Journal of
Plankton Research

academic.oup.com/plankt

J. Plankton Res. (2022) 44(4): 508–521. First published online July 5, 2022 https://doi.org/10.1093/plankt/fbac035

ORIGINAL ARTICLE

Phytoplankton phagotrophy across nutrients and light gradients using different measurement techniques

MARIANA R. A. COSTA[1,*], **HUGO SARMENTO**[2], **VANESSA BECKER**[1,3], **INESSA L. BAGATINI**[4] **AND FERNANDO UNREIN**[5]

[1]PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA, DEPARTAMENTO DE ECOLOGIA, UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE (UFRN), CAMPUS UNIVERSITÁRIO LAGOA NOVA, NATAL, RN 59078-900, BRAZIL, [2]DEPARTAMENTO DE HIDROBIOLOGIA, UNIVERSIDADE FEDERAL DE SÃO CARLOS (UFSCAR), SÃO CARLOS, SP, BRAZIL, [3]DEPARTAMENTO DE ENGENHARIA CIVIL, UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE (UFRN), NATAL, RN, BRAZIL, [4]DEPARTAMENTO DE BOTÂNICA, UNIVERSIDADE FEDERAL DE SÃO CARLOS (UFSCAR), SÃO CARLOS, SP, BRAZIL AND [5]INSTITUTO TECNOLÓGICO DE CHASCOMÚS (INTECH), UNSAM-CONICET, AV. INTENDENTE MARINO 8,200 KM, CHASCOMÚS, BUENOS AIRES 7130, ARGENTINA

*CORRESPONDING AUTHOR: costamra@gmail.com

Received October 15, 2021; editorial decision June 8, 2022; accepted June 9, 2022

Corresponding editor: Beatrix E. Beisner

Mixotrophy is important to ecosystems functioning. Assuming that limiting resources induce phagotrophy in mixotrophs, we used a factorial experimental design to evaluate how nutrient and light affects phagotrophy in two mixotrophic phytoflagellates belonging to different lineages. We estimated cell-specific grazing rates (CSGR) by analyzing prey ingestion using microscopy and flow cytometry (FC). Furthermore, we tested if the acidotropic probe LysoTracker green (LyTG) can be used to differentiate autotrophs from mixotrophs. *Cryptomonas marssonii* (cryptophyte) had higher CSGR in high-nutrient treatments. Although it seems counterintuitive, phytoflagellates likely uses phagotrophy to obtain organic growth factors instead of inorganic nutrients when photosynthesis is more favorable. In contrast, CSGR in *Ochromonas tuberculata* (chrysophyte) increased when light decreased, suggesting that it uses phagotrophy to supplement carbon when autotrophic growth conditions are suboptimal. Measurements of CSGR obtained by FC and microscopy were significantly correlated and displayed the same trend among treatments, although FC rates tended to be higher. Fluorescence with LyTG did not differ from the control in the non-phagotrophic chlorophyte. Contrarily, addition of LyTG significantly increased the fluorescence in chrysophytes and cryptophytes, although no differences were observed among treatments. This approach allowed for differentiation between phagotrophic and non-phagotrophic flagellates but failed to quantify mixotrophy.

KEYWORDS: cryptophyte; chrysophyte; mixotrophy; grazing rates; flow cytometry; LysoTracker green; fluorescent-labeled prey

available online at academic.oup.com/plankt

INTRODUCTION

Interactions among aquatic microorganisms play an important role in the biogeochemical cycle and account for large amounts of carbon transfer among trophic levels (Azam *et al.*, 1983). Mixotrophic organisms, defined here as the combination of photosynthesis and particle grazing (*sensu* Sanders, 1991), act both as producers and consumers in aquatic ecosystems, shaping food-web structure and ecosystem functioning (Stoecker *et al.*, 2017). Mixotrophy in the plankton is now recognized to be widespread in marine and freshwater ecosystems (Unrein *et al.*, 2007; Flynn *et al.*, 2013; Stoecker *et al.*, 2017). Numerous field studies have documented that mixotrophic phytoflagellates can account for, on average, half of the total bacterivory in the sea (Unrein *et al.*, 2007; Hartmann *et al.*, 2012), and also in many freshwater systems (Urabe *et al.*, 2000; Gerea *et al.*, 2018).

Several phytoflagellate groups such as Chrysophyceae, Cryptophyta, Haptophyta and Dinoflagellate, among others include mixotrophic taxa (Carvalho and Granéli, 2010; Gast *et al.*, 2014; Unrein *et al.*, 2014; Fischer *et al.*, 2017). In particular, much of what is known about mixotrophic phytoflagellates comes from studies with chrysophytes that include several photosynthetic species capable of phagotrophy (Caron *et al.*, 1990; Jones and Rees, 1994; Flöder *et al.*, 2006). Pascher (1943) described the uptake of small particles by *Dinobryon*, whereas Bird and Kalf (1989) reported that *Dinobryon* and *Ochromonas* assemblages obtained 79% of their total carbon from phagotrophy. Facultative mixotrophy is recognized by its ability to grow by either phototrophy or heterotrophy (Skovgaard, 1996) and is commonly observed in freshwater and marine cryptophyte as well (Tranvik *et al.*, 1989; Unrein *et al.*, 2007; Izaguirre *et al.*, 2012; Ballen-Segura *et al.*, 2017).

Classifying mixotrophic organisms is not a trivial task, but it is useful to address general patterns to understand mixotrophy as an evolutionary strategy (Mansour and Anestis, 2021). Many classification models have been produced over the years and it is clear that there is a continuum of nutritional strategy among protist, from strict heterotrophy to strict phototrophy, with mixotrophs in between (Jones, 1994; Jones and Rees 1994; Flynn *et al.*, 2013). However, there are some known differences among mixotrophic phytoflagellates, where some species are predominantly autotrophs (e.g. cryptophytes) and some are more heterotrophs (e.g. chrysophytes; Stoecker, 1998; Mitra *et al.*, 2016; Stoecker *et al.*, 2017).

The mixoplankton capacity for phagotrophy confers an advantage over strictly phototrophic species. The benefits of ingesting prey vary among species and must be higher than the costs with sufficient energy gain to cells maintenance (Kirchman and Hanson, 2013), this includes obtaining particulate nutrients from prey (Stoecker *et al.*, 2017). Several factors, like the limitation of carbon supply, nitrogen, phosphorous and iron acquisition, as well as light or prey availability, can stimulate phagotrophy in mixoplanktonic species (e.g. Pålsson and Granéli, 2003; Flöder *et al.*, 2006; Caron, 2016). However, the main drivers promoting phagotrophic behavior might differ among algal groups, and even among species, since organisms have different strategies for survival under distinct environmental conditions. In general, grazing rates increase when inorganic nutrients decrease. This trend was observed for several different phytoflagellates (Nygaard and Tobiesen, 1993; Carvalho and Granéli, 2010; McKie-Krisberg *et al.*, 2015, Ptacnik *et al.*, 2016, Millette *et al.*, 2017), and might explain their dominance in oligotrophic environments (Domaizon *et al.*, 2003; Katechakis and Stibor, 2006; Unrein *et al.*, 2007). However, the effect of different light conditions on grazing rates is not as straightforward as for nutrients, since there is no common pattern established for all flagellates (e.g. McKie-Krisberg *et al.*, 2015). The question is: under what conditions do different groups of mixotrophs rely on phagotrophy to supplement autotrophic growth?

Despite the recognized relevance of mixotrophy to ecosystem functioning, it is hard to quantify its effects and contribution to carbon fluxes due to the very limited data on effective grazing rates measurements and our knowledge of which species are actually phagotrophic (Ward and Follows, 2016; Stoecker *et al.*, 2017). The reasons include the technical difficulties of measuring ingestion rates and the contribution of phagotrophy to the metabolism of different mixotrophs (Beisner *et al.*, 2019).

A technique frequently used to quantify grazing is counting ingested fluorescent particles using epifluorescence microscopy (Unrein *et al.*, 2007; McKie-Krisberg *et al.*, 2015; Gerea *et al.*, 2018). However, it requires long sample processing time and qualified training. Therefore, faster and more automated methods that allow the achievement of good quantification and accuracy would be useful. A suitable alternative for estimating ingestion rates could be the use of flow cytometry (FC) to detect fluorescent-labeled prey ingested by protists (Keller *et al.*, 1994). FC, which can quantify thousands of cells in a few minutes (Shapiro, 1995), has been routinely used in microbial ecology to estimate the abundance of several distinct types of aquatic organisms (Gasol and del Giorgio 2000; Sarmento *et al.*, 2008; Schiaffino *et al.*, 2013). It acquires single-cell information such as size and complexity, as well as fluorescence emitted by natural pigments or

fluorescent probes (Gasol and Morán, 2015). Acidotropic probes that stain digestive vacuoles have been used to quantify heterotrophic nanoflagellates with FC in laboratory cultures (Rose *et al.*, 2004; Carvalho and Granéli, 2006, Sintes and del Giorgio, 2010) in natural samples (Rose *et al.*, 2004), and more recently, to identify phytoflagellates phagotrophy in natural samples (Anderson *et al.*, 2017; Sato and Hashihama, 2019).

The aim of our study was to evaluate how nutrient and light combined affect grazing rates in two freshwater mixotrophic phytoflagellates of different lineages. We compared the phagotrophic activity of a cryptophyte and a chrysophyte in a full factorial experimental design, combining three level of nutrients and three levels of light. We evaluated grazing activity with different approaches: FC and epifluorescence microscopy counts as measurement techniques to estimate grazing rates in short-term ingestion experiments of fluorescent-labeled prey, and LysoTracker Green (LyTG) probe and FC to evaluate the presence of digestive vacuoles. We hypothesized that light and nutrient limitation induce the phagotrophic activity resulting in higher cell-specific grazing rate (CSGR) in short-term ingestion experiments, and also hypothesized that higher grazing activity would result in higher fluorescence signal, meaning that LyTG could be used as a quantitative method.

