Eco-physiological traits of mixotrophic *Strombidium* spp.

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Abstract:

Ciliates represent an important trophic link between nanoplankton and mesoplankton. Many species acquire functional chloroplasts from photosynthetic prey, being thus mixotrophs. Little is known about which algae they exploit, and of the relevance of inorganic carbon assimilation to their metabolism. To get insights into these aspects, laboratory cultures of three mixotrophic *Strombidium* spp. were established and thirty-five photosynthetic algal species were tested as prey. The relative contributions of ingestion and photosynthesis to total carbon uptake were determined, and responses to prey starvation were studied. Ciliate growth was supported by algal species in the 2-12 μm size range, with cryptophytes and chlorophytes being the best prey types. Inorganic carbon incorporation was only quantitatively important when prey concentration was low (3-100 μgCL^{-1}), when it led to increased gross growth efficiencies. Chl*a* specific inorganic carbon uptake rates were reduced by 60 to 90% compared to that of the photosynthetic prey. Inorganic carbon uptake alone could not sustain survival of cultures and ciliate populations declined by 25-30% during five days of starvation. The results suggest that mixotrophy in *Strombidium* spp. may substantially bolster the efficiency of trophic transfer when biomass of small primary producers is low.

Introduction

Oligotrich ciliates are a major component of microzooplankton in pelagic and coastal waters (Johansson *et al.*, 2004; Haraguchi *et al.*, 2018), representing an important trophic link between small primary producers and larger zooplankton (Calbet, 2008). Microzooplankton can consume up to the 75% of the daily primary production (Schmoker *et al.*, 2013), thus microzooplankton productivity and trophic efficiency can have a major impact on element and energy transfer within the planktonic food web and on biogeochemical fluxes (Calbet and Landry, 2004; Buitenhuis *et al.*, 2010). Traditionally, planktonic ciliates in food web and ecosystem models are regarded as size selective heterotroph grazers with a typical growth efficiency of 30-50% (Gismervik 2005; Yang et al. 2015). However, in the euphotic zone, about the 30% of oligotrich ciliates biomass is accounted for species that retain functional plastids from their prey, acquiring the ability to photosynthesize (Stoecker *et al*., 1987; Putt, 1990a; Stoecker *et al*., 2009), and thus being non-constitutive mixotrophs (Mitra *et al.*, 2016).

Mixotrophy can lead to increased growth efficiencies in oligotrich ciliates, especially in conditions of limiting prey availability (Schoener and McManus, 2017). The increase in growth yield gained from photosynthesis is therefore crucial in the parametrization of ciliates carbon budget in plankton ecosystem models (Mitra *et al.*, 2014; Ghyoot *et al.*, 2017). Despite that, very few data on the contribution of photosynthesis to the carbon budgets of mixotrophic ciliates are available in the literature and even less relate it to prey availability. The main reason for this lack of quantitative data is the difficulty in keeping stable laboratory cultures (Gifford, 1985; Jonsson, 1986; McManus *et al*., 2018).

The few studies carried out on the ecophysiology of mixotrophic oligotrichs suggest that although they are prey generalists, not all photosynthetic prey may support their growth (Gifford 1985; McManus et al. 2018). Nevertheless, it is currently not known to which extent these ciliates can grow on different algal groups, or if some degree of prey preference exists. Mixotrophic oligotrichs may have high ingestion rates, in the range of 50-100 prey cells h^{-1} when prey is abundant (Stoecker *et al*., 1988a; Gismervik, 2005). Photosynthesis in these ciliates seems insufficient to sustain cell division but can cover respiratory requirements (Stoecker *et al.*, 1988 a,b; McManus *et al*., 2018). Mixotrophic oligotrichs do not seem to retain prey nuclei (Laval-Peuto and Febvre, 1986; Stoecker *et al*., 1988a), or to express genes related to maintenance of plastids (Santoferrara *et al.*, 2014). Thus, it is possible that the functionality of the sequestered plastids is affected by aging upon

sequestration. For this reason, they seem to be dependent on continuous ingestion of prey, not only for nutrition, but also as chloroplast supply.

Current knowledge of ecophysiology of mixotrophic oligotrichs is built on studies of very few species, and there is a need to study more species to be able to make generalizations.

We therefore established cultures of three species from the field to investigate: 1) the prey size spectra they can exploit, and which algal taxa better sustain their growth, 2) growth, ingestion and inorganic carbon uptake rates at different prey abundances, and 3) effects of prey starvation on growth and photosynthesis

