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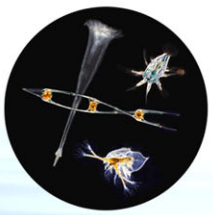

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Growth, grazing, and inorganic C and N uptake in a mixotrophic and a heterotrophic ciliate

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We compared growth, grazing and inorganic carbon and nitrogen uptake in two ciliates, the mixotrophic *Strombidium rassoulzadegani* and the heterotrophic *Strombidinopsis sp.* The mixotroph had over 2-fold higher gross growth efficiencies (fraction of ingestion devoted to growth; GGE) for C at low food concentrations, while the heterotroph's GGE was 2-fold higher under food saturation. Inorganic carbon uptake did not vary significantly with food concentration in the mixotroph, but its importance for growth was highest at low food concentrations. Although there was measurable inorganic carbon uptake in the heterotroph due to still-active algae in food vacuoles, it did not contribute significantly to growth. The two ciliates took up inorganic nitrogen (both nitrate and ammonium) at similar biomass-specific rates, but inorganic nitrogen did not contribute significantly to their N requirements. Mixotrophy with retained chloroplasts provides a significant energy subsidy, especially at low food levels, but maximum growth rates were similar for the mixotroph and the heterotroph we compared, suggesting that the advantage of chloroplast retention diminishes when food concentrations are high.

KEYWORDS: mixotrophy; kleptoplasty; growth efficiency; chloroplast retention

INTRODUCTION

Planktonic ciliates are an important trophic link between metazoa, small algae and bacteria (Sherr *et al.*, 1986; Gifford and Dagg, 1991; Gifford, 1991). About 30% of copepod diets consist of ciliates (Calbet and Saiz, 2005). In addition to being grazers, many ciliates are mixotrophic, capable of both phagotrophy and photosynthesis (Johnson, 2011). These "green" ciliates acquire plastids from their algal prey. The best-studied mixotroph, the litostome *Mesodinium rubrum*, can be the dominant phytoplankton during blooms in estuaries and coastal waters (Crawford, 1989; Kifle and Purdie, 1993; Herfort *et al.*, 2012), but plastidic oligotrich ciliate blooms have also been observed (Burkholder *et al.*, 1967; Dale and Dahl, 1987).

They regularly comprise up to 50% of all ciliates in ocean surface waters (Stoecker *et al.*, 1987; Dolan *et al.*, 1999; Dolan and Perez, 2000).

Although the widespread occurrence of chloroplast retention (also known as kleptoplasty or chloroplast enslavement) has been documented in ciliates, there have been few studies that have measured its importance for ciliate growth, especially in the oligotrichs that dominate plankton assemblages. Furthermore, we know little about how the carbon subsidy obtained via kleptoplast photosynthesis relates to N requirements.

In this study we compared growth, grazing and gross growth efficiency (growth rate divided by ingestion rate; GGE) for both carbon and nitrogen in the mixotrophic ciliate *Strombidium rassoulzadegani* and the heterotrophic ciliate *Strombdinopsis* sp. These two species are similar in size, morphology, growth rates and behavior. Both are commonly encountered in coastal waters worldwide. Currently, we do not have a reliable model ciliate that is culturable as either a heterotroph or a mixotroph, so we chose to compare two similar ciliates, each grown on its optimal food. GGE represents the fraction of ingested material that becomes biomass (Straile, 1997). Values can be different for C, N or P, depending on biomass composition of predator and prey. Measurements of carbon GGE for heterotrophic ciliates range between 0.03 and 0.8, but most values are 0.3–0.4 (Scott, 1985; Stoecker and Evans, 1985; Verity 1985). Mixotrophic ciliate GGEs have been found to range between 0.1 and 11 (Laybourn, 1976; Johnson and Stoecker, 2005). Higher GGEs in mixotrophs, especially values greater than 1, are undoubtedly the result of the inorganic C subsidy from phototrophy (Laybourn, 1976, reviewed in Caron *et al.*, 1990).

We also used radio- and stable isotopes to measure inorganic C and N uptake, respectively, in the two ciliates, and to estimate the amount of respiratory carbon that may be recycled in the mixotroph via photosynthesis. These data were used to evaluate the relative importance of inorganic C and N uptake for growth, compared to the acquisition of these elements via ingestion.

METHOD

Cultures

The ciliates were isolated from Long Island Sound, USA (LIS: 41°16.5'N 72°05.5'W). Based on preliminary observations, each ciliate was fed on the food organism that provided it with consistent high growth. The mixotroph *S. rassoulzadegani* was fed the chlorophyte *Tetraselmis chui* and maintained at 19°C, and 12:12 light cycle at 100 µmol photons m^{-2} s^{-1} in f/2 filtered sea water (FSW) medium (Guillard and Ryther, 1962) at a salinity of 30 (practical salinity scale). The heterotroph *Strombidinopsis sp.* was fed the dinoflagellate *Heterocapsa triquetra* and maintained in f/20 FSW under the same light and temperature conditions as the mixotroph.

To determine ciliate chlorophyll content, *S. rassoulzadegani* cells were grown to high abundance on a diet of *T. chui* at concentrations that were saturating for growth. We then separated the ciliates from their food. This was accomplished by placing the culture in a volumetric flask and filling it to the bottom of the neck with FSW at a salinity of 30. To minimize mixing, the rest of the neck was filled with salinity 28 FSW. The bowl of the flask was then covered with black paper and a light was focused at the top of the neck. *Strombidium rassoulzadegani* is strongly phototactic and thus was quickly concentrated at the top of the neck of the flask. The concentrated ciliates were removed and subsamples fixed with Lugol's iodine were counted to estimate their abundance. Under these conditions, the remaining food organisms were fewer than 1 per milliliter (~0.01% of the total biomass). Ciliates were then collected on glass fiber filters (Whatman GF/F) and extracted overnight in 90% acetone at −15°C. We measured fluorescence with a Turner Designs fluorometer; chlorophyll content was calculated as in Arar and Collins (1997).

Carbon content for ciliates and their food organisms was estimated from the volume to carbon relationships in Menden-Deuer and Lessard (2000). For calculating cell volumes, the shape of *S. rassoulzadegani* was assumed to be a cone with a hemispherical cap. *Strombidinopsis sp.* was treated as a cone. Both algae were assumed to have a prolate spheroid shape. We measured ~10 cells of each organism to calculate the average volumes. Direct measurements of carbon content were also available for both ciliates (see below).

