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# Grazing by microzooplankton and copepods on the microbial food web in spring in the southern Yellow Sea, China

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## Abstract

Assessment of microzooplankton and copepods grazing pressure on picoplankton is a key requirement for resolving the microbial food web efficiency. Although microzooplankton grazing on picoplankton has been extensively studied, the impact of microzooplankton on different groups of picoplankton, i.e., heterotrophic bacteria, *Synechococcus* and picoeukaryotes have rarely been compared. Furthermore, in the very few existing studies there is no consistent evidence of an enhancing or restraining effect of copepods on picoplankton. More studies are needed to improve our understanding of the influence of microzooplankton and copepod on picoplankton. Dilution incubations and copepod addition incubations were performed during a cruise to the southern Yellow Sea on May 16–29, 2007. The bulk grazing of microzooplankton and the calanoid copepod *Calanus sinicus* on phytoplankton, flagellates and picoplankton was estimated. Stations were divided into either eutrophic or oligotrophic according to the nutrient and biological parameters. Picoplankton comprised a large part of the diet of microzooplankton in the central oligotrophic area, while phytoplankton was the main food of microzooplankton in the coastal eutrophic area. In the central oligotrophic area, microzooplankton preferred grazing on *Synechococcus*. After copepod addition, ciliate abundance decreased while *Synechococcus* abundance increased (382%, 64% and 64% at three experimental stations, respectively), indicating strong grazing pressure of microzooplankton on *Synechococcus*. Our results suggest that *Synechococcus* might be an essential carbon source the planktonic food web in the oligotrophic waters of southern Yellow Sea.

**Keywords** Microzooplankton · Dilution incubations · Copepod addition incubations · Ciliate · Picoplankton · Yellow Sea

## Introduction

The planktonic microbial food web is composed of microzooplankton, phytoplankton, including flagellates, microphytoplankton, cyanobacteria and picoeukaryotes, heterotrophic prokaryotes (bacteria and archaea, hereafter referred to “bacteria”) and virioplankton (Azam et al. 1983; Ducklow 1983). There are complex trophic relationships between these microorganisms (Sherr and Sherr 2008). Planktonic ciliates are often the dominant

microzooplankton (Pierce and Turner 1992) and are well recognized as a key component of many plankton food web models (Baretta et al. 1995; Kishi et al. 2007). Microzooplankton can affect picoplankton either directly by grazing or indirectly by feeding on other picoplankton grazers, such as heterotrophic nanoflagellates. These grazing activities demonstrate that microzooplankton are critical trophic intermediates in carbon transfer along the trophic pathway and in remineralization processes (Sherr and Sherr 1994). Over the past 30 years, microzooplankton grazing on picoplankton, i.e., heterotrophic bacteria (HB), cyanobacteria *Synechococcus* (Syn) and photosynthetic picoeukaryotes (PicoE) has been extensively studied (e.g., Callieri et al. 2002; Chen and Liu 2010; Mukhanov et al. 2016; Reckermann and Veldhuis 1997; Šimek et al. 1995; Worden and Binder 2003). However, there are only a few studies that have compared the impact of microzooplankton on different groups of picoplankton. When compared

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with the fluorescently labelled food item method (Livanou et al. 2019), dilution incubations have been more commonly used to study the bulk grazing of microzooplankton on HB, Syn and PicoE (Anderson and Harvey 2019; Brown et al. 1999; Calbet et al. 2008; Kimmance et al. 2007; Morison et al. 2019; Sakka et al. 2000). However, only a few studies using dilution incubations have estimated and compared the grazing impact of microzooplankton on these groups. Copepods are the dominant mesozooplankton in most marine pelagic ecosystems (Verity and Paffenhofers 1996). Copepods cannot efficiently graze on the particles smaller than 5  $\mu\text{m}$  and prefer to graze on ciliates, which will consequently indirectly influence picoplankton and flagellates abundance (Irigoien et al. 2000; Stoecker and Capuzzo 1990; Yang et al. 2019). No consistent enhancing or restraining effect of copepod on picoplankton have been found in the very few existing studies (Böttjer et al. 2010; Burns and Schallenberg 1996; Liu et al. 2005a; Nakamura et al. 1997; Sommer and Sommer 2006; Wetz et al. 2011). Additional studies are therefore needed to improve our understanding of the influence of copepods on picoplankton.

The Yellow Sea (YS) is a temperate marginal sea with a mean depth of 44 m in the northwest Pacific Ocean. The central part of the Yellow Sea is oligotrophic (Zou et al. 2000) and contains a deep trough (maximum depth 87 m). There is a marked seasonal change in the vertical structure of the water column in the YS. Yellow Sea Cold Bottom Water (YSCBW) is formed in the central deep part (> 60 m) during summer due to water column stratification (Hur et al. 1999; Su and Weng 1994; Zhang et al. 2008). The dominant copepod species in YS is *Calanus sinicus*, which over-summer in the YSCBW (Li et al. 2004; Wang et al. 2003).

Most of the historic and current interest in the YS microbial ecosystem has focused on characterizing the dynamics and standing stocks of picoplankton, protozoa and mesozooplankton, as well as the factors regulating their variation (Li et al. 2006; Lin et al. 2013; Zhao et al. 2011, 2013). Dilution incubations have been used to study the microzooplankton grazing rates on bulk phytoplankton (Sun et al. 2013; Zhang et al. 2002). However, not much is known about the principal prey being consumed by microzooplankton in YS. Assessment of the microzooplankton and copepods grazing pressure on picoplankton is a key point to resolve the microbial food web functioning and carbon transfer efficiency. In this paper, we examined the contribution of different picoplankton groups to the microzooplankton diet, as well as the impact of the dominant copepod, *Calanus sinicus*, on picoplankton in the southern YS.

