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Temporal variability of the microbial food web (viruses to ciliates) under the influence of the Black Sea Water inflow (N. Aegean, E. Mediterranean)

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Abstract

The entire pelagic microbial food web was studied during the winter-spring period in the frontal area of the North Aegean Sea. Abundance of viruses, heterotrophic bacteria, cyanobacteria, auto- and hetero-trophic flagellates, and ciliates, as well as bacterial production, were measured at three stations situated along a N-S transect, between the area directly influenced by the inflowing Black Sea water and the area covered by the Levantine water. Samples were collected in December 2009, and January, March, April, and May 2011. Station MD1 exhibited the highest values of abundance and integrated biomass of all microbial groups and bacterial production during all months, and MD3 the lowest. Bacteria dominated the total integrated biomass at all stations and months, followed by cyanobacteria, auto-, hetero-trophic flagellates and ciliates. On a temporal scale, the microbial food web was less important in March as all microbial parameters at all stations showed the lowest values. After the phytoplankton bloom in March, the heterotrophic part of the microbial food web (mainly) strongly increased, although the intensity of the phenomenon was diminished from North to South. Pico-sized plankton was found to be heterotrophic whereas nanoplankton was autotrophic. It appears that the influence of the Black Sea water on station MD1, permanent throughout the study period of early winter to late spring, was reflected in all microbial populations studied, and produced a more productive pelagic food web system, with potential consequences for the upper trophic levels.

Keywords: North Aegean Sea, front, viruses, bacteria, cyanobacteria, flagellates, ciliates.

Introduction

The Aegean Sea is the northeastern extension of the Eastern Mediterranean Sea and it is characterized by particularly complex hydrology, geography and topography (Theocharis *et al.,* 1993). It is connected to the Sea of Marmara through the Dardanelles Strait and to the Black Sea through the Bosporus Strait. This marine area receives freshwater input from rivers and streams discharging along its coastline, cold and brackish Black Sea water through the Dardanelles Strait, and warm and saline water from the Levantine Sea through the Cretan Straits (Poulos *et al.,* 1997).

The inflowing modified Black Sea water (BSW -low salinity S=29 and low temperature) occupies the surface layer (less than 40 m thickness) of the Northeast Aegean Sea; a strong thermohaline front (2 to 4°C difference in temperature and up to ten units difference in salinity) is formed close to the Dardanelles Strait when the BSW meets the waters of Levantine origin (LW) (Zervakis *et al.*, 2000). The position of the front depends on the dominant circulation, which varies temporally even at short scale (Zervakis & Georgopoulos, 2002; Pazi, 2008); however, the water column is permanently stratified in the frontal area.

The BSW inflow in the North Aegean has been found to be rich in dissolved organic carbon and dissolved organic nitrogen (Polat & Tugrul, 1996; Zeri *et al.,* 2014). Their concentrations, as well as those of inorganic nutrients, vary seasonally (Tugrul *et al.,* 2002). Sempéré *et al.* (2002) found high concentrations of total organic carbon in the North Aegean, particularly in the areas where the BSW signature was more pronounced.

Hydrological fronts are usually characterized by high levels of biological activity and active exchanges of energy and matter. The influence of the modified BSW inflow on the planktonic food web of the North Aegean has been examined during the last years. Significant variability occurred among stations for most of the microbial parameters tested, and the planktonic biomass and production revealed a gradual decrease from north to south in parallel with the progressive attenuation of the BSW signal (Christaki *et al.,* 1999; 2003; Pitta & Giannakourou, 2000). Ignatiades *et al.* (2002) reported that phytoplankton population

structure and dynamics are influenced by the different hydrographic conditions in the area, and picoplankton predominated during spring and late summer, accounting for more than 50% of Chl-α. Previous studies in the area have underlined the strong impact of this frontal structure on the zooplankton community (Isari *et al.,* 2006; Zervoudaki *et al.,* 2006; 2007; Siokou-Frangou *et al*., 2009; Siokou *et al*., 2013) and fisheries productivity (Tsagarakis *et al.,* 2010). A large part of the fixed carbon is channelled through the microbial food web (Siokou-Frangou *et al.,* 2002; Zervoudaki *et al.,* 2011).

However, most of the above studies have taken place during two contrasting time periods of the year: March, reflecting the cold mixing period, as opposed to September, reflecting the warm stratification period. To our knowledge, this is the first attempt to study the monthly evolution of the pelagic microbial food web of the highly variable, spatially and temporally, North Aegean Sea (NAS), especially during the phytoplankton pre-bloom, bloom and post-bloom periods, i.e. late winter and spring (Peliz, 2013). Vertical distributions and temporal variability of all microbial components, from viruses to ciliates, were analyzed regarding abundance, biomass as well as bacterial production. An attempt was made to examine the mutual relationships between microbial groups and to depict microbial food web functioning in the North Aegean-Black Sea interface. These new data will fill a gap in the study of the pelagic ecosystem structure and functioning in NAS as influenced by BSW, and could be used for the improvement of ecological models on the effects of BSW inflow variability on the NAS pelagic ecosystem (Petihakis *et al*., 2014).

Materials and Methods

Sampling and description of the study area

Sampling was performed on board the R/V AEGAEO in December 2009, and January, March, April, May 2011 in the eastern part of NAS. The one-year gap between the first and second cruise was due to logistic problems. Samples were collected at three stations MD1, MD2, MD3 (Fig. 1), which were positioned along a N-S transect and selected as representatives of the extreme contrasts across the thermohaline front associated with the presence of the Dardanelles inflow current in the northern part of the domain of interest. MD1 (65 m depth) was selected due to the permanent presence of BSW on its surface. MD2 (87 m) was positioned in an area between the Black Sea and Levantine water masses. Finally, the position of MD3 (290 m) was selected based on the permanent presence of Levantine-originated waters in that area.

