MixITiN Deliverable 3.8 Report

Section 3

Development And Validation Of New Methods For Measuring Predation Rates In Mixoplanktonic Protists

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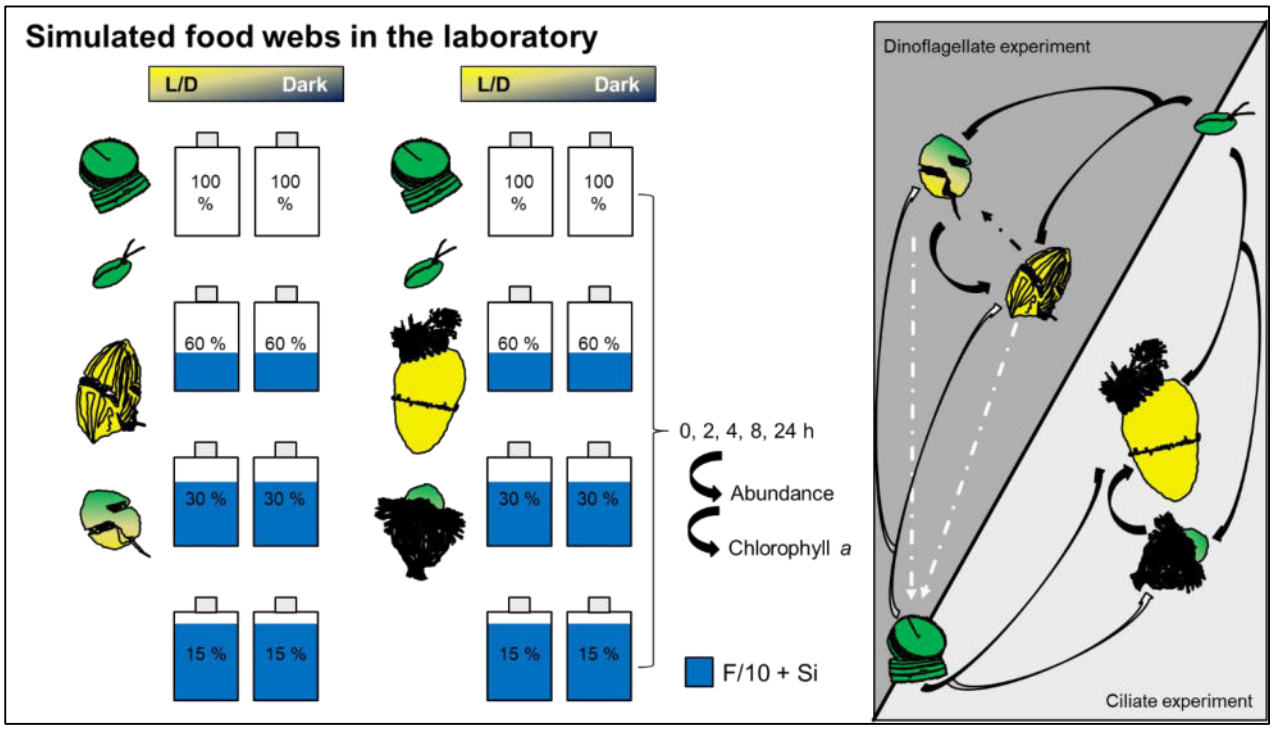

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3.1 Introduction

Despite the supposed relevance of mixoplankton (Stoecker *et al.* 2017, Flynn *et al.* 2019) we still lack standardized techniques to measure their grazing impact in the field. This is mostly because of the difficulty in separating grazers and prey (similar sizes and/or pigments). To date, most approaches to estimate mixoplankton grazing are based on extrapolating to the field the rates obtained in the laboratory (e.g. Yih *et al.* 2004). For instance, to estimate grazing on protists it is common to measure the disappearance of prey during 24h incubations under strictly controlled conditions. Given the impossibility to obtain data for all the species present at sea on a given time, and to predict the particular interactions between groups, the approach is, if not completely erroneous, at least very limited and inaccurate. Moreover, the fact that some species, like *Prymnesium parvum* or *Karlodinium armiger*, release allelopathic compounds (e.g. Shilo & Aschner 1953, Rasmussen *et al.* 2017) that may cause immobilization and lysis of potential prey may introduce further complications to the interpretation of the results.

To overcome this problem, we investigated several existing methods and adapted them to measure herbivory in mixoplankton. For instance, in mixotrophic organisms, stable isotopes have been used to track contributions of symbiotic partners to the growth of their holobiont (reviewed in Ferrier-Pagès & Leal 2019). While the stable isotope approach has been predominantly used in plankton research to assess non-phagotrophic uptake, it has also been used to show feeding, examples include: feeding of invertebrate larvae on both bacteria and diatoms (Leroy *et al.* 2012); phytodetritus uptake by benthic foraminifera (Enge *et al.* 2016) and heterotrophy in corals (Seemann *et al.* 2013). A use of stable isotope probing to quantify contribution of photosynthesis and bacterivory to the metabolic needs of a mixotrophic protist has been reported by Terrado *et al.* (2017). The methodology used here uses a similar approach but attempts to assess phagotrophy on live algal cells.

We also evaluated the pros and cons of using the addition of rotenone (Ferreira & Calbet 2020), a mitochondrial inhibitor, as a means to partition the contribution of protozooplankton and mixoplankton within a standard dilution technique. This chemical has been previously used to get rid of contaminations by heterotrophs in large algal culture facilities. In theory, by inhibiting protozooplanktonic grazing, in a natural ecosystem the remaining one should be result of mixoplankton activity (provide these

organisms are not affected by rotenone). Here, we present our results on the effects of different concentrations of rotenone on survival, growth and grazing rates of several species of mixoplankton and protozooplankton.

Conversely, other traditional methods to estimate protozoan bacterivory or herbivory rely on the addition of fluorescently labelled tracers (usually bacteria, FLB, or algae, FLA) to the population. These fluorochromes enable the estimation of incorporation rates of the tracer and obtain an approximation to grazing (Sherr *et al.* 1987). Yet, this widely used technique to account for bacterivory has been seldom used to estimate feeding activity of mixoplankton (but see for example Unrein *et al.*, 2014 for mixotrophic bacterivory). Even less studies target algae instead of bacteria as prey, mostly because of a discrimination against inert particles (Verity 1991) by protistan grazers, which lead to the replacement of dead FLA (Rublee & Gallegos 1989) for live ones (e.g., Putt 1991, Li *et al.* 1996), with all the due advantages and disadvantages. As such, we used this approach to assess and compare mixoplankton and protozooplankton grazing impacts. Therefore, our experiments included several species of different taxonomic groups, nutritional strategies, and feeding mechanisms. This approach is not novel in itself, however, the implementation of this for understanding mixoplanktonic activity is novel. Therefore, our experiments included several species of different taxonomic groups, nutritional strategies, and feeding mechanisms (Ferreira *et al.* in prep.).

Finally, given the caveats associated to all the previously described methods, we proposed to decipher mixoplanktonic predation in the most widely used technique to estimate protistan grazing rates, the dilution technique (Landry & Hassett 1982). This technique is based on the diminution of encounter rates between grazers and prey, by sequentially diluting the natural planktonic community with particle-free water. The technique relies on two precepts: a constant proportionality between microplankton grazing rates and prey concentration, and a constant rate of instantaneous phytoplankton growth through the dilution series. In the original wording of this technique, the growth of the “phytoplankton” prey is assessed by using chlorophyll a (Chl a) as a proxy for its biomass, and the grazing is assumed to be exclusively due to “microzooplankton” (i.e., de facto protozooplankton). Thus, it becomes clear that the activity of mixoplankton is simultaneously “phytoplankton” and “microzooplankton”, effectively rendering the dilution grazing technique blind to this form of mixotrophy (Paterson *et al.* 2008, Calbet *et al.* 2012) and, therefore, they are usually omitted from the interpretation of the results.

Keeping this in mind, we designed a set of laboratory experiments to feed a mathematical model (to be built) in order to extract the contribution of mixoplankton to the total predation measured in these experiments (Ferreira *et al.* submitted).

3.2 Stable Isotopes for the assessment of prey uptake by eSNCM Acantharia

3.2.1 Methods

The stable isotope methodology was tested with the endosymbiotic Specialist NCM (eSNCM) Acantharia (Radiolaria) and prey *Isochrysis galbana*. Symbiotic Acantharia (clade F3b) were collected, as in Mansour *et al.* (2021), by gentle plankton net tows along the sub-surface in the bay of Villefranche (Mediterranean Sea, Villefranche-sur-Mer, 43°41′10″ N, 7°18′50″ E, France). Acantharia from the genus *Amphistaurus sp.*, *Acanthostaurus sp.* and *Acanthometra sp.* were left to self-clean in Filtered Sea Water (FSW, 0.2-µm-pore-size), and transferred twice to clean FSW. The specimens used for experiments all harboured photosynthetic symbionts which provide the hosts with photosynthates (Anderson 1978, Anderson *et al.* 1983, Decelle *et al.* 2012, Probert *et al.* 2014).

I. galbana (strain RCC 178) was grown in 50 mL K/2 medium without extra nitrogen addition, and subsequently incubated for a minimum of 24 h with 95 µM ^{13}C; 0.12 µM $^{15}NO_3$ and 0.02 µM $^{15}NH_4$. Prior to experimental incubations, *I. galbana* was filtered onto a 0.2 µm filter, rinsed with FSW to remove the remnants of enriched nutrients, and subsequently submerged and re-suspended in FSW. The vitality of the culture was ascertained by the observation of motile cells. A sample of the new (^{13}C/^{15}N labelled) suspension of *I. galbana* was fixed in a final concentration of 0.25 % glutaraldehyde (1 mL total volume) for subsequent flow cytometry cell counts (T0 sample). For the assessment of the enrichment of the prey, a remainder of 30 mL of the culture to be used with Acantharia incubations was filtered on pre-combusted GF/F for isotopic analysis.

Of the originally isolated and acclimated Acantharia cells, a total of 40-60 were transferred to a 6-well culture plate prefilled with 1 mL FSW. The *I. galbana* prey were then added to the well in a volume of 10 mL to start the incubation. After five hours of dark incubation, a sample of *I. galbana* was again fixed in a final concentration of 0.25 % glutaraldehyde (1 mL total volume, T5 sample). The Acantharia were pipetted through three instances of clean FSW to remove attached prey and dilute out any residual prey. Finally, the Acantharia were picked and put on pre-combusted GF/F, and a separate filter with FSW equal in volume to the picked Acantharia. This last sample was taken to assess the contribution of any remaining prey to the Acantharia sample. The experimental

incubations were done in triplicate, and in duplicate with unlabelled prey. All experimental incubations were in an incubator with a 14:10 Light/Dark (L/D) cycle at 24 °C with 100-120 µmol photons m^{-2} s^{-1}. This temperature was the same as in the field at the time of the experiment. All GF/F Filters were directly stored at -20 °C until isotopic analysis. The complete analysis is still pending at the time of writing.

3.2.2 Results

Uptake of prey was investigated by isotopic ratio change in the host after incubations with isotopically labelled prey. Acantharia can clearly uptake ^{15}N and ^{13}C (**Figure 3-1**), which is very likely due to uptake of the algal prey. However, from this analysis alone, prey adherence to the Acantharia cell cannot be excluded. Nevertheless, we expect that adhered prey are rapidly digested. Therefore, as differences between non-enriched and enriched treatments are quite large, it is unlikely that these results are a consequence of coincidental adherence and not ingestion. To confirm whether there is actual ingestion of prey, we will conduct an experiment with LFLA (*I. galbana*) and analyse it with confocal microscopy, to ensure that prey are inside the predator and not just attached. This experiment is yet to be completed at the time of writing.

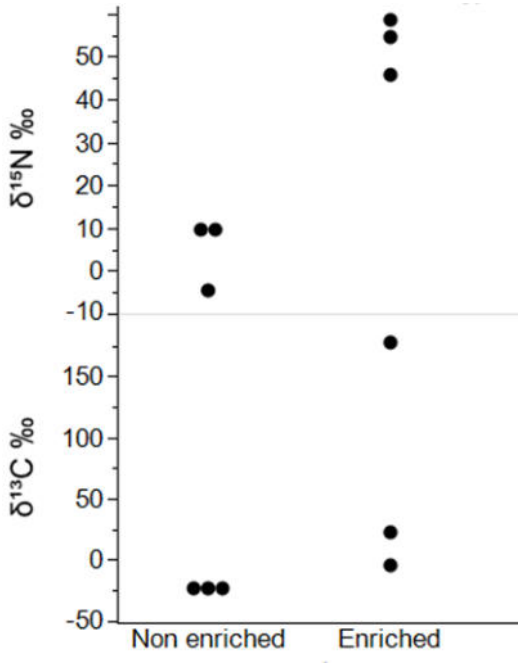

Figure 3-1 $\delta^{15}N$ (top) and $\delta^{13}C$ (bottom) in control samples containing unlabelled prey (non-enriched) and after dark incubation with labelled prey (enriched).

Calculating the algal prey C and N uptake in Acantharia from isotopic data gives the data as per **Table 3-1** for the three samples. By these measurements we calculated prey C

and N uptake as between 468 and 3510 pg C Cell^{-1} h^{-1}, and between 6 and 124 pg N Cell^{-1} h^{-1}. **Table 3-2** shows the carbon and nitrogen mass of samples used during these experiments. Acantharian C and N content averages 122.6 ± 65.6 ng C per cell and 26.5 ± 18.2 ng N per cell, this is coherent with results in Mansour *et al.* 2021.

Table 3-1 Algal carbon and nitrogen uptake of each Acantharia sample as measured by isotopic analysis. The average prey density during the 5 h incubation is also given (sample 1 was incubated 4 h, no prey density was measured at the end of incubation so no average density could be calculated, initial prey density was ca. 3.9x10^4 Cells mL^{-1}).

Sample#	Average prey density (Cells mL^{-1})	Algal N uptake (pg N Cell^{-1} h^{-1})	Algal C uptake (pg C Cell^{-1} h^{-1})
#1	NA	125	3510
#2	321x10^2	4.75	136
#4	337x10^2	6.31	468

Table 3-2 Carbon and nitrogen content per cell and C:N ratio of each sample (Radiolaria and *I. galbana* prey).

Identifier	ng N Cell^{-1}	ng C Cell^{-1}	C:N
0 radiolaria	66.88	255.69	4.67
1 radiolaria	16.17	99.42	5.26
2 radiolaria	18.61	69.43	5.30
3 radiolaria	17.13	135.37	6.58
4 radiolaria	22.48	54.99	6.08
5 radiolaria	17.57	120.71	5.58
1 prey	0.0064	0.081	12.63
3 prey	0.0082	0.055	6.69
4 prey	0.0054	0.058	10.89
5 prey	0.0022	0.027	12.06

3.2.3 Discussion

Work on the eSNCM Radiolaria presents challenges, such as small amount of available material, and only from the field. Typically, these organisms are sampled in “rough-and-ready” conditions. As such, improvements in sustaining the cells would greatly help physiological studies. Especially for bulk isotopic approaches where quantity requirements are too high to effectively do multiple experiments. Prey uptake experiments employing stable isotopes have thus been foregone for traditional prey disappearance techniques (Frost 1972, Heinbokel 1978).

3.3 Can rotenone be used to measure mixoplankton grazing?

3.3.1 Methods

i. Cultures

The experiments were conducted with two protozooplankton, the dinoflagellate *Gyrodinium dominans* (strain ICM-ZOO-GD001) and the ciliate *Strombidium arenicola* (strain ICM-ZOO-SA001); two mixoplankton, the CM dinoflagellate *Karlodinium armiger* (strain ICM-ZOO-KA001) and the pSNCM ciliate *Mesodinium rubrum* (strain DK-2009); two phytoplanktonic flagellates, *Rhodomonas salina* (strain K-0294) and *Tetraselmis chuii*, and one phytoplanktonic diatom, *Conticribra weissflogii* (previously known as *Thalassiosira weissflogii*). All cultures were kept in a controlled-temperature room at 19°C with a 10:14 L/D cycle. Additionally, all cultures were maintained at a salinity of 38.

R. salina, *T. amphioxeia*, and *T. chuii* were kept in f/2 medium (Guillard 1975) under exponential growth conditions. These organisms were irradiated at 100-200 µmol photons m^{-2} s^{-1} provided by cool white fluorescent lights. *C. weissflogii* was grown under the same conditions with the exception that silicate was added to the medium and bubbling was applied to maintain cells in suspension. *G. dominans*, *S. arenicola*, and *K. armiger* were kept at a PFD of 35-55 µmol photons m^{-2} s^{-1} in autoclaved 0.1 µm-filtered seawater that contained EDTA and trace metals in accordance with f/2 medium. *R. salina* was offered as prey to all three species *ad libitum*. *M. rubrum* was grown under the same light conditions though kept in autoclaved 0.1 µm filtered seawater without the addition of metals and supplied with *T. amphioxeia* as prey at a proportion of ca. 5 prey per predator (Smith & Hansen 2007).

ii. Rotenone effects on growth and grazing rates

Rotenone solutions were prepared according to the guidelines provided by El-Sayed (2018). Briefly, a stock solution of 1 g L^{-1} (2.535 mM) was prepared by dissolving 0.05 g of rotenone (≥ 95 %, Sigma-Aldrich) in 50 mL of dimethylsulfoxide (DMSO) (≥ 99.5 %, PanReac AppliChem) and stored at -20°C while not in use. Standard solutions of 50 mg L^{-1} were prepared on the days of the experiments by diluting the stock solution 20 times with deionized water.

The experiments were conducted in 132 mL Pyrex bottles and consisted of three concentrations of rotenone (0.5, 1.0 and 2.0 mg L^{-1}, i.e., 1.27 to 5.08 µM), and two

controls, one containing only water (0 mg L^{-1}) and another with DMSO (solvent control). DMSO controls comprised only the highest concentration of DMSO (ca. 0.2 %) used with the rotenone solutions of 2.0 mg L^{-1}. Additionally, experimental and control suspensions were prepared in f/40 to guarantee that the incubated prey was not nutrient limited during the experimental period.

Each treatment with rotenone was conducted in triplicate experimental (predator and prey) and control bottles (only prey) and mounted on a plankton wheel (0.2 rpm). The incubations begun shortly after the onset of the light period to maximise the odds of survival for chloroplast-bearing organisms by enabling a longer period for ATP synthesis using the light phase of photosynthesis (Dawson *et al.* 1991, El-Sayed *et al.* 2018). The bottles were incubated for ca. 24 h at 19°C with a 10:14 L/D cycle. At the same time, as the preparation of the triplicate experimental and control bottles, a fourth bottle of each treatment was also prepared for sacrifice as an initial bottle. All bottles were filled gradually, in three to four steps, before being capped. The suspension was gently stirred between fillings. The formation of air bubbles during the filling and capping processes was avoided because shear may damage the organisms (Broglio *et al.* 2004) and thus bias the measured rates. Rotenone was added to the bottles with an automatic pipette just before capping them to avoid exposing the organisms to very high concentrations of the compound, though only temporarily.

For mixoplankton and protozooplankton, prey was added at saturating concentrations (**Table 3-3**) to minimise the effect of different food concentrations on the measured ingestion rate. Predator concentrations were adjusted to allow for ca. 30 % prey depletion after the incubation time (Calbet *et al.* 2013). All organisms were counted, and their volumes were assessed using a Beckman Coulter Multisizer III particle counter, with the exception of *M. rubrum*, which can escape the current flow generated by the particle counter due to their shear sensitivity (Fenchel & Hansen 2006). Aliquots of the experiments with *M. rubrum* were therefore fixed in acidic Lugol's (final concentration 5 %) and enumerated manually using a Sedgwick-Rafter counting chamber. A minimum of 300 cells (of both predator and prey) were counted using a 10× objective on an inverted microscope.

Table 3-3 Summary of the prey and predator concentrations used for the acute toxicity assays with rotenone for the mixoplankton *M. rubrum* and *K. armiger*, and protozooplankton *S. arenicola* and *G. dominans*.