## Results

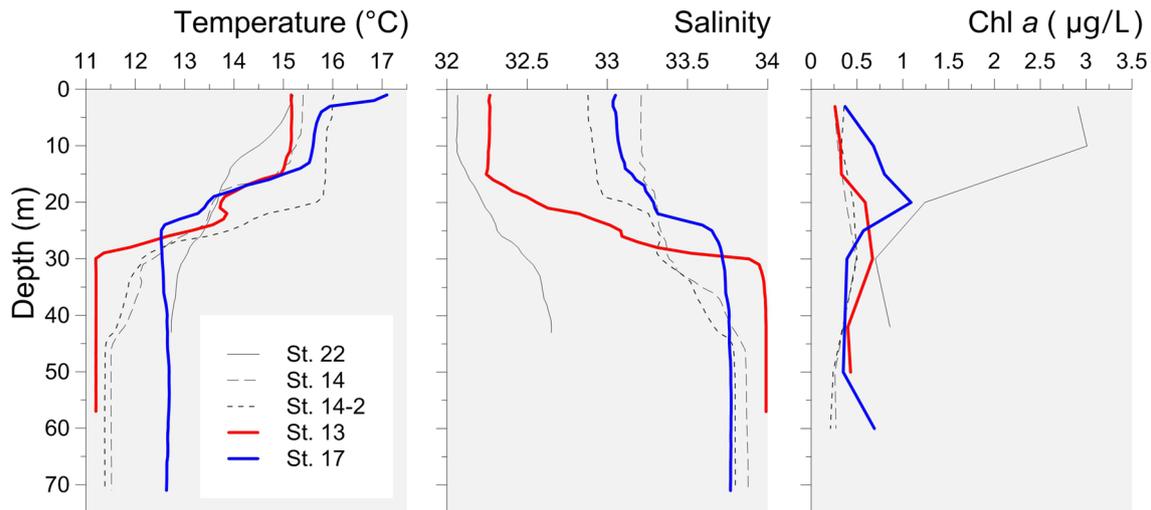
### Hydrographical conditions and microbial abundances

Temperature and salinity profiles (Fig. 1) indicate higher temperature and lower salinity in surface waters than in deep waters at all sampling stations. There was very small difference (< 2°C) between temperatures at the surface and at 10 m layer, which justified the use of surface water for temperature control in the incubation experiments.

Nutrient and microbial abundance data (Table 1) divided the stations into two categories. Station 22, with higher nutrients ( $\text{NO}_3^-$  and Silica) and Chl *a* concentrations, was considered a eutrophic station. All other stations (13, 14, 14-2 and 17), located at the central YS, were oligotrophic. In general, the eutrophic station had higher numbers of ciliates, HB, PicoE and a lower amount of Syn as compared to the oligotrophic stations (Table 1). At St. 22, ciliate abundance was 14,820 ind./L and was dominated by the tintinnid *Tintinnopsis beroidea*. At the oligotrophic stations the ciliate composition was characterized by long, conical shaped aloricate forms. The abundance of TF ranged from 1080 ind./ml at oligotrophic station St. 14, to 2287 ind./ml at eutrophic station St. 13. HF was more abundant than PF (Table 1). HB was the most abundant picoplankton at all stations. In eutrophic water, the HB and PicoE were about two- to threefold more abundant than those at oligotrophic stations. Syn abundance was much lower, with only 1/3–1/10 the abundance of that in oligotrophic waters.

### Microzooplankton grazing on phytoplankton, flagellates and picoplankton

Phytoplankton apparent growth rates estimated from Chl *a* in dilution incubations had good linear regressions ( $R^2 > 0.8$ ) with dilution factors (Fig. 2). At eutrophic St. 22, phytoplankton grew faster than the microzooplankton grazing ( $k = 1.22/\text{day}$ ,  $g = 0.62/\text{day}$ ), resulting in a  $P_p$  value of 0.66 (Table 2); about 2/3 of the phytoplankton production being grazed by microzooplankton. For flagellates, the  $k$  and  $g$  values of TF were 1.02 and 1.01/day, respectively (Fig. 2), indicating about 99% of TF potential production was being grazed by microzooplankton (Table 2,  $P_p = 0.99$  for TF at St. 22). There was no correlation between picoplankton abundance and dilution factor, meaning no microzooplankton grazing rate on picoplankton could be calculated at this station (Fig. 2). These results indicate that phytoplankton and TF were the main microzooplankton food items in eutrophic water of YS. Grazing pressure on standing stock per day ( $P_i$ ) was 46% of phytoplankton and 64% of TF. However,



**Fig. 1** Vertical profiles of temperature (°C), salinity and Chl *a* concentration (µg/L) at the experimenting stations

**Table 1** Initial physical, chemical and biological conditions of 10 m depth waters

Stations	St. 22	St. 14	St. 14-2	St. 13	St. 17
Temperature (°C)	14.49	15.25	15.86	15.15	15.60
Salinity	32.06	33.21	32.91	32.26	33.07
NO <sub>3</sub> <sup>-</sup> (µmol/L)	7.55	0.25	1.00	1.35	0.79
PO <sub>4</sub> <sup>3-</sup> (µmol/L)	0.27	0.26	0.24	0.11	0.16
Silica (µmol/L)	22.13	4.66	3.80	3.20	13.43
Chl <i>a</i> (µg/L)	2.02	0.26	0.33	0.23	0.34
Ciliate (ind./L)	14,820	1203	1446	1713	1309
TF (ind./ml)	1734	1080	1313	2287	1358
PF (ind./ml)	808	378	702	795	430
HF (ind./ml)	926	702	910	1492	928
Syn (cells/ml)	24,884	283,767	112,075	82,417	65,389
PicoE (cells/ml)	28,345	7036	8391	4915	12,899
HB (cells/ml)	2,541,448	1,024,964	1,092,665	658,617	965,348

TF total flagellates, PF pigmented flagellates, HF heterotrophic flagellates, Syn *Synechococcus*, PicoE picoeukaryotes, HB heterotrophic bacteria

with a much higher biomass being grazed, phytoplankton could account for up to 96.54% of the food intake of ciliates and only 3.46% of food resources was contributed by TF in eutrophic water of YS.