Nitrogen content of the algae was estimated using the volume to nitrogen relationships in Verity *et al.* (1992) and Menden-Deuer and Lessard (2000). *Strombidinopsis sp.* carbon and nitrogen content were taken from measurements made for a previous study with the same isolate (Siuda and Dam, 2010). *Strombidium rassoulzadegani* carbon and nitrogen content were measured on a Fisons Instruments Elemental Analyzer, following the method used by Siuda and Dam (2010).

Growth, ingestion and GGE

Measurements of growth and ingestion at different food concentrations were conducted in six-well plates. Algal food ranged from 10^2–10^5 cells mL^{-1} (0.1–17 mgC or 0.01–3 mgN L^{-1}), with values chosen to represent a range of saturated and unsaturated conditions based on

preliminary experiments. Ciliates were acclimated at each food concentration for 24 h prior to the experiment. After acclimation, 15 ciliates were placed in triplicate 10 mL wells within hanging cell culture inserts (Millipore 6 well Millicell, Catalogue number PIEP30R48) with equal initial concentrations of algae inside and outside of the inserts. An 8 μm pore size filter on the bottom of the insert allowed media to pass through, but not ciliates or algae. Preliminary experiments with *Tetraselmis* grown in the presence of *S. rassoulzadegani* suggested that increased algal growth rates under grazing due to remineralization of nutrients by the ciliates could be significant. Thus the algae within the wells but outside the inserts served as the no-grazer control for the ingestion measurements. Light and temperature conditions for these experiments were the same as for culture maintenance.

After 3 days (sufficient time to have measureable signals for both growth and grazing), the contents of the wells were fixed with Lugol's iodine, and ciliates and algae were counted. Specific growth rates (d^{-1}) were calculated assuming exponential growth between initial and final sampling points. Feeding rates were obtained by comparing the growth rates of the algal food with and without ciliate grazers present and were calculated using the equations of Frost (1972) as modified by Heinbokel (1978).

We fit the growth and ingestion rate data to a modified Michaelis–Menten equation (Montagnes, 1996) using the R environment for statistical computing (Fox and Weisberg, 2011; R Development Core Team, 2012),

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

where V is the ciliate growth or grazing rate, $V_{\max}$ is the maximum rate, $[C]$ is the algal concentration, and K_m is a parameter that describes how rapidly V approaches its maximum. T is a feeding threshold (x-intercept). Note that with the addition of the threshold parameter (T), K_m is not precisely equivalent to the food concentration at which growth or ingestion is half the maximum, as in the unmodified equation, but it does approximate that when T is small, as in our case (see below).

We used ciliate and algal carbon content to convert algae ingested per ciliate to a specific ingestion rate (IR, d^{-1}) so that we could compare ingestion rates with specific growth rates and specific inorganic carbon uptake rates. The dimensionless GGE was calculated as specific growth rate divided by specific ingestion rate. Some heterotrophic protists are known to continue dividing under low food conditions without a concomitant increase in biomass (e.g. Fenchel, 1982), but to our knowledge this has not been examined in mixotrophs. To evaluate this possible effect, we measured changes in *S. rassoulzadegani* cell volumes at different food concentrations in a separate feeding experiment, including a starved treatment. At end of the experiment, linear dimensions of seventy five individuals from each of nine food concentrations were measured and biovolumes were compared across subsaturating or saturating food levels.

Inorganic carbon uptake

Carbon uptake rates were measured using ^{14}C-bicarbonate as a tracer (Putt, 1990a, 1990b; Stoecker and Michaels, 1991; Skovgaard *et al.*, 2000). Ciliates were preacclimated at a range of algal food concentrations above and below levels saturating for growth. Twenty ciliates were individually picked with a drawn capillary and placed into 0.25 mL FSW in each of six 20 mL scintillation vials; 0.25 mL of $NaH^{14}CO_3$-spiked FSW was added for a target activity of 0.5 μCi mL^{-1}. Actual activity was measured by taking 100 μL samples and adding 200 μL of phenylethamine, an organic base. Phenylethamine samples received 10 mL liquid scintillation fluid (Opti-Fluor, Perkin Elmer, Inc.) and their activity was measured with a Packard Tricarb 3100TR liquid scintillation counter (LSC). Experimental treatments were incubated at 20°C in the light (100 μmol photons m^{-2} s^{-1}) or the dark for 4 h. Treatment vials were then acidified and dried down to remove excess inorganic carbon, leaving behind the organic carbon fixed by the ciliates (Skovgaard *et al.*, 2000). After they were dry, samples were re-suspended in 0.5 mL deionized water, 10 mL scintillation fluid was added, and ^{14}C activity was measured. Total inorganic carbon was determined as in Parsons *et al.* (1984). Uptake rates were calculated as in Parsons *et al.* (1984) for a 12:12 hour light: dark cycle and converted to specific incorporation rates (d^{-1}) based on ciliate carbon content.

^{14}C pulse-chase

To determine whether the mixotroph recycles a significant amount of inorganic carbon from respiration into photosynthesis, we conducted a pulse-chase experiment, allowing the ciliates to accumulate ^{14}C and then separating them from the activity source. Ciliates were first grown to high abundance with saturating concentrations of algal food, and then separated from the algae by the same method used in the chlorophyll analysis (phototaxis). The algae-free ciliates were spiked with ^{14}C-bicarbonate to a final activity of ~0.1 μCi mL^{-1}. Ciliates were allowed

to accumulate activity for 8 h, and then were separated from the isotope using their phototaxis and transferred to fresh medium. The activity in the medium after this treatment was <0.001 μCi mL^{-1}. Ciliates were then split into light and dark treatments and monitored for changes in ^{14}C over 6 h. Because ciliates in the dark treatment were assumed to not be performing photosynthesis, comparison of the light and dark loss rates indicates the degree to which respiratory $^{14}CO_2$ is recycled into photosynthesis.

Nitrogen uptake

^{15}N-ammonium and ^{15}N-nitrate were used as tracers to measure inorganic nitrogen uptake. Uptake was measured in both ciliates in both the light and the dark. One day prior to the uptake experiment, ciliates were acclimated at food concentrations that were either saturating or subsaturating for growth. Saturating algal food concentrations were 828 μgN L^{-1} for the mixotroph and 807 μgN L^{-1} for the heterotroph. Subsaturating algal food concentrations were 46 μgN L^{-1} for the mixotroph and 77 μgN L^{-1} for the heterotroph. Because we needed higher amounts of ciliates for these experiments, we used a different method to separate them from their food. On the day of the experiment, ciliates were separated from the algae by a repeated dilution with autoclaved filtered seawater followed by reverse filtration through a mesh large enough to allow the algae but not the ciliates to pass through (20 μm). Cycles of dilution and reverse filtration were continued until there remained less than one algal cell per millilitre, or <0.001% of the nitrogen biomass in the containers. In preliminary experiments, controls showed that at these concentrations the residual algae could account for 0.005% of ammonium and 0.008% of nitrate uptake.

