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ORIGINAL RESEARCH ARTICLE

Microbial plankton communities in the coastal southeastern Black Sea: biomass, composition and trophic interactions

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Summary We investigated biomass and composition of the pico-, nano- and microplankton communities in a coastal station of the southeastern Black Sea during 2011. We also examined trophic interactions within these communities from size-fractionated dilution experiments in February, June and December. Autotrophic and heterotrophic biomasses showed similar seasonal trends, with a peak in June, but heterotrophs dominated throughout the year. Autotrophic biomass was mainly comprised by nanoflagellates and diatoms in the first half of the year, and by dinoflagellates and *Synechococcus* spp. in the second half. Heterotrophic biomass was mostly dominated by heterotrophic bacteria, followed by nanoflagellates and microzooplankton. Dilution experiments suggest that nano- and microzooplankton were significant consumers of autotrophs and heterotrophic bacteria. More than 100% of bacterial production was consumed by grazers in all experiments, while 46%, 21% and 30% of daily primary production were consumed in February, June and December, respectively. In February, autotrophs were the main carbon source, but in December, it was heterotrophic bacteria. An intermediate situation was observed in June, with similar carbon flows from autotrophs and heterotrophic bacteria. Size-fraction

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dilution experiments suggested that heterotrophic nanoflagellates are an important link between the high heterotrophic bacterial biomass and microzooplankton. In summary, these results indicate that nano- and microzooplankton were responsible for comprising a significant fraction of total microbial plankton biomass, standing stocks, growth and grazing processes. This suggests that in 2011, the microbial food web was an important compartment of the planktonic food web in the coastal southeastern Black Sea.

1. Introduction

Biogenic carbon transfers from autotrophic to heterotrophic organisms through two main pathways: the classical herbivorous food web and the microbial food web (Azam et al., 1983; Legendre and Rassaulzadegan, 1995; Sherr et al., 1986; Sommaruga, 1995). In the classical herbivorous food web, energy is channelled directly from large diatoms to metazoans (Pomeroy, 1974). In the microbial food web energy is channelled to higher trophic levels from bacteria and small phytoplankton (<20 μm) to nano-microzooplankton (Azam et al., 1983; Calbet and Landry, 2004). Therefore, through the microbial food web, heterotrophic nanoflagellates (HNF) and microzooplankton (<200 μm, especially ciliates and heterotrophic dinoflagellates) play significant roles in structuring plankton communities (Calbet, 2008) and in nutrient regeneration (Calbet and Saiz, 2005). They control lower level production and dynamics (Calbet and Landry, 2004) and are a favourite prey for mesozooplankton in a range of aquatic environments, from the poles to upwelling regions to oligotrophic ocean gyres (Atkinson, 1996; Calbet and Saiz, 2005; Stoecker and Capuzzo, 1990). The microbial food web is less efficient due to energy losses in each trophic step and dominant in oligotrophic waters. However, many productive systems have multivorous food webs where both the classical and microbial food webs play important roles in carbon cycling (Legendre and Rassaulzadegan, 1995). Thus, information on the different trophic compartments and their interactions is important for understanding the functioning of the planktonic food web and its representation in ecological models.

The Black Sea ecosystem has significant potential in terms of fishing among the world oceans, but drastic changes in biogeochemical properties occurred during the last four decades (Besiktepe et al., 1999; Daskalov, 2002; Kideys, 2002; Oguz and Gilbert, 2007; Oguz et al., 2012a). Pollution, eutrophication, over-fishing, climatic cooling/warming and introduction of non-native species altered the Black Sea ecosystem in the 1990s (Oguz and Gilbert, 2007). Nutrient concentrations decreased in the 2000s compared with the eutrophication period, which has been regarded as an improvement of the Black Sea ecosystem state (Pakhomova et al., 2014). However, the ecosystem seems not to have recovered to the classical herbivorous food web of the pre-eutrophication period prior to 1970 and is now characterized by a food web dominated by dinoflagellates and other nano-size phytoplankton species with respect to diatoms, and relatively low levels of phytoplankton (Oguz and Velikova, 2010). Despite improvements, the Black Sea is still under serious environmental threats as a result of uncontrolled coastal pollution and high river discharge of several industrialized countries into this semi-enclosed basin. Climatic changes may have also played a role in shifts towards domination of dinoflagellates and nanoflagellates, reduced frequency and magnitude of phytoplankton blooms, and declines in phytoplankton biomass (Daskalov, 2002; Kideys, 2002; Nesterova et al., 2008; Oguz and Gilbert, 2007; Oguz et al., 2012a). Long-term changes of in situ phytoplankton biomass in the interior basin indicate distinct decadal changes that followed closely temperature variations, with higher (lower) biomass occurred during cold (warm) years (Nesterova et al., 2008; Oguz et al., 2006). It has been speculated that warming over the next decades (Collins et al., 2013) might significantly increase carbon flow through the microbial food web (Caron and Hutchins, 2012). However, there is little information on the importance of the microbial food web in the Black Sea, since previous studies mainly analysed the dynamics of classical food web contributors such as diatoms, dinoflagellates and their mesozooplankton predators, in particular, copepods (BSC, 2008). A number of studies have investigated components of the microbial food web (heterotrophic bacteria, pico-autotrophs, small flagellates and microzooplankton), but these have mostly focused on specific compartments or taxonomic subsets during limited periods and in specific regions (e.g. Becquevort et al., 2002; Feyzioglu et al., 2004; Kopuz et al., 2012; Sorokin et al., 1995; Uysal, 2001). To the best of our knowledge, a simultaneous assessment of the whole microbial community has not been done for the Black Sea. A few studies indicate the importance of nano- and microzooplankton as grazers. Bouvier et al. (1998) measured feeding activity of nano- and micrograzers on heterotrophic bacteria and nanoplankton during summer 1995 in the NW Black Sea based on the uptake of fluorescently labelled-prey, and Stelmakh and Georgieva (2014) reported microzooplankton grazing on phytoplankton based on dilution experiments conducted in the Western Black Sea.

The SE Black Sea is an important part of the Black Sea in terms of fishing. A milder climate provides more favourable spawning and overwintering grounds for the anchovy, and the region currently sustains 80% of the total fish catch in the Black Sea (Oguz et al., 2012b). As such lower trophic levels dynamics should be understood as much as possible. However, there are no studies on trophic interactions within a

microbial food web and on complete community assessments. The present study simultaneously addresses the dynamics of autotrophic and heterotrophic plankton <200 μm during a seasonal progression and discusses the balance between prey and predator in the SE Black Sea. To test the hypothesis that the microbial food web is an important pathway of carbon, the population dynamics of the various planktonic groups are described and the carbon flow within the microbial food web is determined.

2. Material and methods

2.1. Study site and sampling

Sampling was carried out at a coastal monitoring station (40°57′03″N; 40°11′22″E) in the southeastern Black Sea during eight cruises from February to December 2011 on board r/v *KTU Denar I*. This station has been monitored since 2001 (e.g. Agirbas et al., 2014, 2015). The sampling station has a depth of 50 m and is situated 0.5 nautical miles off from the coast (Fig. 1). The region is characterized by a narrow continental shelf compared to the northwestern Black Sea and is influenced by the meandering rim current, the permanent circulation feature that encirculates the Black Sea in a counterclockwise direction, as well by local river discharges. In summer, waters are thermally stratified and in winter, vertical mixing can go as deep as the water column depth. Within the euphotic depth (~30 m), reported concentrations of nutrients are highly variable (Agirbas et al., 2014, 2015). Nitrite + nitrates have been found to vary between 0.2–5 μM. Phosphate is usually very low (~0.01 μM), sometimes not detectable, and silicates around 5 μM. The spring bloom is mainly dominated by diatoms, followed by increases of dinoflagellates and cocolitophores in summer, and autumn blooms of these two groups have also been found (Agirbas et al., 2015). Changes in trophic status based on chlorophyll-*a* have been reported for the last decade in the region (Agirbas et al., 2015).