Species	Target concentration, Cells mL^{-1}		Reference
	Prey	Predator	
Gyrodinium dominans	100 000	1 500	Calbet *et al.* 2013
Mesodinium rubrum	15 000	1 500	Smith & Hansen 2007
Karlodinium armiger	100 000	3 750	Arias, unpublished
Strombidium arenicola	100 000	400	Arias, unpublished

Grazing rates and average prey concentrations were calculated using Frost (1972) equations; the average concentration of grazers in each replicate was used to assess the grazing per predator (Heinbokel 1978). The magnitude of the effects of the different concentrations of rotenone on the grazing impact reduction (GIR, %) on the prey populations was assessed separately for each grazer species by **Equation 3.1**

$$GIR_{species\ A} = 100 - \left(\frac{< C >_i \times I_i}{< C >_0 \times I_0} \times 100\right) \qquad \mathbf{3.1}$$

where <*C*> and *I* are the mean predator concentration (Cells mL^{-1}) during the incubation period and the average ingestion rate (Cells Ind^{-1} d^{-1}) measured for each treatment *i* (<*C*>$_0$ and I_0 refer to the control treatment of 0 mg L^{-1}). The control suspensions with only DMSO added (no rotenone) were considered a treatment. Non-significant ingestion rates (see section *iv.* Statistical analysis) were considered 0 for the calculation of the GIR.

iii. Physiological condition effects on the response to rotenone

Additionally, whether the physiological condition of an organism affected its response to rotenone was assessed. The experiments were conducted with *R. salina,* which was exposed to rotenone both during the exponential and stationary growth phases, with all target concentrations tested at once. The experimental protocol was the same as described above.

iv. Statistical analysis

Species-specific effects of rotenone on growth were analysed using One-Way ANOVAs followed by Tukey's HSD post hoc tests (n = 15 for each treatment; Zar 2010). In the case

of *S. arenicola*, in which the assumptions of homoscedasticity were not met, the non-parametric Kruskal-Wallis test was applied, followed by the Games-Howell post hoc test (n =15; Zar 2010).

Ingestion rates were deemed significant only when the prey growth rates in the control and experimental bottles were significantly different (two-tailed Student's t-test, n = 6 for each treatment) (Saiz *et al.* 2014). Subsequently, the results for this parameter were analysed with the same procedure as described for growth rates, with the normality and homoscedasticity assumptions met and Tukey's HSD post hoc tests applied (Zar 2010). Finally, the effects of rotenone on the exponential and stationary *R. salina* were analysed separately for each growth phase using One-Way ANOVAs followed by Tukey's HSD post hoc tests. The interaction between growth phases and treatment was assessed using a two-way ANOVA followed by Bonferroni post hoc tests due to the unequal sample size between factors (Zar 2010). Calculations were conducted using IBM SPSS Statistics 25, and all the results were considered significant at $p < 0.05$.

3.3.2 Results

i. Rotenone effects on growth rates

The increase in rotenone concentration progressively reduced the growth rates of the two autotrophic flagellates tested (**Figure 3-2a,b**). The response was more drastic in *T. chuii*, which even displayed mortality at the lowest concentration (**Figure 3-2b**). Conversely, at the same concentration, *R. salina* was not significantly affected (**Figure 3-2**a; Tukey HSD, $p = 0.261$). On the other hand, the diatom *C. weissflogii* was unaffected by all concentrations of rotenone (One-Way ANOVA, $p = 0.792$; **Figure 3-2c**). DMSO at ca. 0.2 % had no visible effect in any of the target autotrophic species when compared to the treatment with 0 mg L^{-1} (Tukey HSD tests, $p > 0.05$ in all cases). The mixoplankton *M. rubrum* and *K. armiger* were not significantly affected by the presence of DMSO or by the lowest concentration of rotenone, although a negative tendency was observed for *K. armiger* in this last instance (**Figure 3-3**; Tukey HSD, $p = 0.098$). However, higher concentrations of the chemical compound severely reduced the growth rates of both protists, even resulting in mortality (**Figure 3-3**).

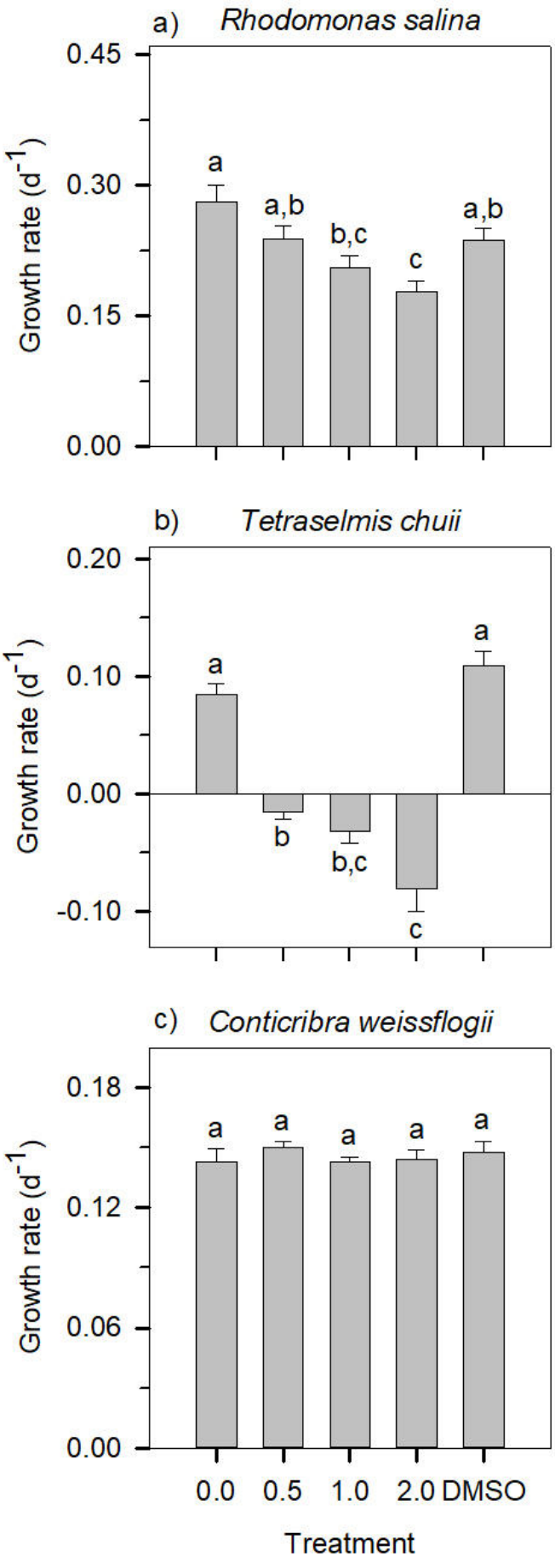

Figure 3-2 Growth rate (d^{-1}) of the phytoplankton species (a) *R. salina*, (b) *T. chuii*, and (c) *C. weissflogii* upon exposure to increasing concentrations of rotenone. The data plotted for *R. salina* include all the results for the experiments with the different grazers. Different letters within the same organism indicate statistically significant differences (Tukey HSD, $p < 0.05$). Error bars ± se.

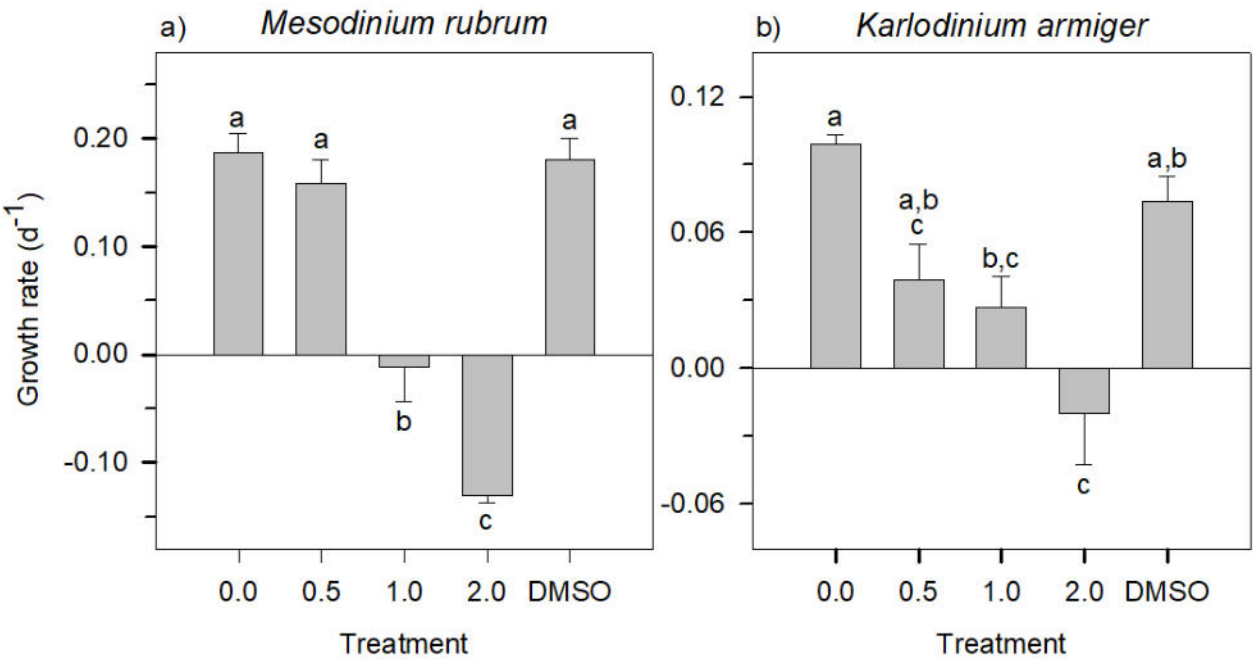

Figure 3-3 Growth rate (d^{-1}) of the mixoplankton species (a) *M. rubrum* and (b) *K. armiger* upon exposure to increasing concentrations of rotenone. Different letters within the same organism indicate statistically significant differences (Tukey HSD, $p < 0.05$). Error bars ± se.

The growth of the heterotrophic species was, on average, the most affected by rotenone (**Figure 3-4**). For the protozooplanktonic dinoflagellate *G. dominans*, the maximum growth inhibition was achieved immediately at the lowest concentration tested (0.5 mg L^{-1}); at this concentration, however, the ciliate *S. arenicola* still exhibited positive growth, although it was almost 80% lower than that under the control condition of 0 mg L^{-1}. This ciliate species appeared to be particularly sensitive, being the only species significantly affected by the sole presence of DMSO in the water (Games-Howell, $p = 0.017$). Further supporting a high sensitivity of *S. arenicola*, the ciliates that remained alive after the 24 h were nearly immotile, suggesting severe deleterious effects. Independent of the trophic mode, all motile species exhibited a reduction in the speed of displacement in the presence of the highest concentrations of rotenone, although the magnitude of the reduction was not quantified.

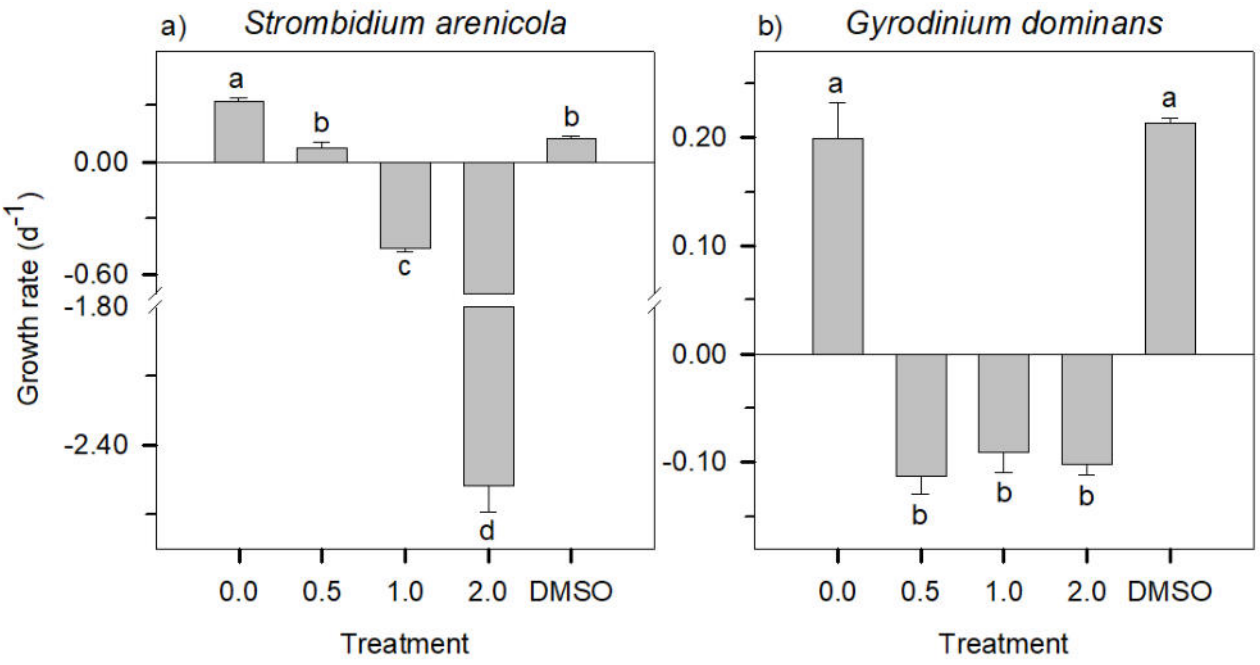

Figure 3-4 Growth rate (d^{-1}) of the protozooplankton species (a) *S. arenicola* and (b) *G. dominans* upon exposure to increasing concentrations of rotenone. Different letters within the same organism indicate statistically significant differences (Tukey HSD, $p < 0.05$). Error bars ± se.

ii. Rotenone effects on ingestion rates

The presence of rotenone impaired feeding in both ciliates, *M. rubrum* (**Figure 3-5a**) and *S. arenicola* (**Figure 3-5c**), regardless of the trophic mode of nutrition. The responses varied between no significant grazing (two-tailed Student's t-tests, $p > 0.05$ in all cases) and significantly negative ingestion rates (Tukey HSD tests, $p < 0.05$ in all cases). A significantly negative ingestion rate (1.0 and 2.0 mg L^{-1} for *M. rubrum*, and 0.5 and 1.0 mg L^{-1} for *S. arenicola*) implies a positive growth of the prey in the experimental with respect to the control bottles, and likely results from an increase in the nutrient pool originating from the dead grazers. The presence of DMSO also deterred the feeding of these two species, with *M. rubrum* being the most affected (**Figure 3-5a,c**). On the other hand, neither the mixoplanktonic *K. armiger* (**Figure 3-5b**) nor the protozooplanktonic *G. dominans* (**Figure 3-5d**) were significantly affected by the DMSO treatment (Tukey HSD tests, $p > 0.05$ in all cases). Rotenone, however, did affect the feeding rates of these dinoflagellates. *K. armiger* displayed no evidence of feeding whenever rotenone was present, and *G. dominans* showed null ingestion rates at 2.0 mg L^{-1} of rotenone (two-tailed Student's t-tests, $p > 0.05$ in all cases).

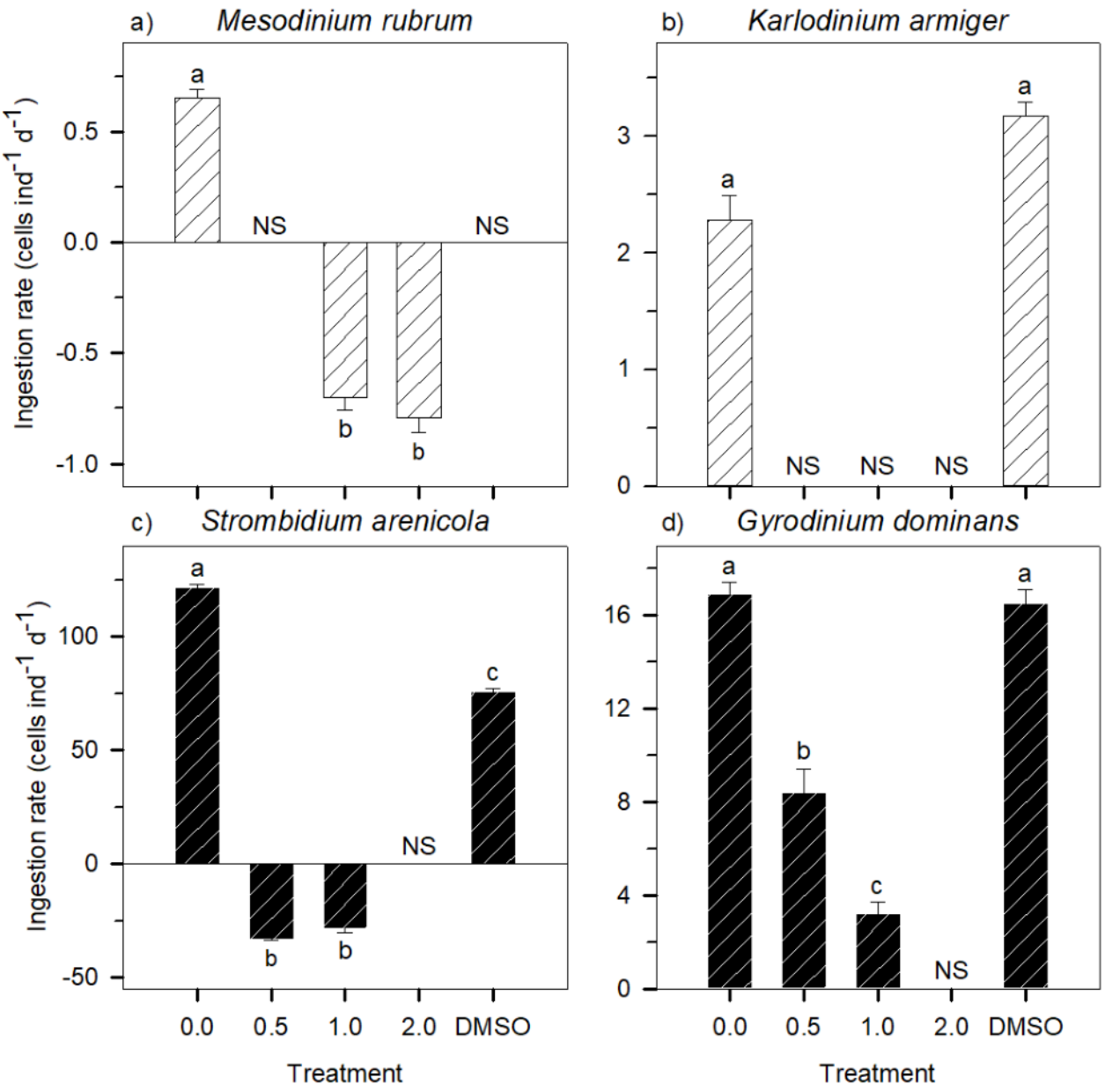

Figure 3-5 Ingestion rate (Cells Ind^{-1} d^{-1}) of the mixoplankton (◸) (a) *M. rubrum* and (b) *K. armiger*, and protozooplankton (◪) (c) *S. arenicola* and (d) *G. dominans* upon exposure to increasing concentrations of rotenone. All organisms were fed *R. salina* during the exposure period. Different letters within the same organism indicate significant differences (Tukey HSD, $p < 0.05$). NS represents the non-significant ingestion rates (two-tailed Student's t-test, $p > 0.05$). Error bars ± se.

iii. Rotenone effects on the overall grazing impact

The reduction in the impact of the chosen predators on the standing stock of *R. salina*, defined using **Equation 3.1** as the combined effect of feeding rates and grazer abundances during the incubations, is summarised in **Table 3-4**. Regardless of the

trophic mode of nutrition, the grazing pressure by dinoflagellates was overall less inhibited by rotenone than that exhibited by ciliates.

Table 3-4 Combined effects of rotenone on grazer survival and on their feeding rates on *R. salina* (GIR, %) throughout the incubation. The values were calculated using **Equation 3.1**. Non-significant ingestion rates were considered 0, and GIR was thus capped at 100% in these situations, with the values highlighted with an *. No effect of the treatment on the overall grazing impact is indicated by a zero in the table.