Materials and methods

Algal cultures

Thirty-five algal cultures were used as prey for the ciliate cultures (Table 1). The cultures were mainly provided by the Scandinavian Culture Collection of Algae and Protozoa (SCCAP), and the Bigelow National Center for Marine Algae and Microbiota (NCMA). Stock algal cultures were maintained in *f*/2 media based on filtered seawater (FSW) from the Øresund, Denmark, at a salinity of 15. Aliquots of dense algal stock culture were diluted in FSW, with no addition of nutrients, before being used as prey for ciliates. The cultures were kept at 15 °C and at an irradiance of 70 μmol photons $m^{-2}s^{-1}$ on a light:dark cycle of 16:8h. Algal growth was monitored and only exponentially growing algal cultures were used as prey. Algae were fixed in Lugol´s (2% final conc.) and enumerated using an inverted light microscope (Olympus CKX53) at a magnification of 100X in Sedgewick-Rafter chambers. The equivalent spherical diameter (ESD) of most of the algal species was measured by a Beckman Coulter Counter Multisizer 3.3. The ESDs of algal species, which size was outside the range of sensitivity of the instrument (<3μm), were measured manually with an Olympus light microscope TH4-200 equipped with Olympus camera DP73 at a magnification of 400x using the software CellSense. ESD measurements allowed the calculation of algal biovolume as: $4/3\pi*(ESD/2)^3$.

Isolation and maintenance of ciliate cultures

Three ciliates species were isolated from natural water samples collected in Roskilde Fjord (South of Frederiksværk, Denmark) on June 2nd, 2018. Individual cells were isolated using a drawn glass capillary pipette under an Olympus SZ61 dissection microscope (X10-50 magnification) and

transferred to sterile-filtered seawater (FSW) from the location several times to remove other protists. In the end, single cells were added to FSW enriched with either monocultures of cryptophytes (*Teleaulax amphioxeia* and *T. acuta*) or green algae (*Nephroselmis rotunda*, *Pyramimonas mitra* and *Tetraselmis chui*), or mixtures of these algae. Successful isolates were subsequently kept in culture in FSW at a salinity of 15, temperature of 15°C and a 16:8 light: dark cycle at an irradiance of 70 μmol photons $m^{-2}s^{-1}$, and fed either *T. amphioxeia* or *N. rotunda*. Cultures were maintained in 24 wells tissue-culture dishes (well volume of 2 mL) and in glass bottles (volume of 50 to 150 mL). The ciliate cultures were subcultured weekly. Any attempt to upscale *Strombidium* sp. 3 into glass bottles failed, so experiments 2 and 3 were only conducted with the two other ciliates species. 28S and 18S gene sequences were used to aid in the identification of the ciliates species (sequences were obtained and analyzed as described in the Supplementary Material).

Experiment 1. Prey size and prey type spectra

Up to 35 different species of photosynthetic algae were tested as monocultures as prey for the three ciliate species to get insights into the algal prey sizes and taxonomic groups that led to successful growth. The prey algae covered the size range: <1 to 15 µm in ESD (equivalent spherical diameter). This experiment was carried out in 24 wells tissue-culture dishes harbouring 2 mL of algal suspension. Each prey species was tested in six replicate wells for each ciliate species. Prey was added at a final prey biovolume of 6.25×10^6 μm^3 mL^{-1}. Ten starved ciliates were subsequently added to each well, using a drawn micropipette. Temperature, irradiance, and medium composition were the same as for cultures maintenance. The ciliates were allowed to grow for five days, during which the plates were inspected by live observation on a stereomicroscope. A value rank was assigned to the growth of each of the ciliates species for each of the tested prey algae: "-1", if less than 10 ciliate cells were still present at the end of the five days, "0" if the number of cells remained constant, "1" if they were ~ 20 cells, and 2 if there were > 20 cells. Prey species that successfully sustained ciliate growth during these 5 days experiments were tested for long term maintenance of ciliate cultures on a single prey species.

Experiment 2. Growth rate, prey ingestion, cellular Chl*a* and photosynthesis of two *Strombidium* species at three different prey concentrations

To measure the contribution of carbon derived from photosynthesis and prey ingestion in presence of different prey concentrations, experiments were set up with three prey concentrations in triplicates. Based on the results from preliminary experiments (see Supplementary Material), cultures of *Strombidium* cf. *conicum* (45 ciliates mL^{-1}) were acclimated to *T. amphioxeia* average concentrations of 3.0, 100 and $1.3x10^3$ $\mu gCmL^{-1}$, while *Strombidium* cf. *basimorphum* cultures (20 ciliates mL^{-1}) were acclimated to *T. amphioxeia* average concentrations of 6, 25 and 800 $\mu gCmL^{-1}$. Average prey concentration were calculated as:

$$Cavg = (C1-C0)/LN (C1/C0) \quad (1)$$

where C0 is the initial prey concentration and C1 is prey concentration after 24h.

Acclimation to the prey concentration was carried out in 500 mL glass flasks having a water volume of 300 mL. Stock cultures were incubated with the desired prey concentration for two days adjusting ciliates and algae concentration every 24h. After the two days of acclimation, the cultures were split into triplicate bottles and incubated for another 3 days, adjusting concentrations of ciliates and prey every 24h. 6 mL samples were withdrawn for cell enumeration, transferred to 24 well tissue culture plates, fixed in Lugol´s and enumerated using an inverted light microscope (Olympus CKX53) at a magnification of 50X. Ciliate growth was measured as change in cell abundance over time and calculated assuming exponential growth (μ, d^{-1}):

$$\mu = \ln (N1/N0)/(t1-t0) \quad (2)$$

Where N1 and N0 are the cell mL^{-1} at time 0 and time 1.