At the oligotrophic stations St. 14 and St. 14-2, almost all the phytoplankton production (97–117%) was grazed

per day (Table 2). For TF, the growth rates (0.64–0.68/day) were higher than grazing (0.42–0.44/day). Only 72–73% of the TF potential production was grazed by microzooplankton (Table 2). Microzooplankton grazing rate on Syn (1.17–4.30/day) was much higher than on HB (0.35–0.44/day) and PicoE (0.35–0.50/day). Picoplankton played an important role in food intake by microzooplankton. This grazed biomass was largely contributed by Syn (44.4–72.4%). Furthermore, 4.4–9.2% and 23.2–46.5% of the food was PicoE and HB, respectively.

### Influence of *C. sinicus* on phytoplankton, flagellate and picoplankton

At the end of the *C. sinicus* addition incubation, ciliate abundances decreased by 35%, 30% and 54% compared to the control bottles at St. 13, St. 14 and St. 17, respectively (Fig. 3). Chl *a* concentration increased by 63%, 13% and 23%, respectively. HF abundance increased by 22% at St. 13, decreased (7% and 3%) at St. 14 and St. 17, respectively. With respect to picoplankton, Syn abundances showed a consistent increase (382%, 64% and 64% at St. 13, St. 14 and St. 17, respectively) in the copepod containing bottles. However, PicoE and HB abundances showed systematic change (in the range of ± 15%, except for PicoE which increased by 74% at St. 17, Fig. 3).

### Discussion

The question of which are the principal preys being consumed by microzooplankton was first proposed by Rivkin et al. (1999). Microzooplankton plays a significant role in structuring the microbial food web through its ability of selecting food and displaying rapid responses to changes in food availability. The determination of the relative

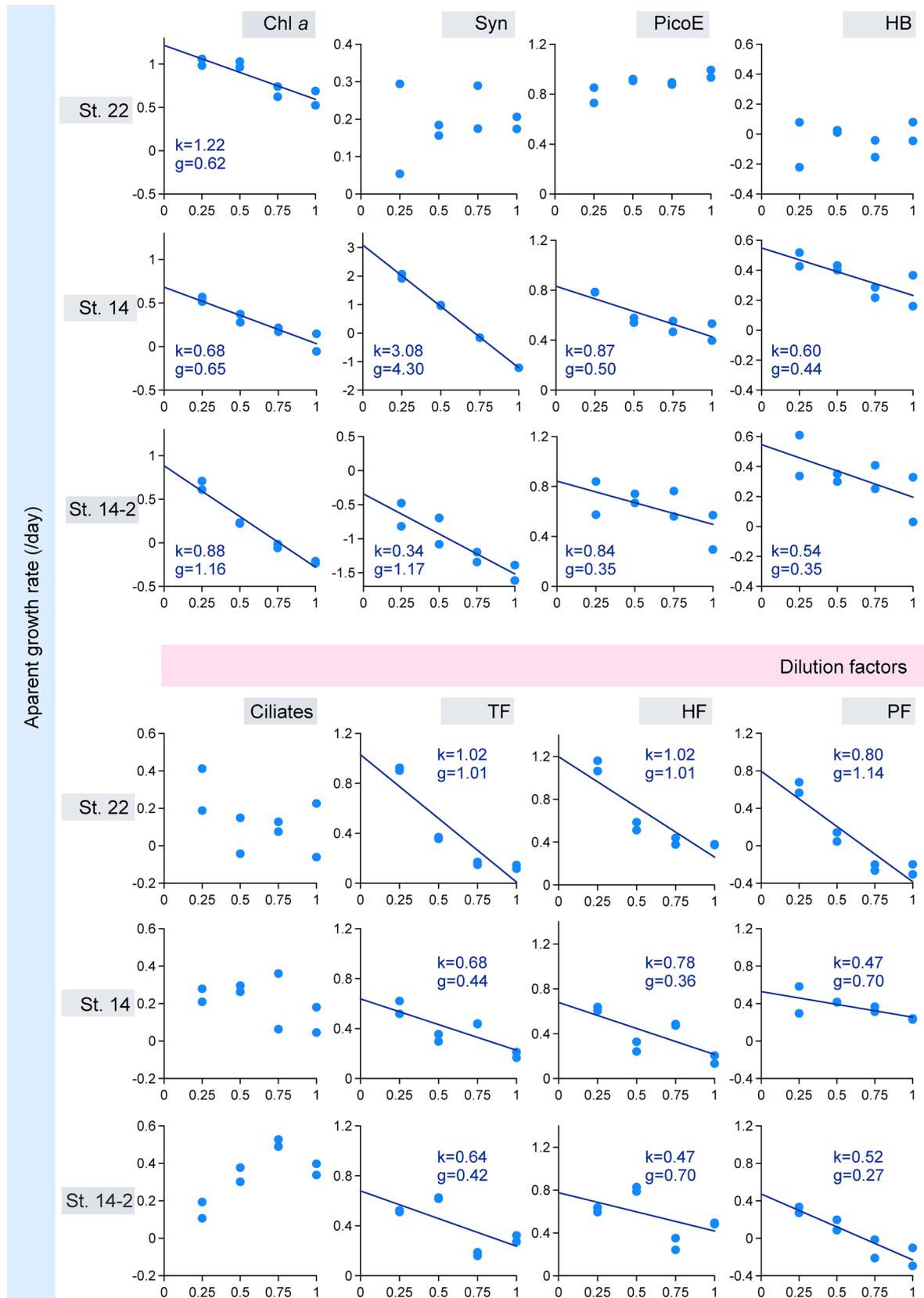


Fig. 2 Regressions between apparent growth rates (/day) and the dilution factors in the dilution incubations

