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Role of the chloroplasts in the predatory dinoflagellate *Karlodinium armiger*

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ABSTRACT: *Karlodinium armiger* is a phagotrophic dinoflagellate that synthesizes several small chloroplasts of haptophyte origin. It depends on light, but it grows very poorly in standard nutrient growth media (f/2) without food. When fed prey in the light, growth rates increase dramatically (μ = 0.65 d^{-1}), suggesting that it relies heavily on phagotrophic nutrition. To explore the reasons for this phenomenon and the role of the plastids for the growth of *K. armiger*, we measured C^{14} fixation rates, chl *a* content and growth rates as a function of irradiance in short- and long-term prey-starved cultures. In starved cultures, rates of photosynthesis were relatively high and increased as a function of irradiance, even though growth rates remained low. In fed cultures, both ingestion and growth rates of *K. armiger* increased as a function of irradiance in a saturating manner. Cellular chl *a* contents and photosynthetic rates were ~40 and ~70% lower, respectively, in long-term compared to short-term starved phototrophic cells at irradiances of 70 to 250 µmol photons m^{-2} s^{-1}. Carbon fixation was important for achieving high mixotrophic growth rates, especially in prey-limited conditions. However, under prey-saturated conditions, *K. armiger* seems to obtain most of its carbon through phagotrophy. Our data suggest that feeding stimulates the photosynthetic machinery under nutrient limitation, and that the poor phototrophic growth capacity of *K. armiger* in the absence of prey seems to be due to a lack or a very poor ability to take up essential inorganic nutrients.

KEY WORDS: Mixotrophy · Photosynthesis · Phytoplankton · Growth · Ingestion · Dinoflagellates · Harmful algae

INTRODUCTION

Eukaryotic microalgae evolved from phagotrophic 'protozoa' through the structural and genetic fixation of photosynthetic machineries, viz. plastids or chloroplasts, derived from prey located inside food vacuoles (Delwiche 1999, Archibald 2009, Raven et al. 2009). The diversity of plastids is best represented within the dinoflagellates, where at least 9 plastid types of different endosymbiont origin have been identified (e.g. Daugbjerg et al. 2000). A special type of chloroplast is found in the recently described dinoflagellate family Kareniacea, including the genera *Karlodinium*, *Karenia* and *Takayama* (Daugbjerg et al. 2000, Takishita et al. 2004, Bergholtz et al. 2006). It differs from the typical peridinin-containing dinoflagellate plastid in that it has replaced the original secondary red-algal endosymbiont with a haptophyte endosymbiont (Patron et al. 2006). These tertiary plastids are permanently incorporated and synthesized in all species of Kareniacea (de Salas et al. 2005, Bergholtz et al. 2006), and most species can survive and grow strictly phototrophically in the light in the absence of prey in standard inorganic nutrient media, such as f/2 (Li et al. 1999, Garcés et al. 2006, Berge et al. 2008a).

A particularly interesting aspect of the kareniacean species is that closely related species and strains seem to cover a large range of nutritional strategies, from primarily phototrophs to primarily phagotrophs

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(Li et al. 1999, Berge et al. 2008a, Bachvaroff et al. 2009, Calbet et al. 2011). Some strains grow just as fast as phototrophic dinoflagellates with the typical peridinin-containing chloroplasts, while others, like *Karlodinium armiger* strain K0688 and probably also strains of *Karlodinium australe,* barely grow in the light even at high irradiances (DeSalas et al. 2005, Berge et al. 2008a,b). Reported carbon-specific ingestion rates are quite high (2.0–5.0 g C g C^{-1} d^{-1}) and in fact similar to that of typical heterotrophic protozoa (Berge et al. 2008b). The 'animal-like' and omnivorous nature of *K. armiger* is best described by its ability to immobilize, kill and feed on much larger copepods and metazoans (Berge et al. 2012). Thus, despite the fact that *K. armiger* always synthesizes plastids and is obligatorily dependent on light for survival, the nutritional configuration seems to be close to the phagotrophic end of the mixotrophic spectrum (Jones 1994, Berge et al. 2008a, Andersen et al. 2015).

Chloroplasts are costly to produce in terms of carbon, nutrients and energy (Raven 1984), which raises the question of why a species like *K. armiger* continues to produce them. Several possibilities exist: (1) The plastids of *K. armiger* may serve essential functions other than carbon fixation, such as ATP or reductant provider for biosynthesis (Geider & Osborne 1989). (2) Photosynthesis may serve as the source of carbon for respiration (Putt 1990, Flöder et al. 2006), while biosynthesis depends on prey-derived carbon. The organism may be mainly heterotrophic, with the chloroplasts only serving for survival when prey is limiting. This has been observed in a few chrysophytes (*Ochromonas*/*Poterioochromonas*), where low photosynthetic rates may only cover basal respiratory needs (Sanders et al. 1990). (3) Rates of carbon fixation are high and can support fast growth rates, but the species depends on ingestion (phagotrophy) of essential inorganic nutrients (Stoecker 1998, Mitra et al. 2016). This nutritional strategy may be important for the recently discovered and ecologically important plastidic mixotrophic nano-picoplankton dominating vast oceanic and oligotrophic areas (Hartmann et al. 2012, Mitra et al. 2014).

