

RESEARCH ARTICLE

Plankton do not care: Minimal effects of ocean liming on plankton growth and grazing in the Eastern Mediterranean

Claudia Traboni ^{1*}, Ariadna C. Nocera ², Filomena Romano ³, Justine Courboulès ⁴,
Christos Chantzaras ⁵, Iordanis Magiopoulos ³, Selene Varliero ⁶, Daniela Basso ⁷, Paraskevi Pitta ³

¹Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy; ²Centro para el Estudio de Sistemas Marinos (CCT CENPAT-CONICET), Puerto Madryn, Argentina; ³Institute of Oceanography, Hellenic Centre for Marine Research, Heraklion, Greece; ⁴Department of Biology, Norwegian Institute of Science and Technology, Trondheim, Norway; ⁵Department of Biology, University of Crete, Heraklion, Greece; ⁶Department of Chemistry, Materials and Chemical Engineering “G. Natta”, Politecnico di Milano, Milan, Italy; ⁷Department of Earth and Environmental Sciences, University of Milano-Bicocca, Milan, Italy

Abstract

Increasing CO₂ emissions have led to the development of CO₂ removal strategies to counteract ocean acidification. Among these, ocean alkalinity enhancement techniques, particularly ocean liming, may represent a promising approach to restore seawater pH and boost CO₂ sequestration. Yet, the impact of liming on plankton communities remains underexplored. In the framework of a mesocosm experiment, we conducted three dilution incubations to assess natural plankton response (abundance, composition, growth, grazing, diet, and food selectivity) to liming, achieved with calcium hydroxide (Ca(OH)₂) additions. Experiments included two liming treatments (low concentration, and high concentration) and a control treatment without Ca(OH)₂. The community was dominated by small-sized plankton (bacteria, *Synechococcus* and pigmented picoflagellates), outnumbering larger diatoms, dinoflagellates, and ciliates. While chlorophyll *a*, heterotrophic bacteria, and pigmented picoflagellates remained stable across treatments, the abundance of *Synechococcus* and dinoflagellates increased, whereas diatoms and ciliates declined particularly under high Ca(OH)₂. Growth and grazing rates were largely unaffected by alkalinity, except for increased growth in pigmented picoflagellates upon liming. Microzooplankton showed low ingestion of *Synechococcus* and pigmented picoflagellates and higher intake of diatoms, dinoflagellates, and ciliates. Food selectivity was unresponsive to liming, as the grazers selected prey based on size, regardless of Ca(OH)₂ concentrations. Increased alkalinity and pH, and the parallel effect of trophic cascades might have driven nutrient fluctuations and shaped downstream trophic interactions. Despite positive responses highlighted in this study, further research is needed to explore liming potentiality on a wider range of food-web components and larger scales in the framework of ocean alkalinity enhancement research.

*Correspondence: claudia.traboni@szn.it

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Microzooplankton (< 200 μm) are key pelagic consumers, and include unicellular heterotrophs (e.g., flagellates, dinoflagellates, ciliates) and metazoan larvae (crustaceans, annelids, mollusks). These organisms graze on bacteria, protozoa and phytoplankton, and are preferred prey for mesozooplankton (> 200 μm) (Calbet 2008; Steinberg and Landry 2017). Microzooplankton can outcompete mesozooplankton in removal of phytoplankton production (Schmoker et al. 2013), due to broad nutritional adaptations and prey capture strategies (Jeong et al. 2010; Saiz et al. 2014; Maselli et al. 2020). Hence they act as a link between microbial and metazoan food webs, influencing remineralization (Sherr and Sherr 1988; Landry

and Calbet 2004), and nutrient transfer in food webs (Steinberg and Landry 2017).

Dissolution of carbon dioxide (CO_2) in seawater lowers pH, a process known as “ocean acidification.” This can affect zooplankton by reducing food quality (higher C:N and C:P ratios) (Caron and Hutchins 2013; Calbet et al. 2014), suppressing growth (Meunier et al. 2017), respiration (Cripps et al. 2016), reproduction, and survival (Zhang et al. 2011), and decreasing anti-predatory defense (Hammill et al. 2018). In spite of this, these organisms can also tolerate elevated $p\text{CO}_2$ (Suffrian et al. 2008; Isari et al. 2015).

Ocean alkalinity enhancement (OAE) has emerged as a group of marine carbon dioxide removal strategies aimed at mitigating acidification impacts on marine ecosystems (Renforth and Henderson 2017; Bach et al. 2019). OAE involves the addition of pulverized silicate, carbonate, or calcium hydroxide minerals to seawater (ocean liming) to increase alkalinity, thus theoretically restoring pH to normal levels, and simultaneously enhance CO_2 sequestration (Renforth and Henderson 2017; Bach et al. 2019).

Marine planktonic calcifiers such as coccolithophores appeared unaffected when exposed to increased limestone within a natural pH range (Gately et al. 2023), but long-term exposure and higher pH significantly reduced growth rate in diatoms and prymnesiophytes (Oberlander et al. 2025). Some dinoflagellates and ciliates well resisted pH between 8 and 9.5, unlike others that eventually declined (Hansen 2002; Pedersen and Hansen 2003a) and showed lower grazing rates at $\text{pH} > 8.8$, likely due to altered prey availability and carbonate chemistry (Deore 2020). Mesocosm experiments showed that phytoplankton, mixoplankton, and protozooplankton biomasses were insensitive to alkalization (Xin et al. 2024; Ramírez et al. 2025), while metazoan microzooplankton suppressed growth (Sánchez et al. 2024). Natural populations did not provide different responses compared to those observed in laboratory and mesocosm setups. No stress signals were reported in response to carbonates in a phytoplankton assemblage of the oligotrophic subtropical Atlantic (Ramírez et al. 2025). While some mixotrophic taxa can tolerate rising alkalinity and pH, heterotrophic ciliates showed declined growth (Pedersen and Hansen 2003b), with possible cascading effects on food webs. The outcome will depend on exposure doses, time, and trophic plasticity, making it hard to draw conclusions on the impact of liming on plankton communities, and to support the implementation of OAE solutions.

Given the variability in responses and limited information on the effect of liming on protistan physiology under OAE scenarios, we investigated the response of natural microzooplankton to slaked lime ($\text{Ca}(\text{OH})_2$) in the oligotrophic eastern Mediterranean Sea. Small-volume incubations were conducted as side experiments of a large-scale mesocosm setup, aimed to assess the effect of liming on community composition, functioning, and productivity from viruses to zooplankton. In our specific study, we aimed to: (1) assess the abundance and growth rates of key functional groups in

the planktonic food web, (2) quantify grazing on pico-, nano-, and microplankton, and (3) determine diet composition and food preference of the grazer community under $\text{Ca}(\text{OH})_2$ exposure. We hypothesized that liming would not significantly affect nano-microplankton performance, supporting its suitability as a potential remedy against ocean acidification.

Materials and methods

Mesocosm setup, treatments, and abiotic factors assessment

The main mesocosm experiment took place at the CretaCosmos facility (<https://www.aquacosm.eu/mesocosms/cretacosmos>) of the Hellenic Centre for Marine Research (HCMR) on the northern coast of Crete (Greece) in May–June 2023. Nine 3-m³ mesocosms were filled on May 26th with natural seawater collected in front of HCMR. Subsurface water (1.5-m depth) was pumped into several 1-m³ high-density polyethylene barrels, which were transported by truck to the mesocosm facility. The water from each one of the barrels was evenly distributed to all mesocosms to ensure homogeneity of initial conditions. Mesocosms were transparent polyethylene bags (1.3 m diameter, 3-m³ capacity), submerged in a concrete pool of 150 m³ with running water to keep the temperature constant during the experiment. Our incubations were conducted as side experiments of the main mesocosm experiment, which lasted in total 14 d (D_1 – D_{14}).

Throughout the experiment, the alkalinity was increased by repeated additions of calcium hydroxide ($\text{Ca}(\text{OH})_2$) to selected mesocosms over multiple days (D_1 , D_3 , D_5 , D_7 , D_9 , and D_{11}), between 11:00 and 11:30 h (Fig. 1). The quantity of $\text{Ca}(\text{OH})_2$ to add was carefully calculated considering the target concentration and the mesocosm seawater volume, taking into account volume reductions due to daily subsampling. $\text{Ca}(\text{OH})_2$ was dosed as a 1.5 M slurry prepared by mixing for about 5 min the desired quantity of $\text{Ca}(\text{OH})_2$ with water sampled from the corresponding mesocosm. The experiment had three triplicate mesocosms per treatment: three control (C_1 – C_2 – C_3) mesocosms (no lime input), three low (L_1 – L_2 – L_3) lime concentration mesocosms (input: 0.00074 g L⁻¹), and three high (H_1 – H_2 – H_3) lime concentration mesocosms (input: 0.0067 g L⁻¹). After sampling the mesocosm, the pH and temperature were measured by a Thermo Scientific Orion Star A111 pH meter. The probe was calibrated according to the NIST scale and then corrected on the total scale (Badocco et al. 2021). After pH measurements, the samples were filtered with a syringe through a 0.2 μm PES filter, stored at 4°C, and sent to the Politecnico di Milano (Italy) for analyses of total alkalinity (TA), estimated by titration (Varliero et al. unpublished; Varliero 2025). Added alkalinity (ΔTA) was calculated as the difference between the TA in treated (X, being L or H) vs. control (C) mesocosms on each sampling day (t_i) as $\Delta\text{TA}_{t_i} = \text{TA}_{(X,t_i)} - \text{TA}_{(C,t_i)}$. The conductivity was measured with a Mettler Toledo Seven Excellence instrument, and the salinity was then calculated according to Lewis and Perkin (1981).

