



# Grazing Pressure Is Independent of Prey Size in a Generalist Herbivorous Protist: Insights from Experimental Temperature Gradients

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## Abstract

Grazing by herbivorous protists contributes to structuring plankton communities through its effect on the growth, biomass, and competitiveness of prey organisms and also impacts the transfer of primary production towards higher trophic levels. Previous evidence shows that heterotrophic processes (grazing rates,  $g$ ) are more sensitive to temperature than autotrophic ones (phytoplankton growth rates,  $\mu$ ) and also that small cells tend to be more heavily predated than larger ones; however, it remains unresolved how the interplay between changes in temperature and cell size modulates grazing pressure (i.e.,  $g:\mu$  ratio). We addressed this problem by conducting an experiment with four phytoplankton populations, from pico- to microphytoplankton, over a 12 °C gradient and in the presence/absence of a generalist herbivorous protist, *Oxyrrhis marina*. We found that highest  $g$  rates coincided with highest  $\mu$  rates, which corresponded to intermediate cell sizes. There were no significant differences in either  $\mu$  or  $g$  between the smallest and largest cell sizes considered. The  $g:\mu$  ratio was largely independent of cell size and C:N ratios, and its thermal dependence was low although species-specific differences were large. We suggest that the similar  $g:\mu$  found could be the consequence that the energetic demand imposed by rising temperatures would be a more important issue than the mechanical constriction to ingestion derived from prey cell size. Despite the difficulty of quantifying  $\mu$  and  $g$  in natural planktonic communities, we suggest that the  $g:\mu$  ratio is a key response variable to evaluate thermal sensitivity of food webs because it gives a more integrative view of trophic functioning than both rates separately.

**Keywords** Activation energy · Microzooplankton · Phytoplankton growth · *Oxyrrhis marina* · Predator-prey relationship

## Introduction

Temperature governs metabolism through its effect on biochemical reactions [1]. A higher environmental thermal

energy entails higher kinetic energy of molecules and, ultimately, increased rates of cellular processes and organism activity [2–4]. Importantly, according to the metabolic theory of ecology (MTE, [2]), the temperature sensitivity of autotrophs (e.g., phytoplankton growth rates,  $\mu$ ) and heterotrophs (e.g., grazing rates,  $g$ ) differs markedly so that heterotrophic processes have a higher temperature dependence (or activation energy,  $E_a$ ) than autotrophic ones [3]. In fact, an analysis by Rose and Caron [5] showed a stronger reduction in herbivore growth, relative to phytoplankton growth, when temperature was < 15 °C, suggesting a different temperature effect on both processes in marine ecosystems. These authors argued that the weaker temperature sensitivity of phytoplankton compared with that of their grazers could allow phytoplankton to temporarily avoid top-down control and help explain the initiation of blooms in cold waters. However, recent studies by Chen and Laws [6] and Wang et al. [7] challenge the above stated assumption by the MTE and suggest that the

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temperature dependence of phytoplankton growth can be as high as that of heterotrophic protists.

Together with temperature, another relevant driver modulating the grazing activity is prey cell size. Phytoplankton blooms worldwide are often dominated by large cells [8, 9]. The most commonly cited explanation for this pattern is that larger cells (> 5–10  $\mu\text{m}$  in equivalent spherical diameter, ESD) suffer less grazing than the smaller ones, which allow them to escape predation and dominate blooms [8]. In addition, larger cells may be less preferred by grazers because they have lower nutritional qualities [10]. However, global-scale observational analyses of microzooplankton grazing pressure ( $g:\mu$  ratios) show losses of similar magnitude (~60–70%) in regions with a widely contrasting phytoplankton size structure [11]. In addition, recent experimental results have found that the grazing pressure was the highest on microphytoplankton when pico-phytoplankton cells were the main contributors to the carbon biomass and on nanophytoplankton when microphytoplankton dominated [12]. By contrast, Dong et al. [13] reported that the strongest grazing pressure occurred on the dominant size class (i.e., micro- or nanophytoplankton) of the community, whereas results from Anjusha et al. [14] based on size-fractionated phytoplankton experiments over a seasonal succession indicated that microzooplankton preferred the nano-size fraction of the phytoplankton community regardless of the dominant size class. Given that the phytoplankton size structure is a key driver of ecosystem functioning (e.g., productivity, nutrient cycling, 9, 15), it is necessary to ascertain the role of temperature and cell size in the control of grazing pressure and trophic structure in planktonic communities.

The intense efforts dedicated to characterize the spatial and temporal (weekly to seasonal) variability in  $g$  rates have highlighted the crucial role of temperature and prey cell size as key drivers of grazing pressure [16–18]. Under the framework outlined above, we would expect a strengthening of the top-down control on phytoplankton as temperature increases for grazers to achieve their nutritional requirements to grow [19, 20]. However, natural communities are composed by multiple different cellular sizes and taxonomic groups. Field grazing studies typically focus on the net effect of different drivers on the community-level responses, but interactions between biotic and abiotic drivers and species-specific processes make it difficult to ascertain the underlying mechanisms behind such response patterns. Given that the combined effect of body size and temperature on individual metabolic processes imposes important constraints on the global carbon cycle [3], experimental approaches dealing with species-specific responses are required to gain insight into the general patterns observed in nature. Most studies have addressed either the effect of temperature [4] or cell size [13], but the concurrent effect of these drivers on grazing pressure remains unresolved.