Species	Grazing Impact Reduction (GIR), %				
	0.0 mg L^{-1}	0.5 mg L^{-1}	1.0 mg L^{-1}	2.0 mg L^{-1}	DMSO
Gyrodinium dominans	0.00	56.83	83.62	100.00*	0.15
Strombidium arenicola	0.00	123.07	114.80	100.00*	45.59
Karlodinium armiger	0.00	100.00*	100.00*	100.00*	0.00
Mesodinium rubrum	0.00	100.00*	197.07	202.77	100.00

iv. Effects of physiological conditions on the resistance to rotenone

Rotenone affected *R. salina* in different ways depending on its physiological condition (**Figure 3-6**). During the deceleration phase, the flagellate was roughly unaffected by the presence of rotenone, independent of the concentration (One-Way ANOVA, $p = 0.071$). On the other hand, during exponential growth, progressively higher concentrations of rotenone diminished the growth of this cryptophyte up to a maximum of ca. 40% lower than that under the control treatment with 0 mg L^{-1} (Tukey HSD test, $p = 0.000$).

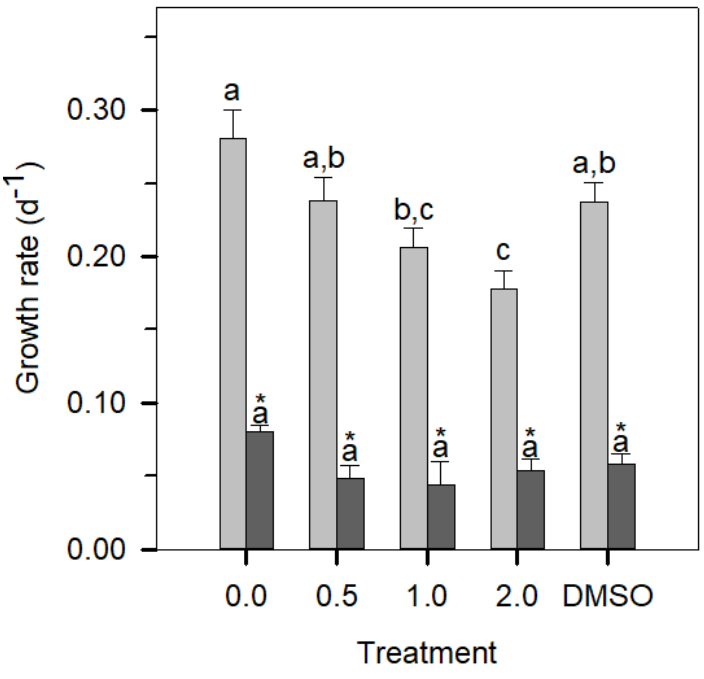

Figure 3-6 Growth rate (d^{-1}) of the phytoplanktonic *R. salina* upon exposure to increasing concentrations of rotenone on the exponential (▒) and deceleration (■) growth phases. The data regarding the exponential phase are the same as those displayed in **Figure 3-2a**. Different letters within the same growth phase indicate significant differences (Tukey HSD, $p < 0.05$). *Indicates statistically significant differences between exponential and deceleration phases for each individual treatment (Bonferroni, $p < 0.05$). Error bars ± se.

3.3.3 Discussion

As expected, phytoplankton species were more resistant to rotenone, with the exception of *T. chuii*. Other species of the genus *Tetraselmis* are also reported to be more vulnerable to rotenone than other algae species, such as the marine *Nannochloropsis oculata* (Van Ginkel *et al.* 2016) and the freshwater *Chlorella kessleri* (Van Ginkel *et al.* 2015). This observation suggests that there may be a factor that is common to the genus *Tetraselmis* that enhances cellular susceptibility to this compound, although it is currently unknown. Conversely, the diatom and *R. salina* were quite resistant to rotenone effects. In the particular case of the cryptophyte, the effects of this compound were only evident during the exponential growth phase.

Cells undergo drastic metabolic changes when switching from exponential to stationary phases. For example, photosynthesis and respiration rates are, on average, higher during the exponential growth phase for phyto- and mixoplanktonic species (López-Sandoval *et al.* 2014). Similarly, protozooplanktonic ciliates and flagellates displayed higher respiration rates when actively growing than when stationary (Fenchel & Finlay 1983). Therefore, it seems that the increased respiratory chain activity during the exponential phase enhances an organism's susceptibility to rotenone. This conclusion aligns with the mechanism of action of rotenone, which, among other effects, is known to inhibit the synthesis of ATP (Palmer *et al.* 1968). A consequence of the reduced pool of available ATP can be seen in the assembly of microtubules, which becomes impaired and ultimately results in mitotic arrest and inhibition of cell proliferation (Srivastava & Panda 2007). Without these processes, the cell cannot divide, which would have a much higher impact on actively growing cells than on those progressing towards stationary phase.

The observed differences between growth phases under exposure to rotenone may have important consequences for the interpretation of laboratory and field experiments on single-celled organisms, not only with rotenone but with other toxic compounds as well. Despite having data exclusively from *R. salina* (which forces caution in the extrapolation of conclusions to other species), the results indicate that the effect of pollutants should always be tested using the same physiological conditions to minimise intra-specific differences. In the laboratory, this can be easily accomplished by controlling sampling times and/or by using a single batch of cultured organisms (as used in the experiments with mixo- and protozooplankton). On the other hand, for field work this may represent a

challenge. Nonetheless, organisms in the field are likely living on an almost constant exponential growth phase (e.g. Dortch *et al*. 1983), diminishing the risk of comparisons.

A major result of the study is the high sensitivity of the ciliate *S. arenicola* to rotenone, in particular compared to the other protozooplanktonic predator tested, the dinoflagellate *G. dominans*. In fact, *G. dominans*, displayed a peculiar response to rotenone, showing negative growth rates and high ingestion rates at the lowest concentration, and similar growth rates and non-significant ingestion rates (two-tailed Student's t-test, $p = 0.114$) at the highest. These results suggest that this dinoflagellate may be able to tolerate the presence of rotenone up to a concentration of 1.0 mg L^{-1} by maintaining key cellular processes (like phagocytosis) active while avoiding expensive ones such as cellular division. This could be a mechanism of survival that enables the endurance of harsh conditions for short time periods. Nonetheless, more data is needed to validate this hypothesis. On the other hand, planktonic ciliates are known to be highly susceptible to several chemical compounds, such as hydrocarbons and chemical dispersants (Almeda *et al.* 2014, Schmoker *et al.* 2016), but also to DMSO, although the toxicity of the latter is usually evidenced at higher concentrations than the ones used in this study (Fok & Valin 1983 Rajini *et al.* 1989). Indeed, the ciliate *S. arenicola* was the only species whose growth was reduced by ca. 60% solely by the presence of DMSO.

Analogous to the results observed for protozooplankton, with the ciliate being more sensitive than the dinoflagellate, the pSNCM *M. rubrum* was more sensitive than *K. armiger*. In fact, the ingestion rate of *M. rubrum* was already negligible even with DMSO as the only added compound (**Figure 3-5a**). Indeed, DMSO hindered the ingestion of prey for both ciliates (**Figure 3-5a,c**). In this regard, a precursor of DMSO, β-dimethylsulfoniopropionate (DMSP), reduced the feeding of protozooplanktonic marine ciliates by 50-75%, whereas for dinoflagellates of the same trophic mode, this reduction was 28-40% (Fredrickson & Strom 2009).

Overall, chloroplast-bearing predators displayed better resilience than protozooplankton at a concentration of 0.5 mg of rotenone L^{-1}. However, their feeding rates were more affected, rendering the overall mixoplankton grazing impact on prey populations considerably lower than that of protozooplanktonic species. In particular, *K. armiger* did not exhibit any evidence of feeding in the presence of rotenone (irrespective of concentration), while displaying only a slight negative growth rate in the highest concentration. These results are in agreement with those of previous studies on this species, in which it has been observed that *K. armiger* can survive long starvation periods

using only chloroplasts for C acquisition, although barely dividing in the absence of food (Berge & Hansen 2016), comparable to the non-significant ingestion rates observed in this study in the presence of rotenone.

One of the main motivations of this study was to test the effectiveness of the use of rotenone combined with the dilution technique to determine mixotrophic grazing. For the method to be useful, protozooplanktonic grazing should be impaired while leaving mixoplankton grazing unaffected. Despite the promising results for the effects of rotenone in terms of growth rates (with perhaps the caveat of the high sensitivity of *T. chuii*), the analysis of the effects of this compound on grazing highlighted severe limitations that were not predicted by the theoretical mechanism of action for rotenone (see **Table 3-4**). For instance, one of the assumptions of the study was that chloroplast-bearing organisms would be less affected by rotenone despite likely displaying a reduced ATP pool. A question that remains unanswered by this assumption is how large is the dependence of mixoplankton grazing processes on the ATP produced by the oxidative phosphorylation. In other words, can the photo phosphorylation supply enough ATP to maintain basal functions while enabling phagocytosis? The answer, at least from the present experiments, seems to be no, as explained next.

At 30 µmol photons m^{-2} s^{-1} (the experimental conditions used in the present study), a well fed *K. armiger* fixes C at a higher rate than the maximum observed for unfed cells, and the chlorophyll *a* content is close to the maximum registered (Berge & Hansen 2016). These observations suggest that this dinoflagellate maximises the use of the chloroplasts in situations akin to those used on the present study. Therefore, it seems plausible to assume that these conditions are less prone to magnify potential negative effects of rotenone on the behaviour of the CM.

For *M. rubrum*, it is known that the photosynthetic capacities depend on the quality of the chloroplasts acquired through the ingestion of cryptophytes from the genera *Teleaulax*, *Plagioselmis* or *Geminigera* (Hansen *et al.* 2012), and peak around 30 µmol photons m^{-2} s^{-1} (Moeller *et al.* 2011). Ultimately, the sequestered chloroplasts require the presence of active cryptophyte nuclei, and start to lose photosynthetic efficiency after 3 days without the adequate food source (Kim *et al.* 2017). Accordingly, during the exposure to rotenone, the chloroplasts of the NCM were also likely close to their full potential despite being fed with *R. salina*.

Contrary to all other tested predators, *G. dominans* was still able to ingest *R. salina* under concentrations of rotenone up to 1.0 mg L^{-1} (although with ca. 9% mortality and a grazing

impact reduction, GIR, of ca. 83%). Indeed, it is important to note that with a concentration of 0.5 mg L^{-1}, the absolute number of *R. salina* cells ingested per *G. dominans* was approximately 3.8× higher than that of *K. armiger* and 13.2× higher than that of *M. rubrum* in their respective control situations.

Thus, despite the fact that the chloroplasts of both mixoplankton tested here should have been in good conditions and that the available ATP pool for *G. dominans* was (likely) severely reduced during the exposure to rotenone, field grazing estimates using rotenone would be, at best, conservative. Indeed, the analysis of the GIR suggests that in a hypothetical dilution setting with rotenone (0.5 mg L^{-1}) and all 3 predators, *G. dominans* would still be the major grazer. Hence, mixoplanktonic grazing is clearly affected by the reduced ATP concentration, although future physiological studies are required to elucidate the actual contribution of the oxidative phosphorylation for mixoplankton and its role on phagotrophy.

An example that further corroborates that protozooplanktonic dinoflagellates can display a substantial grazing impact on natural populations (thus further complicating the use of a protozooplanktonic grazing deterrent such as rotenone) is the fact that some areas of the Mediterranean Sea possess a biomass of protozooplanktonic dinoflagellates approximately 4× higher than that of phytoplanktonic (with high potential for phagotrophy, Jeong *et al.* 2010) and mixoplanktonic species combined when not blooming (Ignatiades 2012). Additionally, the average C-specific ingestion rate of protozooplanktonic dinoflagellates is ca. 4.5× higher than that of their mixoplankton counterparts (see Figure 10 in Calbet *et al*. 2011a and references therein), meaning that one can assume that protozooplankton would impact prey nearly 20× more than mixoplankton. Assuming that *G. dominans* is a good representative of protozooplanktonic dinoflagellates (Kim & Jeong 2004), the presence of 0.5 mg L^{-1} would still render their impact (see **Table 3-4**) on prey populations ca. 11× higher than that of mixoplankton, which would be virtually zero (as per *K. armiger* results). Thus, rotenone cannot be used as an addition to the standard dilution technique for the purpose of deterring heterotrophic predation.

On an ecological note, rotenone has been used for decades to kill undesirable fish species *in situ*, and typical concentrations varied between 0.5 and 5.0 mg L^{-1}, depending on the sensitivity of the target species (Hinson 2000) . However, as evidenced by the results of this study, considerably lower concentrations cause nefarious or even lethal effects on several planktonic species of distinct taxonomic groups. Additionally, the half-life of rotenone in aquatic environments ranges from hours to weeks (Dawson *et al.* 1991)

and depends on several factors, namely temperature and pH, increases in which quicken degradation (El-Sayed *et al.* 2018). Hence, this information, together with the data gathered in this study for protists, and previous studies on zooplankton (Naess 1991, Beal & Anderson 1993) and rotifers (Van Ginkel *et al.* 2015, 2016) suggest that the indiscriminate use of this compound in the past may have had disastrous consequences for aquatic food webs, whose extent is largely unknown.

3.4 Pros and cons of using Live FLA to account for protist grazing

3.4.1 Methods

We wanted to assess the occasions on which Live Fluorescently Labelled Algae (LFLA) could be successfully used to measure protist grazing in situ and, therefore, prepared three independent sets of laboratory-controlled experiments to answer distinct questions.

i. Preparation of LFLA

I. galbana, *T. chuii*, *Heterocapsa sp.* and *M. rubrum* were fluorescently labelled following the guidelines by Martínez *et al.* (2014) with slight modifications. Briefly, cells were stained overnight with the fluorochrome CellTracker™ Blue CMAC (7-amino-4-chloromethylcoumarin), a vital cytoplasmic stain, at a final concentration of 10 µM. After the staining period, excess stain was removed from the medium by centrifugation (1000 g) for 10 min for all species except for *M. rubrum*. The supernatant was discarded and the cells were re-suspended in filtered sea water (0.1 µm). This clean-up process was repeated two times, to reduce the carryover of stain which can enter predator cells and mask the actual ingestion of LFLA. *M. rubrum* was hand-picked and moved through five wells of FSW for the same reason.

ii. Experiment 1 – The effects of prey concentration and incubation time

For the first experiment, we decided to use common protozooplankton and mixoplankton species (encompassing several taxonomic groups and feeding strategies to increase variability and thus, provide a better overview of natural populations) to determine optimal incubation times to measure grazing on tracer concentrations of LFLA. Additionally, we assessed how different prey concentrations affected the estimation of grazing using this technique.

For this experiment, we selected three protozooplankton species which conduct phagocytosis through direct engulfment: the dinoflagellates *Oxyrrhis marina* (strain ICM-ZOO-OM001) and *G. dominans* (strain ICM-ZOO-GD001), and the ciliate *S. arenicola* (strain ICM-ZOO-SA001). Additionally, we selected three mixoplankton species, the CM dinoflagellates *K. armiger* (peduncle feeder, strain ICM-ZOO-KA001) and *K. veneficum* (engulfment feeder, strain ICMB-274), and the pSNCM ciliate *M. rubrum* (strain DK-2009).

R. salina (cryptophyte, strain K-0294) was offered as prey to *G. dominans*, *O. marina*, *S. arenicola* and *K. armiger*. *K. veneficum* and *M. rubrum* were offered *Teleaulax amphioxeia* (cryptophyte, strain K-1837) prey instead. During the experiments, all predators were offered a mixture of the LFLA *I. galbana* (ca. 30 % of the total prey concentration) and the cryptophyte prey. The final predator concentrations were adjusted to avoid the depletion of prey at the target concentration. Additionally, we prepared a second incubation for *M. rubrum* where we gave it only the LFLA (i.e., 100 % of the offered prey were labelled *I. galbana*). All three prey species were kept in f/2 medium (Guillard 1975) at 100-200 µmol photons m^{-2} s^{-1} (provided by cool white fluorescent lights) under exponential growth conditions. All predators were kept in autoclaved 0.1 µm-filtered seawater at 35-55 µmol photons m^{-2} s^{-1}. All cultures were kept in a temperature-controlled room at 19°C with a 10:14 L/D cycle, at a salinity of 38.

Experiments were done in Pyrex bottles and consisted of two concentrations of prey, one saturating ([Prey] = High) and one non-saturating ([Prey] = Low). The experimental bottles were prepared in duplicates, which were filled in two to three steps using a common master suspension containing both predator and prey at the target concentrations. The master suspension was gently stirred between fillings and the formation of air bubbles was avoided (Broglio *et al.* 2004). The bottles were incubated on a plankton wheel (0.2 rpm) at 19°C at 35-55 µmol photons m^{-2} s^{-1}. Each experimental bottle was sampled (9-45 mL depending on final predator concentration) every 20 min during the first 2 h of incubation, and then after 3 and 5 h. The samples were fixed with cold Glutaraldehyde (4°C, final concentration of 1%) for ca. 2 h and then filtered with a vacuum pump onto 2 µm pore-size black polycarbonate filters, which were mounted on microscope slides. The first 100 predators encountered on each slide were examined for the presence or absence of ingested prey, determined as blue-fluorescent inclusions (BFI).

In the incubations where the mixture of prey was within different size ranges (i.e., when the cryptophyte prey was *R. salina*), an initial and a final sample were also collected from each experimental bottle and quantified using a Beckman Coulter Multisizer III particle counter, to calculate clearance rates using Frost (1972) equations as modified by Heinbokel (1978) to account for the growth of protist predators. From the calculated clearance rates we estimated the selection coefficient (Wi) and the electivity index (Ei*) according to Vanderploeg & Scavia (1979). The former is calculated according to the

Equation 3.2

$$Wi = \frac{Fi}{\sum Fi} \qquad \mathbf{3.2}$$

where *Fi* is the clearance rate for a given food type *i* and *ΣF*i is the sum of clearance rates on all food types. The latter is calculated using the **Equation 3.3**

$$Ei^* = \frac{\left[Wi - \frac{1}{n}\right]}{\left[Wi + \frac{1}{n}\right]} \qquad \mathbf{3.3}$$

where *n* is the total number of food types. This index can vary between -1 and 1. Negative values imply active avoidance of prey whereas the opposite implies selection for a given species.

iii. Experiment 2 – The diel effects on prey incorporation and digestion

For the second experiment we decided to focus only on one grazer, the NCM *Strombidium basimorphum*, due to the laborious and time-consuming analysis of the samples. This species was maintained on *T. amphioxeia* before the experiment (Maselli *et al.* 2020) but was allowed to graze down its prey to negligible levels before the onset of the incubations. We conducted two independent incubations with the LFLA *T. chuii* in a proportion of ca. 5 prey per predator under non-saturating concentrations, one incubation during the day and one during the night. *S. basimorphum* were kept in a temperature-controlled room at 15 °C with a 14:10 L/D cycle, at a salinity of 15 in FSW. Both *T. amphioxeia* and *T. chuii* were kept in the same conditions although f/2 media (Guillard 1975) was used instead of FSW. The experiments were always conducted in FSW.

We prepared triplicate experimental bottles containing the mixture of the NCM and the LFLA, which were filled as described above. We coordinated the experiment so that the initial samples for both the day and night periods would be as close as possible to the lights-on/lights-off event in the temperature-controlled chamber. Both incubations lasted 5 hours and were sampled hourly with the exception of the second sampling point, which occurred within 30 minutes of incubation. The fixation and processing of the samples was as mentioned above. The ingested volume of LFLA (μm^3 BFI^{-1}) was obtained from linear dimensions measured on photographs using the Fiji software (Schindelin *et al.* 2012). Carbon (C) contents for *T. chuii* were estimated using the chlorophyte equation of Menden-Deuer & Lessard (2000).

iv. Experiment 3 – The effects of peduncle feeding

The results obtained with *K. armiger* in the first experiment suggested that there could be issues concerning the feeding mechanism of the predator, as suggested before by Archer et al. (1996). Accordingly, we prepared an experiment to ascertain whether or not the feeding mechanism could be an issue when using LFLAs as tracer particles, by selecting known peduncle feeders and offering them labelled prey. This experiment was designed to be qualitative instead of quantitative as the previous two experiments and, thus, prey was always offered in a proportion of ca. 1 prey per predator.