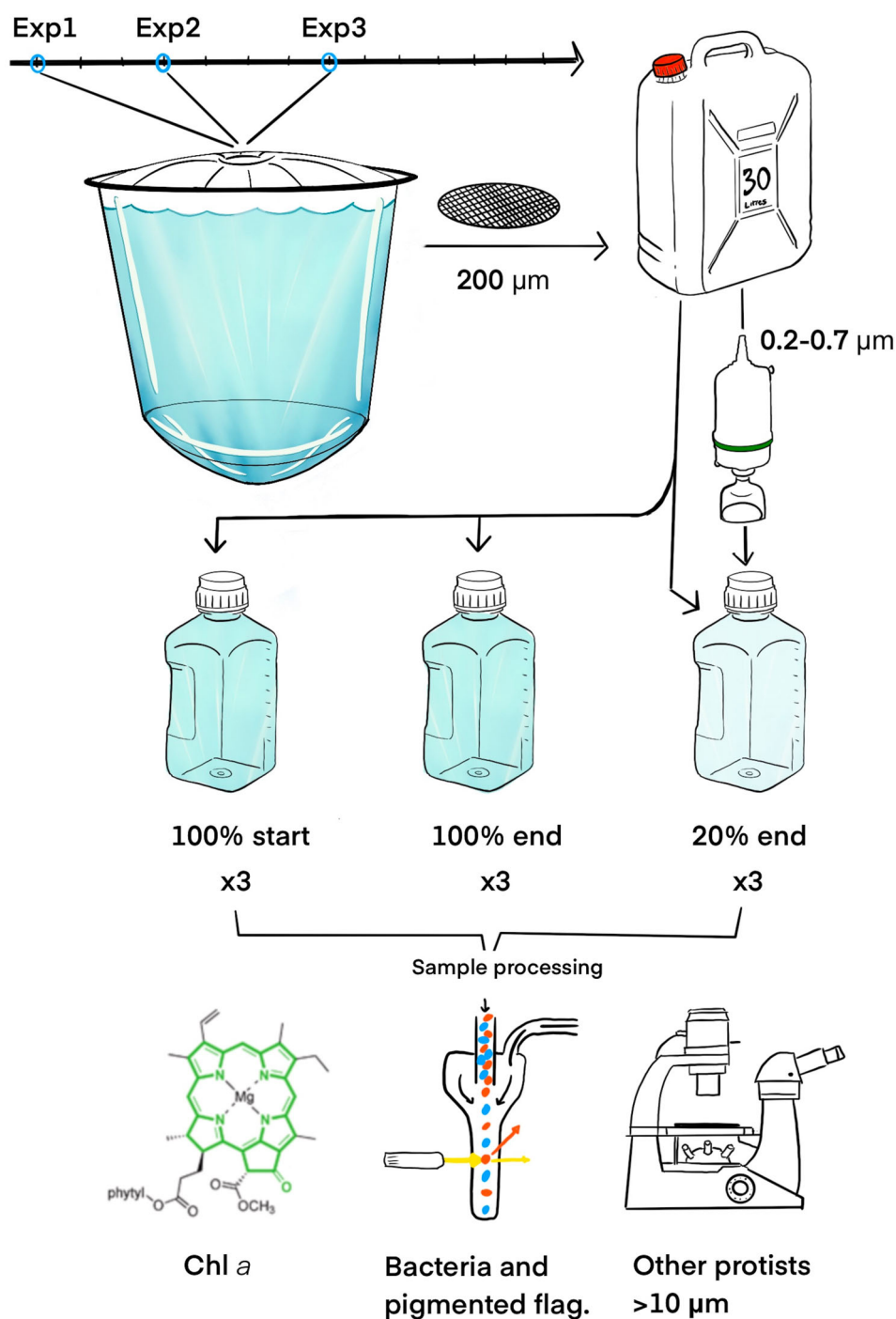


Fig. 1. Timeline of the AlkalOcean mesocosm experiment (14 d, D₁–D₁₄) highlighting the days where the three grazing incubations were conducted (Exp1 on D₁, Exp2 on D₄, and Exp3 on D₈), and a summarized scheme showing the experimental workflow including mesocosm sampling, incubations, and sample processing.

Photosynthetically active radiation measurements were taken by means of a LiCOR sensor. Inorganic nutrients were added to all the mesocosms on D₅ (ammonium, 202 nM; nitrate, 198 nM) and D₇ (phosphate, 5 nM) to ensure nutrient repletion during the course of the experiment and keep their concentrations similar to those measured at the beginning of the experiment.

Microzooplankton grazing experiments

Three grazing experiments, referred to as Exp1–2–3, were conducted on D₁, D₄, and D₈, respectively (Fig. 1). In Exp1, before the lime was added, 3 L of water were collected from each of the nine mesocosms and pooled to have a representative water environment enclosed within each mesocosm

during the initial filling. For Exp2 and Exp3, as the mesocosms had already been lime-treated in the previous days, only one out of three replicate mesocosms was randomly selected to represent each treatment: control (mesocosm C2), low treatment (mesocosm L2), and high treatment (mesocosm H2), but throughout this work, we refer to the general treatments: C, L, and H. To estimate grazing by the nano-microplankton grazers (microzooplankton for simplicity), the dilution technique was applied, based on the prey removal in diluted vs. undiluted treatments (Landry and Hassett 1982). An illustrative scheme of the experimental procedure is provided in Fig. 1.

A water volume of 20–27 L from each mesocosm was collected in 30 L carboys and carefully sieved through 200 μm to remove larger predators. This water was split into two fractions: one fraction was sieved through either 0.7 μm GF/F Whatman filters (Exp1) or 0.2 μm Acropack Nylon membranes (Exp2 and Exp3) to obtain filtered seawater for the dilution experiment, and the other fraction was kept unfiltered, including all grazers < 200 μm . For each experiment and treatment, three replicate 2.375 L Nalgene® bottles were gently filled — one to be sampled immediately (100% initial conditions), one for the 20% dilution treatment (containing 20% seawater and 80% filtered seawater), and one for the 100% treatment (containing undiluted seawater) to be sampled after 24 h. In each bottle, f/2 medium (1 mL L⁻¹) was added (54.6 μL of 50 \times concentrated f/2 stock, Sigma-Aldrich®) to ensure nutrient availability and to compensate for the effect of microzooplankton excretion between 20% and 100% bottles. Bottles were filled up to capacity, sealed with transparent film, and closed with a screw cap to prevent the formation of bubbles. Finally, the Nalgene bottles were incubated in the pool next to the mesocosms under similar light and temperature for 24 h (Table 1).

Sample processing

The three 100% start bottles were immediately subsampled after closing, while the other bottles incubated in the pool were sampled after 22–24 h, representing the final conditions at the end of the incubation. From each bottle, 250–1000 mL were subsampled for the determination of Chl *a* concentration and fluorescence (proxy of phytoplankton biomass); 200 mL were preserved with Lugol's iodine solution (2% final concentration) for the estimation of protistan abundance and diversity. Finally, 1.8 mL samples were collected for flow cytometry analyses to determine the abundance of picophytoplankton and bacteria.

For Chl *a* fluorescence analysis, the seawater was filtered under dim light onto 47 mm GF/F and/or polycarbonate filters (0.2–0.7 μm) at minimum pressure (125 mmHg: 0.166653 bar) with a vacuum pump (Millipore®). After filtration, the filters were folded with tweezers and placed in 15 mL falcon tubes, completely submerged in 6 mL 90% acetone; tubes were covered with aluminum foil and stored at 4°C throughout the extraction time (24 h). After the Chl *a* extraction, samples were left at room temperature (30–40 min), and subsequently, the liquid phase was transferred to 7-mL glass tubes covered with aluminum foil. All samples were measured at the

fluorometer (TD-700, Turner Designs) before and after acidification (500 μL 10% HCl) to determine phaeopigment concentration (Yentsch and Menzel 1963). In case of high Chl *a* biomass, samples were diluted before reading.

Identification and enumeration of protistan cells were performed from the Lugol preserved samples and analyzed under an inverted microscope (IX70, Olympus®, at magnification of 100–200 \times) using Utermöhl sedimentation chambers (Utermöhl 1931). The volume settled for counting (10–50 mL) and the counting strategy (transects, half chamber, or whole chamber) reflected the concentration of cells in each sample. The protists comprising the community were classified into groups (diatoms, dinoflagellates, ciliates) and further identified to the order or genus level whenever possible. Protist trophic mode was assigned following the classification by (Schneider et al. 2020). Only cells > 10 μm were counted and photographed for cell size estimates. The cell C content was determined from literature equations for diatoms, dinoflagellates (Menden-Deuer and Lessard 2000) and ciliates (Putt and Stoecker 1989). The full list of protistan taxa is presented in Supporting Information Table S1.

