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# Journal of Experimental Marine Biology and Ecology

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## Effects of light availability on mixotrophy and microzooplankton grazing in an oligotrophic plankton food web: Evidences from a mesocosm study in Eastern Mediterranean waters

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### ARTICLE INFO

#### Article history:

Received 5 December 2011

Received in revised form 1 May 2012

Accepted 7 May 2012

Available online xxx

#### Keywords:

Dilution experiments  
Eastern Mediterranean  
Light  
Mesocosm  
Microzooplankton  
Mixotrophy

### ABSTRACT

Plankton biomass and composition in the pelagic zone of oceans is exposed to changes in availability of light and nutrients due to large-scale ocean circulation and water column stratification. We hypothesized that displacement of plankton from surface to deeper darker waters would not only favor heterotrophy over time, as previously suggested, but also first rapidly affect the level of mixotrophy and, consequently, overall microbial grazing in plankton food webs. To test this in an oligotrophic marine system we incubated Eastern Mediterranean water (from 10 m depth north of Crete in September 2010) in 2.8 m<sup>3</sup> mesocosms simulating two different light intensities at the sampling station, surface waters (ca. 10 m; mesocosms L1) and deeper layers (ca. 50–60 m; mesocosms L4). The biomass and abundance of the main planktonic groups were monitored either daily or every second day, depending on the group. Microzooplankton grazing rates and the contribution of mixotrophic feeding were estimated by a combination of dilution experiments and incubations with live fluorescently labeled algae (LFLA). Although no nutrients were added to the mesocosms the chlorophyll *a* increased during the first 2 days of the experiment in both treatments. This increase resulted from phytoplankton growth in the light L1-mesocosm (autotrophic biomass was ca. doubled in L1 compared to L4), but was mostly due to photoadaptation of the algae in the L4-mesocosm, as indicated by lower carbon to chlorophyll *a* ratios. By the end of the experiment, the total biomass of protozoan and metazoan grazers in L1 was ca. twofold higher than in L4. The microzooplankton responded within the first 24 h, showing different grazing activity in L1 than in L4. Microzooplankton grazing rates on total Chl *a* were similar in both treatments; however, phytoplankton instantaneous growth rates were higher in the more illuminated mesocosm. This resulted in a closer coupling between both rates in L4, where all production was grazed daily, than in L1. Nevertheless, the overall flux of carbon through the microzooplankton was 33–60% higher in L1 compared to L4 throughout the experiment. The fraction of mixotrophy in the ciliate community varied in L1 (20–50%), but decreased over time in L4 from 50% to 30%. Our results do not support studies from freshwater, postulating that reduced light and nutrient limitation may increase herbivore production due to stoichiometric effects. Finally, 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

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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 (Calbet et al., 1996; Casotti, 2003; Estrada et al., 1993; Ignatiades et al., 2002; Letelier et al., 1996). In these deep layers light is scarce (ca. 1% surface irradiance, Estrada et al., 1993; Ignatiades et al., 2002) 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 trophic characteristics of some algae (i.e., enhancement of mixotrophy); both mechanisms potentially scaling up the food web. However, it is not clear how these communities respond to strong changes in light intensity when vertically displaced in the water column.

In this work we investigate the effects of an abrupt decrease in light supply on natural marine plankton communities collected in light-saturated oligotrophic surface waters. The light intensities were manipulated to represent the light availability at ca. 10 m depth in a mixed surface layer (where water was collected) and to simulate a low light climate equivalent to 50–60 m water column depth, near the base of the thermocline for the season (Casotti, 2003; Ramfos et al., 2006). To understand how light modulates the response of the plankton in the oligotrophic mixed layer of the Eastern Mediterranean we conducted a series of microzooplankton grazing and mixotrophy incubation experiments, and analyzed the composition of the microzooplankton community in a mesocosm experiment using natural water without any nutrient addition. Great care was taken to keep the nutrient levels naturally low. We hypothesized that i) the flow of matter through the food web would be diminished by a reduction in the energy input, and ii) that the plankton community would rapidly adapt to the new, darker, conditions by a fast increase of mixotrophic algae, and perhaps a longer-term negative response of mixotrophic ciliates, subsequently displaced by heterotrophic ciliates.

## 2. Methods

### 2.1. Experimental set up

This study was conducted as a part of a larger international experiment (LightDynamix) funded by the European project MESOAQUA (<http://mesoaqua.eu/>) at the Cretacosmos mesocosm facility of the Hellenic Centre for Marine Research (Crete, Greece). The total LightDynamix experimental design involved 4 light intensities (L1, L2, L3, and L4), duplicated in 2.8 m<sup>3</sup> mesocosms (replicates a, b) and run between 23 September and 1 October 2010. A detailed description of the overall mesocosm design and plankton development in all treatments is provided in Ptacnik et al. (in prep.). In short, the mesocosms were filled with water pumped from 10 m depth into acid cleaned 1 m<sup>3</sup> polyethylene (PE) containers, at a station 6 nautical miles north of Heraklion, Crete (Greece). Within 2–4 h the containers were transported to land and the content distributed evenly between the 8 mesocosm bags immersed in a tank with water at *in situ* temperature, by gravity

siphoning with acid cleaned plastic tubes. Filling took altogether two days. During this time, the mesocosms were kept dark by non-transparent covers. The mesocosms were made of transparent PE bags, with a diameter of 1.32 m and a depth of 2.5 m, surrounded by a double layer of PE black and white bags serving as an optical isolation. Optical lids for light manipulations in the mesocosms were created using 4 different grades of neutral density filters (Lee Filters, UK) and were installed at night after filling the mesocosms. Thus the experimental manipulation started the following morning (day 0). Due to the labor intensity of the incubation experiments and analysis of trophic level of the protists performed in this study we were restricted to focus on two experimental (mesocosm) light intensities only. We used the lightest and darkest, i.e. mesocosms L1a,b and L4a,b, respectively. The grey filters covering these mesocosms attenuated approximately 5 and 87% of the incoming light, resulting in average light intensities of ca. 60% and 6% of incident light intensity inside each of the L1 and L4 mesocosms, respectively. Thus closely mimicking the light intensities at the sampling depth 10 m and near the base of the thermocline for the season (50–60 m, Ramfos et al., 2006). We measured vertical profiles of photosynthetically active radiation (PAR) in 0.5 m steps with a spherical quantum sensor (LI-139SA; Licor, Lincoln, NE, USA) while simultaneously measuring incident PAR (flat quantum sensor LI-190SA) above the water surface outside the mesocosm, both attached to a Licor 1500 data logger. Light inside each mesocosm was calculated as the light intensity averaged over the water column (Imix). The water in the mesocosms was gently mixed by slow bubbling through an airlift pipe. Because ciliates and other plankton may be disturbed by bubbles or any activity creating turbulence, on the one side; but, on the other side, some type of mixing is needed to create homogenous mesocosm contents, we created a mixing system that uses a minimal amount of air, with a maximal but gentle mixing effect. It was modeled after the mixing systems that have been successfully used for over 30 years in the mesocosms at the Norwegian National Mesocosm centre, University of Bergen (see e.g. Williams and Egge, 1998; Nejtgaard et al., 2006), but down-scaled for these smaller mesocosms used here. In addition effort was made to make the bubbles big, to minimize the surface to volume ratio of the bubbles and thus the actually encounter rate between plankton and bubbles.