**Table 2** Results of dilution incubations

Stations	Food item	Concentration/ Abundance	Biomass	<i>k</i>	<i>g</i>	<i>R</i> <sup>2</sup>	<i>P</i> <sub>i</sub>	<i>P</i> <sub>p</sub>	Biomass being grazed	Percentage
St. 22	Phytoplankton (Chl <i>a</i> )	2.02	202.0	1.22	0.62	0.81	0.46	0.66	92.9	–
	TF	1734	1.6	1.02	1.01	0.82	0.64	0.99	1.0	–
	PF	808	0.7	0.80	1.18	0.85	0.69	1.26	0.5	–
	HF	926	0.9	1.20	0.94	0.77	0.61	0.87	0.5	–
	Syn	24,884	2.0	–	–	–	–	–	–	–
	PicoE	28,345	15.0	–	–	–	–	–	–	–
	HB	2,541,448	50.8	–	–	–	–	–	–	–
St. 14	Phytoplankton (Chl <i>a</i> )	0.26	26.0	0.68	0.65	0.89	0.48	0.97	12.5	–
	TF	1080	1.0	0.68	0.44	0.49	0.36	0.72	0.4	–
	PF	378	0.3	0.47	0.70	0.85	0.50	1.34	0.2	–
	HF	702	0.6	0.78	0.36	0.28	0.30	0.56	0.2	–
	Syn	283,767	23.3	3.08	4.30	0.99	0.99	1.03	23.1	72.4%
	PicoE	7036	3.7	0.87	0.50	0.88	0.39	0.68	1.4	4.4%
	HB	1,024,964	20.5	0.60	0.44	0.89	0.36	0.79	7.4	23.2%
St. 14-2	Phytoplankton (Chl <i>a</i> )	0.33	33.0	0.88	1.16	0.95	0.69	1.17	22.8	–
	TF	1313	1.2	0.64	0.42	0.64	0.34	0.73	0.4	–
	PF	702	0.6	0.52	0.27	0.50	0.24	0.58	0.2	–
	HF	910	0.8	0.67	0.46	0.54	0.37	0.76	0.3	–
	Syn	112,075	9.2	–0.34	1.17	0.83	0.69	–	6.3	44.4%
	PicoE	8391	4.4	0.84	0.35	0.38	0.30	0.52	1.3	9.2%
	HB	1,092,665	21.9	0.54	0.35	0.42	0.30	0.71	6.6	46.5%

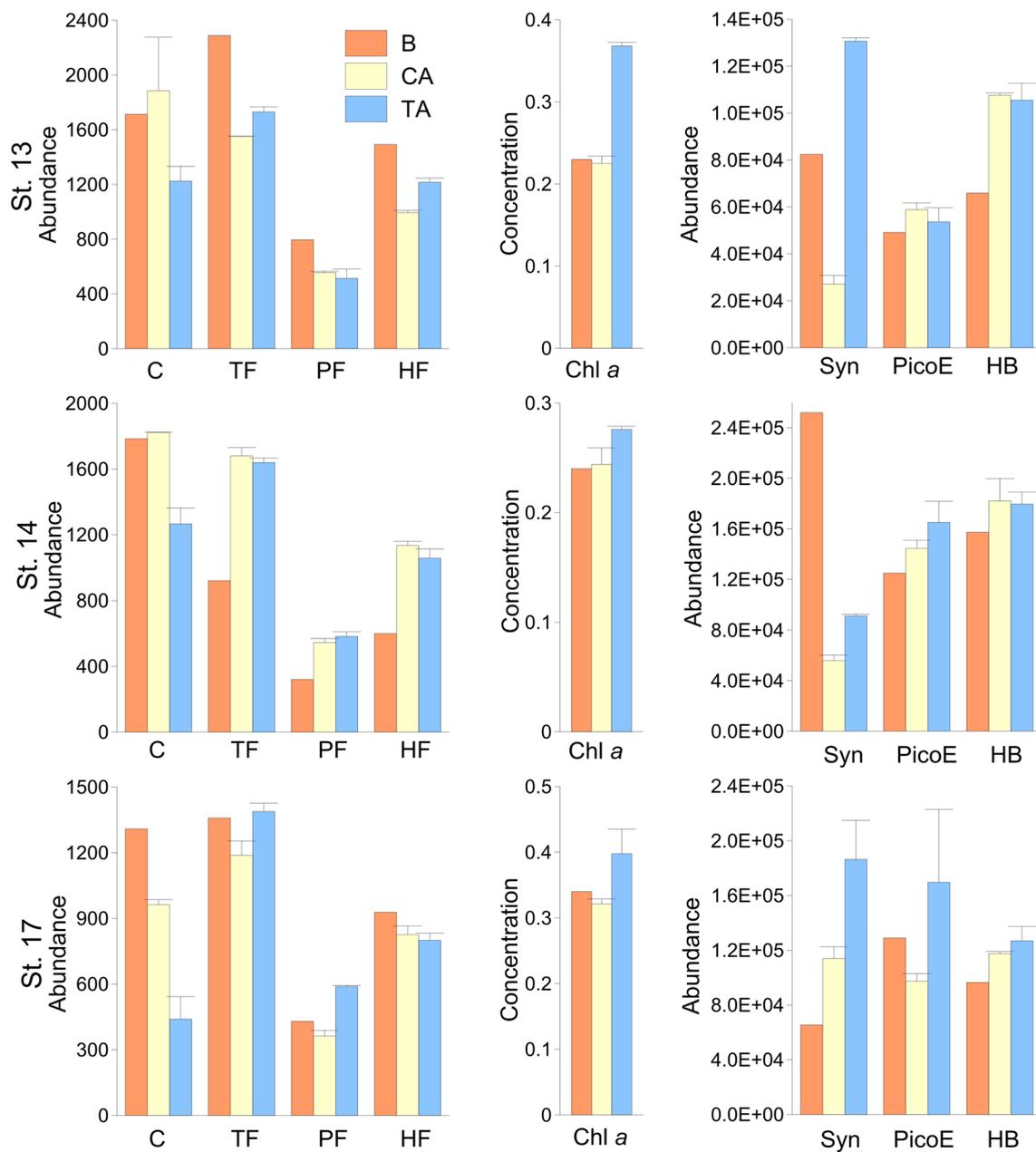
Chl *a* µg/L, TF total flagellates, PF pigmented flagellates, HF heterotrophic flagellates, abundance: ind./ml, Biomass: µgC/L; Syn *Synechococcus*, PicoE Picoeukaryotes, HB Heterotrophic bacteria, abundance: cells/ml, Biomass: µgC/L; *k* potential growth rates, /day; *g* grazing rate by grazers, /day; *P*<sub>i</sub> grazing pressure on standing stock per day, *P*<sub>p</sub> grazing pressure on primary production per day