Samples for flow cytometry were collected in 2 mL cryovials, fixed with glutaraldehyde (2% final concentration), flash-frozen in liquid nitrogen, and long-term stored at –80°C until analysis. After the samples were unfrozen, aliquots of 500 μL were sampled and run on the acoustic focusing cytometer (Attune NxT, Invitrogen Thermo Fisher Scientific) equipped with a 488-nm laser. Flow cytometry was used to quantify autotrophic populations, namely *Synechococcus* and pigmented picoflagellates (picoeukaryotes up to 2 μm) as well as heterotrophic bacteria, stained with SYBRgreen I (Marie et al. 1999), and classified as low nucleic acid (LNA) and high nucleic acid (HNA) bacteria based on cell and genome sizes. More details regarding the gates and channels used are reported in Supporting Information Fig. S1. Due to loss of samples, data are missing regarding growth and grazing rates on heterotrophic bacteria, for which only the initial concentration could be analyzed.

Estimate of plankton growth and microzooplankton grazing rates and selectivity

From the Chl *a* concentration, and the cell counts performed with the flow cytometer and under the microscope, the growth rate of the prey (Chl *a*, pico-, nano-, and microplankton) plus the bacterial population in both 20% (μ , d⁻¹; Eq. 1) and 100% (k , d⁻¹; Eq. 2) bottles were calculated according to the Frost's equation (Frost 1972) as:

$$\mu = \text{LN} \left(\frac{C_{1,\text{prey}20\%}}{C_{0,\text{prey}20\%}} \right) \times \frac{1}{t} \quad (1)$$

$$k = \text{LN} \left(\frac{C_{1,\text{prey}100\%}}{C_{0,\text{prey}100\%}} \right) \times \frac{1}{t} \quad (2)$$

where $C_{0,\text{prey}}$ and $C_{1,\text{prey}}$ are the initial and final prey standing stock (cells mL⁻¹ or cells L⁻¹), respectively, and t is the

Table 1. Abiotic variables measured in the mesocosms selected for the grazing experiments (mesocosm C2 for the control-no input, mesocosm L2 for the low lime concentration, mesocosm H2 for the high lime concentration).

Exp	Treat	Temp	PAR	pH	TA	Salinity	NH ₄ ⁺	NO ₃ ⁻	PO ₄ ³⁻	SiO ₂
		°C	μmol photons m ⁻² s ⁻¹		μmol L ⁻¹		μM	μM	nM	μM
Exp1	C	20.2	2250	8.21	2630	38.5	0.44	0.39	10.4	0.806
Exp2	C	20.6	1389	8.2	2556	37.9	0.1	0.03	9.3	0.513
	L	20.6	2003	8.25	2710	38.2	0.09	0.01	12.9	0.558
	H	20.6	1081	8.47	2864	38.1	0.1	0.01	15.4	1.197
Exp3	C	21.1	1970	8.23	2566	37.8	0.09	0.05	8.0	0.351
	L	20.7	1124	8.3	2694	38.0	0.07	0.1	5.3	0.522
	H	20.8	2209	8.68	2858	37.9	0.2	0.06	3.3	1.548

C, control; Exp, experiment; H, high lime concentration; L, low lime concentration; NH₄⁺, ammonium; NO₃⁻, nitrate; PAR, photosynthetically active radiation; PO₄³⁻, phosphate; SiO₂, silicate; TA, total alkalinity; Temp, temperature; Treat, treatment.

incubation duration (d⁻¹). According to the dilution method (Landry and Hassett 1982), μ reflects the intrinsic growth rate with negligible grazing in diluted bottles, while k (apparent growth rate) incorporates also grazing-induced mortality. The difference between the intrinsic and apparent growth rates of the prey groups gives an estimate of the grazing rate (g ; Eq. 3) on that group by the microzooplankton community:

$$g = \mu - k \quad (3)$$

Only positive grazing rates were considered, and they were set to 0 when rates assumed negative values. Only a few outliers were removed from the analyses. Per each treatment and within each experiment, the percentage values of the contribution of the different plankton groups in the diet (Eq. 4) and/or in the environment (Eq. 5) were expressed as:

$$r_i = \frac{g_i p}{g_{tot}} \times 100 \quad (4)$$

$$n_i = \frac{C_i p}{C_{tot}} \times 100 \quad (5)$$

where $g_i p$ (cells cells⁻¹ d⁻¹) is the grazing rate of the microzooplankton community on the group p , g_{tot} (cells cells⁻¹ d⁻¹) is the grazing by the community on the total cells ingested, $C_i p$ is the cell abundance (cells mL⁻¹) of the group p , and C_{tot} (cells mL⁻¹) is the total cell abundance. Selectivity on specific prey taxa was expressed as the electivity index (E ; Eq. 6), calculated according to Ivlev's equation (Ivlev 1961):

$$E = \frac{(r_i - n_i)}{(r_i + n_i)} \quad (6)$$

where r_i is the percentage ingestion of group i in the diet and n_i the percentage concentration of group i in the environment. E ranges from -1 to $+1$, varying with the preference for a specific prey. When a prey type is highly represented in

the environment, but it is scarcely ingested, $E < 0$. On the contrary, highly ingested prey that occur at low frequency in the environment are selected positively, $E > 0$. Prey that are eaten in proportion to their relative contribution in the environment are neutrally ingested, hence not selected nor avoided ($E = 0$).

Data analysis

To check for differences in standing stock, growth rates, grazing rates, and selectivity between the three treatments in each experiment separately, we performed a non-parametric Kruskal-Wallis test ($n = 3$, $df = 2$). In case of significant results, a post hoc Dunn test for multiple comparisons (C vs. L, L vs. H, C vs. H) was conducted independently for the different prey groups (heterotrophic bacteria, Chl a , pigmented picoplankton, *Synechococcus*, and protists $> 10 \mu\text{m}$, i.e., diatoms, dinoflagellates, and ciliates). Due to lack of data of heterotrophic bacteria in some experiments, comparisons for growth and grazing rates on these groups were not assessed. Redundancy analyses (RDA) were performed to identify the relationship between the vital rates (growth and grazing) and abiotic factors. Prior to RDA, environmental variables were log-transformed to reduce data skewness and correlated variables were excluded from the RDA, namely salinity, pH, and photosynthetically active radiation. Linear regression analyses (log-log transformed data) were conducted to ascertain the dependency of the protist abundance from the abundance of potential metazoan predators in the environment as well as from the abiotic factors. Statistics and data visualization were performed using Rstudio software version 4.2.1 (R Core Team 2022). Differences were considered significant at $p < 0.05$.

Results

Plankton standing stock

The mean Chl a concentration throughout the experiments showed an increasing trend (range: 0.2–0.45 $\mu\text{g Chl } a \text{ L}^{-1}$;

Fig. 2a), but no differences emerged between differently lime-treated incubations within each experiment (Exp2, $p = 0.0608$; Exp3, $p = 0.2881$).

The microbial heterotrophic compartment (Fig. 2b, c) was represented by high nucleic acid bacteria (HNA, range: 219,127–319,460 cells mL⁻¹) and low nucleic acid bacteria (LNA, range: 223,880–286,233 cells mL⁻¹). Both populations showed decreasing trends throughout the experiment. Their abundance was not significantly affected by Ca(OH)₂ in either HNA (Exp2, $p = 0.0991$; Exp3, $p = 0.0991$) or LNA bacteria (Exp2, $p = 0.1479$; Exp3, $p = 0.7326$).

The phytoplankton community was numerically dominated by *Synechococcus* (36,016 ± 1245 cells mL⁻¹) and pigmented picoflagellates (4542 ± 138 cells mL⁻¹). *Synechococcus* gradually increased after Exp1 until Exp3 (Fig. 2d, e). Significant differences in abundance only emerged in Exp3 between L and H incubations ($p = 0.0219$). The pigmented picoflagellates population remained almost unchanged after the first lime addition in Exp2 and without difference as a function of Ca(OH)₂ concentration ($p = 0.7326$) but sharply increased in Exp3, yet non-significantly between treatments ($p = 0.0608$).

Less abundant groups were represented by large (> 10 μm) protists, that is, diatoms, dinoflagellates, and ciliates. Diatoms peaked at the start of Exp1 and accounted for about 52,000 ± 20,000 cells L⁻¹ (Fig. 2f). Over the course of the experiment, the overall diatom stock progressively decreased until reaching abundances as low as 5200 ± 1700 cells L⁻¹ in Exp3. Yet, no significant differences in diatom abundance

emerged as a function of liming in Exp2 ($p = 0.4429$) and Exp3 when comparing C and H treatments ($p = 0.0512$).

Dinoflagellates standing stock was rather low, ranging between 3400 ± 2500 and 7200 ± 2300 cells L⁻¹, showing a slight increase in Exp3 (Fig. 2g). The abundance of dinoflagellates was twofold higher in L treatment compared to C in Exp2 ($p = 0.0338$); on the contrary, in Exp3 no significant variations occurred between treatments ($p = 0.5611$).

