1 **Effects of light availability on mixotrophy and microzooplankton grazing in an**

2 **oligotrophic plankton food web: evidences from a mesocosm study in Eastern**

3 **Mediterranean waters**

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1   **Abstract**

2   Plankton biomass and composition in the pelagic zone of oceans is exposed to

3   changes in availability of light and nutrients due to large-scale ocean circulation and

4   water column stratification. We hypothesized that displacement of plankton from

5   surface to deeper darker waters would not only favor heterotrophy over time, as

6   previously suggested, but also first rapidly affect the level of mixotrophy and,

7   consequently, overall microbial grazing in plankton food webs. To test this in an

8   oligotrophic marine system we incubated Eastern Mediterranean water (from 10 m

9   depth north of Crete in September 2010) in 2.8 m^3 mesocosms simulating two

10 different light intensities at the sampling station, surface waters (ca. 10 m; mesocosms

11 L1) and deeper layers (ca. 50-60 m; mesocosms L4). The biomass and abundance of

12 the main planktonic groups were monitored either daily or every second day,

13 depending on the group. Microzooplankton grazing rates and the contribution of

14 mixotrophic feeding were estimated by a combination of dilution experiments and

15 incubations with live fluorescently labeled algae (LFLA). Although no nutrients were

16 added to the mesocosms the chlorophyll *a* increased during the first 2 days of the

17 experiment in both treatments. This increase resulted from phytoplankton growth in

18 the light L1-mesocosm (autotrophic biomass was ca. doubled in L1 compared to L4),

19 but was mostly due to photoadaptation of the algae in the L4-mesocosm, as indicated

20 by lower carbon to chlorophyll *a* ratios. By the end of the experiment, the total

21 biomass of protozoan and metazoan grazers in L1 was ca. twofold higher than in L4.

22 The microzooplankton responded within the first 24 h, showing different grazing

23 activity in L1 than in L4. Microzooplankton grazing rates on total Chl *a* were similar

24 in both treatments; however, phytoplankton instantaneous growth rates were higher in

25 the more illuminated mesocosm. This resulted in a closer coupling between both rates

1 in L4, where all production was grazed daily, than in L1. Nevertheless, the overall

2 flux of carbon through the microzooplankton was 33-60% higher in L1 compared to

3 L4 throughout the experiment. The fraction of mixotrophy in the ciliate community

4 varied in L1 (20-50%), but decreased over time in L4 from 50% to 30%. Our results

5 do not support studies from freshwater, postulating that reduced light and nutrient

6 limitation may increase herbivore production due to stoichiometric effects. Finally,

7 we discuss how mixotrophy may bias rate estimates in dilution experiments.

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1. Introduction

Light is a major driver of life on Earth, and therefore regulates the production and distribution of phototrophic organisms. This spatial regulation is evident in all aquatic ecosystems as the vertical distribution of the organisms, especially the plankton, is greatly dependent on light availability. One of the most remarkable biological structures related to light in aquatic systems is the establishment of phytoplankton maxima at certain depths, occurring whenever both light and inorganic nutrients are available in sufficient amounts. However, when thermal stratification prevents continuous upwelling of nutrients, the phytoplankton maxima are usually found in deeper layers, where the organisms attain a compromise between nutrient and light availability (Cullen, 1982). An extreme example of this situation is the deep chlorophyll maxima in oligotrophic waters, where phytoplankton can be found at relatively high abundance at depths generally ranging from 50 to 100 m (Estrada et al., 1993; Calbet et al., 1996; Letelier et al., 1996; Ignatiades et al., 2002; Casotti et al., 2003). In these deep layers light is scarce (ca. 1% surface irradiance, Estrada et al., 1993; Ignatiades et al., 2009) and nutrients, although still limiting due to the trophic characteristics of these systems, occur in sufficient amounts to maintain stable phytoplankton communities. On the other hand, the communities that develop in the extensive mixed layers of these poorly productive ecosystems experience both severe nutrient limitation and variable light levels as a result of the vertical displacement due to the circulation of the water masses. In order to cope with these extreme environmental challenges it has been suggested that the algae supplement their nutrient acquisition with mixotrophy during periods of low dissolved nutrient concentrations in the surface mixed layer (Arenovski et al., 1995). Theoretically, light limitation should result in a reduced production of autotrophs, and could modify the

1   trophic characteristics of some algae (i.e., enhancement of mixotrophy); both

2   mechanisms potentially scaling up the food web. However, it is not clear how these

3   communities respond to strong changes in light intensity when vertically displaced in

4   the water column.

5   In this work we investigate the effects of an abrupt decrease in light supply on

6   natural marine plankton communities collected in light-saturated oligotrophic surface

7   waters. The light intensities were manipulated to represent the light availability at ca.

8   10 m depth in a mixed surface layer (where water was collected) and to simulate a

9   low light climate equivalent to 50-60 m water column depth, near the base of the

10   thermocline for the season (Casotti et al., 2003; Ramfos et al., 2006). To understand

11   how light modulates the response of the plankton in the oligotrophic mixed layer of

12   the Eastern Mediterranean we conducted a series of microzooplankton grazing and

13   mixotrophy incubation experiments, and analyzed the composition of the

14   microzooplankton community in a mesocosm experiment using natural water without

15   any nutrient addition. Great care was taken to keep the nutrient levels naturally low.

16   We hypothesized that i) the flow of matter through the food web would be diminished

17   by a reduction in the energy input, and ii) that the plankton community would rapidly

18   adapt to the new, darker, conditions by a fast increase of mixotrophic algae, and

19   perhaps a longer-term negative response of mixotrophic ciliates, subsequently

20   displaced by heterotrophic ciliates.

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22   **2. Methods**

23   *2.1. Experimental set up*

1   This study was conducted as a part of a larger international experiment

2   (LightDynamix) funded by the European project MESOAQUA (http://mesoaqua.eu/)

3   at the Cretacosmos mesocosm facility of the Hellenic Centre for Marine Research

4   (Crete, Greece). The total LightDynamix experimental design involved 4 light

5   intensities (L1, L2, L3, and L4), duplicated in 2.8 m^3 mesocosms (replicates a, b) and

6   run between 23 September and 1 October 2010. A detailed description of the overall

7   mesocosm design and plankton development in all treatments is provided in Ptacnik et

8   al. (in prep.). In short, the mesocosms were filled with water pumped from 10 m depth

9   into acid cleaned 1 m^3 polyethylene (PE) containers, at a station 6 nautical miles north

10   of Heraklion, Crete (Greece). Within 2-4 h the containers were transported to land and

11   the content distributed evenly between the 8 mesocosm bags immersed in a tank with

12   water at *in situ* temperature, by gravity siphoning with acid cleaned plastic tubes.

13   Filling took altogether two days. During this time, the mesocosms were kept dark by

14   non-transparent covers. The mesocosms were made of transparent PE bags, with a

15   diameter of 1.32 m and a depth of 2.5 m, surrounded by a double layer of PE black

16   and white bags serving as an optical isolation. Optical lids for light manipulations in

17   the mesocosms were created using 4 different grades of neutral density filters (Lee

18   Filters, UK) and were installed at night after filling the mesocosms. Thus the

19   experimental manipulation started the following morning (day 0). Due to the labour

20   intensity of the incubation experiments and analysis of trophic level of the protists

21   performed in this study we were restricted to focus on two experimental (mesocosm)

22   light intensities only. We used the lightest and darkest, i.e. mesocosms L1a,b and

23   L4a,b, respectively. The grey filters covering these mesocosms attenuated

24   approximately 5 and 87% of the incoming light, resulting in average light intensities

25   of ca. 60% and 6% of incident light intensity inside each of the L1 and L4

1 mesocosms, respectively. Thus closely mimicking the light intensities at the sampling

2 depth 10 m and near the base of the thermocline for the season (50-60 m, Ramfos et

3 al., 2006). We measured vertical profiles of photosynthetically active radiation (PAR)

4 in 0.5 m steps with a spherical quantum sensor (LI-139SA; Licor, Lincoln, NE, USA)

5 while simultaneously measuring incident PAR (flat quantum sensor LI-190SA) above

6 the water surface outside the mesocosm, both attached to a Licor 1500 data logger.