Monocultures of *T. amphioxeia* in triplicates were also set up allowing for the calculation of prey ingestion rates. Ingestion rate (IR: prey cells $ciliate^{-1}day^{-1}$) were calculated from reduction in prey concentration in grazing treatments compared to control treatments with the prey algae alone, over 24 hours. Frost equations was applied as modified by Heinbokel *et al.*, (1978). Carbon content of *T. amphioxeia* (10 pg/cell) was calculated applying the volume to carbon regression for protist plankton as in Menden-Deuer and Lessard (2000). Prey and ciliates carbon content have been used to convert the IR into carbon specific ingestion rate (d^{-1}) as follow:

$$\text{Carbon specific ingestion} = \frac{\text{cellular carbon content of the prey (pg)} * \text{IR (prey cells ciliate}^{-1}\text{day}^{-1})}{\text{cellular carbon content of the ciliate (pg)}} = d^{-1} \quad (3)$$

Daily, samples (6mL) were also withdrawn for measurements of Chl*a* and photosynthetic rates.

Chlorophyll a measurements. For ciliate Chl*a* measurements, 20 ciliate cells from each experimental bottle were picked with a drawn micropipette, rinsed in FSW several times and added to 2 mL of 96% ethanol. Chl*a* content of the algal control was also measured by collection of 2 mL of algal suspension onto glass microfiber filters (Whatman, GF/F), which was extracted in 5 mL of 96% ethanol. Samples were then stored in the dark at 4°C for 24 hours and Chl*a* was quantified using a Turner Trilogy Fluorometer equipped with a Chl*a* non-acidification insert.

Photosynthetic rate measurements. Photosynthetic rates of ciliates were measured on triplicate samples each one containing twenty ciliates singularly picked from each experimental bottle with a drawn Pasteur pipette, applying the ^{14}C technique by Rivkin and Seliger (1981). Ciliates cells were rinsed in FSW and incubated for 3 hours in 23-mL glass scintillation vials filled with 2mL of FSW in which 20 µL $NaH^{14}CO3^-$ stock solution (specific activity 100 µCi mL^{-1}) was added. Incubations were carried out simultaneously in the light and in the dark to compensate for passive incorporation of the isotope. Specific activity was determined after the incubation by transferring 100 µl from each incubation vial into new vials containing 200 µL phenethylamine . The remaining volume of each sample was acidified with 2 mL 10% glacial acetic acid in methanol and dried overnight on a 65˚C heat plate. Dried samples were re-suspended in 1.5 mL of distilled water. Ten mL of Ultima Gold scintillation cocktail were then added and radioactivity was determined using Tri-Carb 2910 TR, Perkin-Elmer liquid scintillation counter.

Carbon incorporation rates (P= pgC $cell^{-1}$ h^{-1}) were calculated as follows:

$$\mathrm{P} = \frac{[(\text{lightDPM} - \text{darkDPM})/\text{n° of cells}] \ * \ \mu\text{gC/mL} * 10\text{^}6}{\text{DPM specific activity} \ * \ \text{incubation time(h)}} \quad (4)$$

Where DPM is disintegration per minute and μgC/mL refers to the inorganic carbon content of the medium.

The total inorganic carbon in the culture medium has been measured on 25 mL samples collected in glass vials and analysed with a Shimadzu TOC-L analyser. The hourly photosynthetic rate (pgC

206 cell^{-1} h^{-1}) was used to calculate the daily photosynthetic (pgC cell^{-1} d^{-1}). Daily photosynthetic rate
207 was used to calculate carbon specific photosynthetic rate (pgC pgC^{-1} d^{-1}= d^{-1}) and chlorophyll
208 specific photosynthetic rate (pgC pgChl*a*$^{-1}$d^{-1}= C Chla^{-1} d^{-1}). Photosynthetic rates of the algal control
209 were also measured on 2 mL of algae monoculture, spiked with 20 µL $NaH^{14}CO_3^-$ stock solution
210 (specific activity 100 µCi mL^{-1}) and incubated simultaneously in the light and in the dark. At the
211 end of the incubation, specific activity has been determined and the remaining volume of each
212 sample was acidified and processed as described for ciliates. Carbon incorporation rates were
213 calculated according to eq. (4).
214
215 ***Carbon content and gross growth efficiency.*** Gross growth efficiency (GGE) was calculated as the
216 percentage of the ingested carbon (pgC cell^{-1} day^{-1}) effectively converted into new ciliates biomass
217 (pgC cell^{-1} d^{-1}).
218 Ciliate biomass production was calculated as:

219 $$\text{Biomass production} = \mu_y \times C_y \qquad (5)$$

220 where μ_y is growth rate (μ, d^{-1}) and C_y is the average ciliates carbon content (pg C cell^{-1}).