Ciliates were the least abundant group, occurring within the range between 300 ± 70 and 5100 ± 1700 cells L⁻¹ (Fig. 2h), and showing a declining trend over time. Ciliate abundance was significantly different in Exp2 between L and H incubations ($p = 0.0219$), but non-significant changes were observed in Exp3 ($p = 0.1112$).

Growth and grazing rates

Chl *a* showed a higher net growth rate in diluted bottles (20%) compared to that in undiluted bottles (100%) (Fig. 3a), sign of positive grazing by microzooplankton. Overall, the intrinsic Chl *a* growth rate (range: 0.76–1.27 d⁻¹) was similar between treatments and experiments (Exp2 $p = 0.5611$, Exp3 $p = 0.9565$; Fig. 3b). The grazing rate on Chl *a* (range: 0.44–1.06 d⁻¹) did not significantly differ between treatments (Exp2 $p = 0.0794$, Exp3 $p = 0.2881$; Fig. 3c).

Synechococcus had a moderately negative net growth slope in all experiments as a function of the dilution factor (Fig. 4a). The growth rate oscillated between 0.1 and 0.5 d⁻¹ (Fig. 4b) and the community grazing rate on *Synechococcus* accounted for 0.09–0.25 d⁻¹ (Fig. 4c). The growth rate did not change

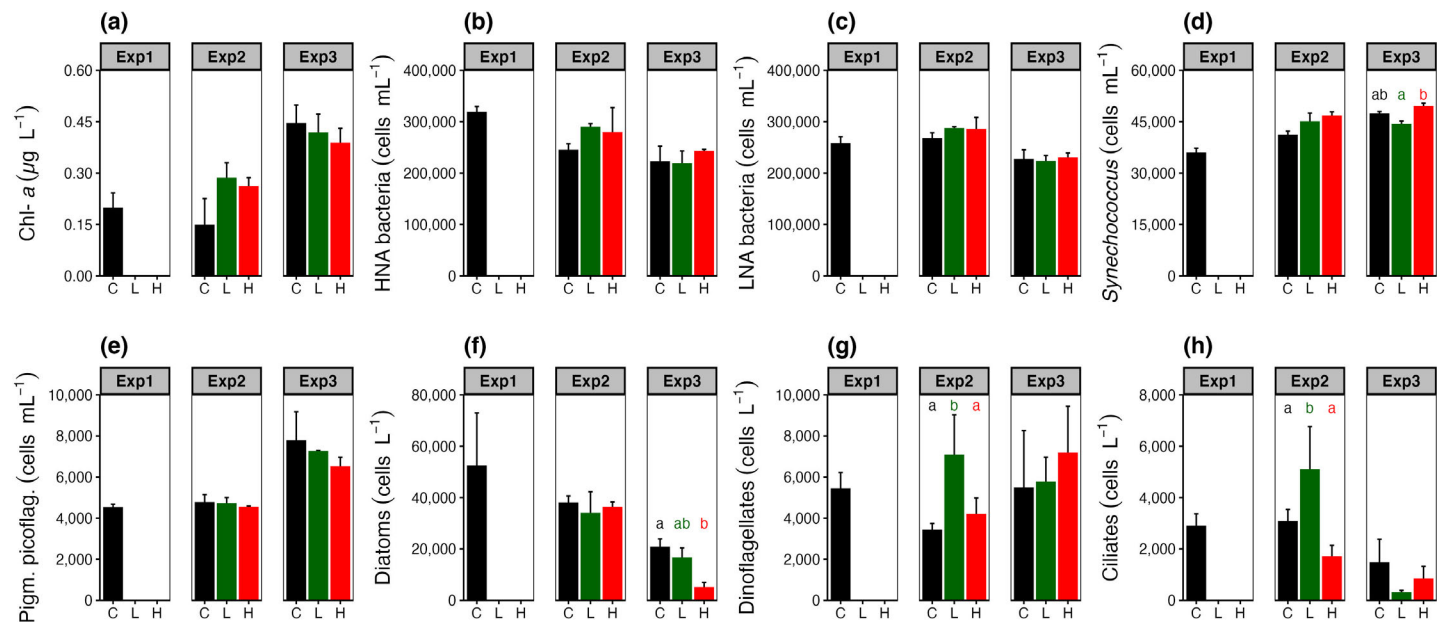


Fig. 2. Initial standing stock of (a) Chl *a*, (b) HNA bacteria, (c) LNA bacteria, (d) *Synechococcus*, (e) pigmented flagellates, (f) diatoms, (g) dinoflagellates, and (h) ciliates during the three grazing experiments (Exp1, Exp2, and Exp3) grouped by treatment (C, L, and H, control, low, and high, respectively). Data are expressed as mean ± SD ($n = 3$), and letters above bars indicate significance of the multiple comparison (Dunn's test) between treatments.

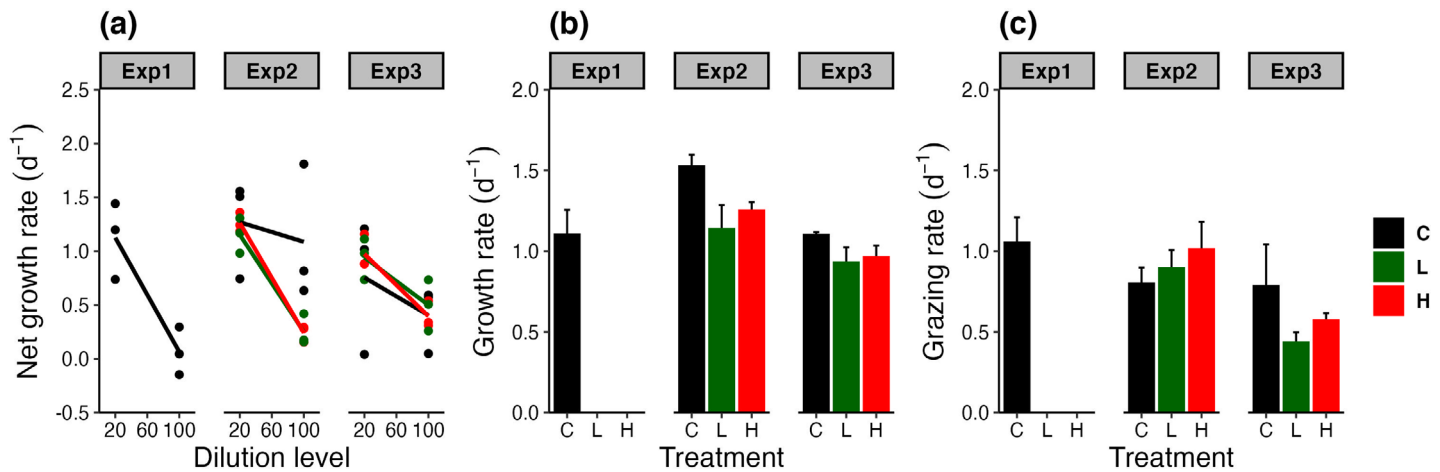


Fig. 3. (a) Instantaneous growth rates of Chl *a* as a function of the dilution level (20% and 100%), (b) mean Chl *a*-specific growth rates, and (c) mean grazing rates on Chl *a* in the three grazing experiments (Exp1, Exp2, and Exp3) grouped by treatment (C, L, and H, control, low, and high, respectively). Data are expressed as mean \pm SD ($n = 3$).

significantly with liming in either experiment (Exp2, $p = 0.0608$; Exp3, $p = 0.0665$), similarly to the grazing rate on this group, which was unaffected by $\text{Ca}(\text{OH})_2$ in both Exp2 ($p = 0.3292$) and Exp3 ($p = 0.0665$).

Pigmented picoflagellates growth exhibited a flat or positive slope in relation to the dilution factor in Exp1 and Exp2, whereas a negative slope indicated mortality by grazing in Exp3 (Fig. 4a). The growth rate oscillated between 0.01 and 0.37 d⁻¹ (Fig. 4b), with significant differences between L and H in Exp2 ($p = 0.0336$) and between C and H in Exp3 ($p = 0.0306$). The grazing effort on this group (0–0.35 d⁻¹; Fig. 4c) did not display significant differences upon liming (Exp2, $p = 0.1054$; Exp3, $p = 0.0549$).

Concerning the three larger protistan groups, mortality by grazing was variable between experiments and treatments (Fig. 5a). Diatoms showed the highest growth rate (range: 0.08–0.99 d⁻¹), followed by dinoflagellates (0–0.42 d⁻¹) and ciliates (0–0.20 d⁻¹) (Fig. 5b). The community grazing rate on these three groups followed a similar pattern as the growth rate (Fig. 5c), with diatoms being the most ingested (range: 0–0.66 d⁻¹), compared to dinoflagellates (0.08–0.51 d⁻¹) and ciliates (0–0.30 d⁻¹). Generally, growth and grazing rates on these groups were never significantly different between treatments ($p > 0.05$ in all cases). The list of significance values for each comparison is reported in Supporting Information Table S2.