7 Light inside each mesocosm was calculated as the light intensity averaged over the

8 water colum (Imix). The water in the mesocosms was gently mixed by slow bubbling

9 through an airlift pipe as described in Ptacnik et al. (in prep.). Because ciliates and

10 other plankton may be disturbed by bubbles or any activity creating turbulence, on the

11 one side; but, on the other side, some type of mixing is needed to create homogenous

12 mesocosm contents, we created a mixing system that uses a minimal amount of air,

13 with a maximal but gentle mixing effect. It was modelled after the mixing systems

14 that have been successfully used for over 30 years in the mesocosms at the Norwegian

15 National Mesocosm centre, University of Bergen (see e.g. Williams and Egge, 1998;

16 Nejstgaard et al., 2006), but down-scaled for these smaller mesocosms used here. In

17 addition effort was made to make the bubbles big, to minimize the surface to volume

18 ratio of the bubbles and thus the actually encounter rate between plankton and

19 bubbles.

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21 *2.2. Community composition and biomass*

22 Samples (125-250 mL) for chlorophyll *a* concentration (Chl *a*) were collected daily

23 from each mesocosm. The water was sequentially filtered onto duplicate 10, 2, 0.65,

24 and 0.2 µm pore-size, 47 mm diameter, polycarbonate filters (GE Water & Process

1   Technologies), to obtain the size-fractions: 0.2-0.65 μm, 0.65-2 μm, 2-10 μm, and >

2   10 μm, respectively. The filters were extracted immediately in acetone overnight at

3   4ºC and measured on a TD Turner 700 (Turner Designs, Sunnyvale, CA), with and

4   without acidification according to Parsons et al. (1984). We collected particulate

5   seston daily by filtering 500 ml sample water onto GF-F filters (47 mm). The filters

6   were dried overnight and placed in petri slides until further analyses. The amount of

7   particulate organic carbon, phosphorus and nitrogen on the filters was analysed by x-

8   ray fluorescence (XRF) of the specific elements using a S4 Pioner (Bruker AXS,

9   Karlsruhe Germany) at the University of Bergen (Norway). We then calculated C:P,

10   C:N molar ratios, and C:Chl *a* mass ratios.

11   Samples (50-100 ml) for analysis of the plankton community composition in

12   the mesocosms were taken every second day, stained with primuline (Direct

13   Yellow 59, Sigma-Aldrich Co.), fixed with 3.6% glutaraldehyde solution with 10%

14   glycerol (final concentrations) and gently filtered onto 0.6-μm pore-size black

15   polycarbonate membrane filters, and frozen until analysis (within 1-2 days). The

16   method is a modification from Grebecki (1962), Hobbie et al. (1977) and Caron

17   (1983) with the glycerol added to reduce the damage of especially small delicate

18   protists during filtration as described in Sazhin et al. (2007). Samples were analysed

19   by epifluorescence microscopy and cell volumes were calculated by approximation of

20   simple geometrical 3D shapes and converted into cell carbon as described in Menden-

21   Deuer and Lessard (2000). In addition, samples for abundance of tintinnids and larger

22   oligotrich ciliates were collected from one replicate bag of each treatment (L1a, L4a)

23   every other day and fixed with acid Lugol's solution (2% final concentration),

24   subsequently settled (250 mL) for 48 h and counted on an inverted microscope at

25   150x magnification. In order to estimate the percentage of mixotrophic ciliates an

1 additional set of samples (250 mL) was fixed with borax-buffered formalin (final

2 concentration 2% formaldehyde), subsequently settled in the dark for 48 h and

3 counted on an inverted epifluorescence microscope at 150x magnification using blue

4 light.

5   At the end of the experiment, mesozooplankton abundance and composition in

6 the mesocosms were determined by pumping the entire volume of each mesocosm

7 through the same 48-µm mesh submerged plankton net. Samples were preserved

8 immediately after collection in 4% borax–buffered formaldehyde solution, with the

9 exception of the L1b-sample, that was lost during the collection process. Species

10 composition analysis and the estimation of total mesozooplankton abundance were

11 carried out under a dissecting microscope in sub-samples taken with a Stempel

12 pipette. Major mesozooplankton taxa (e.g., copepods, cladocerans, doliolids,

13 appendicularians, etc.) were counted, and copepods and cladocerans were further

14 identified to the species level when possible (at least 300 copepods were counted per

15 sample). According to the latest taxonomy (Boxshall and Halsey, 2004), the order of

16 Poecilostomatoida has been transferred to the order of Cyclopoida, hence for the

17 purposes of this work have been presented together.

18

19 *2.3. Microzooplankton grazing and mixotrophy experiments*

20 Every other day we conducted standard grazing dilution experiments (Landry and

21 Hassett, 1982) in one replicate of the lightest and darkest mesocosms (L1a and L4a,

22 respectively). The water for these experiments was collected early in the morning by

23 gravity-filling of 30 L dark containers using silicon tubing. Once in the laboratory, the

24 water was gently siphoned into a 60 L bucket and carefully mixed to avoid generating

1   bubbles that could damage delicate ciliates (Gifford, 1985; Broglio et al., 2004). A

2   known volume of water was gravity-filtered through a Pall Acropak 0.8/0.2 500

3   capsule (previously flushed, including tubing, with diluted HCl and thoroughly rinsed

4   with deionised water) to obtain the dilution series at the proportions 25, 50, 75 and

5   100% of the experimental water. The dilution series was prepared in 1.3 L PC bottles,

6   which were amended with a nutrient mixture (1 μM NH_4Cl, 0.07 μM Na_2HPO_4, and

7   0.5 μM Na_2SiO_3) to guarantee that nutrients were not limiting in any treatment. To

8   assess the natural growth of algae two100% experimental water (i.e., not diluted)

9   bottles without addition of nutrients were also prepared. Special care was taken to

10 work under dim light conditions for the darkest treatments.

11   In order to avoid adverse light effects on the mesocosms when initiating and

12 ending the incubation periods (ca. 24 h) of the grazing experiments, the bottles were

13 incubated in specially prepared mesocosm incubators equipped with an easily

14 opening-closing cover made from the same neutral density filter used for the

15 mesocosms L1 and L4, hence providing temperature (24.3-25.7ºC) and light levels

16 comparable to these mesocosms (same level attenuation; for absolute values see

17 Ptacnik et al. in prep.). The bottles were hanged from a floating wheel at 0.5 m depth,

18 which corresponded to the average light intensity of the mesocosms. The wheel

19 rotated at app. 1 r.p.m propelled by a submerged water pump. This approach assured

20 that the light conditions were homogeneous for all the bottles. However, because

21 these incubators were filled with brackish nutrient rich water from a well at the

22 HCMR, different from the nutrient poor water of the mesocosms, a denser

23 phytoplankton bloom developed at days 4-5. This had consequences for the light

24 intensity at which the experimental bottles were incubated, and is discussed below. In

25 the beginning (initial samples), as well as at the end of the incubations, we took

1 duplicate samples for the quantification of the different size-fractions of Chl *a*, and for

2 the quantification of nanoflagellates (in the initial and unfertilized bottles). For the

3 quantification of nanoflagellates in the dilution grazing experiments, 75-100 mL of

4 glutaraldehyde preserved samples were stored at 4ºC in the dark for 2h and then

5 filtered onto 2 µm black polycarbonate filters, and stained with 4', 6'-diamidino-2-

6 phenylindole (DAPI). We counted 200 to 500 cells under epifluorescence microscopy

7 in each filter. The rates obtained for the cell counts of autotrophic flagellates were

8 compared to Chl *a*-based growth rate estimates. This way we could both estimate the

9 cellular growth rates of the phytoplankton and quantify potential artifacts in pigment

10 based growth rates due to photoadaption changing the cellular Chl *a* contents during

11 the incubations.