MATERIALS AND METHODS

Phytoplankton culture and growth conditions

Cultures of three freshwater non-axenic strains of phytoflagellates were used for the experiments. The chrysophyte *Ochromonas tuberculata* (CCAP 933/27) and the cryptophyte *Cryptomonas marssonii* (CCAP 979/64) were obtained from the Algae and Protozoa Culture Collection (www.ccap.ac.uk). Both strains have been reported in the literature as having mixotrophic metabolism (Katechakis and Stibor 2006; Izaguirre *et al.*, 2012). In addition, a strict photosynthetic chlorophyte (*Chlamydomonas* sp.) from our private collection was used as negative control. The cultures were grown and maintained in sterile WC medium (Guillard and Lorenzen, 1972) at 20 ± 1°C, and 50 μmol of photons m^{-2} s^{-1} irradiance, under a light:dark cycle of 12:12 h. They were kept in an exponential growth phase by diluting biweekly into a fresh medium. The strains grew as flagellated spheroid single cells; the mean dimension of *O. tuberculata* was 8.1 × 7.2 μm (biovolume of 219.9 μm^3), *C. marssonii* was 9.4 × 8.0 μm (315 μm^3) and *Chlamydomonas* sp. was 9.1 × 8.2 μm (480.57 μm^3). The abundance of background bacteria (mainly small bacillus) in phytoplankton cultures, ranged from 2 to 5 × 10^6 cells mL^{-1}. A total of 250 mL of a dense phytoflagellate culture (5 × 10^5 cells mL^{-1}) were used as inoculum in the experiments.

Experimental design

Two kinds of experiments were carried out: (i) a short-term ingestion experiment with a fluorescent surrogate prey to calculate ingestion rates, and (ii) an experiment to detect mixotrophic capability using a food vacuole fluorescent stain.

In both cases, experiments consisted in a 3 × 3 factorial design. We manipulated three levels of light: 95 μmol m^{-2} s^{-1} that we referred to as high light (HL), 10 μmol m^{-2} s^{-1} as low light (LL) and dark condition (D), which was obtained by wrapping the flasks with aluminum foil; as well as three concentrations of nutrients: high nutrients (HN), medium nutrients (MN) and low nutrients (LN). We diluted the WC medium 20 times with deionized water for LN (final concentration 50 μM of N and 2.5 μM of P), five times for MN (final concentration 200 μM of N and 10 μM of P), whereas HN was not diluted (final concentration 1000 μM of N and 50 μM of P). All treatments were performed in triplicates.

To induce mixotrophy in phytoflagellates we acclimated the two species (*C. marssonii* and *O. tuberculata*) before starting the experiments. A volume of 50 mL of exponential-phase culture strain was added to 150 mL of HN, MN and LN culture medium (prepared as explained above). After 5 days of incubation at 20 ± 1°C and 50 μmol m^{-2} s^{-1}, each culture was split in three and further incubated in three different light conditions (HL, LL and D) for 2 more days, resulting in a combination of nine different treatments. In order to check for possible negative effects on the physiology of the cells during the acclimatization period, we used FC data at T0 (after the acclimatization) to estimate the percentage of "healthy" and "unhealthy" cells before starting the experiment. Those cells with the typical SSC-H signal but with a much lower FL3-H (chlorophyll-*a*) were considered "unhealthy" cells. Detailed information on this procedure is provided as supplementary material (Supplementary Fig. S1, see online supplementary data for a color version of this figure and Supplementary Table S1). Regardless of the conditions of light and nutrients for acclimatization, in all the treatments at T0 most cells were in "healthy" condition (on average 96% for *C. marssonii* and 81% for *O. tuberculata*). Estimations of CSGR were always performed considering this fraction. Preliminary tests with *C. marssonii* and *O. tuberculata* (data not shown) were carried out before starting the experiments to confirm that the phytoflagellates were able to ingest fluorescent

Table I: Comparison of mean CSGR, standard deviation (SD) measured by two methodologies: epifluorescence microscopy and FC and phytoflagellates abundance before the beginning of the experiments

		Epifluorescence microscopy	Flow cytometry	
Treatment	**Phytoflagellate**	**CSGR (bact. ind.$^{-1}$ h^{-1}) and SD**	**CSGR (bact. ind.$^{-1}$ h^{-1}) and SD**	**Mean of phytoflagellates abundance in initial time (cels.mL $^{-1}$)**
HN + HL	*Ochromonas*	0.67 (0.25)	1.85 (0.11)	1.87E+04
HN + LL		0.55 (0.19)	2.70 (0.51)	3.07E+04
HN + D		1.05 (0.41)	3.10 (0.69)	2.04E+04
MN + HL		0.29 (0.21)	1.24 (0.51)	2.69E+04
MN + LL		1.10 (0.85)	1.21 (0.41)	5.19E+04
MN + D		1.64 (1.54)	2.57 (1.39)	3.28E+04
LN + HL		0.63 (0.40)	0.74 (0.10)	3.24E+04
LN + LL		0.84 (0.28)	2.48 (0.98)	3.63E+04
LN + D		0.98 (0.36)	3.76 (2.02)	7.20E+04
HN + HL	*Cryptomonas*	4.58 (2.24)	26.85 (6.34)	2.57E+04
HN + LL		3.43 (0.43)	20.15 (0.73)	1.66E+04
HN + D		4.93 (1.85)	21.53 (3.54)	1.37E+04
MN + HL		3.31 (1.26)	1.24 (0.15)	1.26E+04
MN + LL		1.53 (0.91)	2.80 (2.51)	1.13E+04
MN + D		2.29 (0.70)	4.74 (0.22)	2.09E+04
LN + HL		2.74 (0.64)	4.69 (0.72)	2.07E+04
LN + LL		1.39 (0.49)	8.26 (6.8)	2.44E+04
LN + D		2.53 (1.66)	1.67 (0.54)	3.58E+04

Note: Treatments are high nutrient (HN), medium nutrient (MN), low nutrient (LN), high light (HL), low light (LL) and dark (D).

prey. The abundance of *O. tuberculata* at the beginning of the experiments ranged between 1.9 and 7.2 × 10^4 cells mL $^{-1}$ (avg. 3.6 × 10^4 cells mL $^{-1}$), and *C. marssonii* ranged between 1.1 and 3.6 × 10^4 cells mL $^{-1}$ (avg. 2 × 10^4 cells mL $^{-1}$; Table I).

Short-term ingestion experiment

Experiments were performed using the mixotrophic strains of *C. marssonii* and *O. tuberculata*. Fluorescent yellow–green latex beads (1-μm diameter, Fluoresbrite, Polysciences) were used as surrogate prey. A stock bead solution was treated with bovine serum albumin (0.5 mg mL^{-1}) to reduce particle aggregation (Pace and Bailif, 1987).

Besides beads of 1 μm, we also tested beads of 0.5 μm and heat-killed fluorescently labeled bacteria (FLB), prepared as in Unrein *et al.* (Unrein *et al.*, 2007) using a strain of *Brevundimonas diminuta* (ca. 0.065 μm^3) stained with 5-([4,6 dichlorotriazin-2-yl]-amino)-fluorescein (DTAF), final concentration 100 pg mL^{-1}. Unfortunately, the green fluorescence signal of surrogate prey inside food vacuoles could not be observed properly in FC, therefore only beads of 1 μm were further considered. Short-term ingestion experiments were carried out in triplicate 10-mL flasks. A working solution of surrogate prey was added to a concentration ~30% of the background bacterial concentration for each experiment. Samples were taken at initial time and after 45 min. The incubation time was determined based on preliminary tests that confirmed that surrogate prey ingestion remains linear at least during the first 45 min (Supplementary Fig. S2, see online supplementary data for a color version of this figure).

Bead ingestion rates were estimated by two different approaches: using (i) epifluorescence microscope counts, and (ii) FC. For each method, a 1 mL of sample was taken at each sampling time. Samples for epifluorescence microscopy were fixed with filtered (0.2 μm) glutaraldehyde (1% final concentration) and stored at 4°C until processing within the 24 h of fixation. Samples for FC were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde fixation solution (final concentration). Fixed samples were kept in the dark at room temperature for 10 min, flash-frozen in liquid nitrogen and stored at −80°C until analysis.

For epifluorescence microscopy, 1-mL samples were filtered through 0.8-μm black polycarbonate membranes (Maine Manufacturing, LLC) and stained for 5 min with a solution of 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 10 μg mL^{-1}, following Porter and Feig (Porter and Feig, 1980). At least 100 flagellates were counted under 1000× magnification using a Zeiss epifluorescence microscope equipped with an HBO 50 W lamp, and a filter set for blue light, green light and UV excitation. For each phytoflagellate we counted the number of surrogate prey inside food vacuoles (e.g. beads flagellates^{-1}).