Profiles of water column structure were collected with Idronaut Ocean Seven 316 Plus CTD profiler and fluorescence was recorded with Satlantic hyperspectral radio spectrophotometer in order to obtain subsurface chlorophyll maximum (SCM) and euphotic depths. Samples for nutrients ($NO_2 + NO_3$, PO_4, and SiO_2) were taken with 5 m intervals within euphotic zone and analysed using a SEAL AutoAnalyzer. Ammonium was not measured because it is scarce in a surface layer. Samples were collected for pico-, nano-, microplankton from the SCM using 5 l Niskin bottles mounted on a SBE32 Carousel water sampler.

2.2. Abundance and biomass

2.2.1. Pico- and nanoplankton

Pico- and nanoplankton sub-samples (50 ml) were fixed with 1% glutaraldehyde. Samples (10 ml) were drawn under low vacuum (<5 mm Hg) onto 0.2 μm and 0.8 μm black Nuclepore filters, with cellulose nitrate backing filters (1.2 μm) to enhance even cell distribution for pico- and nanoplankton, respectively. Acridine orange (200 μl) solution was added during filtration to stain heterotrophic bacteria and nanoflagellates (Hobbie et al., 1977). Counts for *Synechococcus* spp. were made on unstained preparations due to their autoflorescence property. Filters were mounted on glass slides with a drop of immersion oil between the filter and glass coverslip. They were either processed immediately or frozen for subsequent analysis within 2 weeks. Cell counts were performed under a Nikon E 600 epifluorescence microscope with a filter combination of B-2A (blue excitation, dichroic mirror DM 505, excitation filter EX 450–490, barrier filter BA 520) and G-1A (green excitation, dichroic mirror DM 575, excitation filter EX 546/10, barrier filter BA 580). Bacterial cells were counted in at least 30 microscopic fields. Mean cell volumes were estimated using image analysis system composed of a digital camera, computer and the image analysis software. Heterotrophic nanoflagellates (HNF) were distinguished from autotrophic nanoflagellates (ANF) by the

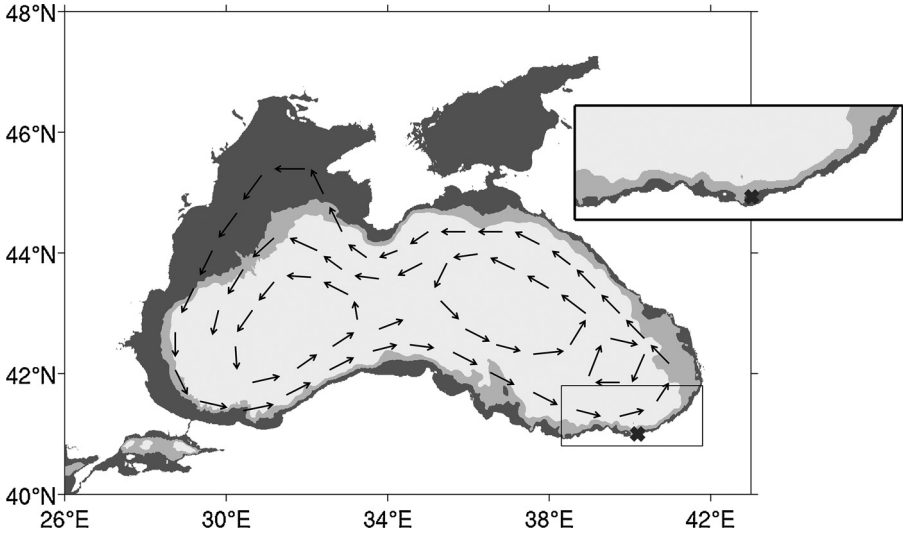

Figure 1 Map showing study area, sampling station, bathymetry and a schematic representation of the general circulation in the Black Sea. The three grey colours represent bathymetric contours of <200 m, 200–1000 m, and >1000 m.

absence of fluorescence. To calculate carbon content of heterotrophic bacteria, *Synechococcus* spp. and nanoflagellates, 77 (Carlson et al., 1999), 123 (Waterbury et al., 1986) and 220 (Børsheim and Bratbak, 1987) fg C per cubic micron were used, respectively.

2.2.2. Microplankton

Microplankton sub-samples (50 or 200 ml depending upon microplankton abundance) were preserved with glutaraldehyde at a final concentration of 1% and stored in the dark (Stoecker et al., 1994). The samples were concentrated by settling for 1 week and siphoning off the supernatant (Karlson et al., 2010). One millilitre of the concentrated samples was placed in a Sedgwick rafter counting chamber and observed using Nikon E 600 epifluorescence microscope at 100–400× magnification. A minimum of 50–100 microplankton observed within 10–20 fields of view were enumerated and grouped into major taxa (diatoms, autotrophic and heterotrophic dinoflagellates, ciliates). In addition, glutaraldehyde fixed samples (0.5% f.c.) were concentrated onto black polycarbonate filters and examined under fluorescence microscopy to estimate the proportion of chlorophyll *a*nd non-chlorophyll containing cells under blue (450–480 nm) light (considered autotroph and heterotroph, respectively) (Karlson et al., 2010). The biomass of each group was estimated by assigning standard geometric shapes or combinations of shapes to specific organisms and measuring the dimensions (Edler, 1979; Hillebrand et al., 1999). Measurements were taken for at least 50 individuals for the abundant taxa and all present individuals for the rare taxa using Image ProPlus 6.2 software (Media Cybernetics, Bethesda, MD). These volume (μm^3) measurements were converted to estimates of carbon content by using the following conversion factors, pg C $cell^{-1}$ = 0.288 × $volume^{0.811}$ or diatoms; pg C $cell^{-1}$ = 0.760 × $volume^{0.819}$ for dinoflagellates (Menden-Deuer and Lessard, 2000); pg C $cell^{-1}$ = (volume × 0.053) + 444.5 for tintinnids (Verity and Langdon, 1984); pg C $cell^{-1}$ = volume × 0.19 for other ciliates (Putt and Stoecker, 1989).

