PhD Thesis
Maira Maselli

Eco-physiology of prey generalist mixoplanktonic ciliates

Supervisor: Per Juel Hansen
Submitted on: 28 May 2021
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Principal Supervisor: Per Juel Hansen, Professor  
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Front-page photos: The ciliate Strombidium cf. conicum (bottom left) and Strombidium cf. basimorphum (top right) and their cryptophyte prey Teleaulax amphioxeia in epifluorescence microscopy. Pictures by Maira Maselli
Preface

This thesis contains the research I have done during the last three years as PhD fellow at the University of Copenhagen. This project has received funding from the European Union’s Horizon 2020 research and innovation program in the Marie Skłodowska-Curie action, Innovative Training Network, MixITN - grant n° 766327. The work was conducted under the supervision of Per Juel Hansen, at the Marine Biological Section in Helsingør, Denmark.

The working title on the initial PhD plan was “Ecophysiology of Key Species of General Non-Constitutive Mixotrophs (GNCMS)”. The aim was to isolate and culture mixoplanktonic ciliates and conduct experiments to produce numerical data usable for modelling, study their physiology and their role into the ecosystem dynamics and interpret results based on the natural distribution recorded in literature and in novel field surveys. The collaboration with different research groups made possible to address the project using different approaches and very much broaden my interests in this research topic.

I owe these organisms my continuously renewed interest for plankton research and the continuous wonder of life science. These ciliates are the obvious evidence that the interactions among (sometimes) very different individuals are what make it so wonderful. They gave me occasions to experience that myself in many different contexts, and still have it in mind while writing this thesis during some long months of self-isolation, dictated by the current circumstances.

Helsingør, May 2021
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II. Hughes EA, Maselli M, Sørensen H, Hansen PJ. Metabolic reliance on photosynthesis depends on both irradiance and prey availability in the mixotrophic ciliate, *Strombidium cf. basimorphum* Manuscript accepted for publication in *Frontiers in Microbiology*.


Additional work done during the PhD not to be included in the evaluation of the thesis

Appendix I

Abstract

Ciliates have a pivotal role in the ecology of marine systems, as they are an important trophic link between the microbial loop and metazoan grazers. Many planktonic ciliates retain functional chloroplasts from the photosynthetic prey that they ingest. This enable them to photosynthesize and thereby being mixotrophs. While some species express a selective behavior towards specific prey types from which they acquire chloroplasts, the majority of species among the oligotrich ciliates can acquire chloroplasts from different algal prey types and are defined as prey generalist. The focus on this thesis was on ecology and the physiology of these organisms based on laboratory experiments on cultures of Strombidium spp. In addition to this, a field survey was carried out on four Greenlandic fjords that receives high amounts of glacier flour to assess the role of mixotrophs in these systems.

Cultures of three Strombidium species were established from single cells isolated from their natural environment and identified via sequencing of their ribosomal subunits genes. The growth of the three Strombidium species in culture was best sustained when they were fed cryptophytes and chlorophytes as prey. However other algal prey types within the size range of 2 and 12 µm could also sustain their growth. Based on these observations, the cryptophyte Teleaulax amphioxea was selected as prey in the subsequent experiments.

The relative contributions of photosynthesis to the total carbon uptake was estimated for S. cf. basimorphum and S. cf. conicum, when acclimated to different prey abundances. Inorganic carbon incorporation via photosynthesis was only quantitatively relevant when prey availability was low and it led to increased gross growth efficiencies in this condition if compared with purely heterotrophic ciliates species. Strombidium cf. basimorphum and S. cf. conicum displayed differences in the ability to maintain the sequestered chloroplasts during prey starvation. Strombidium cf. basimorphum seemed to exploit efficiently the chloroplasts when starved of prey, while that was not the case for S. cf. conicum.

The use of molecular markers demonstrated that genetic material from prey nuclei, nucleomorphs and ribosomes were detectable in S. cf. basimorphum for at least five days after prey ingestion. Transcripts of prey nuclear origin were also found in starved cells of S. cf. basimorphum. The functionality of sequestered chloroplasts in S. cf. basimorphum was affected during prey starvation, when exposed to relatively higher irradiances. However, at higher irradiance levels, the ciliates had higher photosynthetic rates when supplied with prey abundances that saturated its growth.

The availability of dissolved inorganic nitrogen and phosphorus in the media had no effects on the starvation responses in Strombidium cf. basimorphum, as the ciliates seem unable to take up and utilize these inorganic nutrients for growth. Nutrient concentration, however, affected the physiology and the stoichiometry of the prey; thus indirectly affecting the ciliate, which maximized its growth and ingestion rates when grown under nutrient depleted conditions.

The total biomass of mixotrophic ciliates in Greenlandic fjords and their relative abundance compared to the total ciliate biomass was comparable to what has been found in the same season in more open waters in the same area. Indeed, water turbidity and de-eutrophication due to glacier inputs in the fjords are not likely to affect mixotrophic ciliates abundance directly, as they rely on prey ingestion.
to proliferate. In addition, experiments on mixotrophic ciliates in culture demonstrated that inert sediment particles were not ingested at all, thus the sediment carried with the water runoff from glaciers does not seem to have any effects on their growth and chloroplasts turnover.
Dansk resumé


Kulturer af tre Strombidium arter blev etableret fra enkeltceller indsamlet fra deres naturlige miljø og identificeret vha molekylær sekventering af deres ribosomal gene. Væksten af de tre Strombidium arter i kultur var bedst når de blev fodret med rekylalger og grønalger i størrelsespektret 2 - 12 µm. Dog kunne ciliaterne også gro på andre algegrupper inden for dette størrelsespektrum. Rekylalgen, Teleaulax amphioxeia, blev på basis af disse studier valgt som bytte i en række eksperimenter.


Den totale biomasse af mixtrofe ciliater i grønlandske fjorde and deres relative udbredelse i forhold til den totale ciliatbiomasse var af samme niveau som hvad der har været fundet i mere åbne vandområder i Disko Bugt på tilsvarende tid af året. Vandet turbiditet and mindre næringssaltførsel pga gletsjer input til fjordene synes ikke have en direkte effekt på udbredelsen af de mixtrofe ciliater, da de er afhængige af fødeoaptagelse for at kunne formere sig. Forsøg med mixtrofe ciliater i kultur
demonstrerede at de ikke spiste inert sedimentpartikler. Meget tyder således på at gletsjer-input med gletsjermel ikke har nogen effekt på de mixotrofe ciliaters vækst og kloroplast-omsætning i grønlandske fjorde, som er påvirket af gletsjer input.
Introduction

Nutritional modes of planktonic protists

Planktonic protists are traditionally classified in plant-like phytoplankton and animal-like protozooplankton based on the way they obtain nourishment for their survival and growth. The fundamental distinction between these organisms lies in that phytoplankton incorporate inorganic carbon via photosynthesis (are phototrophs), while protozooplankton ingest particulate organic material (are phagotrophs). However, in the last couple of decades, an increasing number of planktonic organisms have been found that can combine both mode of nutrition within a single cell (are mixotrophs). Indeed, among planktonic protists there are many “plants that eat” and “photosynthetic animals” (Mitra, 2018). Such mixotrophic planktonic protists have been collectively termed mixoplankton (Flynn et al. 2019), and differentiated in into two groups: 1) constitutive mixoplankton (CM), which are constitutive phototrophs capable of phagotrophy and 2) non-constitutive mixoplankton (NCM), which are organisms that acquire phototrophy via ingestion and retention of functional phototrophic prey (or parts of them) within their cell body. The uptake of dissolved organic material (DOM) has been documented in all planktonic protist groups, so osmotrophy is not considered in the classification of their trophic mode.

![Figure 1](attachment:image.png)

Figure 1. Schematics showing the distinct differences between different protist plankton physiologies. The protozooplankton are osmo–phagotrophic; they are incapable of phototrophy. The phytoplankton are photo–osmo–mixotrophic; they are incapable of phagotrophy. The constitutive mixoplankton (CM) and non-constitutive mixoplankton (NCMs) are all photo-, osmo- and phago-mixotrophic. However, some mixoplankton have life stages that are not photo- and phagotrophic, with nutrition aligning with that of “phytoplankton” or “protozooplankton”. The generalist GNCMs acquire phototrophy from many phototrophic prey types; pSNCMs are plastidic specialists acquiring phototrophy from specialist prey type(s); eSNCMs are endosymbiotic acquiring phototrophy by harbouring specific phototrophic prey. Note: illustrations are not to scale; in particular, eSNCMs are in relative terms ca. 10 to 100 times larger than the others. From Flynn et al. 2019, with original figure legend.
Non-constitutive mixoplankton (NCM)
Non-constitutive mixoplankton (NCM) are found within the clade of Rhizaria (mainly foraminifera and acantharea), Alveolates (ciliates and dinoflagellates) and Katablepharids, though other non-planktonic protists (such as amoebozoa) and metazoan have also been shown to engage acquired phototrophy (dotted lines in Figure 2).

Figure 2. Synthetic tree of eukaryotic life schematically displaying the phylogeny of NCMs (dotted lines in the yellow area). Photosynthetic capacitation impacted a major and monophyletic part of the tree of eukaryotic life (yellow area). From Stoecker et al. 2009, modified figure legend.

From phylogenetic studies, it appears that a photosynthetic capacity occurred very early on, in a monophyletic branch of eukaryotic life (Fig. 2). Heterotrophic protists may indeed come from lineages that lost their plastid early in the radiation of photosynthetic protists, thus they might have a genomic memory for plastid maintenance or oxygenic photosynthesis (Stoecker et al. 2009). This is probably what makes some NCMs able to exploit symbionts/plastids of different origins. However, different NCMs display different degrees of specialization towards the photosynthetic prey from which they can acquire phototrophy. While some NCMs can exploit many different prey types (generalist NCMs), other are only able to acquire phototrophy from certain prey species (specialist NCMs).
**Generalist non-constitutive mixoplankton (GNCMs)**

Prey generalist non-constitutive mixoplankton (GNCMs) are found among dinoflagellates and ciliates, but still very little is known about their biology and ecology. This is partially because GNCMs are not easily recognized in plankton natural samples. Indeed, GNCMs dinoflagellates are not easily distinguishable from dinoflagellates species that have their own chloroplasts, with exception of few species, which life history and metabolic strategy have been studied on isolates or cultures (Hehenberger et al. 2019; Nishitani et al. 2012). Ciliates, on the other hand, all lack their own chloroplasts, so any chloroplasts-bearing ciliate can be considered NCMs. However, due to their fragility, planktonic ciliates are difficult to collect, preserve and examine. Additionally, plastids-retaining ciliates are difficult to keep in culture, so our knowledge of potential prey selectivity is based on the observations on very few species.

**Studied organisms**

Plastid retention (kleptoplasty) is common among the oligotrich ciliates (Subclass Oligotrichia). This group includes both heterotrophic and mixotrophic species in a size spectrum of 10–200 µm. Plastid retention has been reported within the genera *Cyrtostrombidium*, *Laboea*, *Strombidium* and *Tontonia* (Stoecker et al. 2009). To date, ~30 species of marine oligotrich ciliates have been described to be kleptoplastidic (Stoecker et al. 2009), but this number is generally considered to be heavily underestimated due to the aforementioned difficulty in collection and culturing, but also in their identification. Species in the genus *Strombidium* represent more than the half (~17) of the total number of oligotrich ciliates described as kleptoplastidic so far. Most of the studies on marine GNCM *Strombidium* species date back to the late 80s (Stoecker et al. 2009), while more recently *Strombidium rassoulzadegani* has been proposed as model organisms to study chloroplasts retention in oligotrich ciliates, because it is relatively easy to keep in culture (Mcmanus et al. 2018).

**Identification**

Taxonomy of oligotrich ciliates is traditionally based on cell morphology and ciliary patterns, which are generally studied in vivo and/or by complex staining techniques of fixed cells (Santoferrara et al. 2017). Taxonomy has been complemented with DNA sequences studies recently, though the classification system requires updates. Indeed, for some ciliate families there is still a lack of data on the ciliary patterns, or they have never been sequenced reliably. For other families, data on morphology and DNA sequences do not agree (Santoferrara et al. 2017). For these reasons, it is currently difficult to link taxonomical and ecological studies. However, molecular methods for identification are largely employed in studies on natural populations (Gimmler et al. 2016; Orsi et al. 2018) for which the association of the functional descriptions of publically available DNA sequences is highly demanded (Faure et al. 2019).

In studies on natural populations that are only based on morphology, GNCM ciliates are recognized via the direct observation of chloroplasts in their cell body, thus more easily assigned to a functional (rather than taxonomical) identification. The observation of chloroplasts require the employment of transparent fixatives and epifluorescence microscopy, which are not commonly used to examine natural populations.
Chloroplast retention
Oligotrich ciliates (GNCMs) have been shown to retain chloroplasts from a wide range of algal groups, including chlorophytes, haptophytes, cryptophytes and heterokonts (Laval-Peuto and Febvre 1986; Johnson and Beaudoin 2019; Paper 1). The mechanisms by which chloroplasts are sequestered from the prey and retained by the ciliate after ingestion, are still not completely understood. It has been suggested that the ciliate endoplasmic reticulum is involved in the process, protecting chloroplasts from lytic activities (Laval-Peuto and Febvre, 1986). Once sequestered from the ingested prey, chloroplasts lie free in the ciliate cytoplasm (Laval-Peuto and Febvre 1986; Stoecker et al. 1989). Chloroplasts are not found in food vacuoles and they do not seem to be digested in response to prey starvation (Laval-Peuto and Febvre, 1986; Stoecker and Silver, 1990; Schoener and McManus 2012). Chloroplast retention seems to be a constitutive feature in GNCM species. Specimens collected from their natural environment always seem to contain chloroplasts (Laval-Peuto and Rassoulzadegan 1988) and GNCM ciliates in culture keep chloroplasts also when grown in the dark (Stoecker et al. 1987).

Studies on the GNCM ciliate Strombidium rassoulzadeganii suggest that GNCM ciliates do not express genes related to maintenance and replication of plastids (Santoferrara et al. 2014), so GNCM ciliates are thought to depend on frequent reacquisition of plastids via prey ingestion. Transmission electron microscopy has never revealed the retention of prey genetic material associated to the sequestered chloroplasts (Laval-Peuto and Febvre 1986; Stoecker et al. 1988) so, they are generally considered unable to exert any control of the sequestered chloroplasts.
Photosynthesis
The sequestered chloroplasts in GNCMS have been shown to actively incorporate inorganic carbon (they photosynthesize). Inorganic carbon uptake rates in GNCM oligotrich have been shown to be related to the irradiance levels, and rates can be comparable to those of similar sized algae (Stoecker et al. 1988 (Laboea), 1989 (Strombidium)). Cellular inorganic carbon uptake rates have also been shown to depend on the number of sequestered chloroplasts (Mcmanus et al. 2018), thus potentially on ingestion. Carbon acquired via photosynthesis, seem to be allocated into polysaccharides that are preferential used for respiration (Putt 1990; Stoecker and Michaels 1991), so that photosynthesis can fully cover the respiratory requirements, but is insufficient to sustain cell division (Stoecker et al. 1988; Stoecker and Silver 1990; McManus et al. 2018).

Food requirements and growth
Despite acquiring carbon via photosynthesis, GNCM ciliates cannot grow as pure photoautotrophs in absence of prey (Stoecker et al. 1988; Stoecker and Silver 1990). When prey is available, ingestion rates of GNCMs are comparable to those of purely heterotrophic species (Schoener and McManus 2017). Covering respiration via photosynthesis, they can better withstand period of prey deprivation, and grow more efficiently than purely heterotrophic species in conditions of limited prey availability (Schoener and McManus 2017).

Figure 4. Conceptual drawing displaying differences between heterotrophic ciliates and GNCM ciliates growth in response to prey availability

Despite prey generalists, oligotrich ciliates can only exploit prey items which size fits with the morphological constrains of their feeding apparatus (Jonsson 1986). Regardless from size, however, not all photosynthetic prey seem to support the growth of GNCM oligotrichs in culture (Gifford 1985; McManus et al. 2018).

Utilization and regeneration of dissolved inorganic nutrients
Little is known about the ability of GNCM ciliates to directly uptake inorganic nutrients other than carbon from the extracellular environment. GNCM ciliates are thought to rely on ingestion to obtain essential elements such as N and P (Dolan, 1997). The GNCM species Strombidium rassoulzadegani has been shown to take up inorganic nitrogen (both NO$_3^-$ and NH$_4^+$) at similar rates as a purely heterotrophic ciliate species. However, the uptake does not contribute significantly to its N
requirements (Schoener and McManus 2017). Anyways, nutrients regenerated through heterotrophic activity (catabolism) in GNCM ciliates might be retained inside the cell to compensate for the carbon derived from photosynthesis (Ghyoot et al. 2017), resulting in lower rates of N and P excretion compared to those of heterotrophic species.

Relative abundance and seasonality
In marine waters, GNCM oligotrichs make up, on average, 30% of the total oligotrich biomass (Dolan and Pérez 2000; Stoecker et al. 2009). They are present at all latitudes throughout the water column, though they dominate ciliates assemblages in the euphotic zone of stratified waters (Modigh 2001; Haraguchi et al. 2018; Stoecker and Lavrentyev 2018). In the stratified summer waters of the Mediterranean Sea, GNCMs abundances at surface are comparable to those at the deep chlorophyll maxima (Dolan and Marrasé, 1995; Dolan et al. 1996). In temperate coastal waters, GNCMs oligotrich can represent up to the 90% of the total ciliate biomass at surface, in the period between late spring-early autumn (Haraguchi et al. 2018). In Arctic and subarctic regions GNCMs oligotrich abundance peaks in the late summer, when GNCMs oligotrich may account for up to the 50% of the total chlorophyll (Putt, 1990; Stoecker et al. 2014). At depths below the euphotic zone, GNCM ciliates exhibit patterns similar to their heterotrophic counterparts, and experience less seasonal variability than in well-lit surface waters (Levinsen et al. 2000).

GNCM ciliates are recorded in low levels during winter, especially in temperate and polar regions (Haraguchi et al. 2018; Stoecker and Lavrentyev, 2018). The formation of non-motile resting stages (cysts) that sink out and accumulate in the sediment, has been observed in many oligotrich species (Müller 2000), and is thought to be a strategy to overcome unfavorable winter conditions (low temperatures, low prey and light availability).

Role in ecosystem trophic dynamics
In being one of the more representative groups of microzooplankton in natural communities, oligotrich ciliates represent an important trophic link between small primary producers and higher trophic levels (Calbet 2008). Microzooplankton encompass organisms which size is in between 20 and 200µm, and they can consume up to the 75% of the daily primary production (Schmoker et al. 2013). Thus, this group is of major importance in the understanding of energy fluxes in food webs and biogeochemistry (Calbet and Landry 2004; Buitenhuis et al. 2010). Oligotrich ciliates are traditionally described as heterotrophic consumers, but the extra carbon acquired from photosynthesis from GNCM species can be crucial in the parametrization and output of plankton ecosystem models (Mitra et al. 2014; Ghyoot et al. 2017). This is especially true in mature ecosystem where production is mainly supported by nutrients cycling in the microbial loop (Rassoulzadegan 1993). Mixotrophic organisms might respond differently than heterotrophs and autotrophs to the environmental conditions. Differently from heterotrophs, mixotrophs produce their own carbon. This enable them to cover respiration needs with photosynthesis, allowing mixotrophs to respond better to higher temperatures and prey deprivation. Differently from photo-autotrophs, mixotrophs can rely on ingestion to cover their energetic requirements thus better tolerate low light levels, e.g. due to turbidity. Thus, further understanding on the eco-physiology of mixotrophic oligotrich ciliates would contribute to better depict the overall ecosystem trophic dynamics, also in response to environmental stressor often associated with climate change and/or anthropogenic disturbance.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CM</td>
<td>Constitutive mixoplankton</td>
</tr>
<tr>
<td>ESD</td>
<td>Equivalent spherical diameter</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GNCM</td>
<td>Generalist non-constitutive mixoplankton</td>
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<tr>
<td>I</td>
<td>Irradiance</td>
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<tr>
<td>NCM</td>
<td>Non-constitutive mixoplankton</td>
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<tr>
<td>OBIS</td>
<td>Ocean Biodiversity Information System</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>SNCM</td>
<td>Specialist non-constitutive mixoplankton</td>
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Aims of this thesis

The overall aim of the study was to evaluate the role of acquired phototrophy in the physiology of GNCM (kleptoplastidic) ciliates under different environmental scenarios, and unveil the ecological implications and the molecular mechanisms that stand behind the retention of functional chloroplasts. This required different approaches:

1) Culture-based measurements of physiological rates (growth, ingestion and photosynthesis) of GNCM ciliates under different availabilities of light, prey and dissolved inorganic nutrients
2) Molecular-based study of the potential retention of prey genetic material in cultured GNCM ciliates
3) Field-based study of the abundance and distribution of different microplankton functional groups (including GNCM ciliates) in a geographic area of special interest

Specific aims of each paper

Paper I Ecophysiological traits of mixotrophic Strombidium spp.
The aims were to: 1) Characterize the algal prey sizes and taxonomic groups that GNCM Strombidium species can exploit, 2) Estimate the relative contribution of photosynthesis to the total carbon uptake of GNCM Strombidium species when fed with different amounts of prey and 3) study the effects of prey starvation on growth and photosynthesis of GNCM Strombidium species.

Paper II Metabolic reliance on photosynthesis varies depending on irradiance and prey availability in the mixotrophic ciliate, Strombidium cf. basimorphum
The aims were to: 1) study the effects of irradiance on photosynthesis and survival of the GNCM ciliate Strombidium cf. basimorphum during prey starvation and 2) study the effect of the irradiance on growth and photosynthesis of S. cf. basimorphum when fed with different amounts of prey

Paper III Impacts of inorganic nutrients on the physiology of a mixoplanktonic ciliate and its cryptophyte prey.
The aims were to: 1) study growth, photosynthesis and ingestion of the GNCM ciliate Strombidium cf. basimorphum when cultured in media with different concentrations of NO$_3^-$ and PO$_4^{3-}$, 2) study inorganic nutrients uptake/excretion rates of the GNCM ciliate S. cf. basimorphum and its prey Teleaulax amphioxea and 3) study the effects of different concentrations of NO$_3^-$ and PO$_4^{3-}$ on photosynthesis, growth and on the stoichiometry of the prey.

Paper IV Retention of prey genetic material in the kleptoplastidic ciliate Strombidium cf. basimorphum
The aims were to: 1) detect the presence of prey genetic material in DNA extracted from ciliates and provide a semi-quantitative estimation of its concentration over time following prey depletion, 2) detect prey rRNA in the GNCM ciliate S. cf. basimorphum over time following prey depletion and 3) detect prey transcripts in the GNCM ciliate S. cf. basimorphum after 4 days of prey starvation
Paper V  **Effects of glacier flour on marine micro-plankton: evidences from natural communities of Greenlandic fjords and experimental studies.**

The aims were to: 1) describe microplankton natural communities based their trophic modes in four Greenlandic fjords, 2) study the abundance of different functional groups of microplankton in relation to environmental variables, 3) study the effect of suspended particles addition on the growth of different functional groups of microplankton and 4) study the effects of glacier flour on growth rates and chlorophyll content in GNCM ciliates in laboratory culture.
Results and discussion

Culturing and identification
The experimental studies in this thesis were done on plastid retaining species of the ciliate, genus *Strombidium*. Ciliate cultures were established from single cells isolated from natural seawater samples collected from Roskilde fiord (Denmark) in June 2018, and maintained in laboratory for ~ three years. The isolates were identified based on in vivo morphology and sequence similarity of their LSU and SSU genes, but due to the uncertainty in the method employed for identification (see above, Identification) the conferatur (cf.) designation has been consistently added to the name of the species studied in here. Ciliates were cultured in 24 well tissue-culture plates and offered mixtures of different prey types during the first 2-3 months after initial isolation. Subsequently cultures were up-scaled to glass bottles and maintained in 200mL of filtered seawater on a single prey species in order to minimize variability in measuring ciliate ingestion and photosynthetic rates. The algal species used as single prey was selected based on the ciliates growth responses and because some information about its biology, as well as some molecular tools to study its interaction with ciliates were already available in literature.

Resting stages (cysts) were observed in cultures of all three ciliate species during the first 6 months but cyst formation was lost when cultures were up-scaled to glass bottles. One of the ciliate species initially cultured (*Strombidium* sp.) could not grow in large volumes and/or on a single prey species; the culture crashed after several attempts. Another isolate was identified as *Strombidium* (cf.) *conicum*. *Strombidium* cf. *conicum* grew well on a mono-diet based on the cryptophyte prey *Teleaulax amphioxeia* since the very early stage of culturing. However, *Strombidium* cf. *conicum* growth and ingestion rates decreased after one year of culturing, and therefore it was not used any further for experiments. The reduction of growth rates after some time in culture has previously been reported for oligotrich ciliates and ascribed to the genetic drift in small laboratory populations (Gifford 1985; McManus et al. 2012). The last isolate was identified as *Strombidium* cf. *basimorphum*. This ciliate was successfully maintained on a mono-diet based on *T. amphioxeia* or the chlorophyte *Nephoroselmis rotunda* during the entire study period allowing more extensive experimental studies.

*Strombidium conicum*

*Strombidium conicum* has a worldwide distribution. It was first described by Lohmann in 1908 (as *Laboea conica*), and registered ~2300 times in the OBIS database in the time range 1912-2017 ([https://obis.org/taxon/101289](https://obis.org/taxon/101289)). Most records are from the upper ten meters of the water column at temperatures between 5 and 25 °C, and salinities of 10 to 35. Chloroplast retention and functionality in *Strombidium conicum* was first studied by Jonsson (1987) and Stoecker et al. (1988).
Strombidium basimorphum

*Strombidium basimorphum* was first described from waters samples taken in Canadian fiord by Martin and Montagnes in 1993. Liu et al. (2011) reinvestigated this species via molecular phylogeny on isolates from water samples of Chinese coastal waters. Only the first record of this species (Martin and Montagnes, 1993) is reported in OBIS so far, but *S. basimorphum* seem to be ubiquitous, as it has been reported from China (Liu et al. 2011), in the open water of the North Pacific (Orsi et al. 2018) and in Danish waters (*paper I*). Despite previously hypothesized (Orsi et al. 2018), the retention of chloroplasts in this species has only been reported in the isolates cultured for the studies reported in this thesis (Fig. 4).

**Figure 5.** Distribution of *Strombidium conicum* (yellow dots) according to records registered in OBIS in the time range 1912-2017 ([https://obis.org/taxon/101289](https://obis.org/taxon/101289))

**Figure 6.** Transversal section in transmission electron microscopy of the *Strombidium cf. basimorphum* isolate studied in this thesis. Chloroplasts are visible in the cytoplasm of the ciliate (left picture, arrowed). Higher magnifications (right pictures) show that chloroplasts lie free in the cytoplasm and keep a well-preserved structure. (Pictures by Øjvind Moestrup)
Size and taxonomic group of suitable algal preys
To get insights into the algal prey sizes and taxonomic groups that support growth of the three Strombidium species in culture, thirty-five different algae species were tested as prey (paper I). Similar to other Strombidium species of similar size, the three Strombidium species studied were able to grow on algal prey ranging between 2 and 12 µm in equivalent spherical diameter (ESD). Cryptophytes and chlorophytes best supported the growth of the three ciliate species, whereas the cyanophytes and dinophytes generally did not support growth. Not all haptophytes and stramenophiles within the 2–12 µm size spectrum supported the growth of the ciliates. Thus, factors other than size seem to affect their suitability as prey for GNCMs (e.g. toxin production, food quality or chloroplast quality). It is not possible to 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, as it could be the case in natural population where some algae could be more exploited as food rather than being used as chloroplasts source, and vice versa.

Growth, photosynthesis and ingestion as function of prey availability and irradiance
The effects of prey density on growth, ingestion, and photosynthetic rates of Strombidium cf. basimorphum and Strombidium cf. conicum was studied when fed with the cryptophyte, Teleaulax amphioxeia as prey (paper I). Prey densities were selected based on the functional response of ciliates to represent different conditions: ciliates survival (no growth), prey limited growth and prey saturated growth.

Figure 7: Ingestion and photosynthetic rates of Strombidium cf. basimorphum and Strombidium cf. conicum that have been acclimated to different prey densities.

Strombidium cf. conicum had lower growth, ingestion and photosynthetic rates compared to Strombidium cf. basimorphum at all conditions, but in both species, the growth and ingestion rates increased with prey availability, while photosynthetic rates were lower at high prey densities (Figure 7). The lower photosynthetic rates can be attributed to the self-shading of chloroplasts inside the ciliates when more prey was ingested.

Photosynthesis contributed substantially to the total carbon budget of the studied ciliates when prey availability was limiting for growth and close to what generally observed in the natural standing stocks of nanoplankton in coastal waters (below 100 µg C L⁻¹).
Three prey densities were tested on *Strombidium* cf. *basimorphum* at different irradiances (10, 40 and 120 µmol photons m$^{-2}$ s$^{-1}$) ([paper II](#)). Ingestion rates were lower at the highest of the tested irradiances and at saturating prey availability. Ciliates were significantly bigger in this treatment compared to the other treatments. Higher irradiances prevented chloroplast self-shading in ciliates acclimated to saturating prey availability, resulting in higher photosynthetic rates. However at low prey density, some photo-inhibition might have occurred, resulting in lower photosynthetic rates. At low prey density indeed, chloroplast turnover was probably not sufficiently fast to replace chloroplasts that were more easily damaged by the high light. At the lowest of the tested irradiances, the contribution of photosynthesis to the total carbon uptake was almost null.

These results confirm that mixotrophic *Strombidium* spp. have a competitive advantage compared to purely heterotrophic species in the photic zone of areas with relatively low prey biomass, as typical of the surface layer of stratified waters. The more prey that is available and the less light, the more GNCMs ciliates behave as their heterotrophic counterpart, mostly relying on ingestion for growth.

![Figure 8: Schematic representation of the relative contribution of ingestion and photosynthesis to the total carbon intake of GNCM ciliates as function of prey (Chl a) and light availability (attenuated with depth) in an ideal condition of stratification of the water column.](image)

**Effect of inorganic nutrients concentration on physiological rates**

The effects of the concentration of inorganic nitrogen and phosphorus on growth, ingestion, excretion and photosynthetic rates of *Strombidium* cf. *basimorphum* were studied when fed with the cryptophyte *Teleaulax amphioxeia* as prey ([paper III](#)). Relatively high inorganic nutrient concentrations negatively affect *S. cf. basimorphum* growth (Figure 9) and prey ingestion. This was probably due to changes in prey stoichiometry. The prey contained less carbon when grown in media with high inorganic nutrient concentrations (Figure 9). The relatively high carbon content of the prey in the low nutrient treatments might have induced a compensatory feeding behavior in *Strombidium* cf. *basimorphum*, which ingested more prey to obtain nitrogen and phosphorus. Indeed, *Strombidium*
cf. *basimorphum* seems unable to directly uptake inorganic forms of these two major nutrients from the media and use them for growth. Additionally, *Strombidium* cf. *basimorphum* excreted considerable amounts of ammonium in all the experimental treatments, suggesting that its growth was probably limited by phosphorus in all conditions. However, some internal recycling of phosphorus is conceivable from the elevated growth rates of the ciliates in the low nutrients treatments.

![Strombidium cf. basimorphum growth rates](image1)

![Teleaulax amphioxeia](image2)

**Figure 9:** *Strombidium* cf. *basimorphum* growth rates and the stoichiometry of its prey, *Teleaulax amphioxeia*, in media with different amount of dissolved inorganic nitrogen and phosphorus. (f/200= 8,8 µM NaNO₃ and 0,36 µM NaH₂PO₄; f/100= 17,6  µM NaNO₃ and 0,72 µM NaH₂PO₄; f/40= 44,1 µM NaNO₃ and 1,82 µM NaH₂PO₄; f/20= 88,2  µM NaNO₃ and 3,63 µM NaH₂PO₄).