Phytoflagellates, natural bacteria present in cultures and surrogate prey were counted using a flow cytometer

(FACSCalibur, Becton Dickinson) equipped with a 15-mW argon-ion blue laser (488-nm excitation), at least 20 000 events were acquired in each sample. Natural heterotrophic bacteria stained as described below and surrogate prey (i.e. yellow–green fluorescent latex beads) were detected by their signature in a plot of green fluorescence (FL1-H, 530 ± 15 nm) under blue light excitation (488 nm) versus side scatter (SSC-H, 488 ± 5 nm emission), at a flow rate of 103.6 (μL min^{-1}) for 1 min. For heterotrophic bacteria, a sample of 400 μL was stained with 4 μL of a working solution of SybrGreen I (Molecular Probes Inc., Eugene, OR, USA), diluted 1:100 with DMSO (10^{-4} final concentration from commercial stock), left for ∼10 min in the dark to complete the staining and run in the flow cytometer (Gasol and del Giorgio, 2000). Phytoflagellates were detected on unstained subsamples by their signature in a plot of red chlorophyll-*a* fluorescence (FL3-H, >670-nm emission) under blue light excitation (488 nm) versus SSC-H (Fig. 1A). Samples were run at a flow rate of 103.6 (μL min^{-1}) for 3 min. Gated phytoflagellates were further analyzed in a plot of red (FL3-H) versus green (FL1-H) fluorescence to detect phytoflagellates with and without beads (Fig. 1B). Finally, phytoflagellates with ingested beads were then plotted in a histogram of green fluorescence (FL1-H), where the first peak corresponded to phytoflagellates with one beads inside, the second peak phytoflagellates with two beads inside, and successively (Fig. 1C).

Phytoflagellate CSGR expressed in bacteria ind.$^{-1}$. h^{-1} were estimated through the uptake of fluorescent beads by each phytoflagellate and were calculated as:

$$\mathrm{CSGR} = \frac{(\mathrm{If} - \mathrm{Ii}) \times \left(\frac{\mathrm{HB}}{\mathrm{Beads}}\right)}{t}$$

where "If" and "Ii" are the number of ingested beads per flagellate at the final and initial incubation time, respectively, "HB" is the abundance of background populations of heterotrophic bacteria, "Beads" is the total abundance of inoculated surrogated prey and "*t*" is the incubation time in hours. The CSGR were calculated assuming that native bacteria and surrogated prey were grazed upon at the same rates.

Food vacuoles staining experiment

Phagotrophy by the phytoflagellates was determined using FC and the acidotropic probe LysoTracker Green DND-26 (LyTG; ThermoFisher Scientific), following the protocol by Sintes and del Giorgio (Sintes and del Giorgio, 2010). LyTG is a green fluorescent dye that stains acidotropic compartments of living cells and detects the presence of digestive vacuoles, with a maximum excitation/emission wavelengths of 504/511 nm (Sintes and del Giorgio, 2010; Anderson *et al.*, 2017). Thus, phytoflagellate with LyTG and chlorophyll-*a* fluorescence were considered to be mixotrophs (Anderson *et al.*, 2017; Sato and Hashihama, 2019).

We performed the same 3 × 3 factorial experimental design described above, manipulating three levels of light (HL, LL and D) and three levels of nutrients concentration (HN, MN and LN). Three species of phytoflagellates were tested: two mixotrophic strains (*C. marssonii* and *O. tuberculata*) and one non-phagotrophic strain (*Chlamydomonas* sp.) that were considered as the negative control. All experiments were duplicated.

FC was used to compare each culture, acclimated under different light and nutrient conditions, with and without LyTG addition. To do so, we first run 2 mL of live samples for 5 min using a flow rate of 103.6 μL min^{-1} in the dark. Then we added 10 μL of LyTG working solution (10 μM) in the same tube (final concentration 50 nM) and run for another 5 min (Sintes and del Giorgio, 2010). At least 10 000 events were acquired in each sample. The 90° side-scatter (SSC-H), green fluorescence (FL1-H) and red fluorescence (FL3-H) were obtained for each particle. The same settings were used for all experiments.

We analyzed algae populations by their signature in a plot of FL3-H versus SSC-H (Fig. 2A). Particles without chlorophyll-*a* fluorescence were excluded. The gated population was further analyzed in a FL3-H versus FL1-H biplot. To determine the percentage of cells with labeled food vacuoles, we ran a sample with no added LyTG and determined a FL-1 baseline (Fig. 2A). Cells that exhibited a higher FL-1 after incubation with LyTG (Fig. 2B) were considered positives (i.e. with labeled food vacuoles), and their percentage with respect to the total abundance was calculated for each treatment. The median FL1-H measured before LyTG addition was subtracted from that measured after the LyTG addition.

Data analyses

All statistical analyses were done in R Statistical Software (version 3.5.2, R <www.r-project.org>; R Core Team, 2020). Two-way ANOVA and Tukey's post-hoc test were performed to test differences in CSGR between treatments (light and nutrients) for each experiment, using, respectively, the functions "aov" and "TukeyHSD" from the package "stats" (R Core Team 2020). The CSGR obtained from ingestion experiments with either FC or microscopy epifluorescence were compared with Linear type-II regression using the "lmodel2" function and major axis (MA) method of the package "lmodel2" (Legendre 2018). A *P*-value below 0.05 was consider significant. Prior to each analysis, the data were tested for normality

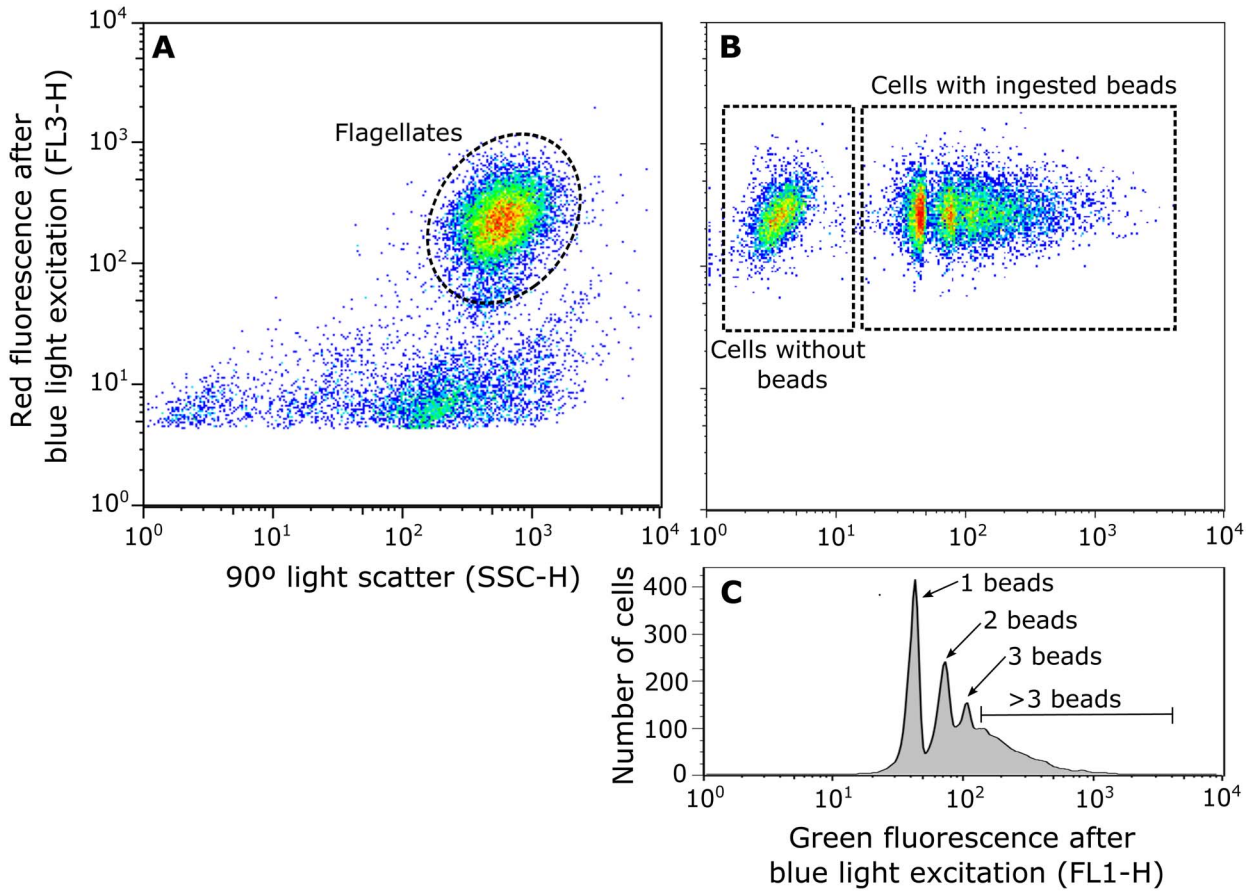

Fig 1. Examples of cytograms of ingestion experiments with *Ochromonas tuberculata*. (**A**) Dotplot of FL3-H (red fluorescence of chlorophyll-*a*) versus 90° side-scatter (SSC-H) showing phytoflagellate population. (**B**) Dotplot of the gated phytoflagellate population showing cells with and without ingested beads. (**C**) Histogram showing FL1-H (green fluorescence) of phytoflagellate cells with ingested bead counts; arrows indicate cells with one bead, two beads, three beads and more than three beads ingested.

and homoscedasticity. Natural log transformation was made when data were not normally distributed.

RESULTS

Short-term ingestion experiments

In general, CSGR of *C. marssonii* were higher than *O. tuberculata*. For *C. marssonii,* the CSGR were significantly higher in HN compared to MN and LN, when estimated by both methods (Fig. 3, Table I and Supplementary Table S3). Significant difference in light treatments was detected only by epifluorescence, i.e. LL was significantly lower than HL and D was not significantly different from HL and LL (Fig. 3, Table I and Supplementary Table S3). Significant interaction among light and nutrients was detected only by FC (see statistical results in Supplementary Table S3).