During the study period, BSW and/or the formed halocline occupied the upper 10-meter layer of station MD1. In this layer, the lowest mean integrated salinity was observed in May 2011 (33.70) and the highest in December 2009 (38.32) (Supp. data, only on the electronic

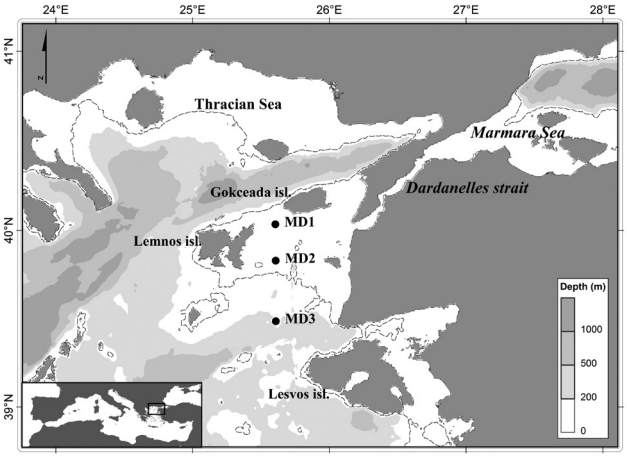

Fig. 1: Sampling stations in the Northeast Aegean Sea.

edition, Fig. S.1). Mean integrated temperature varied from 12.26° C (March 2011) to 17.76° C (December 2009). A strong halocline was found in the lower layer and the relevant values varied between 37.39 (January 2011) and 38.98 (December 2009), and from 13.88° C (March 2011) to 18.34° C (December 2009) (Zervakis, personal communication). At station MD2, the vertical gradient of salinity was very weak and occupied the upper 40-50 m, except in April 2011 when a stronger gradient was observed. Mean integrated salinity over the upper 40 m varied between 37.69 (April) and 39.09 (December and May), while temperature was minimum in March (13.62° C) and maximum in December (18.56° C). The upper 100 m layer of station MD3 was occupied by LW during the entire study period; the lowest mean integrated salinity over 100 m was found in January 2011 (38.9° C) and the highest in December 2009 (39.2), while relevant values of temperature varied from 15.3° C (April) to 18.8° C (December) (Zervakis, personal communication). A general decreasing trend of nitrates and phosphates was observed from MD1 to MD3; concentrations were highest in March, especially in the low salinity layer of MD1, and after a strong decline at all stations in April, a second increase was observed in the lower layer of MD1 in May (Pavlidou, personal communication). Chlorophyll *α* values decreased generally from MD1 to MD3. Chl-*a* peaked strongly in the upper 20 m of MD1 (maximum value 0.88 mg m^{-3}) and at a lesser degree of MD2 in March due to the important abundance of large diatoms; a very weak peak was observed in the upper 30 m of MD3 in April (Psarra, personal communication).

Temperature, salinity and water density were measured with a CTD. A thermo-salinograph, with water intake at 2.5 m below the surface, was used for the mapping of surface values of temperature and salinity, and thus for the assessment of the position and extent of the thermohaline front formed between the two water masses, BSW and LW (Zervakis, unpublished data). Water samples were collected at standard depths from surface to bot-

tom with Niskin bottles attached to a Rosette frame. The following parameters were determined: abundance and biomass of virus-like particles (VLP), heterotrophic bacteria (HBA), autotrophic cyanobacteria (CYANO), autotrophic flagellates (AF), heterotrophic flagellates (HF), ciliates (CIL) and bacterial production.

Virus-like particles, heterotrophic bacteria and cyanobacteria

Samples were preserved on board with glutaraldehyde (1% final concentration) for flow cytometric analysis. They were left for 15-30 min at 4°C, followed by flash freezing in liquid nitrogen (-196°C) and storage at -80°C until analysis. Autotrophic cyanobacteria (*Synechococcus* spp. and *Prochlorococcus*), heterotrophic bacteria, and viruses were enumerated using a FACS Calibur Flow Cytometer, according to Marie *et al.* (1999) and Brussaard (2004). One sample was used for the identification of two groups of autotrophic bacteria by their auto-fluorescence. A second sample was stained with SYBR Green I (1:10000 final solution) in order to enumerate the heterotrophic bacteria and viruses. Their DNA content was indirectly measured according to the proportionate amount of stain incorporated into their genetic material (higher fluorescence emission is considered to be a measurement of higher DNA content). The data were processed using Cell Quest and Paint A Gate software.

Abundance data were converted into C biomass using 20 fg C $cell^{-1}$ for heterotrophic bacteria (Lee & Fuhrman, 1987), 250 fg C $cell^{-1}$ for *Synechococcus* (Kana & Glibert, 1987) and 50 fg C $cell^{-1}$ for *Prochlorococcus* (Campbell *et al.,* 1994).

Bacterial production and bacteria growth rates

Bacterial production (BP) was measured using the [^{3}H] leucine method modified by Smith and Azam (1992). Water samples (1.5 ml) were collected, in triplicate, in 2 ml Eppedorf tubes and 50 µl of [4,5-^{3}H]-l-leucine (Amersham TRK 636, specific activity 165 Ci $mmol^{-1}$) was added at 20 nM final concentration. Controls received 90 µl of 100% trichloroacetic acid (TCA) before injection of tritiated leucine. All samples, including controls, were incubated for 2 h in the dark and at *in situ* temperature. Incubations were stopped with 90 µl of 100% TCA and the samples were stored at 4°C in the dark until further processing in the laboratory. Bacterial production was calculated according to Kirchman (1993), from [^{3}H] leucine incorporation rates. During the cruises, time series experiments were carried out in order to choose the appropriate incubation time for a certain level of activity. Concentration kinetic experiments were also performed in order to verify that the concentration of leucine added (20 nM) was sufficient to saturate incorporation (Van Wambeke *et al.,* 2002).

Specific Growth Rate (SGR) was calculated by dividing bacterial carbon production by bacterial biomass.

Heterotrophic and autotrophic flagellates

Flagellate counts were performed on 30 ml samples, fixed with borax-buffered formalin (final concentration 2% formaldehyde), filtered on black polycarbonate (Poretics) filters with 0.6 µm pore-size, and stained with DAPI (Porter & Feig, 1980) using an epifluorescence microscope. Auto- (AF) and hetero-trophic (HF) flagellates were distinguished using UV and blue excitation, and categorized into five different size-classes (from <3 to >10 µm) using an ocular micrometer. Formulas of approximate geometric shapes were used to calculate the biovolume of heterotrophic flagellates (W^2 L $\pi/6$ where L and W are the measured length and width of the cell). Biovolumes were converted into C biomass using 183 fg C μm^{-3} (Caron *et al.,* 1995).