Here we address these uncertainties by quantifying the temperature dependence of phytoplankton  $\mu$ , microzooplankton  $g$ , and  $g:\mu$  by a generalist herbivore protist feeding on a range of prey sizes. The prey species were chosen to represent both sides of the hump-shaped relationship between the phytoplankton growth and cell size [9, 21]. All experiments were replicated under a full factorial design in which we manipulated: (1) temperature, with a gradient that spanned 12  $^{\circ}\text{C}$ , and (2) the cell size of the phytoplankton population by considering a range of three orders of magnitude in biovolume from pico- to microphytoplankton. By using four model phytoplankton species (as preys) belonging to different taxonomic groups in the presence and absence of a model cosmopolitan and generalist predator [22], the dinoflagellate *Oxyrrhis marina* (as predator), we tested how the interaction between rising temperatures and changes in cell size could alter grazing pressure on phytoplankton and, potentially, the proportion of primary production that is available for higher trophic levels.

## Methods

**Model Strains and Growth Conditions** The dinoflagellate (hereafter, predator) *O. marina* Dujardin (strain B21.89) (mean biovolume  $4800 \pm 222 \mu\text{m}^3$ ) was obtained from the Culture Collection of Algae (SAG) at the University of Göttingen, Germany. The ochrophyte *Nannochloropsis gaditana* L. M. Lubián, the haptophytes *Isochrysis galbana* Parke and *Emiliania huxleyi* (Lohmann) W. W. Hay & H. P. Mohler, and the bacillariophyte *Thalassiosira rotula* Meunier (hereafter, preys) (mean biovolumes,  $9 \pm 1$ ,  $64 \pm 9$ ,  $160 \pm 14$ ,  $2600 \pm 198$  and  $4800 \pm 222 \mu\text{m}^3$ , respectively) were obtained from the Culture Collection of Algae of the CIM-UVigo. Non-axenic semi-continuous cultures (1-L Erlenmeyer flasks) of all species were grown in f/2 medium [23] at 14, 18, 22, and 26  $^{\circ}\text{C}$ , avoiding supra-optimal temperatures [24], with constant aeration and photosynthetically active radiation under a 12 h L:12 h D cycle ( $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; HomePluss®, Garcasso leds, Spain) inside a temperature-controlled environmental chamber (CLIMASlab, model CIR300, Spain). Additionally, we chose the temperature range mentioned above because the previous results with several strains of *O. marina* have shown that it exhibits similar growth rates ( $0.5\text{--}0.6 \text{d}^{-1}$ ) within the 15–26  $^{\circ}\text{C}$  range [22, 25].

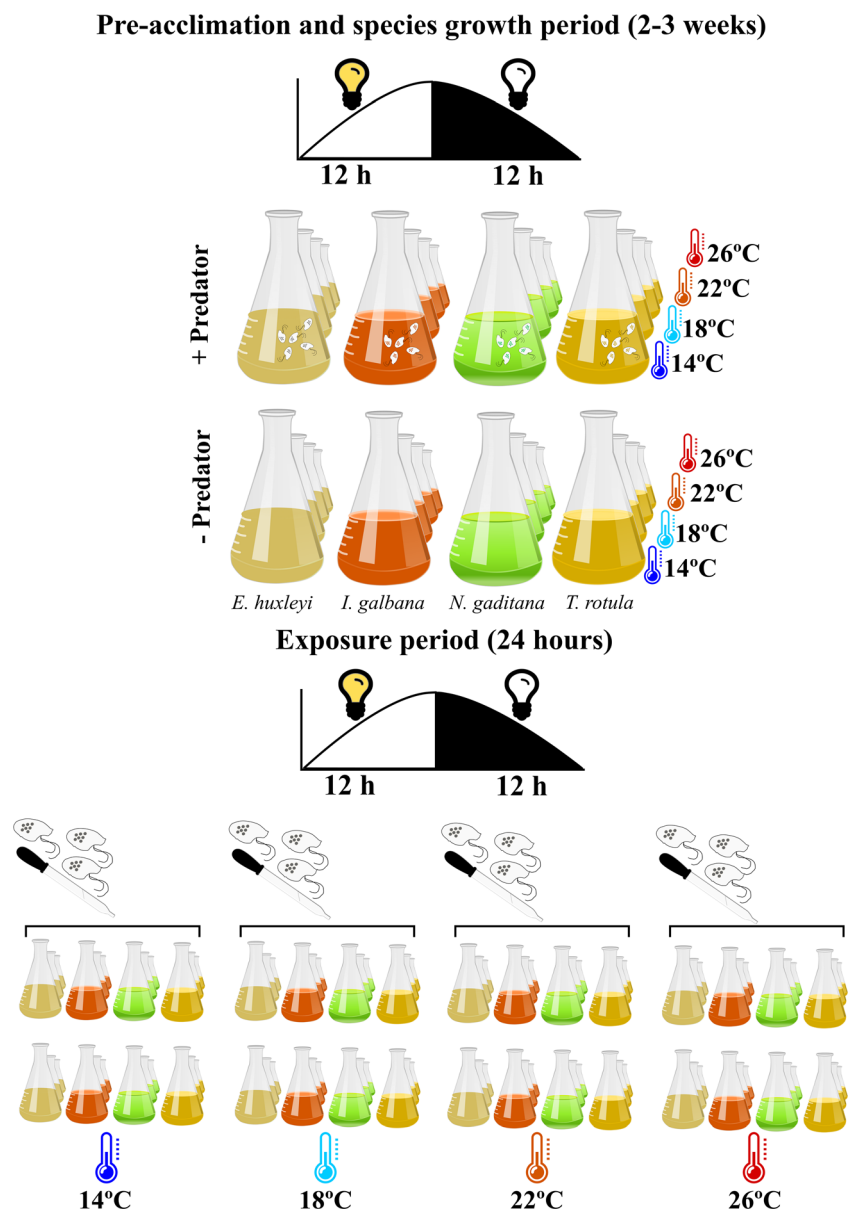
Transfer into fresh medium was carried out at intervals of 3–5 days to maintain exponential growth. Such fresh media was autoclaved the same day that it was replaced into cultures to avoid bacterial proliferation (mean values  $\sim 2 \text{ng C mL}^{-1}$ ; assuming a bacterial C content of 20 fg per cell [26]). The new stock cultures of *O. marina* were grown in 250-mL Erlenmeyer flasks under the same experimental conditions described above (mean abundance  $10^7 \text{cell L}^{-1}$ ) and fed at saturating concentrations with the four target phytoplankton