In the experiment with *O. tuberculata*, CSGR systematically increased when light intensity decreased, regardless of the nutrient concentration (Fig. 3 and Table I). However, differences between LL/D and HL were only significant when CSGR were measured by FC (i.e. *P*-value for light treatment with epifluorescence microscopy was 0.069, DF 2, F 3.22) (Fig. 3D and Supplementary Table S2). Neither nutrients alone nor the interaction between light and nutrients had a significant effect on the CSGR of *O. tuberculata* (Fig. 3 and Supplementary Table S3).

Results of CSGR measured by FC and microscopy epifluorescence were significantly correlated either when the whole data set was used or when each algal was tested separately (Fig. 4). However, CSGR measured by FC tended to be higher than by epifluorescence microscopy, especially for *C. marssonii*.

Food vacuoles staining experiment

In order to detect potential unspecific binding of the dye, we tested the use of LyTG in a non-phagotrophic

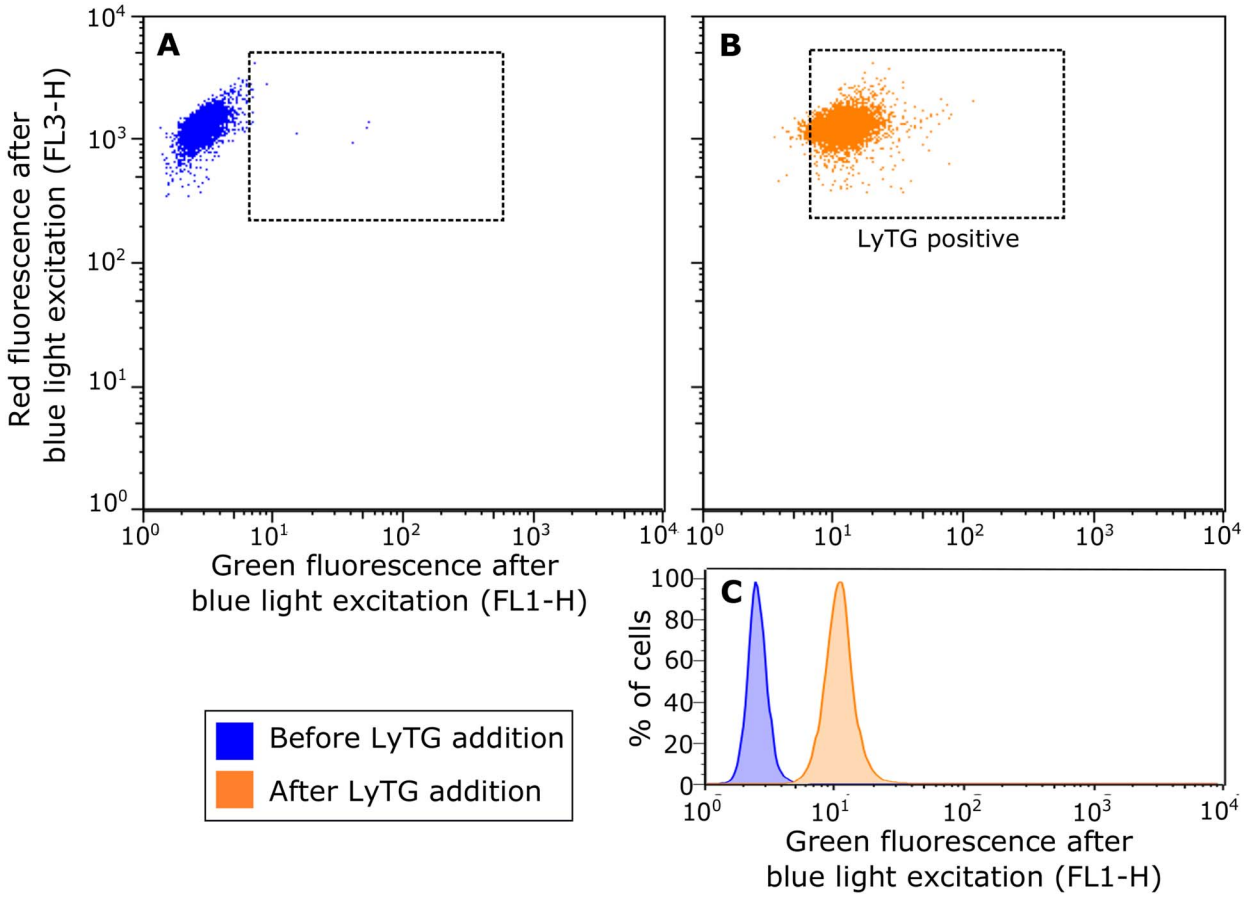

Fig. 2. Examples of cytograms of food vacuole staining experiment with *Ochromonas tuberculata*. (**A**) Dotplot of FL3-H (red fluorescence of chlorophyll-*a*) versus FL1-H (green fluorescence) before LyTG addition showing negative cells, and (**B**) after LyTG addition showing positive cells. (**C**) Overlapped histogram of FL1-H for a mixotrophic strain before and after LyTG addition.

strain of *Chlamydomonas* sp. No measurable LyTG staining could be observed in any treatment. Even though the median of green fluorescence (FL1-H) slightly increased after the addition of LyTG, the overlap of cytograms between samples before and after LyTG addition was close to 100% (Table II), i.e. on average only 1.3% of cells with LyTG staining above the threshold after subtracting blank values (Supplementary Fig. S1a, see online supplementary data for a color version of this figure and Supplementary Table S4). Therefore, there were no cells containing food vacuoles and consequently no feeding activity. Contrarily, in *C. marssonii* and *O. tuberculata*, green fluorescence increased markedly after the addition of LyTG, indicating phagotrophic activity in both cases (Table II). Particularly in *O. tuberculata*, almost all cells were stained after the addition of LyTG, whereas about a quarter of the *C. marssonii* cells were positive. No differences were observed among treatments with different light and nutrient conditions (Supplementary Fig. S1b and c, see online supplementary data for a color version of this figure, Supplementary Table S4).

DISCUSSION

We performed laboratory experiments to evaluate how nutrients and light affect phytoflagellate mixotrophy, estimating grazing rates with epifluorescence microscopy and FC counts, and testing an acidotropic probe to differentiate autotrophs from mixotrophs. We found differences in phagotrophic activity between cryptophyte and chrysophyte strains, with nutrient concentration being the main resource affecting grazing rates for the cryptophyte and light for the chrysophyte. Moreover, the acidotropic probe LyTG was successfully used as a qualitative method to detect mixotrophic nanoflagellates, however, it was not able to quantify phagotrophic activity.