The species that were chosen for this experiment were the protozoan *Lessardia elongata* (strain ICM-ZOO-LSP001), and three mixoplanktonic species, *K. armiger* (CM, strain ICM-ZOO-KA001), *Dinophysis acuminata* (pSNCM, strain FR101009), and the previously thought-to-be phytoplankton *Gymnodinium litoralis* (CM, strain CGA). *L. elongata* and *K. armiger* were maintained with *R. salina* as prey in conditions akin to those described for the first experiment and fed the LFLA *I. galbana* during the experiment. *D. acuminata* was maintained as described by Nielsen *et al.* (2012) and Rusterholz *et al.* (2017). The co-existent unlabelled prey were removed before the incubation with labelled *M. rubrum* using a similar approach as used for the cleaning of extra stain in *M. rubrum*, as described above. Lastly, *G. litoralis* was maintained under exclusive autotrophic conditions before the experiment (see prey maintenance conditions in the experiment 1), and was given the LFLA *Heterocapsa sp.* Yet, phagotrophy in this species was confirmed first on *T. amphioxeia.*

v. Calculations of clearance and grazing rates using LFLA

The average number of blue fluorescent inclusions (BFI) per protist was determined using UV light by epifluorescence microscopy on samples collected at several time points. Plotting this information versus time (h) typically yields a linear relationship for the initial time points, levelling off as the experiment progresses due to digestion of ingested algae (Rublee & Gallegos 1989, Caron 2001). The slope of the linear portion of this regression yields the ingestion of LFLA per predator per hour. This value can be converted to total algae ingested per predator per hour by multiplying it by the unlabelled algae:LFLA ratio assuming that there is no discrimination for or against fluorescently labelled tracers (e.g., Kamiyama 2000, Johnson *et al.* 2003, Martínez *et al.* 2014). Clearance rates (both LFLA-specific and total) are determined by dividing the ingestion rates by the concentration of

LFLA or by the total concentration of prey (Frost 1972, Heinbokel 1978). In the field, estimates of community-level herbivory are obtained by multiplying group-specific clearance rates by the abundance of each group, determining the latter with standard microscopic methods (Caron 2001).

3.4.2 Results

i. Advantages of LFLA

a. Incorporation of LFLA over time

All dinoflagellates exhibited a significantly ($p < 0.01$ for all species) higher Imax (maximum number of Cells per predator) at saturating food conditions than when the concentration of prey was low (**Figure 3-7**). *G. dominans* was the predator with the largest difference between saturating and non-saturating conditions, being followed by *O. marina* (ca. 6.05 and 2.05x more LFLA ingestion at the highest prey concentration, respectively; **Figure 3-7a,b**). Conversely, mixoplanktonic species displayed lower Imax differences between concentrations, being *K. armiger* the species exhibiting the lowest fold-increase (ca. 1.57x). *K. veneficum* increased its Imax by ca. 1.76x.

The differences obtained in the half-saturation constants between saturating and non-saturating conditions were never statistically significant. However, for mixoplankton, the trend was to require more time to achieve food-satiation as the concentration of prey increased (i.e., [High] Km > [Low] Km), whereas the opposite trend was noted in protozooplankton. Overall, all species reached the plateau phase of the tracer incorporation curve within 45 min irrespective of the prey concentration.

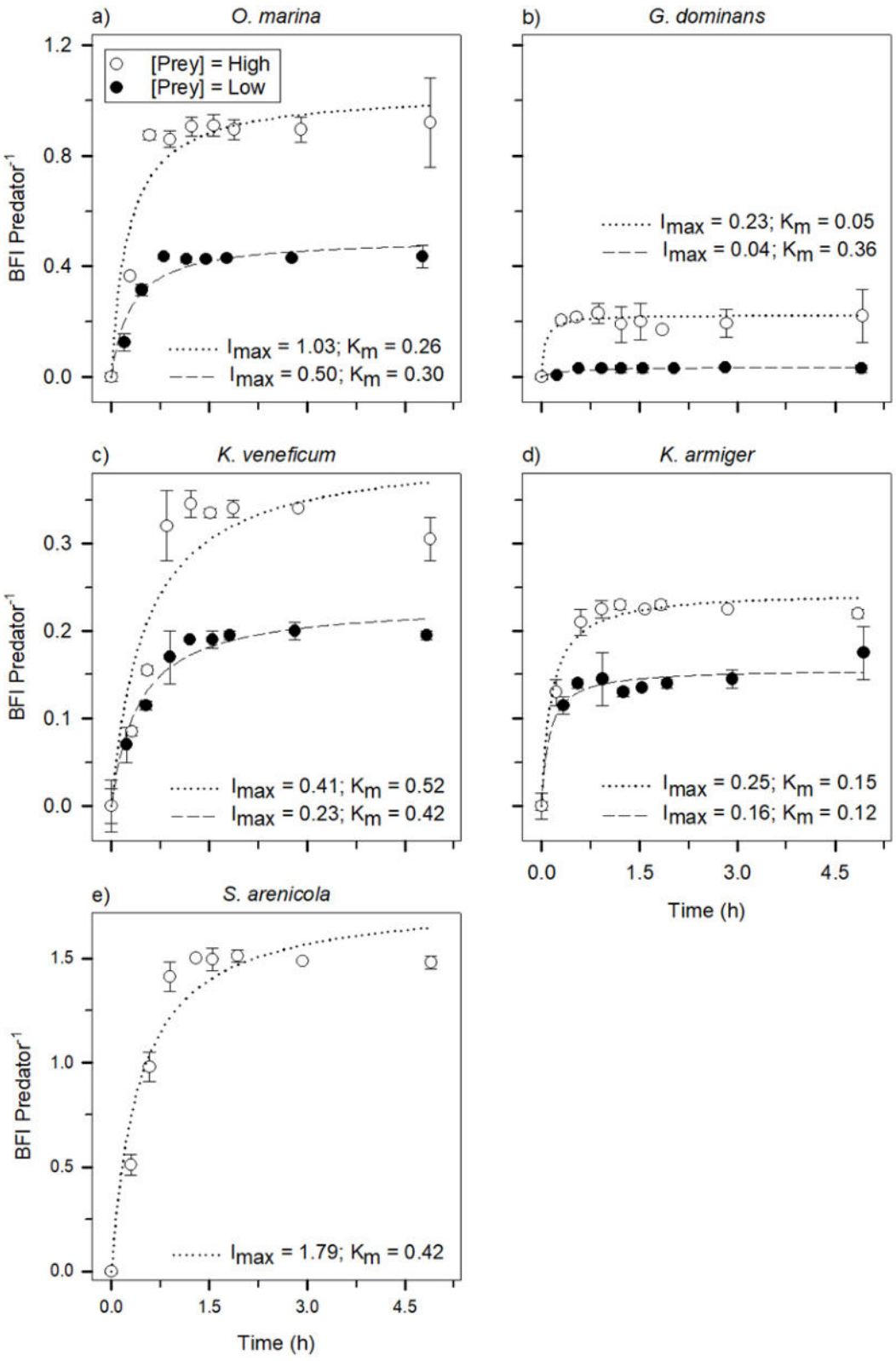

Figure 3-7 Incorporation of prey (BFI predator^{-1}) over time: a, b and e) protozooplankton - *O. marina*, *G. dominans* and *S. arenicola* respectively; c and d) mixoplankton - *K. veneficum* and *K. armiger* respectively. All predators were incubated using two concentrations of prey, one saturating (open circles) and one non-saturating (black circles) with the exception of the ciliate *S. arenicola* which was only followed under saturating conditions. Imax (maximum ingestion rate) and Km (half-saturation constant) were calculated by applying Michaelis-Menten kinetics to the data.

b. Diel ingestion and digestion rates

A very similar procedure was applied to follow the diel incorporation of the LFLA *T. chuii* by the NCM *S. basimorphum* (**Figure 3-8**). The Imax obtained during the day incubation was ca. 1.93x higher than the one obtained during the night ($p < 0.01$) but the half-saturation constants were similar ($p = 0.97$).

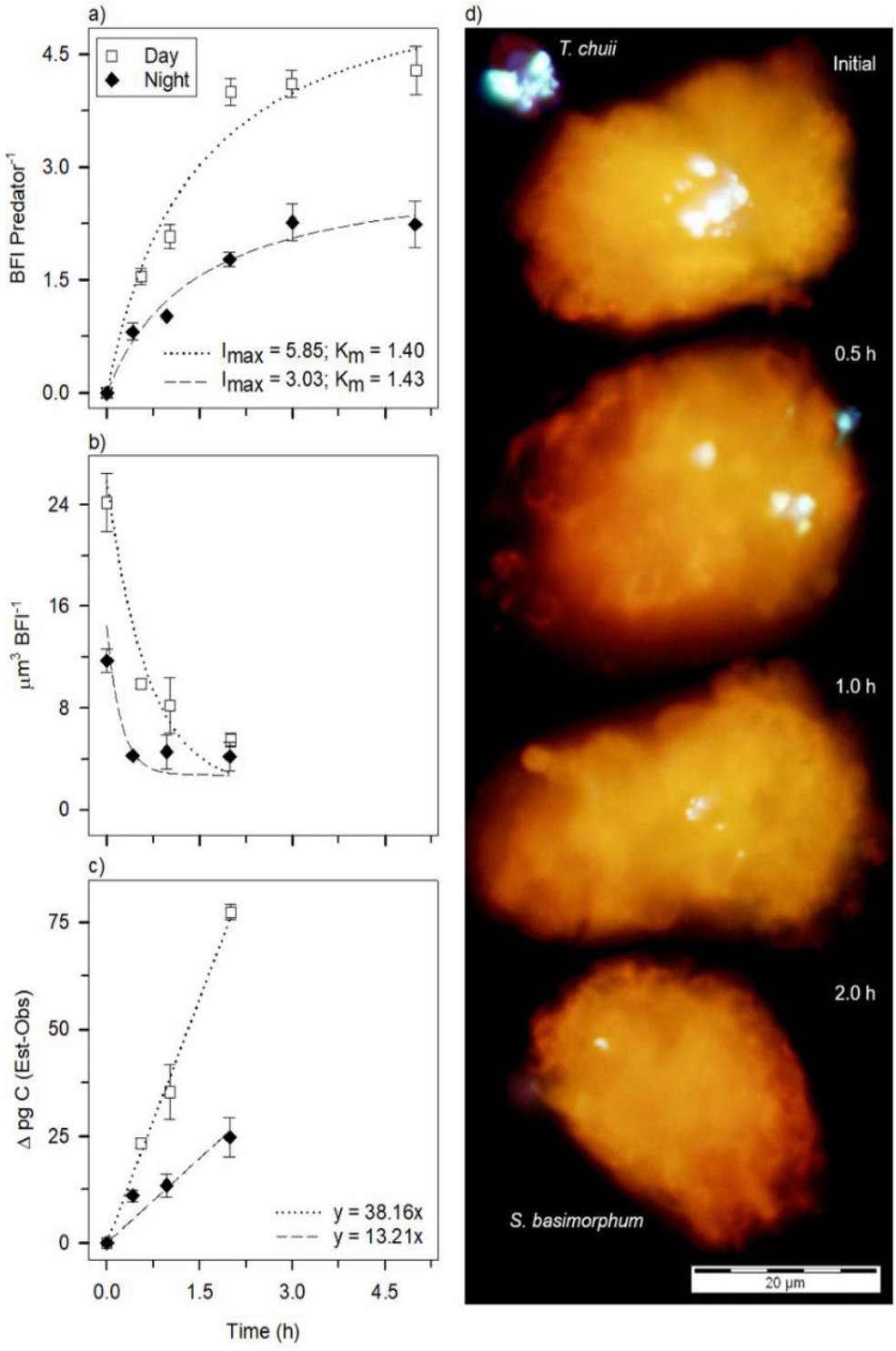

Figure 3-8 Incubation of the CM *S. basimorphum* with the LFLA *T. chuii*. White square points correspond to day samples whereas black diamond points correspond to night ones. a) incorporation of prey over time (BFI predator^{-1}); b) changes in the average fluorescent volume inside a ciliate (µm^3 BFI^{-1}); c) differences between estimated and observed C content in the ingested prey (i.e., digestion rate); d) epifluorescence pictures obtained under UV light excitation where the fluorescent volume inside a given ciliate is decreasing over time.

Regarding volume changes in the ingested BFIs over time (**Figure 3-8b,d**), there was a faster disappearance of fluorescence during the day (18.68 μm^3 over two hours) than during the night (7.55 μm^3 over the same period). Nevertheless, the parameters in both decay curves are not statistically different with the exception of the one controlling the initial volume of the BFI, likely due to the variability of the data. Altogether, from the combined information (and normalized to C units) of the **Figure 3-8a** and **Figure 3-8b**, one can estimate digestion rates by calculating the differences between the estimated and observed pg C per BFI. Despite having statistically insignificant decay parameters, the fact that diurnal ingestion rates were higher than the nocturnal resulted in significantly higher (ca. 2.88x) digestion rates between the day and the night (**Figure 3-8c**, $p < 0.01$).

c. Detection of new mixoplankton species

Another advantage of the use of LFLA is that it enables the direct visualisation of an individual prey inside an individual predator. In particular, it enabled the detection of the phycoerythrin-containing *T. amphioxeia* inside the dinoflagellate *G. litoralis* (**Figure 3-9**), due to the different photosynthetic pigment that each organism possesses. This dinoflagellate can, consequently, be moved from the phyto- into the mixoplankton group.

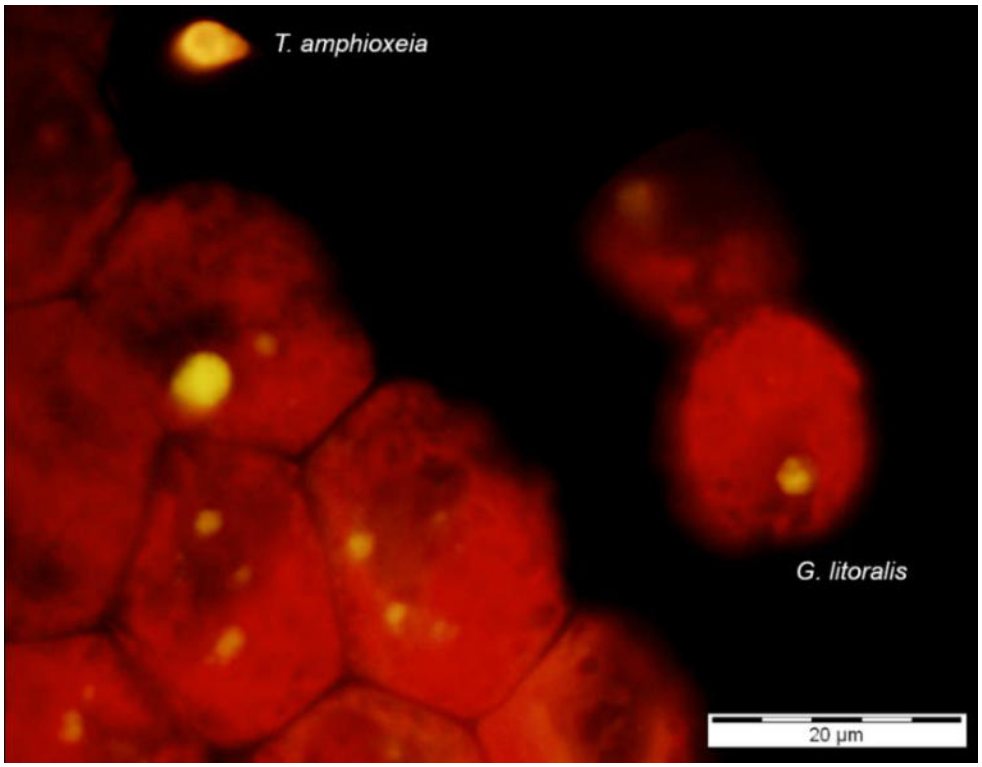

Figure 3-9 Detection of *T. amphioxeia* inside the previously unknown CM *G. litoralis* using epifluorescence microscopy under UV light excitation. *T. amphioxeia* contains phycoerythrin, i.e., seen as orange fluorescent inclusions as opposed to chlorophyll which glows in a bright red tone.

ii. Disadvantages of LFLA

a. Selectivity towards specific species

One of issues that affects experiments using LFLA as tracers for grazing was noticed on the first experiment and concerned the pSNCM *M. rubrum* thus justifying its exclusion from the **Figure 3-7**. This ciliate was, therefore, incubated with LFLA in two independent experiments, one containing the LFLA *I. galbana* as a sole prey and another using it as a tracer, i.e., provided in a mixture containing *T. amphioxeia* as well (**Figure 3-10a** and **3-10b** respectively).

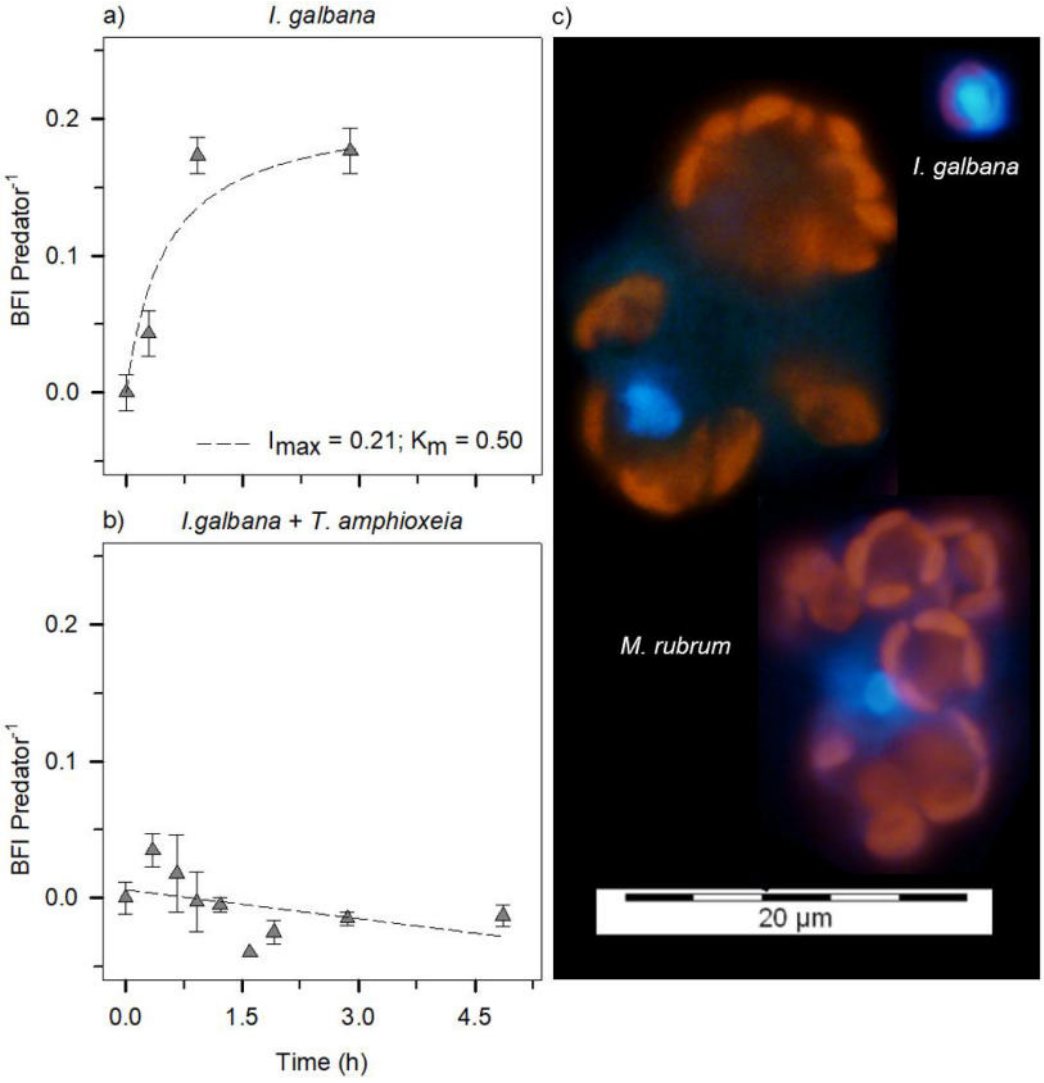

Figure 3-10 Incorporation of labelled *I. galbana* by *M. rubrum* over time: a) *I. galbana* was the only prey offered; b) the LFLA *I. galbana* was offered as a tracer particle, i.e., *T. amphioxeia* was also present in the mixture of prey and c) epifluorescence pictures from the experiment without *T. amphioxeia* obtained under UV light excitation where BFI can be seen inside *M. rubrum*.

