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Effect of invasive ctenophores *Mnemiopsis leidyi* and *Beroe ovata* on low trophic webs of the Black Sea ecosystem

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ABSTRACT

The study focuses on the impact of life excretion and mucus released by the "biological pollutants" invasive ctenophore *Mnemiopsis leidyi* and its predator *Beroe ovata* on the marine environment and lower trophic levels of the Black Sea ecosystem (bacteria, pico-phytoplankton, nano-autotrophic/heterotrophic flagellates, micro-phytoplankton, chlorophyll *a*, primary production (PP), micro-zooplankton). The chemical and biological variables were analysed in two sets of lab experiments with natural communities from mesotrophic (Gelendzhik) and eutrophic (Varna) coastal waters. While both species altered the chemical properties of experimental media, exerting structural and functional changes in the low food-web biological compartments, the results showed a stronger effect of *B. ovata*, most likely related to the measured higher rate of excretion and amount of released mucus. In addition the alterations in the Gelendzhik experiment were more pronounced, indicating that environmental implications on lower food-web are more conspicuous in mesotrophic than in eutrophic coastal waters.

1. Introduction

The ctenophore *Mnemiopsis leidyi* A. Agassiz, 1965 is the most harmful predator that has widely invaded the seas of Eurasia during the last decades (Shiganova et al., 2014). Introduced into the Black Sea by ships' ballast waters from the Gulf of Mexico (Vinogradov et al., 1989; Ghabooli et al., 2010) its rapid colonisation caused cascading effects on the marine ecosystem planktonic and benthic food web structure, ecosystem function and fishery (Shiganova et al., 2004a, b; Daskalov et al., 2007; Katsanevakis et al., 2014). As a zooplanktivorous predator it has greatly decreased edible zooplankton and meroplankton standing stocks, fish eggs and small larvae abundance and species diversity (Oguz and Gilbert, 2007; Costello et al., 2012). Since 1997 with the invasion of its predator ctenophore *Beroe ovata* sensu Mayer 1912 (Konsulov and Kamburska, 1998) the *M. leidyi* pressure significantly relaxed (Shiganova et al., 2014; Finenko et al., 2003). However, two invasive ctenophores still act as biological pollutants being the key drivers of the Black Sea ecosystem functioning. While a wealth of studies focus on the predator-prey interactions (Granéli and Turner, 2002) and their impacts on fish stock recruitment and abundance (Oguz et al., 2008), the effects on water chemical properties and lower trophic level communities are poorly addressed (Dinasquet et al., 2012a). Only some aspects of these processes were studied previously. Among them we may mentioned listed below. Condon et al. (2011) have demonstrated the overall effect of gelatinous plankton on dissolved organic carbon that promotes microbial respiration, further fuelling the microbial loop. Pitt et al. (2009) have documented the influence of jellyfish blooms on carbon, nitrogen, and phosphorus cycling with implications for the biogeochemical regimes of the environment. Daniels and Breitbart (2012) examined the composition and dynamics of bacterial communities associated with ctenophores, suggesting that these microbial consortia may play important roles in ctenophore ecology. In addition in several papers it was shown that gelatinous species may stimulate bacterioplankton growth by direct release of nutrients from tissue, mucus secretion and excretion (Hansson and Norrman, 1995; Titelman et al., 2006; Breitbart et al., 2015).

In other studies have been estimated that excretion of ammonium (NH_4) by jellyfish populations could theoretically support 8% of the nitrogen requirements for phytoplankton in Lake Illawarra, Australia (Pitt et al., 2009), 11% of the requirements in the Kiel Bight, Western

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Baltic (Schneider, 1989), and 10% of the requirements in the Inland Sea of Japan (Shimauchi and Uye, 2007).

A manipulative mesocosm experiment conducted in a saline coastal lake in Australia suggested that blooms of both zooxanthellate and non-zooxanthellate jellyfish deplete mesozooplankton and alter the composition of microzooplankton assemblages via top-down processes, while excretion of nutrients by blooms of non-zooxanthellate jellyfish can greatly increase phytoplankton production and favour algal blooms (West et al., 2009). In another study authors found out that scyphozoan medusa *Aurelia aurita* organic matter excretion affected the ambient bacterial community that induced changes in the bacterial growth rates and community structure (Tinta et al., 2016). Experiment conducted in a nutrient limited estuarine system (The Baltic Sea) showed that direct and indirect short-term effects of *M. leidyi* on the food web is limited to higher trophic levels, indicating that top-down and bottom-up consequences on the microbial loop likely depend on local nutrient conditions (Dinasquet et al., 2012b). So, to date, to our knowledge, there has not been a comparative investigation of the complexity of the effects of predator-prey ctenophores *Mnemiopsis–Beroe* on nutrient concentrations and all lower trophic level components of the marine ecosystem.

The goal of the present study was to investigate the effect of excretion and mucus secretion by the ctenophores *M. leidyi* and *B. ovata* on chemical variables of the environment, and on structural and functional traits of the lower food-web communities of the Black Sea ecosystem: bacteria, pico-phytoplankton, nano-autotrophic/heterotrophic flagellates, micro-phytoplankton, chlorophyll *a*, primary production, and micro-zooplankton in two different environments - mesotrophic and eutrophic coastal areas.

2. Material and methods

2.1. Experimental design

Two sets of lab experiments were conducted in the coastal waters of the Black Sea-NE Black Sea (Blue Bay near the town of Gelendzhik) in August 2013, and the NW Black Sea (Varna Bay) in August 2014. Water for the experiments was collected in the Blue Bay (long. 37.98 E, lat. 44.57 N, depth 7.5 m) and in the Varna Bay (long. 27.92 E, lat. 43.13 N, depth 4.5 m). The Gelendzhik coastal area (G) is an example of mesotrophic waters, with a primary production (PP) of 120–500 mg C m^{-2} day^{-1}, whereas the Varna coastal region (V) is an eutrophic area with PP ranging from 500 to 1200 mg C m^{-2} day^{-1} (Demidov, 2008). The experiments were performed in aquaria in laboratory conditions, designed to analyse the effects of two ctenophores on chemical parameters (pH, O_2, nutrients N, P, Si, and their ratios) and the lower trophic web communities: bacteria, autotrophic/heterotrophic flagellates, phytoplankton species composition, abundance and biomass, including nano-micro-phytoplankton and micro-zooplankton. Triplicates of each experimental treatment: control (C), *Mnemiopsis leidyi* (M), and *Beroe ovata* (B) were prepared in 12 l aquaria and incubated under the same light-dark (12:12 h) and temperature (~24 °C) regime for 5 (Gelendzhik) and 7 days (Varna bay) (Table 2.1). The different manipulated light conditions in the two experimental sets reflected the differences in the initial transparency in two coastal waters environments (the Secchi disk depths in the Varna and Blue bays were about 1.5 and 6 m, respectively).

Ctenophores were collected carefully by hand net. The experimental number of specimen was fixed to concentrations corresponding to the ctenophore specific bloom ranges reported for the coastal waters of the Black Sea (Shiganova et al., 2014; Kamburska and Stefanova, 2005). In each aquarium the number of ctenophores was adjusted to approximately similar total wet weights (Table 2.1).

At the start of the experiments (day 0) fresh marine water was collected from the coastal area, filtered through zooplankton net with mesh size of 180 μm to remove mesozooplankton, then placed in a big plastic tank and mixed well to ensure homogenisation prior to filling the aquaria. All chemical and biological variables were measured before filling the aquaria in 3 replicates to quantify the initial concentrations. During the course of the experiments chemical parameters were measured daily, while the biotic – on day 2, day 4 and day 7 (Varna). Prior to sampling, the media in the aquaria was gently mixed. In Gelendzhik experiment, the water volumes sampled for analyses were as follows: chemistry (0.5 l), phytoplankton (0.5 l), PP and Chl (0.3 l), nanophytoplankton + bacteria (0.05 ml). The total volume of water sampled from each aquarium was 1.3 + 1 = 2.3 l, leaving 9.7 l at the end of experiment, e.g. 74% from initial water volume in the aquaria.

In Varna experiment, the same volumes for analyses were sampled for chemistry (0.3 l), phytoplankton (0.5 l), PP and Chl (0.3 l), nanophytoplankton + bacteria (0.05 ml) plus micro-zooplankton (0.25 l), reducing the initial volume of the experimental media to about 7 l at the end of experiment (e.g. which 60% from initial volume, due to testing an additional parameter, micro-zooplankton).

We intentionally removed mesozooplankton from experimental water through sieve (mesh size 180 μm) to eliminate grazing of zooplankton by *M. leidyi* and to avoid grazing of phytoplankton by mesozooplankton in order to have comparable test conditions in the two ctenophore experiments because *B. ovata* does not feed on zooplankton. Otherwise grazing could distort the effect of ctenophores on lower trophic levels.

2.2. Quantifying of mucus released by ctenophores

Three individuals of each ctenophore species, *M. leidyi* (M) and *B. ovata* (B) were measured, weighted, and each individual placed in sterilized 500 ml aquaria with sterilized water. After 24 h, the ctenophores were removed and the water was filtered through preliminarily weighted 0.2 μm Nucleopore filters. After that the filters were dried at 60 °C until a constant dry weight. The difference between the filter initial and final weight was equal to the dry weight of mucus released by one ctenophore for 24 h. Each ctenophore specimen was also dried in

Table 2.1
Initial ctenophore settings of the lab experiments in Gelendzhik and Varna.

Treatment	Replicate	Gelendzhik				Varna			
		N ind.	Total WW g	T °C	Light intensity μmol quanta m^{-2} s^{-1}	N ind.	Total WW g	T °C	Light intensity μmol quanta m^{-2} s^{-1}
M. leidyi	1	4	39	24.0	300	7	31.78	24.3	120
	2	4	39	24.0	300	7	29.3	24.3	120
	3	4	43	24.0	300	7	29.4	24.3	120
B. ovata	1	3	26	24.0	300	3	22.8	24.3	120
	2	3	22.5	24.0	300	3	22.93	24.3	120
	3	3	22.5	24.0	300	3	20.65	24.3	120

N ind. – number of ctenophore specimen in aquarium; WW – wet weight [g]; T – temperature; 1, 2, 3 - replicates.

order to measure the individual dry weight (DW) and to estimate the amount of released mucus per ctenophore dry weight/24 h. All measurements were done in 3 replicates.