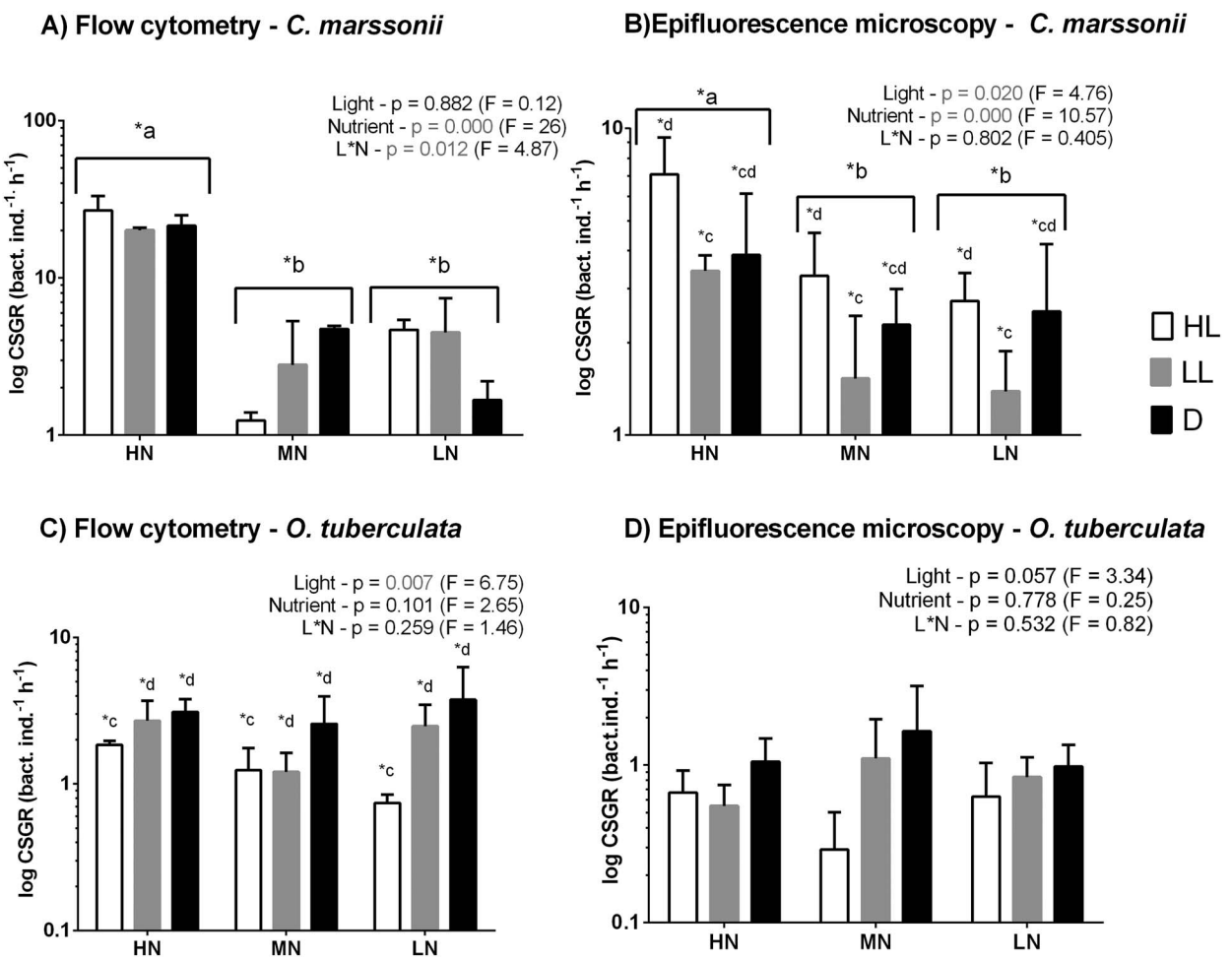

Fig. 3. Cell-specific grazing rates (CSGR, bact.ind-1.h-1) from the short-term grazing experiments for *Cryptomonas marssonii* measured by flow cytometry (**A**) and by epifluorescence microscopy (**B**), and for *Ochromonas tuberculata* measured by flow cytometry (**C**) and by epifluorescence microscopy (**D**). Nutrient treatments: high nutrient (HN), medium nutrient, (MN) and low nutrient (LN). Light treatments: high light (HL), low light (LL) and dark (D). Bars represent the standard deviation ($n = 3$), P is the P-values from the two-way ANOVA, L = light and N = nutrient. Letters "a" and "b" indicate differences by pots-hoc test for the nutrient treatments and letters "c" and "d" indicate differences by the post-hoc test for the light treatments. Significant post-hoc results for L∗N interaction are shown in the Supplementary Table S3.

Table II: Changes in green fluorescence after the addition of Lyso-Tracker Green (LyTG)

	Overlap of cytograms between before and after LyTG addition	% Positives cells	Median of green fluorescence (AU of FL1-H) and SD [a]		Approximated FL1-H increase after the addition of LyTG[b]
			Before LyTG	After LyTG	
***Chlamydomonas* sp.**	98.7%	1.3%	2.13 (0.59)	2.61 (0.46)	1.3 times
Cryptomonas marssonii	75.3%	24.7%	3.46 (0.25)	6.63 (0.70)	2 times
Ochromonas tuberculata	5.9%	94.1%	2.62 (0.45)	12.6 (8.51)	5 times

Note: Summarized results for the three phytoflagellates. Each value represents the average for all treatments and replicates.
[a]Standard deviation (SD) in brackets ($n = 18$). [b]Increase of median green fluorescence (FL1-H).

Cryptophytes are constitutive mixotrophs, although their primary mode of nutrition is phototrophy (Tranvik *et al.*, 1989; Unrein *et al.*, 2014; Mitra *et al.*, 2016). In our experiments we found higher grazing rates for the *C. marssonii* in high-nutrient treatments, measured with both FC and microscope counts, and an interaction between nutrients and light when CSGR was measured by FC. At first, this result could be counterintuitive. If

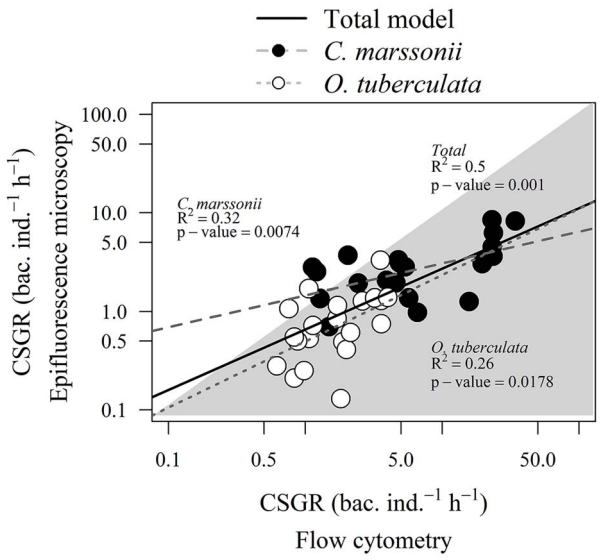

Fig. 4. Type II linear regression comparing cell-specific grazing rates (CSGR) obtained by flow cytometry and epifluorescence microscopy in short-term ingestion experiments with *Cryptomonas marssonii* and *Ochromonas tuberculata*. Solid line is the linear regression for data points of the general total model, dashed lines are the regression model for *C. marssonii* (black dots) and for *O. tuberculata* (white dots). R^2 and *P*-value for each regression model are shown in the graph.

this phytoflagellate is using mixotrophy as an alternative nutrient source, it is not expected that phagotrophy increases with nutrient concentration. Indeed, some *in situ* experiments with cryptophytes observed a negative correlation between grazing rates and nutrients concentration in marine and freshwater systems (Urabe *et al.*, 2000; Unrein *et al.*, 2014; Gerea *et al.*, 2019), suggesting that some cryptophytes uses preferentially inorganic nutrients and rely on phagotrophy when dissolved nutrients are scarce. However, other laboratory experiments with an Antarctic species, showed no effect of nutrient concentration on grazing rates and only a positive response with light-limiting conditions (McKie-Krisberg *et al.*, 2015), whereas *in situ* grazing experiments in a eutrophic floodplain lake showed higher cryptophyte grazing rates in light conditions than in dark conditions (Izaguirre *et al.*, 2012). Consequently, the phagotrophic activity of different cryptophytes as a response to nutrients concentration and light is likely to be species-specific. One possible explanation for our result is that prey would be ingested to obtain organic growth factor rather than essential nutrients, when inorganic dissolved nutrients are sufficient (i.e. HN treatment). Growth factors obtained by phagotrophy may be amino acids (Skovgaard, 2000) or phospholipids (Ishida and Kimura, 1986). This feeding strategy has been long proposed by Stoecker as the Model IIB (Stoecker, 1998) and reinforced as nutritional strategy in mixotrophs in recent simulations (Livanou *et al.*, 2020), and it may explain the high abundance of this species in many nutrient-rich and light-limited ecosystems (Hammer *et al.*, 2002; Izaguirre *et al.*, 2012; Grujcic *et al.*, 2018; Costa *et al.*, 2019).

However, in our experiments it is unlikely that organic factors limited the growth in the HN treatment, as the WC medium contains copious amounts of micronutrients and vitamins. Therefore, our data alone are not sufficient to make this assumption and the reason for this behavior could be related to something other than growth factors. More investigations are needed to understand the driving factors of increased phagotrophy under high-nutrient concentrations.

Ochromonas tuberculata (chrysophyte) grazing rates increased in LL and in dark conditions. The increase in CSGR at decreasing light intensity, regardless of the nutrient concentration, suggests that *O. tuberculata* probably use phagotrophy to supplement carbon or energy when photosynthesis is restricted.

The response of different chrysophytes to light intensity is variable. Although in some cases grazing rate increased at LL intensity (Keller *et al.*, 1994; Holen, 1999; Heinze *et al.*, 2013), in other cases higher light availability promoted phagotrophy (Caron *et al.*, 1993; Jones and Rees 1994). Moreover, some species are able to survive (and grow) in dark conditions when prey concentration is sufficient (Rothhaupt, 1996; Tittel *et al.*, 2003; Pålsson and Daniel, 2004; Wilken *et al.*, 2020), whereas others can grow and survive under limiting light but die in the dark (Keller *et al.*, 1994; Flöder *et al.*, 2006; Wilken *et al.*, 2020). Phagotrophic activity response to light conditions is not straightforward in chrysophytes as it also seems to be linked to prey availability (Rothhaupt, 1996; Palsson and Daniel, 2004; Wilken *et al.*, 2020).

Wilken *et al.* (Wilken *et al.*, 2020) demonstrated that phylogenetically closely related *Ochromonas* strains (belonging to the Ochromonadales-clade) responded differently under distinct light availability and prey densities, evidencing niche differentiation: one was considered an obligate mixotroph (*Ochromonas* CCMP 1393) and the other a facultative mixotroph (*Ochromonas* CCMP 2951). *Ochromonas* is a polyphyletic group (del Campo and Massana, 2021; Groosmann *et al.*, 2016; Wilken *et al.*, 2020). In fact, many strains originally classified as *Ochromonas* are far from the Ochromonadales-clade in phylogenetic trees, and most probably belong to other genera or clades, as is the case of *O. tuberculata* CCAP 933/27 used in our experiments. Here, grazing rate was stimulated under LL conditions (i.e. CSGR increases when light decreases) and was independent of nutrients concentration. Thus, *O. tuberculata* CCAP 933/27 seems to behave more like the *Ochromonas* CCMP 2951 strain

(Wilken *et al.*, 2020), that can replace photosynthesis by phagotrophy and grow heterotrophically in the dark or in limited light conditions; however, from personal observations, the strain used in our experiment is not able to survive during prolonged dark periods.

Estimates of bacterivory by *Ochromonas*-like and *Cryptomonas* spp. in natural communities show a wide range of CSGR, which demonstrates a complex response when different environmental variables are incorporated (Saad *et al.*, 2016; Domaizon *et al.*, 2003, Callieri *et al.*, 2006; Unrein *et al.*, 2014). Our experimental study found comparable values of CSGR with natural systems (Tranvik *et al.*, 1989; Domaizon *et al.*, 2003; Sinistro *et al.*, 2006; Unrein *et al.*, 2014). Saad *et al.* (Saad *et al.*, 2016); sampled different types of environments, from light-limited (turbid, phytoplankton-turbid and humic lakes) and nutrient-limited (oligotrophic lakes), and CSGR ranging from 1.3 to 3.9 bact. ind.$^{-1}$ h^{-1} with higher values by *C. marssonii* in turbid and humic lakes and lower values by *Ochromonas-like* (1.3 and 1.4 bact. ind.$^{-1}$ h^{-1}) in oligotrophic and humic lakes. Sinistro *et al.* (Sinistro *et al.*, 2006) conducted *in situ* grazing experiments under different light conditions, and found higher CSGR for *C. marssonii* (mean 15.4 bact. ind.$^{-1}$ h^{-1}). Therefore, experimental data of feeding by mixotrophic organisms in controlled conditions helps to understand and elucidate information to explore natural systems.