### 2.2. Community composition and biomass

Samples (125–250 mL) for chlorophyll *a* concentration (Chl *a*) were collected daily from each mesocosm. The water was sequentially filtered onto duplicate 10, 2, 0.65, and 0.2 μm pore-size, 47 mm diameter, polycarbonate filters (GE Water & Process Technologies), to obtain the size-fractions: 0.2–0.65 μm, 0.65–2 μm, 2–10 μm, and >10 μm, respectively. The filters were extracted immediately in acetone overnight at 4 °C and measured on a TD Turner 700 (Turner Designs, Sunnyvale, CA), with and without acidification according to Parsons et al. (1984). We collected particulate seston daily by filtering 500 mL sample water onto GF-F filters (47 mm). The filters were dried overnight and placed in petri slides until further analyses. The amount of particulate organic carbon, phosphorus and nitrogen on the filters was analysed by X-ray fluorescence (XRF) of the specific elements using a S4 Pioneer (Bruker AXS, Karlsruhe Germany) at the University of Bergen (Norway). We then calculated C:P, C:N molar ratios, and C:Chl *a* mass ratios.

Samples (50–100 mL) for analysis of the plankton community composition in the mesocosms were taken every second day, stained with primuline (Direct Yellow 59, Sigma-Aldrich Co.), fixed with 3.6% glutaraldehyde solution with 10% glycerol (final concentrations) and gently filtered onto 0.6-μm pore-size black polycarbonate membrane filters, and frozen until analysis (within 1–2 days). The method is a modification from Grebecki (1962), Hobbie et al. (1977) and Caron (1983) with the glycerol added to reduce the damage of especially small delicate protists during filtration as described in Sazhin et al.

(2007). Samples were analysed by epifluorescence microscopy and cell volumes were calculated by approximation of simple geometrical 3D shapes and converted into cell carbon as described in Menden-Deuer and Lessard (2000). In addition, samples for abundance of tintinnids and larger oligotrich ciliates were collected from one replicate bag of each treatment (L1a, L4a) every other day and fixed with acid Lugol's solution (2% final concentration), subsequently settled (250 mL) for 48 h and counted on an inverted microscope at 150× magnification. In order to estimate the percentage of mixotrophic ciliates an additional set of samples (250 mL) was fixed with borax-buffered formalin (final concentration 2% formaldehyde), subsequently settled in the dark for 48 h and counted on an inverted epifluorescence microscope at 150× magnification using blue light.

At the end of the experiment, mesozooplankton abundance and composition in the mesocosms were determined by pumping the entire volume of each mesocosm through the same 48-µm mesh submerged plankton net. Samples were preserved immediately after collection in 4% borax-buffered formaldehyde solution, with the exception of the L1b-sample, that was lost during the collection process. Species composition analysis and the estimation of total mesozooplankton abundance were carried out under a dissecting microscope in sub-samples taken with a Stempel pipette. Major mesozooplankton taxa (e.g., copepods, cladocerans, doliolids, appendicularians, etc.) were counted, and copepods and cladocerans were further identified to the species level when possible (at least 300 copepods were counted per sample). According to the latest taxonomy (Boxshall and Halsey, 2004), the order of Poecilostomatoida has been transferred to the order of Cyclopoida, hence for the purposes of this work have been presented together.

### 2.3. Microzooplankton grazing and mixotrophy experiments

Every other day we conducted standard grazing dilution experiments (Landry and Hassett, 1982) in one replicate of the lightest and darkest mesocosms (L1a and L4a, respectively). The water for these experiments was collected early in the morning by gravity-filling of 30 L dark containers using silicon tubing. Once in the laboratory, the water was gently siphoned into a 60 L bucket and carefully mixed to avoid generating bubbles that could damage delicate ciliates (Broglia et al., 2004; Gifford, 1985). A known volume of water was gravity-filtered through a Pall Acropak 0.8/0.2 500 capsule (previously flushed, including tubing, with diluted HCl and thoroughly rinsed with deionised water) to obtain the dilution series at the proportions 25, 50, 75 and 100% of the experimental water. The dilution series was prepared in 1.3 L PC bottles, which were amended with a nutrient mixture (1 µM NH<sub>4</sub>Cl, 0.07 µM Na<sub>2</sub>HPO<sub>4</sub>, and 0.5 µM Na<sub>2</sub>SiO<sub>3</sub>) to guarantee that nutrients were not limiting in any treatment. To assess the natural growth of algae two 100% experimental water (i.e., not diluted) bottles without addition of nutrients were also prepared. Special care was taken to work under dim light conditions for the darkest treatments.

In order to avoid adverse light effects on the mesocosms when initiating and ending the incubation periods (ca. 24 h) of the grazing experiments, the bottles were incubated in specially prepared mesocosm incubators equipped with an easily opening-closing cover made from the same neutral density filter used for the mesocosms L1 and L4, hence providing temperature (24.3–25.7 °C) and light levels comparable to these mesocosms (same level attenuation; for absolute values see Ptacnik et al. in prep.). The bottles were hung from a floating wheel at 0.5 m depth, which corresponded to the average light intensity of the mesocosms. The wheel rotated at app. 1 r.p.m. propelled by a submerged water pump. This approach assured that the light conditions were homogeneous for all the bottles. However, because these incubators were filled with brackish nutrient rich water from a well at the HCMR, different from the nutrient poor water of the mesocosms, a denser phytoplankton bloom developed at days 4–5. This had consequences for the light intensity at which

the experimental bottles were incubated, and is discussed below. In the beginning (initial samples), as well as at the end of the incubations, we took duplicate samples for the quantification of the different size-fractions of Chl *a*, and for the quantification of nanoflagellates (in the initial and unfertilized bottles). For the quantification of nanoflagellates in the dilution grazing experiments, 75–100 mL of glutaraldehyde preserved samples were stored at 4 °C in the dark for 2 h and then filtered onto 2 µm black polycarbonate filters, and stained with 4', 6'-diamidino-2-phenylindole (DAPI). We counted 200 to 500 cells under epifluorescence microscopy in each filter. The rates obtained for the cell counts of autotrophic flagellates were compared to Chl *a*-based growth rate estimates. This way we could both estimate the cellular growth rates of the phytoplankton and quantify potential artifacts in pigment based growth rates due to photo-adaptation changing the cellular Chl *a* contents during the incubations.

For all experiments, we used Model I linear regressions to obtain the slope ( $m$ ; grazing mortality rate,  $d^{-1}$ ) of the equation relating the fraction of undiluted water ( $x$ ) and the net phytoplankton growth rates ( $y$ ;  $K$ ) estimated from changes in Chl *a* concentration during incubation. Because the intercept of the equation ( $\mu_n$ ) would provide an overestimation of phytoplankton instantaneous growth rates (due to the addition of nutrients in the bottles), the instantaneous *in situ* growth rates ( $\mu$ ) in dilution grazing experiments were derived from net growth in the unfertilized bottles ( $K_0$ ; where no nutrients added) and were corrected for mortality by microzooplankton from dilution experiments when the latter was significant (Landry and Hassett, 1982).

$$\mu = K_0 + m$$

When we found saturated feeding responses we used the linear relationship of the most diluted treatments to obtain the phytoplankton instantaneous growth rates with added nutrients ( $\mu_n$ ) according to Gallegos (1989) and Dolan et al. (2000). The microzooplankton grazing rate ( $m_n$ ) was then calculated for the undiluted nutrient-enriched bottles as

$$m_n = \mu_n - K_n$$

where  $K_n$  is the phytoplankton net growth rate in the undiluted nutrient-enriched bottles. This grazing rate calculated as indicated above does not completely correspond to the *in situ* microzooplankton grazing rate ( $m$ ). According to Moigis and Gocke (2003) and Moigis (2006) the *in situ* microzooplankton grazing rate (i.e., in the undiluted bottles without added nutrients) should be calculated as

$$m = m_n * K_0 * (\exp(K_n * t) - 1) / K_n * (\exp(K_0 * t) - 1)$$

where  $t$  is the incubation time.