There is an aspect that is immediately noticeable by comparing the **Figure 3-10a** and **3-10b**: when *I. galbana* was offered without an alternative prey (**Figure 3-10a,c**), *M. rubrum*

ingests it and the incorporation of BFI per predator followed the typical satiation pattern described above. On the other hand, if *T. amphioxeia* was provided in the mixture of prey (being *I. galbana* used only as a tracer, i.e., using the regular protocol for LFLA), the incorporation of BFI per predator is negligible.

b. Size and concentration-dependent selectivity

The first experiment highlighted a second issue with the LFLA technique, which can be seen in **Figure 3-11**. It concerns the selection for or against the tracer particle.

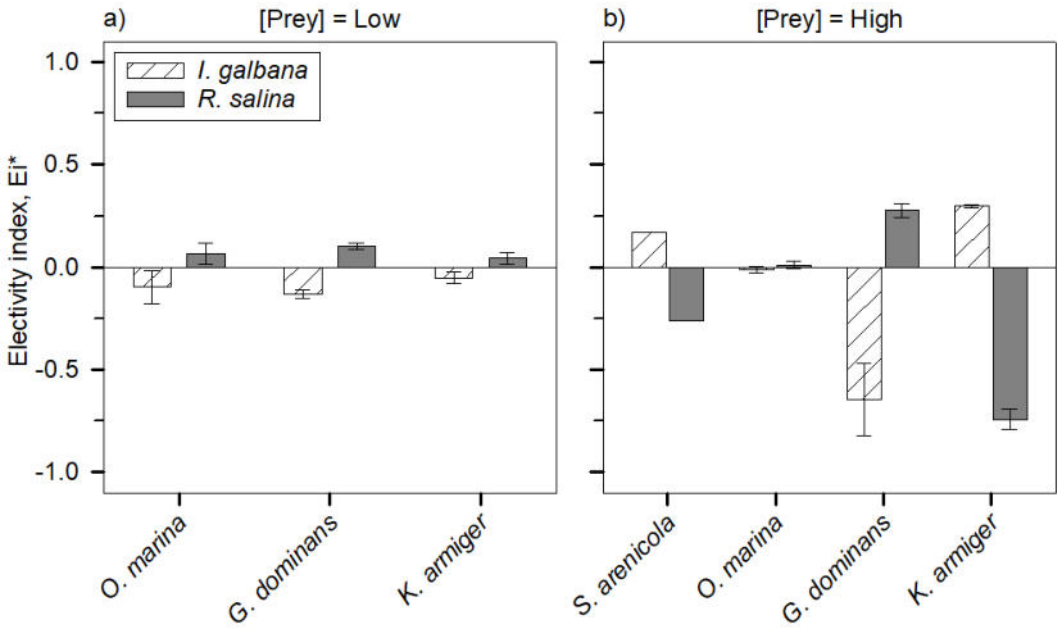

Figure 3-11 Electivity indexes calculated for *I. galbana* and *R. salina* for *S. arenicola*, *O. marina*, *G. dominans*, and *K. armiger* under a) non-saturating food conditions and b) saturating food conditions. This index varies between -1 and 1. Negative values imply a negative selection and vice-versa.

With non-saturating prey conditions (**Figure 3-11a**), the electivity indexes for all three dinoflagellates were close to zero (although positive values for *R. salina* in all cases), which suggests a close-to negligible prey preference in these conditions. Yet, this pattern was not retained when the total concentration of prey reached saturating levels (**Figure 3-11b**). While *O. marina* did not show any prey preference, *G. dominans* increased the electivity index for *R. salina* at the saturating prey concentration, which resulted in a selectivity against *I. galbana*. Also, *K. armiger* shifted from a non-selective predator at low prey concentrations to a highly selective one at saturating conditions, favouring *I. galbana* as prey over *R. salina*. This preference pattern was also shared by the ciliate *S. arenicola*.

c. *Tube, pallium, and toxic mucus trap feeding*

An interesting outcome of the comparison between the experiment with *K. veneficum* and *K. armiger* (**Figure 3-8c,d**) were the higher ingestion rates exhibited by the former. This result made us question whether or not the feeding mechanism could impact the conclusions drawn from an experiment with LFLA. Accordingly, we prepared a qualitative experiment with known tube-feeding dinoflagellates (**Figure 3-12**).

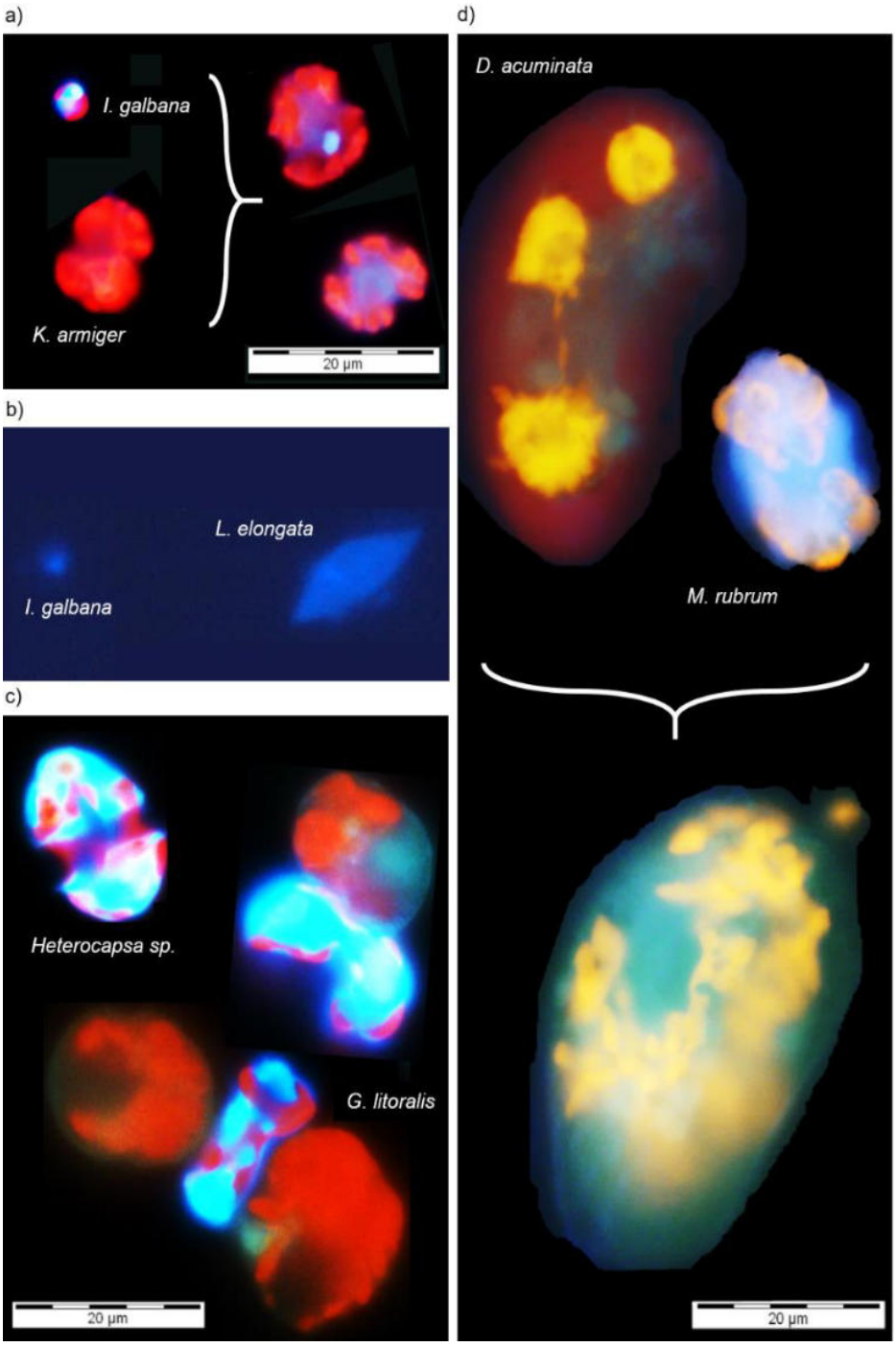

Figure 3-12 Examples of tube-feeding dinoflagellates before and after LFLA addition as seen under UV light in epifluorescence: a) *K. armiger*, where two situations can be found depending on the elapsed time after ingestion b) *L. elongata*, where the BFI cannot be distinguished from the predator c) *G. litoralis*, where the prey is clearly deformed due to the suction of its intracellular content but the predator is either unstained or completely blue, and d) *D. acuminata* where only completely blue cells were found after feeding on labelled prey. The red colour in this figure is due to the autofluorescence of Chl a, orange implies the presence of phycoerythrin, and blue is due to the presence of the fluorochrome CMAC.

The major issue demonstrated by this experiment is the complete cytoplasm staining noticeable irrespective of the chosen predator (i.e., an uncountable amount of prey inside), despite being particularly evident in *D. acuminata* (**Figure 3-12d**). The least affected predator was *K. armiger* (**Figure 3-12a**), whose cytoplasm staining was heavily dependent on the elapsed time after the beginning of the incubation. The experiment with *G. litoralis* (**Figure 3-12c**) demonstrated the same issues as those mentioned above; however, as the offered prey was similarly-sized to the predator, it was common to find half-eaten prey in the filter and/or more than one predator feeding on a single prey, further impairing the estimation of grazing. At last, the experiment with *L. elongata* (**Figure 3-12b**) demonstrated that using LFLA to quantify grazing in peduncle feeding protozooplankton is even worse than in mixoplankton, since the incorporation of LFLA by the former is further masked by the lack of distinguishing pigments themselves.

The analysis of this experiment suggests that dinoflagellates feeding with mechanisms other than direct-engulfment (such as those shown in **Figure 3-13**) may be systematically underestimated by the technique. From the live-pictures, we can presume that that these predators would probably appear either completely stained or completely unstained (**Figure 3-13a** and **3-13b,** respectively) if incubated with labelled prey (prey selectivity issues ignored).

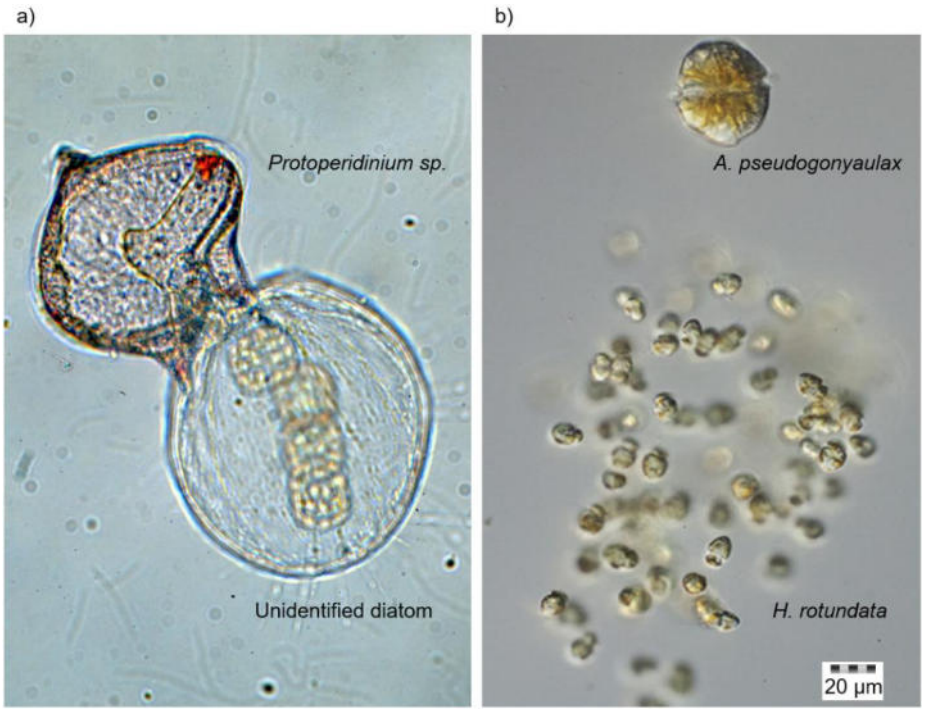

Figure 3-13 Live-pictures of a) the protozooplankton *Protoperidinium sp.* feeding on an unidentified diatom chain through a pallium; b) the mixoplankton *Alexandrium pseudogonyaulax* dragging multiple *Heterocapsa rotundata* which are immobilized inside a toxic mucus trap and eventually ingested. The first photo was taken by Albert Calbet (but see also Gaines & Tailor 1984 for the original description of pallium feeding) and the second by Hannah E. Blossom.

3.4.3 Discussion

The LFLA technique (as proposed by Li *et al.* 1996) offered unique possibilities that distinguished it from other techniques which were developed almost concurrently for the estimation of protist herbivory (e.g., the dilution technique - Landry & Hassett 1992). In particular, it enabled the direct visualisation of organisms, a characteristic which could provide a species/community-specific analysis (Caron 2001). In the particular case of mixoplankton, where knowing who is there is arguably not as important as knowing what are they doing and at what rate (Flynn *et al.* 2019), being able to observe phagocytosis is paramount. Additionally, the LFLA technique did not suffer from a major issue that hindered its predecessor (FLA - Rublee & Gallegos 1989), which was the discrimination against inert or dead particles (e.g., Sherr *et al.* 1987, Nygaard *et al.* 1988, Epstein & Rossel 1995, Jürgens & DeMott 1995) while remaining non-toxic throughout the incubation, as opposed to hydroethidine, the first-ever "vital stain" (Putt 1991). Nevertheless, the technique was not free from issues, as pointed out by its developers.

For instance, Li *et al.* (1996) stated that their stain, the green CMFDA, faded quickly in the light, which would be a major issue if attempting to conduct incubations over large periods of time. Martínez *et al.* (2014) confirmed that the blue stain CMAC (our stain), was better on this specific issue, in particular when following their optimised staining protocols. Indeed, we experienced no issues regarding stain bleaching or fading, both during the actual experiments (light intensities between 35 and 55 µmol photons m^{-2} s^{-1}) but also during the processing of the samples under the epifluorescence microscope. Nevertheless, the fact that predators typically reach the plateau phase within 45 minutes irrespective of prey concentration, trophic mode, and taxonomic group (see **Figures 3-7** and **3-10a**) seems to put this problem on a secondary level. That is, of course, if prey incorporation experiments are conducted instead of prey disappearance ones (see the discussion on FLB by Caron 2001).

The fact that this technique is useful mostly on short-term experiments is both an advantage and a disadvantage. As confirmed by our first experiment, there are a number of protist predators which incorporate LFLA without major issues and under different experimental conditions (**Figures 3-7** and **Figures 3-8**). Nevertheless, even when the incorporation of prey followed the typical satiation pattern, and maximum ingestion rates and half-saturation constants could be estimated, we encountered selectivity issues, both

for and against the tracer algae (**Figures 3-11**). One of the assumptions that is crucial for the estimation of grazing rates is that the tracer is incorporated at similar rates as the remaining prey. In fact, these issues are far from negligible as they shake the very foundations of the technique and, above everything else, are species-specific and unpredictable without experimentation. This is an issue that, as far as we know, has not been described in the past, as most efforts focused on the selection of prey based on the presence of the fluorochrome. For example, Kamiyama (2000) confirmed experimentally that stained and unstained algae were ingested at similar rates by *Schmidingerella taraikaensis* (previously known as *Favella taraikaensis*). Likewise, Martínez *et al.* (2014) demonstrated that *O. marina* grazed on labelled *I. galbana* and *T. chuii* as it did on unlabelled prey. However, in the same study, *G. dominans* seemed to have a slight preference for LFLA over unlabelled prey. In our own experiments, *G. dominans* was the species displaying the largest differences on both Imax and Km between saturating and non-saturating food concentrations. Additionally, this dinoflagellate exhibited a distinct selectivity for labelled *I. galbana* which was dependent on the concentration of prey. Despite not having been strongly emphasized in the past (but see Jürgens & DeMott 1995 and Dolan & Šimek 1999), this change in selectivity could partially explain the enormous differences seen between the afore mentioned parameters. We are unable to confirm whether this result is a direct consequence of size or fluorochrome selectivity (as per Martínez *et al.* 2014), however, we can undeniably state that the measured ingestion rate using the LFLA methodology did not represent the total ingestion of prey by this predator.

Similarly, the results obtained with *Karlodinium* spp. suggest that there could be problems in the extrapolation of community grazing rates for these species. For instance, we know that one *K. armiger* ingests ca. 10 *R. salina* per day at saturating food conditions (Berge *et al.*, 2008b), a value which is ca. 8.9 times higher than the one obtained for *K. veneficum* on the same prey (listed as K21 - Calbet *et al.* 2011a). Yet, the results from the LFLA experiment yielded an ingestion rate of 0.34 BFI *K. veneficum* h^{-1} and 0.38 BFI *K. armiger* h^{-1} (only 1.1 times higher), besides estimating a higher Imax for the former. Additionally, *K. armiger* exhibited a strong preference for *I. galbana* which, if due to a preference for smaller sized prey (Ferreira *et al.* in prep.), could further enhance the differences obtained with both *Karlodinium* spp. since *K. veneficum* is smaller and size preferences are typically correlated with one's own size (e.g., Hansen *et al.* 1994).

Aside from size selectivity, the experiments with the pSNCM *M. rubrum* confirmed that species selectivity is a problem that cannot be circumvented. When its preferential prey was absent (see for example Yih *et al.* 2004 and Smith & Hansen 2007) the number of BFI per *M. rubrum* followed the typical satiation pattern (**Figures 3-10a**). However, when *T. amphioxeia* was present, BFI were mostly absent. In the laboratory, it is possible to control both the grazer and the prey but in the field one cannot chose which species will be present. As the functional group name suggests, *M. rubrum* is a specialised predator and retains chloroplasts mostly from a specific clade of cryptophytes which comprise *T. amphioxeia* among others (e.g. Myung *et al.* 2011, Hansen *et al.* 2012). Still, *M. rubrum* can ingest some other flagellates as well (Park *et al.* 2007, *Hansen et al.* 2012, Ferreira & Calbet 2020, this study), and is mildly able to keep chloroplasts from *Rhodomonas* spp for short periods of time (Myung *et al.* 2011). Accordingly, our results with *I. galbana* are not surprising. Yet, in the field, the presence of *M. rubrum* is typically correlated with the presence of cryptophytes (e.g., Johnson *et al.* 2018), which means that it is extremely unlikely that a field experiment will be able to capture the feeding process of this ciliate using a tracer particle that is not its own preferred prey. This conclusion is likely valid for all the specialised protist predators that exist on Earth.

We have also confirmed the usefulness of the technique on the quantification of diel ingestion and digestion rates on an NCM species. We strongly believe that this is a close-to universal utility of the technique, as pigments/fluorescent tracers have been used numerous times to estimate digestion rates (e.g., Dolan & Šimek 1998, Li *et al.* 2001, Nishibe *et al.* 2002, Setälä *et al.* 2005). Still, even close-to universal approaches have their exceptions and we clearly demonstrated one of them using tube-feeding protozoo- and mixoplanktonic dinoflagellates. Indeed, as confirmed with 4 independent predator-prey combinations from distinct trophic modes, predators that feed using a peduncle tend to become entirely stained themselves instead of displaying individualized (and countable) BFIs. In fact, the low grazing obtained with *K. armiger* may have been due to the fact that this dinoflagellate feeds using a peduncle (Berge *et al.* 2008a). Based on our results with tube feeders, it is likely that some *K. armiger* did contain LFLA but we were unable to see them because of the dispersal of the fluorochrome through the predator's cytoplasm. This issue appears to become more relevant with the passing of time, as some cells did contain easily distinguishable and countable BFIs (see **Figures 3-12a,c**). To further complicate the peduncle feeding matter, it should be mentioned that the

incorporation of ingested material through a peduncle may flow into a single (e.g., Calado & Moestrup 1997) or several (smaller) food vacuoles (e.g., Hansen 1991). Also, small cells may be taken whole through a peduncle in some cases (e.g., Calado & Moestrup 1997), which could also be the reason why some *K. armiger* had countable BFI and some had not (*I. galbana*'s ESD is ca. 4.5 µm).