221 Ciliate carbon content was calculated according to Putt and Stoecker, 1989 as:

222 $$\text{Ciliates C content} = 0.19 \text{ pg} \times \mu m^3 \qquad (6)$$

223 Ciliate biovolumes were calculated from measurement of their linear dimension taken with the
224 Olympus light microscope TH4-200 equipped with Olympus camera DP73 at a magnification of
225 200x using the software CellSense. About 90 cells were measured per each food treatment. The
226 shape of *Strombidium* sp.1 was assumed to be a cone topped with a half sphere and the one of
227 S*trombidium* sp.2 either a sphere or prolate ellipsoid.

228 **Experiment 3. Changes in growth rate, Chl*a* content and photosynthesis of two *Strombidium***
229 **species during prey depletion**
230 Prior to this experiment, cultures of the two ciliates were acclimated for three days to a saturating
231 prey concentration of *T. amphioxeia* (1.0x10^5 cell mL^{-1}, see the Supplementary Material for the
232 determination of saturating prey concentration), then distributed into three flasks (500 mL Blue Cap
233 glass flasks: VWR borosilicate 3.3; 215-1594) each containing a volume of 200 mL and allowed to
234 completely deplete the prey. Cultures were incubated for 1 week at 70 mol photons m^{-2}s^{-1}, day:night
235 cycle 16:8h. Control treatments (flasks with *T. amphioxeia* without added ciliates) were incubated

and sampled similarly, so that ingestion rates could be calculated as described above. Growth rates of both organisms were calculated (eq.2). Measurements of cellular Chl*a* and photosynthetic rates were carried out as described in experiment 2.

Results

Isolation and maintenance of ciliate cultures

The three isolated ciliate species were identified as members of the genus *Strombidium* (order Oligotrichida), based on their morphology (Fig.1) and partial 18S and 28S gene sequences (Supplementary Tables S1 and S2). The different isolates were identified as *Strombidium* cf. *conicum*, *Strombidium* cf. *basimorphum* and *Strombidium* sp. 3. Initially, the ciliate cultures were kept in 24 well tissue-culture plates and maintained on a varied diet of *Nephroselmis rotunda*, *Pyramimonas mitra*, *Teleaulax acuta*, *Teleaulax amphioxeia* and *Tetraselmis chui*. Initial attempts to grow the ciliates on algal monocultures failed, with the cultures dying after 1 to 2 weeks. After some months of mixed prey culture, we finally managed to grow them on algal monocultures in 24 well tissue culture plates. At this stage, cultures were actively growing on a diet of single prey species: either *N. rotunda* or *T. amphioxeia*. To up-scale ciliate cultures into glass bottles, at least 20 cells mL^{-1} were transferred from the tissue-culture dishes in a volume of about 30 mL, and incubated with algal prey previously conditioned to FSW. Resting stages (cysts) were observed in cultures of all three species during the first six months from the isolation date (Fig. 2), but cyst formation was lost when cultures were up-scaled to glass bottles.

Experiment 1. Prey size and prey type spectra

Strombidium cf. *conicum* and *S.* cf. *basimorphum* were able to grow on 13 and 17 out of the tested 35 algal prey species, respectively (Fig. 3 a,b). *Strombidium* sp. 3 was able to grow on 12 out of 27 algal prey species tested (Fig. 3c). With few exceptions, algae below 2 μm and above 12 μm in size did not support the growth of these three ciliates. However, not all the algal prey in the size range of 2-12 μm supported the growth of the ciliates. In the cases of *S.* cf. *conicum* and *Strombidium* sp. 3, only 12 out of 25 and 12 out of 22 of tested algal species in the size range 2-12 μm supported their growth. *Strombidium* cf. *basimorphum* was able to grow on 15 of the tested 22 algal species in that size range.

Some algal groups and species supported the growth of the three ciliates better than others (Fig. 4). Cryptophytes and chlorophytes best supported growth of the three ciliate species, while the cyanophytes and the dinophytes generally did not. Some stramenopiles supported growth, while others did not. The size class <2 μm primarily contained cyanobacteria and small green algae, which, with the exception of *Micromonas pusilla*, (which had an ESD of ~ 2 μm) did not support the growth of any of the ciliates (Fig. 3). The 2-12 μm size range included stramenopiles, haptophytes, chlorophytes, and cryptophytes. In this size range, *Apedinella radians* (stramenopile), *Mantoniella squamata* (chlorophyte) and *Phaeocystis globosa* (haptophyte), *Nephroselmis rotunda* (chlorophyte) and *Teleaulax acuta* (cryptophyte) best supported growth, while *Imantonia* sp., *Prymnesium patelliferum* and *Isochrysis galbana* (haptophytes), *Ochromonas moestrupii* (chrysophyte), and *Thalassiosira pseudonana* (stramenopile) did not support growth. In the prey size fraction exceeding 12 μm, only *Pelagodinium beii* and *Heterocapsa triquetra* (both dinophytes) supported growth of *S.* cf. *basimorphum*, but not *S.* cf. *conicum*.

Experiment 2. Growth rate, prey ingestion, Chl*a* content and photosynthesis of *Strombidium* cf. *basimorphum* and *S.* cf. *conicum* at three different prey concentrations

The two ciliates species showed comparable physiological rates when acclimated to intermediate prey abundances, while *S.* cf. *basimorphum* growth and photosynthetic rates were higher compared to *S.* cf. *conicum* when acclimated to the highest prey concentration (Table2).