In parallel we assessed the amount of herbivory of mixotrophic algae (large flagellates and dinoflagellates) by measuring the uptake of live fluorescently labeled algae (LFLA) during 24 h *in situ* grazing experiments. Aiming at a better representation of the nanoflagellate size spectrum of the mesocosm environment two different types of algae were used to produce the LFLA stocks: *Isocrhysis galbana* (4–6 µm) and *Tetraselmis* sp. (8–10 µm). Algal cultures grown exponentially in f/2 medium at 18 °C in a 12:12 h light: dark cycle were labeled with the live fluorescent vital stain CellTracker® Blue CMAC (7-amino 4-chloromethylcoumarin, Molecular Probes Inc.) according to Teegarden (1999). The latter vital stain makes the cells fluoresce bright blue when excited by UV light at 354 nm.

The incubations were conducted in 650 mL polycarbonate bottles, filled with water from the mesocosm L1a and L4a, and lasted for ca. 24 h. Two sets of triplicate bottles were prepared, one set without nutrient additions and one set amended with nutrients as in the dilution experiments. These bottles were used for an additional estimate of the effect of nutrient limitation. Each experimental bottle was inoculated

with a mixture of fluorescent prey and was subsequently gently mixed. We tried to keep total LFLA concentration inside the bottles below 25% of the abundance at which similarly sized flagellates are typically found in oligotrophic areas of the Mediterranean Sea ( $1.3 \times 10^2$ – $6.0 \times 10^3$  cells mL<sup>-1</sup>) (Christaki et al., 2001). Subsamples were always taken prior incubations to determine exact initial concentration of LFLA in the bottles, which at times was more than expected (see discussion). The incubations took place in the already described incubators, together with the dilution experiment bottles. At the end of each incubation 75–100 mL samples were fixed with glutaraldehyde (final concentration 1%) and processed as above (dilution grazing experiments), with the exception that no DAPI stain was used. Protozoan cells with LFLA prey inside were enumerated under an Olympus BX 40 inverted epifluorescence microscope at a magnification of 400×. Mixotrophic activity was calculated as the percentage of mixotrophs > 10 μm and < 10 μm found with labelled prey inside.

### 3. Results

#### 3.1. Community composition and biomass

In Fig. 1 we show the time course of the total phytoplankton biomass (measured as Chl *a*) along the 8 days of experiment in mesocosms experiencing high (L1) and low (L4) light treatments, as well

as the contribution of the different size fractions to the standing stock of Chl *a*. Both treatments and parallels showed an increase in Chl *a* the first 2–3 days of experiment, from 0.08 to ca. 0.2 μg Chl *a* L<sup>-1</sup>. While values in both the L4 mesocosms were approximately 0.2 μg Chl *a* L<sup>-1</sup> throughout the experiment, the L1 mesocosms showed a rapid decline during days 4 and 5 and ended (on day 8) at concentrations near half the maximum attained. The Chl *a* fractions 0.6–2 μm and 2–10 μm were of similar proportion and together made up 75–90% of the total Chl *a* at all times (Fig. 1). Except for a slight increase in the contribution of Chl *a* > 10 μm during the experiment in all treatments, there were no clear changes in size groups over time in the mesocosms.

The development of the autotrophic protist biomass determined by epifluorescence microscopy (Fig. 2) resembled the pattern of Chl *a* development in the L1 mesocosms. However, in the L4 mesocosms the autotroph biomass showed a development different from Chl *a*, with lower biomass values and less clear peaks ( $0.94 \pm 0.53$  SE μgC L<sup>-1</sup>, Fig. 2). The total biomass peak of heterotrophic eukaryotes in the L1 mesocosms ( $1.7 \pm 0.12$  SE μgC L<sup>-1</sup>) was more than twofold higher than in L4 ( $0.75 \pm 0.07$  SE μgC L<sup>-1</sup>), although the temporal development was similar.

In all mesocosms, mixotrophic ciliates and autotrophic dinoflagellates increased their biomass towards the end of the study, becoming significant components to the community, otherwise dominated by