2.3. Sampling and processing of chemical data

The concentration of nutrients in the water was analysed using standard methods according to Grashoff et al. (1999): inorganic phosphorus (P-PO_4) by colorimetry, using the modified Murphy and Riley method (Hansen and Koroleff, 1999); silicates (Si), colorimetrically according to the blue silicon–molybdenum complex (Koroleff, 1983); nitrites (N-NO_2), colorimetrically (Hansen and Koroleff, 1999); nitrates (N-NO_3), colorimetrically after reduction to nitrite nitrogen on cadmium columns; ammonium nitrogen (N-NH_4), colorimetrically using the Sagi–Solorzano method (Solorzano, 1969). The concentration of dissolved oxygen was measured by Winkler method (Hansen, 1999).

The suspended solids and the 5-day biochemical oxygen demand (BOD) determination was conducted using the procedure outlined in Standard Methods (AWWA, 1960).

2.4. Sampling and processing of bacterioplankton

Water samples (3 ml) were collected by sterile plastic tubes from three discrete locations in each aquarium. Total number of bacteria was analysed with fluorochrome acridine orange (AO). Subsamples (1 ml) of each water sample were fixed with formalin solution (4%) and stained with AO aqueous solution (0.01%). After incubation within 5 min these subsamples were filtered through a black 0.22-μm Nucleopore filter.

Total number of active bacteria was analysed with fluorochrome 5-cyano-2.3-ditolyl tetrazolium chloride (CTC) following Hauer and Lamberti (2006). Water subsamples (2 ml) were incubated with CTC in concentration of 5 mM within 4 h in situ. After incubation, the sample was fixed by formalin solution (4%) and concentrated on a black 0.22-μm Nucleopore filter.

The obtained slides were immediately examined microscopically (FLUOVAL, Carl Zeiss, Yena) using blue excitation (magnification of 1000). Not < 20 fields of vision were counted for each preparation, with not < 200 bacterial cells were counted.

2.5. Sampling and processing of micro-zooplankton

250 ml water from each aquarium was sampled in dark glass vials and immediately preserved to a final concentration of 2% Lugol's solution (Kurilov and Gavrilova, 2015). Samples were settled and concentrated to 25 ml by siphoning out the supernatant. For micro-zooplankton taxonomy and quantification, 1 ml from the concentrated sample was examined under inverted Nikon Eclipse TE2000-U microscope (image analysis L.U.C.I.A. Version 4.8, Laboratory Imaging Ltd., Prague) at 400× magnification in a Sedgwick-Rafter counting chamber. Micro-zooplankton species taxonomic identification was done according to Mordukhay-Boltovskoy (1968), Abboud-abi Saab (2008) and Petz (1999) to the lowest possible taxonomic level. The calculation of biovolume was based on measurements according to the species specific geometric shape (Kurilov and Gavrilova, 2015).

2.6. Sampling and processing of micro-phytoplankton

Water samples (500 ml) from each aquarium were fixed in a 4% formaldehyde solution, buffered to pH 8–8.2 with disodium tetraborate. The samples were concentrated down to 50 cm^3 by slow decantation after storage for at least 20 days in a cool and dark place. Taxonomic identification and cell counts were done under an inverted microscope (Nikon T300E) connected to a video-interactive image analysis system (L.U.C.I.A., Version 4.8, Laboratory Imaging Ltd., Prague) at 400× magnification in Sedgwick-Rafter counting chambers. Four hundred cells were counted in each sample, while rare and large species were checked in the whole counting chamber (Moncheva and Parr, 2005, updated 2015). The individual cell biovolume (μm^3) was derived through the approximation of the cell shape of each species to the most appropriate geometric solid, calculated using the appropriate equations (Edler, 1979; Vadrucci et al., 2007), and averaged over measurements of ten cells from each species. Cell biovolume was converted to weight (ng) following Hosia and Fal Hutchinson (1967). Species identification was done mainly after taxonomic nomenclature according to the on-line data-base of the World Register of Marine Species (WoRMS) - http://www.marinespecies.org/. The QC/QA of the data was performed following the quality control guidelines for phytoplankton (Moncheva, 2010).

2.7. Sampling and processing of pico- and nano-phytoplankton

Small phytoplankton (< 6 μm) was enumerated by epifluorescent microscopy. Aliquots (10 ml) of the collected samples were fixed with glutaraldehyde (up to 1% final concentration). After 20 min, the aliquots were filtered through 0.2 μm Nucleopore black filters, and the algal cells were stained with primulin in the filtering funnel (Caron, 1983). After filtering, slides with the filters were frozen at −22 °C, and transported to a laboratory in Moscow. Within 2–3 weeks, the slides were examined microscopically (FLUOVAL, Carl Zeiss, Yena) using blue excitation. Eukaryotes had a gradation of red color, unicellular cyanobacteria (< 2 μm) fluoresced yellow-orange, and heterotrophic cells were green-colored. Cells were counted at magnification 1000× using transects or microscopic fields of vision. Only a part of the total filter area was examined. In the case of nano-phytoplankton (from 2 to 6 μm), this part corresponded to 0.1–0.4 m1 of the filtered water. In the case of pico-phytoplankton (cell size < 2 μm) the examined volume varied from 0.001 to 0.02 ml.

2.8. Measurements of primary production and chlorophyll

Primary production (PP) was measured by the C^{14} radiocarbon method (Steemann Nielsen, 1952) under simulated light conditions at constant artificial illumination. The water samples (50 ml) were exposed after the addition of 0.05 μCi per 1 ml sample. Exposition (3 h) was carried out in a phyto-incubator with individual LED illumination. Afterwards, incubation flasks were filtered onto 0.45 μm "Vladipore" membrane (Russia). The radioactivity of the samples was determined using a Triathler (Hidex, Finland) liquid scintillation counter.

The chlorophyll *a* concentration (Chl) was measured fluorometrically (JGOFS, 1994). The water samples (300–500 ml) were filtered onto Whatman GF/F glass fibre filters under a low vacuum (~0.3 atm) and extracted in 90% acetone (at 5 °C in the dark, 24 h). The fluorescence of extracts was measured with a MEGA-25 fluorometer (MSU, Russia) before and after acidification with 1 N HCl. The fluorometer was calibrated before and after each experiment using pure Chl (Sigma) as a standard. Chl and the concentration of phaeophytin were calculated according to Holm-Hansen and Riemann (1978).

2.9. Statistical analyses

Standard statistical methods of descriptive, correlation, and *t*-test analyses were applied using PAST software (Hammer et al., 2001, http://folk.uio.no/ohammer/past). The means in this study are presented with the standard error (± SE) or standard deviation (± SD). Correlations are given with the coefficient of correlation (*r*), the number of measurements (*n*), and the probability of the null hypothesis (*p*). One-way analyses of similarities (ANOSIMs; Clark and Green 1988) and *t*-test were used to test for differences among experiments with *B. ovata* and *M. leidyi* based on estimated relative changes (Ratio of (Max value in the B and M treatment − control) / control, normalized to gram ctenophore per liter in %) of abiotic and biotic parameters ($p < 0.05$). Multidimensional scaling (MDS) plots based on

Bray–Curtis similarity index were used to graphically display differences in chemical variables and biological community variables separately, based on the measurements at the end of experiments normalized to the initial values.

3. Results

3.1. Effects of ctenophores on environmental parameters

Experimental measurements of mucus and life excretion released by ctenophores showed substantial losses. On average, one individual of *B. ovata* released 6.54 ± 0.78% mucus per day^{-1} per dry weight, which was 1,71 higher than the 3.82 ± 0.39% mucus per day^{-1}/dry weight in the case of *M. leidyi*. During the experiments, the size of the ctenophores decreased. In Gelendzhik experiment, the size of *M. leidyi* decreased by 5.9 ± 0.4% and of *B. ovata* by 10.78 ± 0.36%. In the Varna experiment, the size of *M. leidyi* and *B. ovata* was initially smaller, and the average decrease was 4.3 ± 0.12% and 9.2 ± 0.38%, respectively.

The results showed that *M. leidyi* and *B. ovata* affected the chemical parameters of the water. The initial content of nutrients in the Gelendzhik area (NO_3 — 0.32 μM; NH_4 — 1.28 μM; PO_4 — 0.12 μM) was significantly lower than in the Varna area (NO_3 — 0.65 μM; NH_4 — 4.35 μM; PO_4 — 1.35 μM), which had a considerable impact on the experimental results.

3.1.1. Gelendzhik experiment

After six days of exposition in both M and B treatments, the content of total suspended solids and all nutrients, with exception of nitrate, increased (Table 3.1.1). Dissolved oxygen (O_2) decreased and, consequently, the biochemical oxygen demand (BOD_5) increased, indicating an active biochemical oxidation of organic and inorganic substances in the water. The pH also decreased, indicating acidification of the medium.

The most significant variation of the chemical parameters was observed during the first 3 days, both in the M and B treatments, and then the gradients weakened, while substantial differences were manifested between the M and B treatments (Fig. 3.1.1a, b). In the B aquaria a sharp and stable increase in the concentration of ammonium, phosphate, and total phosphorus was observed by the end of the experiment. In the M aquaria, these parameters increased during the first 3 days, and then stabilized or decreased slightly on day 6. O_2 decrease was common for M and B treatments (not in C) during the first 3 days, yet more pronounced in the B aquaria and increased again by day 6, indicating that production dominated over consumption.

At the end of experiment, the difference between the concentrations of most nutrients in the B and M treatments was significantly higher compared to the C treatment (Table 3.1.1), while several times higher in the B aquaria than in the M treatments: phosphate — 30.8 versus 4.8 (6.4 times); ammonium — 2.8 versus 0.87 (3.2 times); total phosphorus — 3.05 versus 0.89 (3.4 times). In the M aquaria, only the silica increase was much higher than in the B aquaria (Table 3.1.1).

The observed differences in the nutrient species between the experiments was reflected in the stoichiometric ratios. In the C aquaria, P was almost completely exhausted after the first day, and remained at a minimum level until the end of the experiment. The concentration of dissolved inorganic nitrogen ($N = NO_3 + NO_2 + NH_4$) increased (mainly on the account of NH_4) reaching a peak on day 6. As a result,

Table 3.1.1
Changes of chemical parameters in the experiments.