Ciliates

Samples for ciliates were fixed with acid Lugol's solution (2% final concentration) and stored at 4°C until counting. For the analysis, 100 ml samples were left 24 h for sedimentation and were then examined using an Olympus IX70 inverted microscope, equipped for phase contrast, at 200x magnification. Ciliates were counted, distinguished into size-classes and major taxonomic groups, and identified down to genus or species level where possible. Ciliate cell sizes were measured using an ocular micrometer and converted into biovolumes by approximation to the nearest geometric shape from measurements of cell length and width. Biovolumes were converted into C biomass using 190 fg C μm^{-3} (Putt & Stoecker, 1989).

Statistical analyses

Statistical analyses were performed with STAT Graphics software. All variables compared were log10-transformed in order to attain normality and homogeneity of variances. Regression analyses were used to determine relationships between microbial parameters. The relationship between BP and BB was examined according to Ducklow (1992); the slope of the log-log regression between bacterial production (independent variable) and biomass indicates the strength of bottom-up control (slope $b > 0.6$ = strong BU control; 0.4 - 0.6 = moderate; < 0.4 = weak; < 0.2 = none).

Results

Abundance and biomass of microbial groups

Overall, station MD1 exhibited higher abundances of all groups (except for cyanobacteria) compared to stations MD2 and MD3 during all months (Table 1). Abundances of different groups ranged as follows: 0.5-10.3 x 10^7 VLP ml^{-1}; 3.1-21.6 x 10^5 heterotrophic bacteria ml^{-1}; 0.4-6.4 x 10^4 cyanobacteria ml^{-1}; 0.1-6.8 x 10^3 AF ml^{-1}; 0.04-3.4 x 10^3 HF ml^{-1}; and 0.3-23.6 x 10^2 ciliates L^{-1}. For almost all groups, the minimum value was measured at station MD3.

In addition, station MD1 differed from the two other

Table 1. Abundance (range and mean) of microbial populations and bacterial production (range and mean) at the top 65 m (station MD1) and 75 m (stations MD2 and MD3). VLP: virus-like particles, HBA: heterotrophic bacteria, CYANO: cyanobacteria Synechococcus and Prochlorococcus, AF: autotrophic flagellates, HF: heterotrophic flagellates, CIL: ciliates, BP: bacterial production, SGR: bacterial specific growth rate (bacterial production divided by bacterial biomass), VBR: virus-to-bacterium ratio.

	depth (m)	VLP 10^7 ml^{-1}	HBA 10^5 ml^{-1}	CYAN 10^4 ml^{-1}	AF 10^3 ml^{-1}	HF 10^3 ml^{-1}	CIL 10^2 L^{-1}	BP µg C m^{-3} h^{-1}	SGR day^{-1}	VBR
MD1	65									
Dec09		0.9-1.4 1.0	3.1-4.6 3.8	1.8-6.4 4.3	0.2-1.8 1.2	0.1-1.0 0.6	-	6.9-19.8 12.6	0.026-0.051 0.039	24-31 27
Jan11		1.1-2.4 1.7	5.0-8.6 6.9	0.5-3.9 3.0	0.4-3.7 1.9	0.2-2.1 1.0	0.8-21.0 12.2	2.9-28.4 16.8	0.007-0.053 0.028	20-32 25
Mar11		1.2-3.2 2.2	5.3-6.1 5.8	0.7-0.8 0.7	0.4-2.5 1.4	0.2-1.5 0.9	1.6-5.6 3.7	7.2-26.4 16.1	0.016-0.054 0.033	24-52 37
Apr11		1.5-4.3 2.8	6.7-10.1 8.5	0.7-2.8 1.7	0.9-5.5 2.7	0.3-3.4 1.5	2.2-23.6 12.4	12.1-51.6 31.3	0.022-0.072 0.043	22-47 31
May11		1.2-10.3 4.2	7.2-21.6 11.8	0.8-4.2 2.7	0.6-6.8 3.5	0.3-2.7 1.7	2.8-15.6 7.0	7.7-74.7 31.9	0.012-0.057 0.027	17-71 31
MD2	87									
Dec09		0.9-1.1 1.0	3.4-3.8 3.6	2.2-3.2 2.8	0.1-0.9 0.5	0.1-0.7 0.3	-	1.7-9.5 5.5	0.006-0.031 0.019	25-32 27
Jan11		1.5-1.9 1.7	4.6-5.7 5.3	1.2-2.7 2.1	0.3-3.5 1.5	0.2-1.4 0.6	2.0-22.2 12.4	4.8-20.2 10.8	0.017-0.044 0.027	31-35 33
Mar11		0.7-1.1 1.0	4.6-6.0 5.3	0.4-1.9 1.3	0.3-1.7 1.2	0.3-1.0 0.6	1.2-5.2 3.1	8.2-13.4 11.1	0.021-0.028 0.025	16-20 18
Apr11		1.1-2.9 1.8	6.9-9.1 8.3	0.9-2.7 2.0	0.6-1.7 1.2	0.3-1.3 0.7	3.4-12.0 6.9	3.1-29.3 19.6	0.004-0.04 0.033	12-42 22
May11		0.9-3.0 1.3	5.2-7.5 5.9	2.7-5.2 4.4	1.0-1.9 1.5	0.6-1.4 1.1	2.8-11.6 7.2	11.2-20.8 17.2	0.018-0.048 0.041	16-41 21
MD3	290									
Dec09		0.5-0.7 0.6	3.3-3.8 3.6	2.2-2.6 2.4	0.1-1.8 0.4	0.04-0.4 0.2	-	4.9-7.9 6.5	0.004-0.026 0.023	13-19 16
Jan11		1.0-1.6 1.4	4.3-6.1 5.3	1.8-2.0 1.9	1.1-2.3 1.7	0.6-1.1 0.8	6.2-9.4 7.9	9.4-15.2 12.7	0.012-0.036 0.029	17-34 27
Mar11		0.8-1.0 0.9	2.6-4.7 4.2	1.4-1.5 1.4	0.1-2.5 1.0	0.1-1.4 0.5	0.3-1.0 0.7	3.6-9.1 6.7	0.009-0.031 0.02	18-34 22
Apr11		0.8-1.0 1.0	6.7-9.2 7.6	3.0-4.1 3.7	0.6-1.8 1.1	0.4-1.4 0.7	3.4-6.6 5.0	11.1-22.2 16.0	0.017-0.032 0.025	10-14 13
May11		0.8-1.1 1.0	4.8-5.7 5.0	2.6-4.1 3.6	0.1-1.3 0.9	0.1-1.1 0.6	2.4-16.2 9.1	8.7-17.2 13.5	0.017-0.036 0.032	14-23 21