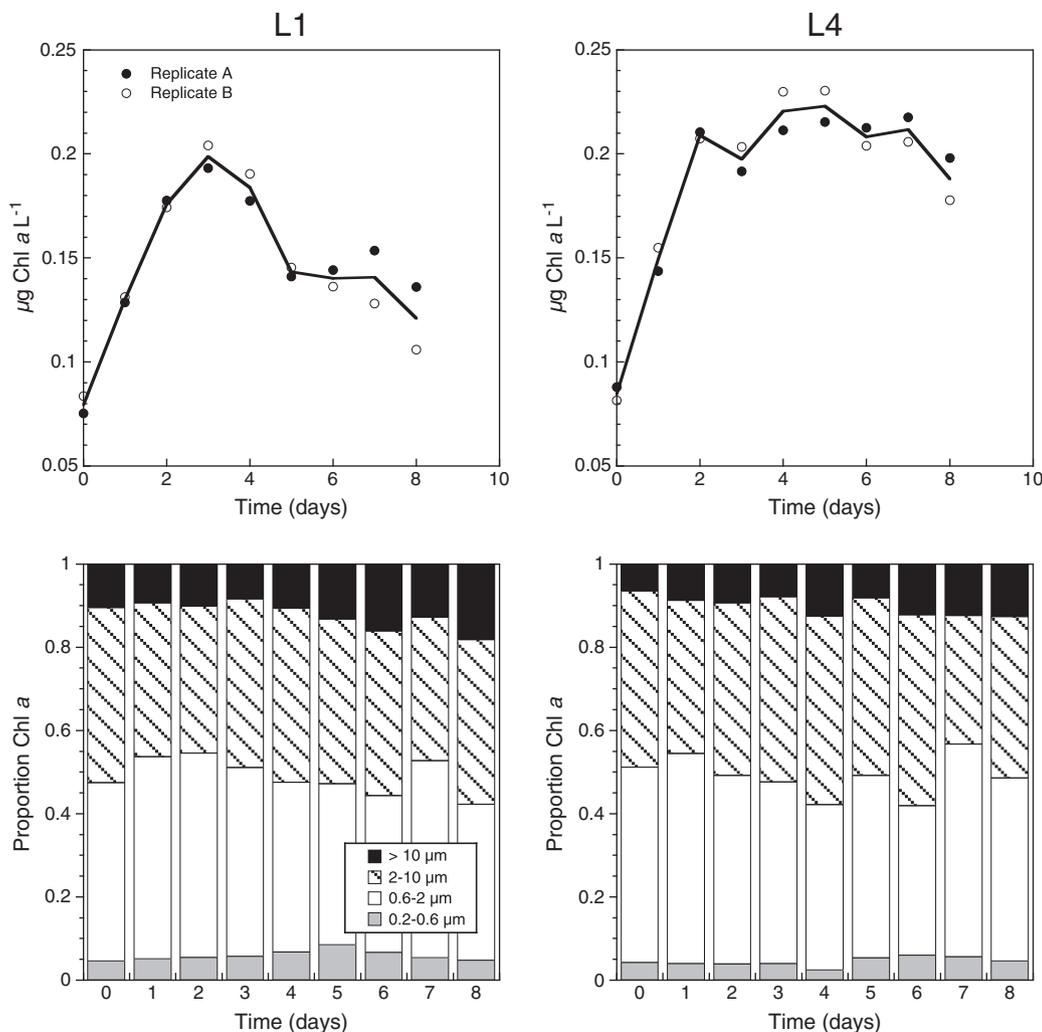
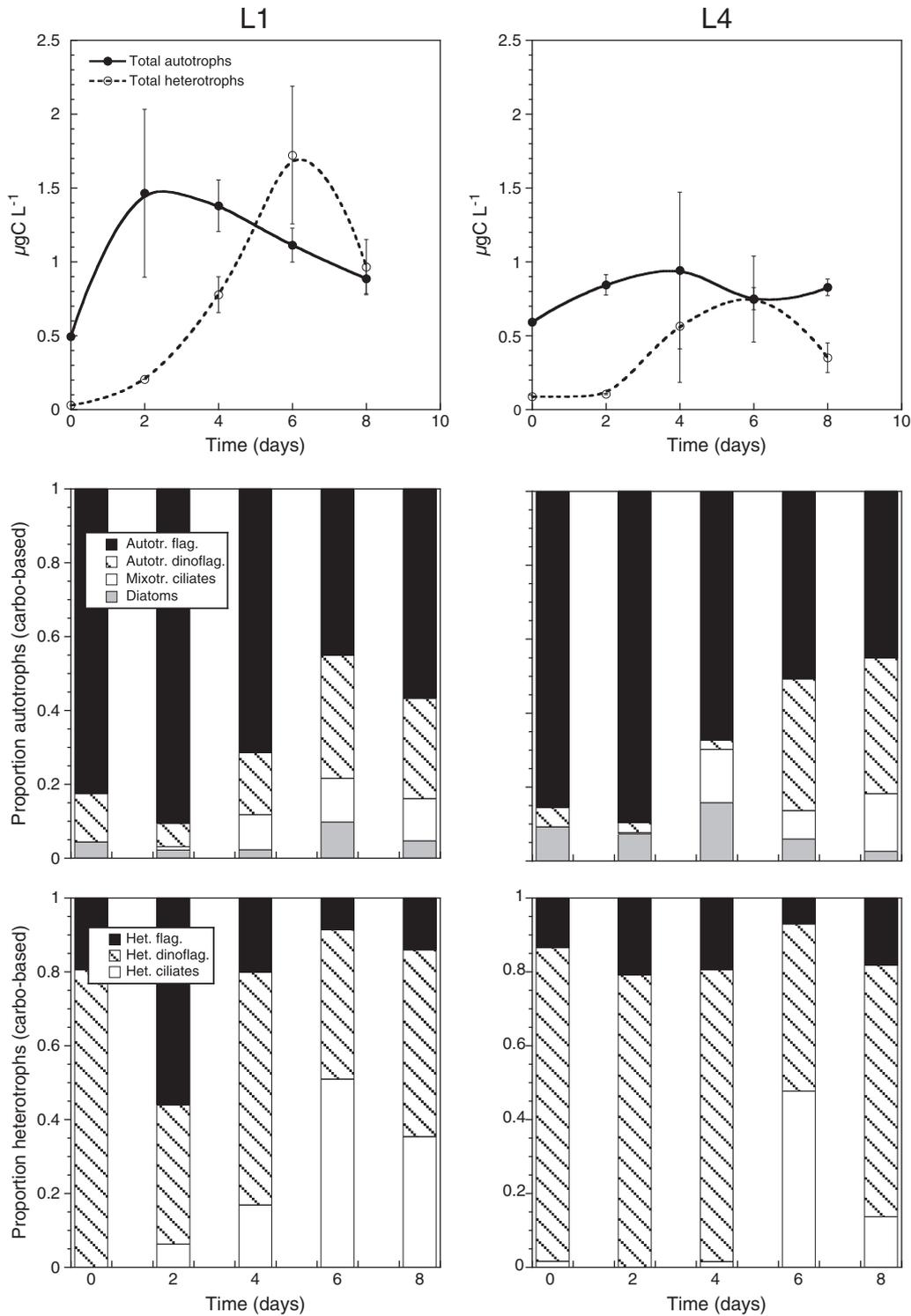


Fig. 1. Above: concentration of chlorophyll *a* (Chl *a*) along the experiment in the mesocosms L1a,b and L4a,b. Curves represent average values. Below: contribution of the different size-fractions of Chl *a*. Left panels L1, right panels L4.



**Fig. 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.

autotrophic flagellates (Fig. 2). In mesocosms L1 heterotrophic flagellates and ciliates dominated the protozoan community-biomass the second and sixth day of the experiment, respectively. In L4 mesocosms, however, the heterotrophic community remained quite stable with the only exception of a peak of ciliates on day 6 (Fig. 2). The Lugol preserved samples showed a progressive increase of ciliates in both mesocosm treatments until day 6 and a slight decrease the last day (Fig. 3). The contribution of mixotrophs to the total abundance

of ciliates consistently decreased in L4a from 50% to 30% and was quite variable in L1a (Fig. 3).

The mesozooplankton biomass community at the end of the experiment was higher in the L1 treatment than in L4 (Fig. 4). The mesozooplankton community was numerically dominated by copepods in all measured treatments with calanoids (mostly *Clausocalanus* spp. and *Calocalanus* spp.) showing ca. double abundance in L1a, being the most abundant group in this mesocosm. Cyclopoida (mostly *Oithona*

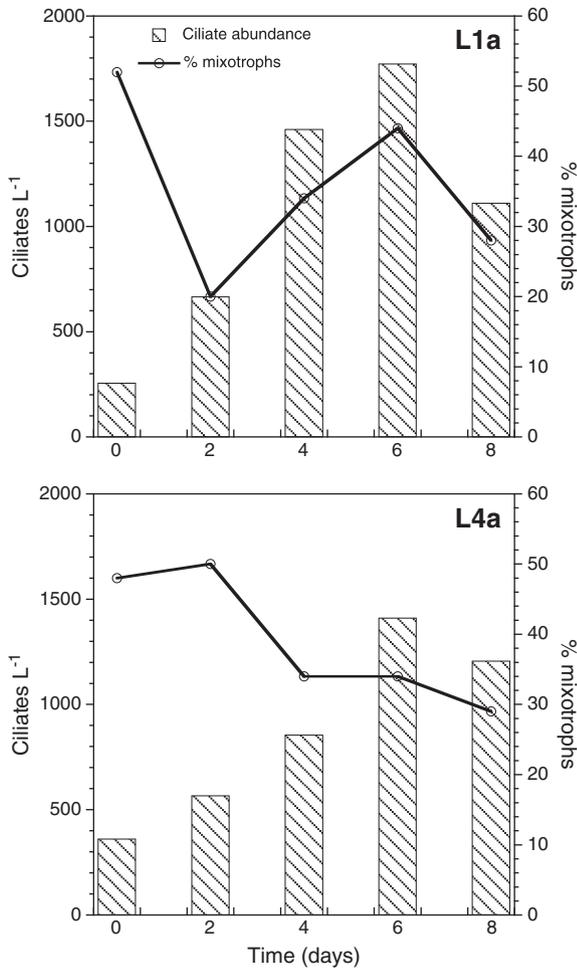


Fig. 3. Abundance of ciliates (Lugol based) and contribution to mixotrophic ciliates (formaldehyde based) in the mesocosms L1a (above), and L4a (below).

spp.) abundance was similar at both light intensities. Other important components, although at much lower abundances were appendicularia and cladocera (Fig. 4).