Low inorganic nutrient availability resulted in reduced chlorophyll a content and chlorophyll a specific photosynthetic rates in the prey, *T. amphioxeia*, which was reflected on the chlorophyll a content and chlorophyll a specific photosynthetic rates of the ciliates. Photosynthesis contributed in a larger proportion of the total carbon uptake in ciliates grown in nutrient replete treatments compared to ciliates grown in the nutrients depleted ones, also due to the differences observed in ingestion rates. *Strombidium* cf. *basimorphum* seems to be very well adapted to exploit its prey, maximizing growth and ingestion when grown in nutrients conditions that resemble those of where it was first isolated (Haraguchi et al. 2018). However, different prey types, with potentially different stoichiometry, would have to be tested to make such an extensive generalization.

**Physiological response and chloroplasts functionality during prey starvation**

The availability of inorganic nutrients in the media had no effects on the starvation response in *Strombidium* cf. *basimorphum* (paper III). Indeed, cultures of *S. cf. basimorphum* declined in abundance as soon as prey was depleted in all experimental treatments, regardless of the dissolved inorganic nutrient availability. The same was observed at different irradiances (paper II) and in *Strombidium* cf. *conicum* (paper I), confirming that GNCMs ciliates are not able to live as pure autotrophs despite they show prolonged survival during starvation compared to heterotrophic species (Montagnes 1996; paper I). These ciliates cannot sustain themselves only with photosynthesis probably because they rely on ingestion to incorporate nutrients other than carbon (Dolan 1997;
paper III). Indeed, the functionality of the retained chloroplasts is kept unaltered in S. cf. basimorphum for at least five days during prey starvation. Chlorophyll a specific photosynthetic rates are similar or even higher during prey starvation compared to chlorophyll a specific photosynthetic rates of feeding cells (papers I, II and III). Chloroplast retention in S. cf. basimorphum appears to be affected by aging during starvation at higher irradiances, as shown by the fast decrease in the cellular chlorophyll a content (Figure 10). Despite this, chloroplasts seem anyways to perform better during starvation, since higher chlorophyll a specific photosynthetic rates were measured after prey depletion at all the tested irradiance levels (paper II).

Figure 10: Chlorophyll a content and chlorophyll a specific photosynthetic rate in Strombidium cf. basimorphum during prey starvation at different irradiances (I= 10, 40 and 120 µmol photons m⁻² s⁻¹). Prey was depleted at day 2-3 in the I10 and I40 treatments, and at day 5 in the I120 treatment. Adapted from paper II.

This response was not observed in Strombidium cf. conicum, which appeared to host a lower number of chloroplasts compared to Strombidium cf. basimorphum, and to lose some of the functionality of the chloroplasts during prey starvation (paper I). Indeed, chlorophyll a content and chlorophyll a specific photosynthetic rate in S. cf. conicum decreased immediately after prey depletion, while, at the same conditions S. cf. basimorphum kept the same chlorophyll a to carbon ratio that had while it was feeding and had higher chlorophyll a specific photosynthetic rates (figure 11).

Figure 11: Chlorophyll a to carbon ratio and chlorophyll a specific photosynthetic rates in Strombidium cf. conicum and Strombidium cf. basimorphum when actively feeding, after prey depletion, and during prey starvation. The vertical lines indicates the day in which prey was depleted. From paper I.
The internal recycling of CO$_2$ and the preferential utilization of recently fixed carbon for respiration can bias the measurements of photosynthetic rates in GNCMs ciliates (Putt 1990; Stoecker and Michaels 1991; Crawford and Stoecker 1996). Differences in the respiration rates of the two ciliates and differences in the respiration rates of fed and starved cells might explain the differences observed in their photosynthetic rates as response to starvation. However, it is also possible that *S*. cf. *basimorphum* has some control of the sequestered chloroplasts that *S*. cf. *conicum* does not have. *S*. cf. *basimorphum* may be able to switch to a more autotrophic metabolism when prey is depleted.

**Retention of prey genetic material**

To investigate if the ability of *Strombidium* cf. *basimorphum* to keep the functionality of sequestered chloroplasts during prey starvation was connected to the retention of prey genetic material, molecular markers specific for the prey, *Teleaulax amphioxeia*, were applied in well-fed cells as well as in cells that had been starved for 1 to 5 days (paper IV). Quantitative polymerase chain reaction (qPCR) demonstrated that genetic material from prey nuclei and nucleomorphs are detectable inside *S*. cf. *basimorphum* for at least five days after the prey had been ingested. However, prey genetic material seems to disappear quite quickly in *S*. cf. *basimorphum* compared to what has been observed in the prey specialist NCM *Mesodinium rubrum* which is able to retain prey nuclei for up to ten weeks (Johnson and Stoecker 2005; Kim et al. 2017). Fluorescence in situ hybridization (FISH) demonstrates that *Strombidium* cf. *basimorphum* contain prey ribosomes even after 5 days of prey starvation (Figure 12). Moreover, single-cell transcriptomic revealed the presence of transcripts of prey nuclear origin in *S*. cf. *basimorphum* after four days of prey starvation.

![Figure 12](image_url): Micrographs of *S*. cf. *basimorphum* cells hybridized with the probe for rRNA of its prey *Teleaulax amphioxeia* (in green, Alexa Fluor 488 dye) and counterstained with DAPI (in blue, ciliate nuclei) in different nutritional phases. A,E) well fed cell; B,F) after prey depletion; C,G) three days starved; D,H) five days starved. A to E: combined light channels. E to H light channel for the FISH probe only. The red is chloroplasts auto fluorescence. Adapted from paper IV.
This study was conducted more than one year after the initial isolation of the ciliate cultures. At this time the growth rates of *Strombidium cf. conicum* cultures had decreased compared to the initial rates. Also, it was not possible to get *Strombidium cf. conicum* cultures to completely remove all the prey, because ciliate cultures already declined in cell density before the prey was completely depleted. For this reason, only FISH assays were conducted on only well-fed cultures *Strombidium cf. conicum*. Despite that *Strombidium cf. conicum* was actively ingesting the prey cells, no hybridization was observed. These results suggest that differences in the functional biology of these two ciliates species exist, and this difference is not only related to their photosynthetic rates, but also concerns the molecular mechanisms that stands behind the interaction with the sequestered chloroplasts. At present it is not known whether these features in both ciliates species arise from strain-specific characteristics selected during culturing. To get deeper understandings, the same techniques would have to be employed on natural specimens and probes for other algal prey types would have to be developed.

**Abundance and spatial distribution of GNCMs in natural populations of Greenlandic fiords**

A field survey was conducted in four fjords on the south-west Greenland coast to investigate the effects of melting glacier discharges on the marine microplankton community (paper V). At all sampling locations, non-constitutive mixoplankton was relatively less abundant in terms of biomass compared to other functional groups (constitutive mixoplankton, autotrophs and heterotrophs). However, the total biomass of mixotrophic ciliates, as well as their relative abundance compared to the total ciliate biomass, was comparable to what found in the same season in more open waters of the same area (Putt 1990; Levinsen et al. 1999, 2000).

Indeed, the alteration of the physico-chemical characteristics of the water column due to the inputs from land-terminating glacier, mainly affect the autotrophic microplankton (diatoms), since in most cases the input from land-terminating glacier lead to high turbidity and de-eutrophication. These factors are not supposed to directly affect mixotrophic ciliates, as they mostly rely on prey ingestion for grow. A possible negative effect of the glacier inputs on mixotrophic ciliates could have come from the ingestion of the glacier flour particles carried by the runoff water. This possible effect has been hypothesized based on the results of an incubation experiments of natural populations sampled in the same location. Mixoplanktonic ciliates showed a negative growth rates since the very first day of incubation. The ingestion of inert particles could have displaced the sequestered chloroplasts and led to a negative effect on the growth of mixotrophic ciliates. This hypothesis was tested on laboratory cultures of *Strombidium cf. conicum* and *Strombidium cf. basimorphum*. Tests on laboratory cultures have not demonstrated such an effect, as ciliates seem not to ingest sediment particles, and to have the same chlorophyll *a* content (pg cell⁻¹) as when incubated without sediment. Actually, in the fjords, at stations closer to the glacier inputs, where the higher load of suspended particles were observed, mixotrophic ciliates biomass was comparable or even higher to what recorded in less turbid waters at the stations towards the open ocean. Other than in few punctual locations, prey generalists mixotrophic ciliates were equally or more abundant than the prey specialist *Mesodinium rubrum*, suggesting that these ciliates would substantially contribute to sustain higher trophic levels in such impacted areas.
Conclusion and future perspectives

Studies on prey generalist non-constitutive mixotrophs can have relevant implications for our understanding of plankton ecology and evolution. From the ecological perspective, plastid retention in ciliates has been previously analyzed in terms of costs and benefits (Dolan and Perez 2000; McManus et al., 2012). However, the potential inclusion of GNCMs as separate functional group in biomass-based ecosystems models would add a level of complexity not completely supported from our current knowledge. The results in this thesis mostly aimed to add to that. Despite providing usable numerical data, studies on cultures have limitations and the data should always be evaluated taking into account the natural variability of different environments. Additionally, long term culturing of the organisms might induce the selection of strain specific traits that also have to be taken into consideration. Undoubtedly, cultures are essential for in-depth studies of physiology and functional biology and more effort should be put into in the establishment of stable cultures of other mixoplanktonic ciliate species. Topics that will need further scrutiny includes:

I. Cysts formation and implications for ecology and modelling

Cyst formation has been previously reported in marine Strombidium species other than all the three species isolated in here. Examples include: Strombidium rassolzadegani (McManus et al. 2018); S. biarmatum (Agatha et al. 2005); S. crassulum (Reid 1987); S. oculatum (Jonsson 1994); S. capitatum (Kim et al. 2008). Clearly mixotrophy is not a prerequisite for cyst formation, but it may subsidize this “energetically costly” life-stage (Dolan and Perez 2000). The loss of the ability to form cysts in culture might lead to biases in the estimation of ciliates physiological rates, when used for modelling purposes. Indeed, in the initial stages of culturing S. cf. basimorphum constitutively produced considerable amount of cysts (up to the 50% of total cells number), which of course did not ingest prey nor divided during dormancy. Thus, growth and ingestion rates might be overestimated when cultures only produce “active” vegetative cells. It is possible that sub-strains that could not produce cysts have been selected in the early stages of culturing. Of course, the lack of potential environmental triggers is also a factor, since cyst formation is commonly thought to be a mechanism to survive unfavorable conditions (Müller 2000). Nevertheless, the topic deserves further studies, as it have important implications in the understanding of the biology of mixotrophic ciliates, and, probably, the seasonality of these ciliates. Additionally further understandings might have applications in facilitating the prolonged maintenance of cultures.

II. Photo-physiology and experiments with temperature

Inorganic carbon incorporation measure net photosynthesis. However, functional chloroplasts also generate reductant equivalents and energetic molecules that could contribute to metabolic routes that eventually do not terminate in carbon fixation, but that would anyways boost gross growth efficiency. Several photosynthetic byproducts might also interfere with the intracellular redox state of the ciliate and have a negative effect on cells physiology, which is not necessarily related to the chloroplast malfunctioning. Chloroplast functionality can actually be better inspected through in vivo fluorescence techniques, which yet have to be adapted for these highly motile and relatively large organisms. The use of chemical inhibitors that selectively interfere with the photosynthetic and the mitochondrial electron transport chains can instead reveal what is, if any, the interdependence of photosynthesis and respiration. This would have important
applications in studying the physiological response to temperature changes in organisms with acquired phototrophy, which would be likely more evident in their respiration rates when photosynthesis is repressed.

III. *Who or what drives chloroplast symbiosis?*

Retention of chloroplasts may be alternatively define as “chloroplast symbioses”, as it is actually not completely clear yet if any of the partners involved (either the ciliate host or the chloroplast itself) actively lead the process. The fact that prey generalist mixoplanktonic ciliates can establish such interaction with chloroplasts of different phylogenetic origins is very intriguing. However, do all prey generalist mixoplanktonic ciliates perform equally well with the same chloroplasts type? or do species specific preferences exist? If so, are those preferences eventually related to the co-occurrence of species in nature? And do “preferences” imply the retention of prey genetic material together with chloroplasts? Does this latter only occur after repeated interactions? And if so, how long would that take? Finally, is it possible that this process has been artificially forced in the *S. cf. basimorphum* culture in here?

Molecular studies on short-term cultured ciliates isolated from natural populations would help to address some of these questions. Some effort should be put in isolating ciliates and potential prey from the same environment and in develop molecular markers specific for those preys.
References


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Papers
Ecophysiological traits of mixotrophic *Strombidium* spp

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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 35 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. Chlα specific inorganic carbon uptake rates were reduced by 60–90% 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 5 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.

KEYWORDS: mixotrophy; kleptoplasty; ciliates; *Strombidium*

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, 2006). 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 heterotrophic 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 (IRs), 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., 1988a, 1988b; 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 ageing 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: (i) the prey size spectra they can exploit, and which algal taxa better sustain their growth, (ii) growth, ingestion and inorganic carbon uptake rates at different prey abundances and (iii) effects of prey starvation on growth and photosynthesis.

METHOD

Algal cultures

Thirty-five algal cultures were used as prey for the ciliate cultures (Table I). The cultures were mainly provided by the Scandinavian Culture Collection of Algae and Protozoa, and the Bigelow National Center for Marine Algae and Microbiota. Stock algal cultures were maintained in 1/2 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 16:8 h. 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 CX53) at a magnification of ×100 in Sedgewick-Rafter chambers. The equivalent spherical diameter (ESD) of algal species, which size was outside the range of sensitivity of the instrument (<3 μm), were measured manually with an Olympus light microscope TH4-200 equipped with Olympus camera DP73 at a magnification of ×400 using the software CellSence. ESD measurements allowed the calculation of algal biovolume as: $4/3\pi \times (ESD/2)^3$.

Isolation and maintenance of ciliate cultures

Three ciliates species were isolated from natural water samples collected in Roskilde Fjord (South of Fredriksverk, Denmark) on 2 June 2018. Individual cells were isolated using a draw glass capillary pipette under an Olympus SZ61 dissection microscope ($\times10$–50 magnification) and transferred to sterile-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. acetab) or green algae (Nephroselmis rotunda, Pyramimonas nitra 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 50–150 mL). The ciliate
Table I: 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>
<thead>
<tr>
<th>Algal group</th>
<th>Species</th>
<th>Culture collection number</th>
<th>Size (ESD, μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanophytes</td>
<td>Aphanocapsa sp.</td>
<td>CCMP2524</td>
<td>2</td>
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<tr>
<td></td>
<td>Chroococcidiopsis sp.</td>
<td>CCMP3281</td>
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<tr>
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<tr>
<td></td>
<td>Synechococcus sp.</td>
<td>CCMP833</td>
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<td>Cryptophytes</td>
<td>Chroomonas mesostigmatica</td>
<td>CCMP1168</td>
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<tr>
<td></td>
<td>Rhodomonas sp.</td>
<td>CCMP318</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Teleaulax acuta</td>
<td>SCCAP K-1486</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Teleaulax amphioxeia</td>
<td>SCCAP K-1837</td>
<td>4</td>
</tr>
<tr>
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<td>SCCAP K-0272</td>
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</tr>
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<td>Emiliania huxleyi</td>
<td>CCMP379</td>
<td>4</td>
</tr>
<tr>
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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 ciliate species. 28S and 18S gene sequences were used to aid in the identification of the ciliate species (sequences were obtained and analysed 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: <1–15 μm in ESD. 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 × 10^5 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 5 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 <10 ciliate cells were still present at the end of the 5 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 Chla 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 1.3 × 10⁻² μgCmL⁻¹, whereas Strombidium cf. basinorum cultures (20 ciliates mL⁻¹) were acclimated to T. amphioxeia average concentrations of 6, 25 and 800 μgCmL⁻¹. Average prey concentration were calculated as:

\[
C_{avg} = \frac{(C_1 - C_0)}{LN(C_1/C_0)}
\]

(1)

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

Acclimation to the prey concentration was carried out in 500 mL glass flasks having a water volume of 300 mL. Stock cultures were inoculated with the desired prey concentration for 2 days adjusting ciliates and algae concentration every 24 h. After the 2 days of acclimation, the cultures were split into triplicate bottles and incubated for another 3 days, adjusting concentrations of ciliates and prey every 24 h. About 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 CKXX3) at a magnification of ×50. Ciliate growth was measured as change in cell abundance over time and calculated assuming exponential growth (μ, d⁻¹):

\[
μ = \ln(\frac{N_1}{N_0}) / (t_1 - t_0)
\]

(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 IRs. 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 h. Frost equations were 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 IR (d⁻¹) as follows:

\[
\text{Carbon specific ingestion} = \frac{\text{cellular carbon content of the prey (pg) } \times \text{IR (prey cells ciliate⁻¹ day⁻¹)}}{\text{cellular carbon content of the ciliate (pg)}}
\]

(3)

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

Chlorophyll a measurements

For ciliate Chla measurements, 20 ciliate cells from each experimental bottle were picked with a drawn Pasteur pipette, rinsed in FSW several times and added to 2 mL of 96% ethanol. Chla 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 h and Chla was quantified using a Turner Trilogy Fluorometer equipped with a Chla non-acidification insert.

Photosynthetic rate measurements

Photosynthetic rates of ciliates were measured on triplicate samples each one containing 20 ciliates singularly picked from each experimental bottle with a drawn Pasteur pipette, applying the ¹⁴C technique by Rivkin and Seliger (1981). Ciliates cells were rinsed in FSW and incubated for 3 h in 23-mL glass scintillation vials filled with 2 mL of FSW in which 20 μL NaH¹⁴CO₃ 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 resuspended in 1.5 mL of distilled water. Ten milliliter 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:

\[
P = \frac{\text{[light DPM} - \text{dark DPM] / no of cells}}{\text{DPM specific activity} \times \text{incubation time (h)}} \times \text{μgC/mL} \times 10^6
\]

(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 cell⁻¹ h⁻¹) was used to calculate the daily photosynthetic (pgC cell⁻¹ d⁻¹). Daily photosynthetic rate was used to calculate carbon specific photosynthetic rate (pgC pgChla⁻¹ d⁻¹) and chlorophyll specific photosynthetic rate (pgC pgChla⁻¹ d⁻¹=C
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MIXOTROPHIC STROMBIDIUM

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

Fig. 2. Cysts of the ciliates: (a) Strombidium cf. conicum; (b) S. cf. basimorphum; (c) Strombidium sp. 3.

Chla$^{-1}$ d$^{-1}$). Photosynthetic rates of the algal control were also measured on 2 mL of algae monoculture, spiked with 20 μL NaH$^{14}$CO$_3$ − stock solution (specific activity 100 μCi mL$^{-1}$) and incubated simultaneously in the light and in the dark. At the end of the incubation, specific activity has been determined and the remaining volume of each sample was acidified and processed as described for ciliates. Carbon incorporation rates were calculated according to equation (4).

Ciliate biomass production was calculated as:

$$ \text{Biomass production} = \mu_y \times C_y \times \mu $$

where $\mu_y$ is growth rate (μ, d$^{-1}$) and $C_y$ is the average ciliate carbon content (pg C cell$^{-1}$).

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

$$ \text{Ciliate C content} = 0.19 \text{ pg C} \mu \text{m}^{-3} \times \mu \text{m}^3 $$

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

Experiment 3: changes in growth rate, Chl$a$ content and photosynthesis of two Strombidium species during prey depletion

Prior to this experiment, cultures of the two ciliates were acclimated for 3 days to a saturating prey concentration of T. amphioxeia (1.0 $\times$ 10$^7$ cell mL$^{-1}$, see the Supplementary Material for the determination of saturating prey concentration), then distributed into three flasks (500 mL Blue Cap glass flasks: VWR borosilicate 3.3; 215-1594) each containing a volume of 200 mL and allowed to completely deplete the prey. Cultures were incubated for 1 week at 70 mol photons m$^{-2}$ s$^{-1}$, day–night cycle 16:8 h. Control treatments (flasks with T. amphioxeia without added ciliates) were incubated and sampled similarly, so that IRs could be calculated as described above. Growth rates of both organisms were calculated (equation 2).
Measurements of cellular Chl \text{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 \textit{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 \textit{Strombidium cf. conicum}, \textit{Strombidium cf. basimorphum} and \textit{Strombidium sp. 3}. Initially, the ciliate cultures were kept in 24 well tissue-culture plates and maintained on a varied diet of \textit{Nephroselmis rotunda}, \textit{Pyramimonas mitra}, \textit{Teleaulax acuta}, \textit{Teleaulax amphioxia} and \textit{Tetraselmis chui}. Initial attempts to grow the ciliates on algal monocultures failed, with the cultures dying after 1–2 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 \textit{N. rotunda} or \textit{T. amphioxia}. 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 6 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

\textit{Strombidium cf. conicum} and \textit{S. cf. basimorphum} were able to grow on 13 and 17 out of the tested 35 algal prey species, respectively (Fig. 3a and b). \textit{Strombidium sp. 3} was able to grow on 12 out of 27 algal prey species tested (Fig. 3c). With few exceptions, algae below 2 \(\mu\text{m}\) and above 12 \(\mu\text{m}\) in size did not support the growth of these three ciliates. However, not all the algal prey in the size range of 2–12 \(\mu\text{m}\) supported the growth of the ciliates. In the cases of \textit{S. cf. conicum} and \textit{Strombidium sp. 3}, only 12 out of 25 and 12 out of 22 of tested algal species in the size range 2–12 \(\mu\text{m}\) supported their growth. \textit{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, whereas the cyanophytes and the dinophytes generally did not. Some stramenopiles supported

Fig. 3. Experiment 1: prey size spectra of the 3 ciliates, \textit{Strombidium cf. conicum} (a), \textit{S. cf. basimorphum} (b) and \textit{Strombidium sp. 3} (c). Each point represents the value assigned to the ciliate growth when incubated with one prey species.
growth, whereas 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, whereas *Imantonia* sp., *Prymnesium pateliferum* and *Isochrysis galbana* (haptophytes), *Ochromonas moestrii* (chlorophyte) and *Thalassiosira pseudonana* (stramenopile) did not support growth. In the prey size fraction exceeding 12 μm, only *Pelagodinium beii* and *Heterocapsa triqueta* (both dinoflagellates) supported growth of *S. cf. basimorphum,* but not *S. cf. conicum."

**Experiment 2:** growth rate, prey ingestion, Chla content and photosynthesis of *Strombidium cf. basimorphum* and *S. cf. conicum* at three different prey concentrations

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

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 II). 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 in *S. cf. basimorphum* and *S. cf. conicum* increased (Supplementary Table S3), whereas rates of photosynthesis decreased, as a function of prey concentration and prey IRs. Consequently, Chla specific photosynthetic rates decreased with prey availability and IRs (Supplementary Fig. S3, Table II). The cellular Chl 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), whereas the photosynthetic rate was 8.82 ± 2.71 pgC cell⁻¹ d⁻¹, leading to a Chla specific photosynthetic rate of 24.7 ± 6.6 C Chl⁻¹ d⁻¹ in the control cultures of *T. amphioxeia*.

The cell size of the ciliates increased with prey availability and prey IRs. *S. cf. conicum* biovolume
ranged from 1.78 to 3.01 × 10^4 μm³, whereas the S. cf. *basimorphum* biovolume ranged from 2.20 to 3.31 × 10^4 μm³. The estimated cellular carbon content ranged from 3.33 to 5.73 × 10^1 pgC cell⁻¹ in S. cf. *conicum* and from 3.23 to 6.30 × 10^1 pgC cell⁻¹ in S. cf. *basimorphum* (Supplementary Table S4). The highest GGE was calculated at intermediate prey concentrations (25–100 μgCL⁻¹). GGE was lower when more prey was available and became almost 0 or even negative at very low prey concentrations (Table II).

### Experiment 3: changes in growth, Chlα 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 IRs of S. cf. *basimorphum* were significantly higher than rates of S. cf. *conicum* (Table III) whereas 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 Chla content and carbon content was almost constant in S. cf. *basimorphum* during starvation, whereas it decreased in S. cf. *conicum* (Table III). Ciliate cells were significantly smaller when starved (see Supplementary Table S4) and cellular Chla 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, whereas become lower in S. cf. *conicum* (Fig 7). Expressing data as Chla specific rates of photosynthesis reveals that in well-fed culture, S. cf. *basimorphum* had a specific rate almost four times higher than that of S. cf. *conicum* (Table III). The data also reveal that the Chla specific rates of photosynthesis were not significantly different in well-fed and in prey starved cells of S. cf. *conicum* (P > 0.1), whereas in S. cf. *basimorphum* the Chla 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, whereas 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 μgCL−1 (Montagnes, 1996; Gismervik, 2005). With prey concentrations (25–100μgCL−1) that resemble the natural standing stocks of nanoplanckton 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, whereas S. cf. basimorphum grew to approximately 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 μgCL−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 when less prey was available. Such GGE percentages are similar to those previously reported for heterotrophic ciliate 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 budgets 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., 1988a and 1988b) 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 Chla specific inorganic carbon uptake rates of the Strombidium spp. were reduced by 60–90% to that of the prey cells. Similar reductions of Chla 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, 1990b) so that up to the 80% of the photosynthates would actually be respired and lost as CO2 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-Preto 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 1 or 2 days of starvation (Montagnes, 1996). S. cf. conicum and S. cf. basimorphum populations only declined by 25–30% in the 3–4 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 ciliate 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 S3) 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. Chla 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, whereas 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 2–12 μm, but as monocultures, some prey species better sustained ciliate growth. Sequestered chloroplasts were functionally active for at least 5 days, but photosynthesis alone could not sustain the growth of the ciliates. IRs increased with prey availability whereas photosynthetic rates appeared lower when more prey was ingested. Highest GGE was observed at prey abundances of 25–100 μGCL−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 nanosized 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.

**SUPPLEMENTARY DATA**

Supplementary data can be found at *Journal of Plankton Research* online.

**FUNDING**

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**DATA ARCHIVING**

Nucleotide sequences are deposited in GenBank, accession numbers: MT349838 and MT420873 (Strombidium sp.1 cf. conicum); MT349841 and MT420874 (Strombidium sp.2 cf. basimorphum); MT349840 and MT420876 (Strombidium sp.3).

**REFERENCES**


Supplementary material
Identification of ciliates

**Methods. DNA extraction and PCR:** For DNA extraction, cells were picked from established cultures with a drawn Pasteur pipette, and added to clean FSW water drops, using a Leitz Labovert FS inverted microscope (X40-100 magnification). For each species, single cells were transferred to 200 μL PCR tubes containing 100μl water and 10% (w/v) Chelex 100 (Sigma-Aldrich #C7901) in ten replicates. The PCR tubes were vortexed for 5s, pelleted in a micro-centrifuge for 10s, and subsequently incubated at 95°C for 20min in order to extract the DNA (Richlen and Barber, 2005). After incubation, the tubes were centrifuged again for 10s and stored at 4⁰C until use in PCR reactions. 2μL of the DNA extract were used as template in the subsequent PCR reactions to amplify the small subunit ribosomal RNA gene (18S) and partly the large subunit ribosomal RNA gene (28S)(see primers in Table S1). PCR reactions were done in 25μL reaction containing, 1.5mM MgCl₂, 0.8mM dNTPs [VWR #733-1363], 0.5 units polymerase [VWR #733-1301], 0.4µM primers using the following reaction settings: 2 min at 95 ºC, followed by 40 cycles: 95 ºC for 30s; 56 ºC for 30s; 72 ºC for 50s; and finally 5 min at 72 ºC.

**Sequencing and Phylogenetic analyses:** PCR products were tested on a 2% agarose gel and sent to Macrogen (Macrogen Europe, Amsterdam, NL) for purification and sequencing in both directions. Sequence analysis (trimming, assembly, BLAST) was carried out using Geneious version 11.1.5. Sequences of the SSU genes were downloaded from GenBank and aligned using MAFFT with subsequent alignment masking, as implemented in GUIDANCE2 (Landan and Graur 2008; Sela et al., 2015). GUIDANCE alignment score was 0.99, the masked alignment (columns below confidence score of 0.93 were removed) was trimmed by hand. The SSU alignment included 1678 characters and was uploaded to ATGC bioinformatics platform in Montpellier for PhyML 3.0 execution with smart model selection (Akaike Information Criterion, 1000 bootstrap replicates) (Guindon et al., 2010; Lefort et al., 2017). The best model was GTR+G+I and the alignment was further used to build trees using, MrBayes (GTR+G+I model, four MCMC run for 1.000.000 generations, sampling every 1000 generation, burn in of the first 25% of trees), and Neighbor Joining (HKY, 10.000 bootstrap replicates) as implemented in the software Geneious Prime 2020.1.2.

**Results and Discussion.** Two of the species aligned with species already described in literature. 18S sequence [MT349838] had 99.6% pairwise similarity with species described as *Strombidium conicum*, while 18S [MT349841] had a 100% pairwise sequence similarity with species described as *S. basimorphum*. The third *Strombidium* species could not be linked to a described species via
sequencing (Table S2). Closest BLAST results of the 18S sequence [MT349840] were with *Strombidium hausmanni* (96.9%) and 93.1% of the 28S [MT420876, MT420877] with *Strombidium rassoulzadegani*. The ciliates in this study with matches > 99.5% to reference sequences deposited in GenBank have been assigned species names qualified by the “cf” designation; the limits of molecular method in the identification of oligotrich ciliates is recognised (Santoferra *et al.*, 2017). Our aim was to provide a functional descriptions of these ciliates which are associable to a species name and sequence, since molecular methods for identification are largely employed in studies on natural populations (Gimmler *et al.*, 2016; Orsi *et al.*, 2018) and the association of the identity with the relative trophic function is actually demanded for the interpretation of environmental data (Faure *et al.*, 2019). The cf (confer, compare to) designation underlines the uncertainty of the identifications, thus avoiding the potential that misidentification would affect future studies of taxonomy or phylogeny with the sequences that have been deposit in GenBank as *Strombidium* sp. 1_cf. conicum, *Strombidium* sp.2_cf. basimorphum and *Strombidium* sp.3.

**Screening for growth of the ciliates with different initial prey concentrations**

**Methods.** Initial experiments on the numerical responses were carried out on ciliates that were not acclimated to the prey concentration to get some insight into the relevant prey concentrations to be used in the successive experiments. Ten starved ciliates were singularly added to 2 mL of *T. amphioxeia* or *N. rotunda* suspensions, in 24 well tissue-culture dishes. Ten different prey concentrations were tested in twelve replicates each. The experimental cultures were fixed after 5 days of incubation in Lugol’s (final concentration 2%), and the cells were enumerated using an inverted light microscope (Olympus CKX53) at a magnification of 50X and 100X, for ciliates and algae, respectively.

**Results.** At the beginning of the five-days incubation period, prey-starved ciliates displayed a lag time of one or two days before cell division took place. In addition, prey concentrations greatly varied within the 5 days incubation of *S. cf. conicum* and *S. cf. basimorphum*, making it impossible to assume exponential growth and associate the change in ciliates abundance with a reliable prey concentration. Nevertheless, these initial experiments gave some crude data on their numerical responses, indicating a prey concentration for maximum growth of ~ 100 μg C L⁻¹ (Supplementary Figure S2).