Contribution of plankton groups and their morpho-trophic traits

The bulk standing stock was numerically constituted by *Synechococcus* and pigmented picoflagellates (> 99% of the plankton abundance in all experiments), whereas the contribution of diatoms, dinoflagellates, and ciliates was negligible (Fig. 6a). The diatom community was dominated by centric and pennate

representatives in similar percentages; dinoflagellates were mostly athecate, and the prevailing ciliates were loricate, progressively replaced by aloricate taxa (Supporting Information Fig. S2a). Trait distribution within groups did not change between treatments. Despite the highly abundant *Synechococcus* and pigmented picoflagellates, the diet composition of the grazers' community indicated a very high ingestion of other groups: diatoms (10–40%), dinoflagellates (25–45%), and ciliates (7–38%) (Fig. 6b). Overall, $\text{Ca}(\text{OH})_2$ addition was not responsible for the diet partitioning, yet a slightly higher ingestion of ciliates in Exp2 (treatment L) and pigmented picoflagellates in Exp3 (treatment H) emerged at the expense of diatoms. Thecate dinoflagellates contributed to the community diet to a higher extent compared to athecates (Supporting Information Fig. S2b), whereas, concerning ciliates, loricate taxa were more ingested in the H treatment compared to C and L (Exp 2). In Exp3, the community and the diet were constituted entirely by aloricate ciliates, regardless of the treatment.

The prey size spectrum showed that small taxa/species (1–10 μm) were the most represented in the natural assemblage (Fig. 6c), represented by *Synechococcus*, pigmented picoflagellates, and some dinoflagellates. Intermediate-sized (10–35 μm) protists included diatoms, dinoflagellates, and ciliates. Large representatives (> 35 μm) were mostly ciliates (Supporting Information Fig. S3a). The highest contribution to the microzooplankton diet (65–80%) was conferred by protists of intermediate size (Fig. 6d), mainly due to the ingestion of diatoms and dinoflagellates, followed by a lower contribution of larger species in these groups, and no changes in the size-specific ingestion within groups were observed as a function of liming (Supporting Information Fig. S3b). Yet, larger ciliates were ingested in lime-treated conditions in Exp2 compared to intermediate ones, but this pattern disappeared in Exp3 due to the low abundance of large ciliates in the seawater.

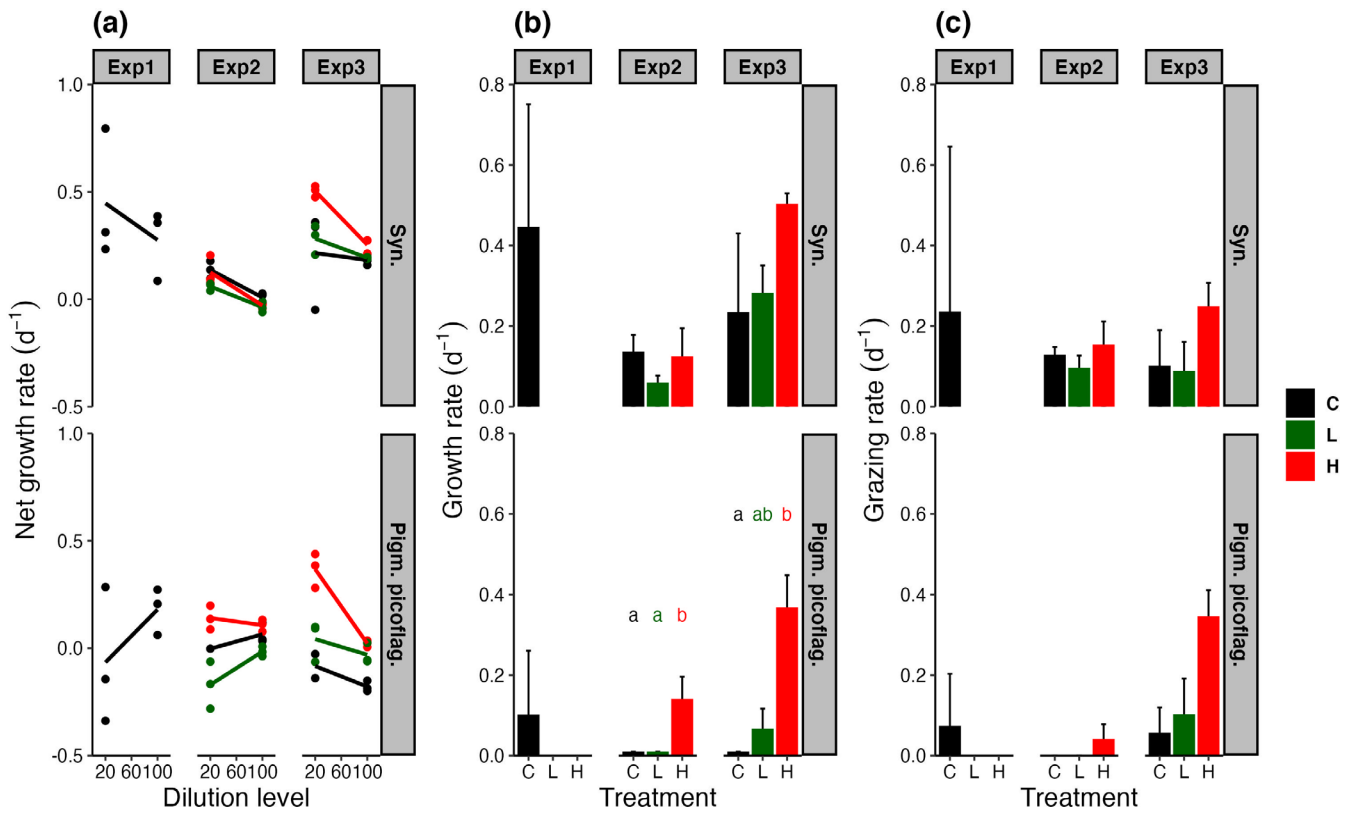


Fig. 4. (a) Instantaneous growth rates as a function of the dilution level (20% and 100%), (b) growth rates, and (c) grazing rates on *Synechococcus* and pigmented picoflagellates in the three grazing experiments (Exp1, Exp2, and Exp3) grouped by treatment (C, L, and H, control, low, and high, respectively). Data are expressed as mean \pm SD ($n = 3$), and letters above bars indicate significance of the multiple comparison (Dunn's test) between treatments.

The plankton community was dominated by strict autotrophs, and putative auto-mixotrophs represented by *Synechococcus* and pigmented picoflagellates, respectively (Supporting Information Fig. S4a). Of the least abundant groups, dinoflagellates showed auto-mixotrophic and heterotrophic nutrition evenly distributed, whereas ciliates were, for the most part, heterotrophic, mixotrophs being not highly abundant. The analysis of ingested taxa revealed that dietary composition consisted of 40–65% of strict autotrophs, 9–42% of strict heterotrophs, and 15–40% of a flexible diet including auto-mixotrophic taxa, in varying amounts between experiments (Supporting Information Fig. S4b). A progressive increased fraction of ingested mixotrophs emerged along the $Ca(OH)_2$ gradient. Specifically, the most ingested dinoflagellates were autotrophic and mixotrophic, whereas heterotrophic taxa dominated the ciliate-based intake. We observed an increased proportion of ingested mixotrophic ciliates in the community diet in Exp3, but these differences were not linked to liming.

Food selection

No differences were observed in selectivity due to liming treatments ($p > 0.05$ for all groups and experiments; Supporting Information Table S3). Despite their abundance,

Synechococcus and pigmented picoflagellates were neglected in proportion to their relative abundance, and therefore $E < 0$ in all cases (Fig. 7a). Diatoms, dinoflagellates, and ciliates were not evenly distributed between environment and community diet; these were poorly abundant but highly ingested; thereby, these three groups were positively selected in all experiments and without significant differences between treatments ($p > 0.05$ for all groups and experiments; Supporting Information Table S3).

Small-sized prey were in most cases discarded or neutrally ingested (Fig. 7b). Both intermediate and large size classes of protistan prey were positively selected. No differences in the size selectivity emerged on the basis of the $Ca(OH)_2$ input ($p > 0.05$ for all size categories and experiments; Supporting Information Table S3).

Forcings on the food web components vital rates and standing stock

The RDA (Fig. 8) showed that the community growth rate did not respond significantly to alkalinity ($p = 0.413$), but to NO_3^- ($p = 0.001$) and temperature ($p = 0.025$). The total proportion of plankton growth rates explained by the constrained variables was 58.4%, the unconstrained variables being