The elemental ratios of C, N, and P, as well as the C:Chl *a* mass ratio are presented in Fig. 5. Light did not significantly affect any of the ratios ( $p > 0.05$ ; grouped ANOVA with repeated measures), however some differences are evident in the proportion of Chl *a* per unit of carbon since day 4 (Fig. 5A), and in the C:N at days 1 and 2 (Fig. 5B). C:P

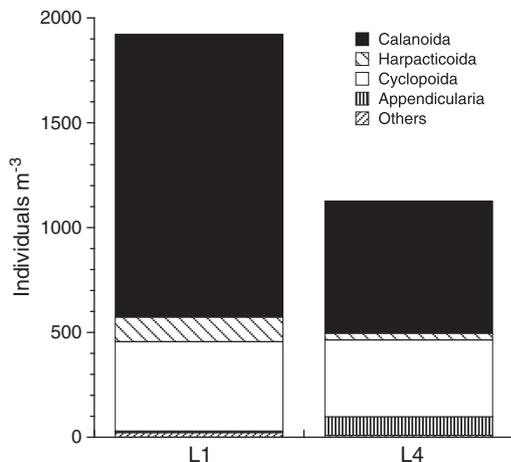


Fig. 4. Final abundance of the different mesozooplanktonic groups in L1a and L4a,b treatments. Note data for L1b are missing.

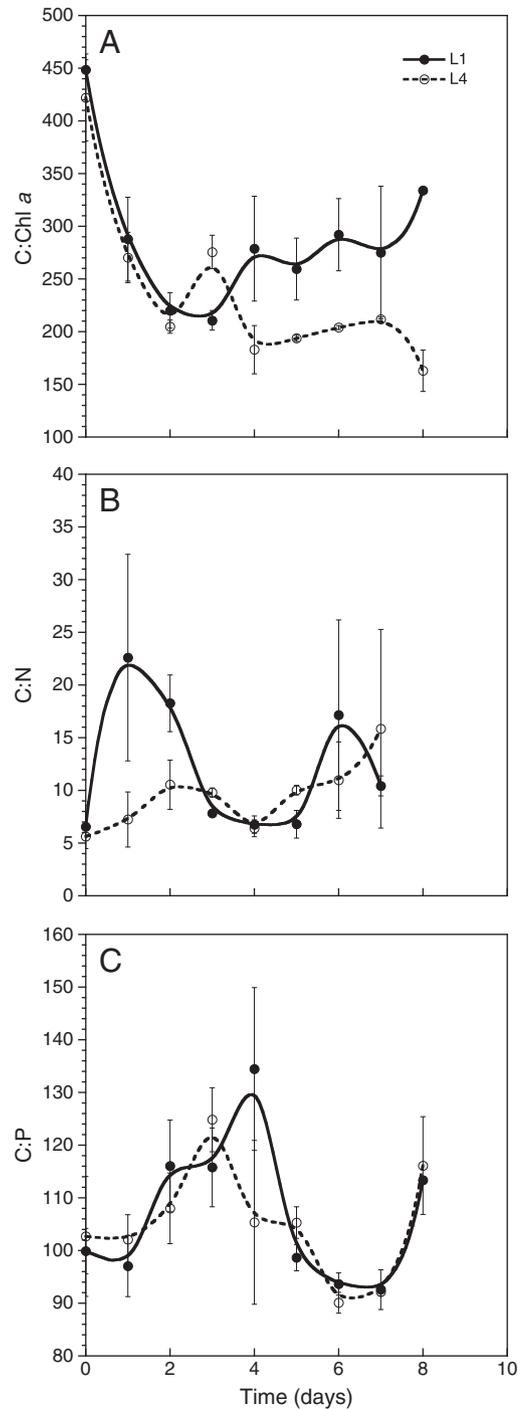


Fig. 5. Elemental and mass ratios. A) Mass ratio of Carbon/Chl *a*, B) Molar ratio of Carbon/Nitrogen, C) Molar ratio of Carbon/Phosphorous. Data are fitted to a smooth curve. Note day 8 for C:N is missing.

ratios followed a similar pattern in both treatments along the experiment, with peaks at days 3 and 4 for L4 and L1, respectively and an increase of the quotient last day in all mesocosms (Fig. 5C).

### 3.2. Microzooplankton dilution grazing experiments

We conducted four dilution-grazing experiments (Landry and Hassett, 1982) in each replicate “a” of the mesocosms L1 and L4. Overall, all (Chl *a*-based) phytoplankton growth and mortality rates observed for total and size-fractionated Chl *a* were quite high, with many saturation-feeding responses at the end of the experiment,

sensu Gallegos (1989) (Fig. 6; Table 1). During day 1 we observed consistent positive slopes for total phytoplankton and all size-fractions in the low light mesocosm (L4a, Fig. 6). In general, mortality rates ( $m$ ) on total Chl  $a$  were similar in both treatments, but the instantaneous growth rates ( $\mu$ ) were higher in L1a mesocosm. While the smallest size-fractions of the phytoplankton (Chl  $a$ ) were more heavily grazed than the largest sizes in L1a, the microzooplankton community grazing severely impacted the 0.65–2  $\mu\text{m}$  size-fraction, but not the 0.2–0.65  $\mu\text{m}$  in L4a.

In order to control for artifacts from potential photoadaptation processes during the incubations we estimated net growth rates of the unfertilized bottles by counting nanoflagellates on 2  $\mu\text{m}$  pore-size filters. These data are compared to the net growth rates in the same bottles for the closest Chl  $a$  size-fraction (2–10  $\mu\text{m}$ ) in Fig. 7. No significant differences were observed for the first 3 experiments in L1a, and for the first 2 in L4a ( $p < 0.05$ ). However, after these dates the differences became significant ( $p > 0.05$ ). Therefore, our data on instantaneous growth rate ( $\mu$ ) in these experiments based on Chl  $a$  appear to be overestimated since day 5 on.

Dilution experiments were also used to assess phytoplankton nutrient limitation during the incubations, by comparing the net phytoplankton growth rates between the fertilized and unfertilized undiluted bottles (Fig. 8). Nutrients appeared to be limiting in all the L1-experiments, except the one conducted the first day ( $t$ -test;  $p < 0.05$ ). For L4a mesocosm the results showed no clear pattern. Differences in this treatment were significant (although not always indicating higher growth in nutrient amended bottles) in all experiments, except the ones at day 5 ( $t$ -test;  $p < 0.05$ ).

By comparing the biomass of eukaryotic autotrophs (Fig. 2) with the grazing rates from microzooplankton (Table 1) we attempted to estimate the autotrophic carbon flux through microzooplankton. Due to the 1-day decoupling between grazing and biomass estimates, we calculated the carbon flux by multiplying the averaged biomass of autotrophs between two consecutive sampling dates ( $C_{\text{average}}$ ) by the corresponding grazing coefficient ( $m$ ), as estimated from the dilution experiments. These values can be converted into total flux by including the total volume ( $V$ ) of the mesocosm in the equation.

$$\text{Carbon flux} = m * C_{\text{average}} * V$$

The results for both mesocosms are presented in Fig. 9. As a whole, the carbon flux through microzooplankton was ca. 50% higher in the high light mesocosms than in the low light treatments.

### 3.3. Identification of major microbial herbivores and mixotrophy

As previously described we identified the major microbial herbivores using live fluorescently-labelled cultivated algae. The percentage of pigmented protists (ANF) with labelled prey inside was low (Table 2), ranging from 3 to 6%. However, the contribution of mixotrophic organisms (flagellates and dinoflagellates) to the total number of grazers on phytoplankton (considering the heterotrophs with labelled prey inside) was considerable (ca. 50%). We tested for significant differences ( $t$ -test) in the percentages of heterotrophs vs mixotrophs for the variables light intensity and nutrient addition. We only found a significant difference at the last day, when we found a moderate increase of 1.6 times in the percentage of mixotrophic flagellates with labelled prey in the L4 bottles compared to L1 ones (light effect), and this difference was only apparent in the unfertilized treatments.