species before being used in experimentation. Before each experimental incubation, *O. marina* cultures were checked under the microscope to make sure that the algal food had virtually been depleted. All cultures were acclimated during 2–3 weeks to the target temperature before being used in experimentation, thus avoiding any short-term thermal shock [27]. The duration of the acclimation period used was based on the fact that the mean number of generations elapsed during this period ranged between 13 and 30, enough generations to consider that our species were acclimated to the target temperatures tested (i.e., 4 generations; [28]).

**Experimental Setup** To assess the interaction between the three factors considered in our experimental design (temperature, presence/absence of predator and prey species), a  $4 \times 2 \times$

4 (in triplicate) matrix was implemented (Fig. 1). The four temperature treatments were 14, 18, 22, and 26 °C; the two predator treatments were the presence and absence of predator, and the four phytoplankton species used were those already described above (see Fig. 1). Initial abundances ( $t_0$ ) of prey species ranged between  $\sim 3 \times 10^5$  and  $2 \times 10^6$  cell  $L^{-1}$ , whereas those of predator were  $\sim 52 \times 10^3$  cell  $L^{-1}$ . The prey:predator biomass ratios, based on cell counting data, used in our experimental setup (*E. huxleyi*  $0.29 \pm 0.11$ ; *I. galbana*  $0.24 \pm 0.09$ ; *N. gaditana*  $0.33 \pm 0.10$ ; *T. rotula*  $0.35 \pm 0.11$ ), are similar to those previously used in other laboratory predator-prey studies with *O. marina* populations [29, 30]. Also, and to ensure that  $g$  was not limited by prey availability, predator was exposed to mean food concentrations ( $\sim 22$  [*E. huxleyi* and *I. galbana*], 37 [*N. gaditana*], and 59

**Fig. 1** Graphic scheme of the experimental full factorial design for each species (preys, *Emiliana huxleyi*, *Isochrysis galbana*, *Nannochloropsis gaditana*, and *Thalassiosira rotula*). The design included four temperature treatments (14, 18, 22, and 26 °C) in the absence and presence of a generalist heterotrophic dinoflagellate (*Oxyrrhis marina*), as predator



[*T. rotula*] mg C L<sup>-1</sup>) that exceeded the half saturation constant ( $k = 0.04\text{--}19$  mg C L<sup>-1</sup>) reported in the literature using functional response curves with different prey cell sizes [31]. Due to the high number of resulting experimental units (96 in total), and the limited space available in the growth chambers, the acclimation and exposure of the different phytoplankton species to the target temperatures, and to the presence/absence of the predator, were done sequentially.

Growth ( $\mu$ ) and grazing ( $g$ ) rates were measured following the dilution method in a 2-point modification with phytoplankton populations in which the grazer was absent and present [32–34]. This abbreviated method provides  $\mu$  and  $g$  rates that are statistically indistinguishable from the multi-point, traditional approach for both linear and non-linear feeding responses in laboratory and field-based experiments [18, 34–36].

We performed a total of 32 independent dilution experiments (i.e., 4 prey species and 4 temperatures in the presence/absence of predator), using 250-mL Erlenmeyer flasks containing 150 mL of culture. For each single experiment, chlorophyll *a* (Chl *a*) concentrations and cell abundances were determined for each flask at  $t_0$  and  $t_f$ , i.e., after 24 h of exposure to the predator, whereas the other response variables were only determined at  $t_0$  (see below). Data treatment for each dilution experiment was focused on using Chl *a* (Supplementary Table S1) and followed procedures described in Morrison and Menden-Deuer [36]. Briefly,  $\mu$  in the presence and absence of predator was calculated as

$$\mu = \ln(C_t/C_0) \times 1/t$$

where  $C_t$  and  $C_0$  are the final and initial Chl *a* concentrations, respectively, and  $t$  is the time elapsed in days.  $\mu$  rates measured in absence of predator were considered to be the intrinsic growth rates unaffected by grazing. Herbivorous grazing rate ( $g$ , d<sup>-1</sup>) was calculated as

$$g = \mu_{\text{absence}} - \mu_{\text{presence}}$$

where  $\mu_{\text{absence}}$  and  $\mu_{\text{presence}}$  are the  $\mu$  in the absence and presence of predator, respectively. Grazing pressure was calculated as the ratio between  $g$  and  $\mu$  rates (in percentage).