*Ecophysiology of mixotrophic Strombidium spp. (supplementary material)*
### Table S1: Primer sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ – 3’</th>
<th>Target region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>25F1</td>
<td>CCGCTGAATTTAAGCATAT</td>
<td>28S forward</td>
<td>(Kogame et al. 1999)</td>
</tr>
<tr>
<td>LSUr (Dir-2CR)</td>
<td>CCTTGTCCTGCTTCAAGA</td>
<td>28S reverse</td>
<td>(Scholin et al. 1994)</td>
</tr>
<tr>
<td>SR4</td>
<td>AGGGCAAGTCTGGTGGCCAG</td>
<td>18S part 2</td>
<td>(Yamaguchi and Horiguchi 2005)</td>
</tr>
<tr>
<td>SR6</td>
<td>GTCAGAGGTGAATTTCTTGG</td>
<td>18S part 3</td>
<td>(Yamaguchi and Horiguchi 2005)</td>
</tr>
<tr>
<td>SR7</td>
<td>TCCTTGGGCAAATGCTTTCGC</td>
<td>18S part 1</td>
<td>(Yamaguchi and Horiguchi 2005)</td>
</tr>
<tr>
<td>SR9p</td>
<td>AACTAAGAAGRCCATGCAC</td>
<td>18S part 2</td>
<td>(Yamaguchi and Horiguchi 2005)</td>
</tr>
<tr>
<td>SSUF (IF)</td>
<td>ACCTGGTTGATCTGCCCAGT</td>
<td>18S part 1</td>
<td>(Medlin et al. 1988)</td>
</tr>
<tr>
<td>SSUR (1528R)</td>
<td>TGATCCTTCTGAGGTTCACCTAC</td>
<td>18S part 3</td>
<td>(Medlin et al. 1988)</td>
</tr>
</tbody>
</table>

### Table S2: GenBank accession numbers and sequence similarities

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank accession numbers</th>
<th>Pairwise sequence identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strombidium sp. 1</td>
<td>MT349838 (18S)</td>
<td>99.6% to Strombidium conicum</td>
</tr>
<tr>
<td></td>
<td>MT420875 (28S)</td>
<td>93.0% to Halteria grandinella</td>
</tr>
<tr>
<td>Strombidium sp. 2</td>
<td>MT349841 (18S)</td>
<td>100% to Strombidium basimorphum</td>
</tr>
<tr>
<td></td>
<td>MT420874 (28S)</td>
<td>99.7% to Strombidium cf. basimorphum</td>
</tr>
<tr>
<td>Strombidium sp. 3</td>
<td>MT349839, MT349840 (18S)</td>
<td>96.9% to Strombidium hausmanni</td>
</tr>
<tr>
<td></td>
<td>MY420876, MT420877 (28S)</td>
<td>93.1% to Strombidium rassoulzadegani</td>
</tr>
</tbody>
</table>

Ecophysiology of mixotrophic Strombidium spp. (supplementary material)
Table S3: Experiment 2. Cellular Chla content and physiological rates (pgC cell d$^{-1}$) of Strombidium basimorphum and S. conicum at three different concentrations of T. amphioxeia.

<table>
<thead>
<tr>
<th>Ciliate species</th>
<th>Prey concentration (µg C L$^{-1}$)</th>
<th>Ingestion (pgC cell d$^{-1}$)</th>
<th>Chla content (pgChla cell$^{-1}$)</th>
<th>Photosynthesis (pgC cell$^{-1}$d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cf. basimorphum</td>
<td>6.0</td>
<td>1.90x10$^3$ ± 100</td>
<td>43 ± 4.5</td>
<td>459 ± 46</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3.83x10$^3$ ± 130</td>
<td>49 ± 9.0</td>
<td>318 ± 110</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>1.16x10$^4$ ± 2.98x10$^3$</td>
<td>113 ± 2.1</td>
<td>389 ± 66</td>
</tr>
<tr>
<td>S. cf. conicum</td>
<td>3.0</td>
<td>210 ± 60</td>
<td>33 ± 0.3</td>
<td>181 ± 86</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.76x10$^3$ ± 413</td>
<td>54 ± 0.8</td>
<td>199 ± 87</td>
</tr>
<tr>
<td></td>
<td>1.3x10$^3$</td>
<td>7.99x10$^3$ ± 1.46x10$^3$</td>
<td>84 ± 5.6</td>
<td>148 ± 66</td>
</tr>
</tbody>
</table>

Table S4: Ciliates carbon content when incubated with different concentrations of prey. Ciliate carbon was estimated converting the biovolumes as: C content= 0.19 pg C µm$^{-3}$.

<table>
<thead>
<tr>
<th>Ciliate species</th>
<th>Prey concentration (µgC L$^{-1}$)</th>
<th>Carbon content (pgC cell$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cf. basimorphum</td>
<td>0</td>
<td>2.08x10$^3$ ± 7.30x10$^2$</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>3.34x10$^3$ ± 1.99x10$^3$</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5.35x10$^3$ ± 1.53x10$^3$</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>6.30x10$^3$ ± 1.48x10$^3$</td>
</tr>
<tr>
<td>S. cf. conicum</td>
<td>0</td>
<td>2.12x10$^3$ ± 7.11x10$^2$</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.61x10$^3$ ± 1.11x10$^3$</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.20x10$^3$ ± 1.91x10$^3$</td>
</tr>
<tr>
<td></td>
<td>1.3x10$^3$</td>
<td>5.73x10$^3$ ± 1.98x10$^3$</td>
</tr>
</tbody>
</table>
Table S5: Experiment 3. Cellular Chla content and physiological rates (pgC cell d⁻¹) of S. cf. basimorphum and S. cf. conicum during the period of exponential growth (day 1 to 3 S. cf. basimorphum and 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

<table>
<thead>
<tr>
<th>Ciliate species</th>
<th>Ingestion (pgC cell d⁻¹)</th>
<th>Chla content (pgChla cell⁻¹)</th>
<th>Photosynthesis (pgC cell⁻¹d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cf. basimorphum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential growth</td>
<td>1.74x10⁴ ± 1.03x10³</td>
<td>72.7 ± 2.2</td>
<td>163.56 ± 76.24</td>
</tr>
<tr>
<td>Starvation</td>
<td>/</td>
<td>36 ± 3.0</td>
<td>288.00 ± 9.24</td>
</tr>
<tr>
<td>S. cf. conicum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exponential growth</td>
<td>7.99x10³ ± 1.46x10³</td>
<td>84.3 ± 5.6</td>
<td>147.85 ± 60.15</td>
</tr>
<tr>
<td>Starvation</td>
<td>/</td>
<td>19.4 ± 0.10</td>
<td>41.24 ± 6.62</td>
</tr>
</tbody>
</table>
Figure S1: Phylogeny of ciliates based on Bayesian inference of 18S rDNA sequences including the three newly isolated ciliates. Support values at nodes show posterior probability of Bayesian inference / bootstrap values of maximum likelihood / bootstrap values of neighbour-joining. Branches with maximum support are marked with (*), support values below 65% are marked with (-). The scale bar corresponds to 5 nucleotide substitutions per 100 nucleotide positions. New sequences produced in this study are marked in bold.
**Figure S2:** Growth of *Strombidium* cf. *conicum*, *S.* cf. *basimorphum* and *Strombidium* sp. 3, initiated at different initial prey concentrations. Data points show ciliates average increase in cell number over prey average concentration, horizontal error bars indicate the deviation from the initial prey concentration, vertical error bars indicate standard deviation among replicates.

**Figure S3:** Experiment 4. The relationship between Chla specific photosynthetic rate and ingestion rates in *S.* cf. *basimorphum* and *S.* cf. *conicum*. 
References


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Metabolic reliance on photosynthesis depends on both irradiance and prey availability in the mixotrophic ciliate, *Strombidium cf. basimorphum*

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Keywords: mixotrophy¹, ciliates², photosynthesis³, ingestion⁴, GNCM⁵, *Strombidium⁶,* irradiance⁷.

Abstract

Many species of the ciliate genus *Strombidium* can acquire functional chloroplasts from a wide range of algal prey and are thus classified as generalist non-constitutive mixotrophs. Little, however, is known about the influence of irradiance and prey availability on their ability to exploit the photosynthetic potential of the chloroplasts, and how this may explain their spatial and temporal distribution in nature. In this study, inorganic carbon uptake, growth, and ingestion rates were measured for *S. cf. basimorphum* under three different irradiances (10, 40, and 120 μmol photons m⁻² s⁻¹) when acclimated to three different prey densities (5×10³, 1×10⁴, and 4×10⁴ cells mL⁻¹), as well as when allowed to deplete the prey. After prey depletion, cultures survived without prey longest (~6 days) at the medium irradiance treatment (40 μmol photons m⁻² s⁻¹), while ciliate density, inorganic carbon uptake rates, and cellular chl-a content declined fastest at the highest irradiance treatment. This indicates that the ciliates may be unable to maintain the chloroplasts functionally without replacement at high irradiances. Ingestion rates were not shown to be significantly influenced by irradiance. The maximum gross growth efficiency (GGE) in this study (1.1) was measured in cultures exposed to the medium test irradiance and lowest prey density treatment (5×10³ cells mL⁻¹). The relative contribution of inorganic carbon uptake to the ciliate carbon budget was also highest in this treatment (42%). A secondary GGE peak (0.99) occurred when cultures were exposed to the highest test irradiance and the medium prey density. These and other results suggest that *S. cf. basimorphum*, and other generalist non-constitutive mixotrophs, can flexibly exploit many different environmental conditions across the globe.
1 Introduction

Mixotrophy in protists is generically defined as a nutritional strategy that combines phagotrophy and phototrophy (Flynn et al., 2019). An increasing number of protist species have been shown to utilize varying degrees of mixotrophy, and thus the perceived prevalence and relevance of this nutritional strategy to the marine food web and nutrient cycles is also rising. Subsequently, more ecophysiological studies of mixotrophic organisms have been conducted in recent years and researchers have been able to incorporate mixotrophic functions into ecosystem models of aquatic environments. Already, the ability of mixotrophic organisms to utilize multiple avenues of energy and nutrient acquisition has been used to predict an increased efficiency in the transfer of biomass through trophic levels (Ward and Follows, 2016).

Mixotrophy in protists may be organized in several different ways. Mitra et al. (2016) categorized mixotrophic strategies into two primary groups: constitutive mixotrophs that have their own innate photosystems and non-constitutive mixotrophs that must acquire or utilize photosystems taken from prey. Non-constitutive mixotrophs can then further be split into two subcategories: generalist non-constitutive mixotrophs (GNCMs) that can acquire the ability to fixate carbon from multiple prey sources and specialist non-constitutive mixotrophs (SNCMs) that can only utilize specific species or genera of prey. While different strategies along the spectrum of mixotrophy may be more prevalent at different latitudes or times of year (Stoecker et al., 2009; Leles et al., 2017), mixotrophy as a whole is exceedingly common and fundamental to many aquatic environments (Sanders, 1991; Mitra et al., 2014; Stoecker and Lavrentyev, 2018).

Non-constitutive mixotrophy is a particularly dominant nutritional strategy within the ubiquitous planktonic phylum, Ciliophora (Stoecker et al., 2009). While ciliates do not have the innate ability to photosynthesize, some can sequester and utilize sequestered chloroplasts from their prey, a mechanism known as kleptoplasty. In marine waters, the most well-studied NCM ciliates are the bloom-forming red Mesodinium spp. These species are considered SNCMs due to the close association between hosts and their sequestered prey organelles. Consequently, red Mesodinium spp. acquire a vast majority of carbon from photosynthesis (as opposed to prey ingestion) (Hansen et al., 2013) and often exhibit the ability to survive for at least one month without food (Johnson and Stoecker, 2005; Smith and Hansen, 2007).

Another pervasive ciliate subclass is Oligotrichia, which contains both heterotrophic and mixotrophic species. Unlike the red Mesodinium spp., the mixotrophic oligotrichs have been shown to retain and utilize chloroplasts from many different species of algal prey (Johnson and Beaudoin, 2019; Maselli et al., 2020), and thus, are generally considered GNCMs. Mixotrophic oligotrichs are believed to depend on frequent reacquisition of prey plastids, as they do not appear to express genes related to plastid maintenance and replication (Santoferrara et al., 2014; Mcmanus et al., 2018). These plastidic oligotrichs make up, on average, 30% of ciliate biomass (Dolan and Pérez, 2000; Stoecker et al., 2009), and during spring- and summer-time peaks, they can even comprise > 90% of ciliate biomass (Bernard and Rassoulzadegan, 1994; Haraguchi et al., 2018). Additionally, plastidic ciliates can represent anywhere from 4 to over 50% of total chlorophyll-a (chl-a) in a system, making them of vital importance for overall primary production (Putt, 1990a; Stoecker et al., 2013; Franzè and Lavrentyev, 2017). Thus, the prevalence of GNCM ciliates within natural ecosystems makes it exceedingly important to better quantify their physiological processes.
To date, most studies on oligotrich ciliates have focused on the effects of prey depletion and subsequent starvation on ingestion, photosynthesis, and growth responses, sometimes comparing responses in the light and in the dark (e.g. Montagnes, 1996; Stoecker & Michaels, 1991). However, as GNCMs likely gain a competitive advantage in nature via their nutritional flexibility, it is important to further elucidate the specific interactions between prey availability and GNCM inorganic carbon uptake rate – a topic largely ignored in current literature. Photosynthesis in GNCM ciliates may be strictly beholden to the number of prey chloroplasts that are available and, therefore, would exhibit a clear positive correlation with prey concentration. However, if the ciliates do have some ability to regulate the amount of photosynthesis performed by the stolen plastids, they may upregulate photosynthetic activity in response to reduced access to organic carbon from prey and downregulate photosynthesis when they are exposed to environments with high prey density.

Additionally, very few papers have explored how the physiological responses of GNCMs may change under different light conditions. Indeed, in one of the only studies to quantify a mixotrophic oligotrich’s responses to light, Stoecker et al. (1988b) showed that unlike the light-dependent grazing seen in many SNCMs (Moeller et al., 2019), Laboea strobila’s ingestion rate appeared to be independent of light. Additionally, L. strobila, as well as several Strombidium species, were shown to exhibit an inorganic carbon uptake rate that increased with increasing irradiance up to a point of saturation (Stoecker et al., 1988b, 1988a). Other than these two studies, little is known about the effect of irradiance on ingestion, growth, photosynthesis, and response to prey depletion in GNCMs. Quantifying this behavior is crucial to the development of a deeper understanding of how mixotrophic strategies interact with the environment and affect the planktonic community structure.

To this end, this study aims to gain an understanding of mixotrophy in Strombidium cf. basimorphum. S. cf. basimorphum is a ubiquitous species that can be found across the globe (Martin and Montagnes, 1993; Liu et al., 2011; Orsi et al., 2018). However, its mixotrophic capabilities have only recently been proven in isolates from Danish coastal water (Maselli et al., 2020). Cultures of the same isolate, identified based on 18S and 28S gene sequences (Maselli et al., 2020, GenBank accession number MT349841 and MT420874), have been used in this study to monitor how light affects: 1) photosynthesis and survival of S. cf. basimorphum when starved after prey depletion and 2) growth and photosynthesis of the ciliate at different prey densities. With this information, we can gain insight into which situations mixotrophy may lend an advantage to S. cf. basimorphum when compared to other planktonic species that employ different nutritional acquisition strategies.

2 Materials and Methods

2.1 Culture of organisms

Strombidium cf. basimorphum cultures were obtained from the culture collection of the Marine Biological Section in Helsingør, Denmark. This species was originally collected and isolated in June 2018 from Roskilde Fjord, Denmark (Maselli et al., 2020). Ciliate cultures were maintained on the cryptophyte prey Teleaulax amphioxeia, which was provided by the Scandinavian Culture Collection of Algae and Protozoa (SCCAP, strain number: K-1837). All cultures were grown at a temperature of 15°C in filtered (Whatman, GF/F) and enriched f/20 seawater medium at a salinity of 15 ± 1 psu. Light was provided by cool-white fluorescent lights at a 14:10 h light:dark cycle with an intensity of 10, 40, or 120 μmol photons m⁻² s⁻¹, depending on the experiment.
2.2 Experiments

To get a comprehensive understanding of the effects of irradiance and prey availability on *S. cf. basimorphum*, two sets of experiments were designed. Both experiments utilized three light levels: 10, 40, and 120 $\mu$mol photons m$^{-2}$ s$^{-1}$; these will henceforth be referred to as $I_{10}$, $I_{40}$, and $I_{120}$, respectively. These irradiances were selected to represent the range of light in which *S. cf. basimorphum* can typically be found. $I_{10}$ represents an irradiance that could be found at the base of the pycnocline, where light levels can often be around 1% of surface irradiance. $I_{40}$ represents irradiance levels potentially found at the top of the pycnocline, and $I_{120}$ is the average that can be found within the upper mixed layers of the euphotic zone (Abdelrhman, 2016). Both experiments investigate changes in growth/mortality, ingestion, and inorganic carbon uptake rates, while also measuring ciliate cell biovolume and chl-a content. The first experiment explores differences in the physiologic responses of well-fed ciliate cells when they are subject to prey depletion at each of the three experimental light levels (the starvation experiment). The second investigates differences in the physiologic responses of ciliates acclimated to three different prey concentrations at each of the three light levels (the acclimation experiment).

2.2.1 Exp. 1: Starvation experiment

For these experiments, effects of irradiance on photosynthetic and survival responses of *S. cf. basimorphum* when starved of prey were studied. The experiments were terminated after 13 days, or after the ciliate density was < 5 cells mL$^{-1}$. For a minimum of five days before the initiation of experiments for each light condition, 800 mL of *S. cf. basimorphum* mixed culture (contained in a 1L glass culture flask) were acclimatized to the experimental irradiance and fed light-acclimatized *T. amphioxeia* cells at prey concentrations that were replenished daily to 4×10$^4$ cells mL$^{-1}$. When the ciliate density reached approximately 175 cells mL$^{-1}$, the experiment was initiated (day 0) by splitting the acclimated culture into three 200 mL triplicates that were contained in 500 mL glass culture flasks. Then, *T. amphioxeia* cells were added to the experimental cultures so that the algal density of the culture reached a saturating prey density of ~3-3.5×10$^4$ cells mL$^{-1}$. Subsamples (5 to 10 mL) were collected, at minimum, on days 2, 5, 7, and 9, as well as every 2 days thereafter until the termination of each experiment. These subsamples were used to measure the densities (individuals mL$^{-1}$), growth rates (cell divisions d$^{-1}$), photosynthetic rates (pg C cell$^{-1}$ h$^{-1}$), and chlorophyll content (pg chl-a cell$^{-1}$) of both ciliates and algae, as well as to measure the ciliates’ cell volumes ($\mu$m$^3$). Monocultures of *Teleaulax amphioxeia* were run in parallel with the mixed cultures until there were no measurable prey densities within ciliate cultures. Algal density and growth were measured in the monocultures before they were diluted with fresh f/20 media on each sampling day to match the algal densities found in the experimental cultures. The monocultures were also sampled for photosynthetic rate and chlorophyll content on the same days as the experimental cultures.

2.2.2 Exp. 2: Acclimation experiment

Experiment 2 was carried out to study the effects of light and prey density on growth, ingestion, and photosynthetic rates of *Strombidium cf. basimorphum* using *Teleaulax amphioxeia* as prey. Cultures were acclimatized to each light condition for a minimum of 5 days prior to the initiation of all experiments. The duration of the experiment was 5 days, with days 1 and 2 serving to acclimatize cultures to the experimental prey densities. Monocultures of *T. amphioxeia* were run as controls alongside mixed cultures. The experiments were carried out at the same light levels as for the previous experiment: $I_{10}$, $I_{40}$, and $I_{120}$. A preliminary experiment was carried out to provide a rough estimate of
the minimum prey density required for culture survival (see Supplementary Material 1 for a detailed outline of this experiment). Then, for each light treatment, three different prey densities were tested: 5×10^3, 1×10^4, and 4×10^4 cells mL\(^{-1}\). These prey densities were chosen to cover a range of ecologically relevant prey concentrations (Levinsen et al., 2000; Haraguchi et al., 2018), with the highest density treatment representing prey concentrations that can occur during spring phytoplankton blooms, and the two lower densities representing prey concentrations more typically found throughout the rest of the year. *S. cf. basimorphum* density was maintained at 15 individuals mL\(^{-1}\). Ciliate and prey densities were adjusted every day by dilution with fresh media and addition of algae from monocultures (Figure 1). During days 1-2, 800 mL of both the mixed cultures and control monocultures were kept in 1 L glass culture flasks. At the end of day 2, the acclimatized cultures were split into triplicates by placing 200 mL of each culture into 500 mL glass culture flasks. Subsamples (5-10 mL) were taken daily at a fixed time for all 5 days of the experiment to discern algal and ciliate densities. On days 3-5 of the experiment, additional subsamples were taken to measure cell volume, photosynthetic rate, and chlorophyll content as described previously.

**Figure 1.** Experiment 2: Acclimation of *Strombidium* cf. *basimorphum* to different prey densities: (A) 50, (B) 100, and (C) 400 ng C mL\(^{-1}\), and irradiances, I\(_{10}\), I\(_{40}\), and I\(_{120}\). Dilutions were made daily to return cultures to the experimental prey levels. Solid, dashed, and dotted lines denote light treatments I\(_{10}\), I\(_{40}\), and I\(_{120}\), respectively. Horizontal grey lines represent the average prey density of each culture over all sampling days. Data points are means (n=3).

### 2.3 Techniques

#### 2.3.1 Enumeration of cells

For enumeration of ciliates, 2 mL samples were placed in 24-well tissue culture plates, fixed in Lugol’s solution (final concentration 1%), and subsequently counted on an inverted light microscope (Olympus CKX53) at 50× magnification. Generally, this was repeated up to six times, until a minimum of 200 individuals was counted. For the very dilute cultures found at the end of the starvation experiment, this procedure was repeated up to 12 times. Algal prey was counted using a CytoFLEX flow cytometer (Beckman Coulter, USA) based on fluorescence patterns and cell size from side angle light scatter (Olson et al., 1991). Algal densities were then converted to their C (carbon) equivalents by assuming that each cell contains 10 pg C, based on average cell volume (Menden-Deuer and Lessard, 2000), as in Maselli et al. (2020).

#### 2.3.2 Ciliate cell volume

The length and width of the ciliate cells preserved with Lugol’s solution were measured using an Olympus TH4-200 light microscope, which was set to a magnification of 200x and connected to an Olympus DP73 camera. Measurements were taken utilizing the CellSense software. *S. cf.
basimorphum cells were determined to be either spherical or ellipsoidal, and biovolume (in $\mu$m$^3$) was calculated accordingly.

2.3.3 Growth and ingestion rates
For both acclimation and starvation experiments, the daily growth rate of Strombidium cf. basimorphum ($\mu_5$) and prey cells ($\mu_0$) were calculated assuming exponential growth (Equation 1):

$$\mu = \frac{\ln N_1 - \ln N_0}{t}$$  
Eq. 1

where $N_0$ and $N_1$ refer to the number of cells mL$^{-1}$ at the start ($t_0$) and end ($t_1$) of each incubation period, respectively, and $t$ is the time interval between samplings (in days unless otherwise noted).

The average algal and ciliate densities in cultures were used to calculate the culture’s clearance rate and, ultimately, the ingestion rate. This method was first suggested by Frost (1972) and later modified by Heinbokel (1978). Within this paper, we calculated the amount of prey ingested throughout a 24 h period. The actual ingestion rate at any given time within each day may vary significantly based on the light:dark cycles and the specific amount of prey available. This is particularly important to note for cultures within the two lowest prey concentration treatments ($5 \times 10^3$ and $1 \times 10^4$ cells mL$^{-1}$), as the prey were drastically depleted to near-imperceptible levels within 24 hours. Thus, the daily ingestion rates in this paper are reported as a function of our three initial prey density treatment levels, which should not be misunderstood as the average prey density over each day. These calculations of daily ingestion rate were also used for calculations of gross growth efficiency and carbon acquisition.

2.3.4 Chlorophyll-a content
For ciliate chl-a measurements, twenty ciliate cells from each experimental bottle were picked with a drawn micropipette, rinsed twice in fresh f/20 media to remove prey cells, and added to 2 mL of 96% ethanol. Algal chl-a content was measured by collecting 5 mL of algal culture onto glass microfiber filters (Whatman, GF/F) before the filters were placed in 5 mL of 96% ethanol. All samples were then stored in the dark at 4°C for 24 hours, after which chl-a was measured using a Turner Trilogy Fluorometer set with a chl-a (non-acid) insert.

2.3.5 Inorganic carbon uptake rate
Inorganic carbon uptake rates were measured using the ‘single cell method’ (Rivkin and Seliger, 1981). For each experimental ciliate culture, two 23 mL glass scintillation vials were prepared with 2 mL of fresh f/20 media. Twenty ciliate cells per culture bottle were then picked using a drawn Pasteur pipette and washed twice in fresh f/20 media to separate them from any remaining prey before being added to the scintillation vials. For experiments in which ciliates were given T. amphioxeia as prey, control monocultures of this algae were maintained alongside mixed cultures. For these control cultures, two scintillation vials containing 2 mL of monoculture were prepared from each control culture. Then, for both ciliate and control algal cultures, 20 $\mu$L of NaH$^{14}$CO$_3^-$ stock solution (specific activity 100 $\mu$Ci mL$^{-1}$) was added to each vial; one vial for every culture was incubated in the experimental light conditions, while the second vial was wrapped in aluminum foil and incubated in the dark. The incubation lasted 3 h, whereafter subsamples of 100 $\mu$L from each of the incubated vials was added to a separate vial containing 200 $\mu$L phenylethylamine. These new vials were used to measure the specific activity of the medium. The remaining 1.9 mL was treated with 2 mL of 10% acetic acid in methanol.
Running Title: Irradiance alters mixotrophic carbon uptake

to remove all the inorganic carbon. The vials were dried overnight at 64°C before the resulting residue was re-dissolved in 1.5 mL distilled water. 10 mL of Ultima Gold™ XR scintillation fluid was added to all vials (including those for specific activity) and activity was measured using a Packard 1500 TriCarb liquid scintillation counter. Daily carbon incorporation rates, P (pg C cell⁻¹ d⁻¹) were calculated by multiplying the hourly rates (Equation 2) by the duration of the daily light period (14h):

\[
P = \frac{[D_l - D_d]}{N} \times C_m \times 14 \times 10^6 \times \frac{SA \times t}{t}
\]

where \(D_l\) and \(D_d\) refer to the disintegration per minute in the light and dark, respectively. SA refers to specific activity (disintegrations per minute) of \(^{14}\)C in the medium. N is the number of cells measured, and \(C_m\) refers to the inorganic carbon content of the medium (µg C mL⁻¹). t is the incubation time in hours. \(10^6\) is used to convert µg to pg.

The total inorganic carbon of the culture medium was measured with a Shimadzu TOC-L analyzer using 25 mL media samples secured in screw-top glass vials so that no air was present in the sample.

2.3.6 Gross growth efficiency and carbon content

Gross growth efficiency (GGE, Equation 6) was calculated as the percentage of the ingested carbon (pg C cell⁻¹ d⁻¹) effectively converted into new ciliate biomass (Equation 5). \(\mu_y C\) is reported in pg C cell⁻¹ d⁻¹ and is the C-specific growth rate. The calculation of ingested carbon (\(C_I\), Equation 3) utilizes the aforementioned assumption that each ingested algal cell contains 10 pg C. Ciliate carbon content (\(C_y\), Equation 4) was calculated by multiplying the previously calculated biovolume by a conversion factor of 0.19 pg C µm⁻³ (Putt and Stoecker, 1989).

\[
C_I = I \times 10
\]

\[
C_y = 0.19 \times B_v
\]

\[
\mu_y C = \mu_y \times C_y
\]

\[
GGE = \frac{\mu_y C}{C_I}
\]
2.3.7 Statistical methods

As data from triplicates in Experiment 1 came from the same acclimatized culture, we did not do formal statistical testing and this data is used for trend description and visualization. As two independent variables were tested in Experiment 2, this data was able to undergo statistical testing. Data were analyzed with a linear mixed model (LMM) with fixed effects of irradiance, prey density, and day (categorical variables, three levels each) and random effects of the source bottle (nine levels) and sample bottle (27 levels). Some outcomes were log-transformed to obtain variance homogeneity. Notice that no interactions were included in the LMM due to the experimental design; thus, only main/overall effects were tested. These tests were carried out as F-tests (with Satterthwaite’s approximation for degrees of freedom, where necessary). Pairwise comparisons were carried out with Tukey-adjusted P-values, and letters were used to communicate statistically significant groups (at significance level 0.05). The thirty measurements of cell volume from each sample bottle did not correspond to specific days, so no fixed effect of Day was included for cell volume. The statistical software environment R version 3.6.3 (R Core Team, 2020) and packages lmerTest (Kuznetsova et al., 2017), emmeans (Lenth, 2020), and multcomp (Hothorn et al., 2008) were used for the analyses. All results are presented graphically as means ± STD and graphs were created using Python’s matplotlib library (Hunter, 2007).

3 Results

3.1 Exp. 1: Starvation experiment

3.1.1 Growth and mortality

Ciliate cultures initially experienced growth while food was still available (between days 0 and 2) at light levels of 40 (I_{40}) and 120 (I_{120}) μmol photons m^{-2} s^{-1}, after which the populations began to die out. In cultures kept at 10 (I_{10}) μmol photons m^{-2} s^{-1}, prey was completely depleted by day 2, at which point populations were already experiencing net mortality (Figures 2A & B). The steepest decline occurred in the I_{120} treatment, which reached an absolute maximum mortality rate on day 7 of -0.68 ± 0.1 d^{-1} (mean ± STD) (Figure 2C). By contrast, cultures experienced a much more gradual decline at I_{10} and I_{40}, with maximum mortality rates of -0.48 ± 0.04 and -0.45 ± 0.05 d^{-1}, respectively. Biovolumes of the cells at all irradiances were highest while ciliates were still well-fed (day 2), though already at this time point, ciliates at I_{10} were notably smaller than their counterparts at higher irradiances. A steep decline in volume was observed between days 2 and 5 at irradiances of I_{10} and I_{120}. At these light treatments, cells also experienced a relatively steep decline in cell biovolumes on the final day of the experiments. Cultures at I_{40} still saw a net decline in biovolume, but it occurred more gradually, and the cells were much larger at the termination of the experiment than in the other two treatments.
Figure 2. Experiment 1: Physiological responses of *S.* cf. *basimorphum* to prey starvation at three different light treatments ($I_{10}$, $I_{40}$, and $I_{120}$) as a function of time. (A) and (B) Cell densities of *T. amphioxeia* and *S.* cf. *basimorphum*, respectively. (C) Growth rate of *S.* cf. *basimorphum*. (D) Biovolume of Lugol-fixed ciliate cells. Solid, dashed, and dotted lines denote light treatments $I_{10}$, $I_{40}$, and $I_{120}$, respectively. Data points are means ± STD ($n=3$).
3.1.2 Chlorophyll-a and photosynthesis

Throughout the experiment, all treatments experienced a steep net decline in cellular chl-a content (Figure 3A). The ciliates’ ability to maintain their cellular chl-a after prey was depleted had an inverse relationship with irradiance. Following the trend exhibited in mortality rate, the fastest decline in chl-a occurred between days 2 and 5 at I_{120}, where cultures went from 64.7 ± 4.6 pg chl-a cell^{-1} on day 2 to 15.3 ± 1.5 pg chl-a cell^{-1} on day 5. Cultures kept at I_{10} exhibited the greatest ability to maintain chl-a levels, starting on day 2 at 68.3 ± 8.3 pg chl-a cell^{-1}, and ending on day 11 at 34.7 ± 4.0 pg chl-a cell^{-1}.

At the two lower irradiances, a notable increase in cellular inorganic carbon incorporation between days 2 and 5 was observed, after which cellular inorganic carbon uptake rates declined until the cessation of the experiments on days 11 and 13, respectively. Across all days of the experiment, photosynthetic rates ranged from 120 to 226 pg C cell^{-1} day^{-1}, and 125 to 252 pg C cell^{-1} day^{-1} at I_{10} and I_{40}, respectively (Figure 3B). Photosynthetic rates at I_{120} were considerably lower than at the other two light treatments and ranged from 16.2 to 65.1 pg C cell^{-1} day^{-1}. Chl-a specific inorganic carbon uptake rates (pg C pg chl-a^{-1} d^{-1}) generally increased in all cultures over the course of the experiment. Cultures kept at I_{10} and I_{40} both experienced an increase in chl-a specific photosynthetic rate until day 5, thereafter seeing only minor fluctuations until the experiments were ended (Figure 3C).

