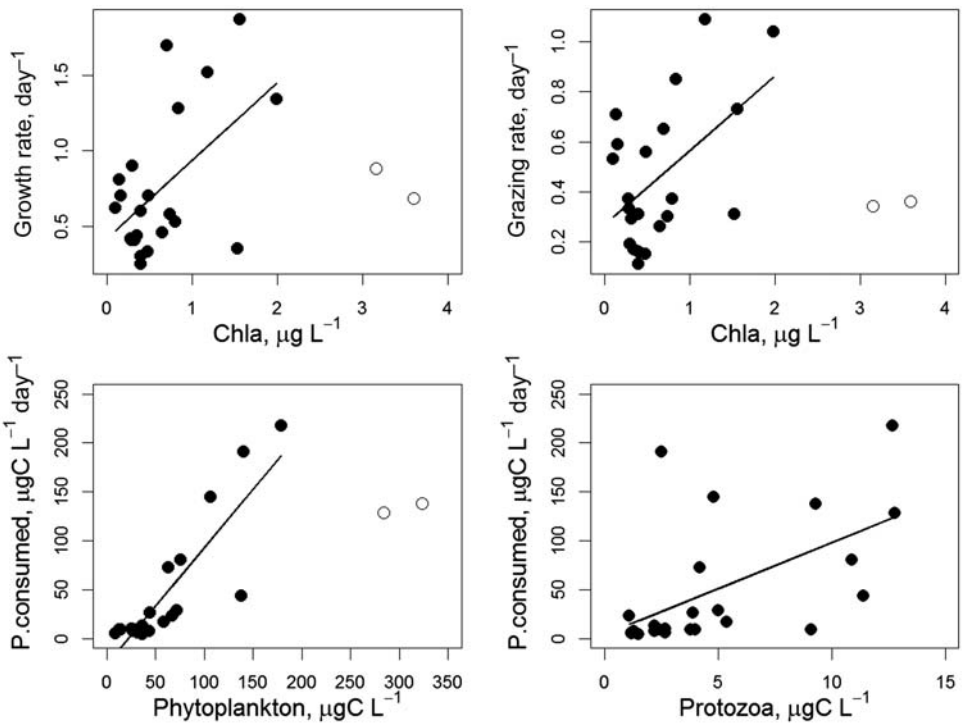

Fig. 12. Chl *a* versus apparent phytoplankton growth rate (day^{-1}) (slope = 0.51, $R^2 = 0.27$, $P < 0.01$), Chl *a* versus protist grazing rate (day^{-1}) (slope = 0.30, $R^2 = 0.24$, $P = 0.01$), phytoplankton biomass versus phytoplankton carbon consumed (P. consumed, μgC L^{-1} day^{-1}) (slope = 1.19, $R^2 = 0.76$, $P < 0.01$), and protist biomass versus phytoplankton carbon consumed (slope = 9.4, $R^2 = 0.36$, $P < 0.01$), with the slope representing the daily ration of carbon (%). The data where chlorophyll a was high (>3 μg L^{-1}) was not included for the regression analyses.

a euphotic depth from the surface to between 20 and 30 m and a C:Chl *a* ratio of 90, this would yield phytoplankton growth of 0.39–0.58 day^{-1}. Chang *et al.* (Chang *et al.*, 2003) reported phytoplankton growth rates of 0.2–0.7 day^{-1} based on the C:Chl *a* ratio and the primary productivity estimated with ^{14}C in the waters of the ECS under the influence of the Kuroshio Current. The specific growth rates estimated from our dilution experiments are in the middle of that range, not substantially different from those determined using the ^{14}C incubation method. Our observed grazing rates (*g*: 0.11, 1.09 day^{-1}, average 0.46 day^{-1}) are similar to the range of Zhang *et al.* of (Zhang *et al.*, 2006) 0.37–0.83 day^{-1}, with a mean grazing rate of 0.63 day^{-1}. Our rates are also comparable with the reported values of *k* (0–3.5 day^{-1}) and *g* (0–2.5 day^{-1}) from previous studies in other waters (Calbet and Landry, 2004).