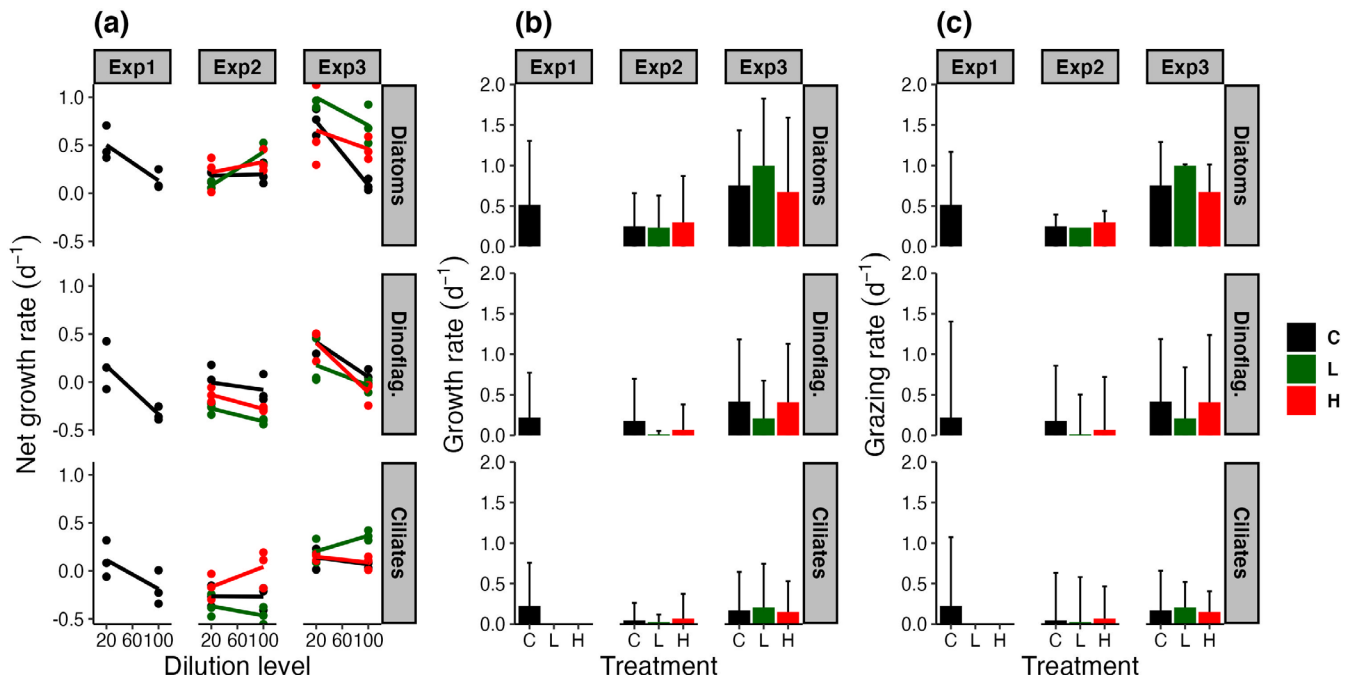


Fig. 5. (a) Instantaneous growth rates as a function of the dilution level (20% and 100%), (b) growth rates, and (c) grazing rates on nanoplankton (diatoms, dinoflagellates, and ciliates) in the three grazing experiments (Exp1, Exp2, and Exp3) grouped by treatment (C, L, and H, control, low, and high, respectively). Data are expressed as mean \pm SD ($n = 3$).

responsible for the remaining 41.6%. As for the growth rate, the community grazing rate was not explained by alkalinity ($p = 0.096$), but influenced by NO_3^- ($p = 0.006$), PO_4^{3-} ($p = 0.038$), and temperature ($p = 0.002$) (Fig. 8). The constrained variables explained 56.8% of the grazing rates while the unconstrained variables accounted for 43.2%.

Linear regressions revealed that temperature, salinity, and PO_4^{3-} represented the three most important abiotic factors influencing the Chl *a* biomass and the abundance of most plankton groups (Supporting Information Table S4). Only two groups significantly responded to alkalinity and pH, namely *Synechococcus* and diatoms (Supporting Information Fig. S6a and Table S4). Total Chl *a* and pigmented picoflagellates increased linearly with the abundance of copepod nauplii, whereas HNA bacteria, LNA bacteria, diatoms, and ciliates showed the opposite trend (Supporting Information Fig. S6b and Table S4).

Discussion

Combining dilution technique and mesocosm experimentation, we tested ocean liming by additions of calcium hydroxide ($\text{Ca}(\text{OH})_2$), with the specific aim to assess stocks, rates, and trophic ecology of natural plankton. The community was dominated by small cells ($< 2 \mu\text{m}$)—bacteria, *Synechococcus*, and pigmented picoflagellates—outnumbering larger diatoms, dinoflagellates, and ciliates. While diatom abundance and pigmented picoflagellate growth rates showed

occasional fluctuations with liming, grazing rates remained unaffected. Dinoflagellates and ciliates showed no major physiological changes, except a general reduction in cell size over time, regardless of $\text{Ca}(\text{OH})_2$. Prey selection by microzooplankton appeared size-driven irrespective of the $\text{Ca}(\text{OH})_2$ treatment. Overall nutrient availability, temperature, and biotic interactions were more influential than liming in shaping community responses. Alkalinity and pH-driven changes in nutrient supply could still affect abundance, cell size, and nutrient quota of protistan prey within the food web. H treatment TA aligned well with the expected lime additions, whereas L treatment showed some variability. Nevertheless, the $\text{Ca}(\text{OH})_2$ inputs produced a measurable increase in TA clearly different between treatments (Table 1), indicating successful addition and no alkalinity loss. This stresses the need to (1) consider titration uncertainty and (2) cautiously draw conclusions only based on a few replicates with an intrinsic variability such as mesocosms.

Plankton food web structure and functional roles

In the ultra-oligotrophic eastern Mediterranean Sea (Med) heterotrophic bacteria represent the most abundant group, and picoplankton dominate primary production (Siokou-Frangou et al. 2002; Romano and Pitta 2021), consistent with our findings. Small autotrophs are more efficient at nutrient uptake, especially under limiting conditions, promoting the formation of a complex microbial food web, unlike larger diatoms thriving in shorter food webs of nutrient-rich upwelling

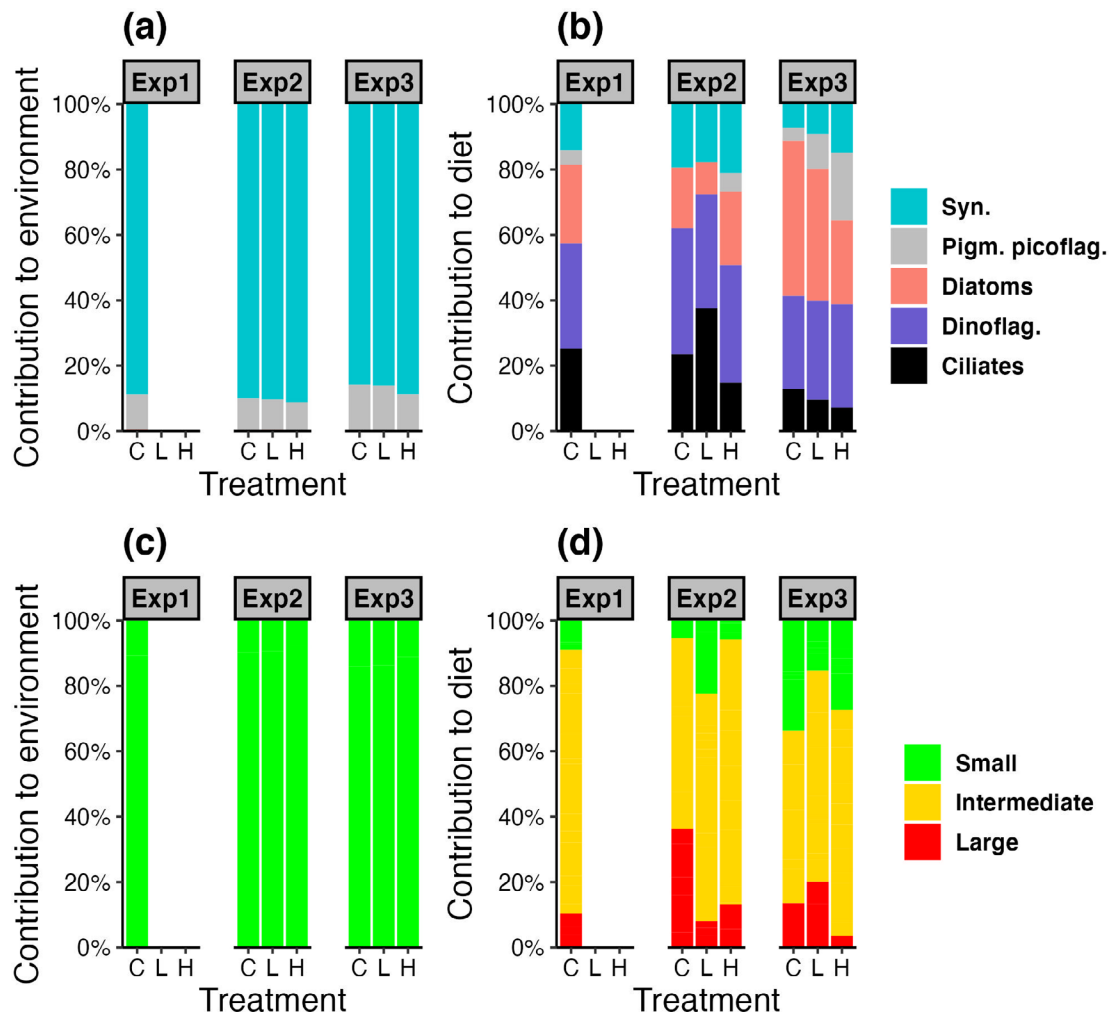


Fig. 6. Relative abundance of plankton in the three grazing experiments (Exp1, Exp2, and Exp3) grouped by treatment (C, L, and H, control, low, and high, respectively). Top panels: abundance of plankton categorized in functional groups between (a) the environment and (b) the diet. Bottom panels: abundance of plankton categorized in size classes between (c) the environment and (d) the diet. Data are expressed as means ($n = 3$).

regions (Sherr and Sherr 1988; Pomeroy 2001; Siokou-Frangou et al. 2010).