**Figure 3.** Experiment 1: Physiological responses of *S. cf. basimorphum* to starvation at three different light treatments (I_{10}, I_{40}, and I_{120}) as a function of time (d). (A) Cellular chl-a content. (B) Cellular photosynthetic rate. (C) Chl-a specific photosynthetic rate. Solid, dashed, and dotted lines denote light treatments I_{10}, I_{40}, and I_{120}, respectively. Data points are means ± STD (n=3).
3.2 Exp. 2: Acclimation Experiment

3.2.1 Growth and ingestion

Growth rates of *S. cf. basimorphum* were significantly influenced by prey density, whereas the statistical significance of irradiance could not be proven in this experiment (Figure 4A). Cultures kept at the lowest irradiance (I$_{10}$) saw the greatest change in growth rate across the range of prey densities. Cultures kept at I$_{40}$ exhibited the highest average growth rates in the low prey density treatment. Ciliates at this intermediate irradiance also experienced some of the highest growth rates at the highest prey density, however, this comparison becomes less striking when biovolume is also considered (Supplementary Figure 2). Compared to cells subjected to other treatments, ciliates grown at the highest irradiance had nearly twice as much biovolume when supplied with a prey density at or above 100 ng C mL$^{-1}$ (Figure 4B). See Supplementary Figure 3 for size comparisons of Lugol-fixed *S. cf. basimorphum* cells and Supplementary Table 3 for the average linear dimensions of cells in all nine treatments.

**Figure 4.** Experiment 2: Physiological responses of *S. cf. basimorphum* at the three light (I$_{10}$, I$_{40}$, and I$_{120}$) and three prey density treatments (50, 100, and 400 ng C mL$^{-1}$). (A) Growth rate (n=9). (B) Biovolume of Lugol-fixed ciliate cells (n=90). (C) Ingestion rate (n=9). (D) GGE (n=9). Black, dark grey, and light grey bars denote light treatments I$_{10}$, I$_{40}$, and I$_{120}$, respectively. Data points are means ± STD. Irradiance was not proven to be statistically significant for any of the displayed variables; the letters, therefore, represent statistically similar groupings based only on the overall effects of prey density across all irradiance treatments. Subplots without letters did not show significant effects of prey density or irradiance.
Only prey density was shown to have a significant effect on ingestion rates. In particular, ingestion rates exhibited a steep increase of 583, 884, and 271 prey predator\(^{-1}\) d\(^{-1}\) between 100 and 400 ng C mL\(^{-1}\) for I\(_{10}\), I\(_{40}\), and I\(_{120}\), respectively (Figure 4C). GGE was consistently lowest at I\(_{10}\), where a maximum efficiency of 0.43 ± 0.12 was seen at 400 ng C mL\(^{-1}\) (Figure 4D). Cultures at I\(_{40}\) expressed an opposite trend, reaching the highest GGE measured in this study (1.10 ± 0.35) at the lowest prey density. Finally, cultures at the highest irradiance and medium prey density also exhibited a high GGE, reaching 0.99 ± 0.43.

### 3.2.2 Cellular chl-a and photosynthetic rate

The prey species, *T. amphioxeia*, exhibited a negative correlation between cellular chl-a and irradiance and a positive correlation between irradiance and both cell-specific and chl-a specific inorganic carbon uptake rates. Cellular chl-a content in *S. cf. basimorphum* was, on average, 231 times greater than in its prey (Figure 5A & D). The large increase in chl-a content observed in the high-irradiance treatment at intermediate and high prey densities can be primarily explained by the substantial increase in biovolume seen in these treatments; biovolume-specific chl-a content at I\(_{120}\) (Supplementary Figure 2) was often actually lower than at the other two irradiances. Chl-a contents were similar between the two highest prey levels at any irradiance. The intermediate light level is the only one to exhibit a decrease in chl-a content from the low to intermediate prey densities.

In the lowest irradiance, regardless of prey density, the cell- and chl-a specific inorganic carbon uptake rates (Figure 5B & C) were extremely low when compared with the other two light levels. Ciliates grown at I\(_{40}\) reached a maximum inorganic carbon uptake rate of 979 ± 125 pg C cell\(^{-1}\) d\(^{-1}\) at the lowest prey density and a minimum of 141 ± 45 pg C cell\(^{-1}\) d\(^{-1}\) at the highest prey density. Cultures kept at the highest light level expressed cellular inorganic carbon uptake rates that increased with increasing prey density to a maximum of 1260 ± 260 pg C cell\(^{-1}\) d\(^{-1}\). The chl-a specific inorganic carbon uptake rate of cultures kept at I\(_{10}\) and I\(_{120}\) did not vary across prey densities. At I\(_{40}\), however, ciliates exhibited a decreasing cell-specific and chl-a specific inorganic carbon uptake rate as prey availability increased.
Figure 5. Experiment 2: Physiological responses of *S. cf. basimorphum* (A, B, C) and *T. amphioxeia* (D, E, F) at the three light (*I*₁₀, *I*₄₀, and *I*₁₂₀) and three prey density treatments (50, 100, and 400 ng C mL⁻¹). (A, D) Cellular chl-a content. (B, E) Daily cellular inorganic carbon uptake rate. (C, F) Daily chl-a specific inorganic carbon uptake rate. Black, dark grey, and light grey bars denote light treatments *I*₁₀, *I*₄₀, and *I*₁₂₀, respectively. Data points are means ± STD (n=9). Prey density was not proven to be statistically significant for any of the displayed variables; the letters, therefore, represent statistically similar groupings based only on the overall effects of irradiance across all prey density treatments. Subplots without letters did not show significant effects of prey density or irradiance.
3.2.3 Carbon acquisition

The percentage contribution of inorganic carbon uptake was found to always be lowest at I\textsubscript{10} (Figure 6). At this irradiance, a minimum of 97.4\% of C came from ingestion. In contrast, at I\textsubscript{120}, ingestion only contributed an average of 77.7\% of C and experienced less variation between the different prey densities. Cultures at I\textsubscript{40} had the greatest contribution from inorganic carbon uptake (41.5\%) at 50 ng C mL\textsuperscript{-1}, and the lowest (1.2\%) at 400 ng C mL\textsuperscript{-1}.

![Figure 6](image)

**Figure 6.** Experiment 2: Percentage of carbon acquired from inorganic carbon uptake and ingestion over the three light (I\textsubscript{10}, I\textsubscript{40}, and I\textsubscript{120}) and three prey density treatments (50, 100, and 400 ng C mL\textsuperscript{-1}).

4 Discussion

4.1 Effects of irradiance on prey-starved *Strombidium* spp.

The ability of *S. cf. basimorphum* to take up inorganic carbon (photosynthesize) led to a prolonged survival during prey scarcity when light was available. During the starvation experiment, cultures kept at I\textsubscript{10} and I\textsubscript{40} did not exceed 50\% mortality until 4 days after complete prey depletion. Mortality rates > 90\% were only reached after 11 and 13 days, respectively (Figure 2B). This is in accordance with observations made on other generalist non-constitutive mixotrophs (GNCMs) (Schoener and Mcmanus, 2012; Mcmanus et al., 2018; Maselli et al., 2020). This prolonged survival without access to prey stands in contrast to purely heterotrophic oligotrichs, which have been shown to reach almost complete culture mortality within 1-4 days (Montagnes, 1996).

While *S. cf. basimorphum* survived longer without prey than comparable heterotrophs, cultures still achieved nearly complete mortality within two weeks of prey depletion and were unable to undergo any further cell divisions at any irradiance. This is in contrast to specialist non-constitutive mixotrophic (SNCM) ciliates, such as red *Mesodinium* spp., who have been shown to not only survive for months without food but even continue to divide 3-4 times after prey is depleted (Smith and Hansen, 2007;
The ability of non-constitutive mixotrophs to survive for prolonged periods is likely dependent on their capacity to keep their acquired photosystems functional. To this end, many SNCMs have been shown to exhibit (among others) two important capabilities that have not yet been discovered in GNCMs: the capacity to additionally sequester prey nuclei and the ability to divide their stolen chloroplasts.

Without this higher level of plastid control, \textit{S. cf. basimorphum} cultures immediately began experiencing mortality once prey was depleted. Despite this, chl-\textit{a} specific inorganic carbon uptake rates were seen to rapidly increase directly following prey depletion and remain at approximately this peak value until experimental cessation (Figure 3C). This increase may be due to physiological starvation response, wherein individuals actively begin to photosynthesize at a higher rate when they are no longer able to ingest enough carbon from prey. A physiological response may also be suggested by the increase in the relative carbon contribution of inorganic carbon uptake to \textit{S. cf. basimorphum}'s total carbon budget at lower prey densities, as seen in this study (e.g., at I\textsubscript{40}). Conversely, this increase in photosynthetic ability may also derive from the ciliates digesting some of their chloroplasts when little or no prey is available, thus reducing the incidence of self-shading and potentially increasing the efficiency of any un-digested chloroplasts.

In the I\textsubscript{120} treatment, the cellular chl-\textit{a} content decreased faster than at the lower light treatments, and inorganic carbon uptake rates were approximately nine times lower than in the mid- and low-light treatments on day five. At the high light level, the ciliate cultures reached over 80\% mortality two days after prey depletion, bringing them closer to rates expressed by heterotrophic species. This fast decay and high turnover may indicate that \textit{S. cf. basimorphum} has a decreased ability to control plastid photoadaptation and repair damages from photo-oxidative stress when compared to organisms that have innate photosystems (Lavaud et al., 2004; Funk et al., 2011). Even SNCMs have been shown to begin to deplete their chl-\textit{a} reserves twice as quickly in high light than in low light (Johnson and Stoecker, 2005).

4.2 Influence of irradiance and prey density on \textit{Strombidium} spp.

4.2.1 Ingestion rate

Evidence of light-dependent ingestion rates has been reported previously among both heterotrophic and mixotrophic SNCMs dinoflagellates (Jakobsen et al., 2000; Li et al., 2000; Kim et al., 2008) and heterotrophic ciliate species (Strom, 2001; Tarangkoon and Hansen, 2011). However, our results did not indicate that ingestion rates were significantly affected by irradiance in \textit{S. cf. basimorphum} and our results were comparable to what has been previously found for this species in other light conditions (Maselli et al., 2020). Similarly, Stoecker et al. (1988b) did not find light effects on ingestion in the only oligotrich ciliate studied previously, \textit{Laboea strobila}. Nevertheless, further research is needed to confirm the presence or absence of this phenomenon in \textit{Strombidium} spp. and other GNCM oligotrichs.

4.2.2 Inorganic carbon uptake rate

It is noteworthy that inorganic carbon uptake rates decreased with increasing prey density in the I\textsubscript{40} treatment (Figure 5B). Maselli et al. (2020) observed a similar pattern in \textit{Strombidium} spp. incubated at 70 \textmu mol photons m\textsuperscript{-2} s\textsuperscript{-1}. However, inorganic carbon uptake rates actually increased as a function of prey density in the I\textsubscript{120} treatment. This may indicate that the ciliates experience self-shading at lower irradiances (\leq 70 \textmu mol photons m\textsuperscript{-2} s\textsuperscript{-1}) when prey is abundant, as has also been suggested in a modeling study (Flynn and Hansen, 2013). These trends, in combination with the low photosynthetic
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rates displayed by starved cultures in higher intensity light conditions, suggest that the ciliate cannot maintain functional chloroplasts without replacement in higher intensity light conditions. This phenomenon may be a result of photoinhibition, as chloroplasts exposed to higher irradiances would work at higher rates and the ciliate may therefore require faster chloroplast turnover - via ingestion - to keep them functional. However, previous studies on other GNCM species have not shown evidence of photoinhibition in ciliates exposed to irradiances up to 500 μmol photons m⁻² s⁻¹ (Stoecker et al., 1988a; Putt, 1990a) – higher than any employed in this study.

Photosynthetic rates based on chl-a are known to be significantly different when comparing organisms with innate photosystems and the non-constitutive mixotrophs that utilize stolen photosystems (Stoecker et al., 1988b; Mcmanus et al., 2012; Maselli et al., 2020). When directly comparing the rate of chl-a specific inorganic carbon uptake in GNCMs and their prey, S. rassoulzadegani was shown to operate its chloroplasts at ~52% efficiency (Mcmanus et al., 2012). In the present study, however, S. cf. basimorphum was only able to exceed 40% efficiency when subject to the lowest prey availability and the lowest two light conditions (I₀ and I₁₀). This may hint again to both the phenomena of self-shading that can occur at lower irradiances when cells are large and well-fed, as well as the ciliate’s inability to efficiently utilize its stolen chloroplasts when subject to higher irradiances.

The rates of inorganic carbon incorporation measured in this study were comparable to what has been previously found in four other Strombidium spp. (Stoecker et al., 1988a), but nearly eight times lower than what was measured in L. strobila when grown in similar conditions (Stoecker et al., 1988b). We did not study inorganic carbon uptake at irradiances > 140 μmol photons m⁻² s⁻¹, but in the cases of L. strobila and the four other Strombidium species, inorganic carbon uptake rates have been shown to increase as a function of irradiance up to 1200 μmol photons m⁻² s⁻¹ (Stoecker et al., 1988b, 1988a).

4.2.3 Growth, biovolume, and GGE

The growth rates of S. cf. basimorphum were positively correlated to prey density at all irradiances. This increase in growth rates was especially apparent when biovolume was accounted for (see Supplementary Figure 2A). Many heterotrophic and mixotrophic plankton have been shown to follow a similar pattern (Montagnes, 1996; Hansen et al., 1997; Smith and Hansen, 2007). GNCM ciliates seem to allocate the carbon acquired from photosynthesis into polysaccharides, which are then preferentially used for respiration (Stoecker and Michaels 1991; Putt 1990). Thus, inorganic carbon uptake can help to sustain ciliate growth by covering part of the respiratory demand. This is of particular relevance when prey availability is low. In the present study, growth rates were generally lowest in the lowest light treatment (I₁₀), when, in fact, cell-specific inorganic carbon uptake rates were significantly depressed. In the I₁₀ treatment, indeed, S. cf. basimorphum appeared to primarily function as a heterotroph. At I₁₂₀ when food was at or above 100 ng C mL⁻¹, ciliate cells were significantly larger than at other light levels. Therefore, the larger GGE (discussed below) exhibited at higher light levels is due to an increase in biovolume and not an increase in cellular divisions. Similarly, L. strobila was also shown to grow to significantly greater sizes in the light, when compared to in the dark (Stoecker et al., 1988b). The highest GGE of S. cf. basimorphum at each level of prey density corresponded with the light level where inorganic carbon uptake was also greatest. When less prey was available (50 ng C mL⁻¹), GGE was highest at I₀. However, at 100 and 400 ng C mL⁻¹, both photosynthesis and GGE instead saw their prey density-specific maximums at I₁₂₀ (Figure 4D). Interestingly, other studies have reported trends in GGE similar to what was shown at I₄₀. Three species of Strombidium (including S. cf. basimorphum) were shown to have increased reliance on inorganic carbon uptake at lower prey densities when grown at 70 μmol photons m⁻² s⁻¹ (Maselli et al., 2020) and 100 μmol photons m⁻² s⁻¹
Running Title: Irradiance alters mixotrophic carbon uptake

(Schoener and Mcmanus, 2012, 2017). However, there is little record of GNCMs having a similarly dramatic increase in GGE at high light levels when prey is plentiful, as was shown in this study.

4.3 Ecological implications

In the field, S. cf. basimorphum, along with other GNCMs, will be subject to competition from other organisms that employ different nutritional strategies. It is likely, however, that GNCMs outperform their heterotrophic counterparts in environments where their GGE is greater than that exhibited by heterotrophs, which generally have a GGE of 0.3-0.4 (Straile, 1997). According to our experiments, this occurs consistently in both prey-saturated high-irradiance and prey-limiting intermediate-irradiance conditions. GNCMs can also capitalize on their prolonged ability to subsist through conditions with low prey availability when pure heterotrophs are unable to survive. Conversely, GNCMs will almost always have a lower GGE compared to SNCMs, which rely more heavily on photosynthesis (Hansen et al., 2013). However, GNCMs would theoretically gain an advantage over SNCMs in situations where the SNCM’s specific prey associate was unavailable or where inorganic nutrients or light were limited, thus restricting phototrophic growth (Stoecker et al., 1988b; Tarangkoon and Hansen, 2011; Hansen et al., 2013).

Our study also indicates that GNCMs like S. cf. basimorphum can grow in low-light conditions when prey concentrations are high enough to support maintenance. Other studies have corroborated these findings in nature, where GNCMs have been shown to maintain biomasses at the dark deep chlorophyll maximum that are comparable to those found in bright surface waters. Even at depths below the euphotic zone, GNCM ciliates can thrive due to their ability to function as completely heterotrophic organisms. In fact, at and below the depth of the chlorophyll maxima, where light is so low that it may strongly limit photosynthesis, GNCM organisms exhibit patterns similar to their heterotrophic counterparts, and experience less seasonal variability than in well-lit surface waters (Levinsen et al., 2000).

In temperate surface waters, from which our isolate of Strombidium basimorphum was collected, SNCMs and phytoplankton utilize the winter build-up of dissolved nutrients to form large spring blooms, reaching abundances nearly 20 times greater than recorded at more southern latitudes (Leles et al., 2017). Then, after phytoplankton biomass increases, GNCMs, exploiting this new prey source along with the available light and dissolved nutrients, begin to grow with high efficiency to from smaller-magnitude blooms in late spring, and achieving the greatest relative contribution to overall ciliate biomass (85-90%) in the summertime (Levinsen et al., 2000; Haraguchi et al., 2018). The ability of GNCMs to outcompete both heterotrophs and SNCMs in the summer can be linked to the flexible nutrition and high growth efficiency that is demonstrated in this study; GNCMs require less prey than heterotrophs for maximal growth and survival if exposed to adequate light and can withstand nutrient-depleted waters better than SNCMs by simply consuming more prey (Stoecker and Lavrentyev, 2018).

5 Conclusion

In this study, the generalist non-constitutive mixotroph (GNCM) S. cf. basimorphum appeared to be most well-adapted to the mid-light condition (40 μmol photons m⁻² s⁻¹) when subjected to prey-starved or limited conditions. However, at slightly higher prey densities, S. cf. basimorphum exhibited the highest inorganic carbon uptake rates and GGE when grown at a higher irradiance. These results indicate that the GNCM is unable to maintain functioning prey chloroplasts without replacement in higher-intensity light conditions. Furthermore, while ingestion rates, growth parameters, and chl-a
content were not shown to vary significantly with irradiance, light consistently influenced both cell-specific and chl-a specific inorganic carbon uptake rates. Our findings suggest that *S. cf. basimorphum* – and indeed, GNCMs in general – have a competitive advantage over specialist non-constitutive mixotrophs in prey-replete conditions, where they can rely more heavily on prey ingestion for growth. Conversely, they can out-compete their heterotrophic counterparts in prey-starved conditions where inorganic carbon uptake can be used to prolong survival or increase GGE.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Funding

This work was supported by EC MSCA-ITN 2019 funding via the project MixITiN (grant number 766327) and by the Danish Independent Research Fund (project number 4181–00484). The Carlsberg foundation funded the flow cytometer used here.

8 Data Availability Statement

The datasets generated and analyzed for this study can be found in the Mendeley Data repository (DOI: 10.17632/wmm2wkcwwz.2) at https://data.mendeley.com/datasets/wmm2wkcwwz/2.
Running Title: Irradiance alters mixotrophic carbon uptake

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Running Title: Irradiance alters mixotrophic carbon uptake


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Supplementary material
Running Title: Irradiance alters mixotrophic carbon uptake
Preliminary Experiment 1

1.1 Materials and Methods

This preliminary experiment was carried out to give a rough estimate of how Strombidium cf. basimorphum may react to different light treatments and to inform the choice of which prey densities to use in Experiment 2. Prey density was not adjusted during the experiment, and thus results could be skewed by early prey depletion in experimental replicates that began at lower prey densities.

Six prey densities (0.5, 1, 2, 5, 10, and $15 \times 10^3$ cells mL$^{-1}$) were tested in three different light conditions (10, 40, and 120 $\mu$mol photons m$^{-2}$ s$^{-1}$, referred to as $I_{10}$, $I_{40}$, and $I_{120}$, respectively). A sample of 20 mL of each experimental prey density was prepared by mixing concentrated algal cultures with filtered sea water. Then, each prepared algal culture was distributed into eight wells, which each contained 2 mL of the culture. The first four wells were assigned to be experimental mixed cultures, and 10 twice-washed ciliate cells were added to each of the four wells. The remaining four wells of the same prey density were designated as control T. amphioxeia monocultures. The experiment was initiated with the introduction and mixing of ciliates and prey in the plates as described and ended after three days when all cultures were fixed in Lugol’s solution and counted in order to determine both the growth and ingestion rates of the ciliates. T. amphioxeia densities were determined using Sedgewick-Rafter chambers, counting a minimum of 200 cells.

The resulting response curve for growth rate was fitted to a Michaelis-Menten equation (Supplementary Equation 1), as modified by (Montagnes, 1996).

$$V = \frac{V_{\text{max}} \times [C - T]}{K_m + [C - T]}$$

Sup. Eq. 1

$V$ is the ciliate’s growth rate, $V_{\text{max}}$ is their maximum possible growth rate, $C$ is the algal/prey density, $T$ refers to the prey density at which ciliate growth rate is 0, and $K_m$ is the prey density at which $V$ is exactly half of $V_{\text{max}}$.

The subsequent Michaelis-Menten Kinetics curves were compared between irradiances using an extra-sum-of-squares F-test. This test separately compared the pooled sum of squares from the curves for each of the light treatments to the extra sum of squares for their combined data set to a single common curve. This was also done separately for parameters $V_{\text{max}}$, $K_m$, and $T$. The null hypothesis is that a single curve or parameter estimate provides a better fit for the three data sets, rather than separate curves or parameters (Motulsky and Ransnas, 1987). A p-value of 0.05 was used for significance testing. Both the curve fitting and subsequent analysis was done utilizing GraphPad Prism version 8.4.2 for Mac.
1.2 Results

As these experiments were meant to give a rough estimation of light and prey effects, prey density was not as strongly controlled as in Experiment 2. The prey density over the three days of this experiment was quite variable (see Supp. Table 1), limiting the statistical power of the subsequent findings.

**Supplementary Table 1.** Prelim. Experiment 1. Initial and final prey densities at the three experimental light treatments. All numbers are in algal cells mL\(^{-1}\) unless otherwise denoted. Standard errors are displayed in parentheses.

<table>
<thead>
<tr>
<th>Initial prey density (10(^3) algal cells mL(^{-1}))</th>
<th>10 ((\mu)mol photons m(^{-2}) s(^{-1}))</th>
<th>40 ((\mu)mol photons m(^{-2}) s(^{-1}))</th>
<th>120 ((\mu)mol photons m(^{-2}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>198 (12.0)</td>
<td>210 (46.6)</td>
<td>162.5 (10.5)</td>
</tr>
<tr>
<td>1</td>
<td>240 (7.36)</td>
<td>300 (45.6)</td>
<td>225 (19.3)</td>
</tr>
<tr>
<td>2</td>
<td>338 (19.0)</td>
<td>375 (26.3)</td>
<td>225 (17.6)</td>
</tr>
<tr>
<td>5</td>
<td>375 (17.6)</td>
<td>300 (31.9)</td>
<td>600 (73.6)</td>
</tr>
<tr>
<td>10</td>
<td>225 (17.6)</td>
<td>350 (20.4)</td>
<td>475 (22.8)</td>
</tr>
<tr>
<td>15</td>
<td>260 (14.7)</td>
<td>950 (119)</td>
<td>650 (22.5)</td>
</tr>
</tbody>
</table>

With that in mind, prey density was shown to have a significant impact on both growth and ingestion rates, while light did not appear to affect either variable (p-value = 0.052). Growth rate (Supp. Figure 1A) increased from 0.03 d\(^{-1}\) at the lowest initial prey density (5 ng C mL\(^{-1}\)) to 0.42 d\(^{-1}\) at the highest prey density (150 ng C mL\(^{-1}\)). Ingestion rates ranged from 26.9 prey cells predator\(^{-1}\) d\(^{-1}\) at the lowest initial prey density and 481 prey cells predator\(^{-1}\) d\(^{-1}\) at the highest.

The growth rate data was fitted to a Michaelis-Menten kinetics curve for each of the three light levels (Supp. Figure 1A, Supp. Table 2). The resulting curves indicated that ciliates grown at 10 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) required the highest prey threshold density (T) to sustain the population. Cultures grown at the highest light treatment, 120 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\), required the lowest density of prey to achieve half their maximum growth (\(K_m\)).
<table>
<thead>
<tr>
<th>Light treatments (µmol photons m$^{-2}$ s$^{-1}$)</th>
<th>10</th>
<th>40</th>
<th>120</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Fit</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$K_m$</td>
<td>7.99 (4.63)</td>
<td>2.73 (1.18)</td>
<td>2.32 (0.96)</td>
<td>0.118</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>1.67 (0.71)*</td>
<td>0.59 (0.12)*</td>
<td>0.69 (0.12)*</td>
<td>0.015</td>
</tr>
<tr>
<td>$T$</td>
<td>0.35 (0.07)</td>
<td>0.30 (0.11)</td>
<td>0.18 (0.095)</td>
<td>0.307</td>
</tr>
</tbody>
</table>
Supplementary Figure 1. Prelim. Experiment: Growth rate $\mu$ (A) and ingestion (B) responses of S. cf. basimorphum across three different light treatments and six different prey densities. Growth rate response curves were numerically fitted to Michaelis-Menten kinetics, and curves in (A) represent the subsequent models. Curves in (B) connect average ingestion rates at each prey density to each other. Solid, dashed, and dotted lines denote light treatments $I_{10}$, $I_{40}$, and $I_{120}$, respectively.
Experiment 2: Data controlled for biovolume

Upon finding large variability in the size of ciliates acclimated to different light and prey conditions (Supplementary Figure 2; Supplementary Table 3), it was clear that simply reporting cell-specific results for variables such as growth rate and chl-a content would not accurately capture the full implications of *S. cf. basimorphum*’s physiologic changes (Supplementary Figure 2). Therefore, to determine the amount of additional volume gained by ciliate cells (in $\mu m^3$ d$^{-1}$) the growth rate (in cellular divisions d$^{-1}$) for each culture was multiplied by the average cell biovolume (Supplementary Figure 3A). Similarly, to compare chl-a content across cells of different sizes, volume-specific chl-a (in pg chl-a $10^{-3} \mu m^3$) was calculated by dividing the cell-specific chl-a content by the average cell biovolume (Supplementary Figure 3B).

**Supplementary Figure 2.** Images of different *S. cf. basimorphum* cells fixed with Lugol solution. The top row of images (A, B, and C) shows examples of small-sized cells that were grown in prey starved or limited conditions. The two bottom pictures (D and E) show cells that were grown in prey replete conditions.
Supplementary Table 3. Experiment 2. Average cell sizes for each of the nine experimental treatments conditions. Standard errors are denoted in parentheses (n = 90).

<table>
<thead>
<tr>
<th>Light Condition (μmol photons m⁻² s⁻¹)</th>
<th>Prey Condition (10^3 cells mL⁻¹)</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Biovolume (10^3 μm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
<td>37.42 (0.518)</td>
<td>34.59 (0.418)</td>
<td>24.16 (0.818)</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>37.42 (0.652)</td>
<td>35.62 (0.894)</td>
<td>27.79 (1.672)</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>39.55 (0.549)</td>
<td>37.55 (0.459)</td>
<td>30.26 (1.162)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>38.04 (0.494)</td>
<td>39.78 (0.433)</td>
<td>32.28 (0.931)</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>35.72 (0.419)</td>
<td>35.12 (0.378)</td>
<td>23.59 (0.734)</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>40.63 (0.526)</td>
<td>39.60 (0.417)</td>
<td>34.18 (1.062)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>37.58 (0.404)</td>
<td>37.15 (0.482)</td>
<td>27.81 (0.828)</td>
</tr>
<tr>
<td>120</td>
<td>10</td>
<td>45.04 (0.512)</td>
<td>45.02 (0.482)</td>
<td>48.97 (1.503)</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>44.48 (0.584)</td>
<td>45.28 (0.549)</td>
<td>50.00 (1.537)</td>
</tr>
</tbody>
</table>
Supplementary Figure 3. Experiment 2: (a) Biovolume-specific growth rate. (b) Biovolume-specific chl-α content across the three experimental light levels and prey densities. Black, dark grey, and light grey bars denote light treatments $I_{10}$, $I_{40}$, and $I_{120}$, respectively.
Running Title: Irradiance alters mixotrophic carbon uptake

References


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 concentration of dissolved inorganic nitrogen and phosphorus. Specifically, we tested the direct and indirect effects of nutrient availability on the ciliate *Stombidium* cf. *basimorphum* fed with the cryptophyte prey *Teleaulax amphioxeia*. We assessed responses in growth, ingestion, photosynthetic rates, inorganic nutrient uptake, and excretion rates. Our results show that low inorganic nutrient concentrations increased *S*. cf. *basimorphum* growth and prey ingestion. This can be associated to the higher carbon content of the prey that supports the growth of the ciliate, while higher carbon:nutrient stoichiometry in the prey may have led to the higher ingestion rates, suggesting the potential for compensatory feeding. The low carbon contents of the prey at high nutrient concentrations resulted in reduced growth in *S*. cf. *basimorphum*, which indicates that photosynthesis in the ciliate cannot compensate for the ingestion of prey. In conclusion, our findings show *S*. cf. *basimorphum* is not able to utilize dissolved inorganic nitrogen and phosphate for growth, while it seems to be very well adapted to exploit its prey when grown in low nutrients 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 are able to keep them functional inside their cell body (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. Such protists have recently been 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 NCMs 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 prey (Smith and Hansen 2007). This is in contrast to the prey generalist NCMs, which have been shown unable to grow autotrophically when starved of prey (Schoener and McManus, 2012; Maselli et al., 2020). The ingestion rates of prey generalist NCMs are much higher than those of M. rubrum, and very similar to those of heterotrophic ciliates species. This suggests that, even if prey generalist NCMs are able to fix carbon via photosynthesis, they rely on ingestion to obtain other essential elements, such as nitrogen (N) and phosphorus (P) (Dolan 1997).

Little is known, however, about the ability of NCM ciliates to directly take up dissolved inorganic nutrients from the external environment. The prey specialist NCM M. rubrum has been shown to take up inorganic nitrogen (NO$_3^-$ and NH$_4^+$) and phosphate at rates comparable to phototrophic protists, and to utilize these nutrients for growth when prey availability is low (Tong et al. 2015). Uptake of inorganic nitrogen (both NO$_3^-$ and NH$_4^+$) also has been documented for the prey generalist NCM Strombidium rassoulzadegani, though at much lower rates as compared to M. rubrum. Reported uptake rates for S. rassoulzadegani are in fact comparable to the rates measured in exclusive heterotrophic ciliates species, and inorganic N uptake does not contribute significantly to its N requirements for growth (Schoener and McManus, 2017).

Impacts of inorganic nutrients on ciliate and prey
The ability of *M. rubrum* to take up inorganic nutrients might lead to depletion of dissolved inorganic nutrients from the external environment when starved by lack of prey, while *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 compensate their phototrophic activity, resulting in lower excretion compared to heterotrophic species (Ghyoot et al. 2017). This has, however, never been empirically demonstrated.

Photosynthetic microalgae that serve as prey for these ciliates exhibit a wide variety of physiological adaptations to nutrient availability. At optimal nutrient conditions, the major elements (C, N and P) are generally present in their biomass in relatively constant proportions, resembling what is known as the Redfield Ratio with an approximate molar C:N:P of 106:16:1 (Redfield et al. 1963). However, if dissolved inorganic N or P becomes depleted, C incorporation though photosynthesis is not balanced by the incorporation of the other nutrients, resulting in an increase in the C:N and C:P ratios of the cells (Berman-frank and Dubinsky 1999; Geider and La Roche 2002; Sterner and Elser 2002). In contrast to autotrophs, the elemental composition of higher trophic levels is usually more constrained because the relative excess of non-limiting nutrients is balanced via excretion (Hessen et al. 2013). Consequently, resource cycling can be driven by the consumers, which selectively retain the limiting resource, excrete more of the non-limiting resource, and may ultimately enhance limitation in the system (Elser and Urabe 1999). Moreover, the elemental composition of the algal prey influences excretion rates of predators (Malzahn et al. 2010), and the metabolic cost for maintaining stoichiometric homeostasis can affect predator growth rate (Boersma et al. 2008; Saikia and Nandi 2010).

