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Impacts of inorganic nutrients on the physiology of a mixoplanktonic ciliate and its cryptophyte prey

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Abstract

Many marine planktonic ciliates retain functional chloroplasts from their photosynthetic prey and use them to incorporate inorganic carbon via photosynthesis. While this strategy provides the ciliates with carbon, little is known about their ability to incorporate major dissolved inorganic nutrients such as nitrogen and phosphorus. Here, we studied how ciliates respond to different concentrations of dissolved inorganic nitrogen and phosphorus. Specifically, we tested the direct and indirect effects of nutrient availability on the ciliate *Strombidium* cf. *basimorphum* fed the cryptophyte prey *Teleaulax amphioxeia*. We assessed responses in the rates of growth, ingestion, photosynthesis, inorganic nutrient uptake, and excretion. Our results show that the prey changed its carbon content depending on the nutrient concentrations. Low inorganic nutrient concentrations increased *S.* cf. *basimorphum* growth and prey ingestion. The higher carbon content of the prey under these low nutrient conditions likely supported the growth of the ciliate, while the higher carbon:nutrient stoichiometry of the prey led to the higher ingestion rates. The low carbon content of the prey at high nutrient concentrations resulted in reduced growth of *S.* cf. *basimorphum*, which indicates that carbon acquired via photosynthesis in the ciliate cannot compensate for the ingestion of prey with low carbon content. In conclusion, our findings show *S.* cf. *basimorphum* is not able to utilize dissolved inorganic nitrogen and phosphorus for growth, and this species seems to be well adapted to exploit its prey when grown at low nutrient conditions.

Keywords: protist, plankton, mixotrophy, inorganic nutrients, ciliates, cryptophytes, prey quality.

Introduction

Planktonic ciliates make up for a significant part of protist communities in marine systems and represent an important trophic link between primary producers and higher trophic levels (Calbet and Landry 2004; Calbet 2008). Many planktonic ciliate species sequester chloroplasts from their photosynthetic prey, and can keep them functional inside the cell (Stoecker et al. 2009). These species acquire the capability for phototrophy via ingestion of prey, and they cover their energetic requirements from both phototrophic and heterotrophic metabolisms. Plankton protists that are capable of obtaining nourishment via both phototrophy and phagotrophy, are defined mixoplankton (Flynn et al. 2019). Since these ciliates do not have the innate ability to photosynthesize, they are termed Non-Constitutive Mixoplankton (NCMs; (Flynn et al. 2019)). Different types of NCMs exist. NCM ciliates, like species within the *Mesodinium rubrum* species complex, are known to only exploit the chloroplasts from cryptophytes within the *Teleaulax/-Plagioselmis/Geminigera* clade (Hansen et al. 2012, Johnson et al. 2016), and are thus defined prey specialist NCMs (pSNCMs). Other species are referred to as generalist NCMs (GNCMs) because they can retain chloroplasts from many different algal groups (Stoecker et al. 1989; Johnson and Beaudoin 2019).

Prey specialist NCM ciliates can exert some control on the sequestered chloroplasts because they also retain prey nuclei (Johnson et al. 2007, Kim and Park 2019). These species can cover > 94 % of the daily carbon uptake from photosynthesis, and live as a complete autotroph for about 4 generations in the absence of prey (Smith and Hansen 2007). This is in contrast to the prey generalist NCM ciliates, which are unable to grow autotrophically when starved of prey (Schoener and McManus, 2012; Maselli et al., 2020). Generalist NCM ciliates in the genus *Strombidium* were shown to only grow when at least 50% of their carbon demands would derive from prey ingestion (Maselli et al. 2020; Hughes et al.

2021). In these *Strombidium* species, photosynthesis is more relevant for their nutrition when prey availability is limiting for their growth (Maselli et al. 2020; Hughes et al. 2021). However, the relative contribution of photosynthetic carbon fixation to the generalist NCMs nutrition has only been quantified in few species so far. Photosynthetic carbon fixation represents at least 16% of the total carbon uptake in the generalist NCM *Strombidium rassoulzadegani* (Schoener and McManus 2017) and 37% in the generalist NCM *Laboea strobila* (Stoecker et al. 1988), when incubated with an excess of prey. The ingestion rates of prey generalist NCM ciliates are similar to those of heterotrophic ciliate species. This suggests that, even if prey generalist NCM ciliates can fix carbon via photosynthesis, they rely on ingestion to obtain other essential elements, such as nitrogen (N) and phosphorus (P).

Little is known, however, about the ability of NCM ciliates to directly take up dissolved inorganic nutrients from the external environment. Uptake of inorganic N (both NO_3^- and NH_4^+) has been documented for the prey generalist NCM *S. rassoulzadegani* at rates comparable to those measured in exclusive heterotrophic ciliate species. However, inorganic N uptake does not seem to contribute significantly to its N requirements for growth (Schoener and McManus, 2017).

Strombidium species, either being mixotrophic or exclusively heterotrophic, have been shown to substantially contribute to the regeneration of inorganic nutrients through excretion (Dolan 1997). Nutrients that are regenerated through heterotrophic metabolism in NCM organisms are thought to be retained inside the cell to match the extra carbon obtained from photosynthesis, resulting in lower excretion compared to heterotrophic species (Ghyoot et al. 2017). This has never been empirically demonstrated, but would suggest the potential for stoichiometric homeostasis in these organisms. Stoichiometric homeostasis refers to the regulatory processes that allow an organism to maintain relatively constant elemental ratios (Hessen et al. 2013). In the context of ecological