## 4. Discussion

### 4.1. Responses of the microbial community to the light conditions

Overall, one order of magnitude reduction in light in the L4 mesocosms compared to the L1 ones resulted in lower peak biomass by a

factor of 0.6 for autotrophs, 0.4 for protozoans ( $p < 0.05$ ; Two-way grouped ANOVA with repetition), and 0.6 for mesozooplankton, and a change in the composition of the community at all trophic levels. Similar reductions in the magnitudes of phytoplankton, micro- and mesozooplankton bloom peaks were observed by experimentally increased stratification depth (reduced light supply) in freshwater mesocosms (Berger et al., 2010) indicating that light is an important driver of primary production scaling up to higher trophic levels.

While the water used to initiate the mesocosms showed typical low Chl  $a$  concentrations for the area (Ignatiades et al., 2002; Psarra et al., 2000), the Chl  $a$  concentration increased faster in the darker mesocosms (L4) than in the more illuminated ones (L1). Although this could suggest e.g. photoinhibition (Neale, 1987) in L1 or increased growth rate of algae in L4 (for unknown reasons), the data reveal that the rapid Chl  $a$  increase was likely due to an increase in pigments per cell, and not to an increase in autotroph cell numbers in L4 (Fig. 2). This can be interpreted as a photoadaptation in response to the dim light conditions in L4 (Cullen and Lewis, 1988; Falkowski, 1980; Therriault et al., 1990). This response was not initially evident in the community C:Chl  $a$  ratio, which was similar for both treatments during the first days of the experiment (Fig. 6). We have to take into account that this ratio includes not only autotrophs, but heterotrophic and detrital carbon as well, therefore not properly describing the variations in the Chl  $a$  contents per cell, but rather mirroring the evolution of heterotrophs and autotrophs in the mesocosms. Higher proportion of heterotrophs by the end of the experiment in L1 resulted in a higher C:Chl  $a$  ratio in this treatment. It is more difficult to interpret the variations in C:N and C:P ratios; neither of them showing any clear difference between treatments. Only, L1 mesocosms showed peaks of higher C:N, indicating an impoverishment of the nutritional quality of the community, as theoretically expected, but the differences were not significant.

The lower autotrophic biomass in the low light (L4) mesocosms could be explained either by lower phytoplankton instantaneous growth rates or higher grazing pressure than in the L1's, or both. The dilution experiments should give us both these rates. Although the dilution experiments for L4a at day 1 cannot be interpreted in a meaningful manner due to the positive slopes (possibly due to trophic cascades during incubations as described by Calbet et al., 2011a), we can compare the rates for day 3, the closest to the peak of phytoplankton biomass in all mesocosms where C:Chl  $a$  ratios did not differ among treatments. At day 3 both mortality and growth rates of total phytoplankton were slightly lower for the L1a mesocosm, indicating a slower circulation of energy through the food web compared to the lower light mesocosms. In L1a, both grazing and growth were similar, indicating that all the potential production was consumed within the day. However, for L4a the measured mortality rates exceeded the potential production. If this was the case, we should expect a reduction of phytoplankton biomass in day 4 for this mesocosm. However, this was not observed in the development of the Chl  $a$ , or in the cellular carbon. Actually, the measured mortality rates exceeded the instantaneous phytoplankton growth rates in several cases, especially in L4. These discrepancies have to be understood in the light of other components of the community that were not considered in the dilution grazing experiments, i.e. mesozooplankton. We sampled water for the dilution grazing experiments from the upper part of the mesocosms, when the sun was up and the mesozooplankton may have remained at the bottom of the bags. At night these mesozooplankton are expected to migrate up the mesocosm and selectively graze on microzooplankton (Calbet and Saiz, 2005; Saiz and Calbet, 2011), thus releasing phytoplankton from their most important grazers (Calbet, 2008; Calbet and Landry, 2004), to a larger extent than in the bottles (Nejstgaard et al., 2001). Because the mesozooplankton community in the mesocosms were rich in carnivorous species, such as *Oithona* spp., they could have significantly predated on the microzooplankton, and thus shaped the abundance of phytoplankton through trophic cascades. Consequently, given

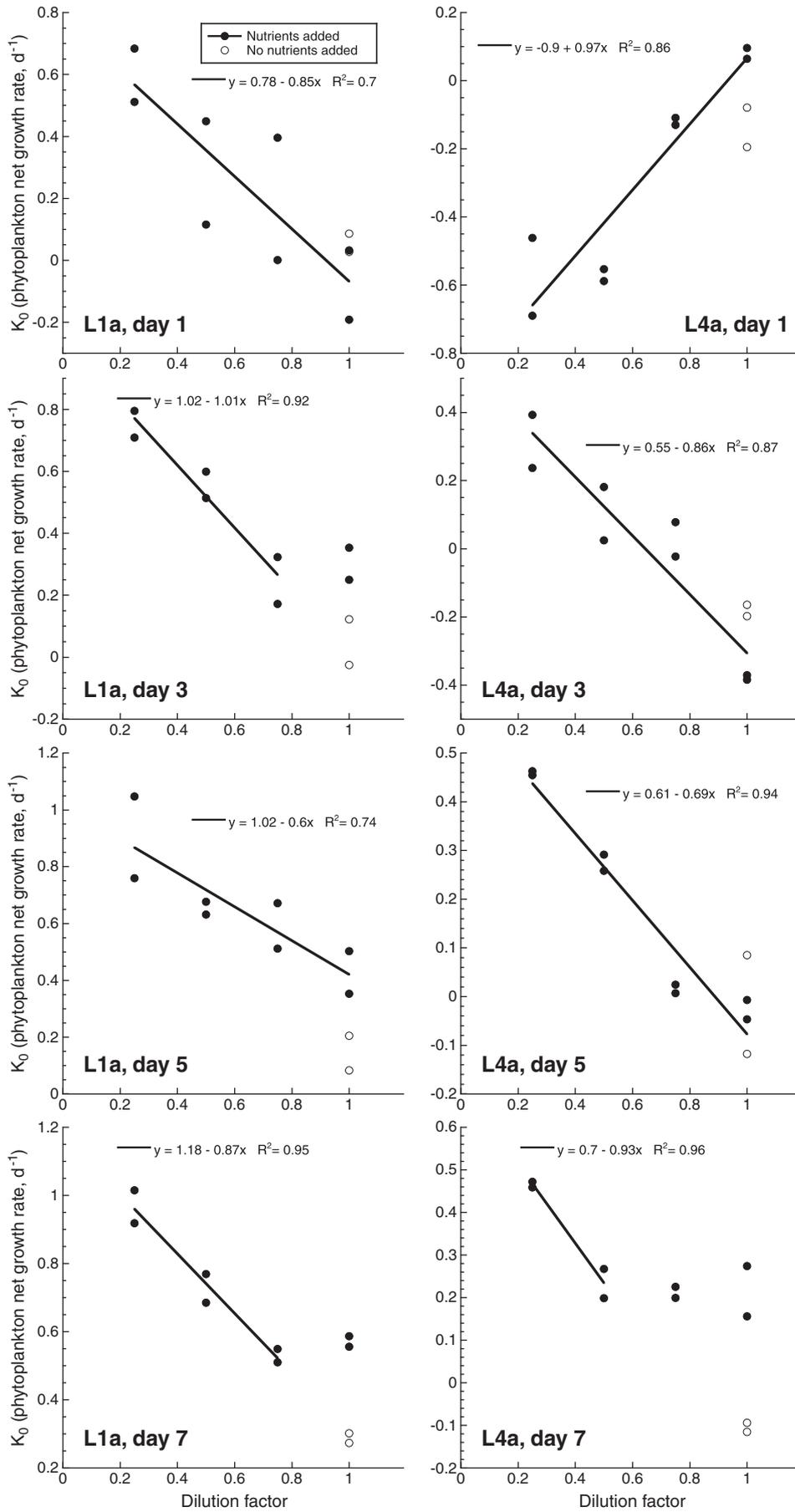


Fig. 6. Example of dilution grazing experiment plots corresponding to total Chl *a* in mesocosms L1a and L4a.