Although dilution experiments have become a standard method for estimating protist community grazing, and are widely used across trophically different marine environments, the reliability of dilution experiments has been questioned. Non-linearities are introduced when initial, undiluted food concentrations are above the levels at which grazing responses saturate. In this situation, the 3-point method (Gallegos, 1989) can be used, in which the grazing rate estimation relies only on the apparent growth in the two most dilute treatments, thus avoiding biases due to saturated grazing at higher phytoplankton concentrations. Visual examination of the relationship between the apparent growth rate and the dilution series showed that grazing saturation might have occurred in our bottles at small dilutions (Fig. 13). For instance, the apparent growth rate for bottles with no dilution was only slightly different from those estimated for bottles of 25% dilution; however, the effects of the grazing saturation seemed limited as indicated by the very high explanatory power of the regression lines fitted to our dilution series (Table II). Analysis of covariance also showed no significant difference between the slopes (i.e. grazing rates, $P > 0.09$ for all analyses) or intercepts (i.e. specific growth rates, $P > 0.22$ for all analyses) of regressions with and without inclusion of the apparent growth rate in whole water. Protist grazing saturation in bottles with no dilution may also be indicated by the estimated daily ration of protists, which varied widely among stations, but showed a slope of 940% of their body carbon each day (Fig. 12). Burkill *et al.* (Burkill *et al.*, 1993) reported that the daily ration of heterotrophic protists was in the range of 100–800%. The oligotrich ciliate, *Lohmanniellaspiralis*, consumes the equivalent of 140–580% of its body volume daily (Rassoulzadegan, 1982), while phagotrophic micro-flagellates ingested about five times their body carbon daily (Landry *et al.*, 1984). Sherr *et al.* (Sherr *et al.*, 1991) reported that ciliates and micro-flagellates only consumed the equivalent of 6–350% of their body volume daily when fluorescently labeled phytoflagellates were provided as food in laboratory experiments. In light of the fact that the ingestion rates of micro-flagellates estimated by Landry *et al.* (Landry *et al.*, 1984) were considered to be food limited, our ration estimate (940% day^{-1}), somewhat higher than reported values, suggests that heterotrophic protists were not likely to be food limited in the present study. However, the ration could also be significantly lower than estimated if we take into account that fixation can result in significant loss of cells (Stoecker *et al.*, 1994; Menden-Deuer *et al.*, 2001). Cell shrinkage by preservation could also lead to underestimation of the total protist biomass. Additional sources of error include the potential presence of mixotrophs and copepod nauplii that also consumed phytoplankton.

We calculated the average individual grazing rate (μL $ciliate^{-1}$ h^{-1}) based on the grazing rate and protist abundance. Their potential clearance rate ranged from 0.71 to 33.81 μL $protist^{-1}$ h^{-1}, with an average of 4.0 μL $protist^{-1}$ h^{-1}. Considering lHDF only, the estimated potential clearance rates ranged from 0.08 to 1.34 μL $protist^{-1}$ h^{-1}, with a mean of 0.60 μL $protist^{-1}$ h^{-1}. With only sHDF considered, the rates were between 0.09 and 1.93 μL $protist^{-1}$ h^{-1}, averaging 0.77 μL $protist^{-1}$ h^{-1}, close to the reported values for the clearance rates for protists of various equivalent spherical diameters (Sherr and Sherr, 2002; Dolan and McKeon, 2005). Our estimate of heterotrophic protist consumption, i.e. an overall mean of 69% of daily Chl *a* production, is in good agreement with the average of 60% reported in coastal waters and also with 70% for the oceanic waters (Landry and Calbet, 2004). Heterotrophic protists are the major consumers of primary production in the northern ECS, and at least in surface waters during our study periods, grazing by mesozooplankton and direct sedimentation were of lesser importance for the fate of phytoplankton. The grazing impact on the phytoplankton biomass by *P. parvus* and *C. sinicus* is low, averaging 8% (range 0.2–29.8%) of the phytoplankton standing stock in the Yellow Sea adjacent to the ECS (Lee *et al.*, 2012b). Such low daily carbon rations (<20% for both copepods) provided by phytoplankton indicate that copepods graze on non-phytoplankton foods. A similar conclusion about the role of mesozooplankton in the grazing of phytoplankton and microzooplankton was drawn by Wu *et al.* (Wu *et al.*, 2010), who showed that meso-zooplankton in the Eastern China Sea preferentially ingest heterotrophic protists over phytoplankton, even during high