stations in terms of vertical distribution of microbial abundances (Fig. 2). The 20 m surface layer at station MD1 demonstrated much higher abundances of almost all groups than the lower layer, compared to stations MD2 and MD3, where planktonic organisms were distributed more uniformly. to stations MD2 and MD3, where planktonic organisms were distributed more uniformly. This trend was more pronounced mainly during April and May. The ratio of abundance at the surface to the abundance at 75m depth (65m for station MD1) was higher at station MD1 for all groups, indicating a sharper decrease of abundance from surface to deeper layers at station MD1 compared to stations MD2 and MD3 (data not shown). In detail, this ratio ranged from 1.7 to 9.2 at station MD1, from 1.0 to 4.3 at station MD2, and from only 0.9 to 2.8 at station MD3.

Integrated biomass was higher at station MD1 compared to stations MD2 and MD3 during all months in the case of heterotrophic bacteria and flagellates, but it was lower in most cases for cyanobacteria and ciliates (Fig. 3). However, this was true only for ciliate biomass, as integrated values of ciliate abundance were constantly, for all months, higher at station MD1 and lower at MD2<MD3.

Heterotrophic bacteria dominated by far the integrated biomass at all stations and during all sampling months (Supp. data Table S.1, only on the electronic edition). On average, heterotrophic bacteria made up 59%, cyanobacteria 20%, autotrophic flagellates 11%, heterotrophic flagellates 7%, and ciliates 3% of total integrated biomass calculated for all stations down to 50 m (data not shown).

Bacterial production

Bacterial production ranged from 1.7 to 74.7 µgC m^{-3} h^{-1}; at station MD1 it was 32% (temporal range 20-36%) and 40% higher (temporal range 22-57%) compared to stations MD2 and MD3, respectively (Table 1). Mean integrated bacterial production was higher at station MD1 during all months (max 1.22 mgC m^{-2} h^{-1} down to 50 m in April) (Fig. 4A). SGR ranged from 0.004 to 0.053

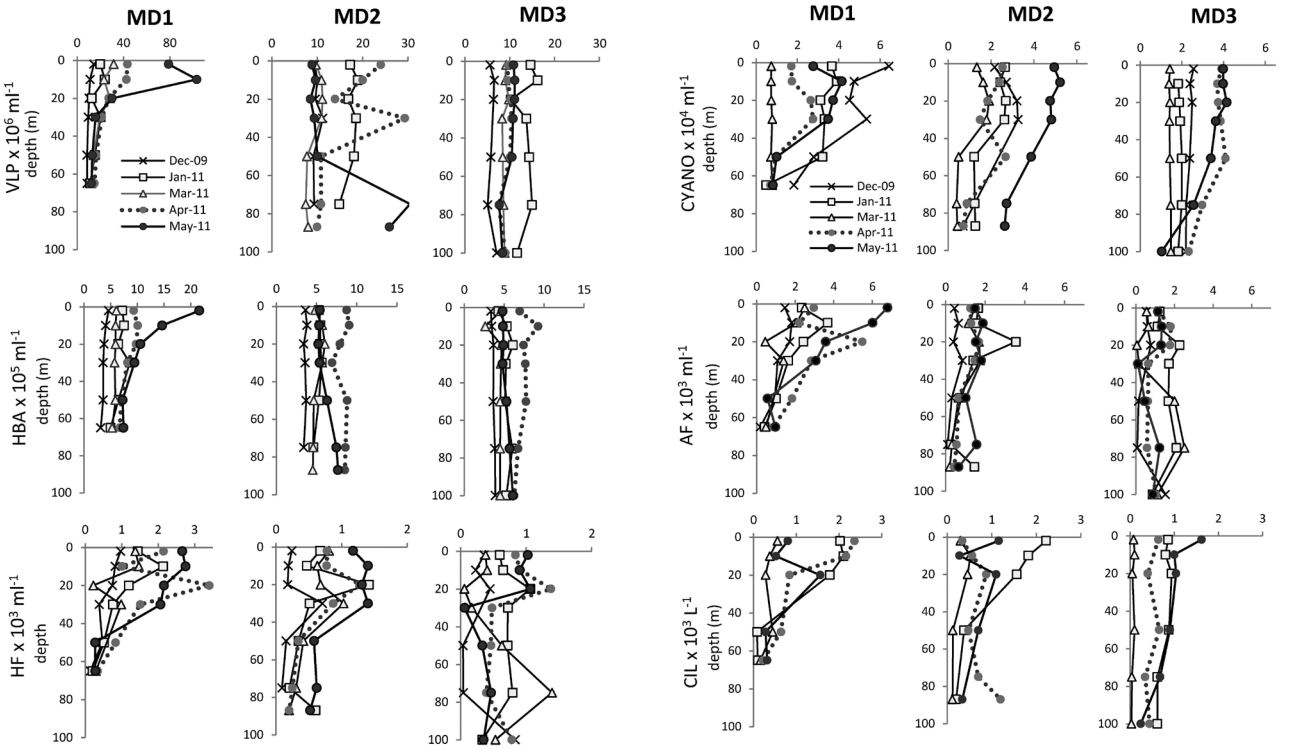

Fig 2: Vertical distribution of microbial population abundance at all stations during sampling months. VLP: virus-like particles, HBA: heterotrophic bacteria, HF: heterotrophic flagellates, CIL: ciliates, CYANO: cyanobacteria *Synechococcus* and *Prochlorococcus*, AF: autotrophic flagellates.