2.3. Size-fractionated dilution experiments

Size-fractioned grazing experiments were performed during February, June and December 2011 to assess the grazing impact of the <200 μm (Landry and Hassett, 1982) and of the <20 μm grazers on autotrophic and heterotrophic plankton. Dilution experiments were planned to cover three distinct periods of the year representing distinct frames of the ecosystem functioning. February would represent the well-mixed period of water column, June a period of thermal stratification characterized by increases in dinoflagellates and cocolitophores following the diatoms spring bloom (e.g. Agirbas et al., 2014; Eker-Develi and Kideys, 2003), and December a period of erosion of the seasonal thermocline. Seawater for each experiment was collected in 5 l Niskin bottles from the subsurface chlorophyll maximum (SCM) based on in situ fluorescence profiles, transferred gently into two 20 l carboys, and then transported immediately to the laboratory where the dilution experiments were conducted. All experimental bottles, silicone tubing, and other materials were acid-washed (10% HCl) and rinsed with Milli-Q water prior to each experiment. Filtered seawater (FSW) for experiments was generated by gentle gravity filtration of the incubation water using cartridge filters (0.2 μm pore size). Depending on the concentration of plankton abundance, the filtration process took approximately 2–4 h. The remaining seawater (initial seawater, ISW) was gently prescreened by syphoning through a submerged 20 μm and 200 μm mesh to remove microzooplankton + mesozoopankton and mesozooplankton, respectively. In parallel treatments, whole seawater (unscreened), containing assemblages of natural plankton was used to assess the impact of screening on microzooplankton and grazing by mesozooplankton. The ISW was diluted by FSW to four target dilutions of 20, 45, 70, and 100% (ISW: ISW plus FSW) in transparent polycarbonate bottles. The incubation volume was 3.2 l and treatments were carried out in duplicate. Some studies add low concentration of nutrients to prevent that phytoplankton growth rates are altered in a result of dilution process (e.g. Calbet et al., 2008; Dupuy et al., 2011). However, nutrient addition might have negative effects on growth coefficients of phytoplankton (Landry and Hassett, 1982; Worden and Binder, 2003) and feeding behaviour of microzooplankton (Worden and Binder, 2003). Therefore, nutrients were not added to the bottles to keep plankton communities close to in situ conditions. The experimental bottles were placed in deck incubators for 24-h period. Incubator was cooled to ambient temperatures with running seawater and screened to ambient light intensity using appropriate light screens. Experimental bottles were gently rotated to avoid sedimentation for 4–6 times during incubation.

Initial and final samples of incubation were collected to enumerate Chl-*a* concentration and carbon biomass of *Synechococcus* spp., HB and HNF as described in Section 2.2.1. Chl-*a* was determined by filtering 250–500 ml of water (depending on season and dilution level) through Whatman glass fibre filters (GF/F, 25 mm diameter). After filtration, the filters were stored frozen at −80°C until fluorometric analysis of acetone extracts (Parsons et al., 1984) using a Turner Designs Fluorometer.

Growth rates of autotrophs, *Synechococcus* spp., HB, HNF and grazing rates of heterotrophic protists were calculated using the exponential model of Landry and Hassett (1982):

$$P_t = P_0 e^{(k-g)t},$$

where P_0 and P_t are the initial and final concentrations of Chl-*a* and prey carbon biomass, and t is the incubation time. The instantaneous coefficients of prey growth (μ) and grazing mortality (g) were estimated by linear regression of apparent growth rate against fraction of unfiltered seawater. The apparent growth rate (k) for each dilution was calculated according to the following equation:

$$k = \frac{1}{t}\ln\left(\frac{P_t}{P_0}\right).$$

Non-significant grazing rates were not excluded following recommendations of Latasa (2014) and Landry (2014). Daily prey production (P) and grazing losses (G) (μg C l^{-1} d^{-1}) (for both significant and non-significant grazing rates) were calculated according to Landry et al. (2000):

$$P = \mu\left(\frac{P_0[e^{(\mu-g)t}-1]}{[\mu-g]t}\right),$$

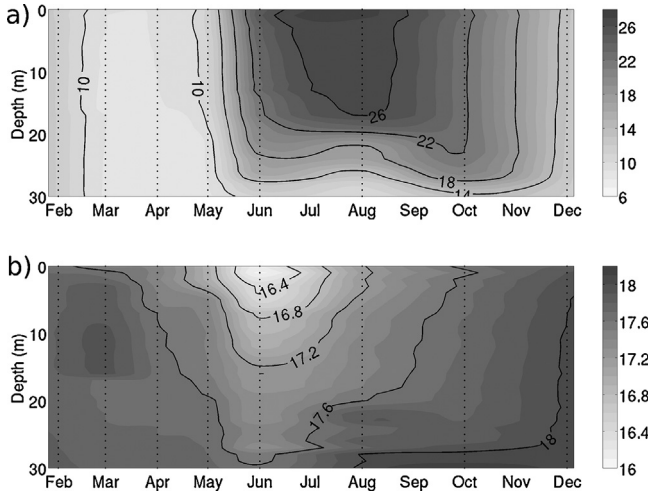

Figure 2 Temperature (°C) (a) and salinity (b) profiles of sampling station between February and December 2011.

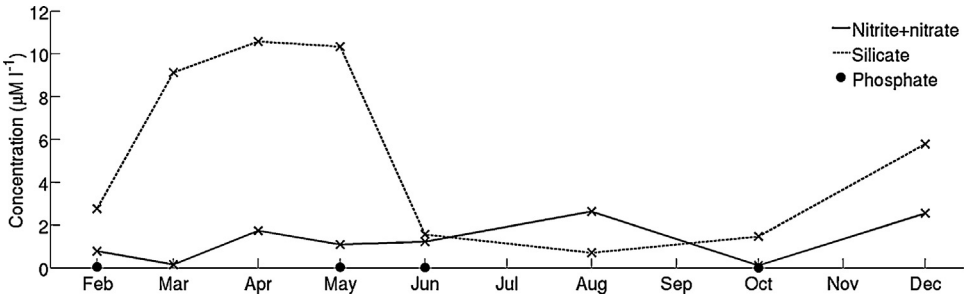

Figure 3 Average nutrient concentrations within euphotic zone between February and December 2011. PO_4 concentrations were below detection limit except for February, May, June and October (<0.04 μM l^{-1}).

$$G = g\left(\frac{P_0[e^{(\mu-g)t}-1]}{[\mu-g]t}\right).$$

Nano- and microzooplankton grazing pressure on initial prey standing stock (P_i, %) was calculated according to the following equations:

$$P_i = \frac{G}{P_0}\times 100.$$

3. Results

3.1. Water column structure

The study area exhibited typical hydrographic conditions of the Black Sea (Oguz et al., 2006). Overall, a well-mixed water column was found from February through April, whereas marked stratification was detected in the summer months, with a thermocline located at ~20 m depth and up to 15°C temperature difference between the surface and deeper waters (Fig. 2a). The highest (27.3°C) and lowest (8.1°C) surface temperatures were detected in August and February, respectively. The presence of low salinity at the surface was occasionally observed, especially in summer (Fig. 2b). The surface salinity was lowest (16.2) in June and highest in February (Fig. 2b). Euphotic depth varied between 21–31 m, being shallower in winter and deeper in summer. Euphotic zone averages of SiO_2 showed a clear seasonal cycle, with concentrations ranging from 0.70 (August) to 10.6 μM l^{-1} (April). Seasonal variations of NO_{2+3} were less clear, varying from 0.11 (October and March) to 2.62 μM l^{-1} (August). PO_4 concentrations were below detection limit except for February, May, June and October (<0.04 μM l^{-1}) (Fig. 3). N P^{-1} ratios in the euphotic zone were 20, 34, 42 and 18 for February, May, June and October, respectively. Averaged SCM concentration ranged between 0.47–2.18 μg l^{-1} reaching maxima in late spring (May) (Fig. 4).

3.2. Autotrophic and heterotrophic carbon biomass and composition

The total plankton carbon biomass was highest (155 μg C l^{-1}) in June, and lowest (~40 μg C l^{-1}) in February, October and December (Fig. 4). The autotrophic carbon (Auto-C) and heterotrophic carbon (Hetero-C) biomasses showed similar trends during the study period, ranging from 8 to 62 μg C l^{-1} and 27 to 93 μg C l^{-1}, respectively (Fig. 5). For all samples, the Auto-C and Hetero-C biomasses averaged (±SD) 20 ± 17 μg C l^{-1} and 49 ± 25 μg C l^{-1}, respectively. In all samples, Hetero-C always comprised more than 60% of total plankton carbon biomass (Fig. 4). As an indicator of the trophic characteristic of a system, the median of Hetero-C to Auto-C ratios was 2.3.