Neither species grew at the lowest prey concentrations used and ingestion and photosynthetic rates were again higher in *S.* cf. *basimorphum* than in *S.* cf. *conicum* (Table 2). At these low prey concentrations, photosynthesis contributed 19% and 46% of the carbon uptake for *S.* cf. *basimorphum* and *S.* cf. *conicum*, respectively (Fig.5). The contribution of photosynthesis to the total carbon uptake dropped to 7.7% and 6.7%, respectively for *S.* cf. *basimorphum* and *S.* cf. *conicum* with intermediate prey availability and further to 4% and 1.8%. with high prey availability (Fig.5).

Cellular Chl*a* in *S.* cf. *basimorphum* and *S.* cf. *conicum* increased (Supplementary Table S3), while rates of photosynthesis decreased, as a function of prey concentration and prey ingestion rates. Consequently, Chl*a* specific photosynthetic rates decreased with prey availability and ingestion rates (Supplementary Figure S3, Table 2). The cellular Chl*a* content of the algal prey was 0.36 ± 0.07 pg (std) on average among all control cultures (N=18: six experiments, three replicates each), while the photosynthetic rate was 8.82 ± 2.71 pgC cell^{-1} d^{-1}, leading to a Chl*a* specific photosynthetic rate of 24.7 ± 6.6 C Chl*a*$^{-1}$ d^{-1} in the control cultures of *T. amphioxeia*.

The cell size of the ciliates increased with prey availability and prey ingestion rates. *S.* cf. *conicum* biovolume ranged from 1.78 to 3.01 x 10^4 μm^3, while the *S.* cf. *basimorphum* biovolume ranged from 2.20 to 3.31 x 10^4 μm^3. The estimated cellular carbon content ranged from 3.33 to 5.73 x 10^3 pgC $cell^{-1}$ in *S.* cf. *conicum* and from 3.23 to 6.30 x 10^3 pgC $cell^{-1}$ in *S.* cf. *basimorphum* (Supplementary Table S4). The highest GGE was calculated at intermediate prey concentrations (25-100μg C L^{-1}). GGE was lower when more prey was available and became almost 0 or even negative at very low prey concentrations (Table 2).

Experiment 3. Changes in growth, Chl*a* content and photosynthesis in *Strombidium* cf. *basimorphum* and *S.* cf. *conicum* during prey depletion

Cultures of *S.* cf. *basimorphum* and *S.* cf. *conicum* were acclimated to saturating prey availability and then allowed to deplete the prey completely (Fig. 6). Growth and ingestion rates of *S.* cf. *basimorphum* were significantly higher than rates of *S.* cf. *conicum* (Table 3) while carbon specific photosynthetic rates were identical during the exponential growth. Cell divisions stopped shortly after the prey was depleted, and the ciliate cultures slowly decreased in cell concentration over time. The ratio between cellular Chl*a* content and carbon content was almost constant in *S.* cf. *basimorphum* during starvation, while it decreased in *S.* cf. *conicum* (Table 3). Ciliate cells were significantly smaller when starved (see Supplementary Table S4) and cellular Chl*a* content was significantly lower in starved cells in both ciliates species (Supplementary Table S5).

Carbon specific rates of photosynthesis increased in *S.* cf. *basimorphum* during prey starvation, while become lower in *S.* cf. *conicum* (Fig.7). Expressing data as Chl*a* specific rates of photosynthesis reveals that in well-fed culture, *S.* cf. *basimorphum* had a specific rate almost 4 times higher than that of *S.* cf. *conicum* (Table 3). The data also reveals that the Chl*a* specific rates of photosynthesis were not significantly different in well-fed and in prey starved cells of *S.* cf. *conicum* ($P > 0.1$), while in *S.* cf. *basimorphum* the Chl*a* specific rates of photosynthesis increased by a factor of ~3 in starved cells compared to the well-fed cells.

Discussion

The suitability of different algal species as prey for mixotrophic *Strombidium* spp.

Strombidium species, whether being mixotrophic or purely heterotrophic, are known to be prey generalist and able to capture preys which size fits with the morphological constrains of their feeding apparatus (Jonsson, 1986). The mixotrophic *Strombidium* spp. studied here ranged from ~30 to ~40 μm in width. Generally they grew only when the offered algal prey were in the size range of 2-12 μm. The tested cyanobacteria and dinoflagellates were generally outside the prey size spectra of the ciliates and consequently could not support growth of the studied *Strombidium* spp. Similar sized *Strombidium* species have previously been found to ingest prey species within the same size range (Jonsson, 1986; Bernard and Rassoulzadegan, 1990; Kivi and Setala, 1995). The *Strombidium* spp. studied here generally grew well on monocultures of cryptophytes and chlorophytes, while not all haptophytes and stramenophiles supported the growth of the ciliates when provided as monocultures despite being within the 2-12 μm size spectrum. Thus, other factors may have impacted their suitability as prey. Some algae, like *Prymnesium,* produce lytic toxins that are known to kill their ciliate grazers (Rosetta and McManus, 2003). Other algae, like *Cyclotella* and *Thalassiosira* form colonies which make the cells functionally larger preventing them from being ingested. Suitability can also be related to strain specific characteristics or growth conditions, which determine food quality even within a certain prey species. For example, the haptophyte *Isochrysis galbana* did not support growth of any of the ciliates tested here nor in other studies (Montagnes, 1996; McManus *et al*., 2012), but this alga has been shown to support the growth of some other mixotrophic ciliates (Stoecker *et al*., 1988a; Crawford and Stoecker, 1996; McManus *et al*., 2018). Finally, we cannot exclude that some of the algae that did not support growth of the ciliates as monocultures, may contribute to growth in mixtures with other algae. Indeed in natural populations some algae could be more exploited as a direct carbon source rather than being used as chloroplasts source, and vice versa, covering different physiological needs of the ciliates. This needs to be explored in future studies.