grazing rates of microzooplankton is critical for understanding the patterns of carbon flow through the pelagic food web. Dilution incubation has been used to estimate microzooplankton grazing on HB, Syn and PicoE in previous studies (see Table 3), although most of these studies only focused on particular parts of the picoplankton. The growth and grazing rates obtained in this study were in the range of those previously found in the literature, however, the growth and grazing rate results of Syn presented here are by one of the highest values.

The interactions of microzooplankton grazers with their prey can be summarized in three situations (Umani and Beran 2003; Zoccarato et al. 2016): (a) strong reduction in microphytoplankton and nanoplankton, with no detectable grazing on picoplankton; (b) partial reduction in microphytoplankton and nanoplankton biomass, with partial grazing impact on picoplankton and (c) microzooplankton directly feed on picoplankton. In the research presented here, the grazing impact of microzooplankton on picoplankton was different under the different trophic environments identified during the cruise. At the YS eutrophic station, microzooplankton chose microphytoplankton as their main food source, i.e., situation (a). At the YS oligotrophic stations, microzooplankton could prey on microphytoplankton,

nanoplankton and picoplankton at the same time, i.e., situation (b), but sometimes, the importance of picoplankton could exceed phytoplankton, as reflected by the biomass being grazed (Table 2). The impact of the trophic environment on microzooplankton grazing was also detected in the Mediterranean Sea (Zoccarato et al. 2016). There, in oligotrophic and mesotrophic conditions, microzooplankton mostly preyed on heterotrophic prokaryotes (56.7% and 60.6%, respectively), which was different from the data presented here. In an analysis of the global impact of microplanktonic grazers on marine phytoplankton, microzooplankton consumption was found to be the main source of phytoplankton mortality, accounting for up to 67% of the daily phytoplankton growth for the full dataset. Under eutrophicated conditions, the potential production of microphytoplankton exceeded the ingestion rate, while under meso- and eutrophic conditions, the ingestion rate on autotrophic prokaryotes exceeded the potential production (Calbet and Landry 2004).

Among the picoplankton groups, the direct impact of microzooplankton was most pronounced on Syn at oligotrophic stations, accounting for about 44.4–72.4% of total biomass being grazed. Syn might thus be the fundamental component of the carbon budget in the oligotrophic water



**Fig. 3** Variation of Chl *a* concentration and abundances of ciliates, flagellates and picoplankton of the copepod addition experiments. *Chl a* Chlorophyll *a*,  $\mu\text{g/L}$ , *C* ciliates, ind./L, *TF* total flagellates, ind./ml, *PF* pigmented flagellates, ind./ml, *HF* heterotrophic flagellates,

ind./ml, *Syn* *Synechococcus*, cell/ml, *PicoE* picoeukaryotes,  $\times 10$  cell/ml, *HB* heterotrophic bacteria,  $\times 10^{-1}$  cell/ml. *B* before incubations, *CA* control bottle after incubations, *TA* copepod addition bottles after incubations

in YS and its variation could affect the carrying capacity of the pelagic food webs.

Data presented here show that microzooplankton did not graze on picoplankton in the eutrophic coastal station and preferred to graze on cyanobacteria in the deep oligotrophic stations. This is the only study to measure microzooplankton grazing rates in all groups of picoplankton. Sakka et al. (2000), using the dilution method, found that grazing rates for HB and PicoE were similar to those reported herein, but

the grazing rate for Syn was much lower (Table 3). In a study of grazing rate on HB and Syn in northern Red Sea, microzooplankton were found to prefer grazing on HB (Sommer et al. 2002). In a study examining grazing rates on PicoE and Syn in Southern California Bight, microzooplankton were found to prefer grazing on PicoE (Worden and Binder 2003). Therefore, for the first time, data presented in this research demonstrate that microzooplankton have a greater preference for Syn than other picoplankton groups.

**Table 3** Growth rate (*k*) and grazing rate (*g*) of marine picoplankton in various oceanic regions obtained in dilution incubation

Region	Incubation time (h)	HB		Syn		Pico E		References
		<i>k</i> /day	<i>g</i> /day	<i>k</i> /day	<i>g</i> /day	<i>k</i> /day	<i>g</i> /day	
Kaneohe Bay, Hawaii	24	1.2-1.9	0.5-1.1	1.98	0.14	–	–	Landry et al. (1984)
Celtic Sea and North Sea	>20-30	0.48-3.12	0.48–5.52	–	–	–	–	Geider (1989)
Northeast Subarctic Pacific	48	0.16-0.57	0.32–0.43	0.12–0.73	0.19–0.42	–	–	Rivkin et al. (1999)
Takapoto Atoll, French Polynesia	24	~0.90	0.25	0.50	0.30	0.80	0.40	Sakka et al. (2000)
The northern Red Sea	36	0.61-0.72	0.73-0.89	–	<0.19	–	–	Sommer et al. (2002)
Gulf of Aquaba		0.86–1.30	0.75–1.06					
Mississippi River plume	24	0.8	0.5	–	0.2–3.0	–	–	Jochem (2003)
Western Arabian Sea	–	–	–	0.18–0.90	0.06–0.73	0.31–0.92	0.18–1.78	Reckermann and Veldhuis (1997)
Northwestern Sargasso Sea	–	–	–	0.54–0.87	0.20–0.33	–	–	Lessard and Murrell (1998)
Subtropical northeast Atlantic (oligotrophic region)	–	–	–	0.44 (average)	0.33	–	–	Quevedo and Anadón (2001)
Sargasso Sea and California Current	–	–	–	0.37–0.67	0.13–0.51	–	–	Worden and Binder (2003)
Southern California Bight	–	–	–	0.52–0.86	0.14–0.39	0.71–1.29	0.17–1.09	Worden et al. (2004)
Western English Channel	–	–	–	0.13–0.50	0.22–0.67	0.34–1.14	0.01–0.81	Kimman et al. (2007)
Indian Ocean west off Australia	–	–	–	–0.12 to 1.34	0–0.45	–0.03 to 0.24	–0.06 to 0.22	Paterson et al. (2007)
Uwa Sea, Japan	–	–	–	0–1.39	0–1.54	–0.60 to 0.92	0.45–0.94	Hirose et al. (2008)
North part of South China Sea	–	–	–	–0.04 to 2.39	0.25–2.44	0.84–2.34	0.37–2.18	Chen and Liu (2010)
Mesotrophic region of Yellow Sea	24	0.54-0.60	0.35–0.44	0.34–3.08	1.17–4.30	0.87–0.87	0.35–0.50	This research