Initial samples were taken, particulates were collected on pre-ashed GF/C filters and the filtrate was kept to measure the initial dissolved inorganic nitrogen concentrations. ^{15}N-nitrogen in the form of ammonium or nitrate was added at a concentration of 100 μM to FSW controls without algae or ciliates, algal controls at 1×10^4 cells mL^{-1}, and to the algae-free ciliates. These were split into three 100 mL replicates per treatment. Experiments were placed in an incubator at 19°C for 6 h. Light treatments were at 100 μmol photons m^{-2} s^{-1}, and dark treatments were in the same incubator but covered so that they would not be exposed to the light. After incubation, particulates were collected on pre-ashed GF/C filters and 20 mL of filtrate was collected in a liquid scintillation vial. ^{15}N atom percent was measured by isotope ratio mass spectrometer located either at the UC Davis core isotope facility, or at the University of Maryland Horn Point Laboratory. Nitrate and ammonium concentrations in the filtrate were measured using a SmartChem wet chemistry system. Uptake rates (V) were calculated as in Dugdale and Wilkerson (1986). The equation used to calculate V does not account for possible dilution of the isotope by internal N pools, so the uptake rates are conservative.

Statistical methods

The fitted curves for growth and ingestion were compared between ciliates using an extra-sum-of-squares F-test (Motulsky and Ransnas, 1987). This test separately compares the pooled sum of squares from curves for each of the ciliates to the extra sum of squares for their combined data fit to a single common curve. This was also done separately for parameters V_{max}, K_m and T. The null hypothesis is that a single curve or parameter estimate provides a better fit for the two data sets, rather than separate curves or parameters (Motulsky and Ransnas, 1987). A P value of 0.05 was used for significance testing.

For multiple comparisons, we used ANOVA followed by multiple pair-wise comparisons with Holm-adjusted P-values. The Holm adjustment is similar to the Bonnferoni correction. Both methods control for the fact that as the number of simultaneous pair-wise comparisons increases the probability of making a type I error also increases. However, the Bonnferoni correction adjusts either α or P-values by the same factor for all comparisons based on the total number of tests (α/k), while the Holm correction first orders the raw P-values from lowest to highest and then compares them from smallest to largest to α/k α/k–1, α/k–2 … α/1 (Holm, 1979). This addresses the problem of falsely rejecting a null hypothesis by chance when doing many comparisons and the problem of the Bonnferoni correction being so conservative that a null hypothesis is accepted erroneously (Montgomery, 2005). All statistics were performed using the R environment for statistical computing (Fox and Weisberg, 2011; R Development Core Team, 2012).

RESULTS

Cultures

Chlorophyll content in the mixotroph *S. rassoulzadegani* was 137 pg $cell^{-1}$ (SD = 19). Its food, *Tetraselmis chui*, contained 1.3 pg $cell^{-1}$ (SD = 0.7). *Heterocapsa triquetra*, the food organism for the heterotroph *Strombidinopsis sp.*, contained 26 pg $cell^{-1}$ (SD = 2.5) (Table I).

S. rassoulzadegani had a measured carbon content of 6.8 ngC cell^{-1} (SD = 0.061) close to the value of 6.5 ngC cell^{-1} (SD = 0.53) calculated from its volume using the relationship in Menden-Deuer and Lessard (2000; their equation for aloricate ciliates). Measured nitrogen content of *S. rassoulzadegani* was 1.3 ngN cell^{-1} (SD = 0.003). The molar carbon to nitrogen ratio of *S. rassoulzadegani* was thus 6.15. This is within the range of 3–8 reported for its similar-sized congener *Strombidium capitatum* (Stoecker *et al.*, 1989). *Strombidinopsis sp.* had a volume-estimated carbon content of 12.2 ngC cell^{-1}, slightly smaller than the previously-measured 13.7 ngC cell^{-1} (Siuda and Dam, 2010). The estimated nitrogen content of *Strombidinopsis* sp., based on the reported C:N of 3.3 and carbon content of 13.7 ngC cell^{-1} (SD = 1.6; Siuda and Dam, 2010) was 4.8 ngN cell^{-1} (SD = 0.55). Carbon and nitrogen contents for the algal food items are in Table I.

Growth, ingestion and GGE

In terms of carbon, numerical response curves (growth vs. food concentration) differed significantly between the mixotroph and the heterotroph (Fig. 1A). In an extra-sum-of-squares *F*-test, two separate models fit the data sets significantly better than a single model ($P < 0.001$). Among the curve parameters, maximum growth rates (V_{max}) were significantly different between the two ciliates, but K_m values were not ($P = 0.057$). The threshold food concentrations below which there was no growth (T) were indistinguishable from zero for both ciliates (Table II).

The carbon functional response curves (ingestion rate vs. food concentration) were also different for the two ciliate species. Two separate models fit the data sets significantly better than one ($P < 0.001$). We also found significant differences in maximum ingestion rates (V_{max}) and K_m, with both being higher for the mixotroph. Threshold food concentrations were not significantly different from zero or from each other ($P = 0.41$) (Fig. 1B and Table II).

Because each ciliate's numerical response curves (ingestion rate vs. food concentration) for C and N are linked by the C:N of the food organisms (essentially a rescaling of the *x*-axis), their shapes are the same. In addition, because the two food organisms had very similar C:N values, the comparisons between ciliates were nearly the same for N as for C. The curves were significantly different overall for the two ciliate species ($P < 0.001$), as were V_{max} and K_m (both higher in the mixotroph), and the estimated threshold food concentration was not different from zero for both ciliates (Table III).

The nitrogen functional response curves were also significantly different ($P < 0.001$) between the two ciliates. The maximum ingestion rates for nitrogen (V_{max}) and K_m were higher in the mixotroph ($P < 0.001$ and $P = 0.006$, respectively). The threshold parameter was significantly different from zero only in the heterotroph (43 μgN L^{-1}; $P = 0.003$).