Relative importance of photosynthesis and food uptake for growth in mixotrophic *Strombidium* spp.

The inorganic carbon uptake was relatively more important (~20-50% of the total carbon uptake) at very low prey concentrations (3-5-µgCL^{-1}). Under these prey conditions, it could make a difference for mixotrophic *Strombidium* in terms of better survival. Indeed, in comparison, some heterotrophic

ciliates experience prey concentration limiting for growth at ~10-50 μg C L^{-1} (Montagnes, 1996; Gismervik, 2005). With prey concentrations (25-100µgCL^{-1}) that resemble the natural standing stocks of nanoplankton in coastal waters (Rassoulzadegan *et al*., 1988), the contribution from inorganic carbon uptake to the total carbon uptake was ~10%. Interestingly, under these conditions *S.* cf. *conicum* grew close to its maximum growth rate, while *S.* cf. *basimorphum* grew to ~half of its maximum growth, and the calculated GGE's were as high as 80%.

The apparent contribution of inorganic carbon uptake to the overall carbon uptake was negligible at high prey concentrations (800-1300 μg C L^{-1}) in both *S.* cf. *basimorphum* and *S.* cf. *conicum*, accounting for only a few percent of total carbon uptake. It is possible that the actual inorganic carbon uptake has been underestimated due to enhanced recycling of carbon derived from the high ingestions rates. Such an underestimation can account to >50% of the gross photosynthesis in mixotrophic ciliates (Stoecker and Michaels, 1991). Nevertheless, the largest contribution of carbon comes from prey ingestion under these conditions. These results and considerations are backed up by the GGE values for the two *Strombidium* species (~30-50%) which were lower then what observed when less prey was available. Such GGE percentages are similar to those previously reported for heterotrophic ciliates species (Gismervik, 2005; Yang *et al*., 2015). Comparable decrease in GGE as function of prey availability has been observed in the mixotrophic species *S. rassoulzadegani* (Schoener and McManus, 2017). Previous estimates on the relative contribution of photosynthesis on ciliates energetic budges are in agreement with our observations that it is mainly relevant in condition of food limitation (Jonsson, 1987; Schoener and McManus, 2017), but absolute photosynthetic rates would of course vary depending on light availability (Stoecker *et al*., 1988 a and b) making this proportion to vary depending of factors other than ingestion.

It was interesting to observe a significant loss of the photosynthetic efficiency of the sequestered chloroplasts. In fact the Chl*a* specific inorganic carbon uptake rates of the *Strombidium* spp. were reduced by 60% to 90% to that of the prey cells. Similar reductions of Chl*a* specific inorganic carbon uptake (50%) has been observed in the mixotrophic *S. rassoulzadegani* compared to its prey (McManus *et al*., 2012). Preferential respiration of recently fixed carbon has been shown to take place in these ciliates (Putt, 1990) so that up to the 80% of the photosynthates would actually be respired and lost as CO_2 rather than incorporated as ciliate biomass (Schoener and McManus, 2017). Additionally, it is possible that the reductant equivalents generated by functional chloroplasts in ciliates could be employed in alternative pathways, which would not result in carbon fixation: i.e. chlororespiration. The redirection of photosynthetically derived electrons on the mitochondrial

respiratory chain could explain the big difference in net carbon fixation of ciliates compared to the prey and would result in higher assimilation efficiencies of the ingested carbon that would not need to be respired (Wilken *et al.*, 2020). The interdependence of photosynthetic electron transport and mitochondrial respiration has been recently assessed in constitutive mixotrophic flagellate species (Wilken *et al*., 2020). So far, the only evidence supporting this hypothesis in kleptoplastidic ciliates is the close association observed between acquired chloroplasts and hosts´ mitochondria (Laval-Peuto *et al*., 1986: *Tontonia appendiculariformis*; Stoecker *et al*., 1988b: *S. capitatum* and *S. chlorophilum*).

Effects of prey starvation on growth and photosynthesis of mixotrophic *Strombidium* spp.