To get insights on how prey generalist NCM ciliates respond to shifts in nutrient availability and assess whether mixotrophy can compensate for the ingestion of potentially nutrient unbalance prey biomass, we studied the responses of *Strombidium cf. basimorphum* along a gradient of N and P concentrations. *Strombidium cf. basimorphum* is a globally distributed ciliate species (Martin and Montagnes, 1993; Liu et al. 2011; Orsi et al. 2018), which recently has been demonstrated to retain functional chloroplasts (Maselli et al., 2020). It can grow on a variety of different algal groups, but chlorophyte and cryptophyte prey types have been shown to best support its growth in culture (Maselli et al., 2020). We measured growth, photosynthetic and nutrient uptake rates as well as ingestion and excretion rates of *Strombidium cf. basimorphum* and the stoichiometry of its cryptophyte prey *Teleaulax amphioxeia* when grown at different concentrations of nitrate and phosphate. Moreover,
we followed the response of the ciliate when its prey became depleted under the different nutrient treatments.

Materials and methods

Culture conditions: The experiments were conducted on cultures of the ciliate *Strombidium* cf. *basimorphum*, established, identified and maintained as in Maselli et al. (2020), fed with the cryptophyte *Teleaulax amphioxeia* (strain K-1837; SCCAP) 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 cryptophyte prey cultures were acclimated for two weeks to the different media prior to being fed to the ciliates. 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 initiation of the experiments. In the last three days of acclimation (day0-day2), ciliates were also acclimated to the prey availability they would have had at the beginning of the experiments (day2). To do that 20 ciliates mL⁻¹ were daily inoculated with 3 x 10⁴ prey cells mL⁻¹. Ciliate cultures were set 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 (Chla) content using media with different content of inorganic nitrogen and phosphate, corresponding to different dilutions of the standard *f/2* media (Guillard 1975): *f/200*, *f/100*, *f/40* and *f/20*. The nutrient concentrations were selected to cover the range of nutrients typically used in culture studies as wells 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*, demonstrated to be optimal in previous studies on *Strombidium* species (Gifford 1985). On day 2, triplicate cultures were fed with the same amount of prey they received during the acclimation period to measure the ingestion rates in the subsequent 24h. Samples for cell enumeration, Chla content, photosynthetic rate and dissolved inorganic nitrogen and phosphorus were collected right after prey addition (day 2) and after 24h more (day 3). Triplicate monocultures of the algal prey were set simultaneously and sampled at the same time. Additionally, samples of prey biomass were collected at day 3 to analyze their elemental composition (C, N, and P). Ciliates grew for one week with no further addition of prey. The sampling was repeated when prey was completely depleted (day 6 and day 7). Ciliates and prey were enumerated on a daily basis 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 counted using a Cyto-Flex flow
cytomter set to discern populations within a scatter plot of PCA5.5A (chlophyll-a auto fluorescence) x FSC-A (forward scatter) and count events 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 magnification of 50x. Growth rates between day 2 and day 3 were calculated assuming exponential growth based on the change in cells abundance over time (μ, d⁻¹):

\[ \mu = \ln \left( \frac{B_3}{B_2} \right) / (t_3-t_2) \]

(1)

Where \( B_2 \) and \( B_3 \) are the cell concentrations (cells mL⁻¹) at day 2 (\( t_2 \)) and day 3 (\( t_3 \)).

Ciliate ingestion rates (prey cells ciliate⁻¹ day⁻¹) were calculated from reduction in prey concentration in the ciliate cultures compared to the prey monocultures, over 24 h as in Heinbokel et. al. (1978).

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

**Photosynthetic rates measurements:** the inorganic carbon incorporation of the ciliates (pg C cell⁻¹ h⁻¹) was measured on triplicate samples, each consisting in 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 NaH¹⁴CO₃ 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:

*Impacts of inorganic nutrients on ciliate and prey*
**Impact of inorganic nutrients on ciliate and prey**

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

(2)

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-\(\alpha\) content of ciliates was used to calculate chlorophyll specific photosynthetic rates (pg C (pg Chl\(\alpha\))\(^{-1}\) 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 2, day 3, day 6 and day 7. Culture samples were filtered through a 0.22 µm filter and stored at -20°C until subsequent analysis. Dissolved inorganic nitrogen and phosphorus 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\(^{-1}\) day\(^{-1}\)) were calculated as:

\[ \text{Nutrient uptake rate} = \frac{(R_2 - R_3)}{B_{avg}} \]  

(3)

where \(R_2\) and \(R_3\) are the dissolved nutrient concentrations (µg L\(^{-1}\)) at day2 and day3 and \(B_{avg}\) is the average cell number (cells L\(^{-1}\)) at these time points calculated as:

\[ B_{avg} = \frac{(B_3 - B_2)}{\ln(B_3/B_2)} \]  

(4)

Where \(B_2\) and \(B_3\) is the cell number (cells L\(^{-1}\)) at day2 and day3.

**Prey elemental composition:** For analysis of particulate organic C, N, and P, at least 1.0 \(\times\) 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 subsample was taken by cutting with a scalpel and used for CN analysis by folding it into a tin cup (D013, Elemental Micro-analysis, Okehampton, UK). Samples were analyzed for particulate C and N on a FLASH 2000 NC elemental analyzer (Brechbuhler Incorporated, Interscience B.V., Breda, The Netherlands). Another quarter subsample of each filter was combusted in a Pyrex glass tube at
550°C for 30 min. Subsequently, 10 mL of persulfate solution (2.5%) was added, and samples were autoclaved for 30 min at 121°C. Digested P (as PO$_4^{3-}$) was measured on a Quaatro auto-analyzer (Seal analytical, Beun de Ronde, Abcoude, the Netherlands).

**Ciliate biovolumes and carbon, nitrogen and phosphorus content:** Linear dimensions of about 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. Biovolumes were calculated from cellular linear dimensions assuming cells had a spherical shape.

Ciliate carbon content was calculated as:

$$\text{Ciliate C content} = 0.19 \times V$$  \hspace{1cm} (5)

according to Putt and Stoeker (1989), where 0.19 is the estimated biovolume specific C content (pg C µm$^3$) and V the ciliate volume (µm$^3$). Ciliate nitrogen and phosphorus content was estimated assuming Redfield ratio (C:N:P = 106:16:1 on a molar basis).

**Ciliate gross growth efficiency and regeneration efficiency:** Gross growth efficiency (GGE) was calculated based on C, N and P as the amount of these elements that are ingested (pg cell$^{-1}$ d$^{-1}$) relative to the amount of these elements effectively assimilated into new ciliate biomass (pg cell$^{-1}$ d$^{-1}$) between day 2 and day 3. Ingested C, N and P were calculated according to the content of each respective element in the prey (pg cell$^{-1}$), and the ciliate ingestion rate (prey cells ciliate$^{-1}$ d$^{-1}$) in each experimental condition. The new ciliate biomass was calculated as the product of ciliate growth rates (d$^{-1}$) at day 3 and estimated content of each element (pg cell$^{-1}$) in the ciliates. As the elemental composition of the ciliate was not measured (see above), the GGE calculation implicitly assumes that ciliate biomass is strictly homeostatic, thus it must be interpreted with caution. Regeneration efficiency was calculated based on nitrogen 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 2 and day 3.

**Statistic**

Differences between treatments were assessed using ordinary one-way ANOVA followed by Sidak’s multiple comparisons test. Differences between treatments in well-fed and prey starved ciliates were assessed using two-way ANOVA followed by Sidak’s multiple comparisons test. For both type of analysis, data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Brown-Forsythe test) using Sigmaplot 14.0. All results are presented as means ± standard deviation among experimental triplicates.

*Impacts of inorganic nutrients on ciliate and prey*
Results

Physiological rates, chlorophyll-a content and elemental composition of the algal prey in monocultures

Monocultures of the cryptophyte *Teleaulax amphioxeia* grew at 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 Chla compared to all other treatments (Table 1). The Chla 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 *Teleaulax amphioxeia* were comparable in all treatments (Table), apart from cultures grown in f/200, which displayed a significantly higher uptake rate of ammonium (p <0.0001), and lower uptake rate of nitrate (p=0.001) compared to the other treatments (Table 1).

<table>
<thead>
<tr>
<th>Media</th>
<th>Growth rate (d⁻¹)</th>
<th>Chla content (pg Chla cell⁻¹)</th>
<th>Phot.rate (pg C (pg Chla⁻¹ h⁻¹))</th>
<th>NH₄⁺ uptake (fg N cell⁻¹ h⁻¹)</th>
<th>NO₃⁻ uptake (fg N cell⁻¹ h⁻¹)</th>
<th>PO₄³⁻ uptake (fg P cell⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f/200</td>
<td>0.42 ± 0.09</td>
<td>0.32 ± 0.00</td>
<td>1.86 ± 0.04</td>
<td>14.8 ± 1.0</td>
<td>74 ± 10</td>
<td>8.1 ± 4.5</td>
</tr>
<tr>
<td>f/100</td>
<td>0.80 ± 0.05</td>
<td>0.41 ± 0.02</td>
<td>1.98 ± 0.10</td>
<td>5.6 ± 2.2</td>
<td>119 ± 25</td>
<td>12.6 ± 1.9</td>
</tr>
<tr>
<td>f/40</td>
<td>0.82 ± 0.06</td>
<td>0.40 ± 0.01</td>
<td>2.02 ± 0.10</td>
<td>5.4 ± 0.2</td>
<td>141 ± 5</td>
<td>17.9 ± 1.6</td>
</tr>
<tr>
<td>f/20</td>
<td>0.65 ± 0.02</td>
<td>0.47 ± 0.03</td>
<td>1.66 ± 0.11</td>
<td>8.1 ± 0.2</td>
<td>128 ± 5</td>
<td>20.1 ± 11.5</td>
</tr>
</tbody>
</table>

When grown in nutrient poor media (i.e. f/200 and f/100), the cryptophyte *Teleaulax amphioxeia* had higher C contents as compared to the nutrient rich media f/40 and f/20 (p <0.0001), while N and P content was not significantly different among any of the experimental treatments (Figure 1a). C:P ratio was significantly higher in the f/200 compared to the f/40 (p=0.0004) and the f/20 (p=0.01) treatments and in the f/100 compared to the f/40 (p=0.01) treatments (Figure 1b).
**Impact of inorganic nutrients on ciliate and prey**

**Content**

(N and P content (pg cell⁻¹))

<table>
<thead>
<tr>
<th>f/200</th>
<th>f/100</th>
<th>f/40</th>
<th>f/20</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
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<tr>
<td>25</td>
<td>20</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

**Figure 1: a)** Carbon, nitrogen and phosphorus content (pg cell⁻¹) of *Teleaulax amphioxeia* in the different experimental treatment. **b)** Molar ratio between elements in *Teleaulax 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).

**Physiological rates and chlorophyll-a content of the ciliate in mixed cultures with prey**

All experiments were initiated with ~20 ciliates mL⁻¹ and ~30 x 10³ prey cells mL⁻¹. The highest ciliate abundances (90-120 cells mL⁻¹) were recorded in all treatments on the days in which the prey was completely depleted, which was day 4 in cultures growing in f/200 and f/100, and day 5 in cultures grown in f/40 and day 6 in cultures grown in f/20 (Figure 2).

Right after prey depletion, ciliates declined in number in all cultures, apart from cultures grown in f/100, which kept the same ciliate abundance for one day longer. Prey abundances were comparable across treatments at day 2, and we therefore estimated the ciliate growth rate between day 2 and day 3. In this time interval, the ciliate growth rates were significantly higher in the f/200 and f/100 treatments (Figure 4a) compared to in the f/40 and f/20 treatments (p = 0.0002), and these rates were comparable to the acclimation period (Supplementary Table1).
Figure 2: Development of cultures in the different experimental treatments as a function of time. Abundances (cells mL$^{-1}$) of the ciliate *S. cf. basimorphum* (a) and of the prey, *T. amphioxeia* (b) during the incubation. Values denote means ± SD (n=3).

Chla content in the ciliates measured on day 3 was significantly less in the f/200 treatment (Figure 3a) compared to ciliates in the treatments with intermediate nutrient levels (i.e. in f/100 and f/40). Likewise, the chlorophyll specific photosynthetic rates were significantly lower on day 3 in ciliates in f/200 treatment compared with ciliates growing in all the other experimental treatments (0.0001 < p < 0.002, Figure 3b). This is in accordance with measurements taken during the acclimation period (Supplementary table 1). Chla content was not significantly different between the treatments in prey-starved ciliates (Figure 3a). Only starved ciliates in f/40 had a significantly higher chlorophyll specific photosynthetic rate compared to starved ciliates of all the other experimental conditions (p < 0.0001; Figure 3). Cellular Chla content declined in starved ciliates in all experimental conditions (Figure 3a).

Figure 3: *Stombidium cf. basimorphum* Chla content (a) and photosynthetic rate (b) measured at day 2 and day 7 in the different experimental treatments. Values denote means ± SD (n=3).
Ammonium excretion rates by the ciliate, calculated between day 2 and 3, were much higher in the f/20 treatment (12.3 ± 2.4 pg N cell$^{-1}$ h$^{-1}$) compared with the f/40 (3.2 ± 2.7 pg N cell$^{-1}$ h$^{-1}$) and the f/100 (5.4 ± 0.45 pg N cell$^{-1}$ h$^{-1}$). The ammonium excretion rate could not be calculated in the f/200 treatment (see discussion). Nitrate uptake rates by the ciliates could not be measured during the first days of the experiments since the decrease in dissolved nitrate concentration measured in the media could not be differentiated from the uptake by the algal prey (Supplementary figure 1b). Ciliate nitrate uptake rates calculated during starvation (day 6-7), were negligible in all treatments, accounting for <0.1% d$^{-1}$ of the cellular nitrogen content.

A decrease in dissolved inorganic phosphate concentration was observed in the mixed cultures between day 2 and day 3 but, as for nitrate, could not be differentiated from the uptake by the algal prey. The dissolved inorganic phosphate concentration stayed at the same level or even increased towards the end of the incubation (day 7; Supplementary figure 1d), indicating that there was no net uptake of phosphate by the ciliate.

Ingestion rates of prey by the ciliates were lower in the f/20 treatment as compared to the other treatments (Figure 4a). Ingestion rates based on carbon (pg C ciliate$^{-1}$ d$^{-1}$) were significantly lower in ciliates growing in f/40 and f/20 compared to f/200 and f/100 (Figure 4b). Ingestion rates based on nitrogen (pg N ciliate$^{-1}$ d$^{-1}$) and phosphorus (pg P ciliate$^{-1}$ d$^{-1}$) were not significantly different among any experimental treatment (Figure 4b).

**Figure 4:** Stombidium cf. basimorphum growth and ingestion rate between day 2 and day 3 in the experimental treatments, with growth as function of prey ingestion (a), and ingestion rates base on C, N and P (b). Values denote means ± SD (n=3).
The total carbon uptake rate (sum of the ingestion and photosynthetic rate) of ciliates grown in the nutrient deprived conditions (f/200 and f/100) were greater than those grown under nutrient rich conditions (Figure 5). Photosynthesis represented a relatively larger proportion of the total carbon uptake in ciliates grown in the f/40 and f/20 (~20%) treatments compared to ciliates grown in the f/100 (~10%) and f/200 (~5%) treatments. This was mainly driven by higher ingestion rates, and partly by reduced photosynthesis under nutrient deprived conditions (Figure 5).

![Figure 5: Daily carbon uptake rates from ingestion and photosynthesis in S. cf. basimorphum between day 2 and day 3 in the different experimental treatments. Values denote means ± SD (n=3)](image)

**Ciliate cell size, gross growth efficiency and inorganic nutrient regeneration**

Ciliate cell size was comparable among experimental treatments (day 2) and declined during prey starvation (day 7) in the same proportion in all conditions (Supplementary Table 2). Ciliate gross growth efficiency calculated based on carbon (GGE<sub>C</sub>) and on nitrogen (GGE<sub>N</sub>) between day 2 and day 3, were very similar (~40-50%) and comparable in all experimental treatments (Table 2), indicating that about 50% of the ingest C and N were converted into new ciliates biomass. GGE calculated based on phosphorus (GGE<sub>P</sub>) in the same time interval exceeded 100% in all treatments apart from f/20 (Table 2), and thus P assimilation was very high and seemed to exceed ingestion.

Nitrogen regeneration efficiencies, based on ciliate ingestion and excretion rates between day 2 and day 3, 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). The nitrogen regeneration efficiency could not be calculated in the f/200 treatment due to differences in the ammonium concentrations between prey monocultures and mixed cultures at day 2 (see discussion). Regeneration of phosphorus was observed at the end of all the experiments, since phosphate concentration was higher at day 7 compared to the concentration at day 2.
(supplementary figure 1d). However, since cultures were not axenic, and bacterial abundance was relatively high at the end of the experiments, this could not solely be attributed to ciliate excretion and regeneration efficiency was therefore not calculated.

**Table 2:** Carbon, nitrogen and phosphorus based gross growth efficiency (GGE) between day 2 and 3 in the different experimental treatments. Values denote means ± SD (n=3).

<table>
<thead>
<tr>
<th>Media</th>
<th>GGE (_C)</th>
<th>GGE (_N)</th>
<th>GGE (_P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f/200</td>
<td>45.7 ± 3.2</td>
<td>43.5 ± 3.0</td>
<td>137.0 ± 9.5</td>
</tr>
<tr>
<td>f/100</td>
<td>40.3 ± 7.3</td>
<td>35.3 ± 9.5</td>
<td>112.5 ± 30.2</td>
</tr>
<tr>
<td>f/40</td>
<td>49.0 ± 10.0</td>
<td>54.1 ± 11.1</td>
<td>122.8 ± 25.0</td>
</tr>
<tr>
<td>f/20</td>
<td>40.5 ± 11.6</td>
<td>38.1 ± 10.9</td>
<td>83.3 ± 23.8</td>
</tr>
</tbody>
</table>

**Discussion**

**Effects of major inorganic nutrients concentration on physiological rates of ciliate**

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 dissolved inorganic nutrient availability. Despite this, inorganic carbon 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. Nitrate uptake detected in *S. cf. basimorphum* when prey starved was very low and similar to rates measured in the mixotrophic *S. rassoulzadegani* and heterotrophic *Strombidinopsis* sp. (Schoener and McManus, 2017). Uptake of dissolved inorganic phosphorus in *S. cf. basimorphum* could not be measured in any of the growth phases, suggesting that it only relies on ingestion to incorporate phosphorus. This is in line with earlier studies on the mixotrophic ciliate species *Strombidium viride* (Taylor and Lean 1981).

Growth rates of *S. cf. basimorphum* were lower in treatments with higher dissolved inorganic N and P content (f/20-40) compared to treatments with lower dissolved inorganic N and P content (f/100-200). A direct negative effect of major nutrients on the growth rate *S. cf. basimorphum* might have resulted from a high sensitivity of the ciliate to the applied concentrations of phosphate or nitrate. However, other *Strombidium* species have been successfully cultured in media with inorganic nutrients concentration even 10 times higher than what used in these experiments (i.e standard f/2 or NEPCC media) and achieved growth rates comparable to the higher growth rates measured here.
Effects of inorganic nutrients on ciliate and prey

The concentration of dissolved inorganic N and P directly affected the elemental content of the prey Teleaulax amphioxeia, as well as its photosynthetic performance. The scarce availability of dissolved inorganic nutrients in the f/200 treatment led to reduced cellular chlorophyll-\(a\) content and chlorophyll specific photosynthetic rates of T. amphioxeia in monoculture (Table1). This was also reflected in the chlorophyll-\(a\) content and chlorophyll-\(a\) specific photosynthetic rates measured on S. cf. basimorphum in the mixed cultures. When the prey T. amphioxeia was grown in nutrient depleted conditions (f/200 and f/100), it also exhibited a higher carbon content compared to the treatments with more inorganic nutrients (f/40 and f/20). This is in accordance with the literature, where it has been shown that photosynthetic organisms under suboptimal growth conditions will accumulate carbon storage compounds, like starch or glucans (Berman-frank and Dubinsky 1999 Malzahn et al. 2010). The relatively higher C:P ratio in the biomass of T. amphioxeia grown in nutrient poor treatments (f/200 and f/100) indicates a higher stoichiometric plasticity for P and thereby the ability of this alga to deal with low inorganic P concentrations. The C:N ratios in the monocultures of T. amphioxeia were similar in all treatments and close to Redfield’s ratio (C:N ~ 6.6, molar basis), suggesting that the species is more homeostatic with respect to N. Following the changes in P, the N:P ratios increased in the low nutrient treatments (double or even triple) than the Redfield ratio (N:P=16) in all experimental treatments. This is likely due to the fact that T. amphioxeia has a relatively high protein content, as has been commonly reported in cryptophytes (Lee et al. 2019; Seixas et al. 2009).

Effects of prey C:N, C:P and N:P ratios on ciliate ingestion, GGE and excretion rates

The C, N, and P contents of the algal prey were reflected in the ingestion rates of the ciliates. Higher ingestion rates were observed in the nutrient poor treatments (f/200 and f/100), where algal cells contained relatively less P compared to C and N. This resulted in carbon specific ingestion rates up

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(Montagnes 1996; Mcmanus et al. 2018). At the same time, similar growth rates were measured in Strombidium species cultured in filtered seawater only enriched with chelated micronutrients at optimal concentration (Gifford 1985). Thus, the effects of dissolved inorganic nutrients on ciliates’ growth rates might rather have indirectly derived from the different prey types used in the previous studies (Gifford 1985; Montagnes 1996; McManus et al., 2012). Prey species are expected to differ in their biochemical composition, and their own physiological adaptation to the experimental conditions may have indirect effects on the physiology of the ciliates.
to 50% higher in the nutrient poor treatments compared with carbon specific ingestion rates measured in nutrient rich treatments. This increase in ingestion rate can be attributed to compensatory feeding that may account for up to 160% of C demands in heterotrophic ciliates feeding on nutrient depleted algal prey (Siuda and Dam 2010).

Calculations of gross growth efficiency in *S. cf. basimorphum* based on phosphorus (GGEₚ), led to values exceeding 100% in most of the treatments. While it cannot be excluded that that actual phosphorus content in ciliates is different from what was calculated assuming a Redfield ratio, the occurrence of some internal recycling of P would explain the elevated growth rates of *S. cf. basimorphum* in the nutrient poor treatments (f/200 and f/100). Indeed, gross growth efficiency based on carbon (GGEₗ) was not lower in the nutrient poor treatments as it would be expected due to the elevated stoichiometric imbalance of the prey biomass. Low GGEₗ is commonly documented for metazoan grazers, which often show the ability for compensatory feeding on nutrient depleted prey (Kisørboe 1989; Siuda and Dam 2010). However, the effect of prey biomass composition on ciliate ingestion and growth has only been studied on very few species so far (Yang et al. 2015; Wickham and Wimmer 2019), which complicates the comparision of those studies with our results. Considering that the ciliate is most likely not strictley homeostatic, the stoichiometric imbalance of the ingested prey can be relatively smaller than what deduced from the GGE calculations in here. However, the assumption of stoichiometric homeostasis in the ciliate biomass does not preclude the clear effect of prey stoichiometric imbalance on *S. cf. basimorphum* ingestion and growth rates.

The relative excess of nitrogen gained from ingestion was excreted as ammonium from *S. cf. basimorphum* at rates comparable to what measured in the heterotrophic species *Strombidium sulcatum* (Ferrier-Pages and Rassoulzadegan 1994). The *S. cf. basimorphum* cultures were not axenic, but nitrogen regeneration from bacteria has previously been shown to be insignificant compared to regeneration associated to the grazing activity of *Strombidium sulcatum* (Ferrier-Pages and Rassoulzadegan 1994), also when taking the concentrations of bacteria into account (see supplementary material). Ammonium concentrations at day 2 in the f/200 treatment were different in the mixed cultures compared to prey monocultures. Therefore, it was not possible to assume that the ammonium uptake rates calculated in *T. amphioxeia* monoculture were the same as in the mixed culture. Consequently, we could not estimate the ammonium excretion rates from ciliates in the f/200 treatment. 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 6-7) in mixed cultures in the f/200 treatment compared to the values measured in the other treatments.
Based on the biomass composition of the prey and the excretion of ammonium from the ciliate, it is conceivable that ciliate growth was P limited in all treatments. In treatments in which the prey contained less carbon, the ciliate was most likely also experiencing some energy deficiency indicated by the reduced growth rates. The potential stoichiometric adjustments of the ciliate biomass have not been inspected in here. However, changes in C:N:P stoichiometry of the ciliate biomass are likely to occur, especially during starvation, when C fixation proceeds while no other nutrients are incorporated.

**Ecological implications**

The ciliate *S. cf basimorphum* has been isolated from a temperate nutrient-rich 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 the 90% of the ciliate biomass in late spring/early summer, when dissolved inorganic nitrogen concentrations are \(< 5 \mu\text{mol (our f/200 and f/100 treatments)}\) and the biomass of potential prey is relatively low (Haraguchi et al. 2018). This suggests that, in this environment where prey is limiting, mixotrophy represent 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 potential use in aquaculture (Lee et al. 2019). Additionally, cryptophyte nucleomorphs contain house-keeping genes that may facilitate the use of the retained chloroplasts, which may render *Tebleaulax* chloroplasts favorable for NCM protists compared to other chloroplast types (Altenburger et al. 2020). It has been shown recently that by retaining *Tebleaulax* nuclei, the specialist NCM *Mesodinium rubrum* acquired the ability to take up inorganic nutrients from the water allowing it to grow autotrophically (Altenburger et al., 2020). This does not seem to be the case for *S. cf basimorphum*, though it has been shown, similar to other mixotrophic *Strombidium* species, to effectively exploit other prey species such as chlorophytes (Stoecker et al. 1989; McManus et al. 2018; Maselli et al., 2020). The suitability of prey type other than cryptophytes and chlorophytes seems to depend on species-specific characteristics and/or interactions (Montagnes 1996; Maselli et al., 2020).

Small photosynthetic flagellates are considered good prey species for prey generalist NCM ciliates (Stoecker et al. 1989; McManus et al. 2018; Maselli et al., 2020), and these 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 in temperate regions are generally dominated by diatoms, which do not support growth of S.cf basimorphum and other prey generalist NCM ciliates (McManus et al., 2012; Maselli et al., 2020). Specialization on prey type render prey generalist NCMs more dependent on prey ingestion compared to specialist NCM. Yet, being generalist may represent a benefit since it potentially provides access to different types of biomolecules coming from a more diverse diet. Indeed, within classes of marine photosynthetic plankton exists a high variability in cellular stoichiometry and biochemical composition (Geider & LaRoche 2002; Garcia et al. 2018), which explains why a mixed diet could support a better growth of grazers, including ciliates, compared to a mono diet (Montagnes 1996; Yang et al. 2019).

Conclusion
Our findings clearly show that the ciliate S. cf. basimorphum is not able to utilize dissolved inorganic nitrate and phosphate for growth, while it might take advantage from some internal recycling of nutrients when feeding on prey with elevated stoichiometric imbalance. Carbon acquired via photosynthesis cannot compensate the lower carbon content of prey acquired via ingestion. Inorganic N and P concentration in the growth medium have indirect effects on the physiology of S.cf basimorphum due to changes in prey elemental composition, notably leading enhanced growth under nutrient limitation due to a higher prey carbon content. Experiments with more prey species, and prey generalist NCM species would have to be carried out at different nutrient concentrations to further elucidate the role of mixotrophy in physiological processes associated with major nutrients other than carbon.

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Impact of inorganic nutrients on ciliate and prey


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https://doi.org/10.3354/meps055229.


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https://doi.org/10.3354/meps130241.


Redfield AC, Ketchum BH, Richards FA. 1963. The influence of organisms on the composition of


Impacts of inorganic nutrients on ciliate and prey


Supplementary material
**Bacterial abundance**

Bacterial abundance (cells mL\(^{-1}\)) was only measured in the most nutrients reach treatment (f/20). Bacterial abundance was measured at the beginning (day2 and day3) and at the end of the experiment (day6 and 7) on 1 mL of the experimental cultures fixed in 0.2% (final volume) glutaraldehyde. Samples were stained with 10µl of sybrGreenI working solution prepared as follow: 10000x sybrGreen I in dimethylsulfoxide (DMSO) diluted by 1:200 in milli Q water. Bacteria were counted using a flow cytometer CytoFLEX (Beckman Coulter), run for 2 minutes with flow rate of 10µL min\(^{-1}\) and threshold event rate of 1000 events per second. Counts were recorded as signals (events µL\(^{-1}\)) on the green fluorescence channel (FITC). Bacterial abundance ranged from about 1.8 x 10^6 cells mL\(^{-1}\) at day2 to 3.6 x 10^6 cells mL\(^{-1}\) at day 7.

**Supplementary Figure 1:** Development of the concentration of dissolved inorganic nutrients (µgL\(^{-1}\)) as a function of time in the mixed cultures in the 4 different treatments: f/20, f/40, f/100 and f/200. (A) Ammonium, (B) Nitrate, (C) Nitrite, and (D) Phosphate.
**Supplementary Table 1:** *S. cf basimorphum* physiological rates and Chla content measured between during the acclimation period in the different experimental treatments. Average ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>media</th>
<th>Growth rate (d⁻¹)</th>
<th>Ingestion rate (prey ciliate⁻¹h⁻¹)</th>
<th>Chla content (pg Chla cell⁻¹)</th>
<th>Chla specific phot.rate (pg C (pg Chla⁻¹) h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f/200</td>
<td>0.93 ± 0.13</td>
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<td>f/200</td>
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<tr>
<td>f/100</td>
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<tr>
<td>f/100</td>
<td>0.89 ± 0.23</td>
<td>42.7 ± 7.2</td>
<td>136 ± 8.4</td>
<td>0.82 ± 0.10</td>
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<td>(day1-2)</td>
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<tr>
<td>f/40</td>
<td>0.64 ± 0.34</td>
<td>32.1 ± 4.2</td>
<td>172 ± 3.0</td>
<td>0.48 ± 0.17</td>
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<td>(day0-1)</td>
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<tr>
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**Supplementary Table 2:** *S.* cf *basimorphum* biovolume, carbon, nitrogen and phosphorous content when feeding on prey (day 2-3) and during prey starvation (day 6-7). Ciliate biovolumes were calculated based on measurements of their dimension. Carbon content was calculated based on biovolumes. Nitrogen and phosphorus contents were estimated assuming Redfield ratio with carbon. Average ± st.dev (n=90).

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<tr>
<th><em>Strombidium cf. basimorphum</em></th>
<th>Day 3</th>
<th>Day 7</th>
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<td>Biovolume ($10^4$ $\mu m^3$)</td>
<td>C content (ng cell$^{-1}$)</td>
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<td>8.7 ± 2.5</td>
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<td>f/100</td>
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<tr>
<td>f/40</td>
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<td>8.9 ± 2.3</td>
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<tr>
<td>f/20</td>
<td>4.2 ± 1.2</td>
<td>7.9 ± 2.3</td>
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</table>
Retention of prey genetic material by the kleptoplastidic ciliate

*Strombidium cf. basimorphum*

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*These authors have contributed equally to this work*
Abstract

Many marine ciliate species retain functional chloroplasts from their photosynthetic prey. In some species the functionality of the acquired plastids is connected to the simultaneous retention of prey nuclei. To date, this has never been documented in plastidic *Strombidium* species. The functionality of the sequestered chloroplasts in *Strombidium* species is thought to be independent from any nuclear control and only maintained via frequent replacement of chloroplasts from newly ingested prey. Chloroplasts sequestered from the cryptophyte prey *Teleaulax amphioxeia* have been shown to keep their functionality for several days in the ciliate *Strombidium* cf. *basimorphum*. To investigate the potential retention of prey genetic material in this ciliate we applied molecular marker specific for this cryptophyte prey. Here, we demonstrate that genetic material from prey nuclei, nucleomorphs and ribosomes is detectable inside the ciliate for at least five days after prey ingestion. Moreover, single-cell transcriptomic revealed the presence of transcripts of prey nuclear origin in the ciliate after four days of prey starvation. These new findings might lead to the reconsideration of the mechanisms regulating chloroplasts retention in *Strombidium* ciliates. The development and application of molecular tools appear promising to improve our understanding on chloroplasts retention in planktonic protists.