Carbon gross growth efficiency (GGE_C) for the heterotroph ranged from 0.1 to 0.3, while that for the mixotroph ranged from 0.1 to 1.0. GGE_C increased very sharply for the mixotroph at low food concentrations (Fig. 2A), while that for the heterotroph decreased at the lowest food levels (Fig. 2A). Nitrogen gross growth efficiency (GGE_N) ranged from 0.3 to 0.8 for the heterotroph, lower than that of the mixotroph at low food concentrations and higher at high food concentrations (Fig. 2B). In the mixotroph, GGE_N ranged from 0.1 to 1.0, with a sharp increase at low food concentrations, as with GGE_C. Both ciliates showed sharp increases in GGE_N at low food concentrations, whereas only the mixotroph showed this for GGE_C. At the lowest food level, GGE_N declined in the heterotroph. The ciliates were thus more similar in nitrogen use than they were for carbon.

In the experiment on effects of food on ciliate size, above a food concentration of ~500 μgC L^{-1}, mixotroph cell volume was approximately constant. With less food, cell volume was lower by about one-third. This is similar to results in the literature on *Strombidinopsis* (e.g. Jeong *et al.*, 1999). Starved *S. rassoulzadegani*, while still retaining some chloroplasts, were decreased by about two-thirds in biovolume, compared to well-fed ones. Because we did not have ciliate volume estimates from

Table I: Algal and ciliate cell size, carbon, and nitrogen content

Species	μm^3 cell^{-1}	pgC cell^{-1}	pgN cell^{-1}	C:N
S. rassoulzadegani[a]		6800 (61)	1290 (3)	6.15
S. rassoulzadegani[b]	33 523 (2793)	6500 (53)	NA	
Strombidinopsis[a] (Siuda and Dam, 2010)		13 700 (1600)	4840 (550)	3.30
Strombidinopsis[b]	63 141 (8519)	12 100 (1600)	NA	
Tetraselmis chui[b]	782 (199)	112 (27)	28 (6.3)	4.67
Heterocapsa triquetra[b]	8642 (712)	1074 (83)	259 (18)	4.83

Values are means with standard deviations in parentheses. [a]measured; [b]estimated from cell volumes.

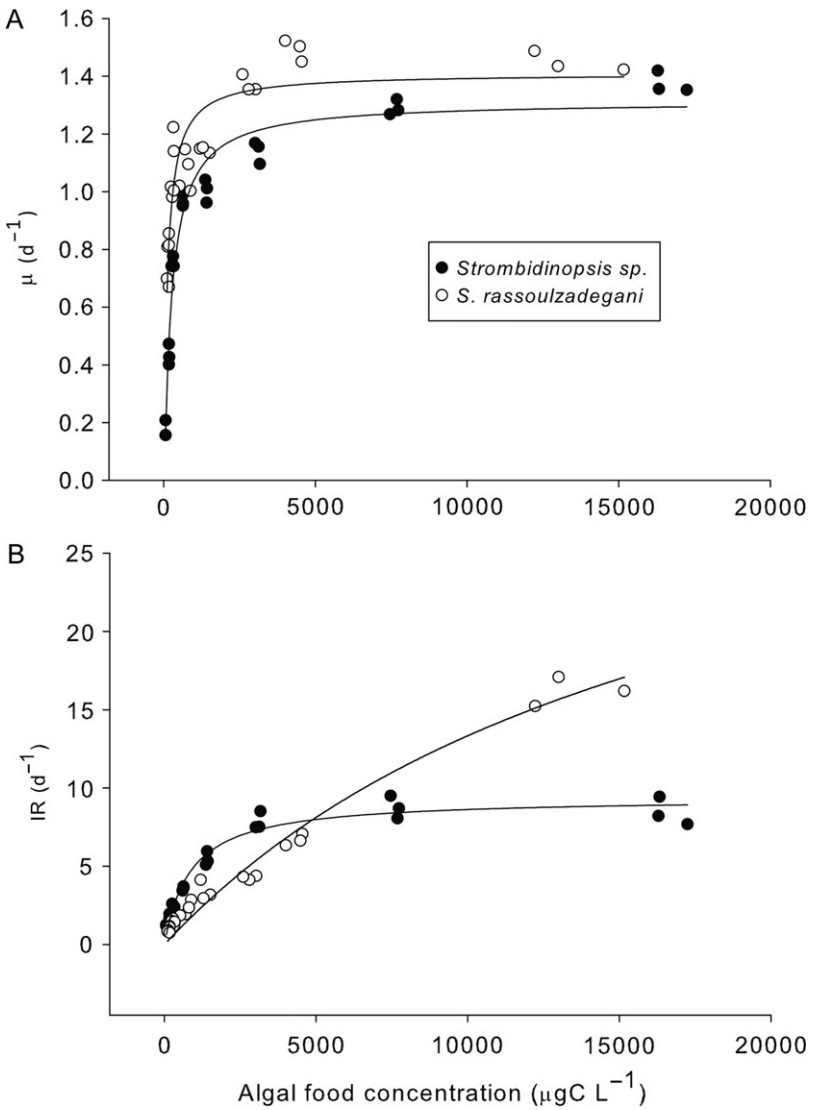

Fig. 1. Growth (**A**) and grazing rates (**B**) vs. algal food concentration for *Strombidinopsis* sp. (filled circles) and *Strombidium rassoulzadegani* (open circles).

the ingestion experiments that were used to calculate growth efficiencies, we opted not to adjust GGE's downward at the lowest food levels, but this experiment indicates that GGEs could have been about one-third lower at the lowest food concentrations (discussed below).

Inorganic carbon uptake

We found no significant difference in inorganic carbon uptake rates for the mixotroph across a range of food concentrations (Fig. 3A). Surprisingly, the heterotroph also had significant inorganic uptake, but only at the higher food concentration (Fig. 3B). When they were transferred to the scintillation vials prior to incubation, we observed intact ingested algal cells inside the heterotrophs that had been acclimated at the higher food concentration. Photosynthesis carried out by intact algal cells inside the ciliate likely explains this uptake.