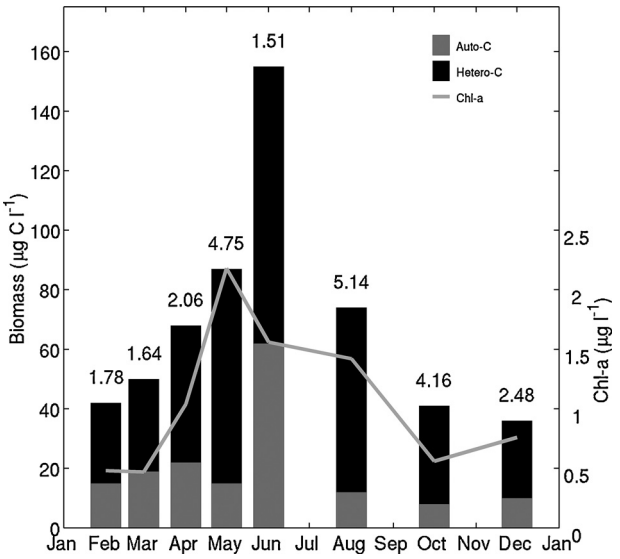

Figure 4 Total carbon biomass of autotrophic (Auto-C) and heterotrophic (Hetero-C) plankton, Chl-*a*, and heterotroph/autotroph ratio (numbers on top of bars) from February to December 2011 at the sampling station.

The Auto-C biomass was comprised by *Synechococcus* spp. (Syn), autotrophic nanoflagellates (ANF), prymnesiophytes (Prym), autotrophic dinoflagellates (A-Dino) and diatoms (Fig. 5). The relative contributions of these groups to Auto-C biomass is shown in Fig. 6a. Autotrophic picoplankton (A-Pico) consisted entirely of *Synechococcus* spp, with biomass ranging from 0.4 to 6.1 μg C l^{-1}. The contribution of A-Pico to the Auto-C biomass increased in May during stratification and remained relatively high until December. The biomass of autotrophic nanoplankton (A-Nano) varied from 0.7 to 18 μg C l^{-1}. A-Nano (mostly ANF) significantly contributed to the Auto-C biomass from February to May, with an average (±SD) of 42 ± 12%. This contribution was particularly high (>50%) in February and March. In June a major bloom of prymnesiophytes, *Emiliania huxleyi*, was observed. After June, the biomass of A-Nano and its contribution to Auto-C biomass was lower. Autotrophic microplankton (A-Micro) biomass ranged from 4.9 to 38 μg C l^{-1}, and its contribution to Auto-C was on average (±SD) 58 ± 11%. The A-Micro biomass was relatively stable throughout the year, with the exception of a pronounced maximum in June during high A-Dino biomass. A-Micro biomass was dominated by A-Dino from June to October (>75%) and diatoms during the rest of

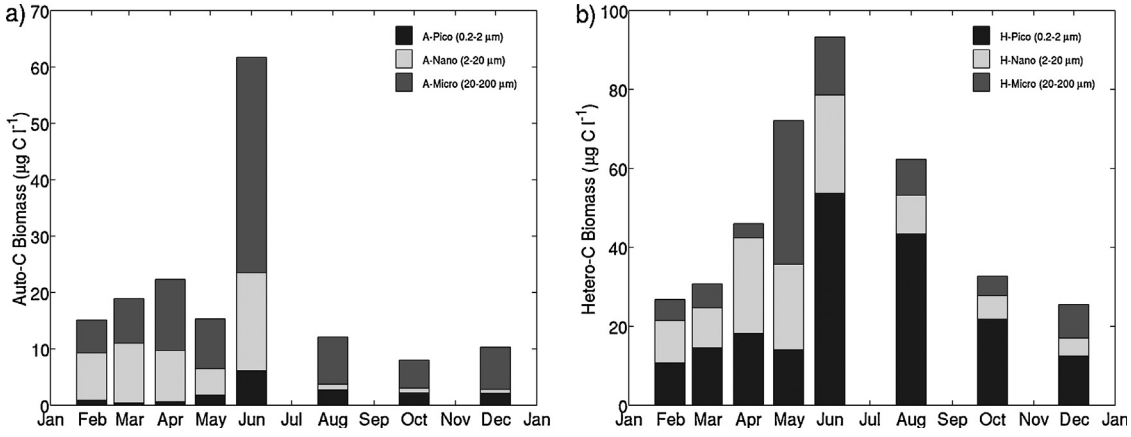

Figure 5 Contribution of size classes to autotrophic (a) and heterotrophic (b) carbon biomass from February to December 2011 at the sampling station.

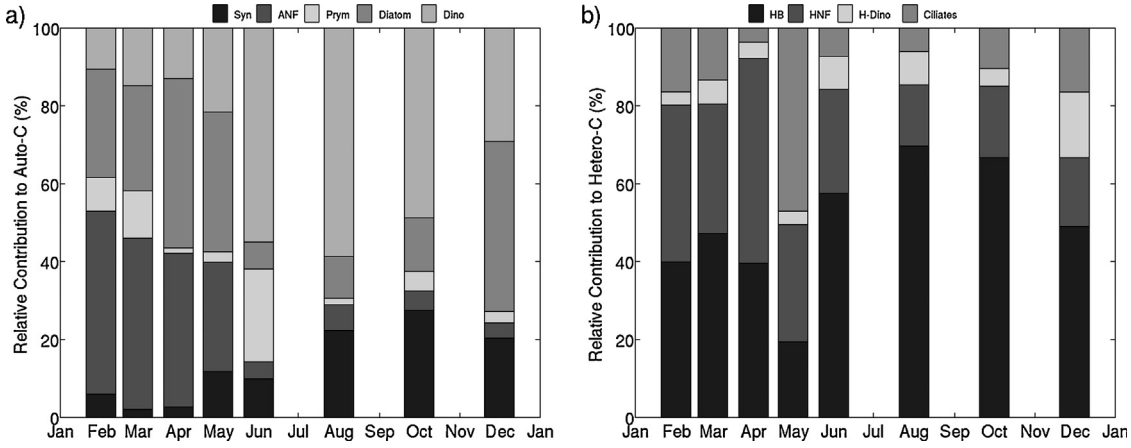

Figure 6 Relative contribution to total Auto-C (a) and Hetero-C (b) biomass <200 μm by groups from February to December 2011 at the sampling station. Auto-C: *Synechococcus* spp. (Syn), autotrophic nanoflagellates (ANF), prymnesiophytes (Prym), autotrophic dinoflagellates (A-Dino) and diatom. Hetero-C: heterotrophic bacteria (HB), heterotrophic nanoflagellates (HNF), heterotrophic dinoflagellates (H-Dino) and ciliates.

the year (>60%). Diatom biomass varied between 1.1–9.7 μg C l^{-1} with maxima in April dominated by the genera *Chaetoceros* and *Rhizosolenia*. A-Dino biomass varied from 1.6 to 34 μg C l^{-1} with maxima in June and main genera were *Gonyaulax*, *Ceratium* and *Prorocentrum*. The C:Chl-*a* ratio was calculated using Auto-C biomass and Chl-*a*, and ranged between 7 and 40 (median of 18). To summarize, we observed one peak of Auto-C biomass in the June sampling, due to a bloom of *E. huxleyi* and A-Dino. In the remaining samplings, the A-Micro biomass was relatively stable (average ± SD of 15 ± 5 μg C l^{-1}) changing from diatoms in winter to dinoflagellates in summer, and the A-Nano and A-Pico biomass showed opposite seasonal patterns. The A-Nano biomass was higher in the first half of the year and the A-Pico biomass was higher in the second half. There was no correlation between Auto-C biomass with temperature, Chl-*a* concentrations, or nutrients ($p > 0.05$). However, Auto-C biomass significantly correlated with salinity ($r^2 = -0.72$, $p < 0.05$).