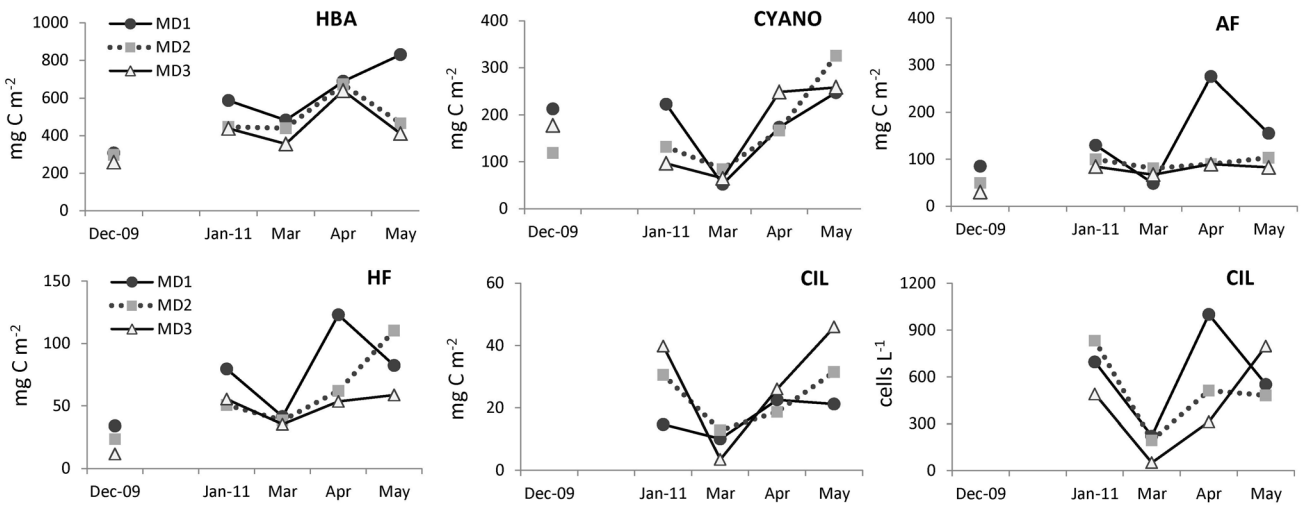

Fig. 3: Integrated biomass of different microbial populations at all stations during sampling months (calculated for the upper 50 m). HBA: heterotrophic bacteria, CYANO: cyanobacteria *Synechococcus* and *Prochlorococcus*, AF: autotrophic flagellates, HF: heterotrophic flagellates, CIL: ciliates. Ciliate abundance (cells L^{-1}) is presented as mean integrated values (divided by the depth of 50m).

d^{-1}; MD1 exhibited the highest values in the upper (0-20 m) layer during all months, decreasing at greater depths (data not shown) and from north to south at stations MD2 and MD3 (Table 1).

Temporal variability of microbial groups

In December 2009, integrated biomass (down to 50 m) for almost all groups (except cyanobacteria) showed the lowest values (Fig. 3). From January to May 2011, the temporal variability did not follow the same pattern for all microbial populations. On the one hand, integrated biomass of heterotrophic bacteria and flagellates, as well as bacterial production, remained constant or decreased in March, and then greatly increased to reach maximum values in April or May. This trend was more noticeable for MD1 and less pronounced for MD2<MD3. On the other hand, the autotrophic part of the microbial food web presented a completely different pattern. After Ja-

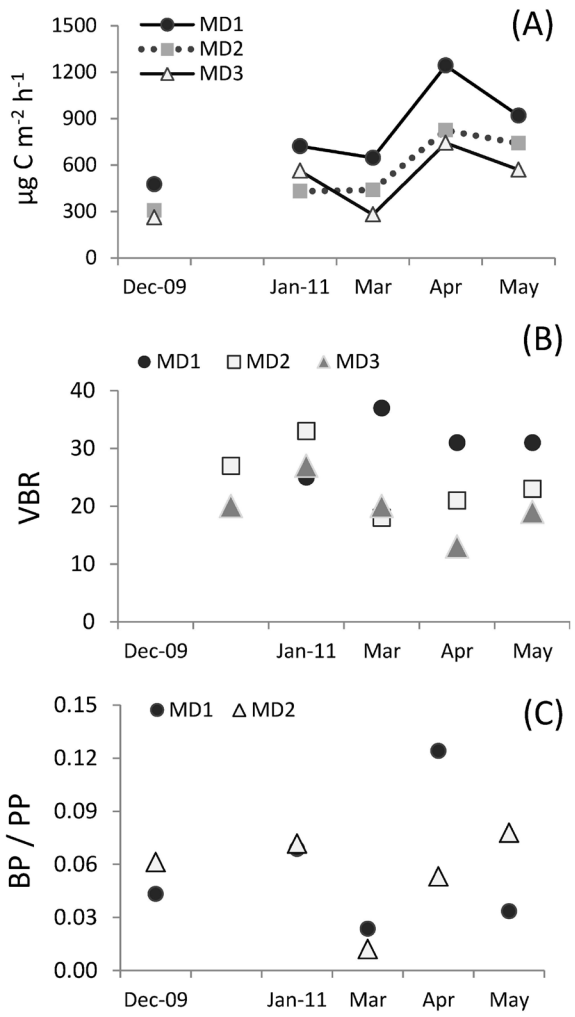

Fig. 4: Temporal variability of A) integrated bacterial production, calculated for the upper 50 m, B) VBR (Bacteria/Viruses abundance ratio) at all stations, C) Bacterial Production (BP)/Primary Production (PP) ratio. For B and C each point is a mean value from all sampling depths.

nuary, biomass did not increase with time. In the case of cyanobacteria, minimum values were measured in March, before and after which values were much higher. In the case of AF, concentration was rather constant, with the exception of station MD1 where integrated biomass increased and reached maximum values in April (Fig. 3). Cells <3µm dominated the AF biomass during this peak (data not shown).

Viral abundance showed minimum values in December at all stations, with low variability from January to May at stations MD2, MD3 (Table 1). In contrast, at station MD1, viruses displayed much greater abundance variability than all microbial groups. It is noteworthy that viruses remarkably increased after January, reaching the highest value in May. The virus-to-bacterium ratio (VBR) ranged from 10 to 71 at all stations (Table 1); higher values were recorded after March at station MD1 (Table 1). The highest ratio was found in May at 10 m depth at station MD1, decreasing when going deeper, and also towards stations MD2 and MD3.