– no information, *HB* Heterotrophic bacteria, *PicoE* Picoeukaryotes, *Syn* *Synechococcus*

At St. 14, an oligotrophic site, the percentage of the picoplankton primary production grazed was 103%, 68% and 79% for Syn, PicoE and HB, respectively (Table 2). This suggests a close predator/prey relationship between picoplankton and microzooplankton in oligotrophic waters and that microzooplankton grazing was potentially a key

controlling factor for the picoplankton biomass. A similar situation was also found in subtropical and sub-Antarctic waters to the east of New Zealand, where picophytoplankton (i.e., PicoE and cyanobacteria) were heavily grazed (86–118%) by microzooplankton (Hall et al. 2004).

Copepods grazing on ciliates has been well studied in many previous studies, with a consensus result that copepods prefer ciliates as their primary food source. There are fewer reports with less consistent results on the influence of copepod on other components of the microbial food web (Table 4). A strong copepod–ciliate–flagellate trophic cascade in the copepod addition incubations in the oligotrophic waters was not found in this study. Copepod additions reduced ciliate abundance. However, a corresponding increase in flagellates abundance was not observed, thus demonstrating a feeding preference of copepods on ciliates rather than flagellates. This weak cascade result is consistent with the previous studies (Sipura et al. 2003). There are several possible explanations of this weak cascade: (1) cross-linkage between flagellates and their predators (copepods, ciliates and flagellate-preying flagellates); (2) HF were regulated mainly by resources, with mortality being only a minor influencing factor; (3) disappearance of the ciliates is a slow process. As a result, the release from ciliate grazing is not obvious in such a short incubation time. HF also needs time to respond. Removal of flagellate predators caused no quick (< 1 day) indirect response (Calbet and Landry 1999; Samuelsson and Andersson 2003) explaining that larger consumers, such as mesozooplankton, exert little net influence on the dynamics at the base of the food web.

Both Chl *a* concentration and Syn abundances showed a consistent increase in the copepod-added bottles while flagellate abundance remained stable. This might indicate that ciliates were in general the main grazers of phytoplankton and Syn in particular. Some indirect evidence supports ciliates being the main grazers of Syn. For example, in waters of the North Pacific, more than two-thirds of the grazing mortality of Syn could be due to > 10 µm heterotrophs (mostly ciliates) (Kudoh et al. 1990). Along the WOCE SR3 line between Tasmania and Antarctica, small ciliates may have a preference for, or may be more competitive grazers of picophytoplankton when prokaryotic picophytoplankton dominates (Safi et al. 2007). Active grazing by ciliates on Syn was also found in lab experiments, although Syn might not be a high-quality food for ciliates (Christaki et al. 1999).

In our results, HB abundance decreased slightly after incubation, which is consistent with Liu et al. (2005b), where HB abundance increased with the increase of ciliates. In another study, the composition and activity of bacterial assemblages was not only a reflection of the substrate supply, but it was also mediated by strong food web interactions (Zöllner et al. 2009).

Although this was a small study with only five stations (one eutrophic and four oligotrophic stations), differences between trophic states were strong and hence our inferences are robust. Clearly future studies should include more stations so statistical differences can be quantified.

## Conclusion

In the southern Yellow Sea, picoplankton comprised a large part of the microzooplankton food source in the central oligotrophic area while phytoplankton was the main food of microzooplankton in the coastal eutrophic area. In the central oligotrophic area, of the three groups of picoplankton (Syn, HB and PicoE), microzooplankton preferred grazing on Syn. A strong copepod–ciliate–flagellate trophic cascade was not found in the copepod addition incubations in the oligotrophic waters. After copepod addition incubations, ciliate abundance decreased while Syn abundance increased, indicating a strong grazing pressure by microzooplankton on Syn. These results suggest that Syn might be a fundamental food source for the carbon budget in the oligotrophic water in YS and its variation could affect the carrying capacity of the pelagic food webs.

## Materials and methods

Dilution incubations and copepod addition incubations were performed during a cruise to the Yellow Sea (Fig. 4) onboard *RV Beidou* on May 16–29, 2007. One station (St. 14) was visited twice. The second visit was labelled as St. 14 -2. Dilution incubations were carried out at St. 22, St. 14 and St. 14-2. Copepod addition incubations were carried out at St. 13, St. 14 and St. 17 (Table 1). At each station, temperature and salinity vertical profiles were measured using a Seabird CTD system (SBE-25). For the determination of Chlorophyll *a* (Chl *a*) concentration, seawater samples were taken at different depths by Niskin bottles mounted on a Rossette water sampler.

At all the stations, a thermocline was observed at 10–30 m depth. Seawater for dilution incubations and copepod addition incubations was collected at 10 m depth, just above the thermocline, with a 60 L large volume water sampler. Filtered seawater (FSW) was made by filtering seawater through Whatman GF/F filters and was assumed to be free of predators (micro- and mesozooplankton). The 200 µm filtered seawater (200 FSW) was made by gravity filtration through a 200 µm pore size mesh. A 100-ml sample was taken to determine nutrients concentrations. Experimental items (25-L polycarbonate carboys, 1.35-L polycarbonate incubation bottles, glass filter bottles, etc.) previously soaked in 10% HCl were rinsed with large amounts of seawater before use.