Parameter	Gelendzhik				Varna			
	Initial conc.	Final conc. ± SE Difference from initial conc. Change (%)			Initial	Final[a] Difference from initial conc. Change (%)		
		Control	*M. leidyi*	*B. ovata*		Control	*M. leidyi*	*B. ovata*
pH	8.46 ± 0.004	8.17 ± 0.004 −0.29 (−3.4%)	8.00 ± 0.01 −0.46 (−5.4%)	7.96 ± 0.02 −0.5 (−5.9%)	8.59	8.45 −0.14 (−1.6%)	8.44 −0.15 (−1.7%)	8.54 −0.05 (−0.6%)
O_2, μM	247.7	236.4 ± 1.9 −11.3 (−4.6%)	212.1 ± 7.2 −35.6 (−14%)	190.8 ± 3.7 −56.9 (−23%)	327.1 ± 1.83	244.6 −82.5 (−25%)	248.2 −78.9 (−24%)	266.1 −61 (−19%)
NO_2, μM	0.04 ± 0.004	0.26 ± 0.01 0.22 (550%)	0.36 ± 0.01 0.32 (800%)	0.38 ± 0.01 0.34 (850%)	0.71 ± 0.03	0.34 −0.37 (−52%)	0.36 −0.35 (−49%)	0.75 0.04 (5.6%)
NO_3, μM	0.32 ± 0.02	0.40 ± 0.03 0.08 (25%)	0.38 ± 0.06 0.06 (19%)	0.37 ± 0.01 0.05 (16%)	0.65 ± 0.02	1.04 0.39 (60%)	0.9 0.25 (39%)	0.41 −0.24 (−37%)
NH_4, μM	1.28 ± 0.05	8.6 ± 0.87 7.32 (572%)	9.91 ± 0.11 8.63 (674%)	27.76 ± 6.1 26.48 (2069%)	4.35 ± 0.06	0.54 −3.81 (−88%)	1.02 −3.33 (−77%)	5.55 1.2 (28%)
PO_4, μM	0.12 ± 0.04	0.06 ± 0.02 −0.06 (−50%)	0.29 ± 0.04 0.17 (142%)	1.85 ± 0.54 1.73 (1442%)	1.35 ± 0.01	0.58 −0.77 (−57%)	0.74 −0.61 (−45%)	2.78 1.43 (106%)
P tot, μM	0.3 ± 0.03	1.19 ± 0.012 0.89 (297%)	1.06 ± 0.36 0.76 (253%)	3.63 ± 0.76 3.33 (1110%)	2.63	2.24 −0.39 (−15%)	2.71 0.08 (3.0%)	7.62 4.99 (190%)
Si, μM	0.99 ± 0.02	1.28 ± 0.12 0.29 (29%)	2.63 ± 0.02 1.64 (166%)	1.75 ± 0.25 0.76 (77%)	10.54 ± 0.04	4.11 −6.43 (−61%)	5 −5.54 (−53%)	4.7 −5.84 (−55%)
TSS, mg/l	1.56 ± 0.54	4.45 ± 0.27 2.89 (185%)	6.67 ± 1.7 5.11 (328%)	6.22 ± 0.27 4.66 (299%)	–	–	–	–
BOD_5, μM	24.1	57.2 ± 3.7 33.1 (37.3%)	87.2 ± 2.5 63.1 (162%)	116.8 ± 6.8 92.7 (285%)	159.8 ± 2.64	160.3 0.5 (0.3%)	166.1 6.3 (3.9%)	253.1 93.3 (58%)

[a] The final measurements were done in one replicate.

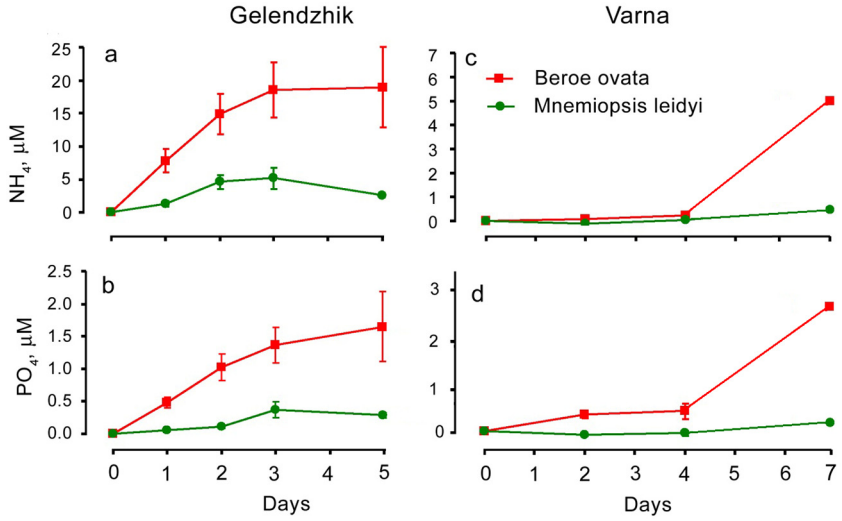

Fig. 3.1.1. Changes of ammonium (a, c) and phosphate (b, d) concentrations in the Gelendzhik and Varna experiments. Data are presented as difference between the experimental aquaria and control. Vertical bars show SE.

the N:P ratio sharply increased compared with the initial value (Fig. S1a). Similar changes, but of a lower magnitude, were observed in the M treatment. In contrast, in the B aquaria the N:P ratio varied in a narrow range from 16 to 25, which was close to the balanced for natural phytoplankton populations ratio of 16 (Redfield et al., 1963; Brzezinski, 1985). A similar pattern was observed for the Si:P ratio. In the C and M treatments, elevated values of the Si:P ratio occurred during the first 2 days and then decreased to the initial level (Fig. S1b). The Si:N ratio decreased from the initial 0.6 in the C, M, and B settings to 0.12, 0.06, and 0.28, respectively.

3.1.2. Varna experiment

Similar to Gelendzhik settings in the Varna experiment ctenophores, *M. leidyi* and *B. ovata*, induced changes in the chemical properties of the water with more pronounced modulations in the B treatment. The O_2 content increased on day 2 reaching the highest value (383 µM) in the B aquarium. On day 4 it dropped to 244 µM, and stabilized until the end of the experiment (Table 3.1.1).

Nitrogen was almost depleted between day 2 and day 4, and then increased more sharply in the B treatment. Ammonium and phosphate showed the same intermittent decrease in all treatments by day 4, and a further increase by day 6, again at higher rate in the B aquaria, where NH_4 and PO_4 values were 5.55 µM and 2.78 µM, respectively (Fig. 3.1.1c, d). Similarly, the increase of total phosphorus was more significant in the B treatment relative to C and M where, significant changes were not found.

Similar to Gelendzhik experiment, in the Varna treatments the concentration of most nutrients increased in comparison with the initial values, reaching much higher once in the B as compared to M treatments: phosphate — 4.8 versus 1.3 (3.7 times); ammonium — 10.3 versus 1.9 (5.4 times); total phosphorus — 3.4 versus 1.2 (2.8 times); nitrite — 2.1 versus 1.1 (1.9 times) (Table 3.1.1). The remaining chemical parameters showed no evident difference between the two treatments.

Regarding nutrients ratios, there was almost no difference in the N:P ratio between all treatments: a decline on day 2 and increase towards the end of exposition but to a level lower than the initial ratio (Fig. S1c). The Si:P ratio exposed totally different patterns with temporal variations during the experiment (Fig. S1d).

3.2. Response of autotrophic plankton to changes of environmental parameters

The initial concentration of micro-phytoplankton in Gelendzhik and Varna experiments differed significantly both in total abundance/biomass and taxonomic structure of the inoculum. The initial total abundance in the Varna experiment was > 200 times higher (1475×10^3 cells l^{-1} versus 7.1×10^3 cells l^{-1}) and the total biomass ~36 times. At the same time, the ratio between the dominant taxa diatoms/dinoflagellates was inversed by biomass (Table S2).

3.2.1. Varna experiment

In Bulgarian eutrophic waters, phytoplankton abundance and biomass were composed of 15 taxonomic classes. The high initial abundance was accounted for mainly by diatoms (40% of the total abundance), *Prasinophyceae* (20%), and *Dinophyceae* species (15%), while in the biomass the contribution of diatoms (~40%) was almost equal to that of dinoflagellates (~50%), with a negligible proportion of the remaining classes (bellow 10%) due to the prevalence of small-size species. The dominant complex of species was rather polyphyletic, composed of diatoms *Cyclotella choctawhatcheeana*, *Lennoxia faveolata*, *Leptocylindrus minimus*, *Thalassiosira complex*, *Thalassiosira minima*, *Nitzschia longissima*, *Cerataulina pelagica*; dinoflagellates *Prorocentrum cordatum*, *Scrippsiella complex*, *Gonyaulax cochlea*; Cryptophytes *Hemiselmis* sp., *Hillea fusiformis*, *Plagioselmis* sp.; and Prasinophytes *Pyramimonas* sp. and *Pachysphaera* sp.

3.2.2. Gelendzhik experiment

In Gelendzhik area, the micro-phytoplankton community was less diverse, represented by species from 11 taxonomic classes. The total biomass was dominated mainly by diatoms (66%), whereas the total abundance was shared between diatoms and dinoflagellates (~46% each). The dominant complex of species was composed of the diatoms *Pseudo-nitzschia delicatissima* complex, *Pseudosolenia calcar-avis*, *Dactyliosolen fragilissimus*, and dinoflagellates *Phalacroma rotundatum*, *Prorocentrum micans*, *Lingulodinium polyedrum*, and *Diplopsalis lenticula*.