12   For all experiments, we used Model I linear regressions to obtain the slope (*m*;

13 grazing mortality rate, d^{-1}) of the equation relating the fraction of undiluted water (x)

14 and the net phytoplankton growth rates (y; *K*) estimated from changes in Chl *a*

15 concentration during incubation. Because the intercept of the equation (μ_n) would

16 provide an overestimation of phytoplankton instantaneous growth rates (due to the

17 addition of nutrients in the bottles), the instantaneous *in situ* growth rates (μ) in

18 dilution grazing experiments were derived from net growth in the unfertilized bottles

19 (K_o; where no nutrients added) and were corrected for mortality by microzooplankton

20 from dilution experiments when the latter was significant (Landry and Hassett, 1982).

21 $\mu = K_o + m$

22   When we found saturated feeding responses we used the linear relationship of

23 the most diluted treatments to obtain the phytoplankton instantaneous growth rates

24 with added nutrients (μ_n) according to Gallegos et al. (1989) and Dolan et al. (2000).

1   The microzooplankton grazing rate (m_n) was then calculated for the undiluted

2   nutrient-enriched bottles as

3    $m_n = \mu_n - K_n$

4   where K_n is the phytoplankton net growth rate in the undiluted nutrient-enriched

5   bottles. This grazing rate calculated as indicated above does not completely

6   correspond to the *in situ* microzooplankton grazing rate (*m*). According to Moigis and

7   Gocke (2003) and Moigis (2006) the *in situ* microzooplankton grazing rate (i.e., in the

8   undiluted bottles without added nutrients) should be calculated as

9    $m = m_n * K_o *(\exp(K_n *t)-1)/ K_n *(\exp(K_o *t)-1)$

10   where *t* is the incubation time.

11   In parallel we assessed the amount of herbivory of mixotrophic algae (large

12   flagellates and dinoflagellates) by measuring the uptake of live fluorescently labeled

13   algae (LFLA) during 24 h *in situ* grazing experiments. Aiming at a better

14   representation of the nanoflagellate size spectrum of the mesocosm environment two

15   different types of algae were used to produce the LFLA stocks: *Isocrhysis galbana* (4-

16   6 µm) and *Tetraselmis* sp. (8-10 µm). Algal cultures grown exponentially in f/2

17   medium at $18^{\circ}C$ in a 12:12 h light: dark cycle were labeled with the live fluorescent

18   vital stain CellTracker$^{®}$ Blue CMAC (7-amino 4-chloromethylcoumarin, Molecular

19   Probes Inc.) according to Teegarden (1999). The latter vital stain makes the cells

20   fluoresce bright blue when excited by UV light at 354 nm.

21   The incubations were conducted in 650 ml polycarbonate bottles, filled with

22   water from the mesocosm L1a and L4a, and lasted for ca. 24h. Two sets of triplicate

23   bottles were prepared, one set without nutrient additions and one set amended with

24   nutrients as in the dilution experiments. These bottles were used for an additional

1 estimate of the effect of nutrient limitation. Each experimental bottle was inoculated

2 with a mixture of fluorescent prey and was subsequently gently mixed. We tried to

3 keep total LFLA concentration inside the bottles below 25% of the abundance at

4 which similarly sized flagellates are typically found in oligotrophic areas of the

5 Mediterranean Sea (1.3×10^2-6.0×10^3 cells ml^{-1}) (Christaki et al., 1999, 2001).

6 Subsamples were always taken prior incubations to determine exact initial

7 concentration of LFLA in the bottles, which at times was more than expected (see

8 discussion). The incubations took place in the already described incubators, together

9 with the dilution experiment bottles. At the end of each incubation 75-100 ml samples

10 were fixed with glutaraldehyde (final concentration 1%) and processed as above

11 (dilution grazing experiments), with the exception that no DAPI stain was used.

12 Protozoan cells with LFLA prey inside were enumerated under an Olympus BX 40

13 inverted epifluorescence microscope at a magnification of 400 x. Mixotrophic activity

14 was calculated as the percentage of mixotrophs >10 μm and <10 μm found with

15 labelled prey inside.

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18 **3. Results**

19 *3.1. Community composition and biomass*

20 In Fig. 1 we show the time course of the total phytoplankton biomass (measured as

21 Chl *a*) along the 8 days of experiment in mesocosms experiencing high (L1) and low

22 (L4) light treatments, as well as the contribution of the different size fractions to the

23 standing stock of Chl *a*. Both treatments and parallels showed an increase in Chl *a* the

24 first 2-3 days of experiment, from 0.08 to ca. 0.2 μg Chl *a* L^{-1}. While values in both

25 the L4 mesocosms were approximately 0.2 μg Chl *a* L^{-1} throughout the experiment,

1   the L1 mesocosms showed a rapid decline during day 4 and 5 and ended (on day 8) at

2   concentrations near half the maximum attained. The Chl *a* fractions 0.6-2 μm and 2-

3   10 μm were of similar proportion and together made up 75-90% of the total Chl *a* at

4   all times (Fig. 1). Except for a slight increase in the contribution of Chl *a* > 10 μm

5   during the experiment in all treatments, there were no clear changes in size groups

6   over time in the mesocosms.

7   The development of the autotrophic protist biomass determined by

8   epifluorescence microscopy (Fig. 2) resembled the pattern of Chl *a* development in

9   the L1 mesocosms. However, in the L4 mesocosms the autotroph biomass showed a

10   development different from Chl *a*, with lower biomass values and less clear peaks

11   (0.94±0.53 SE μgC L^{-1}, Fig. 2). The total biomass peak of heterotrophic eukaryotes in

12   the L1 mesocosms (1.7±0.12 SE μgC L^{-1}) was more than twofold higher than in L4

13   (0.75±0.07 SE μgC L^{-1}), although the temporal development was similar.

14   In all mesocosms, mixotrophic ciliates and autotrophic dinoflagellates

15   increased their biomass towards the end of the study, becoming significant

16   components to the community, otherwise dominated by autotrophic flagellates (Fig.

17   2). In mesocosms L1 heterotrophic flagellates and ciliates dominated the protozoan

18   community-biomass the second and sixth day of the experiment, respectively. In L4

19   mesocosms, however, the heterotrophic community remained quite stable with the

20   only exception of a peak of ciliates on day 6 (Fig. 2). The Lugol preserved samples

21   showed a progressive increase of ciliates in both mesocosm treatments until day 6 and

22   a slight decrease the last day (Fig. 3). The contribution of mixotrophs to the total

23   abundance of ciliates consistently decreased in L4a from 50% to 30% and was quite

24   variable in L1a (Fig. 3).

1 The mesozooplankton biomass community at the end of the experiment was

2 higher in the L1 treatment than in L4 (Fig. 4). The mesozooplankton community was

3 numerically dominated by copepods in all measured treatments with calanoida

4 (mostly *Clausocalanus* spp. and *Calocalanus* spp.) showing ca. double abundance in

5 L1a, being the most abundant group in this mesocosm. Cyclopoida (mostly *Oithona*

6 spp.) abundance was similar at both light intensities. Other important components,

7 although at much lower abundances were appendicularia and cladocera (Fig. 4).