Tube feeding is a common characteristic in both naked and thecate dinoflagellates (Hansen & Calado 1999), suggesting that this caveat of the technique may be a truly important factor to take into consideration when applying this technique in mixed assemblages in the field. One other long-known feeding mechanism in dinoflagellates is through the deployment of a pallium (**Figures 3-13a**), which liquefies the cytoplasmic contents of the prey extracellularly (Gaines & Taylor 1984, Jacobson & Anderson 1986). Accordingly, pallium feeders do not transport particles into the main cell body and thus lack food vacuoles (Archer *et al.* 1996, Hansen & Calado 1999). We did not experiment on pallium feeders and, as such, we can only simply hypothesize on these organisms. Still, it seems reasonable to assume from these past observations and from our own on peduncle feeders that these dinoflagellates would probably appear as a very intense and uniformly bright cell, due to even incorporation of the fluorochrome. On a similar albeit almost species-specific note, comes the feeding by toxic mucus traps. This feeding mechanism has only been reported, as far as we know, in the species *Alexandrium pseudogonyaulax* (**Figures 3-13b**, Blossom *et al.* 2012, 2017) and on a *Prorocentrum sp. A. pseudogonyaulax* consumes whole cells through the sulcus but is unable to capture individual, motile prey cells. Therefore, it deploys a mucus trap that immobilises (but does not kill on a short time scale) prey and, by swimming with it attached to its own body, oftentimes end up entrapping several prey inside. Accordingly, these predators may affect the availability of prey without exhibiting food vacuoles or, even if showing clear and distinguishable BFIs, provide unrealistic grazing rates.

3.5 Extracting mixoplanktonic grazing from dilution grazing experiments

3.5.1 Methods

We constructed several artificial food webs involving protozooplanktonic and mixoplanktonic predators to gain insights into the dynamics of dilution grazing experiments. Being a laboratory experiment, we were able to control variables and unknowns that cannot be controlled in field experiments. In particular, we included prey controls and ascertained single grazer rates at the experimental conditions. These additions enabled us to determine the species-specific contributions to the concentration of chlorophyll and grazing in the mixed dilution grazing experiment. We conducted our experiments with and without light and sampled the bottles at several time points to have a better representation of the grazer and prey dynamics during the incubation.

i. Cultures

We conducted the experiments with the protozooplanktonic dinoflagellate *G. dominans* (strain ICM-ZOO-GD001) and ciliate *S. arenicola* (strain ICM-ZOO-SA001), the CM dinoflagellate *K. armiger* (strain ICM-ZOO-KA001), and the ciliated pSNCM *M. rubrum* (strain DK-2009). As prey for all experiments, we used the cryptophyte *R. salina* (strain K-0294) and the diatom *C. weissflogii* (strain CCAP 1085/18). *R. salina* was offered as prey *ad libitum* for the maintenance and growth of the two dinoflagellates and *S. arenicola*, being replenished upon depletion by the predator. *M. rubrum* was grown and maintained with *T. amphioxeia* (strain K-1837) in a proportion of approximately 5 prey per predator (Smith & Hansen 2007). Both *R. salina* and *T. amphioxeia* were kept in f/2 medium (Guillard 1975) and irradiated at ca. 150 µmol photons m^{-2} s^{-1} provided by cool white fluorescent lights. *C. weissflogii* was kept under the same conditions with the addition of silicate to the medium. All predators were kept in autoclaved 0.1 µm-filtered seawater. Protozooplankton were maintained at ca. 35 µmol photons m^{-2} s^{-1} whereas mixoplankton were kept at ca. 65 µmol photons m^{-2} s^{-1}. The stock cultures were up-scaled and maintained using a discontinuous culture (pseudo-chemostat) approach, i.e., the cultures were diluted every 1-2 days with the respective medium (between 20 and 50 % of the total volume), to maintain them under exponential growth (and within target concentrations) at any moment. Additionally, to avoid an increase in the pH beyond the limits for exponential growth, all cultures were bubbled with 0.2 µm-filtered air (Berge *et al.* 2010). We used a very slow cadence of bubbles (flow rate not measured) to diminish

the chances of stressing the predators (Berge *et al.* 2010). The direct effect of the bubbling process on the growth of the protists was not determined however, if any, it was likely minor as we confirmed that all cultures were healthy and actively feeding before starting the experiments. All cultures were kept in a temperature-controlled room at 19 °C with a 10:14 L/D cycle at a salinity of 38.

ii. Dilution grazing experiments

The dilution grazing experiments were conducted with a paired mixture of predators at a time; i.e., *G. dominans* simultaneously with *K. armiger*, and *S. arenicola* concurrently with *M. rubrum*. Both experiments were conducted with a mixture of *R. salina* and *C. weissflogii* as prey, in an equivalent carbon (C) concentration. Carbon values for all species were obtained from the average volume and C:μm^3 ratio provided by Traboni *et al.* (2020). *M. rubrum* was allowed to deplete *T. amphioxeia* before starting the experiment.

Two dilution series of 60, 30, and 15 % were prepared from the 100 % treatment, in duplicated 1100 mL transparent polycarbonate bottles (Thermo Scientific Nalgene). All bottles contained 200 mL of f/2 + Si per litre of suspension to reach a final concentration equivalent to f/10 + Si. The actual level of dilution was determined from the initial concentration of prey in each dilution relative to the initial concentration of prey in the 100 %. One of the dilution series was incubated with a 10:14 L/D cycle at 100 µmol photons m^{-2} s^{-1} (hereafter termed L/D treatment). The second series was wrapped in aluminium foil and covered with an opaque box (i.e. incubated in complete darkness) during the whole period (hereafter termed D treatment). The times of sampling were the only sources of culture vessel mixing during the incubation.

Additionally, a second, third, and fourth set of duplicated 100 % bottles were prepared under the same nutrient and light conditions mentioned above. The second set contained the two prey and no predators (termed 100prey). These bottles were used as a control and accounted for the net growth rate (both in cell numbers and Chl *a*) of each prey in the absence of grazing. The third and the fourth set of 100 % bottles comprised the two prey and only one of the predators (in the dinoflagellate experiment, 100gyro and 100karlo, or 100strom and 100meso in the ciliate experiment). These bottles eased the interpretation of the more complex mixed experiment by providing the outcome of the presence of a single predator.

All treatments were prepared with a final volume of 1 L per bottle. In the dilution series, the bottles from every dilution level were sampled after 0, 2, 4, 8, and 24 h for both Chl *a* (150 mL) and cell counts (70 mL, 2 % acidic Lugol's solution final concentration). The control bottles were sampled after 0, 8, and 24 h (150 mL for Chl *a* and 50 mL for cell counts). Samples collected after 2 and 4 h were only used to calculate Chl *a* per cell concentrations and are, therefore, not going to be further discussed. The 8 h sample of both L/D and D bottles were collected immediately before the beginning of the night period. For the D treatment, this did not imply any change in the light conditions despite effectively representing a day sample.

The samples preserved with acidic Lugol's solution were stored in the dark at 4 °C for 1-6 months before being counted. After stabilising the samples to room temperature (21±3 °C), the bottles were rotated softly and used to fill 10 mL methacrylate sedimentation chambers. The Utermöhl (1958) method was employed to analyse the samples after 24 h on an inverted microscope (XSB-1A) using a 25× objective. A minimum of 200 individuals of each species were counted per replicate count, being each sample counted twice.

iii. Chlorophyll, growth, and grazing analysis

The total chlorophyll *a* (Chl *a*, µg L^{-1}) was determined by filtering 150 mL of water from every bottle as specified before. The samples were collected into dark bottles and filtered through Whatman GF/C glass fibre filters under dim light conditions immediately after collection. The filters were folded in half twice, wrapped in aluminium foil and then kept at -20 °C for ca. 5 months until the extraction of pigments with 6 mL of acetone 90 %. The extraction was conducted in the dark at 4 °C and lasted ca. 24 h, thus avoiding the need to grind the filters (Holm-Hansen & Riemann 1978). The samples were measured before and after the addition of 100 µL of HCl 10 % (final concentration in the extract ca. 0.05 M) on a Turner Designs Fluorometer (Yentch & Menzel 1963). The fluorometer was calibrated against a pure Chl *a* standard (2.13 mg Chl *a* L^{-1}) of cyanobacterial origin (DHI, Hørsholm, Denmark). Phaeopigments (µg L^{-1}) were determined by dividing the chlorophyll concentration by the acid factor ratio between fluorescence values before and after acidification.

We determined individual species contribution to the total Chl *a* mathematically for each time point in the control bottles. First, 100prey bottles were used to determine Chl *a*

content of *R. salina* and *C. weissflogii*. These concentrations of pigment were then integrated into the controls with one predator and in the dilution series bottles to determine the pigment concentrations within each predator cell. We estimated the intermediate time points (those not directly assessed from control bottles) using linear progression.

Growth, clearance, and grazing rates were calculated for every time point using Frost (1972) equations as modified by Heinbokel (1978). These calculations yielded two different grazing estimates, one for each predator (and, consequently an estimated grazing from their sum), and one for the pooled incubation with two grazers. A third estimate of grazing can be obtained by measuring the slope of the linear regression that correlates the fraction of undiluted water and the apparent growth rates based on the changes in the concentration of prey during the incubation (Landry & Hassett 1982). It is important to notice that the second and third grazing estimates yield non-specific average ingestion rates per predator. Therefore, under these circumstances, individual ingestion rates were estimated by the proportion of measured ingestion rates in the 100gyro, 100karlo, 100strom, and 100meso bottles.

The results of some incubations denoted the presence of saturated feeding responses. Accordingly, under these circumstances, prey growth rates (μ, Chl *a* Chl a^{-1} h^{-1}) were determined from the interception of linear regression with the 3 most diluted treatments. Grazing rates were then calculated using **Equation 3.4**

$$g = \mu - K \qquad \mathbf{3.4}$$

K (Chl *a* Chl a^{-1} h^{-1}) being the apparent growth rates obtained in the undiluted bottles (Gallegos 1989, Dolan *et al.* 2000). We followed the same procedure to determine cell-specific grazing rates with the difference that cell counts were used instead of Chl *a*. For the sake of clarity, we decided to show only the regressions whose slope was significantly ($p < 0.05$) different from zero. Nevertheless, we calculated μ and g for all experiments as recommended by Latasa (2014) and Landry (2014).

3.5.2 Results

i. Dilution grazing experiments

The majority of the dilution grazing incubations yielded non-significant grazing rates ($p > 0.05$) when based on Chl *a* (**Figure 3-14**). The only exceptions were the experiment with dinoflagellates (**Figure 3-14a,c**), but the slopes of the linear regressions were positive on both instances. It is thus impossible to determine the actual grazing mortality using the traditional dilution approach of tracking Chl *a*. Species-specific Chl *a* content can be found in **Table 3-5** for each sampling point.

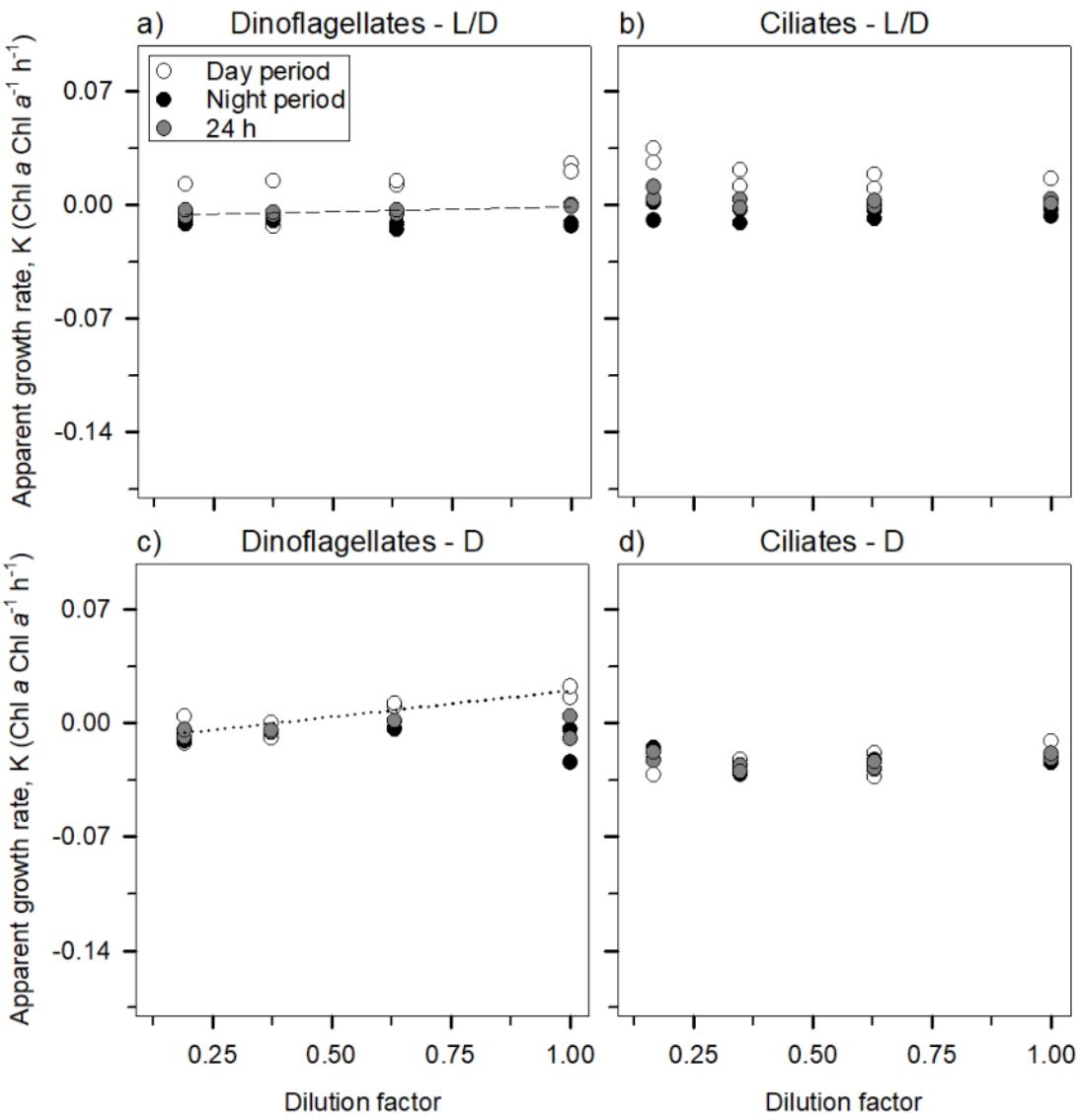

Figure 3-14 Chl *a*-based dilution grazing experiment results. The left panels (a and c) show experiments with dinoflagellates and the right panels (b and d) correspond to experiments with ciliates. The top section is relative to the L/D treatment whereas the bottom one relates to Dark (D) treatment. Plotted linear regressions imply a significant slope ($p < 0.05$). Dotted regression lines correspond to the day and dashed lines to the night.

Table 3-5 Chl *a* content (pg Chl *a* Cell^{-1}) of the target species at each sampling point as calculated from the control bottles. Columns highlighted in grey correspond to the Dark (D) treatment whereas the others correspond to the L/D treatment. The initial samples were the same for both treatments. The + indicates that the calculations yielded a negative value and, as an impossible solution, the value was forced to 0.

Species	Sampling points				
	Initial	Day	Day	Night	Night
R. salina	0.63	1.66±0.11	1.38±0.17	1.22±0.23	1.58±0.11
C. weissflogii	5.59	4.65±0.66	3.40±0.35	3.07±0.51	3.30±1.37
K. armiger	6.71	10.94±0.05	6.70±0.27	17.38±0.80	8.16±0.13
M. rubrum	19.98	21.97±1.14	12.50±2.82	19.97±2.09	15.22±0.74
G. dominans	19.88	15.23±3.90	6.69±0.52	2.74±0.78	1.84±4.58
S. arenicola	10.22	0.62±6.44	13.06±0.24	0.00^{+}	4.52±1.13

Cell-based dilution regressions for dinoflagellates showed very distinct patterns for the two prey (**Figure 3-15**). *R. salina* (**Figure 3-15a,b**) was always ingested irrespective of the period of the day and light conditions (although it had a higher grazing mortality during the day in the presence of light), but the diatom *C. weissflogii* was not (**Figure 3-15c,d**). In fact, the diatom seemed to benefit from the presence of predators, as suggested by the significantly positive slopes both in the L/D and D treatments (see the Methods section for the experimental conditions of each treatment). When the predator community was composed of ciliates instead of dinoflagellates (**Figure 3-16**), *R. salina* was subject to significant grazing mortalities (i.e., negative slope) only during the day in both L/D and D treatments, and in the integrated 24 h in the D treatment (**Figure 3-16a,b**).

All four species of predators showed a lack of response of growth rates to the dilution of the community (**Figure 3-15e,g,h** and **3-16e-h**), except for *K. armiger* in the D bottles (**Figure 3-15f**). It seems then that *K. armiger* was actively ingested by *G. dominans* in the D treatments as ascertained by the significant grazing mortality ($P < 0.05$). A summary of the species-specific growth and grazing rates can be found on **Table 3-6**.

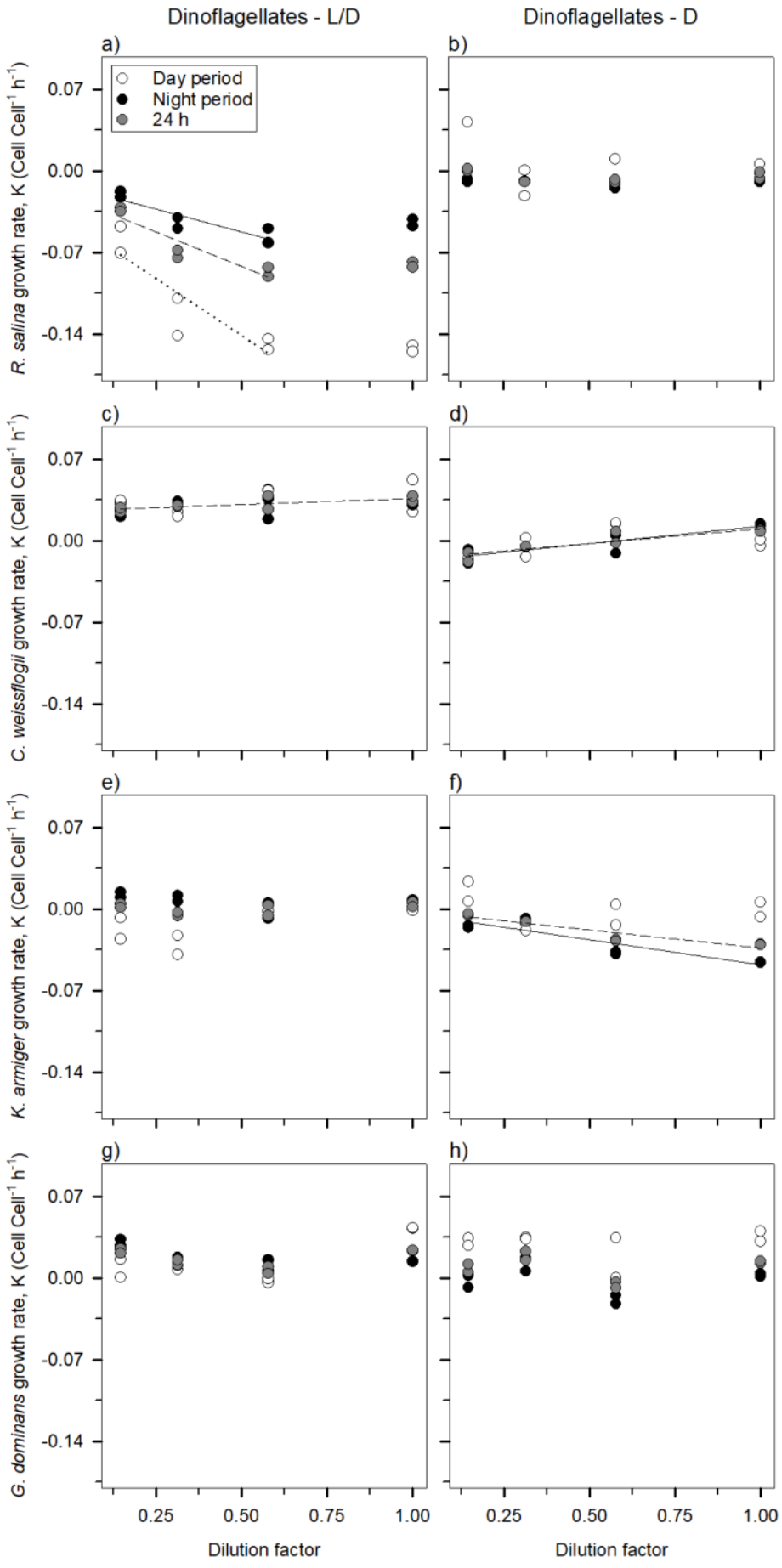

Figure 3-15 Cell-based dilution grazing experiment with dinoflagellates. The left panels (a, c, e, and g) depict the L/D bottles and the right ones (b, d, f, and h) correspond to the Dark (D) bottles. Only linear regressions with a slope significantly ($p < 0.05$) different from zero are plotted. Dotted regression lines, dashed regressions lines, and solid regression lines correspond to the day-time period, night-time period and the 24 h incubations respectively.