To understand the reason for the low phototrophic growth and the role of the photosynthetic machinery in the avid predator *K. armiger*, we measured the growth and photosynthetic rate as a function of irradiance, cell size and chl *a* content in a standard nutrient medium (f/2). We compared these variables in short- and long-term starved cells to investigate to what extent food starvation affects the photosynthetic machinery. To study the relative roles of photosynthesis and feeding in fed conditions (i.e. mixotrophy), we measured ingestion and growth rates of *K. armiger* at low and high prey concentrations as functions of irradiance. Based on the results from these experiments, we calculated and evaluated growth efficiencies (specific growth rate/specific carbon intake) required to explain the observed growth rates through the alternative carbon acquisition pathways: (1) phototrophy, (2) phagotrophy or (3) both (mixotrophy).

MATERIALS AND METHODS

Cultures and conditions

The cultures used in this study were obtained from the Scandinavian Culture Centre for Algae and Protozoa (www.sccap.dk). The stock culture of the mixotrophic dinoflagellate *Karlodinium armiger* (strain K0668) and the primarily phototrophic cryptophyte prey *Rhodomonas marina* (K0435) and *Teleaulax acuta* (K1486) were grown in f/2 medium, based on autoclaved seawater with a salinity of 32 PSU (for constituents of the medium see www.sccap.dk). Temperature was 15°C and the irradiance was 20 to 70 µmol photons m^{-2} s^{-1} based on a 14 h light:10 h dark cycle. Irradiance was measured in air with a spherical sensor (Li185B, Licor). Light was provided from cool-white fluorescent tubes in a 14 h light:10 h dark cycle, and adjusted using optically neutral screening sheets (3M). To obtain high cell concentrations of *K. armiger* for the experiments (see below), the stock culture was fed the cryptophyte *R. marina* every 2 to 5 d for ~2 wk under an irradiance of 70 µmol photons m^{-2} s^{-1}. This culture was diluted into fresh medium and placed together with prey cultures (*T. acuta*) under 5 experimental irradiances (7, 20, 70, 85 and 250 µmol photons m^{-2} s^{-1}) for 14 d of light acclimation. The experimental prey cultures (*T. acuta*) were kept in exponential growth phase during acclimation, before the experiments were initiated. Acclimation to prey in mixed grazing treatments was conducted during the first 3 to 4 d of incubation, before the first sampling occasion.

Experiments and laboratory conditions

To understand the role of the plastids and phagotrophy in *K. armiger,* we conducted 2 experiments in a range of light intensities (7–250 µmol photons m^{-2} s^{-1}). The first focused on phototrophic conditions (no prey) and the second focused on mixotrophic condi-

tions (with prey). Both experiments were initiated by incubating acclimated cells (see above) in polycarbonate flasks (67 ml) illuminated from below. Previous experiments showed no significant difference in ingestion rates between standing and rotating flasks (Berge et al. 2008a). The prey *T. acuta* was chosen because of its much better motility in culture than previously used cryptophytes (e.g. *R. salina* and *R. marina*). Both *K. armiger* and *T. acuta* swam readily, and the cells were evenly distributed in the experimental flasks during the incubation period.

During all experiments, pH was measured using a 2-point calibration pH meter and kept below pH 8.7 for both mixed and monocultures (phototrophic). This was done based on previous observations of the effects of high pH (>8.8) on *K. armiger* (Berge et al. 2008b). Temperature was 15°C and salinity was 32 PSU. All treatments (mono- and mixed cultures) were conducted in 3 replicate flasks. F/2 medium was used throughout the study.