Prey starvation experiments carried out on *S.* cf. *basimorphum* and *S.* cf. *conicum* revealed that as soon as the ciliates had depleted their prey, cell divisions stopped, and the populations slowly declined over time. The observed mortality rates of these two ciliates were constant and lower than what has been observed for heterotrophic species incubated at the same temperature, which virtually die in one or two days of starvation (Montagnes, 1996). *S.* cf. *conicum* and *S.* cf. *basimorphum* populations only declined by 25-30% in the three-four days of starvation, confirming the well-established assumption that mixotrophy enables ciliates to better withstand periods of prey deprivation (Dolan and Perez, 2000).

A difference between the two ciliates species was observed in the ability to keep the sequestered chloroplasts functional during starvation. The cellular chlorophyll content in *S.* cf. *conicum* declines much more than in *S.* cf. *basimorphum* after prey depletion (Supplementary Table S5) and photosynthetic rates in starved *S.* cf. *conicum* were much lower than in starved *S.* cf. *basimorphum,* suggesting that the latter species better retained functional chloroplasts. Chl*a* specific inorganic carbon uptake almost tripled in case of *S.* cf. *basimorphum* during the prey starvation experiment, whereas this was not the case in *S.* cf. *conicum*. Both species were well-fed prior to the initiation of the experiment so photosynthetic rates might have been underestimated due to the internal recycling of carbon as discussed above. Differences in the digestive and respiration rates of the two species during active feeding would lead to the different response to starvation observed in their photosynthetic rates. Anyways, it is also possible that *S.* cf. *basimorphum* undergoes a trophic switch, investing more in photosynthesis when prey is depleted, while *S.* cf. *conicum* lacks of this ability.

Conclusion

The studied mixotrophic *Strombidium* spp. were able to exploit a wide taxonomic range of algal prey in the size range 2 and 12 µm, but as monocultures, some prey species better sustained ciliate growth. Sequestered chloroplasts were functionally active for at least five days, but photosynthesis alone could not sustain the growth of the ciliates. Ingestion rates increased with prey availability while photosynthetic rates appeared lower when more prey was ingested. Highest GGE was observed at prey abundances of 25-100 μg C L^{-1}. These results suggest that mixotrophic *Strombidium* spp. will get a benefit over completely heterotrophic ciliates in the photic zone of areas with relatively low algal biomass dominated by nano-sized algae. This may have important consequences for our understanding of the trophic transfer up the food chain and should be incorporated into planktonic ecosystem and food web models.

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Data archiving

Nucleotide sequences are deposited in GenBank, accession numbers: MT349838 and MT420875 (*Strombidium*_sp.1_cf. *conicum*); MT349841 and MT420874 (*Strombidium*_sp.2_cf. *basimorphum*); MT349840 and MT420876 (*Strombidium*_sp._3).

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Table legends

Table 1: Algal species used as prey for the ciliate cultures, with information on algal group, strain number and size (Estimated Spherical Diameter =ESD). The strains were acquired from the Bigelow National Center for Marine Algae and Microbiota (CCMP strains), Scandinavian Culture Collection of Algae and Protozoa (SCCAP)

Table 2: Experiment 2. Growth rate, carbon specific prey ingestion (C C^{-1} d^{-1} = d^{-1}), carbon specific photosynthesis(C $C^{-1}d^{-1}$ = d^{-1}), Chl*a* specific photosynthesis and gross growth efficiency of *Strombidium* cf. *basimorphum* and *S.* cf. *conicum* at three different abundances of *T. amphioxeia.* Numbers refer to means ± std, n=9.

Table 3: Experiment 3. Chl*a* content and physiological rates (C C^{-1} d^{-1} = d^{-1}), of *S*. cf. *basimorphum* and *S*. cf. *conicum* during the period of exponential growth (day 1 to 3 *S*. cf. *basimorphum* and day 1 to 5 *S*. cf. *conicum*) and the starvation period (day 3 to 5 *S*. cf. *basimorphum* and 5 to 8 *S*. cf. *conicum*). Numbers refer to means ± std, n=3.

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572 **Figure legends**

573 **Fig. 1:** Light microscopy photographs of the three isolates: a) *Strombidium* cf. *conicum*; b) S. cf.
574 *basimorphum*; c) *Strombidium* sp. 3

575 **Fig. 2:** Cysts of the ciliates: a) *Strombidium* cf. *conicum*; b) *S.* cf. *basimorphum*; c) *Strombidium* sp.
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577 **Fig. 3:** Experiment 1. Prey size spectra of the 3 ciliates, *Strombidium* cf. *conicum* (a), *S.* cf.
578 *basimorphum* (b) and *Strombidium* sp. 3 (c). Each point represent the value assigned to the ciliate
579 growth when incubated with one prey species.

580 **Fig. 4:** Experiment 1. The ability of the 3 ciliates to grow on different algal species in different algal
581 groups: Cyanophytes, Dinophytes, Haptophytes, Stramenopiles, Chlorophytes and Cryptophytes.

582 **Fig. 5:** Experiment 2. Percentage of carbon acquired from ingestion and photosynthesis in a)
583 *Strombidium* cf. *basimorphum* and b) *S.* cf. *conicum* using *T. amphioxeia* as prey.