The Hetero-C biomass was comprised of heterotrophic bacteria (HB), heterotrophic nanoflagellates (HNF), heterotrophic dinoflagellates (H-Dino) and ciliates (Fig. 5). The relative contribution of these groups to Hetero-C biomass is shown in Fig. 6b. Micrometazoans <200 μm were only present at low abundance (<1 ind. l^{-1}) so their contributions to Hetero-C < 200 μm were not considered. Heterotrophic picoplankton (H-Pico), composed entirely of heterotrophic bacteria, was the most substantial component of the Hetero-C biomass, with contributions ranging between 19% and 70%. H-Pico biomass ranged from 11 to 54 μg C l^{-1} with highest concentrations in June. The biomass of heterotrophic nanoplankton (H-Nano) composed entirely of HNF, ranging between 4.5–25 μg C l^{-1} and was the second most important contributor to Hetero-C biomass in majority of samples. Heterotrophic microplankton (H-Micro) was represented by H-Dino and ciliates, and their combined biomass ranged from 6 to 61 μg C l^{-1}. In the majority of samples, ciliates were the most substantial component of H-Micro biomass (62% contribution to H-Micro, for all samples). Ciliates were dominated by the genera *Laboea* and *Strombidium*, while H-Dino was dominated by *Protoperidium* and *Gyrodinium*. No significant correlation between Hetero-C biomass and temperature, Chl-*a* concentration or nutrients ($p > 0.05$) were found. However, Hetero-C biomass significantly correlated with salinity ($r^2 = -0.86$, $p < 0.05$).

There was no significant correlation between Hetero-C and Auto-C biomass ($p > 0.05$). The carbon biomass of total grazers (HNF + microzooplankton) correlated significantly with Chl-*a* ($r^2 = 0.79$, $p < 0.05$) but significant correlation was absent for Auto-C biomass ($p > 0.05$). Among the grazers, only H-Dino was significantly correlated with HB ($r^2 = 0.74$, $p < 0.05$), *Synechococcus* spp. ($r^2 = 0.80$, $p < 0.05$) and A-Dino ($r^2 = 0.73$, $p < 0.05$) (Table 1).

3.3. Trophic interactions

The dilution experiments exhibited distinct results during 2011 (Table 2). Growth and grazing rates for the whole phytoplankton community and group-specific (*Synechococcus* spp., HB and HNF) were calculated based on the measured net changes in total Chl-*a* and taxon-specific carbon biomass, respectively. Production and grazing losses were computed based on the estimated growth and grazing rates (including both significant and non-significant data), and carbon biomass.

In the <200 μm fraction, initial Chl-*a* concentration ranged between 0.47–1.52 μg l^{-1}, with highest concentrations in June (Table 3). The phytoplankton growth rates were particularly high in February (1.67 d^{-1}) and June (2.43 d^{-1}), but low in December (0.23 d^{-1}). Grazing rates were moderate in February (0.77 d^{-1}) and June (0.53 d^{-1}), and also low in December (0.07 d^{-1}). Growth rates always exceed grazing rates. Estimates of mean daily PP followed the growth rates, being 43.1 μg C l^{-1}, 289.7 μg C l^{-1} and 3 μg C l^{-1} in February, June and December, respectively. Mean daily removal of primary production (PP) by nano-microzooplankton ranged between 21–46% with the highest value in February. Overall, the unscreened and <200 μm fraction experiments yielded similar results (Table 2). The <20 μm fraction represented 77%, 32% and 39% of the <200 μm Chl-*a* in February, June and December, respectively. Estimated growth rates were similar to the <200 μm fraction, and also always exceed grazing rates. Mean daily PP (<20 μm) were 19.9 μg C l^{-1}, 50.0 μg C l^{-1} and 1.2 μg C l^{-1} in February, June and December, respectively. Mean daily removal of PP (<20 μm) by nanozooplankton ranged between 7–144%, with the highest value in December and the lowest in June (Table 2).

As a result of a low fluorescence of *Synechococcus* spp., the June experiment was not considered. Initial abundance of *Synechococcus* spp. in experimental bottles was 3.37×10^7 cells l^{-1} in February and 2.62×10^7 cells l^{-1} in December. In the <200 μm fraction, estimated growth rates

Table 1 Correlation coefficients between heterotrophic protists and potential preys. Hb, heterotrophic bacteria; Syn, *Synechococcus* spp.; Anf, autotrophic nanoflagellate; Prym, prymnesiophyte; A-Dino, autotrophic dinoflagellates; Auto-C, autotrophic carbon biomass; Hnf, heterotrophic nanoflagellates; H-Dino, heterotrophic dinoflagellates; Cil, ciliate; Hetero-C, heterotrophic carbon biomass; Total grazers (HNF + H-Dino + Cil).

	Hb	Syn	Anf	Prym	A-Dino	Diatom	Auto-C	Chl-*a*
Hnf	0.11	0.09	0.17	0.26	0.26	0.39	0.47	0.33
H-Dino	**0.74***	**0.80***	−0.25	0.55	**0.73***	−0.05	0.50	0.13
Cil	−0.02	−0.01	−0.01	−0.03	−0.02	0.01	−0.01	**0.70***
Hetero-C	0.59	0.57	−0.03	0.46	0.60	0.01	0.53	0.46
Total grazer	0.03	0.09	0.02	0.11	0.12	0.12	0.19	**0.79***

* $p < 0.05$.

Table 2 Mean growth and grazing mortality rates of phytoplankton (Chl-*a*), *Synechococcus* spp., heterotrophic bacteria (HB) and heterotrophic nanoflagellate (HNF) calculated from the size-fractioned dilution experiments. μ, growth rate; g, grazing mortality rate; r^2, the correlation coefficient of the linear regression of apparent growth rate against fraction of unfiltered seawater; P, prey production; G, grazing losses; P_i, percentage of prey standing stock daily removed by grazing.