High and low DNA bacteria and viruses

Two distinct populations of heterotrophic bacteria were recorded and separated by DNA content (Fig. 5). High-DNA content bacteria (mean abundance at all depths for each month) were slightly more abundant than low-DNA ones at all stations, except for December at MD2 and March at MD3. On average, high-DNA bacteria made up 59, 55, and 52% of total bacteria at stations MD1, MD2 and MD3, respectively.

Viruses consisted of three easily-distinguishable sub-populations according to their DNA content (Fig. 6). Low-DNA viruses far dominated at all stations during all months. Contributions of different sub-populations (mean abundance of all depths calculated for each month) varied at all stations with time, with no clear pattern. Station MD1 exhibited a higher contribution of low-DNA viruses (80%) compared to MD2 and MD3 (72 and 66%, respectively).

Community composition of ciliates

In January, the ciliate population was dominated by a small *Mesodinium* species at stations MD1 and MD2, which alone made up 55% and 32% of total ciliate abundance. During March, April and May, small oligotrich ciliates (<30 µm) dominated the ciliate populations (Fig.

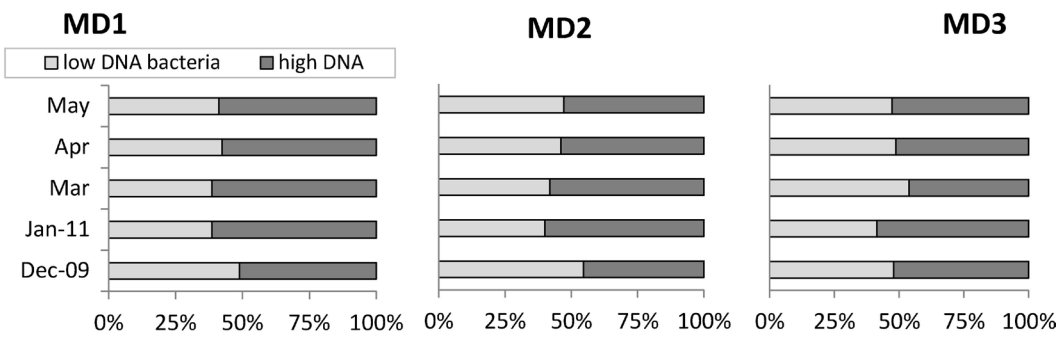

Fig. 5: Contribution of Low- and High-DNA content bacteria to total bacterial abundance at all stations during sampling months (mean value from all sampling depths).

7), more or less similarly, at MD1 and MD2 ; in April, however, oligotrichs even smaller than 20 μm made up the bulk (60%) of ciliate abundance at MD1. In contrast, at station MD3 in April and May, larger ciliates were present, and due to this fact, ciliate biomass showed higher values compared even to MD1, where much higher abundance was recorded.

Heterotrophy vs autotrophy

The picoplankton fraction (heterotrophic bacteria and cyanobacteria) of the food web was heterotrophic at all stations and during the whole period of investigation; heterotrophic to autotrophic biomass ratio varied from 1.6 at MD2 in May to 9.3 at MD1 in March (Fig. 8). On average, the H/A biomass ratio (calculated for the top 65-75m for MD1, MD2 and MD3, respectively) was highest at MD1 (5.3), decreasing to 4.6 at MD2, and reaching 3.2 at MD3. The most pronounced heterotrophic conditions were found at all three stations in March due to the minimum values of cyanobacteria abundance found.

The nanoplankton fraction (auto- and heterotrophic flagellates and nanociliates) was autotrophic in almost all cases except for MD3 in March and MD2 in May (Fig. 8). The heterotrophic to autotrophic biomass ratio (calculated for the top 65-75 m for all stations) did not vary a lot, either between stations or between months, ranging between 0.3 and 0.8.

Discussion

Microbial loop dynamics at the Black Sea - North Aegean interface

Our first objective was to complete documentation on the planktonic food web in NAS, in terms of vertical spatial and temporal distribution of all major components of the microbial food web. Our study is the first to have examined simultaneously all microbial components, from viruses to ciliates, on a temporal scale through the transect of the three stations. Overall, our results were in agreement with the reported north-to-south trends and the increased abundances

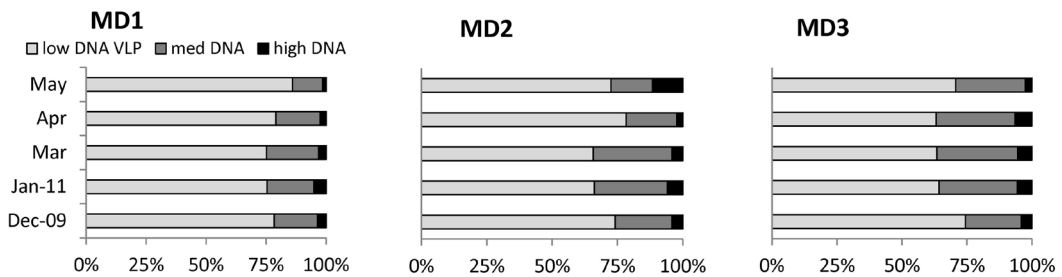

Fig. 6: Contribution of Low-, Medium- and High-DNA content viruses to total virus abundance at all stations during sampling months (mean value from all sampling depths).

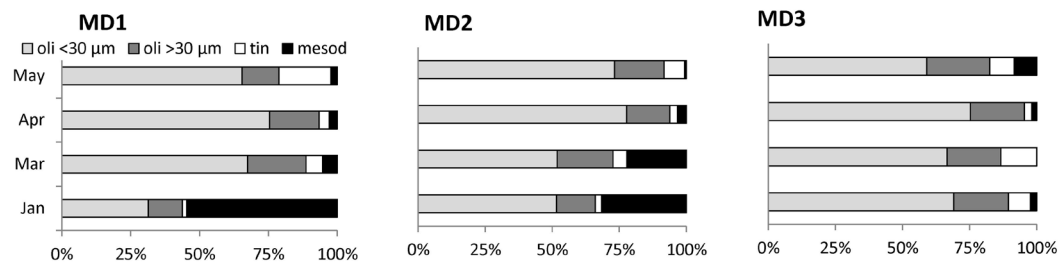

Fig. 7: Contribution of different sized ciliate groups at all stations during sampling months Oli: oligotrich ciliates, Tin: tintinnids, Mesod: *Mesodinium* spp. (mean value from all sampling depths).