In nutrient-poor ecosystems, primary producers are subject to the simultaneous bottom-up nutrient restriction and top-down control by microzooplankton, especially dinoflagellates and ciliates (Pitta et al. 2001; Calbet 2008; Löder et al. 2011). The dominant dinoflagellates in our samples were pigmented Gymnodiniales (2.9 ± 1.2 cells mL^{-1}) and *Gyrodinium* spp. ($27 \mu\text{m}$, 1.4 ± 0.6 cells mL^{-1}), both of intermediate size (10–35 μm). Most Gymnodiniales are autotrophs but include several mixotrophic taxa (Stoecker 1999), whereas *Gyrodinium* spp. are obligate heterotrophs (Jeong et al. 2010). The bulk of ciliates were intermediate-sized (15–35 μm), mostly heterotrophic and mixotrophic aloricates. These represent 85% of the ciliate stock in early summer (Romano et al. 2021), leaving the remaining 15% to larger (> 35 μm) heterotrophic tintinnids (Romano et al. 2021), in line with our results.

Given the strict heterotrophy and large size, tintinnid grazing on phytoplankton exceeds that of aloricates and nude choreotrichs by fivefold in the eastern Med (Pitta et al. 2001). Though the mean low abundance of tintinnids in our samples suggests that the main grazing impact could be exerted by aloricate ciliates and *Gyrodinium* spp.

The effects of liming on standing stock and vital rates

Overall, the addition of $\text{Ca}(\text{OH})_2$ did not significantly affect community abundance nor physiological performance, though we observed occasional group-specific responses, in line with previous findings (Pedersen and Hansen 2003b; Fenderer et al. 2022; Subhas et al. 2022). Laboratory experiments under similar pH gradients showed that cultured phyto-mixoplankton (*Emiliania huxleyi*, *Phaeodactylum tricornutum*, *Heterocapsa triquetra*, *Chaetoceros* spp., and *Prorocentrum minimum*) were unaffected at pH 8–9 when exposed to increased

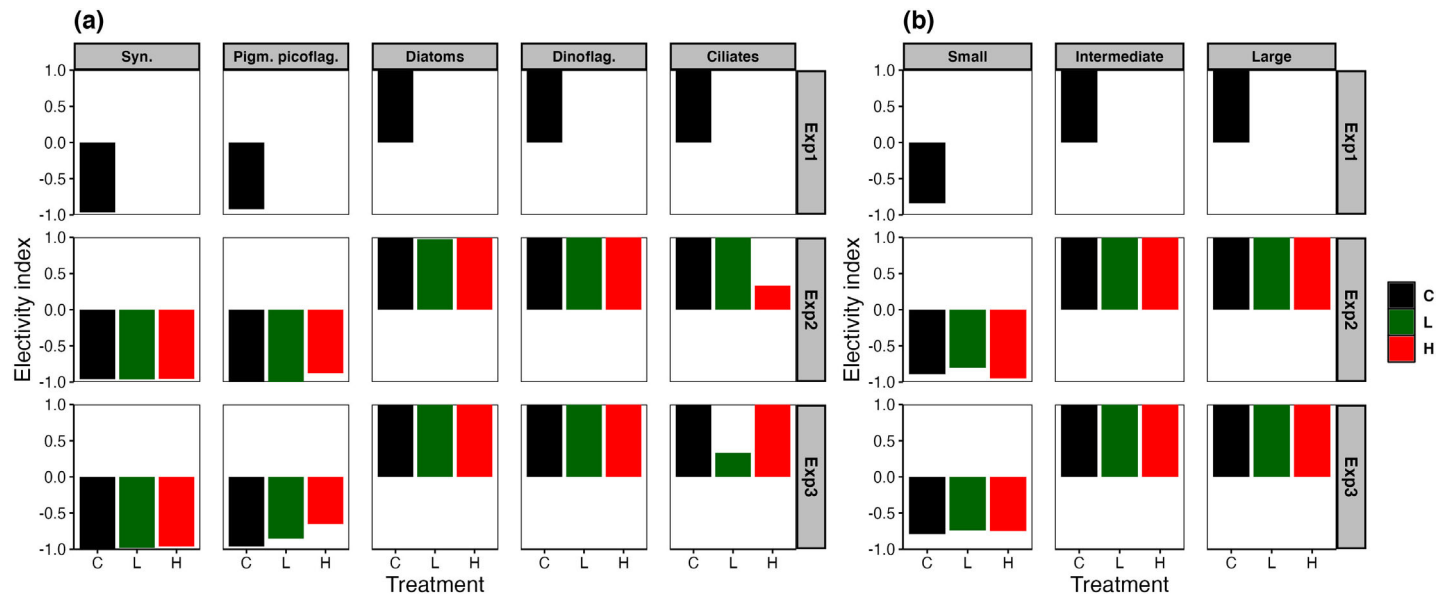


Fig. 7. Electivity index (E) of (a) prey groups, (b) prey size in the three grazing experiments (Exp1, Exp2, and Exp3) grouped by treatment (C, L, and H, control, low, and high, respectively) ($E < 0$, avoidance; $E = 0$, neutral ingestion; $E > 0$, preference). Data are expressed as means ($n = 3$).

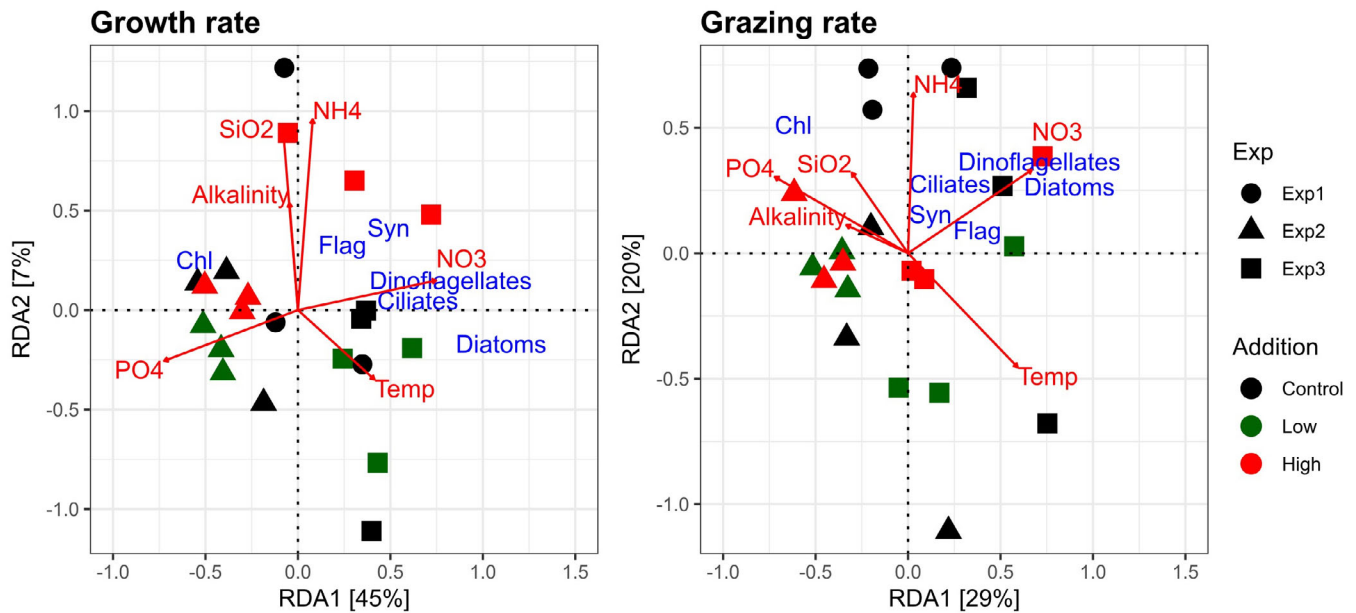


Fig. 8. Redundancy analysis (RDA) showing the growth rates and the community grazing rate on total Chl a and the specific plankton groups (*Synechococcus*, pigmented picoflagellates, diatoms, dinoflagellates, and ciliates; blue labels) as a function of the environmental variables (red vectors). The RDA was conducted on data pooled from the three experiments (Exp1, Exp2, and Exp3). Photosynthetically active radiation, salinity and pH were excluded from the analysis due to their high correlation with other variables (see Data analysis section for more details). NO_3^- : nitrate; NH_4^+ : ammonium; PO_4^{3-} : phosphate; SiO_2 : silicate; Temp: temperature; Chl: chlorophyll a ; Syn: *Synechococcus*; Flag: pigmented picoflagellates.

alkalinity (Hansen 2002; Gately et al. 2023). These taxa may be pre-adapted to transient alkalinity and pH shifts due to natural processes, for example, weathering and CO_2 removal driven by photosynthesis (Hansen 2002; Gately et al. 2023).

Similar findings have been noted in natural plankton (Ramírez et al. 2025), although tolerance to liming depended on exposure duration, pH level, and species-specific traits (Oberlander et al. 2025). While many taxa tolerated pH

fluctuations up to 8.8 (above seawater pH but still within natural ranges), stress responses emerged at pH exceeding 9.5 (Hansen 2002; Pedersen and Hansen 2003b). In our study, pH remained ≤ 8.7 , likely explaining the absence of detectable stress.