8 The elemental ratios of C, N, and P, as well as the C:Chl *a* mass ratio are

9 presented in Fig. 5. Light did not significantly affect any of the ratios ($p > 0.05$;

10 grouped ANOVA with repeated measures), however some differences are evident in

11 the proportion of Chl *a* per unit of carbon since day 4 (Fig. 5A), and in the C:N at

12 days 1 and 2 (Fig. 5B). C:P ratios followed a similar pattern in both treatments along

13 the experiment, with peaks at days 3 and 4 for L4 and L1, respectively and an increase

14 of the quotient last day in all mesocosms (Fig. 5C).

15

16 *3.2. Microzooplankton dilution grazing experiments*

17 We conducted four dilution-grazing experiments (Landry and Hassett, 1982) in each

18 replicate "a" of the mesocosms L1 and L4. Overall, all (Chl *a*-based) phytoplankton

19 growth and mortality rates observed for total and size-fractionated Chl *a* were quite

20 high, with many saturation-feeding responses at the end of the experiment, *sensu*

21 Gallegos (1989) (Fig. 6; Table 1). During day 1 we observed consistent positive

22 slopes for total phytoplankton and all size-fractions in the low light mesocosm (L4a,

23 Fig. 6). In general, mortality rates (m) on total Chl *a* were similar in both treatments,

24 but the instantaneous growth rates (μ) were higher in L1a mesocosm. While the

1   smallest size-fractions of the phytoplankton (Chl *a*) were more heavily grazed than

2   the largest sizes in L1a, the microzooplankton community grazing severely impacted

3   the 0.65-2 μm size-fraction, but not the 0.2-0.65 μm in L4a.

4   In order to control for artifacts from potential photoadaptation processes

5   during the incubations we estimated net growth rates of the unfertilized bottles by

6   counting nanoflagellates on 2 μm pore-size filters. These data are compared to the net

7   growth rates in the same bottles for the closest Chl *a* size-fraction (2-10 μm) in Fig. 7.

8   No significant differences were observed for the first 3 experiments in L1a, and for

9   the first 2 in L4a ($p < 0.05$). However, after these dates the differences became

10   significant ($p > 0.05$). Therefore, our data on instantaneous growth rate (μ) in these

11   experiments based on Chl *a* appear to be overestimated since day 5 on.

12   Dilution experiments were also used to assess phytoplankton nutrient

13   limitation during the incubations, by comparing the net phytoplankton growth rates

14   between the fertilized and unfertilized undiluted bottles (Fig. 8). Nutrients appeared to

15   be limiting in all the L1-experiments, except the one conducted the first day (t-test; p

16   < 0.05). For L4a mesocosm the results showed no clear pattern. Differences in this

17   treatment were significant (although not always indicating higher growth in nutrient

18   amended bottles) in all experiments, except the ones at day 5 (t-test; $p < 0.05$).

19   By comparing the biomass of eukaryotic autotrophs (Fig. 2) with the grazing

20   rates from microzooplankton (Table 1) we attempted to estimate the autotrophic

21   carbon flux through microzooplankton. Due to the 1-day decoupling between grazing

22   and biomass estimates, we calculated the carbon flux by multiplying the averaged

23   biomass of autotrophs between two consecutive sampling dates ($C_{average}$) by the

24   corresponding grazing coefficient (m), as estimated from the dilution experiments.

1   These values can be converted into total flux by including the total volume (V) of the

2   mesocosm in the equation.

3   *Carbon flux = m * $C_{average}$ * V*

4   The results for both mesocosms are presented in Fig. 9. As a whole, the carbon flux

5   through microzooplankton was ca. 50% higher in the high light mesocosms than in

6   the low light treatments.

7

8   *3.3. Identification of major microbial herbivores and mixotrophy*

9   As previously described we identified the major microbial herbivores using live

10   fluorescently-labelled cultivated algae. The percentage of pigmented protists (ANF)

11   with labelled prey inside was low (Table 2), ranging from 3 to 6%. However, the

12   contribution of mixotrophic organisms (flagellates and dinoflagellates) to the total

13   number of grazers on phytoplankton (considering the heterotrophs with labelled prey

14   inside) was considerable (ca. 50%). We tested for significant differences (t-test) in the

15   percentages of heterotrophs *vs* mixotrophs for the variables light intensity and nutrient

16   addition. We only found a significant difference at the last day, when we found a

17   moderate increase of 1.6 times in the percentage of mixotrophic flagellates with

18   labelled prey in the L4 bottles compared to L1 ones (light effect), and this difference

19   was only apparent in the unfertilized treatments.

20

21   **4. Discussion**

22   *4.1. Responses of the microbial community to the light conditions*

1   Overall, one order of magnitude reduction in light in the L4 mesocosms compared to

2   the L1 ones resulted in lower peak biomass by a factor of 0.6 for autotrophs, 0.4 for

3   protozoans ($p < 0.05$; Two-way grouped ANOVA with repetition), and 0.6 for

4   mesozooplankton, and a change in the composition of the community at all trophic

5   levels. Similar reductions in the magnitudes of phytoplankton, micro- and

6   mesozooplankton bloom peaks were observed by experimentally increased

7   stratification depth (reduced light supply) in freshwater mesocosms (Berger et al.,

8   2010) indicating that light is an important driver of primary production scaling up to

9   higher trophic levels.

10   While the water used to initiate the mesocosms showed typical low Chl *a*

11   concentrations for the area (Psarra et al., 2000; Ignatiades et al., 2002), the Chl *a*

12   concentration increased faster in the darker mesocosms (L4) than in the more

13   illuminated ones (L1). Although this could suggest e.g. photoinhibition (Neale, 1987)

14   in L1 or increased growth rate of algae in L4 (for unknown reasons), the data reveal

15   that the rapid Chl *a* increase was likely due to an increase in pigments per cell, and

16   not to an increase in autotroph cell numbers in L4 (Fig. 2). This can be interpreted as

17   a photoadaptation in response to the dim light conditions in L4 (Falkowski, 1980;

18   Cullen and Lewis, 1988; Therriault et al., 1990). This response was not initially

19   evident in the community C:Chl *a* ratio, which was similar for both treatments during

20   the first days of the experiment (Fig. 6). We have to take into account that this ratio

21   includes not only autotrophs, but heterotrophic and detrital carbon as well, therefore

22   not properly describing the variations in the Chl *a* contents per cell, but rather

23   mirroring the evolution of heterotrophs and autotrophs in the mesocosms. Higher

24   proportion of heterotrophs by the end of the experiment in L1 resulted in a higher

25   C:Chl *a* ratio in this treatment. It is more difficult to interpret the variations in C:N

1   and C:P ratios; neither of them showing any clear difference between treatments.

2   Only, L1 mesocosms showed peaks of higher C:N, indicating an impoverishment of

3   the nutritional quality of the community, as theoretically expected, but the differences

4   were not significant.

5   The lower autotrophic biomass in the low light (L4) mesocosms could be

6   explained either by lower phytoplankton instantaneous growth rates or higher grazing

7   pressure than in the L1's, or both. The dilution experiments should give us both these

8   rates. Although the dilution experiments for L4a at day 1 cannot be interpreted in a

9   meaningful manner due to the positive slopes (possibly due to trophic cascades during

10   incubations as described by Calbet et al., 2011a), we can compare the rates for day 3,

11   the closest to the peak of phytoplankton biomass in all mesocosms where C:Chl *a*

12   ratios did not differ among treatments. At day 3 both mortality and growth rates of

13   total phytoplankton were slightly lower for the L1a mesocosm, indicating a slower

14   circulation of energy through the food web compared to the lower light mesocosms.