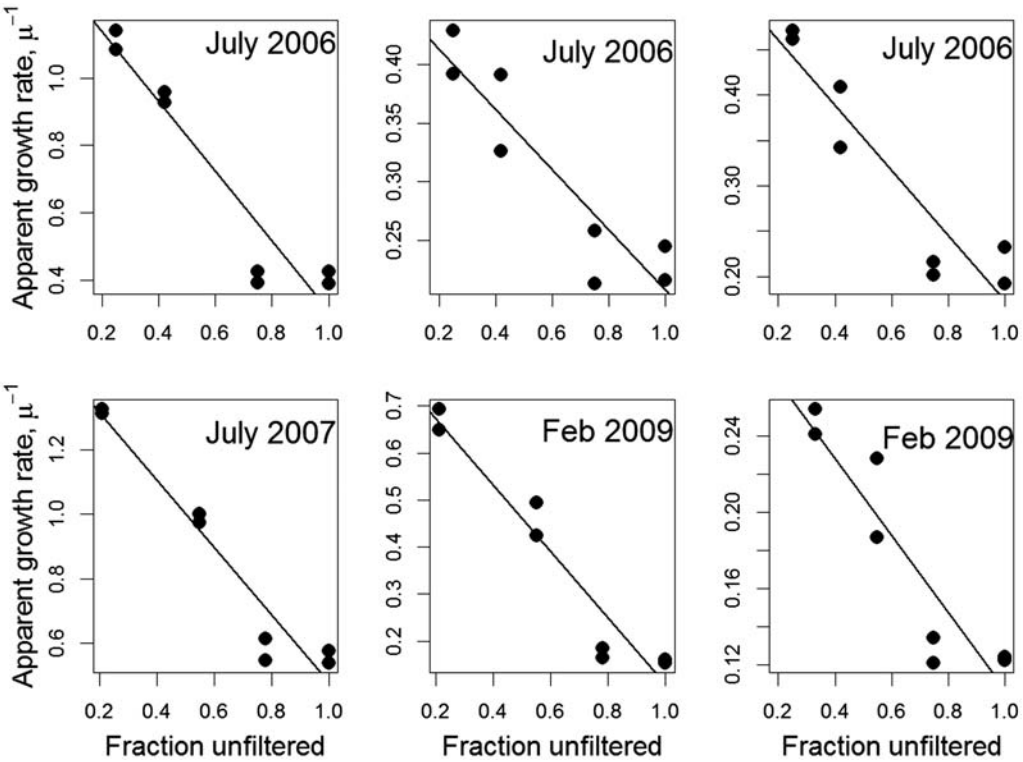

Fig. 13. Apparent growth rate as a function of the fraction of unfiltered water for grazing experiments in which saturated grazing was apparent.

phytoplankton abundance. Our results demonstrate that most primary production passes through at least one heterotrophic protist trophic level before reaching larger consumers. Therefore, the heterotrophic protist assemblage may greatly affect trophic dynamics by controlling the biomass of phytoplankton and they play a pivotal role in mediating the transfer of organic carbon to higher trophic levels in the northern ECS.

ACKNOWLEDGEMENTS

We are indebted to the captain and crew of the R/V *Eardo* who were most helpful in all shipboard operations. The authors would also like to thank three anonymous reviewers for their thoughtful suggestions that significantly improved the quality of the paper.

FUNDING

This work was supported by the KORDI project (PM 56600). E.J. Yang was also supported by the KOPRI project (PN 12030).

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