Both *M. leidyi* and *B. ovata* stimulated phytoplankton growth, resulting in an increase of the total abundance and biomass in all treatments, yet the shape of micro-phytoplankton biomass and abundance response curves and the magnitude of change were different. Phytoplankton growth was much higher in the B treatments in comparison with the C and M settings (Fig. 3.2.1, Table S2). In eutrophic waters (Varna experiment), at its maximum the abundance in the B

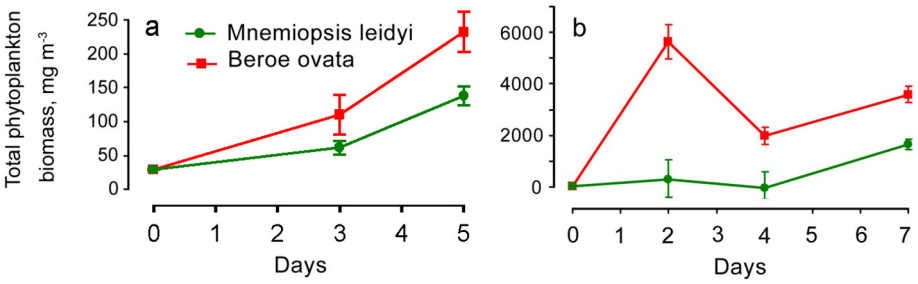

Fig. 3.2.1. Changes in the total micro-phytoplankton biomass in Gelendzhik (a) and Varna (b) experiments. Data are presented as difference between the experimental aquaria and control. Vertical bars show SE.

treatment increased > 12 times up to 1.7×10^6 cells l^{-1}, relative to a ~5 fold increase in the M aquaria (Fig. S2). At the end of the experiment (day 7), the biomass reached 6453 ± 312 mg m^{-3} in the B treatment, ~8 times higher than the initial value, whereas in the M aquaria this difference was very similar to C treatment (~5.5 and 4.8 times), respectively. In mesotrophic waters (Gelendzhik experiment) the final total biomass reached the highest level also in B treatment (~10 times), twice low level in the M and only negligible increase in C.

A substantial difference was observed in the response between the taxonomic groups - diatoms, dinoflagellates, and euglenophytes. In Gelendzhik experiment, on day 3, the diatom biomass was about 3.3 times higher in B as compared to the M aquaria, but by the end of the experiment (day 5) the values were very similar, while on the contrary the increase of dinoflagellates in the B treatment on day 5 was ~3.6-fold higher (Fig. 3.2.2). In Varna experiment, the most significant effect on diatom growth occurred on day 2 with a ~6 times difference between the B and M treatments, but at the end of the experiment (day 7) the dinoflagellates biomass was much higher and the difference between the B and M aquaria was ~4.6 times. Thus, the most pronounced difference occurred due to dinoflagellates in the B aquaria: in Gelendzhik and Varna experiments their biomass increased by ~2000% and ~1000% relative to the initial level respectively (Table S2, Fig. 3.2.2). In the M treatments the range of change varied within 580%–480%. The diatoms growth was also stimulated by the two ctenophores but to a lower degree (~400% in Gelendzhik and ~800% in Varna). The behaviour of euglenophytes differed in Gelendzhik and Varna experiments. They proliferated in the B and M treatments to a similar level in the Gelendzhik experiment (increase > 1000%), while in Varna experiment their growth was apparently stimulated only in the B aquaria (~500% versus < 140% in the M aquaria) (Table S2). Irrespective of the magnitude the differences in the response of micro-phytoplankton observed between M and B treatments in the two

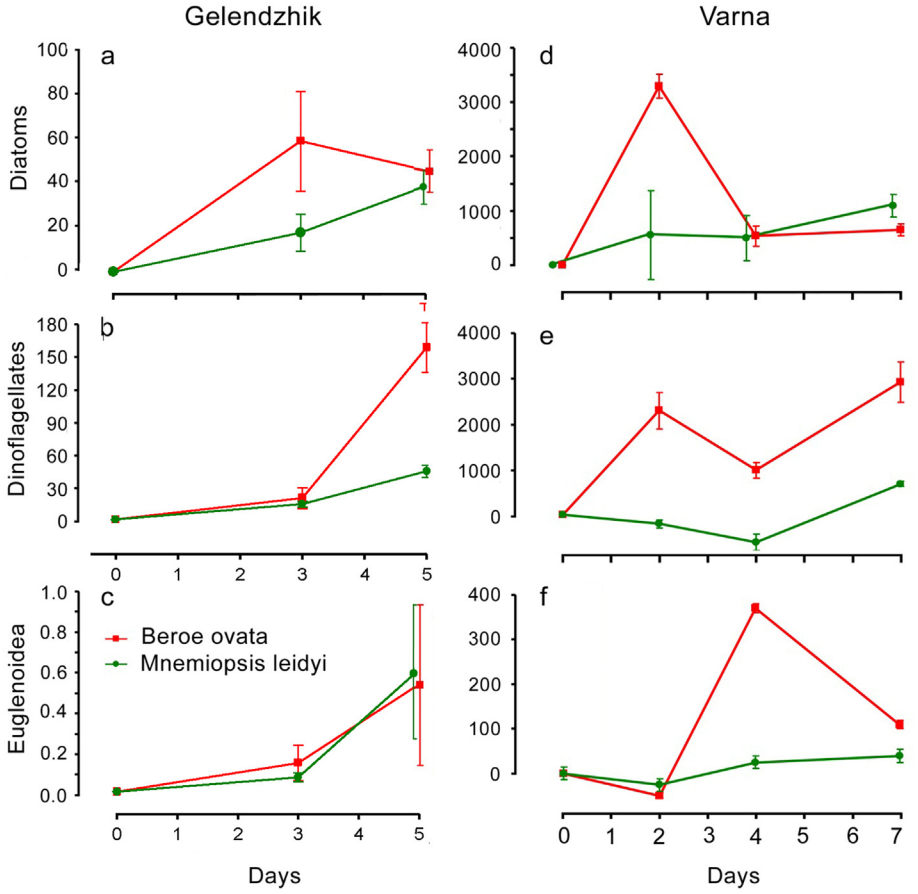

Fig. 3.2.2. Changes in the biomass of diatoms (a, d), dinoflagellates (b, e) and euglenophytes (c, f) in Gelendzhik and Varna experiments. Data are presented as difference between the experimental aquaria and control. Vertical bars show SE.

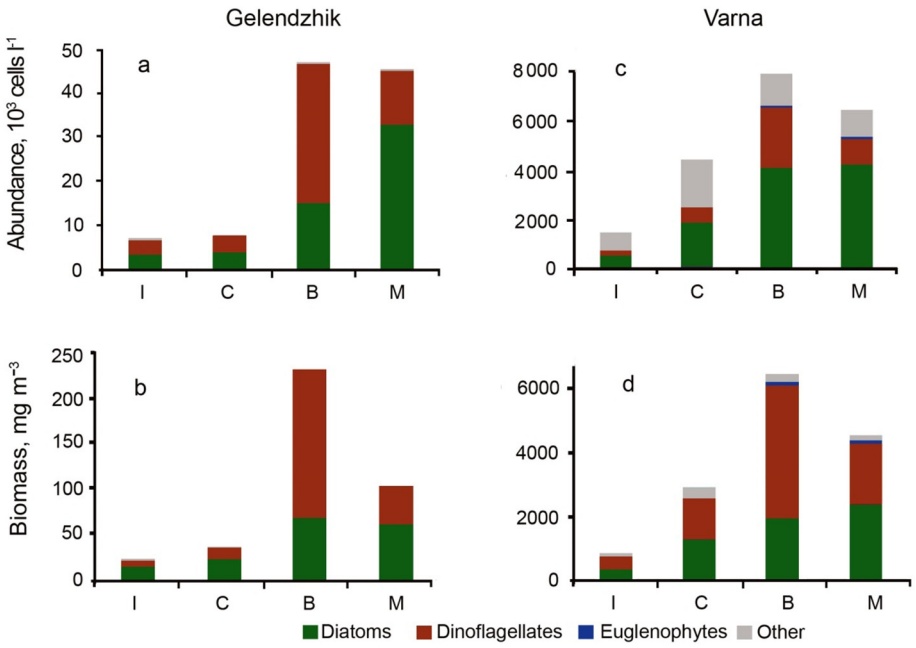

Fig. 3.2.3. Changes in taxonomic structure of micro-phytoplankton in Gelendzhik and Varna experiments. Initial (I) and final structures in aquaria with Control (C), *B. ovata* (B) and *M. leidyi* (M).

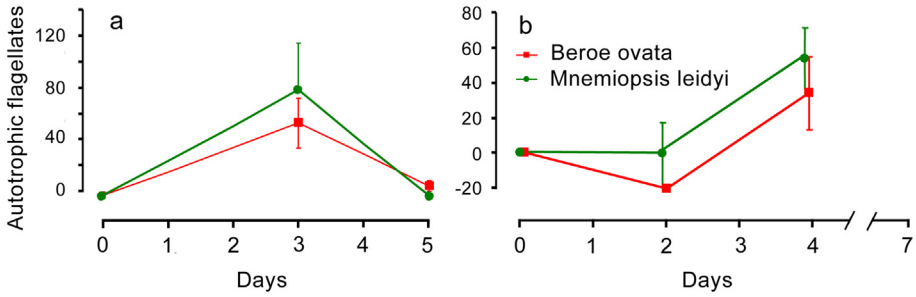

Fig. 3.2.4. Changes of biomass of autotrophic flagellates in Gelendzhik (a) and Varna (b) experiments. Data are presented as difference between the experimental aquaria and control. Vertical bars show SE.

experiments were statistically significant, the only exception was Euglenoidea in Gelendzhik experiment (Table 4.1).

While variations in the abundance and biomass occurred in all treatments, the taxonomic composition displayed a different pattern. In Gelendzhik experiment, almost all groups demonstrated steady growth in the B and M treatments (Fig. 3.2.2). In Varna experiment an increase in diatoms and dinoflagellates were measured on the day 2 in all treatments, declining on the day 4 and then maintained at almost the same level by the experiment termination (Fig. 3.2.2). These variations exerted concomitant alterations in the taxonomic profile of the phytoplankton community (Fig. 3.2.3). Mainly mixotrophic dinoflagellates and heterotrophic euglenophytes species grew in the B aquaria, whereas diatoms predominated in the M aquaria. In the Gelendzhik experiment, taxonomic proportions of diatoms, dinoflagellates, euglenophytes, other taxa in the biomass changed from the initial 67:31:0.2:2.5 to 30:70:0.2:0.1 (B) and to 59:40:0.5:0.2 (M), without obvious change in the C treatment. In the Varna experiment, this proportion shifted from the initial 36:50:3:10 to 29:65:2:4 (B), and to 51:43:1:4 in the M treatment.