15   In L1a, both grazing and growth were similar, indicating that all the potential

16   production was consumed within the day. However, for L4a the measured mortality

17   rates exceeded the potential production. If this was the case, we should expect a

18   reduction of phytoplankton biomass in day 4 for this mesocosm. However, this was

19   not observed in the development of the Chl *a*, or in the cellular carbon. Actually, the

20   measured mortality rates exceeded the instantaneous phytoplankton growth rates in

21   several cases, especially in L4. These discrepancies have to be understood in the light

22   of other components of the community that were not considered in the dilution

23   grazing experiments, i.e. mesozooplankton. We sampled water for the dilution grazing

24   experiments from the upper part of the mesocosms, when the sun was up and the

25   mesozooplankton may have remained at the bottom of the bags. At night these

1   mesozooplankton are expected to migrate up the mesocosm and selectively graze on

2   microzooplankton (Calbet and Saiz, 2005; Saiz and Calbet, 2011), thus releasing

3   phytoplankton from their most important grazers (Calbet and Landry, 2004; Calbet et

4   al., 2008a), to a larger extent than in the bottles (Nejstgaard et al., 2001). Because the

5   mesozooplankton community in the mesocosms were rich in carnivorous species,

6   such as *Oithona* spp., they could have significantly predated on the microzooplankton,

7   and thus shaped the abundance of phytoplankton through trophic cascades.

8   Consequently, given our dilution bottles did not contain representative amounts of

9   mesozooplankton, we will refer to the estimated microzooplankton community rates

10   as "potential", and likely on the higher side.

11   Microbial herbivores were exerting a very high potential grazing pressure on

12   most of the Chl *a* size-fractions considered, except the 0.2-0.6 μm size-fraction in

13   L4a. We should expect to mostly find prokaryotes in this size-fraction, possibly

14   sheltered from heavy predation through trophic cascades in the food web. A similar

15   situation has been described in other oligotrophic areas, where trophic cascade effects

16   masked the grazing on the small phytoplankton (Nejstgaard et al., 1997; Calbet et al.,

17   2001; Calbet et al., 2008b). Surprisingly, we found saturated-feeding responses

18   (Gallegos, 1989) at the end of the experiment. While the concentration of prey

19   increased initially and saturated feeding rates of the microzooplankton could have

20   been expected at that point, we would not anticipate this situation for oligotrophic

21   areas. Although our data are not unique in showing saturated-feeding in dilution

22   grazing experiments of oligotrophic sites (Worden and Binder, 2003; Berninger and

23   Wickham, 2005; Calbet et al., 2008b), given the little biomass of prey attained by the

24   end of the experiment, this fact would imply microzooplankters from oligotrophic

25   sites present a faster-saturating feeding curves than those from productive ones. We

1   are not aware of studies comparing feeding kinetics of protozoans from different

2   ecosystems that can corroborate our results, and thus remains as a hypothesis to be

3   tested.

4

5   *4.2. Microbial community composition and mixotrophy*

6   We have so far discussed how the phytoplankton community as a whole progressed in

7   the different mesocosms. We will now discuss the development of the different

8   groups during the experiment. A conspicuous result was the gradual increase of

9   pigmented dinoflagellates and ciliates (Fig. 2). Both groups are larger in size than the

10   rest of components of the microbial community, and both have the potential to act as

11   mixotrophs (Stoecker, 1999; Johnson, 2011).

12   At organism level, nutrients and light intensity may trigger different feeding

13   responses in obligate mixotrophs, phagotrophic algae, and photosynthetic protozoa

14   (Bird and Kalff, 1989; Skovgaard, 1996; Jones et al., 1997; Legrand et al., 1998;

15   Stoecker, 1998; Stickney et al., 2000. However, at community level, continuous

16   darkness should favour heterotrophic metabolism because their higher efficiencies of

17   feeding and incorporation of organic matter into the cell (Tittel et al., 2003). In our

18   experiments, providing that light and nutrients were limiting, in the L4 treatment we

19   could expect a fast response of existing mixotrophic algae (organism-level response),

20   to be gradually replaced by heterotrophic grazers (community-level response).

21   However, at relatively short time intervals (ca. 24h) we did not detect any significant

22   variation on the mixotrophic contribution to overall grazing on algae (unfortunately,

23   we do not have data on mixotrophic grazing on prokaryotes). At longer time intervals

24   heterotrophic microbial biomass was below that of pigmented organisms (quotient

1   biomass heterotrophs/autotrophs < 1). This indicates dominance of autotrophic

2   processes over the microbial food web. Therefore, our premise was not met.

3   We should be aware, however, that the light intensities chosen in our study

4   were intended to simulate the water circulation in the mixed layer of an oligotrophic

5   highly illuminated sea. Even though in L4 we drastically reduced the irradiance by

6   94% compared to the surface irradiance, the light available in this treatment was still

7   above the light-saturated intensities necessary to develop deep-chlorophyll (90-100

8   μE m^{-2} s^{-1}; Harris, 1986; Ignatiades et al., 2002). Therefore, the community responses

9   of autotrophs and heterotrophs for these light intensities should not be as evident as

10   the ones expected for more limiting irradiance conditions, or even complete darkness.

11   Mixotrophs in L1 treatments should remain unaffected, given the similarity of

12   the experimental conditions to *in situ*, although we cannot disregard a gradual

13   increase in mixotrophy due to nutrient limitation during the experiment. Little is

14   known about the time frame of this process in natural systems, and we did not observe

15   any clear change in mixotrophy of flagellates in the L1 treatments, and mixotrophic

16   ciliates showed either an erratic pattern of abundance or contributed proportionally

17   less to the total abundance of ciliates.

18   Regarding the technique used to estimate mixotrophy, although we intended to

19   keep the LFLA concentration below 25% of the natural abundance of similarly-sized

20   flagellates, the LFLA were added without previous knowledge of the concentrations

21   in the experimental water. Consequently, the contribution of labelled flagellates to the

22   bulk was generally 25-50%, with one very high value of 83% (L1a, day one). These

23   data may thus not estimate ingestion rates or total carbon flux accurately.

24   Nevertheless, in accordance with Li et al. (1996, and references therein) we still argue

they show the percentage of mixotrophs within autotrophic algae, and contribution of

mixotrophic grazing to total grazing, and are overall more natural than the alternative

approach based on dead labelled algae, or inert microspheres.