Experiment	Date	Size fraction	μ [d^{-1}]	g [d^{-1}]	g:μ	r^2	P [$\mu g\ C\ l^{-1}\ d^{-1}$]	G [$\mu g\ C\ l^{-1}\ d^{-1}$]	P_i [%]
Chlorophyll *a*	February 2011	Unscreened	1.72	0.90	0.52	0.76*	43.6	22.8	139.4
		<200 μm	1.67	0.77	0.46	0.67*	43.1	20.0	124.7
		<20 μm	1.14	0.47	0.41	0.82**	19.9	8.2	67.2
	June 2011	Unscreened	2.21	0.74	0.33	0.67*	200.8	67.2	168.6
		<200 μm	2.43	0.53	0.21	0.79*,a	289.7	63.2	158.6
		<20 μm	1.68	0.11	0.07	0.18	50.0	3.3	26.7
	December 2011	Unscreened	0.10	0.02	0.20	0.03a	1.3	0.3	2.1
		<200 μm	0.23	0.07	0.30	0.08	3.0	0.9	7.6
		<20 μm	0.28	0.39	1.44	0.63*,a	1.2	1.7	36.9
Synechococcus	February 2011	Unscreened	0.37	0.48	1.29	0.95**	0.4	0.6	45.5
		<200 μm	0.35	0.68	1.94	0.90**	0.4	0.8	57.8
		<20 μm	0.11	0.72	6.54	0.89**	0.1	0.8	53.7
	December 2011	Unscreened	0.80	0.55	0.68	0.84**	1.0	1.5	70.3
		<200 μm	0.46	0.19	0.41	0.43	1.1	0.4	21.9
		<20 μm	1.27	0.51	0.40	0.56*	3.7	1.5	76.5
HB	February 2011	Unscreened	0.47	0.70	1.50	0.72*	4.6	6.9	62.3
		<200 μm	0.57	0.66	1.16	0.79*,a	6.1	7.1	63.1
		<20 μm	0.15	0.38	2.53	0.62*	1.2	3.1	33.9
	June 2011	Unscreened	0.82	1.08	1.32	0.98**	42.1	55.5	94.7
		<200 μm	0.80	1.20	1.50	0.90**	37.6	56.5	98.8
		<20 μm	0.77	1.22	1.58	0.95**	35.5	56.4	98.6
	December 2011	Unscreened	0.66	0.90	1.36	0.92**	12.6	17.0	79.9
		<200 μm	0.63	0.76	1.20	0.96**	11.1	13.4	71.5
		<20 μm	0.79	0.49	0.62	0.53	18.6	11.6	57.0
HNF	February 2011	Unscreened	0.46	0.36	0.78	0.18a	7.7	6.1	37.7
		<200 μm	0.50	0.39	0.78	0.70*,a	7.4	5.8	41.2
	June 2011	Unscreened	0.93	0.17	0.18	0.02	32.4	5.9	23.7
		<200 μm	0.99	0.42	0.42	0.14	33.0	14.0	56.0
	December 2011	Unscreened	0.21	0.26	1.23	0.30a	0.22	0.3	5.3
		<200 μm	0.25	0.77	3.08	0.67*,a	0.24	0.8	15.0

[a] One outlier removed (Gallegos, 1989).
* $p < 0.05$.
** $p < 0.01$.

Table 3 Initial concentration of Chl-*a* ($\mu g\ l^{-1}$) and contribution of different size groups and physico-chemical parameters from size fractioned dilution experiments.

Time	<20 μm Chl-*a*	20–200 μm Chl-*a*	<200 μm Chl-*a*	Temp. [°C]	NO_{2+3} [$\mu M\ l^{-1}$]	PO_4 [$\mu M\ l^{-1}$]	SiO_4 [$\mu M\ l^{-1}$]	N P^{-1}
February 2011	0.36	0.11	0.47	8.9	0.78	0.04	2.76	20
June 2011	0.48	1.04	1.52	23.7	1.22	0.03	1.57	41
December 2011	0.29	0.45	0.74	12.3	1.94	nd	5.79	2

nd, non-detectable.

of *Synechococcus* spp. were 0.35–0.46 d^{-1}, and grazing rates were 0.19–0.68 d^{-1}. Estimated daily *Synechococcus* spp. production was lower in February (0.4 μg C l^{-1}) than in December (1 μg C l^{-1}). Mean daily removal of *Synechococcus* spp. production by nano-microzooplankton was much higher in February (194%) than in December (41%). Despite differences between the unscreened and <200 μm fraction, estimated mean daily PP by *Synechococcus* spp. was nearly identical due to low initial biomass (Table 2). Comparing the <20 μm fraction with the <200 μm, the grazing rates increased in both June and December, but growth rates decreased in February and increased in December. Daily removal of production by nano-grazers drastically increased to 654% in February, while remained the same in December (40%) (Table 2).

Initial abundance of HB in experimental bottles was 2.67×10^9 cells l^{-1} in February, 5.71×10^9 cells l^{-1} in June and 1.27×10^9 cells l^{-1} in December. HB growth rates were 0.57–0.80 d^{-1} and grazing rates were 0.66–1.20 d^{-1}, in the <200 μm fraction. Both growth and grazing rates were higher in June. Estimates of mean daily BP (bacterial production) ranged between 6.1–37.6 μg C l^{-1} with lowest in February and highest in June. Nano-microzooplankton exerted substantial grazing pressure on bacteria, always removing >100% of daily BP. Overall, the unscreened and <200 μm fraction experiments yielded similar results (Table 2). In the <20 μm fraction, grazing rates were also higher than growth rates, except in December. Mean daily removal of BP ranged between 62–253%, with highest in February and lowest in December (Table 2).

Initial abundance of HNF in experimental bottles was 5.36×10^3 cells l^{-1} in February, 1.98×10^7 cells l^{-1} in June and 1.32×10^3 cells l^{-1} in December. In the <200 μm fraction, growth rates of HNF were 0.25–0.99 d^{-1}, with maxima in June. Grazing rates were 0.39–0.77 d^{-1}, with maxima in December. Mean daily HNF production changed from 7.4 μg C l^{-1} in February to 33 μg C l^{-1} in June to 0.24 μg C l^{-1} in December. Mean daily removal of HNF production ranged between 42% in June to 308% in December. A significant increase of grazing rates in June and December occurred from the unscreened to <200 μm treatments (Table 2).

4. Discussion

4.1. Community structure of autotrophic and heterotrophic plankton

The present study focuses on a monitoring station which could be taken as being representative of the coastal SE Black Sea, a critical fishing area for the Black Sea (Oguz et al., 2012a) and where the regional ecosystem health is considered to be a relatively less degraded ecosystem than the northern shelfs (Oguz et al., 2012b). The hydrography followed an expected pattern, with mixed waters in winter and thermally stratified waters in summer. Lower surface salinity was observed from spring to summer in agreement with seasonality of river discharges (Kara et al., 2008). The nutrients, in particular nitrate, were low, but in agreement with other reports for the southern Black Sea (Eker-Develi and Kideys, 2003).

The autotrophic communities showed clear changes throughout the year. From February to May, diatoms and ANF comprised the majority of Auto-C biomass (combined average ± SD contribution of 73 ± 7%), with similar contributions from both communities. In June, when the water column fully stratified, a major increase in dinoflagellates and coccolithophores (mostly *E. huxleyi*) biomass was observed. Summer blooms of these groups are common in the Black Sea (e.g. Agirbas et al., 2014; Eker-Develi and Kideys, 2003; Oguz and Merico, 2006). A prominent increase in *Synechococcus* spp. carbon biomass was also observed in June, as is the case in nutrient-depleted stratified waters (e.g. Agawin et al., 1998; Feyzioglu et al., 2004; Uysal, 2001). In the following months, the total Auto-C rapidly decreased and it became dominated primarily by A-Dino and secondarily by *Synechococcus* spp. In December, when the thermocline weakened, a return of diatoms was observed. Overall, an unexpected result was the low diatom biomass during the first half of the year when compared to those reported in previous years in the Black Sea (Agirbas et al., 2014; Eker et al., 1999; Eker-Develi and Kideys, 2003). One explanation is that our discrete sampling did not coincide with any bloom. An additional explanation was the warm winter of 2010/2011 in the SE Black Sea, which had the highest sea surface temperature winter-average of the preceding eight winters (Agirbas et al., 2015). The warm winter may have provided favourable conditions for both nutrient limitation and an earlier top-down control on A-Micro (e.g. Caron and Hutchins, 2012). In such conditions, the spring seasonal bloom is expected to either be weakened or absent (e.g. Oguz et al., 2006). Nevertheless, as discussed further on, estimated high growth rates of autotrophs during the February dilution experiment suggest no nutrient limitation, which in turn would point to high-turnover rates as an explanation for the low diatom biomass. The prominence of ANF until May as a contributor to Auto-C may be connected with the low diatom biomass or, perhaps, it is a common winter-period situation outside sporadic diatom blooms. Significant contributions of A-Nano to total Auto-C have been found in other regions during winter and outside seasonal blooms (e.g. Teixeira et al., 2011; Verity et al., 2002). Whether this is a typical feature of the regional lower trophic levels or the result of an anomalous winter, remains unclear and prompts for more studies in the area. In this study, no distinction was made between mixotrophs and strict heterotrophs, therefore the presence of mixotrophic dinoflagellates may have led to an overestimate in autotrophic biomass. However, summer biomass of mixotrophs is reported to be 4% of the total carbon biomass of the phagotrophic organisms in the NW Black Sea (Bouvier et al., 1998).