**Phytoplankton Cell Counts** To determine predator and prey abundances, 5-mL samples were fixed with Lugol's iodine (1%) and examined on an inverted microscope at  $\times 200\text{--}\times 400$  (Nikon® SE type 102, Nikon Instruments Inc., USA) using a 1-mL improved Thomas-Neubauer chamber (Brand®, Brand GmbH + CO KG, Germany). To determine biovolume, 20 predator and prey cells were measured and approximated to their geometric shape [37, 38].

**Chlorophyll *a* (Chl *a*)** Ten-mL samples were taken from each flask, filtered onto GF/F Whatman glass-fiber (25-mm

diameter; Whatman Inc., UK) filters, and stored at  $-20$  °C until analyzed. Afterwards, the filters were thawed and placed in centrifuge tubes with 5 mL of 90% acetone for 24 h at 4 °C in darkness. Chl *a* was measured fluorimetrically with a Turner Designs Fluorometer (Trilogy model, USA) routinely calibrated with pure Chl *a* (Sigma Aldrich, USA) (Supplementary Table S1).

**Photosynthetic Performance** Three-mL samples were taken, on a daily basis, during the pre-acclimation period and before experimentation ( $t_0$ ) for each phytoplankton species to measure photosystem II photochemical efficiency, as an indicator of good physiological status, using a pulse amplitude modulated (PAM) fluorometer (Walz, Water PAM, Effeltrich, Germany). Each sample was dark-adapted for 20 min and then measured three times. The maximal photochemical quantum yield ( $\Phi_{PSII_{\text{max}}}$ ) was calculated using the equation of Genty et al. [39]:

$$\Phi_{PSII_{\text{max}}} = F_v/F_m = (F_m - F_0)/F_m$$

where  $F_m$  is the maximum fluorescence induced by a saturating light pulse ( $\sim 5300$   $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in 0.8 s) and  $F_0$  is the minimal fluorescence induced by applying far-red illumination (peak at 660 nm) in dark-adapted cells.

#### Particulate Organic Carbon (POC) and Nitrogen (PON)

Twenty-mL samples were taken from each flask, filtered through pre-combusted (500 °C for 4 h) GF/F Whatman (25-mm-diameter glass-fiber filters; Whatman Inc., UK), and stored at  $-20$  °C until analyzed. For *E. huxleyi*, filters were exposed to concentrated HCl to remove calcium carbonate. Before the analysis, filters were desiccated at room temperature for 72 h. Samples were analyzed with an elemental analyzer (vario MACRO cube, Elementar Analyses System GmbH, Germany). Blank GF/F Whatman glass-fiber filters were treated as samples to determine background C and N contents (Supplementary Table S2).

**Data and Statistical Analysis** We quantified the effect of rising temperature on  $g:\mu$  for each phytoplankton population by calculating the activation energy ( $-E_a$ ). The ordinary least squares regression was used to determine the slope ( $-E_a$ ) of the linear relationship between  $1/KT$  and the natural logarithm of the  $g:\mu$  ratio, where  $K$  is the Boltzmann's constant (i.e.,  $\sim 8.62 \times 10^{-5}$  eV K<sup>-1</sup>) and  $T$  is the temperature in °K.

A two-way analysis of the variance (ANOVA) was used to examine differences among T treatments and prey species, as factors, on C:N and C:cell ratios as well as on species-specific  $\mu$  and  $g$  and  $g:\mu$  ratios. Mean  $\mu$  and  $g$  rates,  $g:\mu$  ratios, and  $E_a$  and  $\Phi_{PSII_{\text{max}}}$  values measured in each temperature treatment were pooled together to allow the comparison between the four species tested. Before applying a one-way ANOVA with

pooled data, we checked the homogeneity of variances for the above-mentioned variables by Levene's test. Non-linear regression analyses were used to assess the relationship between C:N and C:cell ratios,  $\mu$ ,  $g$  and  $g:\mu$  (%) ratios; and temperature. Normality (by Shapiro-Wilk test) and sphericity (by Mauchly's test) were checked for each data group before the application of ANOVA. A least significant differences (LSD) post hoc test was used to determine significant differences among and within the different factors. A 95% confidence limit was used in all tests, and all analyses were performed with RStudio v. 1.1.463.

## Results

### Temperature × Cell Size Effects on C:N and C:Cell Ratios

The C:N ratios ranged between ~2 and 8 for all species tested in our experiment, and their variability was strongly species-dependent. While C:N increased along the assayed temperature range by 60% for *I. galbana* and *N. gaditana*, it decreased by 50% in *T. rotula* and showed the lowest values at intermediate temperatures in the case of *E. huxleyi* (Fig. 2a). C:cell ratios also exhibited a species-specific response pattern to temperature. Cellular C content decreased with rising temperature (~40% in *E. huxleyi* and ~60% in *N. gaditana*) and increased (~20% *T. rotula*) or did not show any clear trend with temperature (*I. galbana*; Fig. 2b). These responses resulted in a significant Temperature × Species interactive effect on both variables (Table 1).