Besides evaluating how nutrients and light affect two species of mixotrophic phytoflagellates, we also aimed to test different measurement techniques in order to evaluate the use of FC as an alternative tool to estimate grazing rates by mixotrophs in short-term ingestion experiments, and comparing it to the standard time-consuming epifluorescence microscopy counts. FC has been used a few times to estimate grazing rates by mixotrophs in uptake experiments with heat-killed FLBs or natural live prey (Cucci *et al.*, 1989; Keller *et al.*, 1993). Bock *et al.* (Bock *et al.*, 2021) used FC to infer phagotrophy in strains of mixotrophic prasinophytes (i.e. only detect positive and negative cells). However, there are no studies to our knowledge comparing both methods of estimating grazing rates.

The methodological cross comparison indicates that FC can be used to detect mixotrophic phytoflagellates with ingested fluorescent beads. We found a similar trend using both techniques, e.g. lower CSGR in *O. tuberculata* was always found in HL treatments regardless of methodology, although in some cases differences were not significant, e.g. light treatments differed significantly when rates were estimated by FC ($P < 0.05$), but resulted insignificantly with microscopy ($P = 0.069$). Also, the absolute values were systematically higher when estimates were performed with FC. We do not have a definitive answer to these discrepancies, but a potential explanation would be the way each method identifies positive cells (i.e. flagellates with ingested beads). Fluorescent prey attached to cells in the FC could be counted as positive cells, whereas microscope observation would allow the differentiation of beads inside from those attached to the cell; this would cause an overestimation when using the FC.

Besides 1-μm beads, we also performed short-term ingestion incubations with two kind of surrogate prey: heat-killed FLBs and beads of 0.5 μm. Keller *et al.* (Keller et al., 1993) successfully counted ingested FLB with FC; however we could not validate the combination of these techniques. Even though flagellates were able to ingest FLBs (confirmed by microscopic observation) and FLB green florescence was bright enough to be detected with FL1-H, the FLB signal dropped drastically inside phytoflagellates. Probably, the staining procedure was not strong enough, despite DTAF being left overnight to ensure the staining of all cells. A potential solution to this problem could be to conduct the experiments using freshly prepared FLBs, as suggested by Bock *et al.* (Bock *et al.*, 2021). Preliminary tests using beads of 0.5 μm yielded similar results, as we were not able to detect the surrogate prey signal inside the organism, and probably also because of the weak green fluorescence signal of small beads when they were inside a food vacuole.

The use of beads as synthetic prey could be a limitation. Size and morphology of prey (e.g. Jürgens and Matz, 2002), as well as the nature of the prey (e.g. natural vs. artificial beads), are associated with the prey selection by protists. Several studies used beads of different sizes to estimate ingestion rates (Schmidtke *et al.*, 2006; Kamjunke *et al.*, 2007). However, the use of beads may underestimate the ingestion rate due to faster egestion compared to FLBs (Jürgens and DeMott, 1995). Other studies demonstrated prey selection using different kinds of natural prey, such as picocyanobacteria and picoeukaryotes (Tarbe *et al.*, 2011; Izaguirre *et al.*, 2012; Gerea *et al.*, 2018). Bock *et al.* (Bock *et al.*, 2021) explored bacterivory in five strains of prasinophytes using a variety of prey types. They detected bacterivory only when live FLB stained with CellTracker were offered as prey, whereas no feeding was detected when heat-killed bacteria strained with DTAF or magnetic beads were provided. As mentioned above, the use of 1-μm beads demonstrated a better visualization of the signal in FC than smaller beads. Besides being an artificial prey, 1-μm beads are larger than the mean bacterial size usually found in natural systems (often < 1 μm), thus introducing another limitation to the technique. Therefore, in this case the choice of the surrogate prey is a trade-off between obtaining a realistic grazing rate and a good identification of positive phagotrophic cells. Although it is desirable to develop a measuring technique based on

the FC due to it is versatility, the limitation of using 1-μm beads and the discrepancy with the microscope discussed above would allow calculation of only a rough estimation of the CSGR. More experiments are needed to improve this protocol.

The enumeration of phagotrophic protists using acidotropic fluorescent probes that stain digestive vacuoles, developed originally to count heterotrophic flagellates (Rose *et al.*, 2004; Carvalho and Granéli, 2006; Sintes and del Giorgio, 2010), has also been used successfully to identify mixotrophs (Carvalho and Granéli, 2006; Anderson *et al.*, 2017, 2018). Here, we used LyTG to evaluate the presence of food vacuoles in two mixotrophic phytoflagellates and compared with an obligate autotrophic. As expected, when comparing the green fluorescence in the same sample with and without dye, we observed that the obligate autotrophic species was not stained, confirming that *Chlamydomonas* did not have food vacuoles. On the other hand, LyTG stain increased green fluorescence in *O. tuberculata* and *C. marssonii* five and two times, respectively. This suggests the presence of digestive vacuoles in the two mixotrophic species. Few studies determined mixotrophic cells with labeled food vacuoles in cultures and natural samples (Carvalho and Granéli, 2006; Anderson *et al.*, 2017; Sato and Hashihama, 2019). The main advantage of this method is: that it allows a simple and fast assessment of phagotrophic organisms in live samples. Nevertheless, we can list some limitations of this method, as it does not allow for distinguishing old vacuoles from new ones, and also different protists could have distinct digestion times (Gonzalez *et al.*, 1990; Boenigk *et al.*, 2001; Anderson *et al.*, 2017). Another potential constraint is the possibility of unspecific binding of acidotropic probes to acidic organelles other than food vacuoles, such as chloroplasts (Wilken *et al.*, 2019). However, Anderson *et al.* (Anderson *et al.*, 2017) observed no measurable LyTG staining in diverse mixotrophic phytoflagellate strains when grown under nutrient and light replete conditions that favor a strictly photosynthetic growth. Therefore, no unspecific binding was observed by these authors. Similarly, we observed no measurable LyTG signal in our control (*Chlamydomonas*). We hypothesized that different light and nutrient conditions could induce the phagotrophic activity differently and also aimed to evaluate whether these variabilities can be detected by LyTG. However, despite differences in ingestion rates among some treatments, it was not possible to distinguish clear differences between acclimated cultures under distinct light and nutrient conditions, suggesting that the method was not sensitive enough to quantify phagotrophic activity upon vacuoles fluorescence, but it can be used qualitatively to detect the presence or absence of phagotrophy.

The estimation of grazing rates by mixotrophs is commonly based on the detection of putative prey inside the food vacuoles of predators. The gold standard method of identifying prey is epifluorescence microscopy. The comparison with FC performed in this study showed some discrepancies. In addition, the restriction of using only synthetic and relatively large prey (i.e. 1-μm beads) constitutes another limitation. In spite of this, FC could be used with confidence to quantify mixotrophic phytoflagellates using either labeled prey or acidotropic probes, but CSGR estimates should be taken with care. As Wilken *et al.* (Wilken *et al.*, 2019) emphasized in their review, combining the higher throughput attained by FC with the detection of labelled prey or staining of acidic vacuoles could fully utilize the strengths of these techniques in future studies. Therefore, future studies are necessary in order to understand the differences in magnitude when comparing FC versus epifluorescence microscopy measurements.

CONCLUSIONS

The short-term ingestion experiments provide evidence that nutrients and light affected grazing rates of the two phytoflagellates differently. Phagotrophy by the cryptophyte increased with nutrients while the phagotrophy by the chrysophyte increased when light was limited. Grazing rates could be measured by epifluorescence microscopy and FC as well. Both methods showed roughly the same trend, however, grazing rates measured by FC were higher in all treatments. The food vacuoles staining experiments allowed a clear differentiation between phagotrophic and non-phagotrophic flagellates, but failed to detect quantitative differences in grazing activity, consequently LyTG can only be used as a qualitative method.

SUPPLEMENTARY DATA

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

ACKNOWLEDGEMENTS

We thank the editor and three anonymous reviewers for their constructive comments, which helped to improve our manuscript. We thank Dr María Victoria Quiroga for all the support in cytometric analysis and the staff from the Laboratory of Aquatic Ecology (LEA—INTECH) and Laboratory of Microbial Process and Biodiversity (LMPB—UFSCar).

FUNDING

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001 (PDSE no. 19/2016); Fundação

de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Processes: 2014/14139-3 and 2016/50494-8). Argentinean Council of Science and Technology (CONICET) for granting to F.U. the fellowship for young researchers. CONICET and FAPESP funded the Argentina-Brazil bilateral project level 1 to F.U. and H.S. We are grateful to CAPES for granting the Ph.D. scholarship to MRAC. Conselho Nacional de Desenvolvimento e Pesquisa Tecnológica (CNPq research productivity grants 303906/2021-9 and 308652/2019-3, respectively to H.S. and V.B.).

REFERENCES

Anderson, R., Jürgens, K. and Hansen, P. J. (2017) Mixotrophic phytoflagellate bacterivory field measurements strongly biased by standard approaches: a case study. *Front. Microbiol.*, **8**. https://doi.org/10.3389/fmicb.2017.01398.