^{14}C pulse-chase experiment

During the uptake phase of the pulse-chase experiment, the mixotroph accumulated a maximum activity of 0.30 pCi per cell (Fig. 4). After removal from ^{14}C, activity per cell declined but there was no significant difference between light and dark treatments in the rate of decline. When an exponential decay curve was fit to the data the

Table II: Parameter values for numerical (growth) and functional (ingestion) curves for carbon, with standard errors in parentheses

	S. rassoulzadegani	*Strombidinopsis sp.*	*P*
Overall fit, growth	–	–	<0.001
V_{max} (d^{-1})	1.41* (0.14)	1.29* (0.13)	0.011
K_m (μgC L^{-1})	144 (47.12)	256 (58.90)	0.057
T (μgC L^{-1})	0 (48)	0 (33)	1
Overall fit, ingestion	–	–	<0.001
V_{max} (d^{-1})	24* (1.16)	9.6* (1.07)	<0.001
K_m (μgC L^{-1})	2465* (344)	442* (72)	<0.001
T (μgC L^{-1})	66 (45)	18 (26)	0.41

P is the probability that a single value for that parameter would produce a better fit than a separate value for each ciliate (extra-sum-of-squares *F*-test).

Table III: Parameter values for numerical (growth) and functional (ingestion) curves for nitrogen, with standard errors in parentheses

	S. rassoulzadegani	*Strombidinopsis sp.*	*P*
Overall fit, growth	–	–	<0.001
V_{max} (d^{-1})	1.41* (0.14)	1.29* (0.13)	0.012
K_m (μgN L^{-1})	42* (14)	90* (24)	0.003
T (μgN L^{-1})	0 (14)	0 (13)	1
Overall fit, ingestion	–	–	<0.001
V_{max} (d^{-1})	24* (1.14)	6.57* (1.08)	<0.001
K_m (μgN L^{-1})	407* (63)	213* (40)	0.006
T (μgN L^{-1})	0 (13)	43* (9)	0.003

P is the probability that a single value for that parameter would produce a better fit than a separate value for each ciliate (extra-sum-of-squares *F*-test).

decline was 0.04 h^{-1} for the combined light and dark data.

^{15}N uptake in the mixotroph

We measured ciliate ammonium uptake after acclimation at saturating and subsaturating food concentrations, in both light and dark. Specific uptake rates ranged from 0.07 to 0.22 d^{-1}. Using a two-way ANOVA followed by pair-wise multiple comparisons with Holm *P*-adjustment, we found that light did not have a significant effect on ammonium uptake ($P = 0.99$), but uptake was higher in ciliates acclimated at the saturating food concentration ($P = 0.002$). In pair-wise comparisons we did not see differences between light and dark treatments ($P = 0.8$ for saturating food concentrations, and $P = 0.1$ for subsaturating food concentrations; Fig. 5A). There were significant differences between saturating and subsaturating food concentrations ($P = 0.04$ in the light, and 0.003 in the dark). There were no significant interactions between light and food levels ($P = 0.1$).

Nitrate uptake was lower than ammonium uptake in the mixotroph, ranging between 0.008 and 0.07 d^{-1}. There was no effect of light ($P = 0.51$), but uptake was significantly higher in cells previously exposed to subsaturating food concentrations ($P = 0.003$; Fig. 5B). The interaction of light and food concentration was not significant ($P = 0.15$). Pair-wise comparisons showed a significant difference between saturated and subsaturated food concentrations in the light ($P = 0.008$) and in the dark ($P = 0.001$). There was no significant difference between the light and dark treatments at either saturating ($P = 0.6$) or subsaturating ($P = 0.4$) food concentrations (Fig. 5B). All nitrate uptake values were significantly different from zero; *P*-values were 0.0012 and lower.

^{15}N uptake in the heterotroph

We found no significant effect of light ($P = 0.06$) or food concentrations ($P = 0.63$) on ammonium uptake in the heterotroph (Fig. 5C). In pair-wise comparisons there were significant differences between ciliates that were pre-acclimated at saturating food concentrations and then placed in the light and those that had subsaturating food and were then placed in the dark ($P = 0.016$), with subsaturating concentrations having higher uptake. The food-saturated treatment in the light was also significantly lower than the subsaturated light treatment ($P = 0.0006$) (Fig. 5C).

All nitrate uptake rates in the heterotroph were significantly different from zero but small (maximum 0.03 d^{-1}) when compared to the uptake rates of the algal controls (0.17 d^{-1} in the light and 0.045 d^{-1} in the dark) (Fig. 5D). There was a significant effect of both light ($P = 0.015$) and food concentration ($P = 0.004$). Furthermore, there was a significant interaction between light and food concentrations ($P = 0.047$). Uptake was higher in the light than dark at both subsaturated ($P = 0.04$) and saturated food levels ($P = 0.04$).

DISCUSSION

Although observations of pigmented bodies in oligotrich ciliates, and some evidence of ciliate photosynthesis with ingested chloroplasts, had been published earlier (Faure-Fremiet, 1948; Burkholder *et al.*, 1967; Blackbourn *et al.*, 1973), interest in ciliate mixotrophy was rekindled in the mid-1980s due to increased appreciation of ciliates as key trophic links (Laval-Peuto and Febvre, 1986; McManus and Fuhrman, 1986; Stoecker *et al.*, 1987). Many studies since that time have documented the relative abundance of mixotrophs, the sources of algal chloroplasts, rates of inorganic carbon uptake, etc. (Stoecker *et al.*, 1989; Putt, 1990a, 1990b; Stoecker and