### Growth and Grazing Rates as a Function of Temperature and Cell Size

Phytoplankton intrinsic  $\mu$  ranged between 0.14 (*N. gaditana* at 14 °C) and 1.01 (*E. huxleyi* at 22 °C) d<sup>-1</sup> (Fig. 3a), whereas

**Table 1** Results of two-way analysis of the variance (ANOVA) for the effect of temperature, species, and their interaction on carbon:nitrogen (C:N) and carbon:cell (C:cell) ratios, phytoplankton growth ( $\mu$ ) and grazing ( $g$ ) rates, and  $g:\mu$  ratio

Variable	Temperature	Species	Temp × Species
C:N ratio	5.18**	32.84***	5.65***
C:cell ratio	15.64***	212.88***	14.72***
$\mu$	102.99***	170.57***	162.94***
$g$	240.81***	305.77***	247.22***
$g:\mu$ ratio	584.50***	12.62***	593.56***

Values represent  $F$ -test values; \*\* $p$  value < 0.01, \*\*\* $p$  value < 0.001. Degrees of freedom for temperature (3) and species (3) and Temp × Species (9)

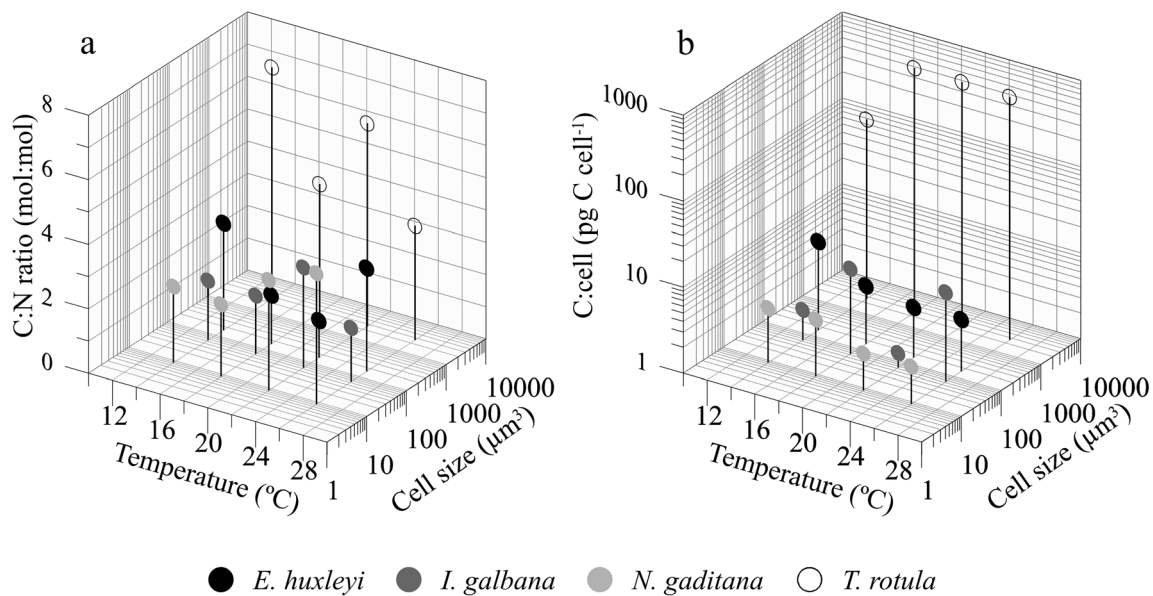
$g$  varied between 0.05 (*I. galbana* at 14 °C, *N. gaditana* at 14 and 18 °C, and *T. rotula* at 14 °C) and 0.45 (*E. huxleyi* at 18 °C) d<sup>-1</sup> (Fig. 3b).  $\mu$  increased with temperature in all species tested with the exception of *I. galbana*, which exhibited the opposite pattern (Fig. 3a). Despite this opposite response, we found relatively high  $\Phi PSII_{max}$  values in *I. galbana* (~0.54 ± 0.02), indicating no depressed photophysiological condition. Indeed, no significant differences were found between  $\Phi PSII_{max}$  values of *I. galbana* and those of the other species (*E. huxleyi* 0.54 ± 0.02; *N. gaditana* 0.53 ± 0.02; *T. rotula* 0.57 ± 0.03) over the temperature gradient considered (LSD post hoc test,  $p > 0.1$ ).  $g$  exhibited two clear response patterns with warming: (1) a significant increasing trend with temperature, such as in the case of *I. galbana* ( $R^2 = 0.98$ ,  $F_3 = 128.28$ ,  $p < 0.01$ ) and *N. gaditana* ( $R^2 = 0.99$ ,  $F_3 = 1166.23$ ,  $p < 0.05$ ), and (2) a unimodal response pattern with  $g$  peaking at intermediate temperatures, as found in *E. huxleyi* ( $R^2 = 0.82$ ,  $F_3 = 2.32$ ,  $p < 0.05$ ) (Fig. 3b).  $g$  on *T. rotula* did not show a significant effect with temperature ( $R^2 = 0.53$ ,  $F_{2,21}$ ,  $p > 0.05$ ). As observed for C:N and C:cell ratios, there was a significant Temperature × Species effect on both rates (Table 1).

The  $g:\mu$  ratio, a measure of grazing pressure equivalent to the fraction of daily primary production removed by microzooplankton grazing, was highly variable (11–77%; mean values ~38%; Fig. 3c).  $g$  did not exceed phytoplankton  $\mu$  in any species or temperature treatment considered. The analysis of the effects of temperature on  $g:\mu$  ratio largely reflected the same response patterns outlined above for  $g$ . The overall fraction of removed primary production increased with increasing temperature in *I. galbana* and *T. rotula* and exhibited a U-shape response pattern in *N. gaditana* (Temperature × Species effect  $F_9 = 593.56$ ,  $p < 0.001$ ), whereas it decreased, exhibiting the lowest  $g:\mu$  ratios at the highest temperature tested in *E. huxleyi* (Fig. 3c).