**Table 1**  
Phytoplankton instantaneous growth rates without nutrient addition ( $\mu$ ;  $d^{-1}$ ) 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.

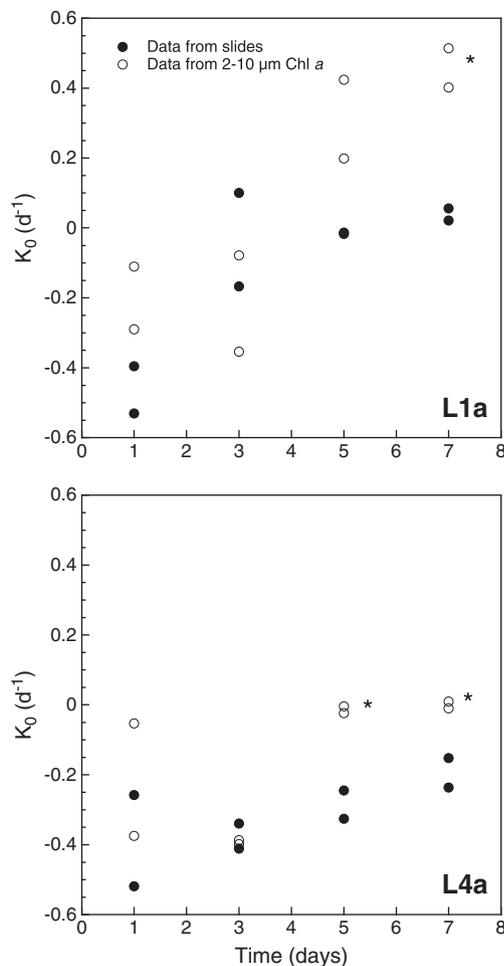
Mesocosm L1a												
Chl <i>a</i> fraction	Day 1			Day 3			Day 5			Day 7		
	<i>m</i>	$r^2$	$\mu$	<i>m</i>	$r^2$	$\mu$	<i>m</i>	$\mu$	$r^2$	<i>m</i>	$r^2$	$\mu$
Total	0.85	0.70	0.90	0.82	0.92 <sup>a</sup>	0.77	0.60	0.74	0.74	0.71	0.95 <sup>a</sup>	0.90
> 10 $\mu m$	0.96 <sup>b</sup>	0.86	1.17	ns	nd	0.13	ns	nd	0.44	0.47	0.79 <sup>a</sup>	0.52
2 to 10 $\mu m$	0.81	0.75	0.61	0.68	0.98 <sup>a</sup>	0.36	ns	nd	0.31	0.49	0.89	0.95
0.65 to 2 $\mu m$	0.85	0.55	0.99	1.07	0.85 <sup>a</sup>	1.19	0.66	0.85	0.52	0.69	0.84 <sup>a</sup>	0.89
0.2 to 0.65 $\mu m$	1.23	0.56	1.93	1.16	0.74	1.46	1.09	0.61	1.31	1.34	0.85 <sup>a</sup>	0.38
Mesocosm L4a												
Chl <i>a</i> fraction	Day 1		Day 3			Day 5			Day 7			
	<i>m</i>	$\mu$	<i>m</i>	$r^2$	$\mu$	<i>m</i>	$r^2$	$\mu$	<i>m</i>	$r^2$	$\mu$	
Total	Posit slope	nd	0.86	0.87	0.68	0.61	0.94	0.67	0.57	0.96 <sup>a</sup>	0.38	
> 10 $\mu m$	Posit slope	nd	0.26	0.62	0.62	0.32	0.73 <sup>a</sup>	0.32	0.49	0.65	0.39	
2 to 10 $\mu m$	Posit slope	nd	0.81	0.808	0.42	0.63	0.96	0.61	0.39	0.74 <sup>a</sup>	0.37	
0.65 to 2 $\mu m$	Posit slope	nd	1.19	0.816	1.09	0.85	0.98 <sup>a</sup>	0.94	0.40	0.85 <sup>a</sup>	0.36	
0.2 to 0.65 $\mu m$	ns	-0.14	ns	nd	-0.0015	ns	nd	-0.09	0.56	0.58	-0.94	

nd = not determined; ns = not significant; posit slope = positive slope.

<sup>a</sup> Feeding saturation

<sup>b</sup> One outlier removed

our dilution bottles did not contain representative amounts of mesozooplankton, we will refer to the estimated microzooplankton community rates as “potential”, and likely on the higher side.



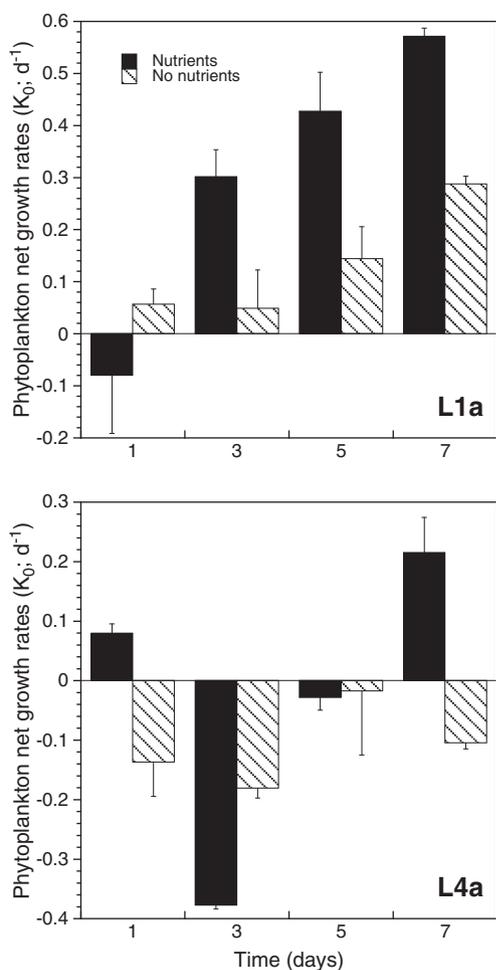
**Fig. 7.** Autotroph growth rates obtained by cell counts (solid symbols) and by Chl *a* analysis of the 2 to 10  $\mu m$  fraction (open symbols) in L1a and L4a mesocosms. Asterisks indicate significant differences ( $p < 0.05$ ).

Microbial herbivores were exerting a very high potential grazing pressure on most of the Chl *a* size-fractions considered, except the 0.2–0.6  $\mu m$  size-fraction in L4a. We should expect to mostly find prokaryotes in this size-fraction, possibly sheltered from heavy predation through trophic cascades in the food web. A similar situation has been described in other oligotrophic areas, where trophic cascade effects masked the grazing on the small phytoplankton (Calbet et al., 2001, 2008; Nejstgaard et al., 1997). Surprisingly, we found saturated-feeding responses (Gallegos, 1989) at the end of the experiment. While the concentration of prey increased initially and saturated feeding rates of the microzooplankton could have been expected at that point, we would not anticipate this situation for oligotrophic areas. Although our data are not unique in showing saturated-feeding in dilution grazing experiments of oligotrophic sites (Berninger and Wickham, 2005; Calbet et al., 2008; Worden and Binder, 2003), given the little biomass of prey attained by the end of the experiment, this fact would imply microzooplankters from oligotrophic sites present a faster-saturating feeding curves than those from productive ones. We are not aware of studies comparing feeding kinetics of protozoans from different ecosystems that can corroborate our results, and thus remains as a hypothesis to be tested.