The initial composition and abundance of small phytoplankton (< 6 μm) in Gelendzhik and Varna experiments were different. Numerical abundance of autotrophic flagellates and cryptomonads was substantially higher in the eutrophic waters of the Varna Bay (Table S1). Heterotrophic cell numbers were similar, whereas the abundance of unicellular cyanobacteria (0.8–1.2 μm) was 3.5 times higher in Gelendzhik region.

In Gelendzhik experiment, the abundance of autotrophic flagellates in all treatments decreased to the end of exposition, whereas in the Varna experiment their abundance increased several times (Table S1). In both experiments the abundance of heterotrophic flagellates increased by several hundred percent. In contrast, the abundance of cryptomonads decreased several times in Varna experiment. Similar results were obtained in both experiments for cyanobacteria. Their cell abundance dropped several-fold both in aquaria with ctenophores and in the C treatment. It should be noted that the maximum difference between control and M and B treatments in many cases was not observed at the end of experiment.

The variation of nano-phytoplankton abundance and biomass in most cases showed an increased growth on day 3, and then a decline to the day 5. A typical example was the changes of the biomass of autotrophic flagellates in Gelendzhik experiment (Fig. 3.2.4a). In the M treatment, on day 3, the biomass of autotrophic flagellates increased five-fold. A similar pattern was observed for all groups in both experiments, with exception of autotrophic flagellates in Varna experiment, where the biomass drastically increased only on day 4 (Fig. 3.2.4b). Despite high concentrations of micro-zooplankton grazers, autotrophic and heterotrophic flagellates and cryptomonads demonstrated high growth rates. The specific growth rate (μ) of these groups during the first three days varied from 0.6 to 1.2 day^{-1}. The population doubling time varied from 12 to 20 h.

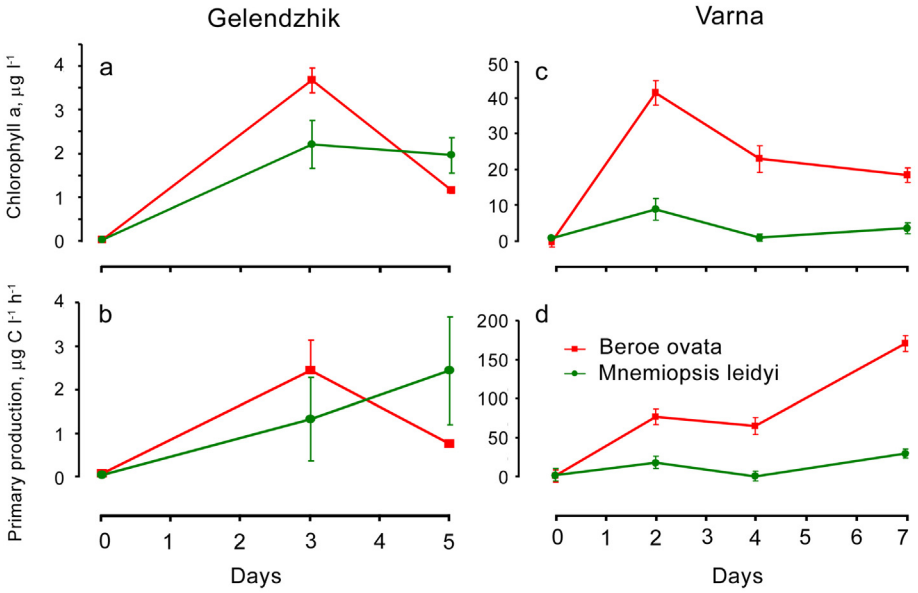

Fig. 3.2.5. Changes of chlorophyll *a* content and primary production in Gelendzhik (a, b) and Varna (c, d) experiments. Data are presented as difference between the experimental aquaria and control. Vertical bars show SE.

3.2.3. Chlorophyll and PP in the Gelendzhik experiment

In Gelendzhik experiment, the initial Chl was 0.242 µg l^{-1}. During the exposition, the Chl in the C aquaria slightly changed (Table S3). In both treatments with the ctenophores, Chl significantly increased on day 2, showing higher values in the B aquaria (Fig. 3.2.5a). By the end of the experiment, Chl decreased both in the B and slightly in the M treatments. The initial PP was 3.0 µg C l^{-1} h^{-1} (Table S4). PP significantly decreased in the C aquaria on day 2, and remained at this level to the end of the exposition. Highest PP was measured in the B aquaria on day 2, which corresponded to the increase in Chl (Fig. 3.2.5b). In contrast, PP in the M treatment reached the maximum increase compared to the control by the end of the experiment. Assimilation numbers (the ratio of PP to Chl) in all treatments sharply decreased from 12 to 0.5–1.0 µg C µg Chl $^{-1}$ h^{-1} on day 2, and then increased to 1–4 µg C µg Chl $^{-1}$ h^{-1} at the end of experiment.

3.2.4. Chlorophyll and PP in the Varna experiment

In Varna experiment, the initial Chl was 4.2 µg l^{-1}. During the experiment, Chl changed in all treatments (Table S3, Fig. 3.2.5c). On day 2 Chl increased in all treatments, with the highest rate in the B aquaria, followed by a decline. Similar variations of Chl with low magnitude were observed in the C treatment. By the end of the experiment, Chl exceeded the initial level about two times in the M and C treatments, and about six times in the B treatment.

The initial PP in the coastal waters of the Varna Bay was 18.6 µg C l^{-1} h^{-1} (Table S4). PP significantly increased in all treatments on day 2, which coincided with the Chl increase. The highest (eight-fold) increase in PP was observed in the B treatment (Fig. 3.2.5d). PP decreased on day 4 and increased on the day 7. This dynamic was typical for all treatments, whereas the range of variations was highest in the B aquaria. By the end of the experiment a ten-fold increase in PP was observed in the B treatment. At the same time, Chl remained at the same level in all treatments between day 4 and day 7. This resulted in a dramatic increase of the assimilation numbers (5.2–8.3 C µg Chl $^{-1}$ h^{-1}) at the end of the experiment in all treatments.

3.3. Response of heterotrophic plankton to changes in environmental parameters

3.3.1. Bacterioplankton

3.3.1.1. Gelendzhik experiment. In Gelendzhik experiment, the initial total number of bacteria (TNB) was 0.43 ± 0.06 × 10^6 cells ml^{-1}. During the experiment TNB in the control increased slightly, and reached a maximum only at the end of the experiment. In the treatments with both ctenophores TNB increased sharply on day 1 of the experiment, compared with the C treatment (Fig. 3.3.1a), with more intense growth in the B aquaria. On day 3, TNB in the B aquaria significantly decreased, while in the M aquaria their abundance did not change. By the end of the experiment, TNB increased again to equal values both in M and B treatments, while in the C aquaria only a slight increase was observed, which did not exceed the natural background level of the Black Sea waters.

The number of the active bacteria changes in a similar way as TNB (Fig. 3.3.1b), whereas the share of active bacteria in the total bacteria abundance (SAB) changed differently. At the beginning of the experiment, SAB in all nine aquaria ranged between 34.5 and 65.1%, averaging 48.4 ± 5.50%. On day 1 in the B aquaria, SAB slightly decreased, but it was higher than in the C and M aquaria. On day 3 in the treatments with the ctenophores, SAB again dropped to about 15%. However, at the end of the experiment SAB in aquaria with both ctenophores slightly increased, whereas in the C aquarium it continued to decrease and reached a minimum value of 9.25 ± 1.15%.

3.3.1.2. Varna experiment. In Varna experiment, the initial concentration was 2.0 ± 0.02 × 10^6 cells ml^{-1}. It was significantly higher than that in Gelendzhik experiment (Table S5). On day 2 TNB increased sharply in the M aquaria, whereas in the B treatment it was low. (Fig. 3.3.1c). On day 4 TNB in the M aquaria increased progressively. In the B and C aquaria, TNB was almost unchanged compared with the values measured on day 2.

At the beginning of the experiment, the number of active bacteria in all aquaria was 0.30 ± 0.03 × 10^6 cells ml^{-1}. On day 2, the number of active bacteria sharply increased in both aquaria with ctenophores, especially in the M treatment (Fig. 3.3.1d). In contrast, the number of active bacteria in the C aquaria slightly decreased. On day 4, the number of active bacteria and SAB in treatments with both ctenophores decreased to minimal values. In the C aquaria, these parameters also slightly declined, but less dramatically than in aquaria with the ctenophores.

3.3.2. Micro-zooplankton

3.3.2.1. Gelendzhik experiment. During Gelendzhik experiment micro-

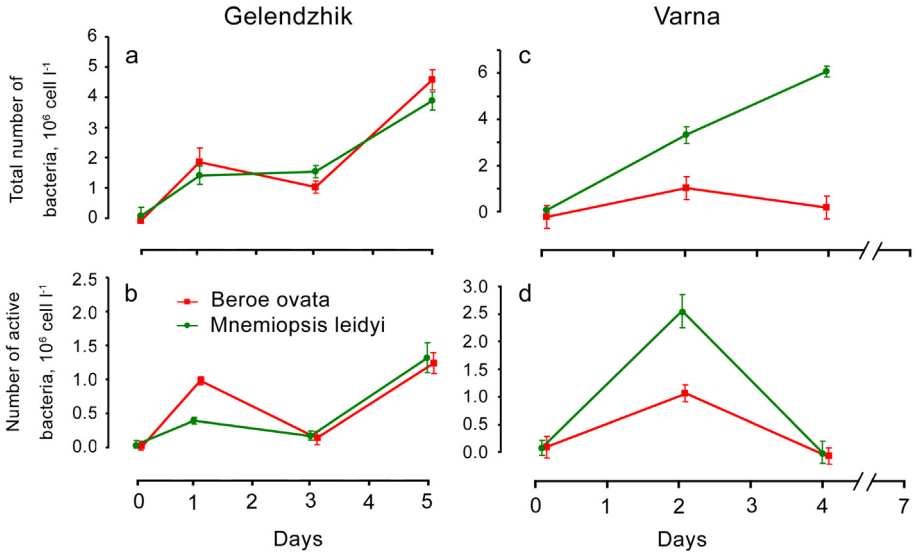

Fig. 3.3.1. Changes of the total number of bacteria (a, c) and number of active bacteria (b, d) in the Gelendzhik and Varna experiments. Data are presented as difference between the experimental aquaria and control. Vertical bars show SE.

zooplankton was not measured.