Azam, F., Fenchel, T., Field, J., Gray, J., Meyer-Reil, L. and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.*, **10**, 257–263. https://doi.org/10.3354/meps010257.

Ballen-Segura, M., Felip, M. and Catalan, J. (2017) Some mixotrophic flagellate species selectively graze on archaea. *Appl. Environ. Microbiol.*, **83**, 1–11. https://doi.org/10.1128/AEM.02317-16.

Beisner, B. E., Grossart, H. and Gasol, J. M. (2019) A guide to methods for estimating phago-mixotrophy in nanophytoplankton. *J. Plankton Res.*, **00**, 1–13. https://doi.org/10.1093/plankt/fbz008.

Bird, D. F. and Kalf, J. (1989) Phagotrophic sustenance of a metalimnetic phytoplankton peak. *Limnol. Oceanogr.*, **34**, 155–162.

Bock, N. A., Charvet, S., Burns, J., Gyaltshen, Y., Rozenberg, A., Duhamel, S. and Kim, E. (2021) Experimental identification and in silico prediction of bacterivory in green algae. *ISME J.*, **15**, 1987–2000.

Callieri, C., Caravati, E., Morabito, G. and Oggioni, A. (2006) The unicellular freshwater cyanobacterium Synechococcus and mixotrophic flagellates: evidence for a functional association in an oligotrophic, subalpine lake. *Freshw. Biol.*, **51**, 263–273.

Caron, D. A., Porter, K. G. and Sanders, R. W. (1990) Carbon, nitrogen, and phosphorus budgets for the mixotrophic phytoflagellate *Poterioochromonas malhamensis* (Chrysophyceae) during bacterial ingestion. *Limnol. Oceanogr.*, **35**, 433–443.

Caron, D. A., Sanders, R. W., Lim, E. L., Marrase, C., Amaral, L. A., Whitney, S., Aoki, R. B. and Porters, K. G. (1993) Light-dependent phagotrophy in the freshwater mixotrophic chrysophyte Dinobryon cylindricum. *Microb. Ecol.*, **25**, 93–111.

Caron, D. A. (2016) Mixotrophy stirs up our understanding of marine food webs. *Proc. Natl. Acad. Sci.*, **113**, 2806–2808.

Carvalho, W. F. and Granéli, E. (2006) Acidotropic probes and flow cytometry: a powerful combination for detecting phagotrophy in mixotrophic and heterotrophic protists. *Aquatic Microb. Ecol.*, **44**, 85–96.

Carvalho, W. F. and Granéli, E. (2010) Contribution of phagotrophy versus autotrophy to Prymnesium parvum growth under nitrogen and phosphorus sufficiency and deficiency. *Harmful Algae*, **9**, 105–115. https://doi.org/10.1016/j.hal.2009.08.007.

Costa, M. R. A., Menezes, R. F., Sarmento, H., Attayde, J. L., Sternberg, L. S. L. and Becker, V. (2019) Extreme drought favors potential mixotrophic organisms in tropical semi-arid reservoirs. *Hydrobiologia*, **2**, 43–54. https://doi.org/10.1007/s10750-018-3583-2.

Cucci, T. L., Shumnway, S. E., Brown, W. S. and Newell, C. R. (1989) Using phytoplankton and flow cytometry to analyze grazing by marinv cbbbbbgnm e organisms. *J. quant. cell scien.*, **10**, 659–669. https://doi.org/10.1002/cyto.990100523.

Campo, D. and Massana (2021) Emerging diversity within Chrysophytes, Choanoflagellates and Bicosoecids based on molecular surveys. *Protist*, **162**, 435–448. doi.org/10.1016/j.protis.2010.10.003.

Domaizon, I., Viboud, S. and Fontvieille, D. (2003) Taxon-specific and seasonal variations in flagellates grazing on heterotrophic bacteria in the oligotrophic Lake Annecy - importance of mixotrophy. *FEMS Microbiol. Ecol.*, **46**, 317–329. https://doi.org/10.1016/S0168-6496(03)00248-4.

Fischer, R., Ptacnik, R., Fischer, R., Giebel, H., Hillebrand, H. and Ptacnik, R. (2017) Importance of mixotrophic bacterivory can be predicted by light and loss rates. *Oikos*, **126**, 713–722. https://doi.org/10.1111/oik.03539.

Flöder, S., Hansen, T. and Ptacnik, R. (2006) Energy-dependent bacterivory in ochromonas minima-a strategy promoting the use of substitutable resources and survival at insufficient light supply. *Protist*, **157**, 291–302. https://doi.org/10.1016/j.protis.2006.05.002.

Flynn, K. J., Stoecker, D. K., Mitra, A., Raven, J. A., Glibert, P. M., Hansen, P. J., Granéli, E. and Burkholder, J. M. (2013) Misuse of the phytoplankton-zooplankton dichotomy: the need to assign organisms as mixotrophs within plankton functional types. *J. Plankton Res.*, **35**, 3–11. https://doi.org/10.1093/plankt/fbs062.

Gasol, J. M. and Del Giorgio, P. A. (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.*, **64**, 197–224. https://doi.org/10.3989/scimar.2000.64n2197.

Gasol, J. M., and X. A. G. Morán (2015) Flow cytometric determination of microbial abundances and its use to obtain indices of community structure and relative activity, p. 159–187. In: McGenity T.J., Timmis K.N., Nogales B. (eds) *Hydrocarbon and Lipid Microbiology Protocols. Springer Protocols Handbooks*. Springer, Berlin, Heidelberg. https://doi.org/10.1007/8623_2015_139

Gast, R. J., McKie-Krisberg, Z. M., Fay, S. A., Rose, J. M. and Sanders, R. W. (2014) Antarctic mixotrophic protist abundances by microscopy and molecular methods. *FEMS Microbiol. Ecol.*, **89**, 388–401. https://doi.org/10.1111/1574-6941.12334.

Gerea, M., Queimalin, C. and Unrein, F. (2018) Grazing impact and prey selectivity of picoplanktonic cells by mixotrophic flagellates in oligotrophic lakes. *Hydrobiologia*, **831**, 5–21. https://doi.org/10.1007/s10750-018-3610-3.

Grujcic, V., Nuy, J. K., Salcher, M. M., Shabarova, T., Kasalicky, V., Boenigk, J., Jensen, M. and Simek, K. (2018) Cryptophyta as major bacterivores in freshwater summer plankton. *ISME J.*, **12**, 1668–1681. https://doi.org/10.1038/s41396-018-0057-5.

Guillard, R. R. L. and Lorenzen, C. J. (1972) Yellowgreen Algae with Chlorophyllide C. *J. Phycol.*, **8**, 1014.

Hammer, A., Schumann, R. and Schubert, H. (2002) Light and temperature acclimation of Rhodomonas salina (cryptophyceae): photosynthetic performance. *Aquat. Microb. Ecol.*, **29**, 287–296. https://doi.org/10.3354/ame029287.

Hartmann, M., Grob, C., Tarran, G. A., Martin, A. P., Burkill, P. H., Scanlan, D. J. and Zubkov, M. V. (2012) Mixotrophic basis of Atlantic oligotrophic ecosystems. *Proc. Natl. Acad. Sci.*, **109**, 5756–5760. 10.1073/pnas.1118179109.

Heinze, A. W., Truesdale, C. L., DeVaul, S. B., Swinden, J. and Sanders, R. W. (2013) Role of temperature in growth, feeding, and vertical

distribution of the mixotrophic chrysophyte Dinobryon. *Aquat. Microb. Ecol.*, **71**, 155–163.

Holen, D. A. (1999) Effects of prey abundance and light intensity on the mixotrophic chrysophyte Poterioochromonas malhamensis from a mesotrophic lake. *Freshwat. Biolog.*, **42**, 445–455.

Ishida, Y. and Kimura, B. (1986) Photosynthetic phagotrophy of Chrysophyceae: evolutionary aspects. *Microbio Sci.*, **3**, 132–135.

Izaguirre, I., Sinistro, R., Schiaffino, M. R., Sánchez, M. L., Unrein, F. and Massana, R. (2012) Grazing rates of protists in wetlands under contrasting light conditions due to floating plants. *Aquat. Microb. Ecol.*, **65**, 221–232. https://doi.org/10.3354/ame01547.

Jones, R. I. (1994) Mixotrophy in planktonic protists as a spectrum of nutritional strategies. *Mar. Microb. Food Webs*, **8**, 87–96.

Jones, R. and Rees, S. (1994) Characteristics of particle uptake by the phagotrophic phytoflagellate, Dinobryon divergens. *Mar. Microb. Food Webs*, **8**, 97–110.

Jürgens, K. and Demott, W. R. (1995) Behavioral flexibility in prey selection by bacterivorous nanoflagellates. *Limnol. Oceanogr.*, **40**, 1503–1507.

Jürgens, K. and Matz, C. (2002) Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Antonie Van Leeuwenhoek*, **81**, 413–434. https://doi.org/10.1023/a:1020505204959.

Kamjunke, N., Henrichs, T. and Gaedke, U. (2007) Phosphorus gain by bacterivory promotes the mixotrophic flagellate *Dinobryon* spp. during re-oligotrophication. *J. Plankton Res.*, **29**, 39–46. https://doi.org/10.1093/plankt/fbl054.

Katechakis, A. and Stibor, H. (2006) The mixotroph Ochromonas tuberculata may invade and suppress specialist phago- and phototroph plankton communities depending on nutrient conditions. *Oecologia*, **148**, 692–701. https://doi.org/10.1007/s00442-006-0413-4.