Considering all rate measurements pooled together for all species, our results show a broadly unimodal relationship between cell size and  $\mu$  over the cellular mean size gradient considered (nearly three orders of magnitude), with significantly higher rates in *E. huxleyi* ( $n = 12$ ; 0.6 d<sup>-1</sup>) than in *I. galbana* = *T. rotula* > *N. gaditana* (LSD post hoc test,  $p < 0.01$ ; Fig. 4a). The highest  $\mu$  rates measured in *E. huxleyi* coincided with the highest values of  $g$  (LSD post hoc test,  $p < 0.001$ ; Fig. 4a). The fraction of primary production consumed by grazing was similar in all cases regardless of the cell size considered (Fig. 4b), taking a value of ~40% (LSD post hoc test,  $p > 0.8$ ) (with the exception of *I. galbana*, in which  $g:\mu$  ratio was ~20%).

### Temperature Sensitivity of $g:\mu$ Ratios Over the Cell Size Range

$E_a$  values showed that the overall temperature effect on  $g:\mu$  ratios was slightly stimulatory although the variability among



**Fig. 2** Mean **a** carbon:nitrogen (C:N) and **b** carbon:cell (C:cell, in pg C cell<sup>-1</sup>) ratios in *Emiliania huxleyi*, *Isochrysis galbana*, *Nannochloropsis gaditana*, and *Thalassiosira rotula* populations exposed to four temperatures: 14, 18, 22, and 26 °C

different prey species was large. The  $E_a$  of  $g:\mu$  was 3-folds higher for *I. galbana* than for the other 3 species (LSD post hoc test,  $p < 0.01$ ), whereas no significant differences were found between the largest (i.e., *T. rotula*) and smallest (i.e., *N. gaditana*) species considered in this study (LSD post hoc test,  $p \sim 0.90$ ; Fig. 5).

## Discussion

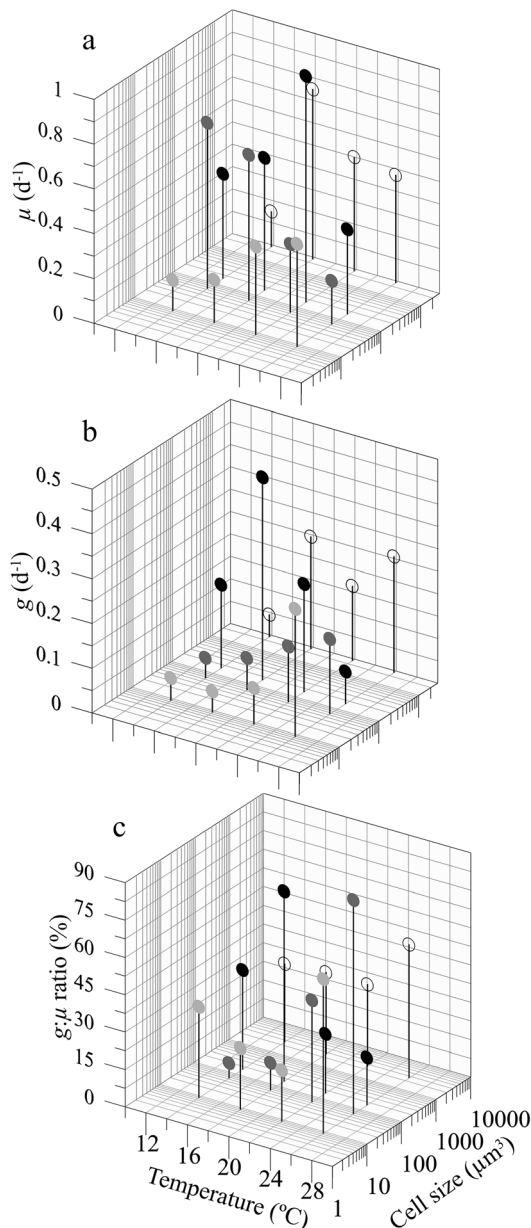
Our work shows that grazing pressure was similar across a wide range of prey cell size and temperatures. These results provide insight into the regulation of microzooplankton grazing pressure by the interaction between temperature and cell size, two drivers whose effect has been often evaluated independently [40].

Previous results by Hansen et al. [29] indicate that *O. marina* prefers larger prey sizes and even can exhibit avoidance of certain species (e.g., on *E. huxleyi*). In addition, the optimal foraging theory for a predator handling individual preys predicts that grazers prefer more energetically valuable organisms (e.g., those with lower C:N ratios) [41]. Following both arguments, it could have been expected that temperature accentuated grazing pressure on larger cells in the first case, but on smaller (more N-rich) cells in the second one. One plausible explanation for the absence of a clear response pattern could be that the energetic demands imposed by increasing temperature, i.e., dominance of catabolic processes requiring a higher food intake to sustain growth [42], would be a more important factor than mechanical constriction to ingestion mediated by prey cell size. Also, it could be that grazing is more dependent on the size-specific  $\mu$  than on cell size itself

[43]. In any event, we can discard that the size-independent grazing pressure found was due either to a limited encounter rate between predator and prey or to a limited maximum growth rate because *O. marina* was exposed to prey biomass that exceeded (up to 3-folds) its half saturation constant [31]. Thus, our results suggest that generalist microzooplankton grazers, such as *O. marina*, have a high ability to ingest preys with variable cell sizes. This feeding versatility could hypothetically confer a competitive advantage relative to more specialist species when potential carbon demands increase, such as may occur under warming conditions [44].