In Experiment 1 (Expt 1), we focused on the role of the plastids in carbon fixation, and growth potential under strict phototrophic conditions (unfed cultures). Our specific hypotheses were: (1) the low growth rate in strictly phototrophic *K. armiger* culture is due to very low rates of carbon fixation, (2) *K. armiger* does not have sufficient control over the plastid to photoacclimate (i.e. regulate chlorophyll levels according to environmental irradiance) and (3) food starvation has no effect on the functioning of the plastid. Thus, we studied the effects of irradiance on growth rates (μ, d^{-1}), cellular carbon fixation rates (C-fix, pg C $cell^{-1}$), cell size (μm^3) and chl *a* concentration (pg chl *a* $cell^{-1}$) in strictly phototrophic cultures (no prey), and compared these in short-term (1 d) and long-term (14 d) prey-starved cells at different irradiances. The long-term prey-starved light treatments were initiated by incubating 2000 cells ml^{-1} of *K. armiger* that had depleted a prey population 14 d prior to the start of the experiment. The short-term prey-starved treatments were initiated by mixing 500 cells ml^{-1} of the *K. armiger* and 2000 *T. acuta* cells ml^{-1} (predator: prey cell ratio of 1:4), and letting the predator deplete the prey population. The measurements of samples for cell size, chl *a* content and C-fixation rate were taken 1 d after the presence of prey cells. Earlier studies on the cell size of *K. armiger* feeding on cryptophytes (see Fig. 7 in Berge et al. 2008a) and the presence of food vacuoles as a function of time showed that cryptophytes were quickly digested, and not kept as active kleptochloroplasts. To confirm the absence of food vacuoles, we carefully examined the short-term prey-starved cells directly before measurements, using inverted and epi-fluorescence microscopy at 200 to 400× magnification. This showed that only under 3 of the 5 irradiance levels provided, i.e. 20, 85 and 250 μmol photons m^{-2} s^{-1}, the incubations lasted long enough to assume no influence of prey cells and prey inside food vacuoles on our measurements.

In Expt 2, we focused on the relative importance of phototrophy and phagotrophy in the carbon acquisition in *K. armiger* at mixotrophic growth conditions. We hypothesized (4) that growth was based on carbon from ingested prey only. We therefore initiated a grazing experiment to measure growth and ingestion rates in prey-saturated and prey-limited cultures under different irradiance levels (i.e. 7, 20, 70, 85 and 250 μmol photons m^{-2} s^{-1}). The feeding experiment was initiated by mixing *K. armiger* and the prey in different initial predator:prey cell concentration ratios (ranging from 1:4 to 1:25) and allowing them acclimate to food for 3 d before the first sampling. Samples for cell concentration were taken every 1 to 2 d for up to a week. Ingestion rates were based on the change in prey concentration in the mixed treatments compared with the change of prey concentration in monocultures (control) during the sampling interval. Prey concentrations were chosen based on the functional response of *K. armiger* feeding on different prey types under high irradiance in the laboratory (180 μmol photons m^{-2} s^{-1}; Berge et al. 2008b). The average prey concentrations in the prey-saturated incubations were from 6000 to 25 000 cells ml^{-1} in the low light treatments and the high light treatments, respectively, corresponding roughly to 480 to 2000 μg C l^{-1}. In the prey-limited incubations, average prey concentrations were from 500 to 4000 cells ml^{-1} in the low and the high light treatments, corresponding to 40 to 320 μg C l^{-1}.

Measurements

Cell concentrations

Cells were fixed in acidic Lugol's (final concentration 1%) and enumerated using a Sedgwick-Rafter counting chamber. At least 300 cells were counted using a 10× objective on a compound microscope. In mixed-culture treatments, containing the *K. armiger* and the cryptophyte prey, all samples were enumerated manually. In monocultures of both *K. armiger* and the prey *T. acuta*, half of the samples were counted manually while *in vivo* fluorescence was measured in all samples. A correlation between cell

concentration and relative fluorescence units (R^2 = 0.98) was used to convert measurements to cell concentration. *In vivo* fluorescence was measured using a Turner Trilogy benchtop fluorometer.

Cell size

Cell dimensions (length and width) were measured by mixing an equal number of cells from 3 Lugol's fixed samples for each treatment (taken at 3 different time points in the exponential growth period). The cells were allowed to settle for at least 5 h in multidish wells (96-well plate). Settled cells were photographed using an inverted microscope with a 20× objective connected to a digital Canon camera. The length and width of the first 30 cells encountered on enlarged micrographs were measured manually, using the ruler tool in Adobe Photoshop CS3. Pixels were converted to µm using a high precision object-micrometer. Biovolume was calculated according to Hillebrand et al. (1999) and assuming ellipsoids with length > width.

Chl *a* content

Cellular chl *a* content was measured according to Skovgaard et al. (2000). Culture samples of 10–20 ml were filtered onto glass fibre filters (Poretics), and 5 ml of 96% ethanol were added to the filters in glass tubes wrapped in aluminium foil. The samples were extracted cold (4°C) overnight in the dark. The filters were ground and the slurry filtered onto new filters, resulting in clear filtrates for measurements in borosilicate glass tubes (Turner). We used a Turner Trilogy bench top fluorometer equipped with blue excitation filter for extracted chl *a*. To allow quantification of chlorophyll in µg chl *a* l^{-1}, the fluorometer was calibrated against a pure chl *a* standard (2.134 mg chl *a* l^{-1}) of cyanobacterial origin (Hørsholm). Triplicate cell counts of the filtered cultures served as basis for the estimation of cellular chl *a* content.