66 stoichiometry, homeostasis is the consumer´s resistance to changes in response to the chemical
67 composition of the food (Persson et al. 2010).
68 Photosynthetic microalgae that serve as prey for ciliates exhibit a wide variety of physiological
69 adaptations to changes in nutrient availability. At optimal nutrient conditions, the major elements
70 (C, N, and P) are generally present in their biomass in relatively constant proportions, resembling
71 what is known as the Redfield Ratio, with an approximate molar C:N:P of 106:16:1 (Redfield 1934;
72 1958). However, if dissolved inorganic N or P becomes depleted, C incorporation through
73 photosynthesis is not balanced by the incorporation of the other nutrients, resulting in an increase in
74 the C:N and C:P ratios of the cells (Berman-Frank and Dubinsky 1999; Geider and La Roche 2002;
75 Sterner and Elser 2002). The elemental composition of the algal prey influences the excretion rates
76 of predators (Malzahn et al. 2010), and the metabolic cost for maintaining stoichiometric
77 homeostasis can affect predator growth rate (Boersma et al. 2008, Saikia and Nandi 2010). While
78 this is well documented in metazoan grazers, less is known about protozoan grazers. Protozoan
79 grazers are more likely to display their regulatory potential at the level of prey capture and ingestion
80 rather than at digestion as, differently from metazoan, they lack the gut where assimilation takes
81 place (Mitra and Flynn 2005). The strength of stoichiometric regulation seems to be different
82 among different protozoan grazers, where ciliates seem more strictly homeostatic compared to
83 flagellates and dinoflagellates (Golz et al. 2015). Additionally, mixotrophic protists were suggested
84 to be even more flexible in their stoichiometry and display an intermediate homeostatic ability,
85 where they are more flexible compared to strict heterotrophs, but less variable compared to strict
86 photoautotrophs (Katechakis et al. 2005, Chrzanowski et al. 2010, Moorthi et al. 2017).
87 To understand how prey generalist NCM ciliates respond to shifts in nutrient availability, we
88 studied the responses of *Strombidium* cf. *basimorphum* along a gradient of N and P concentrations.

Strombidium cf. *basimorphum* is a globally distributed aloricate ciliate species (Martin and Montagnes, 1993; Liu et al. 2011; Orsi et al. 2018), which recently has been shown to retain functional chloroplasts (Maselli et al., 2020). It can grow on a variety of species from different algal groups, but chlorophyte and cryptophyte prey species have been shown to best support its growth in culture (Maselli et al., 2020). Here, we measured growth, photosynthetic and nutrient uptake rates as well as ingestion and excretion rates of *S.* cf. *basimorphum* and the stoichiometry of its cryptophyte prey *Teleaulax amphioxeia*, when grown at different concentrations of nitrate and phosphate. Moreover, we monitored the response of the ciliate when subjected to prey starvation under the different nutrient treatments. In doing so we aimed to test the following hypotheses:

1) Reduced concentrations in dissolved inorganic nutrients in the medium will cause and increase in C:N and C:P stoichiometry of the cryptophyte prey.
2) The ciliate will respond to changes in prey stoichiometry by modulating its physiological rates.
3) Photosynthesis in *Strombidium* cf. *basimorphum* will compensate for the ingestion of prey biomass with relatively low C:N and C:P stoichiometry.
4) Despite being a mixotroph, *Strombidium* cf. *basimorphum*, cannot use dissolved inorganic N and P for growth, and dissolved inorganic nutrient conditions will therefore not influence the growth of *Strombidium* cf. *basimorphum* during prey starvation.

Materials and methods

Culture conditions: The experiments were conducted on cultures of the ciliate *Strombidium* cf. *basimorphum.* The culture was established from single cells isolated from Roskilde Fjord (Denmark) and identified via microscopy and molecular methods (Maselli et al. 2020). Cultures

were maintained for about two years in filtered seawater enriched with repeated additions of small volumes (1 to 10%) of *f*/2 media (Guillard 1975) with a salinity of 15. The ciliate was fed the cryptophyte *Teleaulax amphioxeia* (strain K-1837; SCCAP), which was separately grown in *f*/2 media (Guillard 1975) with the same salinity. All cultures were kept at 15°C, at a photon flux density of 70 µmol photons m^{-2} s^{-1} in a light: dark cycle of 16:8 h. The experiments were performed at the same salinity, light and temperature conditions used for cultures maintenance. The cryptophyte prey cultures were acclimated for two weeks (about 10-15 generations) to the different media before being used as prey for the ciliate. Prey cultures were daily diluted by 50% of volume with fresh media to keep stable nutrient concentrations. Ciliate cultures were acclimated in the same way to the different media for one week before the initiation of the experiments. During the acclimation period, culture of prey and ciliates were daily diluted with comparable volumes of fresh media to ensure that the nutrient concentration in the media was the same at the beginning of the experiment. The last three days of acclimation, ciliates were also acclimated to the prey availability they would have had at the beginning of the experiments (day0). To do that 20 ciliates mL^{-1} were daily inoculated with 3 x 10^4 prey cells mL^{-1}. Ciliate cultures were set up in triplicate of 300 mL each in 500 mL glass flasks (VWR borosilicate 3.3; 215-1594).

Experimental design: Experiments were carried out on ciliate growth, ingestion, photosynthesis and cellular chlorophyll-*a* (Chl*a*) content using media with increasing concentrations of inorganic N and P, corresponding to different dilutions of the standard *f*/2 media (Guillard 1975): *f/200* (very low nutrient conditions), *f/100* (low nutrient conditions), *f/40* (high nutrient conditions), *f/20* (very high nutrient conditions). The nutrient concentrations were selected to cover the range of nutrients typically used in culture studies as well as those found in natural environments. Micronutrients were added in the same amount in all the experiments in a concentration equivalent to what they would

have in *f*/100, which was demonstrated to be optimal in previous studies on *Strombidium* species (Gifford 1985). On day 0, triplicate cultures were fed with the same amount of prey they received during the acclimation period and ingestion rates were measured in the subsequent 24h. Samples for cell enumeration, Chl*a* content, photosynthetic rate and dissolved inorganic N and P were collected right after prey addition (day 0) and after 24h more (day 1). Triplicate monocultures of the algal prey were set simultaneously and sampled at the same time. Additionally, samples of prey biomass were collected on day 0 to analyze their elemental composition (C, N, and P). Ciliates grew for one week with no further addition of prey. The sampling for cell enumeration, Chl*a* content, photosynthetic rate and dissolved inorganic N and P was repeated when prey was completely depleted (day 4 and day 5). Ciliates and prey were enumerated daily for the duration of the experiment.