3.3.2.2. Varna experiment. In the Varna experiment, a total of 18 species/taxa of micro-zooplankton from six taxonomic subclasses were identified (Table 3.3.2). Numerous ciliates (phylum Ciliophora) were unidentified to a lower taxonomic level.

In terms of relative abundance and biomass, the oligotrichids dominated the communities (60–90%), especially during the first days of the experiment, along with the ciliates (10–35%), haptorids (5%), and hyptotrichids (Figs. 3.3.2, S3). It should be noted that the relative biomasses of oligotrichids in the communities were higher (Fig. 3.3.2), compared to their relative abundances (Fig. S3). On day 2, the abundance of *Haptoria* (*Mesodinium* sp.) increased from the initial value of 1974 ± 522 ind. l^{-1} by 3, 6, and 73 times, respectively in the C, M, and B treatments. Furthermore, it dropped close to the initial level. However, the biomass was negligible due to their small size. On the same day (2), new taxa (*Euplotes* sp., *Acineta* sp., and a few unidentified ciliates), which were probably very rare initially, proliferated to high numbers-reorganizing community structure. For example, *Euplotida* (*Hypotrichia*) was higher represented in the B treatment (21%) in comparison with the C (9%) and M (12%) settings, while for ciliates the corresponding figures were 38% in C aquaria, 15% (M), and 52% (B) (figure not shown). At the end of the experiment, micro-zooplankton complexes in the C, M, and B treatments were dominated in abundance by *Hypotrichia* (34%, 14%, and 42%), *Haptoria* (14%, 1%, and 9%), and ciliates (29%, 9%, and 14%). In the biomass, ciliates prevailed and were well-presented in the control (57%), while oligotrichids – in ctenophore's aquaria (Fig. 3.3.2). The initial abundance and biomass of micro-zooplankton were low, respectively 7000 ind. l^{-1} and 89 mg m^{-3} (Table S6). Changes in the total abundance and biomass showed similar

Table 3.3.2
Taxonomic composition of micro-zooplankton in Varna experiment.

Subclass	Species/taxa
Hypotrichia	*Euplotes* sp.
Oligotrichia	*Eutintinnus* sp., *Tintinnidium* sp., *Strobilidium* sp., *Strombidinopsis* sp., *Strombidium* sp., *Laboea strobila*, *Tontonia* sp.
Haptoria	*Mesodinium* sp.
Suctoria	*Acineta* sp.
Monogononta	*Trichocerca* sp., *Keratella* sp.
	Unidentified ciliates

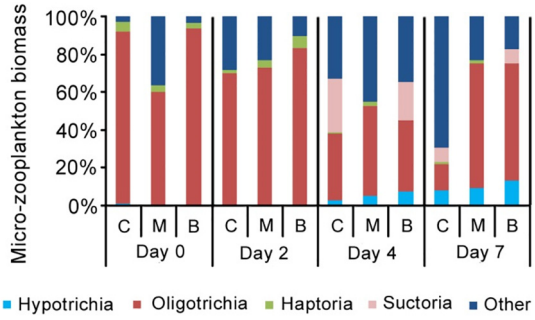

Fig. 3.3.2. Taxonomic structure of micro-zooplankton biomass (%) in aquaria with the control (C), *M. leidyi* (M) and *B. ovata* (B) in Varna experiment. Other - include taxa of subclasses Monogononta and unidentified ciliates. Numbers from 0 to 7 show days of sampling.

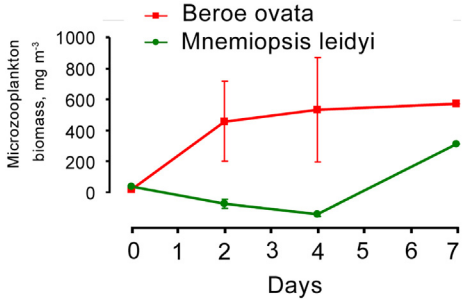

Fig. 3.3.3. Changes of micro-zooplankton biomass during Varna experiment. Data are presented as difference between the experimental aquaria and control. Vertical bars show SE.

trends in all treatments, with the highest increase in the B treatment — about 18-fold in abundance (130 ± 56 × 10^3 ind. l^{-1}) and 12-fold in biomass (823 ± 362 mg m^{-3}) — but with the weaker alterations in the M aquaria (Table S6, Figs. 3.3.3, S3). On day 2, much of the abundance increase was associated with *Haptoria* (101 ± 47 × 10^3 ind. l^{-1}), whereas the increase in biomass was related to *Oligotrichia* (*Strobilidium* - 309 ± 71 mg m^{-3}). Between day 4 and 7 of the experiment, the biomass in the B aquaria was still maintained high due to the development of *Oligotrichia* (*Strombidinopsis* sp.), *Hypotrichia* (*Euplotes* sp.) and new taxa of *Suctoria* (*Acineta* sp.).

4. Discussion

Despite the variations in the response of the chemical and biological variables our study showed a clear bottom-up effect of ctenophores *Beroe ovata* and *Mnemiopsis leidyi* on the productivity and structure of lower trophic levels in the Black Sea coastal waters in spite of experimental design with unfed ctenophores. The natural components of ctenophore mucus are macromolecular organic compounds of protein and polysaccharide, their complexes and derivatives. Hydrolytic enzymes (including proteases, and amylases) are natural catalysts for their degradation (Korneeva and Shiganova, 1995, 1998). In seawater, extracellular enzymes are generated through the activity of microorganisms such as bacteria. They play a key role in the processes of the transformation of organic matter and contribute to the rapid inclusion of suspended organic matter in the lower food web, activating the microbial loop (Korneeva and Shiganova, 1995, 1998).

It was shown that *M. leidyi* excretes phosphorus, dissolved organic carbon, ammonia, and other compounds of dissolved inorganic and organic nitrogen (Kremer, 1977; Shiganova et al., 2004a). The majority of excreted substances consist of nitrogen: 66% of NH4 and 34% of dissolved organic nitrogen (Kremer, 1977, 1982). As both respiration and excretion rates are a direct linear function of animal weight and are very sensitive to temperature, the nitrogen excretion is of high importance when evaluating the contribution of *M. leidyi* to nutrient recycling. At high ctenophore abundances, the contribution of *M. leidyi* excretion to the ammonium pool was estimated to range between 3% and 15% day^{-1} in temperate Narragansett Bay (Kremer, 1977). In the Black Sea, the reported weight-specific nitrogen excretion was 0.00173 ± 0.00121 mol NH_4 gDW^{-1} h^{-1} at T 25 °C (Shiganova et al., 2004a). Our experimental results showed that the excretion rate (loss in dry weight) was two times higher in the case of *B. ovata*. Consequently, the higher amount of nitrogen excretion and released mucus may explain the higher intensity of processes and related changes in the experiments with *B. ovata*, compared with *M. leidyi* and the control. Therefore, from the beginning it was expected that the effect of *B. ovata* on the experimental environment should be stronger.

The experiments in Gelendzhik and Varna demonstrated generally similar patterns of nutrient alterations under the influence of ctenophores. To a certain extent the dissimilarities in the course of the experiments originated from the differences in the initial conditions. In Gelendzhik, the initial content of inorganic nutrient species (especially PO_4 of 0.12 μM) was not enough to support active growth of phytoplankton (Table 3.1.1). Most likely the intensive increase of nutrient content observed in the first three days of the experiment was due to mineralization of organic matter secreted by ctenophores (Fig. 3.1.1a, b). After day 3 of the experiment, the chemical composition of the water was additionally modified due to the increased activity of phytoplankton - increased concentration of oxygen and associated assimilation (decline) of nutrients (Table 3.1.1).

In Varna area, initial concentrations of nutrients and phytoplankton biomass were much higher than in Gelendzhik area (Table 3.1.1). As a result, intensive phytoplankton growth (Fig. 3.2.1b) and nutrient uptake occurred from the very beginning of the experiment in all treatments, O_2 increased on day 2 in all treatments. After the exhaustion of inorganic nutrients (primarily nitrogen) on day 4, the increase in nutrient content could be attributed to ctenophore excretion (more apparent in the B treatment) as evidenced in the final phase of the exposition (Fig. 3.1.1c, d). This resembled the “scenario” in the Gelendzhik experiment, and corresponded to an initial phase of mineral nutrient accumulation. Thus, to some extent, the final phase of the Varna experiment corresponded to the initial phase of the Gelendzhik experiment. In the C aquaria, this process was less manifested, indicating the significant role of ctenophores in the formation of nutrient conditions favourable for phytoplankton growth both in mesotrophic and eutrophic environments.

It should be underlined that the phytoplankton growth-limiting nutrients were different in the two experiments. According to the stoichiometric ratios in the Gelendzhik experiment, P and Si were at a relative minimum, whereas in the Varna experiment these were N and Si (Fig. 1S). Presumably, during the process of regeneration the released nutrients were supplied in a proportion close to natural ratios C:Si:N:P = 106:15:16:1 (Redfield et al., 1963; Brzezinski, 1985), that completely altered the chemical composition of the experimental media. In the mesotrophic waters near Gelendzhik, the experiment showed a strong influence of the excretion of N (mainly NH_4) due to secretion of mucus by *M. leidyi* and *B. ovata* on stoichiometric ratios. In aquaria with the ctenophores, the N:P ratio dropped down from 250 to a more balanced 20–80 (Fig. S1). In the eutrophic waters near Varna, against the background of high initial nutrient concentrations, the addition of ctenophores' regenerated nutrients did not affect the total pool of nutrients. This is well supported by the lack of difference between the experimental setting and control (Fig. S1). Only at the end of the experiment in the B treatment did the regenerated ammonium affect the N:P ratio. At the same time, the active nutrient uptake by phytoplankton resulted in “stoichiometric modulations” typical for experimental conditions (Mitra and Flynn, 2007).