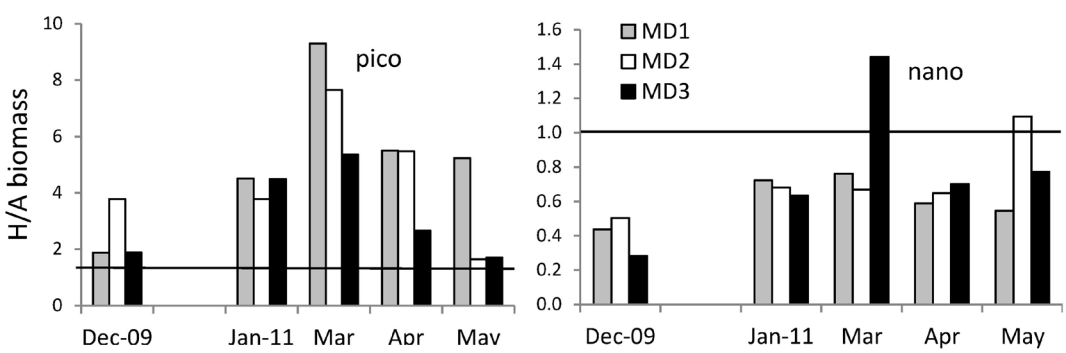

Fig. 8: Ratio of heterotrophic to autotrophic biomass for the pico- and nanofractions, at all stations during sampling months (calculated as mean value of the top 65-75 m at each station).

of different plankton groups in the northern area compared to other regions of the N. Aegean and to the S. Aegean (Christaki *et al*., 1999; 2003; Pitta & Giannakourou, 2000; Ignatiades *et al*., 2002; Siokou-Frangou *et al*., 2004; Isari *et al*., 2006; Zervoudaki *et al*., 2007), due to the impact of the inflowing BSW and the resulting North Aegean front.

Heterotrophic bacteria dominated the integrated biomass at all stations and months. That is not surprising, and has been observed in many other studies and places (Gasol *et al.,* 1997). Studies from frontal areas in the Mediterranean Sea, irrespective of the frontal origin, show an increase in bacterial production in the frontal zone (Christaki *et al.,* 1999; Moran *et al*., 2001; Van Wambeke *et al*., 2004). Bacterial biomass at station MD1 was 17% (temporal range 9-44%) and 25% higher (temporal range 7-51%) compared to stations MD2 and MD3, respectively, and SGRs were two-fold higher. This range in values is similar to that in the western part of the Mediterranean (Vaqué *et al.,* 2001; Weinbauer *et al.,* 2003; Christaki *et al.,* 2011; Van Wambeke *et al*., 2011), whereas at MD2 and, mainly, MD3 (Levantine water mass), slower-growing bacteria (Lykousis *et al.,* 2002) are probably subjected to P-limited conditions (Thingstad & Rassoulzadegan, 1999; Van Wambeke *et al.,* 2002). Results from temporal variation highlighted that increased bacterial activity at station MD1 is more or less a permanent characteristic throughout the sampling period and is related to the special hydrological conditions prevailing in the area.

The different vertical distribution among the three stations studied constitutes evidence of the influence of the BSW on the plankton food web of the studied area. Bacterial abundance at the front station MD1 was so strong in the surface layers (0-10 m) that the integrated values down to 50 m were still high. This pattern was observed to a lesser extent at station MD2, depending on the occurrence of the BSW, while the vertical variability in the upper 100 m of station MD3, more influenced by the Levantine waters, was less remarkable.

The slope of the log-log regression between bacterial biomass and production, as a criterion to assess the strength of bottom-up control (Ducklow, 1992), indicated a weak relationship at all stations (Table 2). Interestingly, as bacterial abundance does not seem to depend on resources, we could hypothesize a top-down control. High bacterial activity in frontal areas is usually consistent with an active heterotrophic community and top-down population control through viral lysis and protist grazing pressure (Hagström *et al.,* 1984; Bratbak *et al.,* 1990). At station MD1, a strong predator-prey dependence was indicated, as 69% of the HB population was regulated by HF (R^2=0.69, $p<0.001$). This dependence was much weaker for station MD3 (R^2=0.13 $p<0.01$) and not evident for station MD2 ($p>0.1$) (Table 2). It is well established that the impact of grazing on bacterial abundance is substantial in productive regions where bacteria are metabolically active and fast-growing (Sanders *et al.,* 1992). Del Giorgio *et al.* (1996) and Vaqué *et al.* (2001) reported that HF selectively consumed metabolically active bacteria, which were grazed more than four times faster than inactive ones. However, the lack of a strong HB – HF relationship at stations MD2 and MD3 may be due to a cascade effect.

Based on differences in the intensity of the SYBR Green I DNA-stain fluorescence, i.e. changes in nucleic acid content, two bacterial populations may be distinguished: High-DNA and low-DNA content bacteria. High-DNA bacteria are considered to be the most metabolically active prokaryotes, responsible for most of the activity in the microbial communities (Gasol *et al.,* 1999; Gasol & del Giorgio, 2000; Lebaron *et al.,* 2001, 2002), and are preferred by viruses (Bonilla-Findji *et al*., 2009). However, high-DNA bacteria abundance at station MD1 was more or less comparable to MD2 and probably due to cell losses from more effective grazing.

Virus densities were in general within the range found in the Mediterranean Sea (Bettarel *et al*., 2002; Weinbauer, 2004; Boras *et al.,* 2009). However, the lowest values never dropped below $3x10^6$ VLP ml^{-1}, in contrast to other parts of the Mediterranean Sea (Weinbauer *et al.,* 2003; Magagnini *et al,*. 2007; Magiopoulos & Pitta, 2010; Christaki *et al.,* 2011). The highest values recorded at station MD1 far exceeded the ones mentioned by these authors in the oligotrophic areas of the Mediterranean

Table 2. Linear regressions between microbial variables. BP: bacterial production, HBA: heterotrophic bacteria, VLP: virus-like particles, HNF: heterotrophic flagellates. Analyses were carried out with log10-transformed data; *b* = slope, *R-squared* = coefficient of determination, *p* = probability, *n* = number of points (all 5 month samplings).

	variable (x)	*variable (y)*	R^2	*b*	*p*	*n*
MD1	HBA	BP	0.51	0.392	<0.001	30
MD2	HBA	BP	0.39	0.290	<0.001	30
MD3	HBA	BP	0.31	0.213	<0.001	45
MD1	HBA	VLP	0.70		<0.001	30
MD2	HBA	VLP	0.24		<0.001	30
MD3	HBA	VLP	0.20		<0.001	45
MD1	HBA	HF	0.69		<0.001	30
MD2	HBA	HF	0.13		<0.01	30
MD3	HBA	HF	0.18		0.29	45

Sea, indicating the impact of the BSW enrichment.