**Keywords:** kleptoplasty, ciliates, *Strombidium*, mixotrophy, plankton.
Introduction

Kleptoplasty is the non-permanent acquisition of chloroplasts from a photosynthetic organism by an otherwise heterotrophic organism (de Vries and Archibald, 2018). The phenomenon is common among marine ciliates (Stoecker et al., 1987; Stoecker et al., 2009). Since they acquire phototrophy from prey, plastidic ciliates are termed non-constitutive mixotrophs, or non-constitutive mixoplankton referring to planktonic species (Mitra et al. 2016; Flynn et al., 2019). Acquired phototrophy gives mixotrophic ciliates a competitive advantage over purely heterotrophic species when prey concentrations are low and light is available (Dolan and Perez 2000; Schoener and McManus, 2017).

Kleptoplastidic species in the *Mesodinium rubrum* species complex are known to only exploit chloroplasts from cryptophytes within the *Teleaulax/Plagioselmis/Geminigera* clade, from which they also retain the nuclei (process known as karyoklepty) and other prey organelles (Hansen et al., 2012; Johnson et al., 2016; Kim et al., 2017). The retention of prey nuclei allows the host to maintain some genetic control of the acquired chloroplasts through the transcription of plastid-related genes from the kleptokaryon. Other than the ability to photosynthesize, *Mesodinium rubrum* acquires from the prey the potential to metabolize several essential compounds including amino acids and vitamins (Altenburger et al., 2020). This enables *Mesodinium* species to retain fully functional plastids and live as a complete autotroph for about 4 generations in the absence of prey (Smith and Hansen, 2007). Such phenomena of kleptoplasty and karyoklepty have been also recorded in some dinoflagellates (Onuma and Horiguchi, 2015; Onuma et al., 2020).

Kleptoplastidic ciliates in the genus *Laboea, Strombidium* and *Tontonia*, can instead exploit chloroplasts derived from a much wider range of algal groups, including chlorophytes, haptophytes, cryptophytes and heterokonts (Laval-Peuto and Febvre, 1986; Johnson and Beaudoin, 2019). These ciliates have much higher prey ingestion rates than *Mesodinium rubrum*, and thus, potentially a fast turnover of sequestered prey plastids. Photosynthesis contributes much less to the total carbon uptake compared to *M. rubrum*, and they cannot grow autotrophically when prey is not available (Schoener and Mcmanus, 2012; Maselli et al., 2020). Transmission electron microscopy studies on kleptoplastidic ciliates in the genus *Laboea, Strombidium* and *Tontonia* have never revealed the retention of any algal prey nuclei (Laval-Peuto and Febvre, 1986; Stoecker et al., 1988). The function of the sequestered chloroplasts in these ciliates is thus currently thought to be dependent on their innate robustness and ability to survive inside the ciliate host. Based on studies on the kleptoplastidic *Strombidium rassoulzadegani*, kleptoplastidic ciliates in the

*Ciliate retains prey genetic material*
genus *Strombidium* are thought to depend on more frequent reacquisition of prey plastids compared to *M. rubrum*, because they do not express genes related to plastid maintenance and replication (Santoferra et al., 2014; McManus et al., 2018).

*Strombidium basimorphum* is a worldwide distributed species, first morphologically described in Canadian waters (Martin and Montagnes, 1993), and then reinvestigated through molecular systematic in a Chinese population (Liu et al., 2011). It has been shown to significantly contribute to grazing on photosynthetic picoeukaryotes in a north Pacific ocean region (Orsi et al., 2018) but the retention of functional chloroplasts in this species has only recently experimentally ascertained on an isolate of *Strombidium cf. basimorphum* from Danish coastal water (Maselli et al., 2020).

This ciliate seems to more efficiently exploit chloroplasts for photosynthesis when ingestion is suppressed by the unavailability of prey. Its photosynthetic rates are kept relatively high and constant during at least five days of prey starvation (Maselli et al., 2020). *Strombidium cf. basimorphum* can thus maintain chloroplasts functionality unaltered for several days when chloroplasts are not replaced via the ingestion of prey. To get some insight into molecular mechanisms that stand behind the retention of functional chloroplasts, here we tested the ability of the Danish isolate of *Strombidium cf. basimorphum*, to also retain prey genetic material. We studied this in well-fed cells as well as in cells that had been starved for 1 to 7 days. Molecular techniques such as quantitative polymerase chain reaction (qPCR), Fluorescence *in situ* Hybridization (FISH) and single-cell transcriptomics were applied. Quantitative PCR and FISH, as applied in here on cultures, were recently developed to detect the presence of prey genetic material in *Mesodinium cf. major* in field samples (Herfort et al., 2017).

**Materials and methods**

**Culture conditions and experimental design:** Cultures of *S. cf. basimorphum* were established from single cells isolated from natural sea water samples from Roskilde fjord (Denmark). The isolate was identified and cultured as described in Maselli et al. (2020) and maintained for about one year feeding it the cryptophyte *Teleaulax amphioxeia* (SCCAP, K-1837). The experiment was conducted in *f*/20 media (a 1:10 dilution of the standard *f*/2 media from Guillard, 1975), at a salinity of 15, at 15°C, with a photon flux density of 100 μmol photons m⁻² s⁻¹ in a light:dark cycle of 14:10 h. Ciliates were allowed to grow exponentially for 5 days in borosilicate bottles (3.5 L culture in 5 L flasks), by daily restoring the prey concentration that saturates their growth (*T. amphioxeia*: 1.0 x 10⁴ cell mL⁻¹, as in Maselli et al. 2020).
At the fifth day, ciliates were fed for the last time and split in three replicates of 1 L into 2 L Blue Cap glass flasks (VWR, Denmark). Cells were harvested for DNA extraction and FISH the day after, when prey was still available (T0), after 48h, when prey was depleted (T2) and after 5 and 7 days, during prey starvation (T5 and T7).

**DNA extraction:** *S. cf. basimorphum* cells were collected from 200 mL of the experimental cultures onto Nitex nylon filters (Millipore by Merck, Darmstadt, Germany) with a mesh size of 10 µm, allowing the separation of the ciliates from prey (*T. amphioxeia* length: ~5 µm). Filters were subsequently rinsed with clean culture media to make sure that no *T. amphioxeia* cells were retained. Samples of *T. amphioxeia* triplicate monocultures were collected on 0.2 µm polycarbonate filters (Whatman Nuclepore, Cytiva, Freiburg, Germany). DNA from both ciliates and prey samples was extracted using the NucleoSpin Soil DNA isolation kit (Macherey-Nagel, Düren, Germany), following the manufacturer’s instructions. Elution was performed using a small volume (35 µL) of the elution buffer provided by the kit. The concentration of the DNA was estimated using Nanodrop spectrophotometer (ND-1000 Peqlab, Erlangen, Germany).

**Quantitative PCR:** *Teleaulax amphioxeia* nuclear 28S rDNA D2 Unique Sequence Element (USE) primers (TxD2 1F and TxD2 USE 2R) and nucleomorph 28S rDNA D2 USE primers (TxNm 1F and TxNm 1R) were used in qPCR assays to detect the presence of prey genetic material in DNA extracted from ciliates and provide a semi-quantitative estimation of its concentration over time, following prey depletion and starvation. The primers (Supplementary Table 1) were designed and checked for their specificity by Herfort et al. (2017). All qPCR assays were run in technical triplicates on a StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA). One ng of ciliate DNA was added to the following PCR mixture: 5 µL FAST SYBR Green Master Mix (Applied biosystem by Thermo- Fisher Scientific, Waltham, USA), 0.125 µL of each primer (final concentration 125 nM) and nuclease free water to a final volume of 10 µL.

Technical triplicates assays of each of the *T. amphioxeia* DNA replicate were run at the same DNA concentration as for the ciliate DNA. Quantitative PCR reactions were run as follows: 40 cycles of 95°C for 3 s, and 60°C for 30 s; followed by a melting curve protocol (95°C for 15 s, 60°C for 1 min and 0.3°C increments with a 15 s hold at each step). Control assays as a general control for extraneous nucleic acid contamination were also subjected to qPCR amplification with purified water in place of DNA.
**Fluorescence in situ hybridization (FISH):** to investigate the potential transcriptional activity of prey nuclear material in *S. cf. basimorphum*, ciliates were hybridized a FISH probe for the *Teleaulax amphioxeia* nuclear-encoded 28S rRNA D2 USE (TxD2 RNA, Table1) designed by Herfort et al. (2017). Twenty mL of experimental cultures in the different growth phases (T0, T2, T5 and T7), were fixed in paraformaldehyde (4% final concentration) and stored at 4°C for 1h, prior the collection of the ciliates on 3µm polycarbonate filters (Whatman Nuclepore). Filters were incubated for 1h in 1mL of 50% dimethylformamide (DMF) to reduce chloroplast autofluorescence (Grobenc and Medlin, 2005). Filters were subsequently hybridized for 3h at 37°C in a buffer with 30% formamide according to Herfort et al. (2017), washed for 10 min at the same temperature with a second buffer (1x SET buffer: 150 mM. NaCl, 1mM EDTA, 20mM Tris/HCL), and counter-stained with 4´, 6-diamidino-2-phenylindole (DAPI). Samples were inspected using the Olympus BX50 microscope equipped with a CoolLED pE-300 light source on 400 x magnification with appropriate wavelengths for DAPI (excitation 350 nm; emission 450 nm), Alexa488- (excitation 480 nm; emission 530 nm) and chloroplast fluorescence (excitation 600 nm; emission 650 nm). Images were acquired by Olympus DP71 camera using the software CellSense. *S. cf. basimorphum* samples from cultures fed with the green alga *Nephroselmis rotunda* were treated in the same way and used as negative control to check for the specificity of the probes.

**Single cell transcriptomics:** to further validate the presence of prey transcripts in the ciliate, single-cell transcriptomic was performed. Eight single cells were isolated from the experimental cultures after 4 days of prey starvation. Each cell was individually picked with a drawn Pasteur pipette, washed three times by transferring it in clean drops of sterile filtered media, and then transferred in the Lysis buffer provided by the extraction RNAqueous™-Micro Total RNA Isolation Kit (Ambion, USA). The cDNA libraries were generated using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio, USA) and cDNA was quantified using the Agilent High Sensitivity Kit (Agilent, USA). Adapter and index ligation was done using the Nextera® XT DNA Library Preparation Kit (Illumina, USA). The raw sequences were de-multiplexed with bcl2fastq and their quality and potential contamination with adapters was checked using FastQC. Reads were trimmed using TrimGalore with the default settings and rRNA was removing with SortMeRNA. The final reads were mapped towards the reference transcriptome of *T. amphioxeia* generated by Altenburger et al. (2020), using the kallisto software (Bray et al., 2016). The reference assembly included the functional annotation of the transcripts with assigned KEGG orthologues. To consider a *T. amphioxeia* transcript present in the ciliate, a threshold of ≥50 reads
summed from all 8 cells was set. The raw read sequences have been deposited at NCBI (National Center for Biotechnology Information) under the BioProject PRJNA718746.

Results

The *Strombidium cf. basimorphum* cultures decline in cell concentration immediately after prey was depleted. Ciliates concentration changed from ~130 to 110 cells mL\(^{-1}\) during the two first days of the incubation to further decrease to ~85 cells mL\(^{-1}\) after 3 days of prey starvation (T5) and to ~60 cells mL\(^{-1}\) after 5 days of prey starvation (T7) (supplementary Figure 1).

Despite the differences in harvested amount of cells at each time point, the yield of DNA extraction was similar ranging between 7.7 and 5.2 ng µL\(^{-1}\). However, 1 ng of template DNA extracted from ciliates that were actively feeding (T0) was sufficient to detect *T. amphioxeia* nuclear and nucleomorph 28S rDNA using the qPCR assays. The relative concentration of these prey genes appear to be lower in DNA extracted from ciliates subjected to prey deprivation and only very low residual signals were detected after 3 days of starvation based on the average cycle threshold (Ct, Table 1). The amplification products of DNA extracted from ciliates at T7 were not reliable, so results of the qPCR assays for this time point are not shown. The reliability of the amplification products was assessed inspecting the melt curve of each of the replicate. Replicates that displayed multiple or shifted peaks in their melt curve have been omitted. The average cycle threshold (Ct) values for the nuclear 28S rDNA range between 29.2 ± 0.4 at T0 and 35.5 ± 0.8 at T5, while average Ct for the nucleomorph 28S rDNA is 27.4 ± 0.4 at T0 and 32.9 ± 1.4 at T5 (Table 1).
**Table 1:** Average cycle threshold (Ct) values: average among replicates of the number of cycles required for the fluorescent signal to exceed background level. (Average ± standard deviation).

<table>
<thead>
<tr>
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<th>Nuclear 28S rDNA</th>
<th>Nucleomorph 28S rDNA</th>
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<tbody>
<tr>
<td></td>
<td>n° of replicates</td>
<td>Average Ct</td>
</tr>
<tr>
<td><em>T. amphioxeia monoculture</em></td>
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<td>20 ± 0.4</td>
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<tr>
<td>T0, <em>S. cf basimorphum</em> <em>(Actively feeding)</em></td>
<td>9</td>
<td>29.2 ± 0.4</td>
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<tr>
<td>T2, <em>S. cf basimorphum</em> <em>(Prey depleted)</em></td>
<td>9</td>
<td>34.1 ± 1.3</td>
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<tr>
<td>T5, <em>S. cf basimorphum</em> <em>(Three days starved)</em></td>
<td>7</td>
<td>35.5 ± 0.8</td>
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</table>

Average cycle thresholds (Cts) of the nuclear and nucleomorph genes are significantly different in *S. cf basimorphum* at T0 (*p* < 0.0001), while they are not different in the *T. amphioxeia* monoculture.

The morphology of ciliates collected on filters was quite well preserved (Figure 1). The fluorescent signal obtained upon hybridization with *Teleaulax amphioxeia* rRNA probe was clearly detectable within the ciliate cytoplasm (Figure 1C-D and Figure 2A-H).

Prey rRNA is quite spread inside the ciliate cells but the fluorescent signal is more intense around the ciliate nuclei (Figure 1C-D; Figure 2B-C), or in localized clusters within the ciliate cytoplasm (Figure 2 A, E). The fluorescent signal of the prey rRNA probe could be detected in ciliates at all time points (Figure 2) and its intensity seem comparable among individuals sampled at different time points (Figures 2 E-H). Individual cells that contained labeled rRNA as wells as individual cells that did not were found in all samples. However, the percentage of positive hybridized cells (over total cell numbers) was not determined. Negative controls of *S. cf. basimorphum* fed with a different prey item (*N. rotunda*) did not show any signal of hybridization, confirming the specificity of the probes.
Figure 1: Micrographs of *Strombidium* cf. *basimorphum* (A) light microscopy of a S.cf. 371 *basimorphum* cell in liquid suspension (prior the collection on filter) (B) bright field micrograph of a 372 S.cf. *basimorphum* cell on filter (C) micrograph of the same cell on filter, acquired with combined 373 light channels. The cell is hybridized with the probe for the prey rRNA (green) and counterstained 374 with DAPI (blue). Sequestered chloroplasts are visible in red. (D) micrograph of the same cell 375 acquired with a single light channel for the FISH probe, showing prey rRNA (E) micrograph of the 376 same cell acquired with a single light channel for DAPI, showing S.cf. *basimorphum* nucleus.
Figure 2: Micrographs of S. cf. basimorphum cells hybridized with the probe for the prey rRNA (Alexa Fluor 488 dye labelled) and counterstained with DAPI at different time intervals. Combined light channels: (A) T0, actively feeding cells; (B) T2, prey depleted; (C) T5, prey starved; (D) T7, prey starved. Single light channels are shown below each cell: (E-F-G-H) light channel for the FISH probe, showing prey rRNA (I-J-K-L) light channel for DAPI, showing ciliates nuclei (M-N-O-P) light channel for chlorophyll autofluorescence, showing chloroplasts. The scale bar in the left bottom corner is 20 µm and refer to all panels. Blue: ciliates nuclei, green: prey RNA, red: chloroplasts.

Ciliate retains prey genetic material
The transcriptomic analysis of the ciliate single cells revealed the presence of transcripts of prey nuclear origin. For each ciliate cell, an average of ~17 million reads were generated and mapped against the reference transcriptome of *T. amphioxeia*. The mapping revealed the presence of 282 transcripts of prey nuclear origin (Table 2). The prey transcripts are associated to biological functions including metabolic as well as genetic information and cellular processes. Within the general category of metabolism, transcripts are involved metabolism of amino acids and general energy. Moreover, genetic information pathways included transcripts related to transcription and translation of the prey nucleus within the host. Cellular processes like cell growth and death could be related to the degradation of the prey nucleus within the ciliate cells. A detailed list of the retrieved transcripts and their functional annotation is provided in the supplementary table 2.

**Table 2:** Number of *T. amphioxeia* transcripts found in *S. cf basimorphum* and their functional annotation according to KEGG

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<td>Protein families: genetic information processing</td>
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<tr>
<td>Protein families: metabolism</td>
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<tr>
<td>Protein families: signaling and cellular processes</td>
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<tr>
<td><strong>Cellular Processes</strong></td>
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<td>Cell growth and death</td>
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<tr>
<td>Cell motility</td>
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<tr>
<td>Cellular community – eukaryotes</td>
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<td>Cellular community – prokaryotes</td>
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<tr>
<td><strong>Environmental Information Processing</strong></td>
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<td>Membrane transport</td>
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<td>Signal transduction</td>
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<td><strong>Genetic Information Processing</strong></td>
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<td>Folding, sorting and degradation</td>
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<tr>
<td>Replication and repair</td>
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*Ciliate retains prey genetic material*
Table 2: continued from the previous page

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Discussion

The retention of prey genetic material is here documented for the first time in a kleptoplastidic *Strombidium* species through the use of specific molecular markers and single-cell transcriptomics. *Strombidium cf. basimorphum* is shown to retain genetic material from prey nuclei and nucleomorphs. The observation of prey rRNA and other transcripts of prey nuclear origin, suggest that prey genetic material is transcriptionally active inside the ciliate.

Quantitative PCR results suggest that prey DNA disappears quite quickly after ingestion in *S. cf. basimorphum*, contrary to what has been observed in *Mesodinium rubrum*, which is able to retain prey nuclei for up to ten weeks (Johnson and Stoecker 2005; Kim et al., 2017). The relative concentration of the prey nucleomorph gene was higher compared to that of the prey nuclear gene (lower Ct values) in DNA extracted *S. cf. basimorphum*, but not in the DNA extracted from the prey monoculture, suggesting that prey nucleomorphs are better preserved in the ciliates compared to the prey nuclei. The reason for that may be attributed to the location of the nucleomorph in between the membranes of the chloroplasts of *T. amphioxia* (Garcia-Cuetos et al., 2010). The location of the nucleomorph in between chloroplasts membranes would eventually preserve it from degradative processes in the ciliate cytoplasm. The presence of the nucleomorph could actually render *Teleaulax* chloroplasts favorable in comparison to other chloroplast types (Altenburger et al., 2020).

The strong fluorescent signal obtained upon hybridization with the prey rRNA probe, proves the presence of prey ribosomes into the ciliate. It is not unequivocally proven that those ribosomes are being actively transcribed from the prey nuclear gene. Indeed, ribosomes could have been sequestered from the prey together with chloroplasts. It is possible that rRNA clusters visualized with FISH in some of the ciliate cells, are in fact food vacuoles. However, the fluorescent signal was persistent and diffuse all over the ciliate cytoplasm even after five days of prey starvation, suggesting that prey ribosomes are at least somehow maintained in the ciliate, and are not only contained concentrated in food vacuoles. Nevertheless, a fraction of cells (not quantified) did not show any fluorescence upon hybridization. This can be ascribed to a dilution of the sequestered genetic material due to cell division, as has been described in *Mesodinium rubrum* (Kim et al., 2017).
The transcriptional activity of the prey genetic material is proven by the results of the ciliate single-cell transcriptomics. The functional annotation of prey transcripts found in *S. cf. basimorphum* revealed the presence of genes involved in metabolic processes related to photosynthesis as well as to processes related to transcription and translation. All these processes argue for an active, at least partially remained nuclei of the prey with transcription, translation and aligned photosynthetic related gene expression. These results will deserve further and extensive studies to elucidate the responses of the host towards functions related the kleptoplasts (Uzuka et al., 2019), as well as the presence of photosynthesis related genes (and eventually their evolutionary origin) within the genome of the host (Hongo et al., 2019; Hehenberger et al., 2019).

The fact that *S. cf. basimorphum*, unlike *Mesodinium*, is not able to survive as pure autotroph in absence of prey could be explained by its need to incorporate through ingestion nutrients other than carbon. Further investigations of its transcriptome and the transcriptional activity of prey genetic material would provide further insight on the metabolism and potential dependency to prey metabolites of this ciliate.

Our study demonstrates the retention of prey genetic material in a *Strombidium* species; to which extent this is true for all plastidic *Strombidium* spp is presently unknown. If retention of prey genetic material is indeed found in all plastidic *Strombidium* species, it would indicate that is essential for the survival of plastids inside these ciliates. The ability (or lack of) to retain prey genetic material may also explain why kleptoplasts are not found in all *Strombidium* species and other ciliate groups living in the photic zone of the sea. To get deeper understandings, the same techniques would have to be employed on natural specimen and probes for different algal preys would have to be developed.

**Funding**

This research was supported by EC MSCA-ITN 2019 funding via the project MixITiN (grant number 766327). Further financial support was provided by the PACES research program of the Alfred Wegener Institute as part of the Helmholtz Foundation initiative in Earth and Environment, and by the project CoCliME. Project CoCliME is part of ERA4CS, an ERA-NET initiated by JPI Climate, and funded by EPA (IE), ANR (FR), BMBF (DE), UEFISCDI (RO), RCN (NO) and FORMAS (SE), with co-funding by the European Union (Grant 690462).
Acknowledgments

We thank Michele Laval-Peuto for her insightful opinions on the interpretation of the experimental results.

Reference List


Ciliate retains prey genetic material
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Ciliate retains prey genetic material
**Supplementary Table 1:** primers and probes used in this study as designed by Herfort et al. 2017

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*Ciliate retains prey genetic material*
Supplementary Figure 1: Changes in cell concentrations of *S. cf basimorphum* and *T. amphioxeia* in the experiment. Errors bars represent standard deviation among the three biological replicates.
**Supplementary Table 2:** detailed list of the retrieved transcripts and their functional annotation

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*Ciliate retains prey genetic material*
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| Tamp_06552 | K02145 | hypothetical protein; K02145 V-type H+-transporting ATPase subunit A [EC:7.1.2.2] |
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| Tamp_14315 | K06630 | hypothetical protein; K06630 14-3-3 protein epsilon |
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_Ciliate retains prey genetic material_
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| Tamp_57449 | K02699 | [pt] photosystem I subunit XI; K02699 photosystem I subunit XI |
| Tamp_25930 | K00021 | Hydroxymethylglutaryl-coenzyme A reductase; K00021 hydroxymethylglutaryl-CoA reductase (NADPH) [EC:1.1.1.34] |
| Tamp_64034 | K18461 | WAS protein family homolog 1-like; K18461 WAS protein family homolog 1 |
| Tamp_05020 | K06185 | predicted protein; K06185 ATP-binding cassette, subfamily F, member 2 |
| Tamp_01394 | K18626 | predicted protein; K18626 trichohyalin |
| Tamp_09943 | K16911 | hypothetical protein; K16911 ATP-dependent RNA helicase DDX21 [EC:3.6.4.13] |
| Tamp_06798 | K02899 | 50S ribosomal protein L27; K02899 large subunit ribosomal protein L27 |
| Tamp_13336 | K15171 | transcription elongation factor SPT4-like protein; K15171 transcription elongation factor SPT4 |
| Tamp_46860 | K02519 | translation initiation factor IF-2; K02519 translation initiation factor IF-2 |
| Tamp_27519 | K19607 | ubc9; ubc9, isoform B; K19607 RNA-binding protein UBC9 |
| Tamp_44268 | K02927 | Ubiquitin/60s ribosomal protein L40 fusion protein; K02927 large subunit ribosomal protein L40e |
| Tamp_71351 | K02707 | psbE; photosystem II PsbE protein; K02707 photosystem II cytochrome b559 subunit alpha |
| Tamp_17271 | K15423 | hypothetical protein; K15423 serine/threonine-protein phosphatase 4 catalytic subunit [EC:3.1.3.16] |
| Tamp_19089 | K05522 | DNA glycosylase; K05522endonuclease VIII [EC:3.2.2.49.99.18] |
| Tamp_32489 | K04529 | APBB1; amyloid beta A4 precursor protein-binding family B member 1; K04529 amyloid beta A4 precursor protein-binding family B member 1 |
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| Tamp_54516 | K10206 | hypothetical protein; K10206 LL-diaminopimelate aminotransferase [EC:2.6.1.83] |
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*Ciliate retains prey genetic material*
Ciliate retains prey genetic material
Ciliate retains prey genetic material

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Ciliate retains prey genetic material
Effects of glacial flour on marine micro-plankton: evidences from natural communities of Greenlandic fjords and experimental studies

Maira Maselli1*, Lorenz Meire2,3, Per Juel Hansen1

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3 Greenland Climate Research Centre, Greenland Institute of Natural Resources, Nuuk, Greenland
Abstract

The meltwater runoff from glaciers carries clay particles, glacial flour, that in several ways may affect plankton and the functioning of the downstream marine ecosystems. Microplankton is at the base of marine food webs and consequently plays an important role in sustaining ecosystem services such as carbon sequestration and fisheries. To assess the effect of glacial flour on autotrophic, heterotrophic and mixotrophic microplankton, the spatial distribution of these trophic groups were studied along transects of four Greenlandic fjords during summer. The spatial distributions in the fjords suggest that the abundances of the autotrophic microplankton were directly affected by the glacier inputs close to the source point due to decreased light penetration and reduced nutrient availability, while heterotrophic and mixotrophic microplankton were equally or more abundant in such locations. Additionally incubation experiments were conducted on the natural population as well as on laboratory cultures of two mixoplanktonic ciliate species. These experiments revealed that none of the trophic group was directly affected by the glacial flour particles themselves at a concentrations of 50 mg L\(^{-1}\). We observed no indication that glacial flour particles were ingested by the mixoplanktonic filter-feeding ciliates, and their growth rates were not affected. These results suggest that the heterotrophic and mixotrophic microplankton may play an important role in sustaining the productivity in highly turbid and nutrients depleted areas impacted by glacial meltwater input.

Keywords: Melting glaciers, glacial flour, microplankton, trophic group, planktonic community
INTRODUCTION

The increase in atmosphere and ocean temperatures in the last decades has led to high loss of Greenland ice sheet (Straneo and Cenedese, 2015). Retreating glaciers discharge large volumes of freshwater into the ocean that often carry high loads of sediment derived from the ice-rock abrasions, named glacial flour. This results in the alterations of the physical and chemical properties of the water column that consequently affect marine primary production and important ecosystem services such as carbon sequestration and fisheries (Hopwood et al., 2020). Planktonic organisms of 20-200µm (i.e. microplankton) are at the base of marine food webs and play central role in marine biogeochemistry both as primary producers and primary consumers (Azam et al., 1983). Additionally many protists can combine autotrophic and heterotrophic modes of nutrition, being thus mixotrophs (Mitra et al., 2016). Such organisms have recently been referred to as mixoplankton (Flynn et al., 2019). Mixoplankton can be further split into constitutive mixoplankton (CM) with innate phototrophic capacity (e.g. many dinoflagellates), and non-constitutive mixoplankton (NCM) which either retains functional chloroplasts from their prey (e.g. kleptoplastidic dinoflagellates and ciliates) or retains entire endosymbionts (symbiont-bearing radiolarian, ciliates and dinoflagellates) (Mitra et al. 2016; Flynn et al. 2019).

The growth of autotrophic organisms is strictly related to the availability of light and dissolved inorganic nutrients, which are both affected by glacial meltwater input in marine ecosystems. Generally meltwater is poor in nitrate and phosphate but is a source of silicate and iron (Meire et al., 2016; Hopwood et al., 2020) potentially favoring the growth of purely autotrophic diatoms. However, the sediment transported by glacial meltwater, limits light penetration into the water column, exerting the opposite effect (Meire et al., 2017; Szeligowska et al., 2021). This might have indirect effects on the growth of heterotrophic organisms as well, as they might rely on photosynthetic preys to feed. Suspended sediment particles can directly affect the growth of non-selective grazers interfering with uptake of food particles (Sommaruga, 2014). The ingestion of inert sediment particles can thus potentially affect both heterotroph and mixotroph microplankton. With its dual mode of nutrition, mixoplankton might be less affected than purely autotrophic organisms by light and nutrients limitation and less affected than heterotrophic organisms by limited prey abundances. On the other end, the common conception about mixoplankton is that it is inferior to their purely autotrophic and heterotrophic counterparts, because of its lack in specialization toward photosynthesis, nutrient uptake or feeding (Flynn et al., 2019). Mixoplankton is ubiquitous in mature ecosystems, where planktonic production in the photic zone is mainly based on regenerated nutrients due to the
stratification of water column (Mitra et al. 2014). Conversely, purely autotrophic protists, such diatoms, are known to dominate the planktonic community in marine systems with high hydrodynamics and availability of inorganic nutrients (Sarthou et al., 2005). Indeed, highly specialized autotrophs as diatoms, are well adapted to quickly adjust to the variable light conditions due to the vertical mixing of the water masses in which they proliferate (Lavaud, 2014). Most of the non-constitutive mixoplankton species are considered unable to do that, as they cannot exert any control on the acquired photosynthetic machineries (Stoecker et al., 2009). Most of the constitutive mixoplankton species are obligate phototrophs which, differently from purely heterotrophic species, cannot grow in the dark because their ingestion rates are relatively low and can only supplement their energetic requirements (Stoecker et al., 2017).

In Greenland, most of the glaciers discharge transits though fjords. Glacial fjords are the links between the ice sheet and the large-scale ocean (Straneo and Cenedese, 2015). Fjords are a pathway along which glaciers inputs are transformed by many processes, including the mixing with adjacent coastal waters (Hopwood et al., 2020). Depending on the geological and hydrological characteristics of each fjord, more or less pronounced gradients are expected in the physical and chemical properties of the water column in moving away from the glacier toward the open waters. Consequently, microplankton assemblages would be characterize by functional groups which nutritional mode would better suit the environmental conditions. The aim of the survey described in here was actually to provide a description of the microplankton communities along such gradients in four different Greenlandic fjords. Additionally, incubation experiments were conducted to assess the effect of glacial flour particles additions on a natural planktonic community and on laboratory cultures of two mixoplanktonic ciliate species. The results would provide some understanding on how the trophic structure of planktonic communities vary in coastal areas subject erosion and freshwater inputs, which are expected to increase under the current climate change scenario.