Slightly lower abundances of *Synechococcus* and diatom were observed under liming during Exp3, accompanied by peaks in dinoflagellates and ciliates in the L treatment (Exp2); though these differences were likely a result of sample variability, pinpointing the need to ensure even water distribution when filling bottles. Pigmented picoflagellates increased under high $\text{Ca}(\text{OH})_2$ levels in both Exp2 and Exp3. This was likely not a direct benefit of liming but rather an indirect effect: reduced competition from diatoms and metabolic adjustments. These flagellates can engage in mixotrophy by preying on bacteria under nutrient shortage (Unrein et al. 2014; Hansen et al. 2019), gaining a competitive edge over strict photoautotrophs (i.e., *Synechococcus*, diatoms).

Dinoflagellates and ciliates showed no consistent changes in growth or grazing rates with lime addition. This evidence disagrees with other laboratory (Pedersen and Hansen 2003a) and field experiments (Pedersen and Hansen 2003b) in which high pH affected protozooplankton in a dose- and species-specific manner. Yet, for the small pH fluctuations (more realistic in OAE scenarios), there are no clear effects on their physiology, in line with these studies, given that some species can endure pH increases better than others, especially at $\text{pH} > 8.8$ (Hansen 2002; Pedersen and Hansen 2003a, 2003b). In our study, the maximum pH recorded was 8.7, within the tolerance range of most dinoflagellates and ciliates.

Although liming did not directly affect the community, it might have limited productivity by reducing nutrient solubility (Otsuki and Wetzel 1972) through mineral precipitation (Gately et al. 2023; Iglesias-Rodríguez et al. 2023). The formation of white precipitates in H mesocosms (Exp3) supports this hypothesis. As the RDA shows, NO_3^- and PO_4^{3-} availability and temperature were the main drivers of growth and grazing responses. Nonetheless, treatments and exposure time did not stress the plankton community overall.

Microzooplankton diet composition and food selection

Microzooplankton diet was unaffected by liming, but it rather responded to prey availability and environmental conditions (i.e., nutrients and temperature). The grazing community here studied appeared to discard smaller prey (*Synechococcus* and pigmented picoflagellates) in favor of intermediate-sized and large prey (diatoms, dinoflagellates and ciliates). This aligns with known prey: predator size ratios where excessively small prey may avoid detection and capture, while very large prey exceed the handling and ingestion capacity of the predator or possess a faster escape response (Hansen et al. 1994). This might explain why the selected prey were found in the intermediate size class. For the same reason, more thecate dinoflagellates and loricate ciliates were ingested

as they were sensibly larger compared to their nude counterparts in Exp2. A slightly different diet composition emerged in the contribution of pigmented picoflagellates and diatoms in the H treatment compared to C and L in Exp3. A lower contribution of diatoms in the microzooplankton diet might result from lower diatom abundance in the H treatment, decreasing the prey–predator encounter rate (Holling 1966).

The role of biotic interactions and trophic plasticity in the food web

A moderate increase in *Synechococcus* and a stronger increase in pigmented picoflagellates was recorded over time, unlike diatoms, ciliates, and bacterial populations that experienced a progressive decline. We hypothesized that a trophic cascade might have influenced the food web, impacting in opposite fashion on consecutive trophic levels (Löder et al. 2011). The analysis of the metazoan zooplankton community conducted during the mesocosm experiment showed that copepod nauplii gradually became the dominant grazers in all mesocosms since D_6 (90% of total zooplankton stock; $p > 0.05$). These young copepods completely outnumbered tintinnids by D_8 , when the water was sampled for Exp3 (Nocera et al. unpublished). Considering their small size ($118 \pm 35 \mu\text{m}$), nauplii were the most abundant microzooplankton grazers during Exp3, likely consuming diatoms, ciliates, and dinoflagellates. This could have relieved the pressure on *Synechococcus* and pigmented picoflagellates, normally eaten by protistan grazers that exert the major removal of phytoplankton production in oligotrophic regions (Schmoker et al. 2013). Additionally, the release of picoflagellates from protistan grazing coupled with nutrient limitation may have led to increased bacterivory (Unrein et al. 2014; Li et al. 2024), reducing HNA and LNA bacterial populations by Exp3. We argued that the combination of mixotrophy, naupliar-driven trophic cascade, and the nutrient exhaustion experienced by the community on D_8 (Exp3, Supporting Information Fig. S5) likely contributed to the observed abundance trends. Unfortunately, we did not directly measure the flagellate phagotrophic activity to entirely corroborate this hypothesis. Moreover, this reasoning must be well contextualized, given the small incubation volume and the few mesocosm replicates selected.

The decline in diatoms and ciliates recorded from Exp1 to Exp3 might seemingly be due to the combined effect of (1) nutrient limitation for autotrophs (Supporting Information Fig. S5), and (2) top-down control, especially by dinoflagellates. *Gyrodinium* sp. is known to ingest both colonial diatom chains (Calbet 2008) and ciliates (Jeong et al. 2010), and consume prey of very large size, diverging from typical 10 : 1 prey : predator size ratios (Hansen et al. 1994).

Dinoflagellates were totally unaffected by liming but became smaller over the experiment. Size reduction could enhance nutrient uptake and growth (Finkel et al. 2010) and dinoflagellates can store internal nutrient reserves (Elgavish et al. 1980; Dagenais-Bellefeuille and Morse 2013), explaining

their unresponsiveness to low nutrient availability. Dinoflagellates display several feeding modes, diets and wide prey size spectrum (Jeong et al. 2010), and span autotrophy, strict heterotrophy and mixotrophy (Stoecker 1999), gaining energetic benefits and higher survival chances. Mixotrophic adaptation to nutrient depletion is frequent in Gymnodiniales (Li et al. 2000; Traboni et al. 2021), very abundant in our samples. Dinoflagellate intrinsic growth rate normally exceeds that of ciliates (Calbet 2008), possibly masking the effect of predation, and supporting the unresponsiveness to naupliar abundance. Another reason for the stable dinoflagellate population may have hydrodynamic bases: copepod nauplii detect prey that generate stronger fluid disturbance, typically large motile cells (Turner et al. 2001; Saiz et al. 2014). In our study, ciliates were larger than dinoflagellates, increasing their detectability (Saiz and Kiørboe 1995; Almeda et al. 2018) and potentially releasing dinoflagellates from predation (Supporting Information Fig. S6). The bulk of heterotrophic ciliates may prove more nutritious due to stoichiometric similarity with nauplii (Stoecker and Capuzzo 1990), making them preferred over Gymnodiniales. Yet, naupliar feeding was not assessed in this study, hence these remain speculative interpretations.

Conclusions

Working in small volume incubations, we found no generalized obvious effect of liming on the community composition and vital rates, or different microzooplankton dietary composition and altered selectivity patterns. Occasional group-specific changes in abundance and growth appeared co-influenced by limiting nutrients, and presumably shaped by trophic interactions. Notably, dinoflagellates showed remarkable unresponsiveness to $\text{Ca}(\text{OH})_2$ inputs, likely due to a combination of trophic strategy, reduced predation, and high growth potential. While liming may alter inorganic nutrient supply and food quality through mineral precipitation, its effects on food web components did not emerge to a substantial extent in this study. Our results support the evidence that liming at the tested $\text{Ca}(\text{OH})_2$ concentrations does not significantly alter plankton vital rates under a semi-natural pH gradient (8.2–8.7). Application of this approach in situ may be even mitigated considering the dilution effect and the capability of zooplankton to be displaced actively (migration) and passively (lateral advection), avoiding constant exposure. Despite the promising response that emerged in our small-scale study, the global effect of liming remains underexplored since responses are in most cases species-specific and system-dependent. This variability complicates broader speculations, particularly in complex multi-level food webs typical in the oligotrophic eastern Mediterranean Sea. Further experiments are required to ascertain the feasibility of these remedial actions using larger incubation volumes and more replicate mesocosms, and assessing zooplankton grazing by upper trophic levels in both laboratory and field setups, as well as

medium- and long-term trials to evaluate the recovery of communities after liming.

Author Contributions

Daniela Basso conceived the mesocosm experiment of alkalization by liming, with the assistance of Iordanis Magiopoulos and Paraskevi Pitta. Conceptualization and design of the experiment on plankton growth and grazing: Claudia Traboni, Justine Courboulès, Ariadna C. Nocera, Filomena Romano, Iordanis Magiopoulos, Paraskevi Pitta; sampling operations: all authors; experimental procedures: Claudia Traboni, Ariadna C. Nocera, Justine Courboulès, Selene Varliero; sample processing: Claudia Traboni, Ariadna C. Nocera, Justine Courboulès, Filomena Romano, Christos Chantzaras, Selene Varliero; analysis and interpretation of results: Claudia Traboni, Filomena Romano, Ariadna C. Nocera, Justine Courboulès; manuscript writing: Claudia Traboni; review and editing of the manuscript: all authors; funding and hosting: Daniela Basso, Paraskevi Pitta.

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Conflicts of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

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