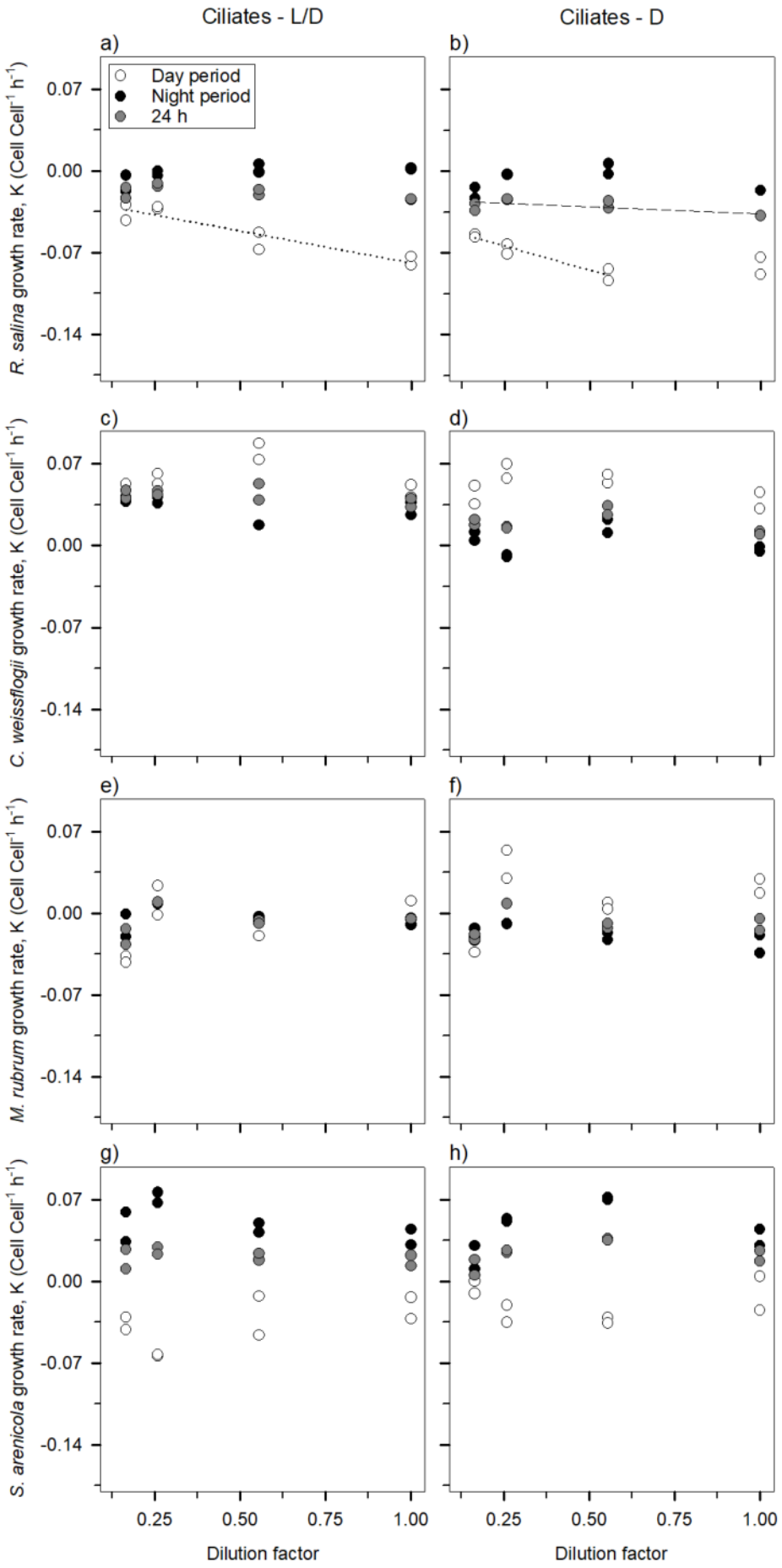

Figure 3-16 Cell-based dilution grazing experiment with ciliates. Legend as in **Figure 3-15**.

Exp	Rate, Species	Day	R^2	Day	R^2	Night	R^2	Night	R^2	24 h	R^2	24 h	R^2
Dinoflagellates	μ, Total Chl *a*	-0.0059	0.47	-0.0121	0.74**	-0.0081	0.31	-0.0032	0.13	-0.0071	0.60*	-0.0061	0.12
	g, Total Chl *a*	-0.0285		-0.0321		0.0049		0.0079		-0.0059		-0.0053	
	μ, *R. salina*	-0.0431	0.77*	0.0065	0.03	-0.0132	0.80*	-0.0102	0.06	-0.0229	0.85**	-0.0039	0.03
	g, *R. salina*	0.1091#		0.0102		0.0311#		-0.0025		0.0575#		0.0025	
	μ, *C. weissflogii*	0.0280	0.15	-0.0107	0.21	0.0249	0.19	-0.0172	0.76**	0.0259	0.50*	-0.0150	0.79**
	g, *C. weissflogii*	-0.0126		-0.0167		-0.0089		-0.0296		-0.0104		-0.0253	
	μ, *K. armiger*	-0.0278	0.48	0.0022	0.03	0.0110	0.23	-0.0049	0.81**	-0.0019	0.09	-0.0024	0.87**
	g, *K. armiger*	-0.0311		0.0073		0.0096		0.0431		-0.0039		0.0312	
	μ, *G. dominans*	-0.0043	0.49	0.0285	0.00	0.0249	0.33	-0.0004	0.01	0.0152	0.01	0.0093	0.00
	g, *G. dominans*	-0.0377		-0.0019		0.0147		0.0028		-0.0027		0.0012	
Ciliates	μ, Total Chl *a*	0.0277	0.36	-0.0281	0.13	-0.0056	0.00	-0.0233	0.00	0.0052	0.16	-0.0248	0.03
	g, Total Chl *a*	0.0171		-0.0087		0.0001		0.0010		0.0047		-0.0022	
	μ, *R. salina*	-0.0241	0.87**	-0.0432	0.92**	-0.0081	0.36	-0.0075	0.01	-0.0134	0.42	-0.0247	0.51*
	g, *R. salina*	0.0548		0.0382+		-0.0122		0.0033		0.0103		0.0120	
	μ, *C. weissflogii*	0.0585	0.00	0.0582	0.17	0.0417	0.24	0.0043	0.01	0.0474	0.27	0.0224	0.09
	g, *C. weissflogii*	0.0014		0.0152		0.0131		0.0025		0.0092		0.0068	
	μ, *M. rubrum*	-0.0225	0.17	-0.0028	0.11	0.0002	0.10	-0.0097	0.49	-0.0073	0.01	-0.0073	0.00
	g, *M. rubrum*	-0.0266		-0.0275		0.0089		0.0168		-0.0030		0.0020	
	μ, *S. arenicola*	-0.0540	0.33	-0.0204	0.00	0.0625	0.28	0.0404	0.03	0.0235	0.08	0.0200	0.07
	g, *S. arenicola*	-0.0322		-0.0024		0.0243		-0.0098		0.0053		-0.0075	

Table 3-6 Summary of growth (μ, h^{-1}) and grazing (g, h^{-1}) rates calculated from the slopes of dilution grazing experiments at the different periods of the day. The significance of the slope of the linear regressions is also listed. Columns highlighted in grey correspond to the Dark (D) treatment whereas the remaining correspond to the L/D ones. Values marked with an # showed saturation and g was then calculated according to Gallegos (1989) and Dolan *et al.* (2000). R^2 values marked with a * or ** are significant, i.e., $p < 0.05$ and $p < 0.01$ respectively.

ii. Incubation experiments

By having control bottles held under the same conditions as the dilution series, we were able to determine individual grazing rates for each predator species. Therefore, it was possible to: i) calculate the individual ingestion rate of each predator on both prey (**Table 3-7**), ii) combine the previous information to estimate what would be the merged ingestion rate per pair of predators, iii) calculate the actual ingestion rate for each pair of grazers by comparing data from the 100 % bottles and controls without grazers, and iv) calculate the ingestion rates based on the slopes of the dilution regressions.

Table 3-7 Carbon-specific ingestion rates (pg C pg C^{-1} h^{-1}) for each predator on both prey items as ascertained by the control bottles with a single predator. NS implies that the measured ingestion rate was not significantly different from 0. Columns highlighted in grey correspond to the Dark (D) treatment whereas the remaining are relative to L/D. Different letters within a given prey row imply statistically significant differences between treatments (One-Way ANOVA, Tukey HSD, $p < 0.05$).

Predator	Prey	Ingestion rates (pg C pg C^{-1} h^{-1})			
		Day	Day	Night	Night
G. dominans	*R. salina*	0.17 ± 0.02^{a}	0.02 ± 0.01^{b}	0.02 ± 0.00^{b}	NS
	C. weissflogii	-0.02 ± 0.01^{a}	0.00 ± 0.00^{a}	NS	-0.01 ± 0.00^{a}
K. armiger	*R. salina*	0.04 ± 0.00^{a}	0.03 ± 0.00^{a}	0.04 ± 0.00^{a}	NS
	C. weissflogii	-0.02 ± 0.01^{a}	NS	0.02 ± 0.00^{a}	-0.01 ± 0.00^{a}
S. arenicola	*R. salina*	0.16 ± 0.01^{a}	0.21 ± 0.00^{b}	0.06 ± 0.00^{c}	0.02 ± 0.00^{d}
	C. weissflogii	-0.04 ± 0.00^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	-0.04 ± 0.03^{a}
M. rubrum	*R. salina*	0.03 ± 0.02^{a}	NS	-0.02 ± 0.00^{b}	$0.01\pm0.01^{a,b}$
	C. weissflogii	-0.10 ± 0.01^{a}	-0.08 ± 0.06^{a}	NS	-0.07 ± 0.03^{a}

Most comparisons resulted in non-significant (i.e., not different from 0, two-tailed Student's t-test, $p > 0.05$) ingestion rates on *C. weissflogii*, with the exception of *S. arenicola* in some treatments, and *K. armiger* during the night-time in the L/D treatment (**Table 3-7**). In the L/D treatment, *G. dominans* clearly consumed more *R. salina* during the day than at night (Tukey HSD, $p < 0.05$), a pattern shared by all grazers except *K. armiger*, whose differences between day and night periods were negligible. *M. rubrum*

was the species with the largest day/night differences, as it was the only species displaying a significantly negative ingestion rate on *R. salina* during the night.

The D treatments affected the grazers in a different manner: *G. dominans* and *K. armiger* decreased their ingestion rates during the day (despite being significant only in the former) and displayed non-significant ingestion rates at night. Conversely, *S. arenicola* benefitted from the D treatment during the day (Tukey HSD, $p < 0.05$) despite having its ingestion rate decreased during the night (Tukey HSD, $p < 0.05$). Finally, ingestion rates by *M. rubrum* decreased to negligible levels during day-time in the D treatments (two-tailed Student's t-test, $p > 0.05$). The nightly ingestion rates of the D treatment were significantly positive whereas the same period in L/D yielded significantly negative ingestion rates (**Table 3-7**), however, this difference was not significant due to high variability of the data (Tukey HSD, $p > 0.05$). Protozooplankton displayed higher 24 h integrated ingestion rates on *R. salina* than did mixoplankton regardless of the light conditions. This difference was more evident in the presence of light but not negligible in its absence. In the L/D treatment, *G. dominans* exhibited carbon-specific ingestion rates ca. 1.5 times higher than *K. armiger*, and *S. arenicola* completely outcompeted *M. rubrum* with an ingestion rate ca. 21.4 times superior. In the D treatment, the differences were only ca. 1.3 and 6.7 times, respectively for dinoflagellates and ciliates.

The integrated 24 h period grazing for each predator tandem calculated as explained before is summarised in **Figure 3-17**. Since *C. weissflogii* was often not consumed in the experiments, we have shown only the data regarding *R. salina*. The estimated ingestion rates (obtained from the grazing impact of each individual grazer) were higher than those measured in the undiluted bottles against the respective controls. Additionally, ingestion rates calculated from the dilution slope (without controls) tend to be lower than those measured using the control bottles containing both grazers. However, the differences between methods used to ascertain ingestion rates were only significant in the L/D treatments.

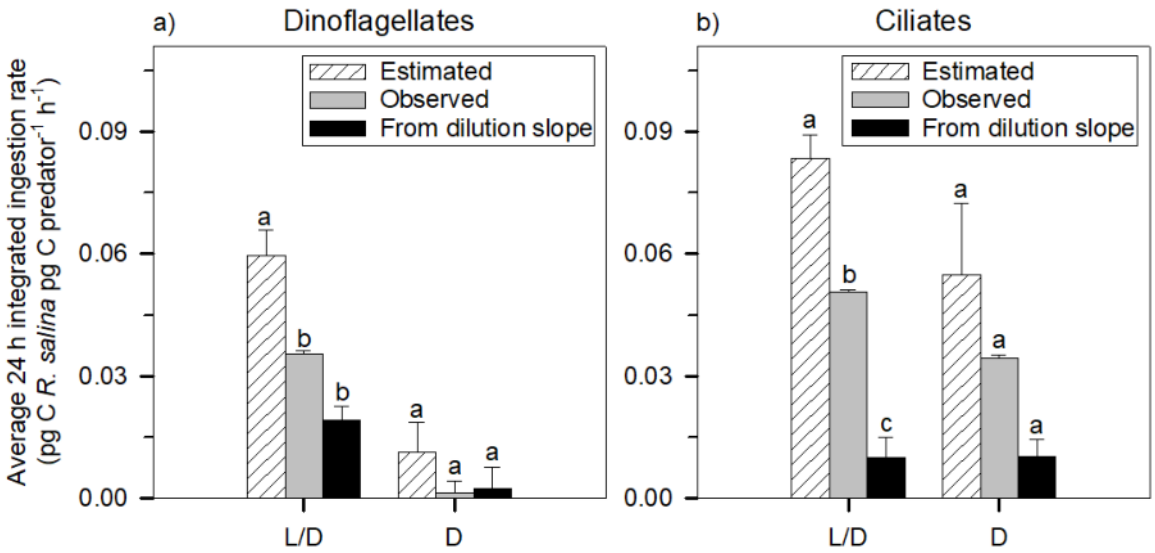

Figure 3-17 Comparison between estimated (▨), observed (▩), and dilution-measured (■) ingestion rates (pg C *R. salina* pg C predator^{-1} h^{-1}) in the L/D and Dark (D) bottles over a 24 h period: a) experiment with dinoflagellates and b) experiment with ciliates. Notice that dilution-measured ingestion rates were calculated using g values listed in Table 2. See the Methods for a detailed explanation of the calculation of each value. Different letters within each group of bars (i.e., L/D evaluated independently from D bottles) imply statistically significant differences (One-Way ANOVA, Tukey HSD, $p < 0.05$).

To further understand the Chl *a* dynamics that shaped the outcome of the dilution experiments based on this proxy, we evaluated the contribution of each species to the total Chl *a* pool (**Figure 3-18** and **3-19**) both in the undiluted and most diluted treatments. Regarding the dinoflagellate experiment (**Figure 3-18**), both the diatom and *K. armiger* became more relevant to the total Chl *a* as time passed, in particular in the undiluted L/D treatment (**Figure 3-18a**) where they increased their contribution to the total Chl *a* by ca. 9.3 and 31.7 % respectively.

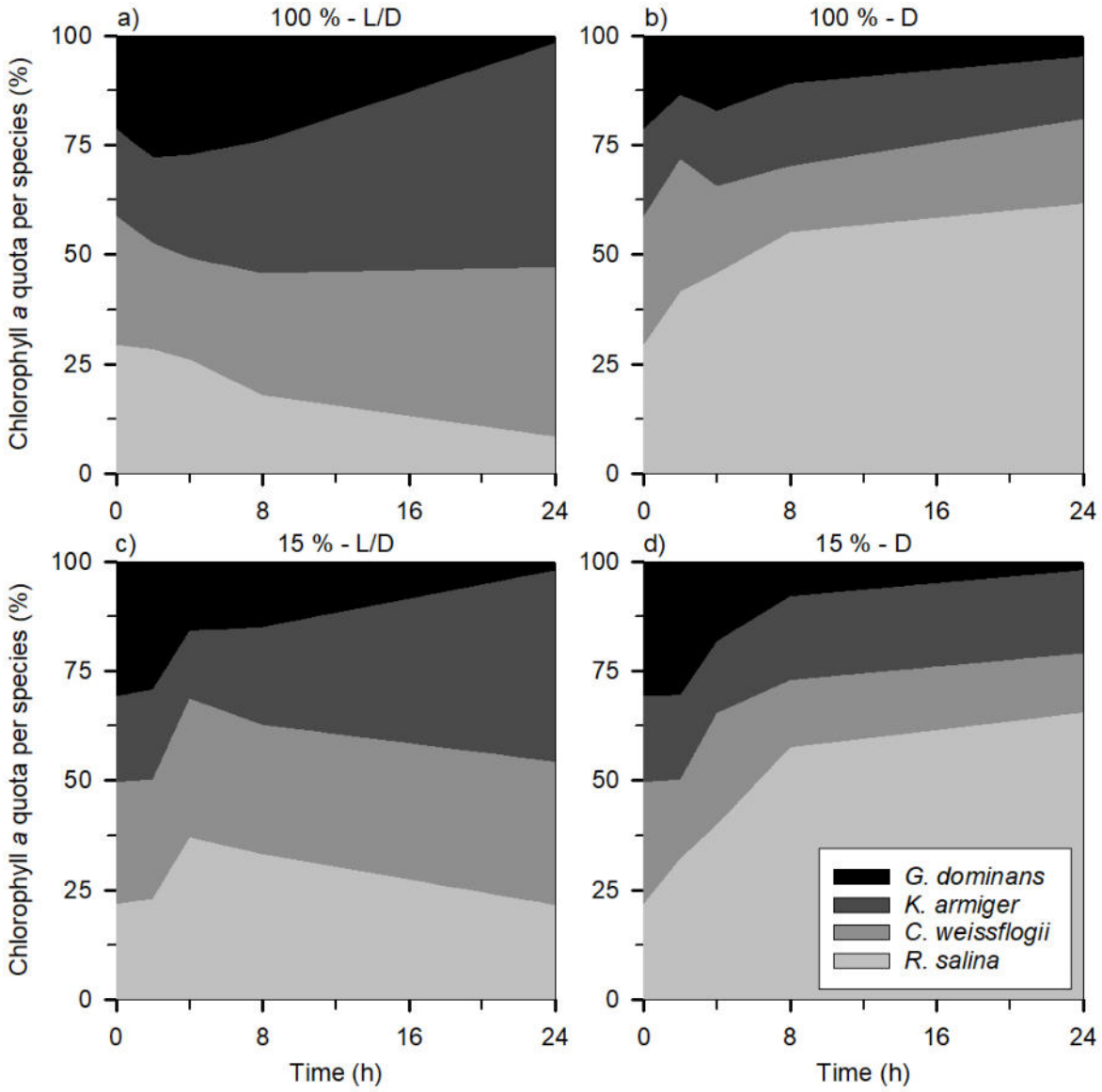

Figure 3-18 The proportion of the total Chl *a* (%) represented by each species (in different colours) in the dinoflagellate experiment throughout the incubation: a and c) L/D treatment with the undiluted and most dilute communities respectively; b and d) Dark (D) treatment with the undiluted and most dilute communities respectively.