4.3. Possible mixotrophy-related artifacts in our experimental set up

Mixotrophs, as any other grazer, may show positive slopes during dilution assays, due

to higher prey encounter rates and thus higher feeding/growth rates in the least diluted

treatments (Dolan et al., 2000). It can be argued this may confound Chl *a* based

estimates of phytoplankton growth rates by flattening the slope of the Chl *a*-based

regression and artificially reducing the estimates of microzooplankton grazing

(Landry, 1995; Schlüter, 1998; Calbet et al., 2008b). Unfortunately, the impact of this

process in a standard dilution experiment is quite difficult to predict because it will

depend on the biomass of mixotrophs, and their growth and grazing rates. In our

experiments, the contribution of mixotrophs to total number of grazers was on

average 50%, which is similar to other values found for other areas (Hall et al., 1993;

Safi and Hall, 1999; Unrein et al., 2007). However, this does not imply that these

values are equivalent to half the microzooplankton grazing impact. It is well known

that mixotrophs are less efficient in consuming prey than the heterotrophs (Stoecker,

1998; Jeong et al., 2010). For instance, for Mediterranean coastal waters, maximum

specific ingestion rates of autotrophic flagellates of 5-20 μm were of the order of 3.6

bacteria h^{-1}, whereas for heterotrophic flagellates were 15.4 bacteria h^{-1}, i.e. a factor of

4 (Unrein et al., 2007). For mixotrophic dinoflagellates specific ingestion rates of prey

were estimated to be 4 times lower than heterotrophic ones (Calbet et al., 2011b). If

we assume a similar 4 times lower biomass specific ingestion efficiency by the

mixotrophs (50% of the active grazers according our data), compared to the

heterotroph microzooplankton in our experiments, the mixotrophs would only account

for ca. 12.5% of the total estimated ingestion here. Converting this relative portion of

microzooplankton community grazing efficiency into autotrophic biomass consumed,

based on a gross growth efficiency of 30%, the 12.5% of total ingestion translates into

3.75% of expressed mixotroph production. This is a relatively modest production that

would not be expected to alter the accuracy of the rates determined by the dilution

experiments, or to explain the positive curve in the L4a, day1 experiment (Fig. 6).

4.4. Scaling up the results to the food web dynamics

Theoretically, a limitation in light intensity should result in a reduction in the attained

biomass of autotrophs (Huisman, 1999; Diehl, 2002), which should have negative

consequences for consumers, and further on higher trophic levels. This theory was

practically demonstrated in comparative lake studies (e.g., Berger et al., 2006).

However, it has been suggested for nutrient-limited lakes that reduced light increases

herbivore production due to a stoichiometric effect of the balance between light and

nutrients (Urabe and Sterner, 1996; Urabe et al., 2002). The mechanism proposed is

based on an increase of the nutrient contents per alga relative to carbon within alga for

light-limited organisms, and the opposite for well-illuminated ones (Diehl et al.,

2002). Therefore, improved food quality would enhance production of grazers at

lower light intensities (Diehl, 2007). Even though this theory could apply also to

marine oligotrophic systems, the differences between lake and marine food webs may

make the effect less relevant. In lakes, at least in those where the hypothesis was

tested, primary producers were directly consumed by crustacean grazers

1 (cladocerans); therefore, very susceptible to variations in nutrient composition of the

2 prey (Urabe et al., 2002). In marine oligotrophic systems, on the other hand, the

3 complexity of the food web makes it more likely that the effect of nutrient imbalances

4 is buffered through homeostasis in intermediate trophic levels. Moreover, the

5 relevance of mixotrophy (Stoecker, 1999; Stoecker et al., 2009), feeding selection on

6 nutritious prey (Cowles et al., 1988; Saiz and Calbet, 2011), nutrient tunneling effects

7 (Thingstad and Cuevas, 2010), and the ability of many protozoans to upgrade food

8 quality (Klein Breteler et al., 1999; Broglio et al., 2003; Veloza et al., 2006) would

9 make any nutrient deficiency of algae grown under high light conditions dampened

10 within the food web.

11   In our experiments the decrease in light intensity resulted in a reduction on the

12 final biomass of both autotrophs and heterotrophs (including metazoans) by a factor

13 of ca. 0.5. This contradicts the theory proposed of a lower zooplankton production in

14 highly illuminated lakes (Urabe et al., 1996; Urabe et al., 2002). Even though we

15 could not detect a clear nutritional response of the different light treatments, the C:N

16 of the more illuminated mesocosms tended to be higher than the one of the darkest.

17 Additionally, since the fourth day of the experiment the data indicate a possible

18 decrease in nutritional quality of phytoplankton in the most illuminated mesocosms

19 (higher C:Chl a ratios), but this likely impoverishment of the diet was not reflected in

20 a lower final abundance of grazers (both micro- and mesozooplankton). Therefore,

21 any effect of poor nutritional value of the prey might have been dampened before

22 reaching higher order consumers. This could be due to a variety of mechanisms (e.g.,

23 intermediary trophic links, food upgrading, mixotrophy, nutrient tunnelling), many of

24 them not examined in this study, but will have to be resolved in future studies in order

25 to better understand the effect of light availability in oligotrophic aquatic food webs.

1

2 **Acknowledgements**

18

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Table 1. Phytoplankton instantaneous growth rates without nutrient addition (μ; d^{-1}) and in situ mortality rates (m; d^{-1}) obtained from dilution grazing experiments for the different size-fractions of Chl a in mesocosms L1a and L4a.

Mesocosm L1a

Chl *a* fraction	Day 1 *m*	*r²*	μ	Day 3 *m*	*r²*	μ	Day 5 *m*	*r²*	μ	Day 7 *m*	*r²*	μ
total	0.85	0.70	0.90	0.82	0.92 **	0.77	0.60	0.74	0.74	0.71	0.95 **	0.90
> 10 μm	0.96 *	0.86	1.17	ns	nd	0.13	ns	nd	0.44	0.47	0.79 **	0.52
2 to 10 μm	0.81	0.75	0.61	0.68	0.98 **	0.36	ns	nd	0.31	0.49	0.89	0.95
0.65 to 2 μm	0.85	0.55	0.99	1.07	0.85 **	1.19	0.66	0.85	0.52	0.69	0.84 **	0.89
0.2 to 0.65 μm	1.23	0.56	1.93	1.16	0.74	1.46	1.09	0.61	1.31	1.34	0.85 **	0.38

Mesocosm L4a

Chl *a* fraction	*m*	μ	*m*	*r²*	μ	*m*	*r²*	μ	*m*	*r²*	μ
total	posit slope	nd	0.86	0.87	0.68	0.61	0.94	0.67	0.57	0.96 **	0.38
> 10 μm	posit slope	nd	0.26	0.62	0.62	0.32	0.73 **	0.32	0.49	0.65	0.39
2 to 10 μm	posit slope	nd	0.81	0.808	0.42	0.63	0.96	0.61	0.39	0.74 **	0.37
0.65 to 2 μm	posit slope	nd	1.19	0.816	1.09	0.85	0.98 **	0.94	0.40	0.85 **	0.36
0.2 to 0.65 μm	ns	-0.14	ns	nd	-0.0015	ns	nd	-0.09	0.56	0.58	-0.94

* one outlier removed; ** feeding saturation; nd = not determined; ns = not significant; posit slope = positive slope.

Table 2. Percentage of flagellates and dinoflagellates with ingested prey. The data have been obtained by multiplying the percentage of grazers with labeled prey times the proportion of labeled prey out of total prey. The experiments were conducted under the addition of excess of nutrients and without nutrients. ANF = autotrophic nanoflagellates, HNF = heterotrophic nanoflagellates, SE = standard error

Mesocosm	Nutrients	Day	%ANF	SE	%HNF	SE
L1a	No	1	2.59	0.21	7.59	0.19
L1a	No	3	5.61	1.25	11.67	0.84
L1a	No	5	4.80	0.71	8.88	2.18
L1a	No	7	2.71	0.19	8.19	0.54
L1a	Yes	1	2.44	0.41	4.59	0.73
L1a	Yes	3	4.06	0.31	8.43	1.58
L1a	Yes	5	4.02	0.62	4.85	0.78
L1a	Yes	7	2.83	0.69	12.05	1.77
L4a	No	1	4.13	0.23	8.35	0.81
L4a	No	3	5.19	0.58	9.14	1.04
L4a	No	5	4.19	1.14	7.52	0.74
L4a	No	7	4.08	0.45	9.93	1.13
L4a	Yes	1	4.56	0.37	8.59	0.76
L4a	Yes	3	5.51	1.18	10.33	0.84
L4a	Yes	5	4.10	0.92	7.85	1.30
L4a	Yes	7	3.58	0.56	11.63	1.15

Figure legends

Figure 1. Above: concentration of chlorophyll *a* (Chl *a*) along the experiment in the mesocosms L1a,b and L4a,b. Curves represent average values. Below: contribution of the different size-fractions of Chl *a*. Left panels L1, right panels L4.