## Dilution incubation

Dilution incubations were carried out according to the protocols described in the literature (Calbet et al. 2008; Landry and Hassett 1982). Samples were taken for the determination

**Table 4** Previous published results in the research of copepod addition experiments

Region	Incubation time	Copepods	Chl <i>a</i>	Ciliates	HF	HNF	PF	PNF	Syn	PicoE	HB	References
NW Mediterranean Sea	24 h	nd			↓		–			↑		Van Wambeke et al. (1996)
San Pedro Channel, California	24 h and 72 h	<i>Clausocalanus</i> spp. and <i>Oithona</i> spp. in August; <i>Acartia</i> spp. in March.	↑↑ in Aug –in Mar	↓↓ in Aug – in Mar	↑ in Aug – in Mar	↑↑	– in Aug ↓↓(72 h) in Mar	↑↑ in Aug and ↓↓(72 h) in Mar	↓(72 h) in Aug	↑↑	↓(72 h) in Aug	Schmetzer and Caron (2005)
Semi-enclosed marine lagoon of the Trondheimsfjord, Central Norway	1 week	<i>Calanus finmarchicus</i>		↓↓		↑↑					↓	Zöllner et al. (2009)
Coastal Gulf of Alaska	nd	<i>Neocalanus cristatus</i>	↓	↓	↑		↑		↑			Liu et al. (2005a)
Oligotrophic open ocean (subtropical North Pacific)	12-18 h	nd	↑↑								↑	Calbet and Landry (1999)
Oligotrophic region in Yellow Sea	24 h	<i>Calanus sinicus</i>	↑	↓	*				↑↑	*	*	This research

nd no data, ↑ increased, ↑↑ significantly increased, ↓ decreased, ↓↓ significantly decreased; \* diverse change, HF heterotrophic flagellates, HNF heterotrophic nanoflagellates, PNF pigmented flagellates, PicoE pigmented nanoflagellates, Syn Synchococcus, PicoE Picoeukaryotes, HF Heterotrophic bacteria

of initial conditions  $P_0$ , including total Chl  $a$  concentration, flagellates, ciliates and picoplankton abundances.

200  $\mu\text{m}$  FSW was poured into four 4-L polycarbonate bottles. FSW was then added to create treatments of 100%, 75%, 50% and 25% 200 FSW (dilution factor,  $c$ ). After mixing, water from the 4-L polycarbonate bottles was transferred into duplicate 1.35-L polycarbonate bottles. The 1.35-L polycarbonate bottles were then incubated in a surface water flow-water-incubator on the deck for 24 h. A neutral plastic mesh was used to dim natural sunlight so that the irradiance of the incubation was similar to that at a depth of 10 m, normally about 30–50% of the irradiance at the surface. After incubation, subsamples were taken for determination of final conditions  $P_1$  (Chl  $a$  concentration, flagellate, ciliate and picoplankton abundances).

### Copepod addition incubation

The calanoid copepod, *Calanus sinicus* were collected by vertically towing a plankton net (mesh size 500  $\mu\text{m}$ ) from

2 m above the seabed to the surface. Contents of the cod-end were carefully poured into a container filled with 200 FSW.

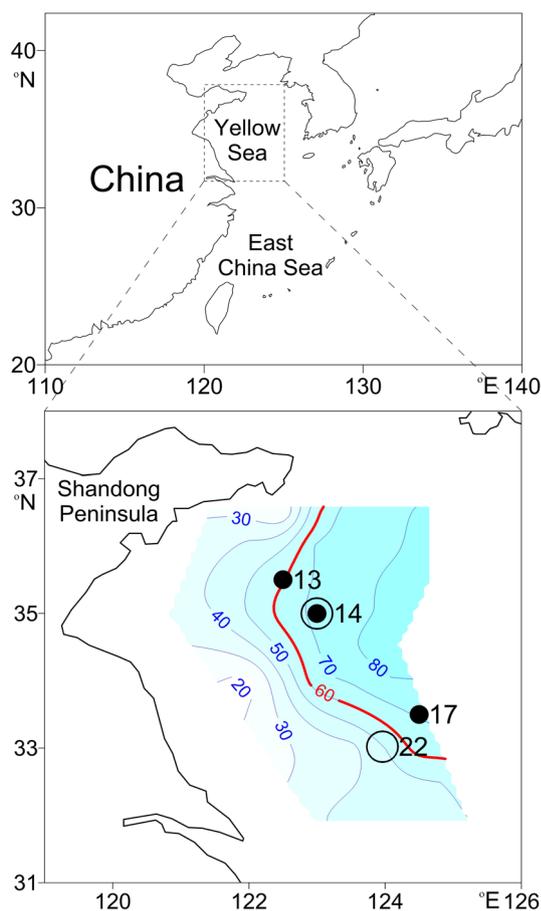
Copepod addition incubation was carried out using two 1.35-L polycarbonate bottles filled with well-mixed 200  $\mu\text{m}$  FSW. Ten healthy active adult females of *C. sinicus* were pipetted into each bottle and sealed without bubbles. Two control bottles without copepods were set up simultaneously. Nutrients (10  $\mu\text{mol/L}$   $\text{NaNO}_3$ , 10  $\mu\text{mol/L}$   $\text{Na}_2\text{SiO}_3$  and 1  $\mu\text{mol/L}$   $\text{NaH}_2\text{PO}_4$ ) were added to each bottle. These four bottles were incubated in surface water flow-water-incubator on deck for 24 h. During the incubation, these bottles were inverted gently about every 4 h. After incubation, the *C. sinicus* were checked and no deaths were found. Before and after copepod addition incubation, subsamples were taken to determine Chl  $a$  concentration, flagellate, ciliate and picoplankton abundances as in the dilution incubation experiment.