MATERIALS AND METHODS

Study site

The study was conducted in July 2019 in Disko Bay, on the West Greenland coast in four fjords impacted by runoff of land-terminating glaciers (Figure 1). In this area, the well-developed sediment plumes allowed to follow gradients in the total load of suspended particles. The sites show different geology, resulting in different chemical imprint on downstream waters.
**Sampling**

Samples were collected along transects from the inner part of the fjord to the mouth (Figure 1B). At each sampling station, profiles of temperature, salinity, fluorescence and turbidity were collected using a SBE19plus CTD. Water was collected from sub-surface (1 m depth) and at the deep chlorophyll maximum (DCM; variable depth) using a 5 and 10L Niskin bottles and siphoned off with a silicon tube to reduce organisms loss due to mechanical disturbance. For organisms identification and count, two samples of 200mL were collected from each depth in 250mL amber glass bottles and fixated with two different fixatives: a Lugol’s solution (1% final concentration) and a glutaraldehyde solution (2% final concentration). For chlorophyll analysis, 1L of water was collected and split in two equal subsample of 500mL from which chlorophyll contents of two different size classes were obtained as described below (chlorophyll analysis). Water samples were also collected for dissolved macro-nutrients (nitrate, phosphate, and silicate) analysis. Samples of 10 mL were kept frozen at -20°C until further analysis.
**On-board incubation experiment of the natural community**

Simultaneously to the sampling in Transect 1, an incubation experiment was conducted to determine the direct effects of particles addition on the planktonic community resident in the nearest offshore area not impacted by the sediments plume. 30L of surface water were collected just before entering the fjord (station 1, Figure 1B) and 20L of surface water were collected at the innermost station (station 2, Figure 1B) where a visibly high amount of glacial flour was present in suspension. Two different treatments were set-up (Figure 2). In treatment 1, the glacial flour containing water was added to the water collected outside the fjord in a volume corresponding to 30% of the total final volume (Figure 2C). In treatment 2, the glacial flour containing water was filtered through Whatmann GF/F glass microfiber filters to remove the suspended particles, and used to dilute the water collected outside the fjord in the same proportion as in the other treatment. Then 50 mg L⁻¹ of glacial flour were added to the suspension (Figure 2D). A control treatment was set-up in the same way as treatment 2, but without the addition of any glacial flour (Figure 2B). This served to account for the effect on the organisms of the mixing of the two water masses with different salinities and potentially different nutrients concentrations. The glacial flour added in treatment 2 was previously collected from sediment samples from different locations of the same area. Sediment samples were mixed into a common representative sample. This sample was dried, sieved through a 200µm net mesh, and weighted by Micro-Analytical Balance.

Each experimental treatment was distributed into fifteen 500mL borosilicate glass bottles (VWR 215-1594), which were mounted on a plankton wheel and incubated at 5°C. Light was provided with cool white led at an irradiance of 110µmol photons m⁻² s⁻¹. Triplicate samples from each treatment were withdrawn from the remaining volume (T0) for enumeration of organisms and chlorophyll measurement. The incubation lasted five days and subsamples were withdrawn daily. From each bottle, a 150 mL subsample was fixed in Lugol´s solution (1% final concentration) for enumeration of organisms, while a 150 mL subsample was collected for total chlorophyll a analysis. On alternate days a 150mL subsample was withdrawn and either fixed in glutaraldehyde (2% final concentration) or used for measurements of chlorophyll a in the <15µm size class as described below.

The growth rates of each functional group were calculated over the five days incubation as:

\[
\text{Growth rate (µ)} = \frac{\text{LN of the biomass at T5 (µg C L}^{-1}) - \text{LN of the biomass at T0 (µg C L}^{-1})}{5 \text{ days}}
\]
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Figure 2: Schematic description of the incubation experiment set-up: A) collection sites of the offshore water (1) and the glacier flour containing water (2); B) Control treatment: glacial flour containing water (2) was filtered to remove particles in suspension, and then added to the offshore water (1) in 30% V/V; C) Treatment 1: glacial flour containing water (2) was added to the offshore water (1) in a 30% V/V; D) Treatment 2: 50 mg L⁻¹ of dried glacial flour, previously collected from sediment samples, were added to the offshore water (1) diluted with filtered water from station 2 (30% V/V).

Incubation experiment on laboratory cultures of non-consitutive mixoplanktonic ciliates

The effect of glacial flour particles was tested on laboratory cultures of two non-consitutive mixoplanktonic ciliates species: Strombidium cf. conicum and Strombidium cf. basimorphum, which were previously isolated and maintained as in Maselli et al. (2020). The experimental treatment consisted in the addition of 50 mg L⁻¹ of glacial flour (prepared as described above) to ciliates in mixed cultures with their cryptophyte prey, Telealulax amphioxeia. The experimental treatment was incubated in parallel with two control treatments. The first control treatment (C1) was set in the same way as the experimental treatment, but with no addition of glacial flour. It served to compare ciliates growth and chlorophyll content when incubated in absence of sediment particles. The second control treatment (C2) consisted in culture of the cryptophyte prey alone with glacial flour, added in the same amount as in the experimental treatment. It served to account for possible interactions of glacial flour particles with the algal prey and to compare the algal growth with that in the mixed cultures, to verify
that ciliates were ingesting it. All treatments were set in 1.2L volume and then split in 15 replicates, each of 50 mL, in tissue culture flasks that were mounted on a plankton wheel and incubated in the same condition used for cultures maintenance (15°C, ~70μmol photons m⁻² s⁻¹ in a 14:10 light: dark cycle). In all treatments, the initial algal density was 2.0x10⁴ cells mL⁻¹. In the experimental treatment and the C1 the initial ciliate density was of 10 cells mL⁻¹. The incubation lasted five days and subsamples (3 replicates from each treatment) were withdrawn daily and used for cells and particles enumeration. Cellular ciliate chlorophyll a content was measured after three and 4 days from the beginning of the incubation as described below (see Chlorophyll a analysis).

Organisms and particles enumeration

Planktonic protists with a cell diameter of >15μm were enumerated in the transects samples and the on-board incubation experiment on 50 mL of the Lugol’s samples, using sedimentation chambers (Hydrobios) in accordance with Utermöhl (1958). Cells were counted on an inverted light microscope Olympus (BX 40) equipped with the camera Olympus DP73 at 200x magnification. For enumeration of ciliates in the incubation experiment on laboratory cultures, 5 to 20 mL samples were fixed in Lugol’s solution (final concentration 1%), and counted using sedimentation chambers (as above) on an inverted light microscope (Olympus CKX53) at 50× magnification. A minimum of 200 individuals was counted for each replicate. Algal prey and sediment particles in this experiment were counted using a CytoFLEX flow cytometer (Beckman Coulter, USA) calibrated and set to discriminate and count particles based on fluorescence (photosynthetic pigments) and forward and side angle light scatter, proxy for particle size and complexity (Olson et al., 1991). It was not possible to enumerate glacier flour particles on fixed samples from transects and the on-board incubation experiment.

Protist community analysis

The linear dimension (length and width) of the planktonic protists in the transect samples and the on-board incubation experiment were measured using CellSense software. Cellular biovolumes were calculated using geometric formulas for spheres, cylinders, prolate spheroids or cones according to Hillebrand et al. (1999) and converted into cellular carbon content according to Menden-deuer and Lessard (2000); this allowed calculations of the biomass (µg C L⁻¹) of the individual protist functional groups. Protists were assigned to functional categories (heterotrophs, mixotrophs and phototrophs) according to unequivocal literature records (Schneider et al., 2020) or further analysis of the glutaraldehyde-preserved samples. Glutaraldehyde-preserved organisms were collected on
polycarbonate filters (pore size 2µm). Filters were stained with Calcofluor (Andersen and Kristensen, 1995) and DAPI (Porter and Feig, 1980), and inspected with an epifluorescence microscopy (Olympus BX 50) equipped with UV, Green and Blue excitation filters prior and after the count of the Lugol sample. This filter set allowed the detection of DAPI, Calcofluor, chlorophyll and phycoerythrin pigments, thus a deeper characterization of the organism morphotypes observed in the Lugol samples. All samples were enumerated by the same person to eliminate observer bias. Triplicate samples from the incubation experiment were averaged for each time point.

**Chlorophyll a analysis**

The total chlorophyll a (total chla) content of the waters samples as well as the chla content in the size fraction <15µm (fractionated chla) were analyzed. For total chla analysis, biomass was directly collected via filtration on Whatmann glass microfiber filters GF/F, while for the fractionated chla, samples were first sieved through a 15µm net mesh. Filters were stored at -80°C until further processing. Chla samples were extracted in 5 mL 96% ethanol for 24 h in the dark at 4°C and quantified using a Turner Trilogy Fluorometer.

Ciliate chlorophyll a content in the laboratory experiment on cultures was measured on twenty cells individually picked with a drawn micropipette from each experimental bottle. Ciliates were rinsed twice in clean media and added to 2 mL of 96% ethanol. Samples were kept for 24 h in the dark at 4°C and chla was quantified as above.

**Dissolved inorganic nutrients analysis**

Subsamples (10 mL) for nutrients were filtered through 0.45 µm filters (Q-Max GPF syringe filters) and directly frozen at -20°C until analysis. Nutrients were measured using standard colorimetric methods on a Seal QuAAtro autoanalyzer.

**Statistical analysis**

Multiple t-tests were conducted to assess the significance of differences observed in the biomass of each functional group between treatments and between sampling points in the incubation experiment. The statistical significance was determined using the Holm-Sidak method (with alpha=5.000%) with the software Graphpad Prism6. Unpaired t-test were conducted with the same software to compare ciliates chlorophyll a content in the incubation experiment on cultures.
RESULTS

Description of the water column along each transect

At the time of sampling, the four fjords were characterized by different physical and chemical conditions. Transect 1 was generally warmer than the others and had a warm surface layer in in the upper 10-15 meters with temperatures of up to 11°C, which was 5 °C higher than temperatures at 20m depth (Supplementary figure 1). Salinity was slight lower at the surface compared to what was below 5m depth in the all transect (Supplementary figure 2). Turbidity was low in all the stations (Table 1; supplementary figure 3). Dissolved inorganic nutrients concentration was also quite low and quite homogeneous along the transect, both at surface and at DCM (Table 1).

Transect 2 showed a strong salinity gradient (Supplementary figures 1-2). Salinity increased with depth and along the transect into the Disko Bay, varying from about 10 at surface in the innermost station to about 30 at the outermost stations at all depths. The three innermost stations were also very turbid. Turbidity decreased with depths and along the transect (Table 1; supplementary figure 3). Silicate concentration decreased from inner part of the fjord to the mouth, while dissolved inorganic nitrogen and phosphorous concentrations did not show a clear trend along the transect.

Transect 3 showed both a temperature and salinity gradient along the transect in the upper 5 m of the water column (Supplementary figures 1-2). Salinity varied from about 10 to 30 and temperature varied from 11 to 6°C. Below 5m depth, salinity and temperature were quite homogenous at depth and along the transect being respectively 31 and about 5 °C. The five upper meters were also the more turbid at the innermost stations (Table 1; supplementary figure 3). Silicate concentration in the surface water of the innermost stations was much higher than in the other transects. Dissolved inorganic phosphates and silicates concentrations decreased along the transect in surface water, while phosphates and nitrate concentrations at the DCM were only different (higher) in the mid part of the fjord.

Transect 4 was characterized by cold and homogeneous temperatures (around 1°C) and limited variation in salinity (Table 1; supplementary figure 1-2). Turbidity was generally quite low (Table 1; supplementary figure 3). The concentration of dissolved inorganic nutrients decreased along the transect into Disko Bay and was very similar at surface compared with the DCM (Table 1).
Table 1: DCM depth (m) and surface and DCM temperature (Temp), salinity, turbidity (arbitrary units) and concentrations of nitrate, phosphate and silicate (μM) at each station.

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Effects of glacial flour on marine micro-plankton

Description of the protists community and Chlorophyll a concentration along each transect

The biomass of organisms >15 \( \mu \text{m} \) (\( \mu \text{g C} \ \text{L}^{-1} \)) and the total chl \( a \) content (\( \mu \text{g L}^{-1} \)) were generally higher at the outermost stations at the 4 transects, where mainly autotrophs dominated. Biomass content and composition strongly differed among transects (Figure3).

Transect 1 had the lower (total) biomass (Figure 3), despite total chla (\( \mu \text{g L}^{-1} \)) was comparable to transect 2 and 3 (Figure 4 and supplementary figure 4). The largest fraction of the biomass in the surface water was allocated in the heterotrophic compartment, and almost 80\% of the chla was allocated in the <15\( \mu \text{m} \) size fraction (Figure 4). Transect 1 was also characterized by a very deep chlorophyll maximum at the outer stations (50m), so that the community composition strongly differed from surface to DCM, being dominated by heterotrophs at the surface and by diatoms at the DCM (Figure3).

The community composition in transect 2 did not greatly vary from surface to the DCM, nor among stations especially in the terminal part of the transect, from station 36 to station 47 (Figure 3). Biomass was much lower in the innermost stations, where the higher turbidity was recorded. At the intermediate station (station 33) the autotrophic biomass was much higher at the DCM (5m) than surface water. Chla in the <15\( \mu \text{m} \) size fraction represented a smaller proportion of the total chlorophyll in the terminal stations compared to the innermost stations (Figure 4). This was due to a dominance of chain forming diatoms. Mixotrophs dominated Transect 3, especially in the surface water and the shallower DCMs of the innermost stations (Figure3). At the outermost stations, the DCM was deeper (supplementary figure 4) and the protist community changed in being dominated by heterotrophs at surface and by autotrophs at the DCM (Figure3).

Transect 4 differed from the other transects in that the biomass was much higher and dominated by chain forming diatoms along the entire transect (Figure3); indeed, Chla in the <15\( \mu \text{m} \) size fraction was almost null (Figure 4). The biomass of heterotrophs was comparable to the other transects, while mixotrophs made up a very small fraction (Figure3).

Heterotrophs were represented by ciliates and dinoflagellates in all samples. Although some radiolarian were found, the rare individual cells did not represent a significant proportion of the total biomass in any of the samples. Heterotrophic dinoflagellates were consistently more abundant than heterotrophic ciliates, which represented less than one third of the total heterotrophic biomass in the
The majority of the samples (Table 2). The most abundant and widespread heterotrophic dinoflagellates belonged to the genera, *Gyrodinium* and *Protoperidinium*. A-loricate ciliates were generally more abundant than loricate ciliates (tintinnids), and dominated by the genera *Strombidium*, *Strobilidium*, *Monodinium*. Despite numerically abundant in some samples, the biomass of small heterotrophic ciliates (~20µm) did not account for a significant proportion of the total heterotrophic biomass in any of the samples (Table 2).

Mixotrophs were generally less abundant than heterotrophs (about ¼ of the heterotrophs biomass on average) and mostly represented by mixotrophic ciliates. Mixotrophic dinoflagellates only exceeded mixotrophic ciliates biomass in: a) surface samples of transect 3 - where dinoflagellates in the genera *Alexandrium* and *Heterocapsa* were abundant; b) two stations in transect 1 (10 DCM and 12 surface), where *Dinophysis* and *Heterocapsa* contributed to the relatively high biomass of mixotrophic dinoflagellates. Mixotrophic ciliates belonging to the genera *Laboea*, *Strombidium* and *Mesodinium* almost equally contributed to the biomass of mixotrophic ciliates in all samples. However, *Mesodinium rubrum/major* accounted for most the mixotrophic ciliate biomass on a few occasions (Table 2).
Figure 3: Biomass (µg C L⁻¹) of heterotrophs (in black), mixotrophs (in grey) and autotrophs (striped) and depth of the DCM (dots) at all stations along each transect.
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Figure 4: Total Chlorophyll $a$ concentration and fraction of Chla concentration in the size category <15µm in all stations at surface and DCM.
Table 2: Biomass (µgC L⁻¹) of the most representative protists groups (dinoflagellates, ciliates and diatoms) at selected stations at (a) surface and (b) DCM. Protists were grouped according to their trophic mode. (Gyrod. = Gyrodinium; Protop. = Protoperidinium; Small spp = ~20µm; Heteroc. = Heterocapsa; Dinoph. = Dinophysis; Mesod. = Mesodinium)

### a) Surface samples

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Effects of glacial flour on marine micro-plankton
Development of the protist community during the on-board incubation experiment

The biomass of organisms >15µm was significantly higher at T0 in treatment 1 (addition of 30% V/V of glacial flour containing water) compared to the control and treatment 2 (addition of 50 mg/L of glacial flour). The higher total biomass in treatment 1 was mainly ascribable to the heterotrophic dinoflagellates, added with the glacial flour containing water used in this treatment (Supplementary figure 5). The chla < 15µm at T1 was also significantly higher in treatment 1 compared to the control and treatment 2 (Supplementary figure 6). At T5, chla < 15µm was significantly increased in the control and in treatment 2, but not in treatment 1 (Supplementary figure 7, p< 0.0001).

Only dinoflagellates (both heterotrophs and mixotrophs) grew in all treatments (Figure 5). Diatoms biomass slightly increased in all treatments until day 3 to then decreased again from day 3 to day 5 (supplementary figure 6), thus diatoms growth rates calculated over the 5-days of incubation were not really different from zero in the control and treatment 1 despite some growth was observed during the experiment. Mixotrophic ciliates expressed negative growth rates in all treatments, including the control treatment (Figure 5). Mixotrophic ciliate biomass displayed a rapid drop especially in the first 3 days in treatment 1 (Supplementary figure 5). Finally, the biomass of heterotrophic ciliate did not significantly vary during the experiment in any of the treatment (Supplementary figure 5) so growth rate was not different from zero in any of the treatments.

**Figure 5:** Growth rates of each functional group in the on-board experiment, calculated over the five day incubation.
Development of cultures during the laboratory incubation experiment

The growth rate of both ciliate species was not statistically different between the experimental treatments that contained glacial flour compared to the control treatment not containing glacial flour (supplementary figure 7). Sediment particles of 2-15µm in size decreased in number in the same proportion in the experimental treatment and in the control where glacial flour was added to the algal prey only (C2) (Figure 6).

Figure 6: Development of sediment particles concentration in non-constitutive mixotrophic ciliates cultures (*Strombidium cf. basimorphum* and *Strombidium cf. conicum*) in presence of prey and 50 mg L⁻¹ of glacial flour (treatment) compared with cultures of the prey only incubated with the same amount of glacial flour (C2). Particles (2µm < particles < 15µm) were counted by means of flow cytometry.

As visible from the flow cytometry scatter plots and microscopy, glacier flour particles agglomerated forming clamps of bigger dimensions, which size was not accessible to ciliates for ingestion (data not shown). The algal prey density had similar trends in the experimental treatment and the control mixed cultures (C1) and removal due to ciliates ingestion was evident from comparison of the algal density with the algal monoculture control (C2). Ciliates chlorophyll a content in the experimental treatment was not different from chlorophyll a content of ciliates incubated without glacier flour (C1) (supplementary figure 8).
Discussion

Distribution of protist groups along the four transects

Gradients associated with the glacier inputs were distinguishable in the chemical and physical qualities of the water column in all four different fjords. The sediment plume was only evident at stations that were close to the glacier input. Indeed, in accordance with previous observations in the area (Meire et al., 2017), a large fraction of glacial sediments settles within a few kilometers from the input. The relatively low protist biomass in the innermost station can only partially be attributed to turbidity caused by suspended particles. Among other reasons there is the fact that suspended particles tend to agglutinate to protists and bacteria, thereby sinking them out from the photic layer (Szeligowska et al., 2021).

Additionally, the freshening of the upper part of the water column lead to a strong stratification of water column that reduces the vertical mixing and create a nutrient-poor surface layer. This limits the growth of autotrophic organisms and lead to the deepening of the chlorophyll maxima (Holding et al., 2019; Hopwood et al., 2020). This was less evident on transect 4 due to the presence of marine-terminating glaciers that could have led to the upwelling of nutrient rich bottom water (Meire et al., 2017). Diatoms dominated the protist communities on transect 4 and in most of the offshore stations on the other transects. This is in accordance with the fact that diatoms typically make-up the biomass of planktonic protist communities in the marginal ice zone in the artic productive season (Stoecker and Lavrentyev, 2018).

The heterotrophic and mixotrophic protists were more abundant at the innermost stations of transect 1-3. Their relative abundance in terms of biomass, exceeded that of the autotrophs in stations where the dissolved inorganic nitrogen concentrations were relatively low (<2µM). On such stations chla was mainly found in the <15µm fraction, suggesting that smaller phototrophic organisms, which have a higher surface to volume ratio compared to phototrophic microplankton, were favored in nutrient limiting conditions (Stolte and Riegman, 1995). Moreover, many photosynthetic nanoplankton species (<20µm in size) other than diatoms, are known to be mixotrophs and to sustain their metabolism feeding on bacteria (Stoecker et al., 2017). Bacteria growth may be boosted by the presence of suspended particles (Szeligowska et al., 2021). A similar predominance of chla in the small size fraction have previously been observed in the inner location of other Western Greenlandic fjords (Arendt et al., 2010, 2016). The predominant grazing activity on primary producers in such locations is likely attributed to the microplankton rather than copepods, which instead play an important role in the coastal zone (Arendt et al., 2010, 2016). Similarly, especially on transect 1 and
transect 3, heterotrophic and mixotrophic microplankton abundance seemed to be associated with the smaller size fraction of primary producers (the chla fraction <15µm).

Heterotrophic dinoflagellates were also found on more offshore stations where most of the chla was due to chain forming diatoms (functionally >15µm). Dinoflagellates in the genera *Protoperinidium* and *Gyrodinium* have already been recorded to be dominant in summer in Greenland and associated to diatom blooms (Krawczyk et al., 2015). Heterotrophic ciliate biomass, instead, was very low or even null in such samples. This is likely explained by differences in the feeding mechanisms in these two groups. The ciliates species found in this survey were mostly filter feeders. Thus, their grazing potential was limited to particles which size fits the morphological constrains of their feeding apparatus (Jonsson, 1986). The feeding mechanisms of dinoflagellates are more diverse. Many thecate species, like *Protoperinidium* and the *Diplosalis* group use a pallium to unveil the prey, while some other species, like *Phalacroma* and many small heterotrophic species use peduncles (feeding tubes). These feeding mechanisms allow the organisms to ingest prey items exceeding their own size (Jacobson and Anderson, 1986; Hansen and Calado, 1999).

Mixotrophic microplankton was relatively more abundant in the mid part of the fjords, especially in areas where the water column was strongly stratified. This is especially evident on transect 3, where stratification was induced by both salinity and temperature. Peaks in the relative abundance of mixotrophic microplankton were formed by constitutive mixotrophic species (*Heterocapsa* spp. and *Alexandrium* spp.), while non-constitutive mixoplankton never dominated the microplankton communities. The reasons for that can be found in biotic factors such as the top-down control from metazoan grazers and specific interaction among microorganisms. Non-constitutive mixoplanktonic ciliates in particular, are a preferred prey of copepods (Stoecker and Lavrentyev, 2018), while most constitutive mixotrophic dinoflagellates produce toxins (Burkholder et al., 2008) that can defend them from predation.

The only identifiable non-constitutive mixotrophic dinoflagellate species were *Dinophysis* spp, which are prey specialist grazers that can only acquire phototrophy by feeding on the non-constitutive mixotrophic ciliate *Mesodinium rubrum* (Hansen et al., 2013). Not surprisingly, *Dinophysis* spp were only found in sample where the mixotrophic *Mesodinium* spp were also present. Non-constitutive mixotrophic ciliates in the *Mesodinium rubrum* species complex are also prey specialist grazers that can only acquire chloroplasts via feeding on cryptophytes within the *Teleaulax/Plagioselmis/Geminigera* clade (Hansen et al., 2013). Differently from many other mixotrophic ciliates, *Mesodinium rubrum* can take up and utilize inorganic nutrients for growth and
go through up to 4 cell divisions without prey (Tong et al., 2015; Kim et al., 2017). *Mesodinium* spp only dominated the mixotrophic ciliates biomass in few of our samples. Indeed, *Mesodinium* biomass is usually low under non-bloom conditions, which tend to occur in localized patches (Crawford, 1989), as actually evident from the distribution of this ciliate found in these fjords. Except for a few locations, prey generalists mixotrophic ciliates were equally or more abundant than *Mesodinium*, as typical in polar waters (Levinsen and Nielsen, 2002; Stoecker et al., 2009; Leles et al., 2017). The total biomass of mixotrophic ciliates was in the low range of what it could be in summer in more open waters of the same area, but their relative abundance compared to the total ciliate biomass (from about 30 to about 70 %) was comparable to those previous records (Putt, 1990; Levinsen et al., 2000; Levinsen and Nielsen, 2002).

**The glacial flour addition in the incubation experiments**

The glacial flour addition in the incubation experiments did not seem to have any effect on any of the microplankton functional group. This suggests that sediment particles themselves, in the concentrations used here, do not directly interfere with the organisms. Mixotrophic ciliates were the only group that seemed affected by the incubation during the experiment on the natural community. At first, this could indicate an effect of ingestion of sediment particles by the cells, which could have displaced the sequestered chloroplasts, thereby causing a negative effect on their growth. Tests on laboratory cultures done after the cruise, however, did not demonstrate such an effect. In fact the two non-constitutive mixoplanktonic ciliates species tested in the laboratory experiments on cultures, did not seem to ingest sediment particles, and were able to maintain the same chla content (pg cell⁻¹) as when incubated without sediment. Also, the negative growth of mixotrophic ciliate in the control treatment during the on-board incubation with the natural population suggests that the negative effect observed may be due to reasons other than the ingestion of suspended particles. Such reasons include competition for prey with other functional groups, direct predation by other microorganisms, or an effect due to the mixing of water in the bottles during the incubation. Ciliates, especially, *Mesodinium* and oligotrotrich ciliates have previously been shown to be affected by incubation in bottles (Hansen et al., 2019).

**Conclusion**

The way in which the glacial flour inputs affect microplankton communities depends on the chemical-physical properties of the runoff water and the hydrology of the specific location. Indeed, glacial flour
particles their self, had no effect during the incubation at the concentrations used in our experiments. As previously suggested, the functional classification of plankton has to be adequate in order to underline different response of organisms to the impact of glacier discharges in the marine ecosystem (Szeligowska et al., 2021). Diatoms abundance seems to be more influenced by the glacier inputs compared to the heterotrophic and mixotrophic microplankton that are not directly affected by turbidity and the nutrients limitation. Microplanktonic filters feeders (ciliates) do not seem not ingest glacial flour particles. The freshening of marine coastal waters, associated with increased turbidity, aggregate formation, and de-eutrophication, may lead to a shift from fast growing photoautotrophic microplankton communities to less productive communities dominated by heterotrophic and mixotrophic microplankton species.

Acknowledgements

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Effects of glacial flour on marine micro-plankton


Supplementary material
Effects of glacial flour on marine micro-plankton
Supplementary figure 1: Vertical profile of temperature (°C) along the 4 fjord transects.

Supplementary figure 2: Vertical profile of salinity along the 4 fjord transects.
Supplementary figure 3: Vertical profile of turbidity (Formazin Turbidity Unit, FTU, uncalibrated) along the 4 fjord transects.

Supplementary figure 4: Vertical profile of fluorescence (uncalibrated) along the 4 transects.
Supplementary figure 5: Development of the biomass (µg C L⁻¹) of each functional group during the incubation experiment.
**Supplementary figure 6:** Development of Chla concentrations in the <15μm size fraction during the incubation experiment
**Supplementary figure 7:** Development of the mixed cultures of non-constitutive mixotrophic ciliates (*Strombidium* cf. *basimorphum* and *Strombidium* cf. *conicum*) in a treatment where ciliates were incubated with both their prey (*Teleaulax amphioxea*) and 50 mg L\(^{-1}\) of glacier flour (treatment), and in a treatment where the ciliates were incubated with prey only (control).
Supplementary figure 8: Chlorophyll $a$ content in non-constitutive mixotrophic ciliates cultures (Strombidium cf. basimorphum and Strombidium cf. conicum) when incubated in a treatment with both prey and 50 mg L$^{-1}$ of glacier flour particles (treatment) and when incubated in a treatment with prey only (control) after 3 days of incubation.
HORIZONS

Mixotrophic protists and a new paradigm for marine ecology: where does plankton research go now?

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Many protist plankton are mixotrophs, combining phototrophy and phagotrophy. Their role in freshwater and marine ecology has emerged as a major developing feature of plankton research over recent decades. To better aid discussions, we suggest these organisms are termed “mixoplankton”, as “planktonic protist organisms that express, or have potential to express, phototrophy and phagotrophy”. The term “phytoplankton” then describes phototrophic organisms incapable of phagotrophy. “Protozooplankton” describes phagotrophic protists that do not engage in acquired phototrophy. The complexity of the changes to the conceptual base of the plankton trophic web caused by inclusion of mixoplanktonic activities are such that we suggest that the restructured description is termed the “mixoplankton paradigm”. Implications and opportunities for revision of survey and fieldwork, of laboratory experiments and of simulation modelling are considered. The main challenges are not only with taxonomic and functional identifications, and with measuring rates of potentially competing processes within single cells, but with decades of inertia built around the traditional paradigm that assumes a separation of trophic processes between...
different organisms. In keeping with the synergistic nature of cooperative photo- and phagotrophy in mixoplankton, a comprehensive multidisciplinary approach will be required to tackle the task ahead.

KEYWORDS: mixotrophy; protist; mixoplankton; phytoplankton; protozooplankton; microbial loop; allometry

INTRODUCTION

Mixotrophy is the combination of autotrophy and heterotrophy in the same organism. The means by which this combination occurs in different organisms varies, but the form that has so fascinated scientists (and captured the public attention; Wyndham, 1951) has been that which combines photo(auto)trophy with carnivory, or as applied to protists, with phago(hetero)trophy. Long considered by most aquatic scientists to be of minor significance, or just a curiosity, we now recognize that protist plankton engaging in photo- and phago-mixotrophy are common and important members of the global plankton community (Leles et al., 2017, 2019; Faure et al., 2019).

Even though photo- and phago-mixotrophy in planktonic protists has been studied by many scientists over many decades in freshwater and marine systems (e.g. Biecheler, 1936; Blackbourn et al., 1973; Bird and Kalff, 1986; Estep et al. 1986; Laval-Peuto and Febvre, 1986; Sanders et al., 1990; Sanders, 1991; Jones, 1994; Stoecker et al., 1987; Jeong et al., 2010; Hansen, 2011), appreciation that these organisms play a core role in especially marine ecosystems has been slow to mature. From 2011 to 2013 a series of workshops (funded by the Leverhulme Trust, U.K.) were held in Sweden (Kalmar), UK (Swansea) and the USA (Horn Point), bringing together experts across the field of marine planktonic protists. Outputs from those meetings explored the false dichotomy of the traditional phytoplankton–zooplankton paradigm (Flynn et al., 2013), advantages of acquired phototrophy (Flynn and Hansen, 2013), the role of mixotrophy in shaping the biological carbon pump (Mitra et al., 2014b), stoichiometric implications for mixotrophy (Landgren et al., 2016), the functional classification of planktonic protists (Mitra et al., 2016) and the biogeographies of the different types of mixotrophic protist plankton (Leles et al., 2017, 2019). Many additional publications have also raised the profile of photo- and phago-mixotrophic plankton over the last two decades (e.g. Stickney et al., 2000; Burkholder et al., 2008; Zubkov and Tarran, 2008; Carvalho and Granéli, 2010; Brutemark and Granéli, 2011; Sanders, 2011; Hartmann et al., 2013; Wilken et al., 2013; Saad et al., 2016; Selosse et al., 2017; Stoecker et al., 2017), and brought the subject to a wider audience (Mitra, 2016, 2018; Glibert et al., 2019).

Why is this subject important? Because the combining of primary and secondary production in a single organism radically changes biogeochemical and trophic dynamics involving those organisms (Hitchman and Jones, 2000; Mitra et al., 2014b; Caron, 2016; Ward and Follows, 2016; Ghyoot et al., 2017a, 2017b; Leles et al. 2018). It alters the flows of energy and materials in and out of organisms that form the base of the food chain, and changes the way that we understand and thence simulate processes from harmful algal blooms (HABs) and fisheries to global climate change.

In all areas of science, there comes a point when the weight of new evidence warrants a fundamental reassessment of the paradigm in which we express our understanding. Within marine ecology, while the components of the microbial loop had been known about for decades before the mid 1970s, the broad importance of the concept was only brought to wide attention with works such as those of Pomeroy (1974), Williams (1981) and Azam et al. (1983), with increasing acceptance following that (e.g. Pomeroy et al., 2007; Fenchel, 2008). We suggest that a similar reassessment point has now been reached concerning the importance of photo- and phago-mixotrophic plankton, and its potential to radically reshape marine ecology. To consider such a reshaping represents a very significant, and most likely controversial task, cutting across all sectors of plankton research. Here we present some ideas on how this task could be undertaken. However, we start by considering naming conventions and some misconceptions that surround the topic of evolution and functional traits of planktonic mixotrophs. We then continue by exploring the implications for marine ecology, and then consider various specific challenges for plankton research.