Virus relative significance compared to bacteria (i.e. the VBR) varied over a wide range. Viral abundance shows seasonal variations, usually following the host's abundance variations (Weinbauer, 2004 and references therein). Additionally, the much higher VBR found at station MD1 after March indicates a high ability of viruses to find new hosts, as increased bacterial growth rates allowed phage replication; the opposite of the slower-growing bacteria at stations MD2 and MD3. Weinbauer & Hofle (1998) reported that viruses prefer to infect metabolically active cells. The high VBR found at station MD1, reaching ~70 in surface waters, suggested that viral abundance was influenced by BSW inflow and nutrient enrichment via the presence of a higher abundance of their prokaryotic hosts. Bongiorni *et al.* (2005) also reported higher VLP abundance in more "eutrophic" areas. Yang *et al.* (2010) reported that deviations from the 10:1 ratio suggest enhanced viral-mediated bacterial mortality in bulk seawater. Virus abundance showed a tight correlation with heterotrophic bacteria at all stations, implying that heterotrophic bacteria were important virus hosts as virus abundance explained 70% of bacteria variance. At stations MD2 and MD3, viruses explained 23.5% and 20.2% of HB variance, respectively (Table 2).

In general, our results agree with previous studies where the variability in BP, relative to PP, is higher in the frontal areas (Pedros-Alio *et al.,* 1999; Cho *et al.,* 2001). The observed distribution of BP/PP ratios at stations MD1 and MD2 varied from 0.012 to 0.124 (Fig. 4C), being within the range of 0.001 to 1.90 reported for coastal and open-ocean waters (Cole *et al*., 1988; Cho *et al*., 2001 and references therein). The decrease in March coincided with the phytoplankton bloom, when measurements of Chl-*a* and primary production showed maximum values (Psarra, unpublished data). A striking observation is the increase of the BP/PP ratio in April at station MD1, consistent with the increase of BP values. It seems that enhanced bacterial growth occurred after large phytoplankton had consumed available nutrients (by March), driving bacterial population growth and the development of their potential predators, namely heterotrophic nanoflagellates and ciliates. This pattern was more evident in the upper layer of station MD1, probably due to adequate quality of labile-dissolved organic carbon from phytoplankton exudates or DOC availability through the inflowing BSW. Furthermore, DOC minimum values were found in March and increased until May (Zeri, personal communication).

We can hypothesize that organic material produced at the surface during the spring bloom sinks to underlying layers. These sinking particles serve as an important site for bacterial mineralization of organic matter within the water column, and thus contribute to nutrient recycling. According to Souvermezoglou *et al*. (2014) the nutrient content of BSW decreases en route from the frontal area in the North Aegean area for the production of organic material. This is confirmed by the increased values of BP, PP and copepod production observed in the area closest to the Dardanelles Strait (Siokou-Frangou *et al.*, 2002), as well as from the fact that POC exported from the BSW layer increased progressively along a drifter track (Frangoulis *et al.,* 2010) during a Lagragian experiment in the area. The analysis of a 30-year nutrient data set from this area (Souvermezoglou *et al.* 2014) confirms that the degradation of sinking organic matter, carried by the BSW and/or produced in the surface layer, enriches the underlying Levantine water (LIW) with inorganic nutrients.

High viral abundance, tightly linked to that of their prokaryote hosts (mainly station MD1), can play a significant role in the NAS microbial food web. If the 'kill-the-winner' hypothesis is true, the dense phage population could rapidly induce dramatic lytic effects in the bacterial community. Viral-mediated mortality of bacteria can lead to DOM and carbon recycling within the microbial loop (Weinbauer & Peduzzi, 2004), allowing the retention of nutrients in the water column (Bratbak *et al*., 1990). As pointed out in Middelboe *&* Jørgensen (2006), viral lysates constitute a significant source of D-amino acids. It is interesting to note that Zeri *et al.* (2014) observed considerable enrichment of a tyrosine-like fluorophore in the N. Aegean waters, which is not only transported to the N. Aegean through the Dardanelles but was also produced *in situ*. Aminoacids are linked to this fluorophore, according to Yamashita & Tanoue (2006).

High mesozooplankton standing stock values are considered a characteristic of the layer occupied by fresh BSW or of the halocline layer of the frontal area (Zervoudaki *et al.,* 2007). Ciliate abundance followed the same pattern of decreasing abundance from station MD1 to MD3; however, mean depth integrated biomass was kept at low levels after the phytoplankton bloom in March (<25 mgC m^{-2} down to 50 m), mainly at the frontal station MD1. Mesozooplankton abundance and biomass was higher by far at MD1 compared to the other stations (Siokou *et al.,* 2014) suggesting a top down control on ciliate populations.

Conclusion

In general, our observations support the idea that viruses, pico-, nano- and micro-plankton constitute an important component of the planktonic food web in the BSW-influenced North Aegean; they are important producers, consumers, and mineralizers of organic material. On a spatial scale, at station MD1, directly influenced by the BSW inflow in the NAS, an active microbial food web explained the high microbial standing stocks and rates observed with decreasing intensity along the N-S gradient. On a temporal scale, the microbial food web was less important in March when all microbial parameters at all stations showed the lower values. Earlier in the winter (January), or even a year earlier in December,

microbial components showed higher values. After the phytoplankton bloom in March, the heterotrophic part of the microbial food web (mainly) strongly increased, although the intensity of the phenomenon diminished with decreasing influence of BSW. It appears that recycling processes mediated by heterotrophs and bacteria (particularly) as well as viruses are crucial for maintaining the structure and functioning of the planktonic community in NAS. On the other hand, carbon supply from the microbial food web could sustain higher trophic levels such as mesozooplankton (Siokou *et al.,* 2014).

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