As demonstrated by the MDS analysis, chemical variables (from Table 3.1.1) by the end of experiments differed between the ctenophores treatments and the control, the highest difference observed between B treatments and the controls in both experiments (Fig. 4A, Table 4.1). Differences in chemical properties were also observed between the M treatments and the controls in the two lab experiments, but they were lower than those in the B treatments. The alterations in the chemical variables were translated in differences in gross biological parameters (the total phytoplankton biomass, chlorophyll *a*, primary production and total bacteria biomass) between ctenophore treatments

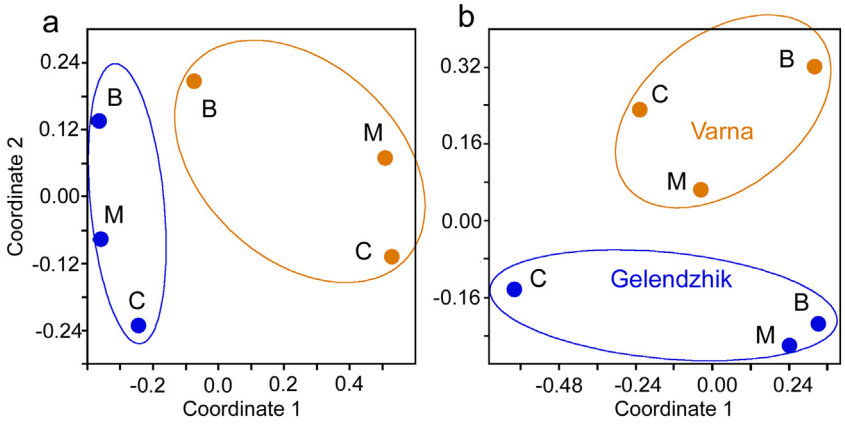

Fig. 4. MDS plot of similarity in chemical (a) and biological parameters (b) in two sets of experiments in Gelendzhik and Varna. C-Control; B-*B. ovata*, M-*M. leidyi*.

Table 4.1
Relative changes (%) of abiotic and biotic parameters versus gram ctenophore per liter. Ratio (Max value − control) / control between values in ctenophore treatments and control during the experiments.

Parameters	Gelendzhik			Varna		
	M. leidyi	p	*B. ovata*	*M. leidyi*	p	*B. ovata*
NH_4	22 ± 7	**0.0035**	135 ± 31	35[a]	–	526[a]
PO_4	124 ± 32	**0.0054**	1602 ± 465	2[a]	–	213[a]
Bacteria	97 ± 3.6	**0.0004**	193 ± 14.4	81 ± 3.13	**0.0001**	25 ± 4.7
Microzooplankton		–		81[a]	–	169 ± 131
Total phytoplankton biomass	65 ± 27	**0.050**	303 ± 153	2.3 ± 2.6	**0.0016**	65 ± 14
Diatoms	19 ± 9.7	**0.0011**	101 ± 14	12 ± 6	**0.050**	72 ± 37
Dinoflagellates	76 ± 34	**0.010**	637 ± 210	8.3 ± 2.7	**0.0001**	89 ± 8
Euglenoidea	455 ± 617	0.605	879 ± 1160	5 ± 13	**0.016**	702 ± 303
Autotrophic flagellates	127 ± 79	0.7621	147 ± 72	33 ± 15	0.871	30 ± 26
Cryptophytes	244 ± 65	0.7927	221 ± 126	24 ± 15	**0.0141**	109 ± 32
Chlorophyll	93 ± 22.5	**0.0006**	265 ± 20.6	22 ± 7.6	**0.0001**	149 ± 12.4
Primary production	120 ± 62.6	**0.0370**	318 ± 92.2	36 ± 6.1	**0.0001**	298 ± 12.5

p - probability (*t*-test) of difference between effect of two ctenophores. Bold text marks significant difference.
[a] One replicate.

and the control by the end of both experiments (Fig. 4a). The highest difference occurred in Gelendzhik experiment; while in Varna experiment the difference was lower, but statistically significant (Fig. 4b, Table 4.1).

Initial differences in nutrient conditions in Gelendzhik and Varna experiments most likely affected the response of phytoplankton assemblages. In Gelendzhik experiment, the initial concentration of P (0.12 μM) was limiting for phytoplankton growth of most species (Riegman et al., 2000; Ou et al., 2008). A sharp increase of P in the ctenophore treatments triggered the growth of almost all algae, in contrast to the C aquaria (Fig. 3.2.1, Table S2, and Fig. 3.2.2a, b). On the contrary, in the Varna experiment all nutrients were of high initial concentrations (Table 3.1.1) and did not significantly limit algal growth at the onset of the lab experiment. As a result, a lower difference between treatments with *M. leidyi* and the control was observed (Fig. 3.2.2d, f). Presumably, the high nutrient content and sufficient irradiance (close to natural light conditions) promoted the high growth of algae in all treatments. Nevertheless, in the case of *B. ovata* the higher concentrations of P and NH_4 in the experimental media (Table 3.1.1) were reflected in a higher maximum biomass and abundance of micro-phytoplankton (Figs. 3.2.1, 3.2.2d–f; Table S2).

In Gelendzhik experiment, autotrophic flagellates demonstrated an increase during the first two days, and a decrease to the initial level by the end of the experiment (Fig. 3.2.4a), most likely associated with the rapid development of their consumers-micro-zooplankton. As it was shown, in Varna experiment, an increased growth of micro-zooplankton occurred on day 2 (Fig. 3.3.3) and coincided with a decrease of autotrophic flagellates in the B treatment (Fig. 3.2.4b). On this basis it could be assumed that the decrease of autotrophic flagellates by the end of the exposition in Gelendzhik treatments (Fig. 3.2.4a; Table S1) could also be explained by the increased abundance of micro-zooplankton. It seems that in the Varna experiment, the growth rate of autotrophic flagellates exceeded its grazing rate which resulted in a several-fold increase in abundance and biomass (Table S2, Fig. 3.2.1b; Table S1).

The decrease in the cell numbers of cyanobacteria in both experiments might be related to a several factors: relatively low growth rates (Table S1), the maximal specific growth rate of *Synechococcus* (the most probable representative of cyanobacteria) is lower (0.8–1.0 day^{-1}) than that of other microalgae (2–3 day^{-1}) (Banse, 1982; Campbell and Carpenter, 1986); they might be overgrazed by actively growing ciliates; and another possible reason could be the light inhibition of growth. In the Black Sea, in summer, cyanobacteria showed maximal abundance in the seasonal thermocline (Rat'kova, 1989) at irradiances, which were much lower than in both experiments.

In both experiments, the presence of ctenophores led to substantial increases in N and P in comparison with the control (Fig. 3.1.1a, b) which boosted prominent changes in phytoplankton taxonomic composition (Figs. 3.2.2, 3.2.4). Both diatoms and dinoflagellates increased their biomass at the end of the experiments to similar levels in the M aquaria, whereas in the B aquaria dinoflagellates reached the higher proportion (Table S2). The affinity to use different nitrogen substrates differs significantly across species and major taxonomic groups (Eppley et al., 1969; Smayda, 1997; Litchman et al., 2004). While the nitrate flow is often associated with the proliferation of diatoms, increased ammonium content generally favours dinoflagellates (Margalef, 1978). Our results are generally in line with this assumption. The increase in NH_4 was much higher in the B treatments, which could be the reason for the predominance of dinoflagellates in the final community composition. Thus, the relative importance of ctenophores on the taxonomic structure of phytoplankton is likely to vary, depending on the gelatinous species and the associated species-specific excretion, as also reported by West et al. (2009), Condon et al. (2011).

Chl and PP variations were coupled with the observed trends in the numerical abundance and biomass of phytoplankton (Figs. 3.2.1, 3.2.5a, c, S2). In aquaria with both ctenophores, the experiments revealed an increase in all of these variables at the end of the experiment in comparison with the initial values and with the control. In addition to structural traits, functional characters such as PP also demonstrated clear increase (Fig. 3.2.5b, d). In the Varna experiment, assimilation numbers reached 6–8 μg C μg Chl^{-1} h^{-1}. These values are in the upper range of assimilation numbers reported for most regions of the world's oceans (Vedernikov, 1976) and for the Black Sea (Mikaelyan et al., 2015), indicating a substantial positive effect of ctenophores on the physiological state of phytoplankton.

The addition of organic matter released by the ctenophores presumably did not change the “food” matrix significantly. This is supported by the fact that, in Gelendzhik experiment, TNB increased 10 to 12-fold, whereas in Varna experiment it increased by 70–360% only (Table S5). High bacteria numbers are typical for Varna Bay area, ranging from 3.5 to 4.5 × 10^6 cells ml^{-1} (Altman et al., 1990), associated with the high concentration of organic matter in eutrophic waters. It is interesting that even at the end of Gelendzhik experiment, the TNB in the C aquarium didn't exceed the maximum TNB recorded previously in these waters (Mosharova and Sazhin, 2007). By the end of the experiments the SAB in all treatments decreased in comparison with initial levels. At the same time, in Gelendzhik experiment, SAB and the number of active bacteria in the aquaria with ctenophores were significantly higher than in the control (Table S5), suggesting that the presence of the ctenophores affected the bacterioplankton growth more strongly in the mesotrophic waters.

Although the micro-zooplankton was not examined in Gelendzhik, on the basis of the experiment in Varna a similar increase in various micro-zooplankton species could have been expected to occur in the aquaria with the ctenophores (Fig. S3). Processes determining the proportion of active and inactive cells in bacterioplankton are not well understood, but there are several factors, besides temperature, which may result in cell inactivation. Among these are viral infection (Proctor and Fuhrman, 1990), substrate limitation, and grazing of protozoa. It is well known that protists may selectively remove both large bacteria and dividing cells (Sherr et al., 1992). The assumption that active cells are generally larger than inactive cells has been confirmed (Gasol et al., 1995). Therefore, it can be assumed that the decline in SAB on the third and fourth day of the experiment (Fig. 3.3.1b, d) was due to their selective predation by ciliates, whose number possibly increased on the same day (according to the Varna experiment).