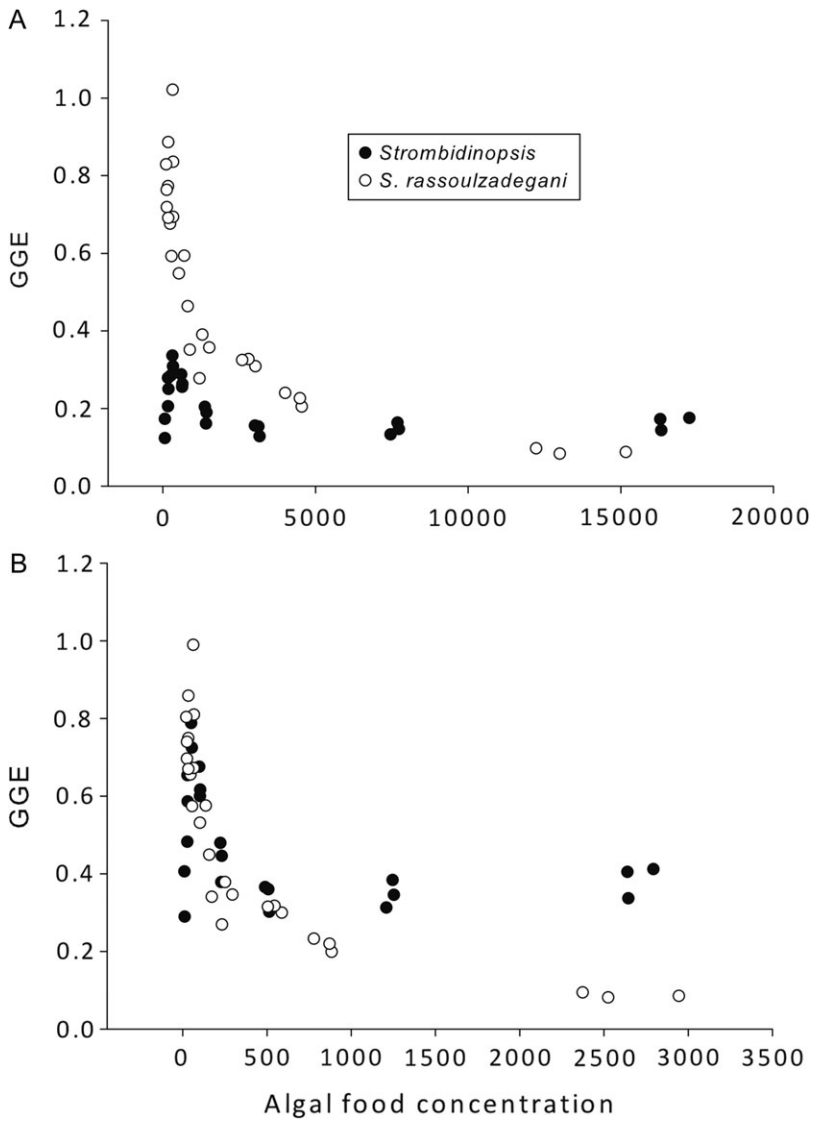

Fig. 2. Gross growth efficiencies for carbon (GGE_C) (**A**) and nitrogen (GGE_N) (**B**) at different algal food concentrations, expressed in terms of $\mu gC\ L^{-1}$ or $\mu gN\ L^{-1}$.

Silver, 1990; McManus *et al.*, 2004) and attempts have been made to incorporate mixotrophy into trophic models (Mitra *et al.*, 2016). Despite this information, we still know little about the linkages between C and N metabolism in mixotrophs, and there have been few studies of the possible costs of retaining chloroplasts (Dolan and Perez, 2000; McManus *et al.*, 2012). By contrasting feeding and growth in a mixotroph with that in a closely related heterotrophic ciliate, our goal was to elucidate how phototrophy with acquired chloroplasts is incorporated into the familiar C and N metabolism of a grazing organism.

We expected the mixotroph would have a higher maximum growth rate (due to the subsidy from phototrophy) and a lower K_m (because previous studies had shown it to be efficient at filling up with chloroplasts, allowing it to reach its maximum growth more quickly; McManus *et al.*, 2004). We also expected that T, the food level below which growth will not occur, would be lower for the mixotroph and possibly even negative (positive growth due to phototrophy even at zero food). These expectations were partially met, though the contrast with the heterotroph was surprisingly small. Estimated V_{max} for growth was less than 10% greater for the mixotroph than for the heterotroph; K_m was lower, but not significantly so, and T was indistinguishable from zero in both ciliates. We did not see evidence of positive growth in food-free conditions,

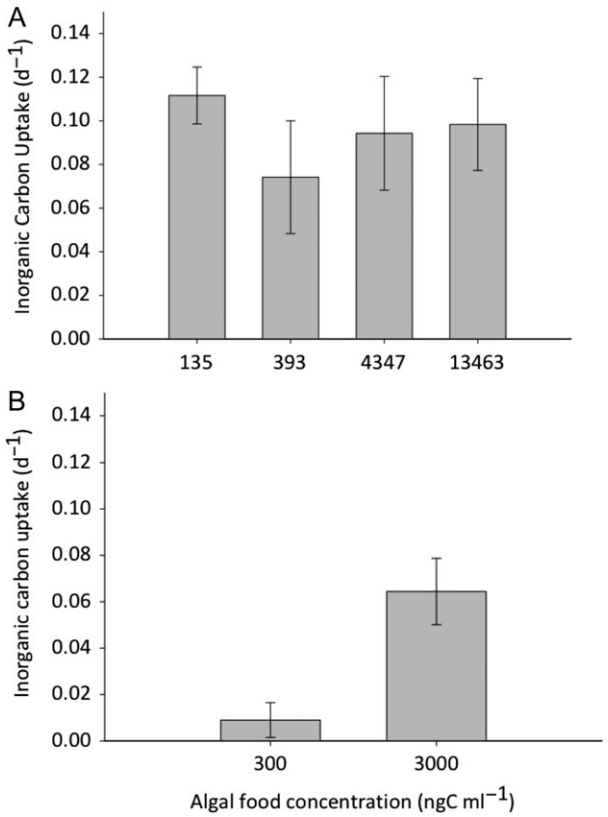

Fig. 3. Specific inorganic carbon uptake rates after acclimation at different algal food concentrations for *S. rassoulzadegani* (**A**) and *Strombidinopsis sp*. (**B**). Bars are means with standard errors. (A) For the mixotroph, there was no significant difference among treatments in a one way ANOVA followed by Holm-Sidak multiple comparison ($P = 0.29$). (B) For the heterotroph, uptake was significantly higher after feeding at higher concentrations (*t*-test; $P = 0.026$).

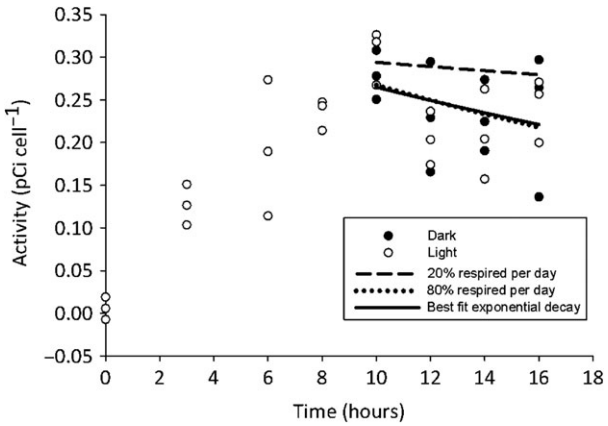

Fig. 4. The mixotrophic *S. rassoulzadegani* was allowed to incorporate $^{14}CO_2$ for 8 h and then transferred to filtered seawater. Repeated measures on ranks found no significant effect of light vs. dark on subsequent loss of isotope ($P = 0.781$). The solid line is a best fit exponential decay curve for combined light and dark treatments. The dotted and dashed lines represent exponential decay curves for 0.80 d^{-1} and 0.20 d^{-1} respiration, respectively.

confirming earlier observations that this ciliate cannot grow without food, even in the light (McManus *et al.*, 2004). The fact that this is true for both C- and N-based curves reinforces our observation that dissolved inorganic N uptake is not significant for growth in the mixotroph and indicates that acquisition of these elements is primarily from food, with plastids providing an energy subsidy.