Heterotrophic bacteria contributed on average half of the Hetero-C biomass reaching the highest values in the summer months. Similar high (>50%) contributions of HB to Hetero-C have been found in other coastal regions (e.g. Dupuy et al., 2011; Linacre et al., 2012). Our HB carbon biomass fall within the range of previous studies in the Black Sea, though these were only focused on the spring and summer periods (Bouvier et al., 1998; Kopuz et al., 2012; Sorokin et al., 1995). Among the grazers, both HNF and H-Micro presented a seasonal succession with highest biomass in the spring-summer period. Generally, HNF carbon biomass was higher, with a notable exception in May during a ciliate bloom. These results are in agreement with other studies that report higher contributions of small heterotrophic flagellates outside productive

seasons (e.g. Calbet et al., 2008; Teixeira et al., 2011). The ciliate bloom on May might have been triggered by the onset of thermal stratification as previously reported (e.g. Becquevort et al., 2002; Dupuy et al., 2011). Between May and June, the reduction in H-Micro biomass, in particular, ciliate may reflect a top-down control by mesozooplankton, since ciliates can sometimes be a favoured food items for copepods (e.g. Atkinson, 1996; Calbet and Saiz, 2005).

Overall, the seasonal trajectories of <200 μm carbon biomass of autotrophic and heterotrophic plankton were similar. Significant correlations were found between ciliates and chlorophyll-*a* (Table 1), but no significant correlation is found between ciliates and the autotrophic groups exposing the weaknesses and difficulty of interpreting these correlations. An interesting finding for this coastal system was the high ratio of heterotrophic to autotrophic carbon. Instances where the biomass of heterotrophs exceeds that of autotrophs (e.g. Cho and Azam, 1990; Fuhrman et al., 1989; Gasol et al., 1997; Simon et al., 1992; Sorokin, 1977) are typical of the oligotrophic ocean and have been explained by high turnover rate of the autotrophic pool (Odum, 1971) or low nutrient input (Duarte et al., 2000). In this highly dynamic coastal region, the year of 2011 might have been an unusual year with low primary production and high recycling, a situation which can occur naturally because of climate variations. The excess of heterotrophic biomass, in particular HB, might also have occurred due to the high dissolved organic carbon concentrations in the Black Sea (~2 times higher than the open ocean; Ducklow et al., 2007).

4.2. Food web interactions

Our dilution experiments present valuable data on the growth and grazing dynamics within the microbial food web in the coastal SE Black Sea. This supplements the few such studies so far in the Black Sea (Bouvier et al., 1998; Stelmakh, 2013; Stelmakh et al., 2009; Stelmakh and Georgieva, 2014). Our experiments lack temporal coverage to resolve a complete seasonal cycle, but they do represent three contrasting scenarios of the microbial food web structure in 2011. Initial concentrations in the dilution experiments of the autotrophic and heterotrophic communities are shown in Fig. 5. In February, there was a high contribution (~50%) of ANF to total Auto-C biomass, thus representative of a mid-winter, non-bloom situation. The June experiment was performed during the most productive sampling, coincident with a bloom of prymnesiophytes and dinoflagellates, thus representative of the typical early-summer blooms in the SE Black Sea (e.g. Agirbas et al., 2014; Eker-Develi and Kideys, 2003; Oguz and Merico, 2006). Finally, the December experiment was characterized by a relatively low Auto-C biomass. A common situation to all experiments was the high ratio of HB to Auto-C biomass (0.7, 0.9 and 1.2 for February, June and December, respectively).

Nano- and microzooplankton play an important role in the carbon transfer to higher trophic levels and have been estimated to consume ~60% of daily PP in coastal waters (Calbet, 2008). In the Black Sea, annual removal of PP was reported as 65% (Stelmakh and Georgieva, 2014). In this study, considering the whole autotrophic community (<200 μm fraction experiment) the percentage of PP consumed by nano-microzooplankton was 46%, 21% and 30% in February, June and December, respectively. While the February estimate was within reported ranges, the June and December estimates were relatively lower than those in the literature. A possible reason for low grazing in June might be related to the concurrent bloom of *E. huxleyi*. Our size-fraction dilution experiments indicate that almost no production of <20 μm was consumed by nano-grazers. Low grazing rates concomitantly with a high biomass of *E. huxleyi* was also reported in the Northern Black Sea (Stelmakh, 2013; Stelmakh and Georgieva, 2014) and other regions (e.g. Fileman et al., 2002; Fredrickson and Strom, 2009; Strom et al., 2003). Nevertheless, we note that despite low grazing (21%), still a large portion of carbon (63 μg C l^{-1} d^{-1}) was being channelled to the grazers, indicating an active microbial food web. During December, the low community grazing might have been related to the very low growth rate, which in turn may indicate low nutritional quality of the autotrophic prey. Overall, for the three experiments, the grazing rates were always lower than growth rates, allowing the standing stock to grow. Estimated growth rates in February and June were high, which suggests autotrophs were not nutrient-limited although nutrients were scarce. Low nutrients do not necessarily imply that phytoplankton growth is under strong nutrient control since the nutrient reservoirs could be quickly renewed through remineralization by heterotrophs, especially during the high abundance of autotrophs in June. In December, although nitrate and silicate were higher than February and June, the phosphate was undetectable, which may have been the cause of the lowest growth recorded. Dilution experiments are not free of problems (Schmoker et al., 2013) and the high growth rates of February and June may have resulted from artefacts such as an increase in the nutrient pool (and remineralization) due to broken cells during filtration. Nevertheless, the experiments were meticulously done, filtration was slow (by gravity) and we have no reason to suspect this was the case. Also, the question remains of whether or not phytoplankton growth rates were affected during incubations as a result of no nutrient amendments. Another uncertainty in our data is related to mixotrophy. In the northwest Black Sea, based on uptake of fluorescently labelled prey, Bouvier et al. (1998) reported an absence of mixotrophic nanoflagellates and the occurrence of mixotrophic dinoflagellates in only one station, with grazing activity mainly on heterotrophic bacteria and nanoplankton. Since mixotrophs can be either grazers or prey they can confound our results. Some known mixotrophic ciliates (e.g. *Laboea* and *Strombidium* spp.) were found during microscopic examination (<15%), but no chloroplasts were observed.