Cell enumeration, growth rates and ingestion rates: Daily samples of 10 mL were taken for cell enumeration from all experimental flasks. The algal prey cells were enumerated using a Cyto-Flex flow cytometer set to discern populations within a scatter plot of PCA5.5A (chlorophyll-a autofluorescence) x FSC-A (forward scatter), and count events were monitored for 60-120 seconds at a flow rate of 20 µL min^{-1}. Ciliates were enumerated in subsamples of 2 mL collected in a 24-well tissue culture plate and fixed with a 1% Lugol solution, using the inverted light microscope Olympus CKX53 at a magnification of 50×. Growth rates of both ciliate and algae were calculated between day 0 and day 1 assuming exponential growth, based on the change in cells abundance over time (µ, d^{-1}):

$$\mu = \ln (B_1/B_0)/(t_1-t_0) \quad (1)$$

Where B_0 and B_1 are the cell concentrations (cells mL^{-1}) at day 0 (t_0) and day 1 (t_1).

157 Ciliate ingestion rates (prey cells ciliate^{-1} d^{-1}) were calculated from the reduction in prey
158 concentration in the ciliate cultures compared to the prey monocultures over 24 h, as in Heinbokel et.
159 al. (1978):

160 $$\text{Ciliate ingestion rates} = B_{avg} \cdot F \quad (2)$$

161

162 were B_{avg} is the average algae concentration (cell mL^{-1}) in the mixed culture and F is the clearance
163 rate, respectively calculated as follow:

164

165 $$B_{avg} = (B_1 - B_0)/(\ln B_1 - \ln B_0) \quad (3)$$

166

167 $$F = (k-g) / P \quad (4)$$

168

169 in which k is the growth rate of the prey in the prey monoculture, g is grazing coefficient (growth
170 rate of prey in the mixed culture), P is the average ciliate concentration in the mixed culture
171 (calculated as for the prey in eq. 3).

172 ***Chlorophyll-a measurements:*** Ciliate Chl*a* content was measured on triplicate samples, each
173 consisting of 20 cells individually picked from the experimental bottles. Single ciliates were rinsed
174 in sterile-filtered media and directly added to 2 mL of 96% ethanol. The Chl*a* content of the algal
175 prey was measured by collecting 5 mL samples of the algal monocultures on glass microfiber filters
176 (Whatman, GF/F) and extracted in 5 mL of 96% ethanol. All samples were stored in the dark at 4°C
177 for 24 hours before being measured using a Turner Trilogy Fluorometer equipped with a Chl*a* non-
178 acid insert.

Photosynthetic rate measurements: the inorganic carbon incorporation of the ciliates (pg C $cell^{-1}$ h^{-1}) was measured on triplicate samples, each consisting of 20 cells individually picked from the experimental bottles, rinsed in clean media, and added to 2 mL of the same media spiked with 20 µL $NaH^{14}CO_3^-$ stock solution (specific activity 100 µCi mL^{-1}). Samples were incubated for three hours in the light. The passive incorporation of the isotope was also measured on triplicate samples prepared in the same way but incubated in the dark, and then subtracted from the light incubated values. The specific activity of all the samples was determined after the incubation by transferring 100 µl from each incubation vial into new vials containing 200 µl phenylethylamine. The incubation was interrupted by the addition of 2 mL 10% glacial acetic acid in methanol. Samples were dried overnight on a 65˚C heat plate, then re-suspended in 1.5 mL of distilled water to which 10 mL of Ultima Gold scintillation cocktail were added. Radioactivity was determined using Tri-Carb 2910 TR, Perkin-Elmer liquid scintillation counter. Carbon incorporation rates (C_{inc} = pg C $cell^{-1}$ h^{-1}) were calculated as follows:

$$C_{inc} = \frac{[(\text{lightDPM} - \text{darkDPM})/B] \ * \ \text{DIC} * 10\text{^}6}{\text{DPM specific activity} \ * \ \text{incubation time}} \quad (5)$$

Where DPM is disintegration per minute, B is cell concentration (cells mL^{-1}) and DIC is the inorganic carbon content of the medium (µg C mL^{-1}), which has been measured on 25 mL samples using a Shimadzu TOC-L analyzer. Incubation time is in hours.

Chlorophyll-*a* content of ciliates was used to calculate chlorophyll-specific photosynthetic rates (pg C (pg Chl*a*)$^{-1}$ h^{-1}). Photosynthetic rates of the algal control were measured in the same way on 2 mL of algal monocultures.

Dissolved inorganic nutrients and uptake rates: For assessing the concentration of inorganic nutrients and their uptake rates, 30 mL aliquots were collected from each experimental culture at day 0, day 1, day 4 and day 5. Culture samples were filtered through a 0.22 µm filter and stored at -20°C until subsequent analysis. Dissolved inorganic N and P were measured on a Seal Analytical Autoanalyzer, model AA3HR according to (Koroleff 1970) and (Solórzano and Sharp 1980). Nutrient uptake rate per cell (pg cell^{-1} d^{-1}) in the algal monocultures (H_{algae}) was calculated as:

$$H_{algae} = \frac{(R_0 - R_1)}{B_{avg}} \quad (6)$$

Where R_0 and R_1 are the dissolved nutrient concentrations (µg L^{-1}) at day0 and day1 and B_{avg} is the average algae number (cells L^{-1}) in the monoculture calculated as in (3).

The nutrient uptake rates of the algae in the monocultures (H_{algae}) were assumed to be the same for the algae in the mixed cultures and used to account for the nutrient removal of algae in the mixed culture. Algal nutrient removal was subtracted to the initial concentration of nutrient in the mixed culture to calculate ciliate uptake rates as follow:

$$H_{ciliate} = \frac{R_0 - (H_{algae} * B_{avg}) - R_1}{P} \quad (7)$$

Where B_{avg} here is the average algae number (cells L^{-1}) in the mixed cultures calculated as in (3) and P is average ciliate number (cells L^{-1}).

Prey elemental composition: For analysis of particulate organic C, N, and P, at least 1.0 x 10^7 prey cells from each experimental condition were collected in triplicate on glass microfiber filters (Whatman, GF/F) and stored in Petri dishes wrapped in tinfoil at -20°C. From each filter, a quarter

222 subsample was taken by cutting with a scalpel and used for CN analysis by folding it into a tin cup
223 (D013, Elemental Micro-analysis, Okehampton, UK). Samples were analyzed for particulate C and
224 N on a FLASH 2000 NC elemental analyzer (Brechbuhler Incorporated, Interscience B.V., Breda,
225 The Netherlands). Another quarter subsample of each filter was combusted in a Pyrex glass tube at
226 550°C for 30 min. Subsequently, 10 mL of persulfate solution (2.5%) was added, and samples were
227 autoclaved for 30 min at 121°C. Digested P (as PO_4^{3-}) was measured on a Quaatro auto-analyzer
228 (Seal analytical, Beun de Ronde, Abcoude, Netherlands).