The functional response curves (ingestion rate vs. food concentration) provided a greater contrast between the two ciliates. We had expected lower values of maximum ingestion rate and K_m for the mixotroph, based on the idea that chloroplasts limit the intracellular space available for food processing, and that the threshold for ingestion (T) would be greater than zero and similar for the two ciliate species. In contrast, we observed that the mixotroph never reached a maximum feeding rate in our experiments. Even at food concentrations greater than 10^4 μgC L^{-1}, much higher than what is commonly found in nature, ingestion rate continued to increase for the mixotroph, while the heterotroph reached saturation at ~5×10^3 μgC L^{-1}. A number of studies have found that ciliate ingestion rates can continue to rise after growth saturation (Jeong *et al.*, 2002, 2007). In heterotrophs, this phenomenon may be due to luxury consumption. When food is plentiful, some heterotrophs may simply become less efficient because it is energetically expensive to digest food to its maximum nutritional value. In the mixotrophic *S. rassoulzadegani*, on the other hand, such high ingestion rates beg the question, what happens to all those ingested chloroplasts? Based on its maximum observed ingestion rate, plus its Chl*a* content and that of its prey, we estimate that the mixotroph ingests 8.7 pgChl*a* (pg ciliate Chl*a*)$^{-1}$ d^{-1}, more than six times its V_{max} for growth. At very high food concentrations, it would thus be turning over plastids much more rapidly than the 0.5 d^{-1} previously measured at growth-saturating food levels (Schoener and McManus, 2012). Since apparently not all kinds of algae can be used as chloroplast donors (McManus *et al.*, 2012), the presence of suitable chloroplasts may be intermittent in the food environment and the ciliate may discard even recently obtained ones if fresher suitable chloroplasts are available. On the other hand, because ingestion is measured from the disappearance of cells, it is possible that at very high concentrations the mixotroph assimilates only a fraction of the nutrients in an ingested cell, egesting its unwanted chloroplasts with other unassimilated material. Without a detailed chloroplast budget in the cultures, these two possibilities cannot be resolved.

Mixotrophic ciliates need to ingest food not only to obtain chloroplasts for growth but also to replace them

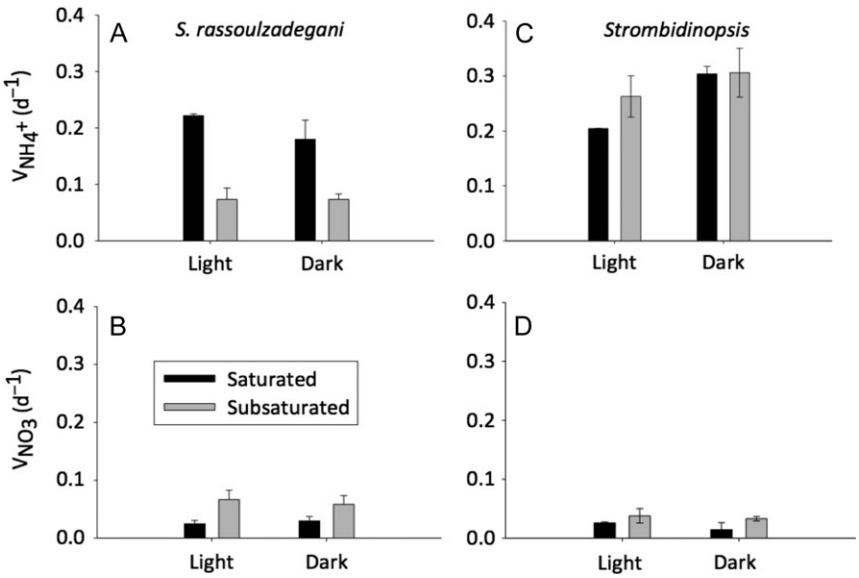

Fig. 5. Inorganic N uptake in the mixotroph (**A**: ammonium; **B**: nitrate) and the heterotroph (**C**: ammonium; **D**: nitrate). There were no effects of light in any of the comparisons. (A) Prior high food concentration led to higher ammonium uptake. (B) Prior low food concentration led to higher nitrate uptake. (C) Prior food concentration did not affect ammonium uptake. (D) Prior low food concentrations had a small but significant effect on nitrate uptake.

even when not dividing. Replacing chloroplasts is thus a "tax" on growth efficiency and the ciliate may minimize this by slowing chloroplast turnover at subsaturating food concentrations. This is supported by previous work demonstrating that *S. rassoulzadegani* retains plastids rather than digesting them when starved and replaces them quickly when food is available (Schoener and McManus, 2012).

At low food concentrations, carbon gross growth efficiencies (GGE_C) were greater for the mixotroph than for the heterotroph. The highest measured GGE_C was ~1.0 (i.e. 100% of ingested food becomes new ciliate biomass). Under food-saturated conditions, ciliates were ~50% larger than at lower food levels, but this extra volume may be partly due to the presence of more chloroplasts. It could be argued that being more full of chloroplasts (or food vacuoles for that matter) is not the same as having synthesized more cell constituents, which are what determines true growth. The presence of free chloroplasts thus complicates the calculation of growth efficiency because biomass is added directly without biosynthesis. Even with the caveat from our biovolume experiment that the highest GGE may be closer to 0.7 if we account for reduced ciliate size at low food levels, it is still considerably higher than that of the heterotroph. However, because the mixotroph continued to increase its ingestion rate when saturated for growth, it had much lower GGE than the heterotroph at high food concentrations. The higher growth efficiency of the mixotroph at low food concentrations was due primarily to lower ingestion rather than markedly higher growth and this indicates another paradox of mixotrophy. At low food concentrations, the mixotroph needs to ingest food for essential nutrients and to obtain chloroplasts for daughter cells, but because it cannot grow mixotrophically in the dark, even with abundant food, its growth is limited to the light period (McManus *et al.* 2012). Growth of the heterotroph, on the other hand, is independent of light, so it can achieve similar daily growth rates even at low food. Thus, while mixotrophy might seem to be an ideal trophic strategy, the ciliate's inability to control its stolen chloroplasts, or possibly even to digest them, limits the benefits of this nutritional mode. One cautionary note to this is that laboratory results may not fully reflect what happens in the environment, where diverse food sources may support some dark growth in mixotrophs.