In other regions, picophytoplankton have been shown to be an important contributor to the microbial food web (e.g. Azam et al., 1983). In the Black Sea, the biomass of *Synechococcus* spp. can be an important contributor to Auto-C (e.g. Kopuz et al., 2012), but the consumption by nano-micrograzers has not yet been reported. In this work, although they were significantly grazed (194% of daily *Synechococcus* PP was removed by nano-micro grazers in February), because of the low biomass and growth rates, carbon flow to grazers was lower than other groups. However, the importance of *Synechococcus* spp. to grazers might increase deeper in the water column since maximal biomass has been reported at the euphotic depth (~30 m) in the Black Sea

especially during thermal stratification (Kopuz et al., 2012; Uysal, 2001).

The importance of HB production and its consumption by microbial grazers has been documented for other coastal regions in the world (e.g. Linacre et al., 2012; Teixeira et al., 2011) but it is still poorly documented in the Black Sea (Bouvier et al., 1998). A clear pattern from our dilution experiments was that HB were always heavily grazed by nano-microzooplankton (daily removal of >100% BP) thus top-down control appear to play an important role in regulating HB biomass. Despite heavy predation, we observe relatively high HB biomass throughout the year, which may be maintained by the high dissolved organic carbon concentrations in the Black Sea (Ducklow et al., 2007). HB biomass may be controlled by several sources other than local phytoplankton production such as river-transported materials, terrestrial runoff, anthropogenic discharges, benthic fluxes, cycles of sediment resuspension and seasonal re-emergences of subsurface coloured dissolved organic matter (CDOM) accumulations (Lee et al., 2001). Non-planktonic sources may explain the imbalance observed in the experiment of December when there was not enough primary production to be converted to HB (considering 25% growth efficiency).

Our experimental data (<200 μm) revealed that autotrophic prey were more important than HB as a carbon source for grazers in February, but the reverse was observed in December when the grazing on autotrophs was not significant (for <200 μm), and the vast majority of total carbon comprised HB (~95% of total) (Fig. 7). However, carbon flow from autotrophs in particular from *Synechococcus* spp. was observed in <20 μm size fractioned. The preference of HB in December appears related as a strategy for available food resources since the total daily production (>200 μm autotrophs + HB = 14.3 μg C l^{-1} d^{-1}) was dominated by HB production (79%). In June, an intermediate situation was observed, with similar contributions of autotrophs and HB to total biomass. Both growth rates of autotrophs and HB were high, and a possible explanation for the bacterivorous pattern is that heterotrophic protists, in particular <20 μm, preferred to prey on the metabolically active bacteria (e.g. Gonzalez et al., 1990) rather than *E. huxleyi*, which might deter grazing. In February, low growth rates of HB and high growth rates of autotrophs, in particular ANF as indicated by the <20 μm size fraction experiment, appear sufficient to explain the preference of grazers towards autotrophs. There are no studies in the Black Sea comparing the carbon flow from autotrophs and heterotrophs to grazers, but our results indicate that nano- and micro-grazers seem to be sustained by more than one pattern of autotrophic and heterotrophic preys.

Our <20 μm size fraction experiment indicates that HNF seems to be an important grazer within the microbial food web. HNF grazed heavily on pico-sized prey in particular HB, which confirms the role of HNF as an important consumer in the Black Sea as reported in other regions (e.g. Andersen and Fenchel, 1985; Calbet et al., 2008; Dupuy et al., 2011; Fenchel, 1982). Comparing the experiments with and without grazers >20 μm, the small differences in HB grazing losses indicate that HB was mostly grazed by HNF. This is most notable in June and December when HB grazing losses where higher, HNF seem to be an important link of transferring HB carbon within the microbial food web. The importance of HNF as a consumer of small autotrophs was only evident in February when grazing losses of the <20 μm fraction were half of the <200 μm fraction, suggesting that a significant amount of carbon was transferred to grazers by small autotrophs. In June, these experiments suggest that grazers mainly preyed on >20 μm autotrophs, and in December, as previously described, the main carbon source was from heterotrophs. Given the high bacterial biomass of the region and high consumption of HNF production, then HNF would be a key organism to transfer this energy to microzooplankton which in turn are favourite prey for mesozooplankton (e.g. Atkinson, 1996; Calbet and Saiz, 2005; Stoecker and Capuzzo, 1990).

5. Conclusions

Heavily exploited fisheries and multiple ecosystem regime shifts in the Black Sea during the last half-century call for an understanding of the structure and functioning of regional microbial plankton communities. This is a first attempt to

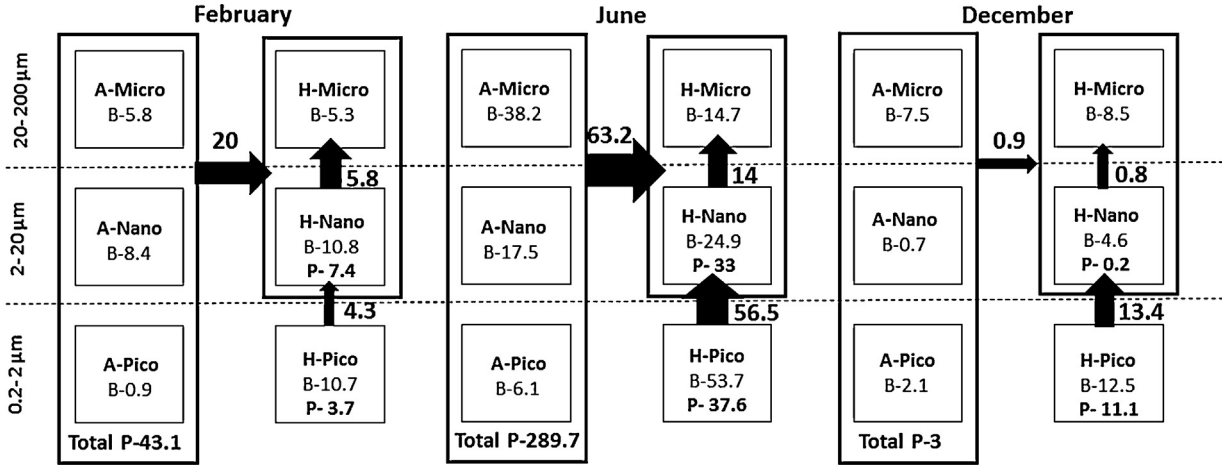

Figure 7 Schematic representation of carbon flow within microbial food web in the SE Black Sea during 2011. Arrows show daily grazing losses [μg C l^{-1} d^{-1}] according to estimation of <200 μm experiments. The thickness of arrows corresponds to amount of carbon flow from autotrophs, H-Pico and H-Nano. B, biomass [μg C l^{-1}]; P, daily production [μg C l^{-1} d^{-1}].

investigate the relative importance of pico-, nano- and microplankton communities in the Black Sea and the major trophic interactions between them. Small autotrophs and heterotrophic bacteria comprised an important compartment of plankton biomass and were important carbon sources for nano- and microzooplankton. Since nano- and microzooplankton are available for direct mesozooplankton ingestion, they might be considered as an important link between lower level production and higher trophic levels in the SE Black Sea, in particular in the years of low autotrophic production. The distinct carbon pathways found in the three experiments indicate that the system is complex and that it varies throughout the year, and possibly between years. There is a need to continuously monitor microbial plankton communities and understand their contribution to mesozooplankton diet, in particular copepods, which in turn support planktivorous fish stocks in the region. Dedicated experiments with continued long-term monitoring at fixed times and locations, with standardized techniques and additional measurements of CDOM pool are necessary. This study emphasizes that for realistic approaches to carbon cycling in the Black Sea, it is essential to consider trophic interactions between the full spectrum of prey and predator.

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