Ciliate biovolume and carbon content:

Linear dimensions of ~90 ciliate cells for each experimental condition were acquired with the Olympus light microscope TH4-200 equipped with Olympus camera DP73 at a magnification of ×200 using the software CellSense. Cell biovolumes were calculated from cellular linear dimensions assuming cells had a spherical shape. Attempts to measure ciliate C, N, and PP contents failed. We were unable to separate the ciliates from the algal prey in the mixed cultures. Reverse filtration was initially tested to separate ciliates from prey, but it resulted in massive loss of ciliate biomass as cells broke on the filter, and the cellular content was washed out during filtration, thus preventing a reliable estimation of the ciliate cellular C, N, and P content.

Ciliate carbon content was calculated according to Putt and Stoecker (1989):

$$\text{Ciliate C content} = 0.19 \times V \quad (8)$$

where 0.19 is the estimated biovolume specific C content (pg C μm^{-3}) and V the ciliate volume (μm^3).

Ciliate gross growth efficiency and regeneration efficiency: Gross growth efficiency (GGE) was calculated as the amount of C that is ingested (C_I = pg $cell^{-1}$ d^{-1}) relative to the amount of C effectively assimilated into new ciliate biomass (μ_c = pg $cell^{-1}$ d^{-1}) between day 0 and day 1:

$$GGE = \frac{\mu_c}{C_I} \quad (9)$$

Ingested C was calculated according to the C content in the prey (C $cell^{-1}$), and the ciliate ingestion rate (prey cells $ciliate^{-1}$ d^{-1}, as in eq.2) in each experimental condition:

$$C_I = (\text{pg C cell}^{-1}) \times \text{ciliate ingestion rate}$$

(10)

The new ciliate biomass (μ_C) was calculated between day 0 and day 1 as the product of ciliate growth rates (μ, d^{-1}) at day 1 and the estimated C content (pg C $cell^{-1}$) in the ciliates:

$$\mu_C = \mu \times \text{ciliate C (pg cell}^{-1}\text{)} \quad (11)$$

As the carbon content of the ciliate was not measured (see above), the GGE calculated here implicitly assumes that ciliate carbon content does not vary in the different experimental conditions, thus it must be interpreted with caution. Regeneration efficiency was calculated based on N as the amount of the ingested N (pg N $cell^{-1}$ d^{-1}) relative to the amount of N excreted by the cells (pg N $cell^{-1}$ d^{-1}) between day 0 and day 1. To take into account the simultaneous removal of ammonium by the algal prey in the mixed culture, the ammonium uptake rate of the algae was multiplied by the average algal concentration between day 0 and day 1, and subtracted to the initial ammonium concentration at day0, as for the calculation of ciliate uptake rates in the mixed cultures (see above, eq.7).

Statistical analysis

Differences between treatments were assessed using ordinary one-way ANOVA followed by Tukey´s multiple comparisons test at significance level 0.05. Differences between treatments in well-fed and prey starved ciliates were assessed using two-way ANOVA followed by Tukey´s multiple comparisons test at significance level 0.05. For both types of analysis, data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Brown-Forsythe test) using Sigmaplot 14.0. Statistically significant differences are represented by letters on the respective plots. All results are presented as means ± standard deviation among experimental triplicates.

Results

Algal prey

Physiological rates and chlorophyll-a content

Monocultures of the cryptophyte *Teleaulax amphioxeia* grew at a lower rate in f/200 treatment compared to all other treatments (Table 1). Also, monocultures of *T. amphioxeia* in the f/200 treatment contained less Chl*a* compared to all other treatments (Table 1). The Chl*a* specific photosynthetic rates in *T. amphioxeia* monocultures were higher in the f/100 and f/40 treatments compared to in the f/200 and f/20 treatments (Table 1).

Inorganic nutrient uptake rates of *T. amphioxeia* were comparable in all treatments (Table *1*), apart from cultures grown in f/200, which displayed a significantly higher uptake rate of ammonium, and lower uptake rate of nitrate compared to the other treatments (Table 1).

Elemental composition

When grown at very low and low nutrient conditions (i.e. f/200 and f/100 media), the cryptophyte *T. amphioxeia* had higher C contents as compared to high and very high nutrient conditions, in f/40 and f/20, while N and P contents were not significantly different among any of the experimental treatments (Figure 1a). C:P ratios were significantly higher in the f/200 and f/100 treatments compared to the f/40 and in the f/200 compared to the f/20 (Figure 1b).

Ciliate predator

Physiological rates and chlorophyll-a content

All experiments were initiated with ~20 ciliates mL^{-1} and ~30 x 10^4 prey cells mL^{-1}. Ciliate abundance changed as function of prey availability. The highest ciliate abundances (90-120 cells

294 mL^{-1}) were recorded in all treatments on the days in which the prey was completely depleted, which
295 was on day 4 in cultures growing in f/200 and f/100, and day 5 in cultures grown in f/40 and day 4
296 in cultures grown in f/20 (Figure 2).
297 Right after prey depletion, ciliates declined in numbers in all cultures, apart from cultures grown in
298 f/100, which kept the same ciliate abundance in the following 24h. Prey abundances were
299 comparable across treatments at day 0, so ciliate growth rates calculated between day 0 and day 1
300 are not dependent on prey availability and comparable among treatments. Ciliate growth rates
301 between day 0 and day 1 were significantly higher in the f/200 and f/100 treatments (Figure 3a)
302 compared to the f/20 treatment, and these rates were comparable to the acclimation period
303 (Supplementary Table1). Thus, the ciliate seems to grow better in the low nutrient treatments.
304 Chl*a* contents in the ciliates measured on day 1 were significantly lower in the f/200 treatment
305 (Figure 4a) compared to ciliates in all other treatments on the same day. Likewise, the chlorophyll-
306 specific photosynthetic rates were significantly lower on day 1 in ciliates in f/200 treatment
307 compared with ciliates growing in all the other experimental treatments (Figure 4b). This is in
308 accordance with measurements done during the acclimation period (Supplementary Table 1), and
309 reflect the lower chlorophyll contents and photosynthetic rates of the prey when grown in the most
310 nutrient depleted treatment (f/200). Chl*a* contents were only significantly different between the
311 f/200 and the f/20 treatments in prey-starved ciliates at day 5 (Figure 4a). Only starved ciliates in
312 f/40 had significantly higher chlorophyll-specific photosynthetic rates compared to starved ciliates
313 of all the other experimental conditions (Figure 4b). Cellular Chl*a* contents declined in starved
314 ciliates in all experimental conditions (Figure 4a).
315 Prey ingestion rates by the ciliates were lower in the f/20 treatment as compared to the other
316 treatments (Figure 3a), which was also the treatment in which the prey contained less C. Ingestion