Figure 2. Above: biomass of autotrophs and heterotrophs along the experiment in the mesocosms L1a,b and L4a,b (error bars are SE). Middle: contribution of the different groups of autotrophs to total autotrophic biomass. Below: contribution of the different groups of heterotrophs to total heterotrophic biomass. Left panels L1, right panels L4. Note that ciliates may not be well sampled with epifluorescence microscopy slides; however, they have been included in the plot for comparative purposes. Data are fitted to a smooth curve.

Figure 3. Abundance of ciliates (Lugol based) and contribution to mixotrophic ciliates (formaldehyde based) in the mesocosms L1a (above), and L4a (below).

Figure 4. Final abundance of the different mesozooplanktonic groups in L1a and L4a,b treatments. Note data for L1b are missing.

Figure 5. Elemental and mass ratios. A) Mass ratio of Carbon/Chl *a*, B) Molar ratio of Carbon/Nitrogen, C) Molar ratio of Carbon/Phosphorous. Data are fitted to a smooth curve. Note day 8 for C:N is missing.

Figure 6. Example of dilution grazing experiment plots corresponding to total Chl *a* in mesocosms L1a and L4a.

Figure 7. Autotroph growth rates obtained by cell counts (solid symbols) and by Chl *a* analysis of the 2 to 10 µm fraction (open symbols) in L1a and L4a mesocosms. Asterisks indicate significant differences ($p < 0.05$).

Figure 8. Total Chl *a* based phytoplankton growth rates in the nutrient amended and unamended bottles in L1a and L4a mesocosms. Error bars are SE.