Despite that we did not observe any clear response pattern of grazing pressure with cell size, we found that it was  $\sim 20\%$  higher in one of the two haptophytes considered (i.e., *E. huxleyi* and *I. galbana*). The differences reported within both species could be explained by two non-mutually exclusive facts. First, *E. huxleyi* does not have an active movement ability. The absence of flagella can increase the probability to be captured compared with a mobile species, such as *I. galbana*; and second, *O. marina* has been reported to show a positive preference for calcified compared with non-calcified cells [29].

Our population-level results that grazing pressure is similar in small and large cells are consistent with the patterns identified by Calbet and Landry [11]. These authors found a similar grazing pressure by microzooplankton in open ocean and estuarine and coastal areas (60–70% respect total) although such communities are dominated by pico-phytoplankton and nano-/microphytoplankton cells, respectively [9]. Also, they are consistent with the observations of Schulhof et al. [45] in a freshwater ecosystem, where they found that when the effects of temperature on the predator-prey interaction are considered,



● *E. huxleyi* ● *I. galbana* ● *N. gaditana* ○ *T. rotula*

**Fig. 3** Mean **a** phytoplankton growth ( $\mu$ , in  $\text{d}^{-1}$ ), **b** herbivorous grazing rates ( $g$ , in  $\text{d}^{-1}$ ), and **c**  $g:\mu$  ratios (in percentage) in *Emiliania huxleyi*, *Isochrysis galbana*, *Nannochloropsis gaditana*, and *Thalassiosira rotula* populations exposed to four temperatures: 14, 18, 22, and 26 °C

the higher grazing pressure on smaller rather than larger cells become similar, regardless of the dominant size class in the community. The absence of preference by a specific prey size found in our work could be supported by the fact that our approach did not allow preferential selection. Although this response pattern does not naturally occur in nature, we explicitly performed predator-prey pairs to test how the predator behaves when no food choice exists. Based on these results, we could speculate that the dominance of phytoplankton blooms by larger cells is not only due to the selective pressure

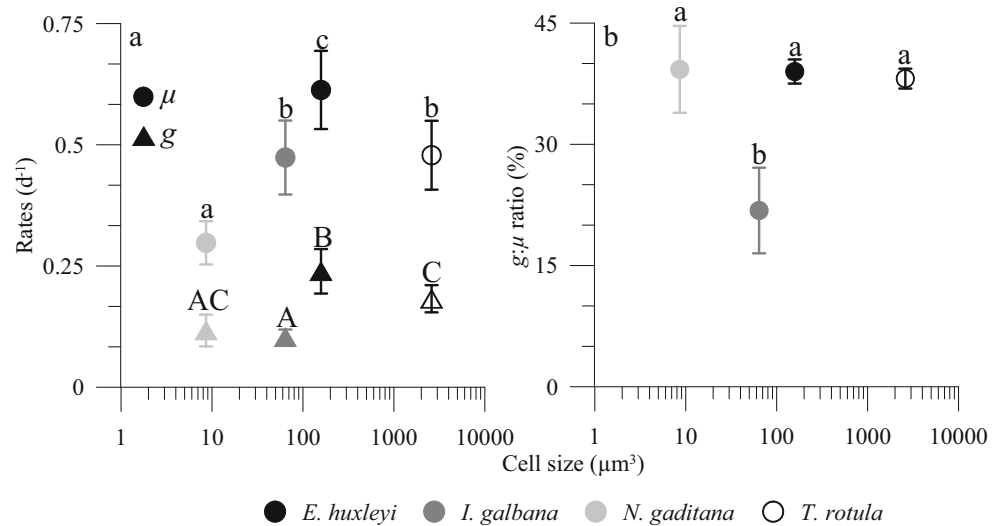
exerted by grazers on small cells [8, 10], but it could also be a consequence that small cells have lower maxima growth rates [46].

Our measurements of  $\mu$  and  $g$  showed a similar (although highly variable) temperature dependence of both processes (i.e.,  $0.47 \pm 1.33$  vs.  $0.40 \pm 1.48$  eV, respectively) and indicated that the changes in these rates were more or less similar in magnitude. These findings contrast with previous reports that suggested a stronger thermal dependence of heterotrophic compared with autotrophic processes in aquatic [5, 47] and terrestrial [3] ecosystems; however, they are consistent with the idea recently proposed by Wang et al. [7] that the thermal dependence of growth in unicellular autotrophs and protist heterotrophs could be similar, contrary to predictions by the MTE. As discussed by Wang et al. [7], this pattern could be related with the fact that  $\text{CO}_2$  solubility in water is 30 times higher than that of  $\text{O}_2$  [48] and the existence of carbon-concentrating mechanisms in protists which increase  $\text{CO}_2$  and  $\text{HCO}_3^-$  within cells by 10–100-folds [49, 50]. Both facts could attenuate the negative effect that increasing temperature exerts on carboxylase:oxygenase activity ratios, because oxygenation has a higher free  $E_a$  than carboxylation [51, 52], thus explaining that the growth of phytoplankton could be as sensitive to temperature as that of heterotrophic protists.