317 rates based on C (pg C ciliate^{-1} d^{-1}) and on N (pg N ciliate^{-1} d^{-1}) were significantly lower in ciliates
318 growing in f/40 and f/20 compared to f/200 and f/100 (Figure 3b). Ingestion rates based on P (pg P
319 ciliate^{-1} d^{-1}) were not significantly different among any experimental treatments (Figure 3b),
320 suggesting some potential of the ciliate to regulate ingestion rates based on the prey stoichiometry.
321 The total C uptake rates (sum of the ingestion and photosynthetic rates) by ciliates grown under the
322 lower nutrient conditions (f/200 and f/100) were greater than those grown under nutrient rich
323 conditions (Figure 5). Photosynthesis represented a relatively larger proportion of the total C uptake
324 in ciliates grown in the f/40 and f/20 (~20%) treatments compared to ciliates grown in the f/100
325 (~10%) and f/200 (~5%) treatments. This was mainly driven by higher ingestion rates, and partly by
326 reduced photosynthesis under lower nutrient conditions (Figure 5).

327 ***Cell size, gross growth efficiency and inorganic nutrient uptake and regeneration***

328 Ciliate cell sizes were comparable among experimental treatments (day 0) and declined during prey
329 starvation (day 5) in the same proportion in all conditions (Supplementary Table 2). Nitrate and
330 phosphate uptake by the ciliates was very low (e.g. <0.1% d^{-1} of the cellular N content), and in
331 some cases too low to be calculated given the algal nutrient uptake. Ciliate gross growth efficiency
332 (GGE) calculated between day 0 and day 1, were similar (~40-50%) and comparable across
333 experimental treatments, indicating that about 50% of the ingested C was converted into new ciliate
334 biomass. Specifically, GGE was 45.7 ± 3.2 in the f/200 medium, 40.3 ± 7.3 in the f/100, 49.0 ± 10.0 in the
335 f/40 and 40.5 ± 11.6 in the f/20.
336 Ammonium excretion rates by the ciliate, calculated between day 0 and 1, were much higher in the
337 f/20 treatment (12.3 ± 2.4 pg N cell^{-1} h^{-1}) compared to the f/40 treatment (3.2 ± 2.7 pg N cell^{-1} h^{-1})
338 and the f/100 treatment (5.4 ± 0.45 pg N cell^{-1} h^{-1}). The ammonium excretion rates could not be
339 calculated in the f/200 treatment, because the ammonium concentration at day 0 was lower in the

prey monoculture compared to the mixed culture. For this reason, it was not possible to assume that the algae would have had the same uptake rate in these two different cultures. Consequently, N regeneration efficiency in the f/200 treatment was not calculated either.

For the other treatments, N regeneration efficiencies based on ciliate ingestion and excretion rates between day 0 and day 1 were ~4% in the f/100 and f/40 treatments (3.8 ± 0.9 and 3.6 ± 3.2, respectively) and ~ 20% in f/20 (18.5 ± 8.6). Regeneration of P was observed at the end of all the experiments since phosphate concentration was higher at day 5 compared to the concentration in the previous time points (Supplementary Figure 1d). However, since cultures were not axenic and bacterial abundance was relatively high at the end of the experiments (see Supplementary material), this could not solely be attributed to ciliate excretion, and regeneration efficiency was therefore not calculated.

Discussion

Effects of major inorganic nutrients availabilities on physiological rates of ciliates

Our results clearly indicate that the ciliate *Strombidium* cf. *basimorphum* is not able to utilize major dissolved inorganic nutrients (N and P) for growth. Indeed, cultures of *S.* cf. *basimorphum* declined in abundance as soon as prey was depleted in all experimental treatments, regardless of the availability of dissolved inorganic nutrients. This pattern is commonly observed in heterotroph protists. Despite this, inorganic C was still incorporated and Chl*a* specific photosynthetic rates were similar or even higher during prey starvation compared to Chl*a* specific photosynthetic rates of feeding cells. A similar increase of the chlorophyll-specific photosynthetic rate during starvation has been observed in previous studies on this ciliate species (Maselli et al. 2020, Hughes et al. 2021). This might have resulted from reduced self-shading of the chloroplasts within the cells, as the number of chloroplasts is likely to be lower in starved cells compared to cells that feed and can

363 sequestrate chloroplasts from the prey. Nitrate uptake rates detected in *S.* cf. *basimorphum* when
364 prey starved were very low and similar to rates measured in the mixotrophic *S. rassoulzadegani*
365 and heterotrophic *Strombidinopsis* sp. (Schoener and McManus, 2017). Uptake of dissolved
366 inorganic P in *S.* cf. *basimorphum* could not be measured in any of the treatments, suggesting that it
367 only relies on ingestion to incorporate P. This is in line with earlier studies on the mixotrophic
368 ciliate, *Strombidium viride* (Taylor and Lean 1981).

369 Growth rates of *S.* cf. *basimorphum* were lower in treatments with higher dissolved inorganic N and
370 P concentration (f/20-40) compared to treatments with lower dissolved inorganic N and P
371 concentration (f/100-200). Other *Strombidium* species have been successfully cultured in media
372 with inorganic nutrient concentrations even 10 times higher than what was used in these
373 experiments (i.e standard f/2 or NEPCC media), and achieved growth rates comparable to the
374 higher growth rates measured here (Montagnes 1996, Mcmanus et al. 2018). At the same time,
375 similar growth rates were measured in *Strombidium* species cultured in filtered seawater only
376 enriched with chelated micronutrients at an optimal concentration (Gifford 1985). Thus, the effects
377 of dissolved inorganic nutrients on ciliate growth rates might rather have indirectly derived from the
378 different prey types used in the previous studies (Gifford 1985; Montagnes 1996; McManus et al.,
379 2012). Different prey species are expected to exhibit a different biochemical composition, which
380 would change depending on the experimental conditions, with indirect effects on the physiology
381 and growth of the ciliates.