#### 4.2. Microbial community composition and mixotrophy

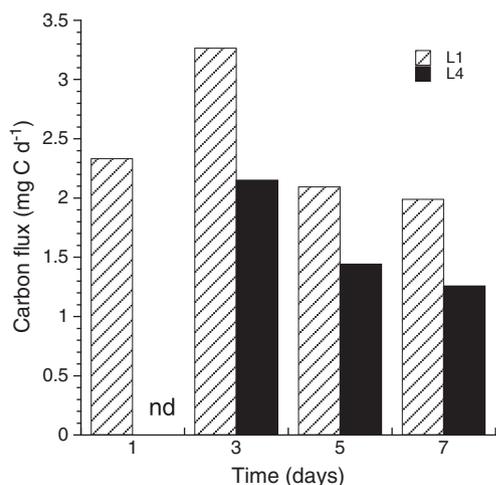
We have so far discussed how the phytoplankton community as a whole progressed in the different mesocosms. We will now discuss the development of the different groups during the experiment. A conspicuous result was the gradual increase of pigmented dinoflagellates and ciliates (Fig. 2). Both groups are larger in size than the rest of components of the microbial community, and both have the potential to act as mixotrophs (Johnson, 2011; Stoecker, 1999).

At organism level, nutrients and light intensity may trigger different feeding responses in obligate mixotrophs, phagotrophic algae, and photosynthetic protozoa (Bird and Kalff, 1989; Skovgaard, 1996; Jones, 1997; Legrand et al., 1998; Stoecker, 1998; Stickney, 2000). However, at community level, continuous darkness should favor heterotrophic metabolism because their higher efficiencies of feeding and incorporation of organic matter into the cell (Tittel, 2003). In our experiments, providing that light and nutrients were limiting, in the L4 treatment we could expect a fast response of existing mixotrophic algae (organism-level response), to be gradually replaced by heterotrophic grazers (community-level response). However, at relatively short time intervals (ca. 24 h) we



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

did not detect any significant variation on the mixotrophic contribution to overall grazing on algae (unfortunately, we do not have data on mixotrophic grazing on prokaryotes). At longer time intervals heterotrophic microbial biomass was below that of pigmented organisms (quotient biomass heterotrophs/autotrophs < 1). This indicates



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

**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

dominance of autotrophic processes over the microbial food web. Therefore, our premise was not met.

We should be aware, however, that the light intensities chosen in our study were intended to simulate the water circulation in the mixed layer of an oligotrophic highly illuminated sea. Even though in L4 we drastically reduced the irradiance by 94% compared to the surface irradiance, the light available in this treatment was still above the light-saturated intensities necessary to develop deep-chlorophyll ( $90\text{--}100\ \mu E\ m^{-2}\ s^{-1}$ ; Harris, 1986; Ignatiades et al., 2002). Therefore, the community responses of autotrophs and heterotrophs for these light intensities should not be as evident as the ones expected for more limiting irradiance conditions, or even complete darkness.

Mixotrophs in L1 treatments should remain unaffected, given the similarity of the experimental conditions to *in situ*, although we cannot disregard a gradual increase in mixotrophy due to nutrient limitation during the experiment. Little is known about the time frame of this process in natural systems, and we did not observe any clear change in mixotrophy of flagellates in the L1 treatments, and mixotrophic ciliates showed either an erratic pattern of abundance or contributed proportionally less to the total abundance of ciliates.

Regarding the technique used to estimate mixotrophy, although we intended to keep the LFLA concentration below 25% of the natural abundance of similarly-sized flagellates, the LFLA were added without previous knowledge of the concentrations in the experimental water. Consequently, the contribution of labelled flagellates to the bulk was generally 25–50%, with one very high value of 83% (L1a, day one). These data may thus not estimate ingestion rates or total carbon flux accurately. 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 (Calbet et al., 2008; Landry et al., 1995; Schülter, 1998). 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 (Jeong et al., 2010; Stoecker, 1998). For instance, for Mediterranean coastal waters, maximum specific ingestion rates of autotrophic flagellates of 5–20  $\mu\text{m}$  were of the order of 3.6 bacteria  $\text{h}^{-1}$ , whereas for heterotrophic flagellates were 15.4 bacteria  $\text{h}^{-1}$ , 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 et al., 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 (cladocerans); therefore, very susceptible to variations in nutrient composition of the prey (Urabe et al., 2002). In marine oligotrophic systems, on the other hand, the complexity of the food web makes it more likely that the effect of nutrient imbalances is buffered through homeostasis in intermediate trophic levels. Moreover, the relevance of mixotrophy (Stoecker, 1999; Stoecker et al., 2009), feeding selection on nutritious prey (Cowles et al., 1988; Saiz and Calbet, 2011), nutrient tunneling effects (Thingstad and Cuevas, 2010), and the ability of many protozoans to upgrade food quality (Broglio et al., 2003; Klein Breteler et al., 1999; Veloza et al., 2006) would make any nutrient deficiency of algae grown under high light conditions dampened within the food web.

In our experiments the decrease in light intensity resulted in a reduction on the final biomass of both autotrophs and heterotrophs (including metazoans) by a factor of ca. 0.5. This contradicts the theory proposed of a lower zooplankton production in highly illuminated lakes (Urabe and Sterner, 1996; Urabe et al., 2002). Even though we could not detect a clear nutritional response of the different light treatments, the C:N of the more illuminated mesocosms tended to be higher than the one of the darkest. Additionally, since the fourth day of the experiment the data indicate a possible decrease in nutritional quality of phytoplankton in the most illuminated mesocosms

(higher C:Chl *a* ratios), but this likely impoverishment of the diet was not reflected in a lower final abundance of grazers (both micro- and mesozooplankton). Therefore, any effect of poor nutritional value of the prey might have been dampened before reaching higher order consumers. This could be due to a variety of mechanisms (e.g., intermediary trophic links, food upgrading, mixotrophy, nutrient tunnelling), many of them not examined in this study, but will have to be resolved in future studies in order to better understand the effect of light availability in oligotrophic aquatic food webs.

#### Acknowledgments

This work was funded through projects CTM2009-08783 from the Spanish Ministry of Science and Innovation assigned to A.C. and projects LightDynamix, LiMic and LightCopFed from the European Union Seventh Framework Program (FP7/2007-2013) under grant agreement no. 228224, MESOQUA, assigned to R.P. (LightDynamix), A.C., R.A.M., A.S., A.G. (LiMic) and S.I. (LightCopFed). We are indebted to G. Piperakis for his inspired technical assistance throughout the experiment, to S. Zivanovic, E. Dafnomili and Dr. M. Tsapakis for assistance with chemical analyses, and to I. Santi, P.A. Gazihan-Akdoglu, and P. Dimitriou for conducting the Chl *a* analyses. The captain and the crew of the R/V Philia are thanked for their assistance during the transportation of water to the mesocosms. We also wish to thank Panagiotis Vavilis, Dimitris Apostolakis, Zacharias Grigorakis, Manolis Ntoumas and Manos Pettas for their immediate and willing response to our continuous requests for help with the zodiac. Finally, we want to thank the rest of participants of the mesocosm experiment: K. Altoja, P. Laas, S. Moorthi, R. Ptacnikova, S.J. Royer, S. Schabhueettl, M. Striebel, and T. Tanaka, for their help and support. [SS]

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