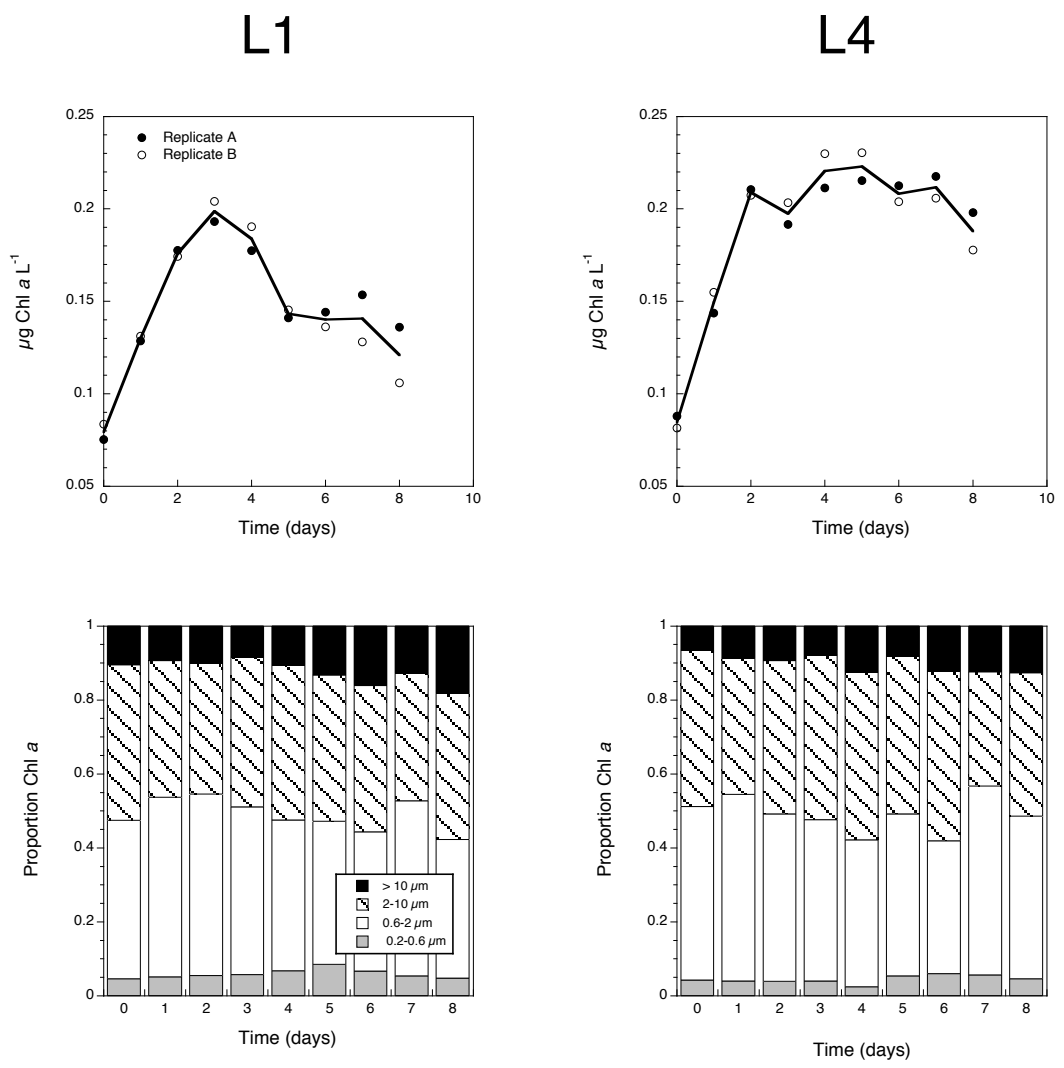**Figure 9.** Autotrophic carbon flux through microzooplankton in L1 and L4 mesocosms (see results); nd: not determined because positive slope in the microzooplankton grazing experiments (see results).
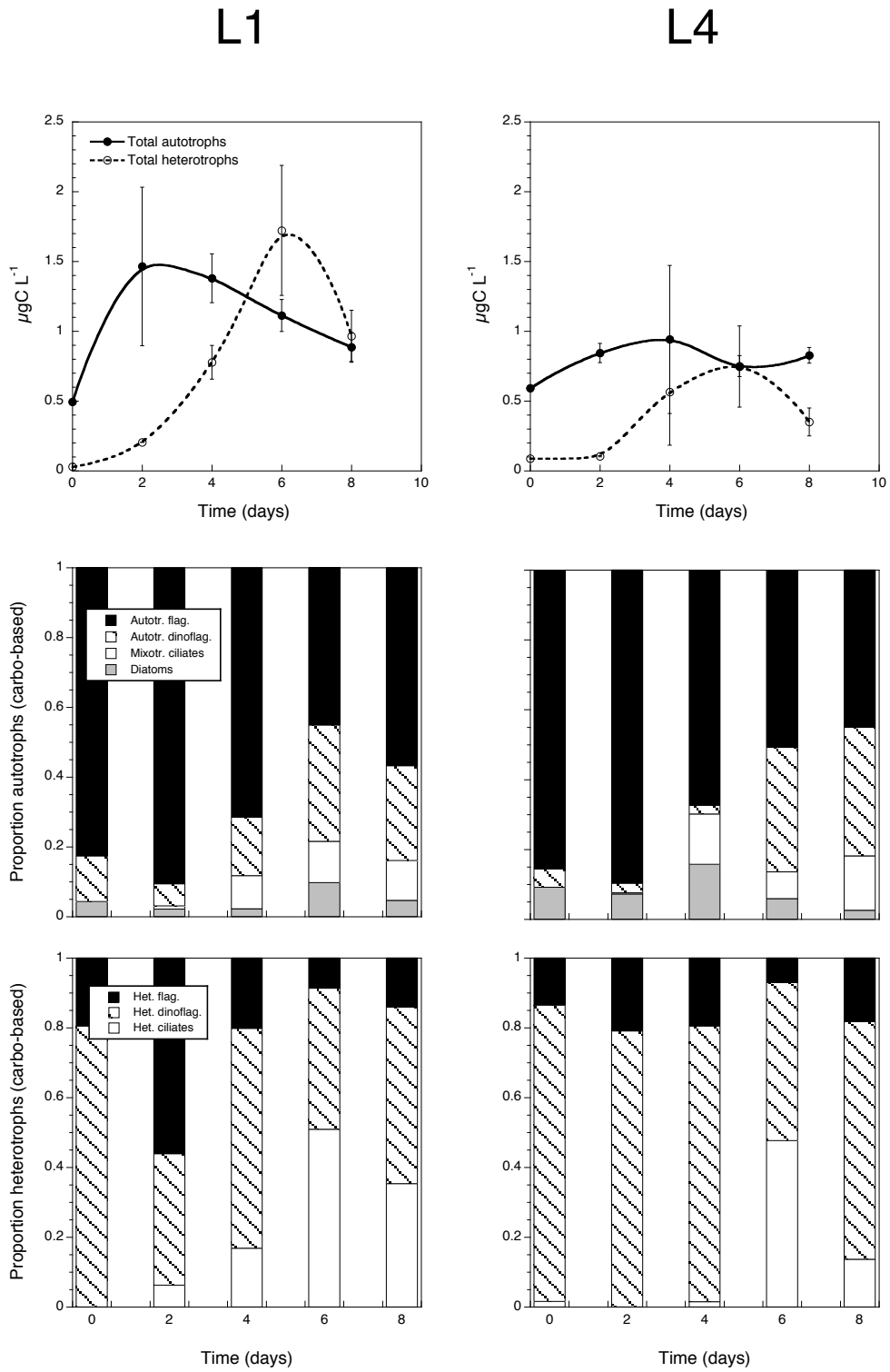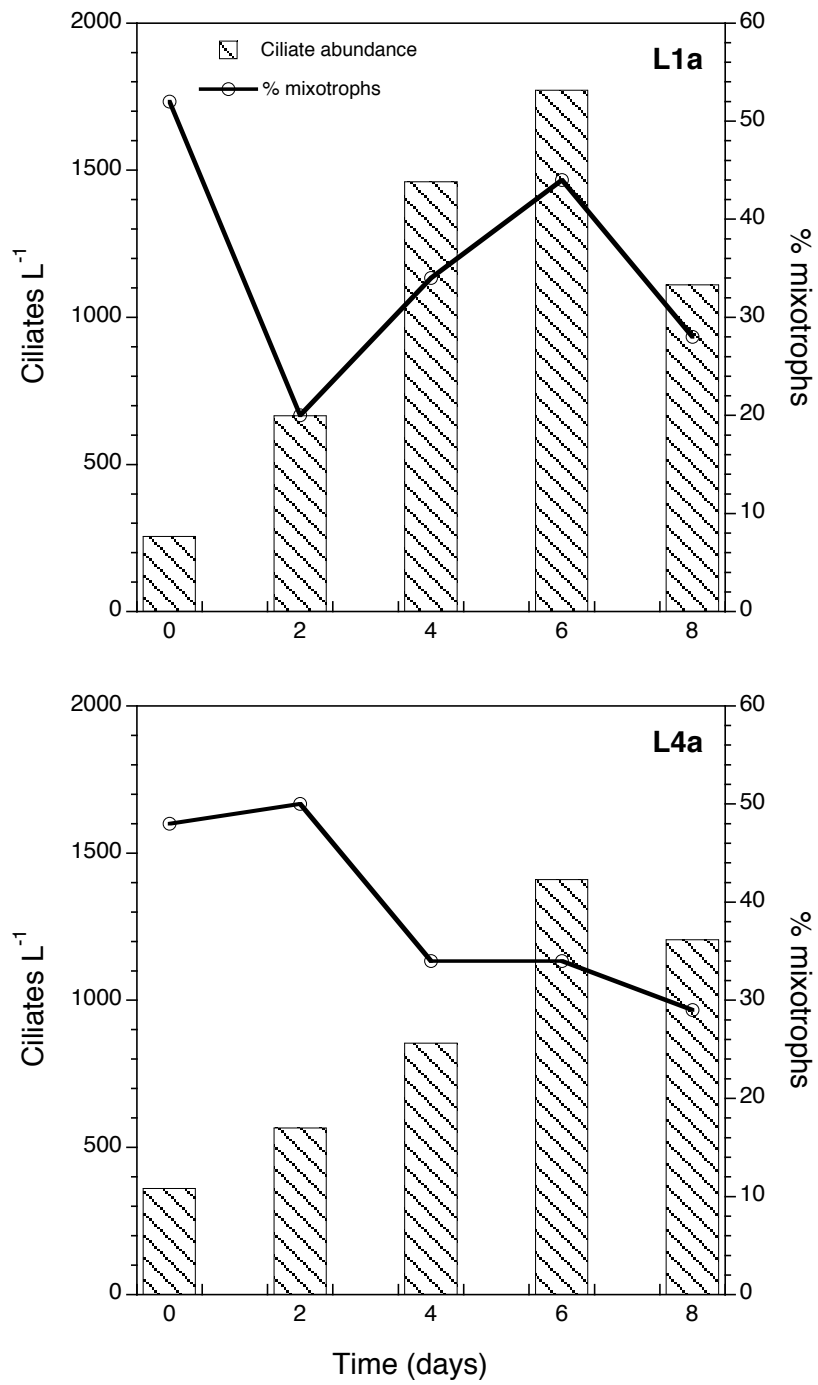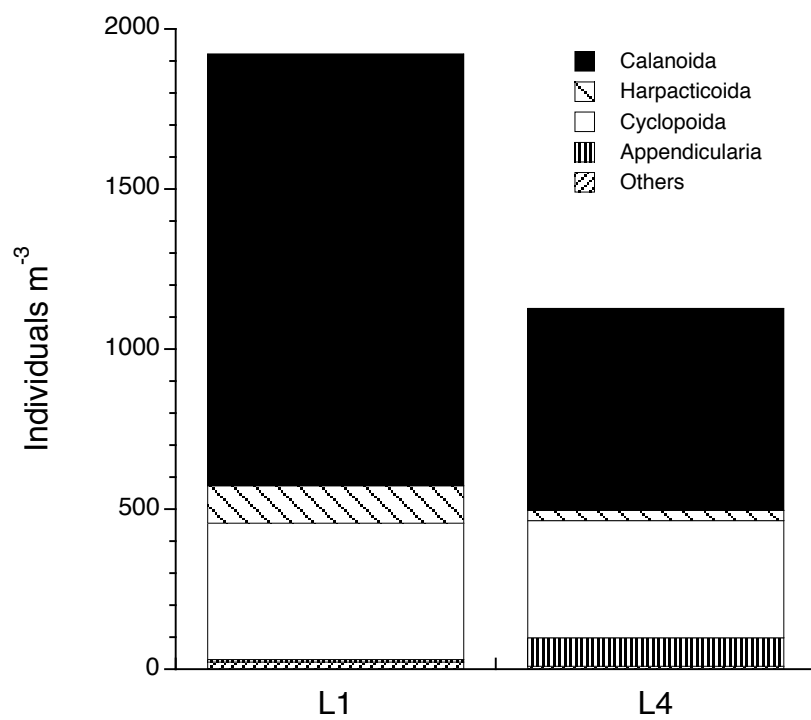
Fig. 1

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