382 **Effects of major inorganic nutrients availabilities on prey quality**

383 The concentration of dissolved inorganic N and P directly affected the elemental content of the prey
384 *Teleaulax amphioxeia*, as well as its photosynthetic performance. The low availability of dissolved

385 inorganic nutrients in the f/200 treatment led to reduced cellular chlorophyll-*a* content and
386 chlorophyll-*a* specific photosynthetic rates of *T. amphioxeia* in monoculture (Table1). This was also
387 reflected in the chlorophyll-*a* content and chlorophyll-*a* specific photosynthetic rates measured on
388 *S. cf. basimorphum* in the mixed cultures. When the prey *T. amphioxeia* was grown in nutrient
389 depleted conditions (f/200 and f/100), it also exhibited a higher C content compared to the
390 treatments with more inorganic nutrients (f/40 and f/20). This is in accordance with the literature,
391 where it has been shown that photosynthetic organisms under suboptimal growth conditions will
392 accumulate C storage compounds, like starch or glucans (Berman-frank and Dubinsky 1999
393 Malzahn et al. 2010). The relatively higher C:P ratio in the biomass of *T. amphioxeia* grown in low
394 nutrient treatments (f/200 and f/100) indicates a higher stoichiometric plasticity for P and thereby
395 the ability of this alga to deal with low inorganic P concentrations. The C:N ratios in the
396 monocultures of *T. amphioxeia* were similar in all treatments and close to the Redfield ratio (i.e.
397 C:N ~ 6.6, molar basis), suggesting that the species is more homeostatic for N. Following the
398 changes in P, the N:P ratios increased in the low nutrient treatments, and was up to three times the
399 Redfield ratio (i.e. N:P=16) in all experimental treatments.

400 **Effects of prey stoichiometry on ciliate ingestion, GGE and excretion rates**

401 The C, N, and P contents of the algal prey were reflected in the ingestion rates of the ciliates.
402 Higher ingestion rates were observed in the low nutrient treatments (f/200 and f/100), where algal
403 cells contained relatively less P compared to C and N. Phosphorus-specific ingestion rates were not
404 different among treatments despite the different C:P ratios of the prey. This suggests that the ciliate
405 can adjust its ingestion rate to satisfy its P requirements, through what has been described as
406 positive stoichiometric modulations (Mitra and Flynn 2005). Similar to positive stoichiometric
407 modulation, compensatory feeding has already been observed in heterotrophic ciliate species (Suida

and Dam 2010). In nutrient rich treatments, where the prey had relatively lower C:P, *S. cf. basimorphum* seems to display a negative stoichiometric modulation (Mitra and Flynn 2005) by decreasing its ingestion rates. The modulation of ingestion based on prey stoichiometry has already been observed in other ciliates, which showed to preferentially feed on prey with relatively higher C:P (Gruber et al. 2009). The lower ingestion and growth rates in the nutrient rich treatments can actually be attributed to differences in the stoichiometry of the prey. Carbon acquired from photosynthesis was apparently not sufficient to compensate the lower C acquired via ingestion in the nutrient rich treatments and sustain cell division. Changes in C:N:P stoichiometry of the ciliates are likely to occur, especially during starvation when C fixation proceeds while no other nutrients are incorporated. Unfortunately, the potential stoichiometric changes of the ciliate biomass could not been tested here, since we were unable to separate ciliates from the algal prey, which is required to obtain reliable estimates of ciliate C, N and P contents (see methods section).

Ingested N was obviously in excess in all treatments, and *S. cf. basimorphum* excreted it as ammonium at rates comparable to what was measured in the heterotrophic species *Strombidium sulcatum* (Ferrier-Pages and Rassoulzadegan 1994). The *S.* cf. *basimorphum* cultures were not axenic, but N regeneration from bacteria has previously been shown to be insignificant compared to regeneration associated with the grazing activity of *Strombidium sulcatum* (Ferrier-Pages and Rassoulzadegan 1994), also when taking the concentrations of bacteria into account (see supplementary material). As the ammonium concentrations at day 0 in the f/200 treatment were lower in the mixed cultures compared to prey monocultures, we could not estimate the ammonium excretion rates from ciliates in the f/200 treatment. However, a higher uptake rate of ammonium by the algal prey might be one reason for the lower ammonium concentration measured at the end of

the experiments (day 4-5) in mixed cultures in the f/200 treatment compared to the values measured in the other treatments.

Ecological implications

The ciliate *S.*cf *basimorphum* was isolated from a temperate estuary, Roskilde Fjord (Denmark), where ciliates dominate the microplankton community throughout the year (Haraguchi et al. 2018). In Roskilde Fjord, prey generalist NCM ciliates constitute up to 90% of the ciliate biomass in late spring/early summer, when dissolved inorganic N concentrations are < 5 µM (our f/200 and f/100 treatments), and the biomass of potential prey is relatively low (Haraguchi et al 2018). This suggests that in this environment, where prey is limiting, mixotrophy represents a competitive advantage for ciliates over heterotrophy. Moreover, this may explain why *S.*cf *basimorphum* seems to be well adapted to exploit its prey, maximizing growth and ingestion rates, when dissolved inorganic nutrient concentrations are low.

The cryptophyte *T. amphioxeia,* is known to represent a good prey for grazers because of its high nutritional value, also reflected by its use in aquaculture (Lee et al. 2019). Similar to other mixotrophic *Strombidium* species, *S.* cf *basimorphum* effectively exploits other prey species such as chlorophytes (Stoecker et al. 1989; McManus et al. 2018; Maselli et al., 2020). The suitability of prey types other than cryptophytes and chlorophytes seems, however, to depend on species-specific characteristics and/or interactions (Montagnes 1996; Maselli et al., 2020).