To aid this discussion we present a glossary (Table I).

WHAT IS IN A NAME?

Traditional descriptions of plankton most obviously comprise phytoplankton and zooplankton, with increasing inclusion of bacterioplankton from the 1980s. Often these descriptors attract add-on terms donating organism size (pico-, nano-, micro-, meso-; e.g. Sieburth et al., 1978). Over a century of marine research has been built upon terminologies largely of terrestrial origin, dividing primary and secondary production neatly between plant-like phytoplankton and animal-like zooplankton. Thus, phytoplankton may be referred to as the “grass of the sea”,
and their copepod predators as “herbivores” or “insects of the sea” (e.g. Cushing, 1975), with classic models (e.g. NPZ: Fasham et al., 1990; Franks, 2002), their complex successors (e.g. DARWIN: Follows et al., 2007; MEDUSA: Yool et al., 2013; ERSEM: Barretta et al., 1995; Butenschön et al., 2016) and indeed climate change (Arora et al., 2013) and fisheries management models (Plagányi, 2007), also deploying this classic dichotomy. The growing realization that plankton communities often contain organisms that combine photo- and phagotrophy (i.e. primary and higher production), requires that we more readily and clearly discriminate between organisms that are actually or potentially photo- and phagomixotrophic and organisms that are not. We need greater clarity during our conversations, in conferences, on posters, in scientific publications and critically also in teaching. The problem is that the terminology currently referencing these protist plankton is unwieldy and/or ambiguous. And the problem starts with the word “mixotroph” (the combining of autotrophy and heterotrophy in one organism; Table I).

For protist plankton, the mode of autotrophy is phototrophy, while heterotrophy may be enabled by osmotrophy and/or phagotrophy (Fig 1; Table I). There is a profound difference between heterotrophy supported by osmotrophy versus that by phagotrophy, both for trophic dynamics and also for food web structuring. Phagotrophy typically involves the killing of other organisms through the process of capture, ingestion (either partially or totally) and digestion. In contrast, osmotrophy does not require the act of killing. Indiscriminate use of the word “mixotroph” fails to draw attention to this important difference.

Osmotrophy, the ability to use dissolved organic compounds such as amino acids and vitamins (auxotrophy; Droop, 2007), is common in plankton typically considered as phototrophs. In aquatic ecology the subject has a long and controversial history (see Flynn and Butler, 1984 for a review of older discussions on the ecological importance to plankton); for many species in nature the role of osmotrophy probably acts mainly to mitigate against metabolite leakage (Flynn and Berry, 1999).
Nonetheless, phytoplankton are typically mixotrophic in this sense, and indeed various modelling explorations of mixotrophy also refer to photo- and osmo-mixotrophy (e.g. Våge et al., 2013). This mode of mixotrophy (photo(auto)trophy + osmo (hetero)trophy) in protistan and cyanobacterial microalgae is also exploited for biotechnology (Chojnacka and Noworyta, 2004; Liang et al., 2009; Bhatnagar et al., 2011; Morales-Sánchez et al., 2013). Confusingly the mode of mixotrophy being studied is neither always apparent from the title nor in the abstract of many scientific publications (e.g. Yelton et al., 2016), and indeed both osmotrophy and phagotrophy may be implicated (Burkholder et al., 2008).

We need to be able to differentiate readily between generic “mixotrophy” and specifically that which involves photo-phagotrophy.

Because of the likely ubiquity of osmotrophy, use of this trait in the functional classification of protists is compromised (Flynn et al., 2013). Accordingly, Mitra et al. (2016) presented a revised classification of the protist plankton based on functional types, centred on the potential for and the mode of expressing photo- and phagotrophic mixotrophy. That publication differentiates between the “pure” photo(auto)trophs (phytoplankton), the mixotrophs that have a constitutive ability to photosynthesise (“constitutive mixotroph”, CM), those that need to acquire their phototrophic capability from their prey (“non-constitutive mixotrophs”, NCM) and the “pure” phago- and heterotrophs (protozooplankton).

NCMs are then themselves split between generalists (GNCM), plastidic specialists (pSNCM) and endosymbiotic specialists (eSNCM) according to their mode of acquiring phototrophic capabilities. All these groups are fundamentally different functional types (Fig. 2). While the terms CM and NCM are gradually gaining increased usage (e.g. Johnson and Moeller, 2018; Hansson et al., 2019; Naselli-Flores and Barone, 2019), what we are still missing is a term akin to the traditional terms—phytoplankton and zooplankton—to use to collectively refer to all photo- and phago-mixotrophic planktonic protists.

As such a short descriptive term, we propose that the word “mixoplankton” be used to specifically refer photo- and phagotrophic protists. This term already appears in various online teaching resources as well as on Fishbase website (https://www.fishbase.de/glossary/Glossary.php?q=mixoplankton), where mixoplankton are defined as “Planktonic organisms that can be classified at several trophic levels. For example, some ciliates can be photosynthetic but also can ingest other plankton and are heterotrophic.” Such a description perhaps does not make it clear that one and the same organism is engaging simultaneously or alternatively in photo- plus phago-“trophic activities. Nor does this description exclude reference to mixotrophs that are incapable of phagotrophy. We thus suggest that this definition is reworded to read:

“mixoplankton: planktonic protist organisms that express, or have potential to express, phototrophy and phagotrophy.”
The word “potential” in the definition of mixoplankton is also important, because while some mixoplankton are obligatory photo- and phago-mixotrophs (or have life stages that are), other species (specifically many CMs) may not show such an obligatory requirement (Sanders et al., 1990; Jones, 1997; Calbet et al., 2011; Berge and Hansen, 2016; Stoecker et al., 2017).

Usage of the overarching term “mixoplankton” has various advantages:

- It is short and not hyphenated, amenable to the addition of an allometric prefix (e.g. nano-mixoplankton).
- There is a clear linkage to plankton; mixotrophy as a mode of nutrition is distributed across many organism groups besides plankton (e.g. corals, the sea slug Elysia viridis, the Venus flytrap Dionaea muscipula).
- Importantly, “mixoplankton” discriminates between the mixotrophy expressed in protists unable to phagocytose (i.e. photo- and osmo-mixotrophs; as conducted by diatoms for example), versus that expressed by protists that can conduct phagotrophy (i.e. the mixotrophy of the CM and NCM functional groups described by Mitra et al., 2016). We can readily realign the original descriptions of Mitra et al., 2016 by modifying the meaning of the abbreviations CM and NCM to “constitutive mixoplankton” and “non-constitutive mixoplankton”, respectively.
- Perhaps of equal importance to the above is that phytoplankton are then characterized as being incapable of phagotrophy, while protozooplankton are incapable of phototrophy (Table I; Fig. 2).

From here on, we shall use the term “mixoplankton” in reference to photo- and phago-mixotrophic planktonic protists, including where the original referenced paper termed such organisms as “mixotrophs”.

MISCONCEPTIONS ON THE EVOLUTION AND TRAITS OF MIXOPLANKTON

To help understand why different organisms occupy the niches that they do occupy requires a full understanding of their functional traits, and also of those organisms.
with which they interact. A common conception is that mixoplankton are inferior to their phytoplankton or protozooplankton counterparts with respect to their expression of traits such as photosynthesis, nutrient uptake and feeding (Dolan and Pérez, 2000; Litchman et al., 2007; cf. Calbet et al., 2011). This assumes that an organism that specializes in two nutritional routes is a generalist in its expression of each of those routes. This argument could be considered as a trait-trade-off (TTO), a concept which has found considerable support in the plankton research community, especially with respect to plankton modelling (Litchman and Klausmeier, 2008; Litchman et al., 2013).

Such TTO arguments must only be applied to organisms from the same environment (Litchman et al., 2007), and TTOs are in any case questionable from an evolutionary standpoint (Flynn et al., 2015).

Clearly, we expect traits expressed by phytoplankton and protozooplankton to be advantaged in the environment in which they evolved, just as those species that combine photo- and phagotrophy must be advantaged by possessing both phototrophy and phagotrophy in other environments. Questions of which trait is better or not in a given environmental setting have been answered by evolution. To better clarify this matter requires a consideration of the evolution of the protists.

The genetic diversity of protists is vast, equalling that of all other eukaryotes combined (Burki 2014; Adl et al., 2019). The evolution of eukaryotes (eukaryogenesis) was based on the phagotrophic acquisition of an alphaproteobacteria into a host, which subsequently lead to the formation (evolution) of mitochondria (Hampel et al., 2019). The formation of mitochondria provided the cells with extra energy that allowed the evolution of eukaryotic cell structures (Martin et al., 2017). One prerequisite for this event is that the host must have had an actin filament system that enabled the formation of protrusions and the subsequent engulfment of bacteria (Yutin et al., 2009). Whether the fast eukaryotic common ancestor was an Archea or not is still not resolved (Martijn and Ettema, 2013), but under any scenario phagotrophy evolved very early in life on Earth. Moreover, secondary endosymbiotic events gave rise to photosynthesis within eukaryotes, which appears to have been lost and (re)gained several times in many protist groups (Archibald, 2009). Extant protozooplankton thus include organisms that gained and then lost their scope for phototrophy (i.e. that had ancestors that were mixoplankton).

Protist evolution saw many cycles of gaining and losing capabilities (Figuerola-Martinez et al., 2015; Hampel et al., 2019). Mixoplankton did not arise as a coming together of protist phytoplankton plus protozooplankton traits into one organism, and protist phytoplankton did not fail to acquire the capability for phagotrophy. On the contrary, the most profound event in protist plankton evolution in some ways was the loss of the ancestral trait of phagotrophy. Not only did this remove an important nutritional pathway, but it also removed a major route through which protists have acquired genetic and physiological variety of importance for their evolution.

Ecologically the most important of those protist groups in contemporary aquatic environments that lost phagotrophy are the diatoms, which also evolved a non-cellulosic cell wall (frustule of silica) that was likely incompatible with phagotrophy from an early stage in their evolution. Planktonic diatoms are also effectively non-motile (they cannot swim) within the plankton. Motility is rather an important trait for a planktonic predator; the low motility of the mixoplanktonic Foraminifera and other Rhizaria are compensated for in this regard by the use of pseudopods for feeding. The environment in which diatoms flourish is also typically an immature one, of high turbulence, relatively high inorganic nutrient concentrations, and lacking in the prey that could otherwise provide nutrients. A similar set of arguments could be made for the often-assumed absence of phagotrophy in the ecologically dominant calcified form of coccolithophorids (cf. Rokitta et al., 2011). In contrast, mixoplankton dominate in more mature systems containing competitors that are also potential prey, and in systems in which nutrients (which can include light) are supplied in unbalanced proportions, if not limiting amounts (Mitra et al., 2014b). These are also the situations in which HABs occur (Granéli, 2006; Granéli et al., 2008; Gilbert et al., 2018), where mixoplankton are often dominant species.

There is significant variation across different mixoplankton types with respect to their core physiology (Fig. 2), and allied variation in feeding types, and hence in the form of trophic interactions. Despite this, the traits of these organisms have often been referenced in the literature in a rather indiscriminate fashion, as if all mixotrophic plankton are effectively the same rather than being different (CM vs GNCM, vs SNCM etc.). This is especially so in the conceptual and modelling-orientated literature (e.g. Ward and Follows, 2016). The allometric “rule”-based analysis of Andersen et al. (2016), suggesting that mixoplankton are mechanistically aligned (optimized) at a position intermediate between smaller phototrophic phytoplankton and larger phagotrophic micro (proto) zooplankton, does not align with reality. In fact, mixoplankton span size ranges that encompass the whole breadth of protist phytoplankton and protozooplankton (Fig. 3); this range exceeds that similar to that of ant-to-cow. Equally important though, there appears to be important functional-allometric differences between the CMs, GNCMS and SNCMs, which is also seen in their biogeography (Leles et al., 2017, 2019).
In conclusion, mixoplankton should not be collectively viewed as inferior, nor indeed superior, to phytoplankton and protozooplankton. They are just different (and different within themselves—Fig. 2). They also, in consequence, have the potential to fulfil different functional roles within ecology, exploiting different niches. Before we appreciated the global abundance of mixoplankton we could perhaps justify ignoring their contribution. We can no longer afford to do so.

PLACING MIXOPLANKTON WITHIN MARINE ECOLOGY

Researching and understanding the role of mixoplankton within marine plankton systems has the potential to completely reform our understanding of marine ecology. However, the development of plankton science in this regard has been complicated by:

(i) the failure to appreciate the implications of different modes of mixoplankton functioning; the differing degrees with which mixotrophy is obligatory (Sanders et al., 1990; Jones, 1997; Stoecker et al., 2017; Gomes et al., 2018) even within the same or closely related species (Calbet et al., 2011; Berge and Hansen, 2016);

(ii) the afore-mentioned confusion in terminologies.

There is in consequence something of a legacy of published works that ignore or misrepresent mixoplankton and are in retrospect questionable as to whether they are best considered as incomplete, or explorations in theoretical biology or ecology rather than of reality. The implications of the advance in our understanding and appreciation of mixoplankton within plankton ecology thus have similarities with the rise to prominence of the “microbial loop” (Azam et al., 1983) and the microbial food web in the 1980s, and the inclusion of the “viral shunt” (Wilhelm and Suttle, 1999) in the 1990s. Before the 1980s, marine ecology saw at its base a system dominated by net-sized phytoplankton (i.e. cells retained in plankton nets (mesh size typically 20–35 μm) and meso-zooplankton (mesh size typically >200 μm; Cushing, 1975). The pre-1980s paradigm can be seen to have been augmented by the addition of microbial food webs; the form of this revised trophic structure is indicated in Fig. 4a. The whole web contributes in various ways to the “biological carbon pump” (Turner, 2015) and to the “microbial carbon pump” (Jiao et al., 2010; Legendre et al., 2015).

The enhanced appreciation of the importance of the microbial food web was brought about in large measure by discovering and identifying the importance of certain types of biological entities (e.g. Prochlorococcus, viruses, etc.), as well as the overdue recognition of the importance of known groups (bacteria, cyanobacteria, heterotrophic nanoflagellates etc.). The recent expansion of interests in mixoplankton could be argued as just the overdue recognition of facets of ecology known for over a century (e.g. Biecheler, 1936; Blackbourn et al., 1973; Jones, 1997; Stoecker, 1998; Jones, 2000). However, the inclusion of mixoplankton as a frequent and at times dominant
activity in plankton biogeography (Leles et al., 2017, 2019; Faure et al., 2019; Gutierrez-Rodriguez et al. in preparation) and trophic dynamics (e.g. Hartmann et al., 2013) marks a radically different development in marine science compared to the inclusion of either the microbial loop or the viral shunt. It is different because it sees the bulk of the known members of the traditional trophic web description actually undertaking different (additional) trophic roles. Thus, most of the protist primary producers traditionally labelled as “phytoplankton”, other than those in immature ecosystems (notably diatoms), are actually also part-time grazers, while half those organisms traditionally labelled as “micro (proto)zooplankton” in the photic zone are also photosynthesising (Fig. 4b; see review by Stoecker et al., 2009). This situation operates across the whole microbial plankton allometric scale, from picoflagellates (Zubkov and Tarran, 2008; Stoecker and Lavrentyev, 2018) to mm-dimension SNCMs (Decelle et al., 2012; Biard et al., 2017) (Fig. 3). The trophic linkages are redrawn and new links added.

One of the most frequent questions that arises in scientific discussions on mixoplankton is, how important is mixotrophy and when are these organisms functionally mixotrophic? Whether we need to know when they are eating, or whether it is sufficient just to know that they may do so if the need or opportunity arises, is a non-trivial question to answer. The ability to eat may supply small but vital amounts of nutrition to a mixoplanktonic organism that would be unavailable to non-mixoplankton competitors at critical stages. At the same time competitors are removed by a combination of phagotrophy and production of compounds with allelopathic effects (John et al., 2002, 2015; Skovgaard and Hansen, 2003; Tillmann, 2003); every difference is a difference. We suspect that many laboratory cultures of these organisms lose their ability to express different nutritional mechanisms over the years of them being held in constant light and high nutrient conditions (e.g. Blossom and Hansen in preparation). It thus follows that if organisms in nature can express a trait then that trait is, at least occasionally, exploited to advantage in nature.

Of the protist functional groups identified by Mitra et al. (2016), the NCMs must be regularly and significantly mixotrophic as many have to feed to acquire chloroplasts to support their obligatory need to undertake photosynthesis. The challenge is primarily with identifying when CM species are eating. Some CM species are perhaps only rare feeders in nature, while others (given the voracious behaviour of those brought into culture) are most likely regular feeders (reviewed by Jeong et al., 2010; Hansen, 2011). The former group include species such as *Tripos furca* (=*Ceratium furca*; Smalley et al., 2003, 2012), *Prymnesium parvum* (Johnson, 2015), *P. minimum* (Jeong et al., 2005), while the latter include species such as *Pseudonitzschia* (Skovgaard and Hansen, 2003; Carvalho and Granéli, 2010), *Karlodinium* (Berge et al., 2008a and b; Calbet et al., 2011) and *Fragilidium* spp (Jeong et al., 1999; Hansen et al., 2000; Skovgaard et al., 2000).
As evidence of the importance of differentiating between the CM and NCM forms, it is perhaps noteworthy that the microbial food web interfaces directly with many CMs (Burkholder et al., 2008; Jeong et al., 2010; Hartmann et al., 2013; Stoecker and Lavrentyev, 2018), while the NCMs generally tend to be larger (eSNCMs up to mm dimensions, e.g. green Noctiluca; Saito et al., 2006; Stoecker et al., 2009) (Figs 3 and 4b). That said, some NCMs are nano-sized, at least in the oligotrophic ocean (Pitta and Giannakourou, 2000; Pitta et al., 2001).

Although we do not yet know the full significance of mixoplanktonic activity for community structure and dynamics, or indeed for the physiology and growth of most individual species, the structural difference between Fig 4a and b appears so profound as to represent a paradigm shift. The mixoplankton paradigm is not a “loop”, or a “shunt”, appended to the pre-2010 trophic web (Fig 4). Indeed, mixoplankton activity has potentially important implications for the functioning of the microbial food web (Fig 4b). Is it perhaps a “multiplier”, improving assimilation efficiency and the allometrics of trophic transfer? We do not know. Perhaps until it becomes much clearer, we should just refer to it as simply the “mixoplankton paradigm”.

**IMPLICATIONS OF THE MIXOPLANKTON PARADIGM FOR PLANKTON RESEARCH**

The ramifications of the mixoplankton paradigm for plankton research are wide ranging. Some implications are perhaps more obvious than others, but taking a fresh look at everything may be the safer route forward. To really understand the implication of mixoplankton for marine ecology, future research will have to answer many questions such as: when and where are mixoplankton important? How and from where/who do mixoplankton acquire their energy and nutrients? What activity rates can be associated with mixoplankton? Which circumstances promote mixotrophy? Only a multidisciplinary approach can answer those questions; below we commence such a review, though it is readily apparent that the task will proceed for likely several decades.

**Survey and fieldwork**

Survey and fieldwork are ideally suited to answer questions such as when, where and how many organisms occur. These are still highly relevant questions regarding mixoplankton. However, survey methods, irrespective of the approach and technology (remote sensing, autobuoys, Continuous Plankton Recorder, etc.) have been designed and optimized essentially for organisms that are traditionally labelled as “phytoplankton” or “zooplankton” (both groups of which we now appreciate include mixoplankton), and typically for species that are nearest the surface, physically robust and relatively large. Many of these approaches are best suited to organisms with clear physiological traits (pigment signatures in cyanobacteria, reflective signatures in coccolithophorids, high structural integrity in diatoms). Many mixoplankton (flagellates and ciliates) are small, not structurally robust or easily sampled, nor amenable to identification. This means that suitable cross calibration with presence/absence of other plankton to glean some information. More profound, perhaps, is the need to detach a signal for chlorophyll from an automatic and exclusive association with primary production supported by inorganic nutrient acquisition, when in mixoplankton that same Chl signal is directly linked to primary production coupled to secondary production.

The paucity of suitably trained researchers who can readily identify mixoplankton not only hinders survey work directly, but will likely restrict the ground truthing of other technologies. Crucially this includes checking the veracity of molecular methodologies such as metabarcoding (who is there?) and metatranscriptomics (what might they be doing?); see discussion in Leles et al. (2019) and Faure et al. (2019). The challenge of exploiting molecular methods becomes even greater when one considers the presence of non-self genetic material within mixoplankton, as nucleic acids from their prey, in acquired plastids and/or in symbionts; more single-cell analyses are needed, including single-cell polymerase chain reaction (PCR) and genomic studies (Tai et al., 2013; Kolisko et al., 2014). Beisner et al. (2019) gives a review of the current states of the art in this subject arena, specifically for nano-sized mixoplankton. Molecular approaches have contributed much to elucidating patterns of mixotrophy in plankton (Burns et al., 2015; McKie-Krisberg et al., 2018; Faure et al., 2019). Nonetheless, “old fashioned” techniques, such as optical and electron microscopy, also have important continuing roles and have been, and still are, frequently deployed in research on mixoplankton (Anderson, 1978; Swanberg and Caron, 1991; Mafra et al., 2016; Kim et al., 2017).
Experimental work

Knowing what is there, is one thing. Knowing what it is actually doing there and at what rate is more challenging, and arguably more important (Flynn et al., 2018). Marine scientists have spent decades developing and deploying field and laboratory techniques for measuring primary production (photosynthesis, nutrient uptake) and, with markedly lesser success (Mitra et al., 2014a), for grazing activities and secondary production by zooplankton. Researchers have studied laboratory cultures with emphasis on using axenic strains, and most frequently using strains that have been maintained in culture for decades in high inorganic nutrient, constant temperature/light environments (and thus have likely evolved, e.g. Martins et al. 2004). These approaches, although undertaken for perfectly sound reasons under the traditional paradigm of considering these phototrophic protists as phytoplankton, can now be seen as simplified too far. Our “laboratory rats”, as single strain isolates, have indeed not represented the true physiology of natural populations, which consists of a high heterogenic diversity, but for reasons far beyond just adapting to constant-temperature room conditions (e.g. Kremp et al., 2012; Alpermann et al., 2010; Brandenburg et al., 2018).

Simply determining whether a mixoplankton is operating at that instant as a photo- and phago-mixotroph, and what its rate of grazing is upon what prey organisms, presents a profound challenge that few researchers and laboratories are well placed to even attempt to confront. We need to know those rates of photosynthesis, grazing, nutrient regeneration, respiration, etc.; without such data we cannot verify the performance of simulation models. To complicate things further, we need similar data for their prey species as well (which could also be mixoplankton), growing in the same water at the same time. There are very few studies of mixoplankton physiology that are adequately executed to provide data describing the dynamics of growth and trophic activity (e.g. Skovgaard et al., 2000, 2003), and even fewer provide the necessary information on both the mixoplankton and its prey (Adolf et al., 2003, Lin et al., 2018); see discussion in Flynn and Mitra (2009). Simply catching these organisms in the act of eating is a problem (Anderson et al., 2017). Consider a nano-mixoplankton eating bacteria; it may only need to eat one bacterium a day to acquire its P or Fe quota. Did we observe that event in our shipboard incubation? Do we know whether the time of day is important for the event? Is the prey presented for possible consumption in an experiment an appropriate species, and in the correct nutritional state?

Training in phytoplankton physiological ecology was perhaps at its zenith during 1970–1980. Since the emergence of molecular biology, however, the literature has seen far fewer papers reporting all-engrossing experiments reporting time series incubations and cultures growth dynamics, etc. Molecular biology and genomics have played a pivotal role in elucidating the evolution of phagotrophy and eukaryogenesis, which is strongly linked to functionality of mixoplankton (Burns et al., 2015). Those approaches were particularly useful in revealing features of the trophic interactions between NCMS (Takishita et al., 2002). Emphasis has been placed upon who is there (at best only semi-quantitatively), and determining the scope for action (as implied from DNA and RNA data). Even meta-transcriptomic approaches can only indicate the potential contribution of genetic functionality at community level rather than the actual rate of action (Stewart et al., 2012; Wohlrab et al., 2018). The absence of genetic capability indicates an absence of functional ability; molecular biology can at best indicate potential capability for rate activity, but it (neither DNA sequences data, nor RNA data) cannot provide the actual rates. And will molecular biology be able to discriminate between a bacterial–mixoplankton interaction where the bacteria are external to the protist (as a contaminant or part of its associated microbiome) rather than internal having been eaten? There are many potential sources of conflicting signals even in cultured organisms. For example, membrane production and (auto)digestion processes occur in cells that are not phagocytic. So, to what extent molecular methods can provide help in establishing rate processes and relative contributions of photo- versus osmo- versus phagotrophy in mixoplankton is far from clear at present, though various studies have explored the topic (Santoferrara et al., 2014; McKie-Krisberg et al., 2018).

In short, there is much to do in the laboratory and in the field, and that applies whether these studies also involve reappraisals of species formally labelled as just phytoplankton or protozooplankton, or as prey, as competitors, or as predators. Critically, while phytoplankton are amenable to study in axenic conditions, or under conditions in which autecology dominates, studies of mixoplankton require a simultaneous study of their prey. This greatly complicates matters, generating problems comparable if not more complex, to those encountered in studies of zooplankton (Mitra et al., 2014a).

Conceptual and simulation models

Survey and fieldwork, as well as experimental studies, are very important for providing information needed to support successful conceptual and numerical modelling of plankton ecology; they provide data and understanding to aid the construction and validation of the models. The added value of models, especially of numerical
simulation models, is the scope for their use to explore cause-and-effect questions, such as why and how are the pathways in trophic dynamics as they are. What is apparent is that modelled mixoplankton activity has clear scope to radically alter simulated trophic dynamics and biogeochemical flows (Flynn and Mitra, 2009; Mitra et al. 2014b, 2016; Ward and Follows, 2016; Ghyoot et al., 2017b; Leles et al., 2018). The question is how closely those projections simulate reality, or whether they simply reflect the conceptual basis of the mixoplankton description. So, do we understand the physiology and functional roles of mixoplankton well enough to construct robust models to test hypotheses?

Mixotrophy in plankton has stimulated a curiosity-driven exploration of simulated ecology (Jost et al., 2004; Hammer and Pitchford, 2005). Mixotrophy in such models has often been photo- and osmotrophic, rather than photo-, osmo- and phagotrophic. Modelled mixotrophs have often had traits that have been “traded” to prevent them from becoming superior and dominating over their pure phototrophic or heterotrophic counterparts (Jost et al., 2004; Ward et al., 2011). The rationale behind TTO models describing phytoplankton activity (Bruggeman and Kooijman, 2007; Follows et al., 2007; Litchman and Klausmeier, 2008; Smith et al., 2016) comes under a different spotlight if we consider that these organisms are also predators. Factors that appear of benefit for phytoplankton (e.g. small size favouring nutrient acquisition) or for consumers (e.g. larger size than the prey, and to minimize predation), and the relative importance of bottom-up and top-down factors become confused when we consider mixoplankton ecology and physiology. Then there are also the arguments against TTO approaches mentioned above. For modellers who wish to exploit TTO concepts for pragmatic computational reasons, at present we simply do not have sufficient empirical evidence from which to construct robust TTO arguments.

Other approaches (Flynn and Mitra, 2009; Ghyoot et al., 2017a) have specifically sought to align model construction with the functional type descriptions that we observe (Mitra et al., 2016). It is important that we recognize the importance of different functional forms of mixoplankton (Fig. 2). We need to do so at least for the forms that are most frequently dominant in terms of biomass (i.e. CM vs GNCM), though pSNCM and eSNCM forms are also important groups in terms of HABs and biogeochemistry. All mixoplankton types appear to be of significance across large areas of the oceans (Leles et al., 2017, 2019). How does such knowledge shape a reappraisal of works considered as exemplars for global-scale plankton modelling, such as the DARWIN model approach of Follows et al. (2007), which sought to describe global “phytoplankton” productivities according to standard phototrophic trait approaches, dominated by bottom-up controls? Equally problematic, is that plankton models that do not describe mixotrophic activity have had their skill considered with reference to data collected for natural populations that will most likely have included, on occasion, significant mixoplankton presence and activity.

Over the last decade, plankton simulation science has gone from the extreme of ignoring mixotrophy, to the opposite extreme of assuming that all plankton are mixotrophic (Ward and Follows, 2016). Ward and Follows (2016) deployed an allometric-linked sliding scale of mixoplanktonic capability. However, as noted above, far from being confined as an optimal strategy for mid-sized protists (Andersen et al., 2016), significant levels of photophag-mixotrophy operates in some of the smallest phototrophic flagellates (Hartmann et al., 2013; Hansen and Hjorth, 2002; Anderson et al., 2018), through 1 mm diameter green Noctiluca (Hansen et al., 2004; Gomes et al., 2018), to the very largest colonial Radiolaria (Collodaria; Swanberg, 1983; Biard et al., 2016). There is a clear difference in the alignment of allometry and different mixoplankton functional types as well (Fig. 3). We should perhaps guard against developing and exploiting simple explanations that may lead to premature conclusions.

For sure, the modelling of protist plankton activities requires a reappraisal. We need computationally efficient models that describe these different organisms (Fig. 2), in a fashion that is acceptable in the eyes of those scientists who study the physiology and ecology of mixoplankton, before we place them in ecosystem simulators. To do otherwise, to describe theoretical mixoplankton forms that do not simulate the behaviour of real organisms, is surely at least as questionable as to continue to ignore the existence of mixoplankton.

Conceptual models of planktonic ecology also extend far beyond the form indicated in Fig. 4, to more overarching concepts, which also warrant a revisit under the mixoplankton paradigm. Thus, we can reconsider the underpinning of Margalef’s mandala (Margalef, 1978) and perhaps even revisit the conceptual basis of Longhurst (2007) provinces. When working on Leles et al. (2017, 2019) we sought to place biogeographies of CM and NCM protists within the Longhurst province descriptions aware that what we were doing was using a structure built in the era of the traditional paradigm, and allied concepts, to attempt to explain the biogeography of very different functional groups. It may come to pass that a merging of a mixoplanktonic centric revision of Margalef’s mandala (Gilbert, 2016) and the Longhurst-style provinces will see a reforming of biogeographic interpretations to provide an enhanced holistic understanding of ocean life.

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CONCLUSION

There is clearly much work to do across the entire gamut of plankton research in the context of mixoplankton. It is possible that our holistic view of how planet-scale processes operate may ultimately be little changed. Maybe, to borrow from Williams (1984) commentary upon how the microbial loop was not impacting the science of plankton ecology as much as one may have expected, the mixoplankton paradigm will prove to be just another of the “Emperor’s New Suit of Clothes” (i.e. de facto invisible). The microbial loop is still not included commonly in extant plankton models. While mixoplankton are ubiquitous, and we will eventually understand better how their food web operates, perhaps ultimately it is just a wheel spinning inside other wheels of greater consequence, with microbial primary, secondary and bacterial productions being so integrated that we would be better considering them as a whole (Flynn, 1988).

On the other hand, perhaps the mixoplankton paradigm will bring into sharper relief that the microbial loop and the allied bacterial food web, with its role in the microbial carbon pump generating refractory dissolved organic matter (DOM) (Jiao et al., 2010; Lechtenfeld et al., 2014), is indeed collectively worthy of more detailed inclusion in models. The important role of bacteria-mixoplankton interactions in oligotrophic systems is clear (Hartmann et al., 2013; Mitra et al., 2014b). By similar arguments, maybe our concepts for the management of HABs will need to be reappraised, recognizing the importance of alternative trophic interactions (including the needs of some species for certain prey) in addition to the primarily bottom-up light/inorganic-nutrient processes that are currently focussed upon (Glibert et al., 2018; Shumway et al., 2018).

If such events come to pass, then the mixoplankton paradigm will develop into more than just recognizing the importance of photo- and phago-mixotrophic protist plankton. We are only going to find out if we look, and we can only attain that goal by combining the different field of research in plankton ecology in a multidisciplinary approach. We will need a combination of laboratory and field rate determinations, biochemistry (stoichiometry) and molecular approaches, new methodologies for field and survey work and new models to test the mixoplankton paradigm. And, of course, we need to train the next generation of scientists to deliver this.

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