The abundance of heterotrophic flagellates increased similarly in all treatments, including C aquaria (Table S1), most likely related to the elevated abundance of bacteria (Table S5). The difference between the two experiments was manifested by the higher increase in the Varna experiment, which may be associated with the higher content of organic matter released by the higher phytoplankton biomass (Table S2).

Micro-zooplankton, by grazing, removes a large proportion of the small phytoplankton and bacterial production, as shown earlier (Verity et al., 2002; Stelfox-Widdicombe et al., 2004). In this way, it plays an important role in nutrient regeneration (Caron and Goldman, 1990) releasing dissolved organic carbon (Strom et al., 1997) which contributes to the microbial loop. Among micro-zooplankton, tintinnids are responsible for grazing most of the algal production, as well as for most of the nutrient regeneration, and they are an important food resource for higher trophic levels (Dolan et al., 2012). As a consumer of small bacteria and nano-phytoplankton, which is the food of larger mesozooplankton, micro-zooplankton act as trophic intermediates between them (Gifford, 1991). In Varna experiment, and hypothetically in Gelendzhik experiment, these prey-predator interactions most likely induced the observed oscillations in bacterial abundance and SAB component in particular (Fig. 3.3.1).

Varna experiment showed the substantial effect of both ctenophores on the taxonomic structure of ciliates (Figs. 3.3.2, S.3). An illustrative example was *Euplotes* sp. from the order *Euplotida*. This species is an opportunistic predator of bacteria. It often grows in seawater enrichments; it is not typically pelagic and it is associated mainly with the flow of substrate or the sediment re-suspension (Jiang et al., 2013). Most probably, mucus released by *M. leidyi* and *B. ovata* served as an appropriate substrate and food supply for bacterial growth that were grazed by *Euplotes* sp.

Another example is the haptorids group (*Mesodinium* spp.), which developed in the experiment. Presumably, there are multiple mixotrophic, as well as heterotrophic, species within this group, and the ecological roles of the different species may diverge (Moestrup et al., 2012). It is hypothesized that the micro-zooplankton, consisting mainly of ciliates in the M and B aquaria, were fed by bacteria. Bacterial concentrations exert a considerable influence on the development of ciliates, but consequently bacterial proliferation is counterbalanced by grazing (Gast, 1985). Additionally, Jiang et al. (2013) found a strong relationship between the *Strombidium* and *Mesodinium* sp. with nutrients (negative correlation) and Chl a (positive correlation). The results presented here are in line with this assumption, showing an increase in the abundance of haptorids on the second day (Figs. 3.3.2, S.3), which coincided with the concomitant increase of its potential food-bacteria and phytoplankton (Fig. 3.3.1c, d).

Summarizing the results of this study, obviously during the experiments, complex processes of nutrient release by ctenophores and nutrient consumption by micro-phytoplankton took place, while the growth of all groups of micro-phytoplankton and bacteria was partly balanced by their grazing by micro-zooplankton. At the same time, the consumption of small phytoplankton and bacteria by micro-zooplankton led to the excretion of nutrients and organic matter, which in turn affected phytoplankton growth. Due to the complexity of these multiple interactions, the observed changes and the difference between the examined parameters in the treatments and the control at the end of experiments would not necessarily reflect the real single effect of ctenophores. Therefore it was suggested that the maximum difference between a parameter in the experimental treatment and the control would be a better expression of the real bottom-up impact of ctenophores on the ecosystem. Calculated this way, the maximum relative changes (%) of abiotic and biotic parameters versus the gram wet weight of ctenophore per liter demonstrated that, in most cases, *B. ovata* affected all the studied variables more apparently than *M. leidyi* (Table 4.1). It was calculated that the addition of 1 g of wet weight of ctenophores resulted in an average increase of all biological parameters in the B treatments by 340%, and 170% in the Gelendzhik and Varna experiments, respectively (Table 4.1). We have to take into consideration that all ctenophores were starved so the effect could have been higher if they were fed.

In the M treatments the same values were 170% and 30%, respectively. This comparison also showed that ctenophores had a much greater effect on the mesotrophic ecosystem in the Gelendzhik coastal waters than in the Varna eutrophic coastal area. This means that similar biomass of ctenophores will cause more evident changes in mesotrophic ecosystems. For example, the highest biomasses of *B. ovata* and *M. leidyi* observed in Gelendzhik coastal waters during the bloom in late July–August 2001 were 0.4 and 2.3 $g\,l^{-1}$, respectively (Shiganova et al., 2014). On the basis of experimental results and the maximum abundances observed in the two regions, the possible effect of both ctenophores on the environment could be projected (Table 4.2). For example, the maximum increase of bacteria would be approximately 200% under the influence of both ctenophores. The same increase in the total phytoplankton biomass is projected to about 150% under the influence of *M. leidyi*, and 120% in the case of *B. ovata*, in spite of its lower biomass. The increase in diatoms and dinoflagellates, as well as in chlorophyll and primary production, would also be significant in both cases (Table 4.2). Generally, as *M. leidyi* reached much higher biomasses than *B. ovata* in the Black Sea, its effect would be stronger on majority of measured variables. In reality the effect could be stronger if ctenophores were fed.

During the last few years, *B. ovata* appeared in higher densities and

Table 4.2
Estimated impact (%) of ctenophores on the abiotic and biotic parameters of ecosystem at maximum and current biomasses (B, $g\,l^{-1}$) of *M. leidyi* and *B. ovata*[a] in natural environments of the two studied areas.

Parameter	Gelendzhik				Varna			
	M. leidyi		*B. ovata*		*M. leidyi*		*B. ovata*	
Year	2001	2016	2001	2016	2004	2016	2004	2016
B, $g\,l^{-1}$	2.3	0.016	0.4	0.03	1.2	0.02	0.003	0.015
NH_4, μM	51	0.35	54	4	42	0.02	1.58	7.9
PO_4, μM	285	2	641	48	2.4	0.04	0.006	3.2
Bacteria, $\mu g\,l^{-1}$	223	1.6	77	5.8	97.2	3.3	0.1	0.4
Microzooplankton, $\mu g\,l^{-1}$	–	–	–	–	97.2	3.3	0.51	2.5
Total phytoplankton biomass, $\mu g\,l^{-1}$	149	1	121	9.1	2.8	0.6	0.2	1
Diatoms, $\mu g\,l^{-1}$	43	0.3	40	1.6	14.4	0.3	0.22	1.1
Dinoflagellates, $\mu g\,l^{-1}$	175	1.2	254	19	17.4	0.2	0.3	1.3
Chlorophyll, $\mu g\,l^{-1}$	214	1.5	106	7.9	26.4	0.4	0.45	2.2
Primary production, $\mu g\,C\,l^{-1}\,day^{-1}$	276	1.9	127	9.5	43.2	0.7	0.9	4.5

[a] Estimations were made on the basis of experimental results of maximal impact and field data on ctenophore biomasses obtained in Gelendzhik (late July–August 2001, 2016) and Varna coastal areas (July 2001, 2016).

started to graze on *M. leidyi* much earlier (phenological shift). As a result, *M. leidyi* did not reach a high biomass. Consequently, *B. ovata* through the predator-prey interactions would not grow in high abundance. Therefore, the effect of the two ctenophores on the ecosystem currently is much lower. For example, the highest biomasses of *B. ovata* and *M. leidyi* observed in Gelendzhik coastal waters in July 2016 were 0.03 and 0.016 $g\,l^{-1}$, respectively. Consequently, the expected effect on all studied parameters would be considerably lower (Table 4.2). In the coastal waters of Varna in 2016, the maximum biomasses of *M. leidyi* and *B. ovata* were only 0.02 and 0.015 $g\,l^{-1}$, respectively and the estimated possible effect on the ecosystem would be negligible.

5. Conclusions

The objectives of the experiments in Gelendzhik and Varna were focused on study of the effect of two invasive ctenophores *M. leidyi* and *B. ovata* on phyto- and microzooplankton, e.g. to focus on the bottom-up effect. Mesozooplankton was excluded from experiments, Despite that both species were not fed during experiments they demonstrated generally similar patterns of influence indicing significant alterations of nutrient cycling and microphyto- and microzooplankton components. Mineralization of secreted organic matter and the direct release of nutrients by ctenophores resulted in an intensive increase in nutrient content in the experimental environment. Both species, especially *B. ovata*, contributed to the increased concentration of inorganic nitrogen (N) and phosphorus (P), and the decreased pH, however a stronger effect was observed in the mesotrophic waters. Both species considerably changed nutrient ratios, and maintained them at the level closer to balanced stoichiometry (N:P = 16–25) in comparison with that in the control treatment (~200).

The phytoplankton species composition also changed significantly. The more remarkable growth of phytoplankton was observed in the *B. ovata* aquaria, most likely related to the higher amount of released mucus (about two times higher than in the case of *M. leidyi*). This difference exerted concomitant alterations in the taxonomic structure of phytoplankton community: the development of primarily mixotrophic dinoflagellates and heterotrophic euglenophytes in the *B. ovata* aquaria, and mainly diatoms in the *M. leidyi* aquaria. Chl and PP variations generally were in accordance with the observed changes in the numerical abundance and biomass of phytoplankton. Experiments showed an increase in Chl and PP in aquaria with *M. leidyi*, yet a more apparent maximum increase in aquaria with *B. ovata*.

Originally, micro-zooplankton consists of multiple populations, each characterized by different traits and adaptations which make it difficult to identify with certainty what nutritional mode they use at any given time based on structural analysis only. The highest abundance was reached by *Euplotes* sp., which is an obligate bacteria grazer. The impact of micro-zooplankton was also well observed on the total, and particularly on the active bacteria abundance which oscillated due to growth and intensive grazing. The effect was more considerable in aquaria with *B. ovata*.

The results give ground to conclude that *B. ovata* more evidently affected the restructuring of the lower pelagic food-web, and the intensity of related processes. However, the estimated effect of the mass development of ctenophores in the studied regions demonstrated a more important role of *M. leidyi* due to its higher abundance. The influence of both ctenophores' on the ecosystem low trophic web was more significant in the mesotrophic waters than in the eutrophic. These should be taken into consideration for further assessment of effects on the lower trophic levels of ecosystems during mass development of ctenophores and other gelatinous species.

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Appendix A. Supplementary data

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