Small photosynthetic flagellates are considered good prey species for generalist NCM ciliates (Stoecker et al. 1989; McManus et al. 2018; Maselli et al., 2020), and small flagellates are typical of mature ecosystems, where primary production is supported by nutrient recycling within the microbial loop (Falkowski et al., 1998). Highly productive and rapidly developing systems (i.e. the spring bloom) in temperate regions are generally dominated by diatoms, which do not support the

453 growth of *S.*cf *basimorphum* and other prey generalist NCM ciliates (McManus et al., 2012; Maselli
454 et al., 2020). Indeed, while peaks in biomass of both heterotrophic and mixotrophic grazers occur in
455 temperate regions as a response to the typical spring bloom, the relative contribution of mixotrophic
456 ciliates to the total ciliate biomass is much greater during mid-summer, when nutrient
457 concentrations are low (Haraguchi et al. 2018). The lack of specialization on any prey type renders
458 prey generalist NCMs more dependent on prey ingestion compared to specialist NCM. Yet, being
459 generalist potentially provides the benefit to access to different types of biomolecules coming from
460 a diverse diet. Indeed, a high variability in cellular stoichiometry and biochemical composition
461 exists within classes of marine photosynthetic plankton (Geider & LaRoche 2002; Garcia et al.
462 2018), which explains why a mixed diet could support better growth of grazers, including ciliates,
463 compared to a mono-diet (Montagnes 1996; Yang et al. 2019).

464 **Conclusion**

465 Our findings show that the ciliate *S.* cf. *basimorphum* is not able to utilize dissolved inorganic N
466 and P for growth. Dissolved nutrient concentrations have indirect effects on the ciliate growth,
467 imputable to the prey elemental composition. The C that the ciliate acquires via photosynthesis
468 cannot compensate for the lower C content of prey acquired via ingestion under nutrient rich
469 conditions. Lastly, *Strombidium* cf. *basimorphum* expresses some stoichiometric regulatory
470 potential at the level of prey capture, adjusting ingestion rates depending on prey elemental
471 composition.

472 **Declarations**

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477 **Conflicts of interest/Competing interests**

478 The authors declare that they have no conflict of interest.

479 **Ethics approval**

480 Not applicable

481 **Consent to participate**

482 Not applicable

483 **Consent for publication**

484 Not applicable

485 **Availability of data and material**

486 All data produced for this study are provided in this manuscript.

487 **Code availability**

488 Not applicable

489 **Authors' contributions**

490 MM and PJH conceived and designed the experiments. MM performed the experiments. DVW
491 analyzed the biomass samples. MM analyzed the data and drafted the manuscript; DVW and PJH
492 contributed to write the final version of the manuscript.

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643

644 Table 1 Growth rates, chlorophyll-a content, photosynthetic rates, and ammonium, nitrate and
645 phosphate uptake rates by the cryptophyte *Teleaulax amphioxeia* in the different experimental
646 treatments. Values denote mean ± SD (n=3)

***T. amphioxeia* in monocultures**						
Media	**Growth rate (d^{-1})**	**Chl*a* content (pg Chl*a* $cell^{-1}$)**	**Phot.rate (pg C (pg Chl*a*)$^{-1}$ h^{-1})**	**NH_4^+ uptake (fg N $cell^{-1}$ h^{-1})**	**NO_3^- uptake (fg N $cell^{-1}$ h^{-1})**	**PO_4^{3-} uptake (fg P$cell^{-1}$ h^{-1})**
f/200	0.42 ± 0.09	0.32 ± 0.00	1.86 ± 0.04	14.8 ± 1.0	74 ± 10	8.1 ± 4.5
f/100	0.80 ± 0.05	0.41 ± 0.02	1.98 ± 0.10	5.6 ± 2.2	119 ± 25	12.6 ± 1.9
f/40	0.82 ± 0.06	0.40 ± 0.01	2.02 ± 0.10	5.4 ± 0.2	141 ± 5	17.9 ± 1.6
f/20	0.65 ± 0.02	0.47 ± 0.03	1.66 ± 0.11	8.1 ± 0.2	128 ± 5	20.1 ± 11.5

647

Figure legends

Fig. 1 a) Carbon, nitrogen and phosphorus content (pg $cell^{-1}$) of *Teleaulax amphioxeia* in the different experimental treatments. b) Molar ratio between elements in *T. amphioxeia* biomass in the different experimental treatment (to be compared with theoretical Redfield values C: N =6.6; C: P= 106; N: P=16). Values denote means ± SD (n=3). Letters represent statistically similar groupings across treatments. Columns without letters did not show significant differences among treatments

Fig. 2 Development of cultures in the different experimental treatments as a function of time. Abundances (cells mL^{-1}) of a) the ciliate *Strombidium* cf. *basimorphum* and b) the prey, *T. amphioxeia* during the incubation. Values denote means ± SD (n=3)

Fig. 3 *Strombidium cf. basimorphum* growth and ingestion rate between day 0 and day 1 in the experimental treatments, with growth as function of a) prey ingestion, and b) ingestion rates base on C, N and P. Values denote means ± SD (n=3). Letters represent statistically similar groupings across treatments. Columns without letters did not show significant differences among treatments

Fig. 4 *Strombidium* cf. *basimorphum* a) Chl*a* content and b) photosynthetic rate, measured at day 0 and day 5 in the different experimental treatments. Values denote means ± SD (n=3). Letters represent statistically similar groupings among treatments and among fed and starved cells

Fig. 5 Daily carbon uptake rates from ingestion and photosynthesis in *S.* cf. *basimorphum* between day 0 and day 1 in the different experimental treatments. Values denote means ± SD (n=3)