The D treatment has a completely different pattern, with *R. salina* benefiting the most, in particular when the predator concentration was low (**Figure 3-18d**), becoming ca. 65.4 % of the total Chl *a* of the system at the end of the incubation (as compared to 21.8 % in the beginning). Irrespective of the light conditions, *G. dominans* displayed a particularly significant contribution to the total Chl *a* (up to 30.8 %) at the beginning of the incubation. The experiment with ciliates (**Figure 3-19**) followed a similar trend for the diatom and the protozooplankton (**Figure 3-19a**), albeit to a slightly larger extent in the former (an increase of ca. 10.9 %) and smaller in the latter (maximum contribution of ca. 12.7 %).

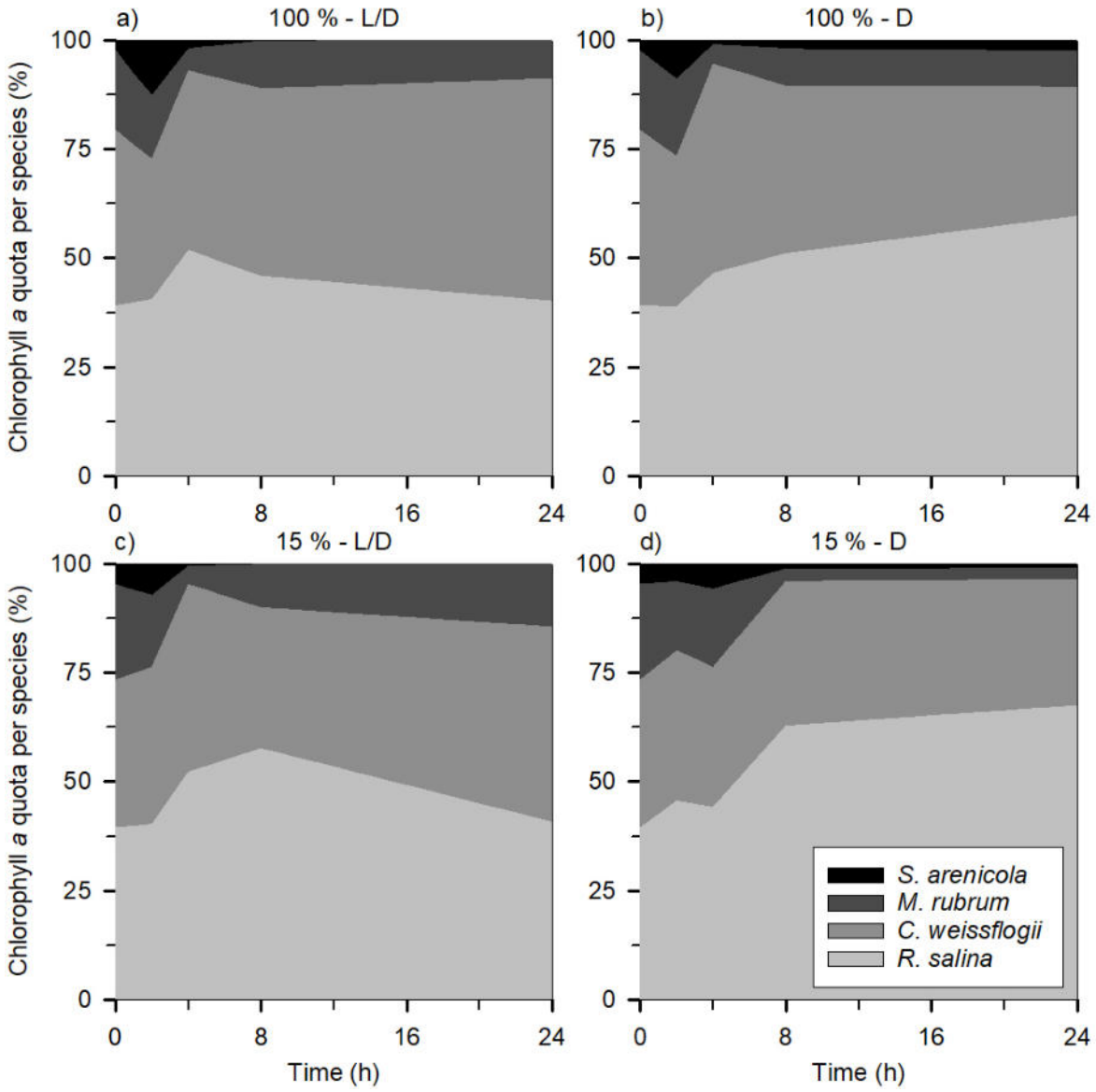

Figure 3-19 The proportion of the total Chl *a* (%) represented by each species (in different colours) in the ciliate experiment throughout the incubation. Legend as in **Figure 3-18**.

M. rubrum, in contrast with its dinoflagellate counterpart, decreased its contribution to the total Chl *a* by ca. 9.2 % (**Figure 3-19a**). Concerning the D treatment, *R. salina* was also the species that fared better with an increase of ca. 28.0 % in the diluted treatment (**Figure 3-19d**). In general, when the incubation contained only one predator species, the calculated individual Chl *a* content was, on average, ca. 8.5 % higher than when two predators were incubated together. Additionally, the magnitude of this effect differed between undiluted bottles, and the most diluted treatments (raw data not shown but incorporated in **Figures 3-18** and **3-19**).

3.5.3 Discussion

i. Consequences of using the wrong proxy for phytoplankton biomass and growth

Our results show that Chl *a* alone is not an adequate proxy for prey growth rates in dilution grazing experiments when mixoplankton are present (Paterson *et al.* 2008, Calbet *et al.* 2012). Chlorophyll is, in any case, a poor proxy for phototrophic plankton biomass (Kruskopf & Flynn 2006) because of inter-species variations, and also for the photoacclimation abilities of some species (for which very significant changes can occur within a few hours). The problem extends to the involvement of mixoplanktonic prey and grazers. Nevertheless, even very recent studies continue to rely on this parameter for quantifications of grazing despite acknowledging the dominance, both in biomass and abundance, of mixoplanktonic predators in their system (Morison *et al.* 2020). Moreover, the detailed analysis of the species-specific dynamics revealed that different prey species are consumed at very different rates. In our experiments, and contrarily to expectations (see Berge *et al.* 2008a and Nakamura *et al.* 1995), *C. weissflogii* was only actively ingested in the ciliate experiment and, according to the results from the control bottles (**Table 3-7**). Certainly, it is not the first time that a negative selection against diatoms has been seen; for example, Burkill *et al.* (1987) noticed that diatoms were less grazed than other phytoplankton species, as assessed by a dilution technique paired with High-Performance Liquid Chromatography for pigment analysis. Calbet *et al.* (2011b), in the Arctic, also found only occasional grazing over the local diatoms. In our study, diatoms were not only not consumed, but the presence of dinoflagellates appeared to contribute to their growth, this relationship being partly dependent on the concentration of the predator (see **Figures 3-15c,d**). This result could be a direct consequence of assimilation and use of compounds (e.g. Admiraal *et al.* 1984, Armbrust *et al.* 2004, Olofsson *et al.* 2019) released by microplankton such as ammonium (e.g. Caperon *et al.* 1979, Gao *et al.* 2018) and urea (e.g. Solomon *et al*. 2010), which were not supplied in the growth medium, but which would have supported prey growth. One *C. weissflogii* cell contains, on average, ca. 2.5 times more Chl *a* than one *R. salina* cell (initial value excluded, see **Table 3-5**). Taken together with the preference for *R. salina* it is not surprising that the proportion of total Chl *a* represented by the diatoms increased over time, in particular in the L/D treatment (**Figures 3-18a,c** and **Figures 3-19a,c**).

Another factor clearly highlighted by our experiments, but invariably ignored in a traditional dilution experiment, is that protozooplankton themselves contribute a significant portion of the total chlorophyll of the system (due to ingested Chl *a*), in particular at the beginning of the incubation (see **Figures 3-18** and **Figures 3-19**). Once again, this is not novel information, since Chl *a* and its degradation products have been found inside several protozooplankton species from different phylogenetic groups immediately after feeding (Kashiyama *et al.* 2012) and even after some days without food (Aristizábal 2009). An increase in intracellular Chl *a* concentrations immediately after feeding has also been found in mixoplankton (Johnson & Stoecker 2005, Berge & Hansen 2016), on which this increase is derived both from ingested prey as well as from new synthesis of their own Chl *a*. Additionally, several experiments with LFLA show that predators (irrespective of their trophic mode) seem to maximise the concentration of intracellular prey shortly after the initiation of the incubation (e.g. Rublee & Gallegos 1989, Setälä *et al.* 2005, see Section 3.4). Indeed, some authors have even been able to measure photosynthesis in protozooplankton, like the ciliates *Mesodinium pulex* (Tarangkoon & Hansen 2011) and *Strombidinopsis sp.* (Schoener & McManus 2017).

ii. When the whole is lower than the sum of the parts

In the previous section, we highlighted the need for a proxy other than Chl *a* to estimate phytoplankton biomass in dilution grazing experiments. This can be fairly easily solved by the quantification of the prey community abundance (e.g. Lawrence & Menden-Deuer 2012) by microscopy or by the use of signature pigments for each major phytoplankton group. The latter method, however, is not as thorough as the former, since rare are the cases where one pigment is exclusively associated with a single group of organisms (e.g. Jeffrey & Vesk 1997, Landry *et al.* 2000, Garcés *et al.* 2006). In any case, any pigment-based proxy is subject to the same problems, as identified by Kruskopf & Flynn (2006). Irrespective of the quantification method, it has been made evident that the different algae are consumed at different rates (e.g. pigments - Burkill *et al.* 1987, Paterson *et al.* 2008, Grattepanche *et al.* 2011; microscopy - Stelfox-Widdicombe *et al.* 2004, Calbet *et al.* 2011b, Arias *et al.* 2020b).

Prey selection in protistan grazers is a common feature (e.g. Flynn *et al.* 1996, Peltomaa & Johnson 2017, Johnson *et al.* 2018, Maselli *et al.* 2020). Given the diversity of grazers in natural communities and the array of preferred prey that each particular species

possesses, it is logical to think that dilution experiments will capture the net community response properly. Likewise, grazers interact with each other through allelopathy, competition, and intraguild predation among other factors. An example of intraguild predation could be the observed on *K. armiger* by *G. dominans* (see **Figure 3-15f** and **Table 3-6**), which caused an average loss of ca. 18.72 pg of *K. armiger* carbon per *G. dominans* per hour in the D treatment. Interestingly, in the same treatment, a slight negative effect of *K. armiger* on its predator *G. dominans* can also be deduced (i.e., positive g, **Table 3-6**), resulting in an average loss of ca. 0.33 pg *G. dominans* carbon per *K. armiger* per hour. This could be a consequence of allelopathy since *K. armiger* is a known producer of karmitoxin (Rasmussen *et al.* 2017), whose presence can have negative effects even on metazoan grazers (Berge *et al.* 2012). Regarding ciliates, none of the species used is a known producer of allelopathic compounds, which suggests that the average loss of ca. 1.25 pg *M. rubrum* carbon per hour in the D treatment was due to to *S. arenicola* predation. Altogether, it seems clear from our data that intraguild predation cannot be ignored when analysing dilution experiments. Furthermore, our results clearly show that single functional responses cannot be used to extrapolate community grazing impacts, as evidenced by the differences in estimated and measured ingestion rates based on the disappearance of prey in combined grazers experiments (**Figures 3-17**). Nevertheless, this is a common procedure (e.g. Kim & Jeong 2004, Yih *et al.* 2004, Lee *et al.* 2014). Often in modelling approaches, individual predator's functional responses have been used to extrapolate prey selectivity and community grazing responses (Ryabov *et al.* 2015); in reality complex prey selectivity functions are required to satisfactorily describe prey selectivity and inter-prey allelopathic interactions (Mitra & Flynn 2006).

It is however also evident that the measured ingestion rates in combined grazers experiments were not the same as those calculated from the slope of the dilution grazing experiment. This raises the question why was that the case? It is well known that phytoplankton cultures, when extremely diluted, show a lag phase of different duration (Fogg 1957, Aliyu *et al.* 2021) which has been attributed to the net leakage of metabolites (Flynn & Berry 1999). Assuming that the duration of the lag phase will be dependent on the level of dilution, it seems reasonable to deduce that after ca. 24 h the instantaneous growth rates (μ) in the most diluted treatments will be lower than that of the undiluted treatments. This has consequences, not only for the estimated prey growth rates, but also

for the whole assessment of the grazing rate, due to the flattening of the regression line (i.e., the decrease in the computed growth rate). This artefact may be more evident in cultures acclimated to very particular conditions (as the laboratory cultures used in this study) than in nature.

iii. When the lights go off

Another important finding of our research is the importance of light on the correct expression of the feeding activity by both mixoplankton and protozooplankton. We noticed that irrespective of the light conditions, all species exhibited a diurnal feeding rhythm (*R. salina* panels in **Figures 3-15** and **3-16**), which is in accordance to earlier observations (e.g. Strom 2001, Ng *et al.* 2017, Arias *et al.* 2020a, 2021). The presence of light typically increased the ingestion rates. Additionally, the ingestion rates differed during the night period between L/D and D treatments, which implies that receiving light during the day is also vital in modulating the night behaviour of protoozoo- and mixoplankton. In particular, mixoplankton grazing is usually affected by light conditions, typically increasing (e.g. Jakobsen *et al.* 2000, Li *et al.* 2000, Berge *et al.* 2008a, Kim *et al.* 2008), but also sometimes decreasing (Myung *et al.* 2006, McKie-Krisberg *et al.* 2015) in the presence of light. Different irradiance levels can also affect the magnitude of ingestion rates both in protozoo- and mixoplankton (Moeller *et al.* 2019 and references therein).

Therefore, we hoped for a rather consistent pattern among our protists that would help us discriminate mixoplankton in dilution grazing experiments. As a matter of fact, based on the results from Arias *et al.* (2020a), we expected that in the dinoflagellate experiment, the D treatment would have inhibited only the grazing of *K. armiger*, enabling a simple discrimination between trophic modes. The reality did not meet the expectations since the day and night-time carbon-specific ingestion rates (as assessed using the control bottles, **Table 3-7**) of *K. armiger* were respectively higher and equal than those of *G. dominans*. Conversely, in the ciliate experiment, protozooplankton were the major grazers in our incubations regardless of the day period and light conditions. This response was not as straightforward as one would expect it to be because *M. rubrum* has been recently suggested to be a species-complex containing at least 7 different species (Drumm *et al.* 2021 and references therein), which hinders any possible conjecture on their grazing impact. Indeed, the uneven responses found between and within trophic modes precluded such optimistic hypothetical procedure.

The D treatment in the present paper illustrated the importance of mimicking natural light conditions, a factor also addressed in the original description of technique by Landry & Hassett (1982). It is crucial for the whole interpretation of the dilution technique that incubations should be conducted in similar light (and temperature) conditions as the natural ones to allow for continued growth of the phototrophic prey. However, here we want to stress another aspect of the incubations: should they start during the day or the night? Considering our (and previous) results on diel feeding rhythms, and on the contribution of each species to the total Chl *a* pool, it is clear that different results will be obtained if the incubations are started during the day or the night. Besides, whether day or night, organisms are also likely to be in a very different physiological state (either growing or decreasing). Therefore, we recommend that dilution experiments conducted in the field should always be started at the same period of the day to enable comparisons (see also Anderson *et al.* (2017) for similar conclusions on bacterivory exerted by small flagellates). Ideally, incubations would be started at different times of the day in order to capture the intricacies of the community dynamics on a diel cycle. Nevertheless, should the segmented analysis be impossible, we argue that the right time to begin the incubations would be during the night, as this is the time where ingestion rates by protozooplankton are typically lower (e.g. Strom 2001, Ng *et al.* 2017, Arias *et al.* 2020a, 2021, this study) and would, consequently, reduce their quota of Chl *a* in the system.

3.6 Conclusions

Despite the inability of acting as a deterrent of protozooplanktonic grazing (noticed by the stronger effect on mixoplanktonic predation), rotenone can still be used as a good algal crop protector, especially if the predator is a sensitive organism like a ciliate (Section 3.3) or a rotifer (Van Ginkel *et al.* 2015, 2016). Nevertheless, future measures should always assess the effect of rotenone on the specific organism that is plaguing the algal culture, as differences in the sensitivity towards the compound are expected, as highlighted in the Section 3.3. Similarly, the sensitivity of the algal culture itself should also be acknowledged, as phytoplankton species are not all immune to this compound as expected. Factors such as the growth phase may be exploited to minimise the nefarious effects of the presence of rotenone in non-target organisms.

Regarding the second approach, the LFLA technique (as described in the Section 3.4) appears to be only directly applicable to organisms that feed by direct engulfment of prey. Indeed, all other feeding mechanisms (present both in protozoo- and mixoplanktonic dinoflagellates) result in biased or even unmeasurable ingestion rates. A way to avoid the size selectivity issue demonstrated by our results could be to use two distinct sizes of prey (Martínez *et al.* 2014). Ideally, both prey would be simultaneously labelled with fluorochromes whose emission spectrum would coincide but differing in the emitted fluorescent colour (Shields & Smith 2008, Nelson *et al.* 2009). For example, *I. galbana* could be labelled with CMFDA (green fluorescence, viable labelling - e.g., Li *et al.* 1996) and *T. chuii* could be simultaneously labelled with CMAC (blue fluorescence, viable labelling - e.g., Martínez *et al.* 2014). Still, even if a sample is dually-labelled there may be a selection for or against the labelled prey due to the presence of the fluorochrome. On these cases, an alternative could be to incorporate the fluorescent tracer on the genome of the tracer algae (for example, by fusing a green fluorescent protein, GFP, to a housekeeping gene). This is an advantage even over vital stains (Epstein & Rossel 1995) and is getting attention from the scientific community since the GFP vector is very bright and easily detectable, besides from being photostable (Bochdansky *et al.* 2015). Nevertheless, despite highly promising, this approach is only available for bacteria at the time of writing (e.g., Ishii *et al.* 2002, Fu *et al.* 2003, Bochdansky *et al.* 2015). At last, we have provided clear evidence that there is one issue that is likely never going to be accounted for using this technique. As seen in the *Teleaulax-Isochrysis-Mesodinium* incubation (and, on a qualitative extent, also on the *Mesodinium-Dinophysis* interaction),

this predator exhibits a species-specific selectivity, which hindered the quantification of ingested tracer prey.

To end on a positive note, let us now consider the Section 3.5. We are aware that our study does not represent natural biodiversity because our experiments were conducted in the laboratory with a few species. Nevertheless, we attempted to use common species of wide distribution for each major group of protists to provide a better institutionalisation of our conclusions. Indeed, our incubations contained cryptophytes and diatoms as prey, and both mixoplanktonic and pure protozooplanktonic predators, namely a dinoflagellate and a ciliate for each trophic mode. With these laboratory experiments, we have presented evidence calling for a cessation of the use of chlorophyll in dilution grazing experiments (Paterson *et al.* 2008, Calbet *et al.* 2012), and we have highlighted the need to observe the organismal composition of both initial and final communities to better understand the dynamics during the dilution grazing experiments (Lawrence & Menden-Deuer 2012). This approach will not incorporate mixoplanktonic activity into the dilution technique *per se* however, if combined with LFLA (see Calbet *et al.* 2012 and Martinéz *et al.* 2014), a semi-quantitative approach to disentangle the contribution of mixoplankton to community grazing could be achieved (although not perfect). An alternative (and perhaps more elegant) solution could be the integration of the experimental technique with *in silico* modelling. The modelling approaches of the dilution technique have already been used, for example, to disentangle niche competition (Beckett & Weitz 2017) and to explore nonlinear grazer responses (Sandhu *et al.* 2019). We believe that our experimental design and knowledge of the previously indicated data could be of use for the configuration of a dilution grazing model, which could then be validated in the field (and, optimistically, coupled to the ubiquitous application of the dilution technique across the globe). We cannot guarantee that having a properly constructed model that mimics the dilution technique will be the solution to the mixoplankton paradigm. However, it may provide a step towards that goal as it could finally shed much-needed light on the mixo- and protozooplanktonic contributions to the grazing pressure of a given system. To quote from the commentary of Flynn *et al.* (2019), it could provide the answer to the question of whether mixoplankton are *de facto* “another of the Emperor’s New Suit of Clothes” or, “on the other hand (…) collectively worthy of more detailed inclusion in models”.

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