584 **Fig. 6:** Experiment 3. Prey depletion experiment. Changes in cell concentrations of ciliates and the
585 prey, *T. amphioxeia*. a) *S.* cf. *basimorphum* and b) *S.* cf. *conicum*. Vertical line indicates time of prey
586 depletion. Error bars indicate standard deviation.

587 **Fig. 7:** Experiment 3. Chl*a* content and photosynthetic rate for a) *S.* cf. *basimorphum* and b) *S.* cf.
588 *conicum* during the prey starvation experiment. Photosynthetic rate is expressed as ratio between the
589 amount of carbon fixed in one day divided by the cellular carbon content. Chl*a* content is expressed
590 as Chl*a* to carbon ratio. Vertical line indicates prey depletion. Error bars indicate standard deviation.

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592 **Table 1**

Algal group	Species	Culture collection number	Size (ESD, μm)
Cyanophytes	*Aphanocapsa sp.*	CCMP2524	2
	Chroococcidiopsis sp.	CCMP3281	3
	Prochlorococcus marinus	CCMP1986	0.7
	Synechococcus sp.	CCMP833	1.5
Cryptophytes	*Chroomonas mesostigmatica*	CCMP1168	5
	Rhodomonas sp.	CCMP318	7
	Teleaulax acuta	SCCAP K-1486	8
	Teleaulax amphioxeia	SCCAP K-1837	4
Haptophytes	*Chrysocromulina simplex*	SCCAP K-0272	3
	Emiliania huxleyi	CCMP379	4
	Imantonia sp.	SCCAP K-0624	2
	Isochrysis galbana	SCCAP K-1355	3
	Phaeocystis globosa	CCMP1805	5
	Prymnesium patelliferum	SCCAP K-0374	7
Stramenopiles	*Apedinella radians*	SCCAP K-0077	3
	Cyclotella meneghiniani	CCMP335	4
	Ochromonas moestrupii	SCCAP K-1766	4
	Thalassiosira pseudonana	CCMP1335	5
Dinophytes	*Alexandrium minutum*	CCMP113	15
	Amphidinium massartii	CCMP1342	14
	Heterocapsa rotundata	SCCAP K-0483	10
	Heterocapsa triquetra	CCMP449	13
	Pelagodinium beii	CCMP3395	11
	Prorocentrum balticum	CCMP1787	12
	Thoracosphaera heimii	CCMP1069	9
Chlorophytes	*Chlorella minutissima*	CCMP3451	2
	Mantoniella squamata	SCCAP K-0284	3
	Micromonas pusilla	CCMP485	2
	Nannochloropsis oculata	CCMP525	2
	Nephroselmis pyriformis	SSCAP K-0557	4
	Nephroselmis rotunda	SCCAP K-0251	4
	Pyramimonas melkonianii	SCCAP K-0628	5
	Pyramimonas mitra	SCCAP K-0241	7
	Tetraselmis chui	PLY429	9
	Tetraselmis wettsteinii	CCMP1722	12

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599 **Table 2**

Ciliate species	Prey (μg C L^{-1})	Growth (μ, d^{-1})	C specific Ingestion (d^{-1})	C specific Photosynthesis (d^{-1})	Chl*a* specific photosynthesis (C Chl*a*$^{-1}$ d^{-1})	GGE (%)
S.* cf. *basimorphum	6.0	0.01 ± 0.05	0.73 ± 0.04	0.18 ± 0.03	11.7 ± 0.96	2,5
	25	0.53 ± 0.07	1.18 ± 0.04	0.10 ± 0.02	6.33 ± 1.26	75
	800	0.90 ± 0.10	3.07 ± 0.78	0.10 ± 0.01	3.43 ± 0.55	48
S.* cf. *conicum	3.0	-0.01 ± 0.01	0.11 ± 0.03	0.09 ± 0.03	5.35 ± 2.35	-10
	100	0.55 ± 0.30	0.87 ± 0.13	0.06 ± 0.03	3.59 ± 1.40	85
	$1.3x10^3$	0.39 ± 0.18	2.42 ± 0.44	0.04 ± 0.02	1.56 ± 0.68	30

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617 **Table 3**

Ciliate species		μ (d^{-1})	C specific Ingestion (d^{-1})	C specific Photosynthesis (d^{-1})	Chl*a* content (Chl*a*C^{-1})	Chl*a* specific photosynthesis (C Chl*a*$^{-1}$ d^{-1})
S.* cf. *basimorphum	Exponential growth	1.37 ± 0.08	4.59 ± 0.27	0.04 ± 0.02	0.02 ± 0.00	2.38 ± 0.90
	starvation	-0.13 ±0.05	/	0.14 ± 0.00	0.02 ± 0.00	8.00 ± 0.05
S.* cf. *conicum	Exponential growth	0.33 ± 0.04	2.53 ± 0.33	0.04 ± 0.02	0.02 ± 0.00	1.72 ± 0.53
	starvation	-0.09 ±0.03	/	0.02 ± 0.00	0.01 ± 0.00	2.1 ± 0.3

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