Figure 1

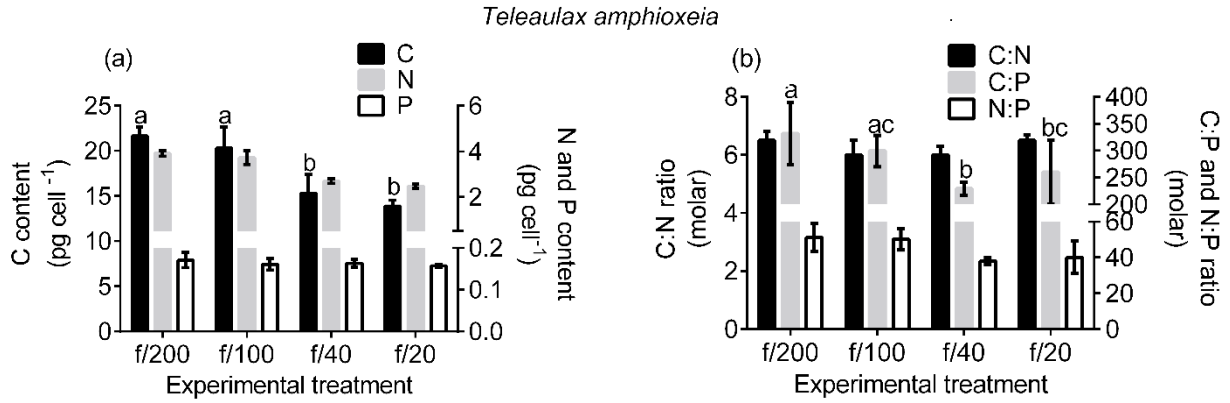
Teleaulax amphioxeia
(a)
C
N
P
C content (pg cell-1)
N and P content (pg cell-1)
a
a
b
b
f/200
f/100
f/40
f/20
Experimental treatment
(b)
C:N
C:P
N:P
C:N ratio (molar)
C:P and N:P ratio (molar)
a
ac
b
bc
Experimental treatment

Figure 2

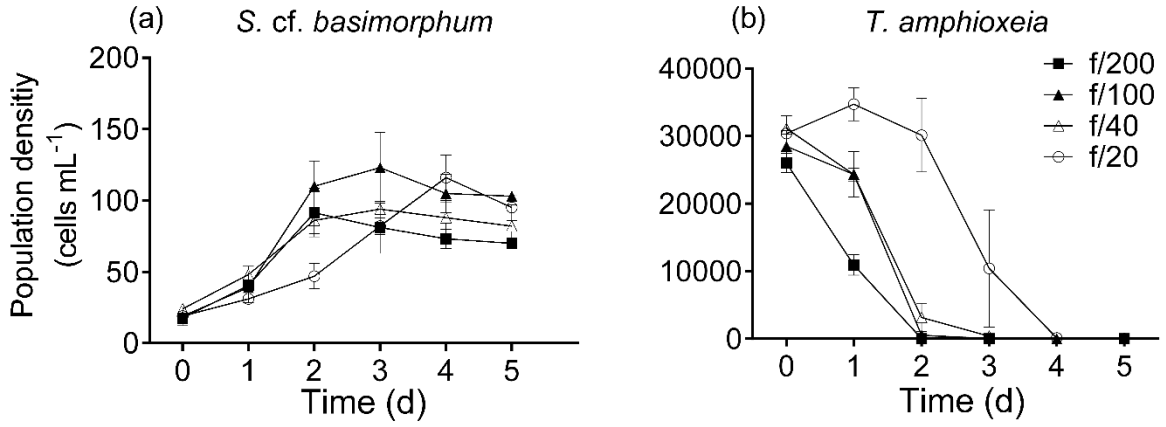

Figure 3

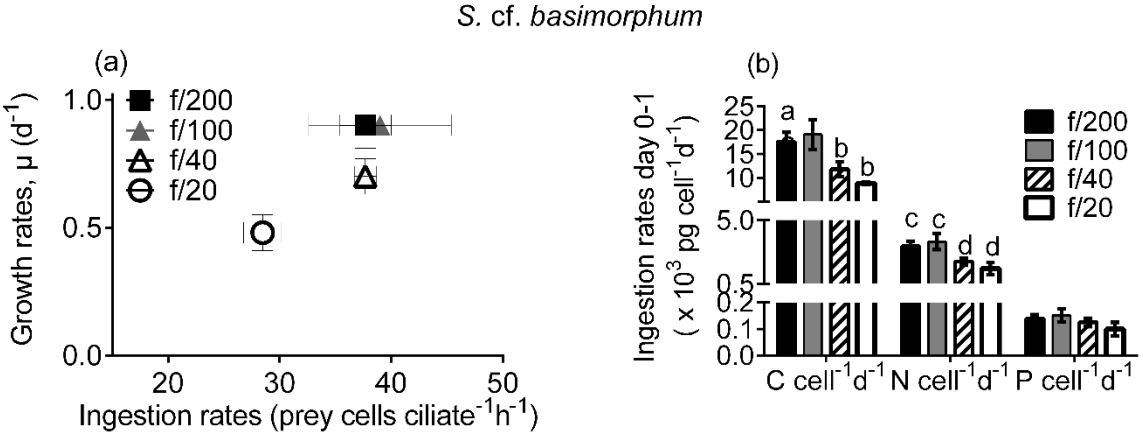

Figure 4

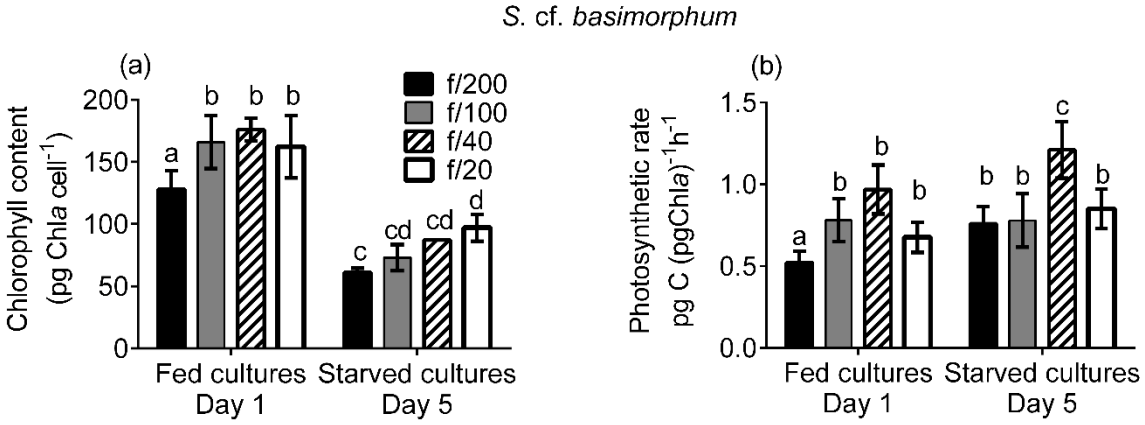

Figure 5