### Sample collection and analysis

To determine the nutrient concentrations in the dilution incubations, 100 ml samples were filtered through 0.45  $\mu\text{m}$  filters. The filtrates were poisoned with saturated  $\text{HgCl}_2$ .  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$  and dissolved silica concentrations were determined photometrically (Grasshoff et al. 1999) using an autoanalyzer (Model: Skalar SAN<sup>plus</sup>) with a precision of < 5–10% in the lab.

For total Chl  $a$  concentration determination, seawater samples of 250 ml were filtered onto GF/F filters. The filters were frozen at  $-20^\circ\text{C}$  until laboratory analysis. Chlorophyll was extracted with 90% acetone at  $-20^\circ\text{C}$  in the dark for 24 h. The concentrations were determined using a Turner Designs (Model II) fluorometer calibrated with a pure Chl  $a$  standard material (Sigma) (Strickland and Parsons 1972). Total phytoplankton carbon biomass per unit volume was estimated from Chl  $a$  concentration assuming a constant C: Chl  $a$  ratio of 100 (mg: mg) (Gasol et al. 1997).

For flagellate abundances, 40–100 ml samples were preserved in a glutaraldehyde solution (1% final concentration), filtered onto 2  $\mu\text{m}$  pore-size black polycarbonate membrane filters and stained with DAPI (5  $\mu\text{g/ml}$  final concentration) for 5 min. The filters were examined under an epifluorescence microscope (Leica DM4500). At least 200 cells were counted and classified as either pigmented flagellates (PF) or heterotrophic flagellates (HF), according to the presence or absence of red fluorescence induced by the chlorophyll. Total flagellate (TF) abundances were defined as the sum of PF and HF. To estimate the biovolume, cell dimensions of flagellate were measured with Leica DM4500 self-carried software and transformed to biovolume by analogy to geometrical forms (Sun and Liu 2003). Conversion to carbon biomass was made using a factor of 220  $\text{fg C}/\mu\text{m}^3$  for flagellates (Børshiem and Bratbak 1987).



**Fig. 4** Study area and experimenting stations. White circle: dilution incubation stations; black circle: copepod addition incubation stations; contour shows the bottom depth (m)

To determine ciliate abundances, 150 ml samples were fixed with 1% acidic Lugol's solution and stored in the dark at 4 °C until processing in the laboratory. The samples were settled for at least 24 h and the upper water was slowly siphoned out to leave a 20 ml aliquot. The concentrated samples were then settled in 20 ml Utermöhl chambers. The whole chamber was counted under an Olympus CKX41 inverted microscope at 100× or 200× magnification following the Utermöhl method (Utermöhl 1958). The volume of each cell was determined by measuring cell dimensions with an ocular micrometer, assuming appropriate geometric shapes (ellipsoid, cone, cylinder, ball, semi-ellipsoid and their combinations). The carbon content of each cell was then obtained using the carbon to volume conversion factor 190 fg C/ $\mu\text{m}^3$  for ciliates (Putt and Stoecker 1989).

Picoplankton samples (5 ml) were fixed with paraformaldehyde (final concentration 1%), immediately frozen in liquid nitrogen until analysis with a FACS Vantage SE (Becton–Dickinson, USA) flow cytometer in the laboratory. Fluorescent beads (2  $\mu\text{m}$  Fluoresbrite microspheres, Polysciences) were used as internal standard for the instrument set-up and enumeration of picoplankton cells (Olson et al. 1993). Syn and PicoE were characterized according to their scatter, red and orange fluorescence intensities. To determine HB abundance, samples were diluted 5-fold with TE buffer (Tris–EDTA, 100 mmol/L Tris–Cl, 10 mmol/L EDTA, pH 8.0, Sigma), incubated for 20 min with the SYBR Green I nucleic acid dye (Molecular Probes, final dilution  $10^{-4}$ ) in the dark, at room temperature. HB were resolved on the basis of their green fluorescence vs. sideward scatter signals (Marie et al. 2000a, b). Picoplankton abundances were expressed in terms of carbon biomass by using conversion factors from the literature, i.e. 82 fg C/cell, 530 fg C/cell (Worden et al. 2004) and 20 fg C/cell (Lee and Fuhrman 1987), for Syn, PicoE and HB, respectively.

## Data elaboration

Apparent growth rate ( $u$ , /day) of Chl  $a$ , picoplankton and flagellates in the dilution incubations were calculated using the equation  $u = \ln(P_t/P_0)/t$  (Landry and Hassett 1982) where  $t$  was incubation time (day),  $P_0$  and  $P_t$  were the density of Chl  $a$ , picoplankton and flagellates at the beginning and after the end of the incubation, respectively. According to Landry and Hassett (1982),  $u$  is the net result of grazing ( $g$ , /day) and growth rates ( $k$ , /day):

$$u = k - c \times g.$$

Values of  $k$  and  $g$  were determined from linear regression of the apparent growth rates against dilution factors ( $c$ ).

Here, we considered only the results with significant linear regression for the preys considered ( $R^2 > 0.8$ ).

Microzooplankton grazing pressure on standing stock ( $P_i$ ) and primary production ( $P_p$ ) were calculated according to Verity et al. (1993):

$$P_i = 1 - e^{-gt} \times 100\%$$

$$P_p = \frac{e^{kt} - e^{(k-g)t}}{e^{kt} - 1} \times 100\%.$$

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**Author contributions** WZ, LZ and YZ designed this program and supervised the entire project. WZ, YZ, LZ, YD, HL, SL and LH conducted the shipboard experiments and analyses. YZ and YD integrated all the data, finalized the figures and wrote the original draft of the manuscript. GG reviewed and edited the manuscript. The final manuscript was approved by all the authors.

## Compliance with ethical standards

**Conflicts of interest** All the authors declare that they have no conflict of interest

**Animal and human rights statement** This article does not contain any studies with human participants or animals performed by any of the authors.

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