Inorganic carbon uptake by the mixotroph ranged from 0.08 to 0.11 d^{-1}, similar to other mixotrophic Strombidiidae. For example, Stoecker *et al.* (1989) found that *Strombidium chlorophilum* and *S. capitatum* had uptake rates of 0.16 d^{-1} and 0.08 d^{-1}, respectively. *S. rassoulzadegani* had lower chlorophyll-specific uptake than *S. conicum* or *S. capitatum* (Supplemental Table 1), but very similar cell-specific uptake rates. The mixotrophic oligotrich *Laboea strobila* has highly variable uptake, ranging from 0.025 to 0.85 d^{-1} (Stoecker *et al.*, 1988; Putt, 1990a, 1990b) (Supplemental Table 1). At the lowest

growth rate we observed ($0.68\ d^{-1}$), inorganic uptake was ~16% of the total carbon budget.

Mixotroph inorganic carbon uptake did not vary with food concentration. This is not surprising because chloroplast content in the ciliate appears to be relatively invariant across food levels (Schoener and McManus, 2012). It suggests that in the short term photo- and heterotrophy are not coupled, and the photosynthetic rate does not depend on ingested nutrients. Thus, the relative importance of inorganic carbon uptake for growth declines as growth rate increases, emphasizing the overarching importance of ingestion for growth.

There was a significant impact of prior high food concentration on inorganic carbon uptake in the heterotroph, *Strombidinopsis* sp., likely due to still-active ingested algal cells in food vacuoles. Another study found a similar effect in the ciliates associated with brown band disease in coral; the ingested coral zooxanthellae were still actively photosynthesizing within the ciliate (Ulstrup *et al.*, 2007). The net effect is small compared to ingestion, but this carbon subsidy was equivalent to ~5% of V_{max} in our study. The important contrast here is that inorganic carbon uptake is most significant for the mixotroph at low food concentrations and for the heterotroph when food is abundant. The phenomenon of heterotrophs performing photosynthesis with ingested algae points out that all grazers are potentially mixotrophs and that incorporation of inorganic carbon by grazers exists on a continuum between almost pure heterotrophy (*Strombidinopsis* at low food concentrations) to almost pure phototrophy (*M. rubrum* in blooms).

Another way to assess the potential impact of ingested chloroplasts on the mixotrophic ciliate's carbon budget is to compare GGE to total growth efficiency (TGE), defined as the proportion of the total carbon uptake (ingestion plus inorganic) that results in new biomass. TGE was calculated as growth rate divided by ingestion rate plus inorganic carbon uptake rate. In the $^{14}CO_2$ uptake experiments, the difference between TGE and GGE was negligible for the heterotroph because inorganic uptake was very small, as discussed above. In the mixotroph, the maximum TGE was 0.73–0.78 in the two low food treatments. This is similar to reported levels from pure autotrophy (Herzig and Falkowski, 1989) and even higher than the value of 0.6 we estimated for the food alga *Tetraselmis* (Supplemental Table 1). A large part of an autotroph's energy budget is expended on chloroplast maintenance (Raven, 1997), so the mixotroph's strategy of replacing chloroplasts from food, rather than maintaining them or synthesizing their own, may provide a carbon efficiency advantage even over a strict autotroph. On the other hand, the mixotrophic ciliate *M. rubrum* has a maximum TGE of 0.74 even though it has the ability to synthesize chloroplasts (Johnson and Stoecker, 2005). Thus there remains much to be learned about mixotrophic energy budgets in relationship to chloroplast acquisition and functioning.

In the pulse-chase experiment, we did not see a significant difference between light and dark treatments. Therefore, the mixotroph likely does not recycle a significant amount of respiratory inorganic carbon into photosynthesis. Given that respiratory carbon is not recycled into photosynthesis, if all carbon (both ingested and taken up via phototrophy) that does not go to growth were excreted, then ~22% of the inorganic uptake should be excreted at the lowest food concentration, based on the TGE of 0.78. The best fit to our data more closely resembles an 80% respiration scenario (Fig. 4), leading us to conclude that most photosynthate in the mixotroph is respired. This agrees well with previous work with *L. strobila*. In that mixotroph, most inrganic carbon went into sugars, which were subsequently respired, with only ~22% of the photosynthate going into structural elements (Putt, 1990a, 1990b).

Inorganic nitrogen uptake was small and similar in the two ciliates. We do not have direct evidence for the mechanism, but ammonium transporters have been found in the transcriptome of the mixotrophic ciliate *M. rubrum* (Qiu *et al.*, 2016), so it seems likely that active uptake is involved. Though not as dramatic as for carbon, the mixotrophic ciliate also had higher nitrogen growth efficiency (GGE_N) at low food concentrations and lower GGE_N at high food concentration, compared to the heterotroph.

As a percentage of the mixotroph's total N input (uptake plus ingestion), ammonium uptake was 3.4% at saturating food concentrations and 4% at subsaturating food concentrations; nitrate uptake was only 0.18% and 1.72%, respectively. Inorganic uptake in the heterotroph was generally similar to that of the mixotroph, with ammonium representing 2.3% of the total uptake at saturating food concentrations and 8% when food concentrations were subsaturating. Nitrate uptake as a percentage of the total nitrogen was similar to that of the mixotroph, 0.3% and 1.4% at saturating and subsaturating food concentrations, respectively. We do not have any data on the incorporation of inorganic N into cellular constituents in the ciliates, so these are maximal estimates of its contribution growth.

CONCLUSIONS

Despite the energy subsidy received by mixotrophs via photosynthesis in chloroplasts retained from their food

(up to 16% of total C intake), maximum growth rates were similar to those of a heterotroph. The largest difference between the two ciliates was in GGE, which was much higher in the mixotroph at low food levels, but lower than the heterotroph at high food levels. Although inorganic carbon uptake was measurable in the heterotroph when they were feeding at high algal concentrations, it made a negligible contribution to growth. Inorganic carbon uptake was invariant with food level in the mixotroph. Both ciliates were able to take up dissolved N as nitrate or ammonium but these did not contribute significantly to growth. While the energy subsidy from retained chloroplasts is significant, mixotrophs pay a price relative to heterotrophs in diminished growth in the dark, leading to similar overall growth via the two metabolic modes.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Plankton Research* online.

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