Keller, M. D., Shapiro, L. P., Haugen, E. M., Cucci, T. L., Sherr, E. B. and Sherr, B. F. (1994) Phagotrophy of fluorescently labeled bacteria by an oceanic phytoplankter. *Microb. Ecol.*, **28**, 39–52.

Kirchman, D. L. and Hanson, T. E. (2013) Bioenergetics of photoheterotrophic bacteria in the oceans. *Envir. Microb. Rep.*, **5**, 188–199.

Legendre, A. P. (2018) lmodel2: Model II Regression. *R package version 1.7-3*. https://CRAN.R-project.org/package=lmodel2.

Livanou, E., Barsakis, K., Psarra, S. and Lika, K. (2020) Modelling the nutritional strategies in mixotrophic nanoflagellates. *Ecol. Model.*, **428**, 109053.

Mansour, J. S. and Anestis, K. (2021) Eco-evolutionary perspectives on mixoplankton. *Front. Mar. Sci.*, **8**. https://doi.org/10.3389/fmars.2021.666160.

McKie-Krisberg, Z. M., Gast, R. J. and Sanders, R. W. (2015) Physiological responses of three species of antarctic mixotrophic phytoflagellates to changes in light and dissolved nutrients. *Microbiol. of aquatic Syst.*, **70**, 21–29. https://doi.org/10.1007/s00248-014-0543-x.

Millette, N. C., Pierson, J. J., Aceves, A. and Stoecker, D. K. (2017) Mixotrophy in Heterocapsa rotundata: a mechanism for dominating the winter phytoplankton. *Limno. Ocean.*, **62**, 836–845.

Mitra, A., Flynn, K. J., Tillmann, U., Raven, J. A., Caron, D., Stoecker, D. K., Not, F., Hansen, P. J. *et al.* (2016) Defining planktonic protist functional groups on mechanisms for energy and nutrient acquisition: incorporation of diverse mixotrophic strategies. *Protist*, **167**, 106–120. https://doi.org/10.1016/j.protis.2016.01.003.

Nygaard, K. and Tobiesen, A. (1993) Bacterivory in algae: a survival strategy during nutrient limitation. *Limnol. Oceanogr.*, **38**, 273–279.

Pace, M. L. and Bailif, M. D. (1987) Evaluation of a fluorescent microsphere technique for measuring grazing rates of phagotrophic microorganisms. *Mar. Ecol. Prog. Ser.*, **40**, 185–193.

Pålsson, C. and Daniel, C. (2004) Effects of prey abundance and light intensity on nutrition of a mixotrophic flagellate and its competitive relationship with an obligate heterotroph. *Aquat. Microb. Ecol.*, **36**, 247–256.

Pålsson, C. and Granéli, W. (2003) Diurnal and seasonal variations in grazing by bacterivorous mixotrophs in an oligotrophic clear- water lake. *Arch. Hydrobiol.*, **157**, 289–307. https://doi.org/10.1127/0003-9136/2003/0157-0289.

Pascher, A. (1943) Zur Kenntnis vershiedener Ausbildungen der planktontischen Dinobryon. *Int. Rev. Gesamten Hydrobiol. Hydrogr.*, **43**, 11 O-l 23.

Ptacnik, R., Gomes, A., Royer, S. J., Berger, S. A., Calbet, A., Nejstgaard, J. C. and Pitta, P. (2016) A light-induced shortcut in the planktonic microbial loop. *Sci. Rep.*, **6**, 1–10.

Porter, K. G. and Feig, Y. (1980) The use of DAPI for identifying aquatic microflora. *Limnol. Oceanogr.*, **25**, 943–948.

Raven, J. A. (1995) Comparative aspects of chrysophyte nutrition with emphasis on carbon, nitrogen and phosphorus. In Sandgren, C. D., Smol, J. P. and Kristiansen, J. (eds.), *Chrysophyte Algae: Ecology, Phylogeny and Development*, Cambridge University Press, Cambridge, UK, pp. 95–118.

Rose, J. M., Caron, D. A., Sieracki, M. E. and Poulton, N. (2004) Counting heterotrophic nanoplanktonic protists in cultures and aquatic communities by flow cytometry. *Aquat. Microb. Ecol.*, **34**, 263–277. https://doi.org/10.3354/ame034263.

Rothhaupt, K. (1996) Laboratorary experiments with a mixotrophic chrysophyte and obligately phagotrophic and photographic competitors. *Ecology*, **77**, 716–724.

Saad, J. F., Unrein, F., Tribelli, P. M., López, N. and Izaguirre, I. (2016) Influence of lake trophic conditions on the dominant mixotrophic algal assemblages. *J. Plankton Res.*, **00**, 1–12. https://doi.org/10.1093/plankt/fbw029.

Sanders, R. (1991) Mixotrophic protists in marine and freshwater ecosystems. *J. Protozool.*, **38**, 76–81.

Sarmento, H., Unrein, F., Isumbisho, M., Stenuite, S., Gasol, J. M. and Descy, J. P. (2008) Abundance and distribution of picoplankton in tropical, oligotrophic Lake Kivu, eastern Africa. *Freshw. Biol.*, **53**, 756–771. https://doi.org/10.1111/j.1365-2427.2007.01939.x.

Sato, M. and Hashihama, F. (2019) Assessment of potential phagotrophy by pico- and nanophytoplankton in the North Pacific Ocean using flow cytometry. *Aquat. Microb. Ecol.*, **82**, 275–288. https://doi.org/10.3354/ame01892.

Schiaffino, M. R., Gasol, J. M., Izaguirre, I. and Unrein, F. (2013) Picoplankton abundance and cytometric group diversity along a trophic and latitudinal lake gradient. *Aquat. Microb. Ecol.*, **68**, 231–250. https://doi.org/10.3354/ame01612.

Schmidtke, A., Bell, E. M. and Weithoff, G. (2006) Potential grazing impact of the mixotrophic flagellate *Ochromonas* sp. (Chrysophyceae) on bacteria in an extremely acidic lake. *J. Plankton Res.*, **28**, 991–1001. https://doi.org/10.1093/plankt/fbl034.

Shapiro, H. M. (1995) *Practical Flow Cytometry*, Third edn, Wiley-Liss, New York.

Sintes, E. and DEL Giorgio, P. A. (2010) Community heterogeneity and single-cell digestive activity of estuarine heterotrophic nanoflagellates assessed using lysotracker and flow cytometry. *Environ. Microbiol.*, **12**, 1913–1925. 10.1111/j.1462-2920.2010.02196.x.

Stoecker, D. K. (1998) Conceptual models of mixotrophy in planktonic protists and some ecological and evolutionary implications. *Eur. J. Protistol.*, **34**, 281–290. https://doi.org/10.1016/S0932-4739(98)80055-2.

Stoecker, D. K., Hansen, P. J., Caron, D. A. and Mitra, A. (2017) Mixotrophy in the marine plankton. *Annu. Rev. Mar. Sci.*, **9**, 311–335. https://doi.org/10.1146/annurev-marine-010816-060617.

Skovgaard, A. (1996) Mixotrophy in Fragilidium subglobosum (dinophyceae): growth and grazing responses as functions of light intensity. *Marine Ecol. Progre. Ser.*, **143**, 247–253.

Skovgaard, A. (2000) A phagotrophically derivable growth factor in the plastidic dinoflagellate Gyrodinium resplendens (Dinophyceae). *J. Phycol.*, **36**, 1069–1078.

Tarbe, A., Unrein, F., Stenuite, S., Pirlot, S. and Sinyinza, D. (2011) Protist herbivory : a key pathway in the pelagic food web of Lake Tanganyika. *Microb. Ecol.*, **62**, 314–323. https://doi.org/10.1007/s00248-011-9817-8.

Tittel, J., Bissinger, V., Zippel, B., Gaedke, U., Bell, E., Lorke, A. and Kamjunke, N. (2003) Mixotrophs combine resource use to outcompete specialists: implications for aquatic food webs. *PNAS*, **100**, 12776–12781. https://doi.org/10.1073/pnas.2130696100.

Tranvik, L. J., Porter, K. G. and Sieburth, J. M. (1989) Occurrence of bacterivory in Cryptomonas, a common freshwater phytoplankter. *Oecologia*, **78**, 473–476.

Unrein, F., Gasol, J. M., Not, F., Forn, I. and Massana, R. (2014) Mixotrophic haptophytes are key bacterial grazers in oligotrophic coastal waters. *ISME J.*, **8**, 164–176. https://doi.org/10.1038/ismej.2013.132.

Unrein, F., Massana, R., Alonso-Sáez, L. and Gasol, J. M. (2007) Significant year-round effect of small mixotrophic flagellates on bacterioplankton in an oligotrophic coastal system. *Limnol. Oceanogr.*, **52**, 456–469. https://doi.org/10.4319/lo.2007.52.1.0456.

Urabe, J., Gurung, T. B., Yoshida, T., Sekino, T. and Nakanishi, M. (2000) Diel changes in phagotrophy by Cryptomonas in Lake Biwa. *Limnol. Oceanogr.*, **45**, 1558–1563.

Ward, B. A. and Follows, M. J. (2016) Marine mixotrophy increases trophic transfer efficiency, mean organism size, and vertical carbon flux. *Proc. Natl. Acad. Sci.*, **113**, 2958–2963. https://doi.org/10.1073/pnas.1517118113.

Wilken, S., Choi, C. J. and Worden, A. Z. (2020) Contrasting mixotrophic lifestyles reveal different ecological niches in two closely related marine protists. *J. Phycol.*, **56**, 52–67. https://doi.org/10.1111/jpy.12920.