The similar thermal dependence of  $\mu$  and  $g$  rates resulted in a relatively low ( $0.41 \pm 0.83$  eV) overall thermal dependence of grazing pressure. We stress the need to assess the effect of temperature on grazing pressure rather than on phytoplankton growth rate and herbivore grazing rate separately, because each process is influenced by the other. These findings agree with recently published reports that showed a low thermal dependence of grazing pressure with rising temperature in tropical [12], subtropical [18], and polar [34] plankton communities. However, it contradicts the view by Rose and Caron [5] that growth rates of algal prey are less constrained by decreasing temperatures  $< 15$  °C than those of their grazers (i.e., different thermal dependence), allowing phytoplankton to temporarily escape top-down control and explaining the occurrence of phytoplankton blooms in cold waters. An alternative explanation is that there is generally an inverse relationship between temperature and nutrient availability in the ocean [53]. The fact that cooler waters (i.e., polar and temperate regions) are subjected to more frequent nutrients inputs into euphotic zone, mediated by vertical mixing and/or upwelling processes, favors rapid phytoplankton growth, and explains that blooms tend to occur more often in those regions [54].

When we pooled together the rates obtained over the 12 °C temperature gradient tested, we found that the highest  $\mu$  rates occurred at intermediate cell sizes. The  $\mu$  pattern is consistent with the unimodal size scaling of the phytoplankton growth found by Marañón et al. [46], where species with intermediate cell sizes (roughly 10–1000  $\mu\text{m}^3$  in cell volume) sustain faster

**Fig. 4** Mean ( $\pm$  SD) **a** phytoplankton growth ( $\mu$ , in  $d^{-1}$ ) and herbivorous grazing rates ( $g$ , in  $d^{-1}$ ) and **b**  $g:\mu$  ratios (in percentage) in *Emiliania huxleyi*, *Isochrysis galbana*, *Nannochloropsis gaditana*, and *Thalassiosira rotula* populations exposed to four temperatures: 14, 18, 22, and 26 °C. Note that all temperatures were pooled together in each species. Letters on symbols denote significant differences by the LSD post hoc test



growth rates than larger and smaller cells. In this connection, several lines of evidence indicate that during conditions of high resource availability, intermediate-size species (nanophytoplankton) tend to be the main contributors to the total biomass of the community [9, 55, 56]. In some cases, as Anjusha et al. [14] found in tropical coastal waters, nanophytoplanktons even constitute the optimal prey size for microzooplankton. More generally, the phytoplankton groups that are consumed at higher rates usually exhibit faster  $\mu$  rates as well [57–59]. Thus, the coupling between  $\mu$  and  $g$  rates reported unveils a highly dynamic nature of species interactions in planktonic populations [60].

Although our approach considered only individual predator-prey interactions, it is relevant given that it was based on the experimental determination of  $\mu$  and  $g$  rates using the same growth conditions avoiding uncertainties associated

with data compiled from the literature, included realistic cell abundances often found in coastal, productive waters during bloom periods [61], and prey populations with a wide size range and variable C:N ratios. Finally, we avoided any potential interference related with a selective grazing on preys by exposing the predator to a single prey species [62, 63]. Nevertheless, our experiments were performed under nutrient replete conditions, and therefore the temperature effects reported on  $\mu$  (and indirectly on  $g$ ) could be smaller if nutrients are limiting for phytoplankton growth [64].

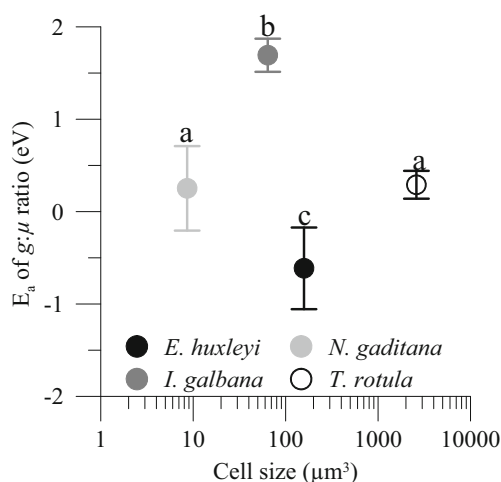
In conclusion, we have shown that predation pressure (i.e.,  $g:\mu$  ratio) on phytoplankton by a generalist microzooplankter was similar regardless of prey cell size and that the stimulatory effect of temperature on such trophic interaction was overall low, although species-specific differences in size classes were large. Despite the difficulties of quantifying phytoplankton growth and herbivorous grazing rates, particularly in natural plankton communities, our results emphasize the need to address grazing pressure, instead both rates separately, to obtain a more integrative view on how thermal dependence and body size could affect trophic interactions in planktonic food webs.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.



**Fig. 5** Mean ( $\pm$  SE) activation energies ( $E_a$ , in eV) for growth:grazing ratios ( $g:\mu$ , in percentage) in *Emiliania huxleyi*, *Isochrysis galbana*, *Nannochloropsis gaditana*, and *Thalassiosira rotula* populations exposed to four temperatures: 14, 18, 22, and 26 °C. Letters on symbols denote significant differences by the LSD post hoc test



**Ethical Approval** This article does not contain any studies with animals performed by any of the authors.

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