Photosynthesis

Carbon fixation rates (pg C $cell^{-1}$ d^{-1}) were determined by measuring C^{14} fixation rates according to Riisgaard & Hansen (2009). Samples (2 ml) were transferred into 20 ml glass scintillation vials. For 3 replicate samples per treatment, 20 µl of $NaH^{14}CO_3$ was added and incubated for 3 h. Dark vials were wrapped in aluminium foil while light vials were incubated at the experimental light levels. Specific activity was measured by adding 100 µl of the mixture to new vials containing 200 µl phenylethylamine. Carbon fixation was measured in the 1.9 ml culture by adding 2 ml of 10% glacial acetic acid in methanol and drying overnight at 60°C, before resuspension in 2 ml of distilled water and 10 ml Packard Insta-Gel Plus scintillation cocktail. Caps were replaced (Skovgaard et al. 2000) before the activities were measured using a Packard Tri-Carb liquid scintillation analyser. ^{14}C fixation rates (C-fix, pg C $cell^{-1}$ d^{-1}) were calculated according to Parsons et al. (1984). Dissolved inorganic carbon in the medium was measured with an infrared gas analyser (225-Mk3, Analytic Development).

Calculations and statistics

Growth rates

Exponential growth rates were calculated as the logarithm of change in cell concentrations, dx divided by the incubation period (d$t = t_2 - t_1$):

$$\mu = dx / dt = [\ln(X_{t2}) - \ln(X_{t1})] / (t_2 - t_1) \quad (1)$$

where X_{t1} and X_{t2} are the cell concentrations at the start and end of the 1 to 3 d period, respectively. Balanced growth rates were estimated by averaging the growth rates over at least 3 sampling points corresponding to 3 to 8 d of growth in each replicate flask.

Ingestion rates

Cellular ingestion rates (U) were calculated based on the disappearance of prey in the mixed flasks compared to the prey growth in monocultures, using the method described by Skovgaard et al. (2000). Here, the 2 following equations were used to iteratively (steps of 0.01 h) calculate ingestion rate over the sampling interval (t = 1 to 3 d):

$$dX/dt = \mu X \quad (2)$$

and

$$dY/dt = \mu_y Y - IR X \quad (3)$$

where Y is the prey concentration, μ_y is the prey growth rate in monoculture, X is the predator concentration (cells ml^{-1}), µ is the exponential growth rate of the predator during the incubation period, and IR is the ingestion rate (cells $cell^{-1}$ d^{-1}). To estimate

balanced ingestion rates, ingestion rates were averaged over a period of at least 3 d, corresponding to at least 2 sample intervals. Over this sample interval, the change in prey concentration was less than 36%. Averaging over several days was done to overcome problems of estimating growth rates of protists associated with daily cycles of cell division and ingestion.

Potential carbon sources and growth efficiencies

To understand the relative roles of photosynthesis and feeding in carbon acquisition during mixotrophy (Expt 2), we evaluated theoretic growth efficiencies necessary to explain the observed growth rates in food-saturated and food-limited mixotrophic cultures, for 3 alternative nutritional configurations: (1) phagotrophy as the only carbon source for growth, (2) phototrophy as the only carbon source for growth and (3) both pathways as carbon sources for growth. Growth efficiencies were calculated as carbon-specific growth rate (μ) / carbon-specific intake rate (based on 1, 2 and 3 above), multiplied by 100%. To estimate carbon-specific intake rates (pg C pg C^{-1} d^{-1}) through both photosynthesis and ingestion, we used the relationships between cellular biovolume (V, μm^3) and carbon (C) described by Menden-Deuer & Lessard (2000) for protists in general (excluding diatoms):

$$C\,(\text{pg C cell}^{-1}) = 0.239\ V^{0.939} \quad (4)$$

(1) *Phagotrophy as the only carbon source.* Assuming that carbon for growth only enters through the phagotrophic pathway, the theoretical growth efficiency of ingested prey required to explain the observed growth rates (μ_{mix}) in the grazing experiment (GE_{phago}) was calculated as:

$$GE_{phago} = 100\% \cdot \frac{\mu_{mix}}{IR} \quad (5)$$

where IR is the daily carbon specific ingestion rate.

(2) *Carbon from phototrophy only — phagotrophy for nutrients.* Phototrophic growth efficiency (GE_{photo}) in the non-fed phototrophic treatments was calculated as:

$$GE_{photo} = 100\% \cdot \frac{\mu_{mono}}{\text{C-fix}} \quad (6)$$

where C-fix is the daily carbon specific photosynthetic rate (pg C pg C^{-1} d^{-1}) and μ_{mono} is the phototrophic growth rate. To evaluate the potential carbon gains through phototrophy during mixotrophy in Expt 2, we used the results for photosynthetic rates in Expt 1. We estimated theoretic phototrophic growth efficiencies that would explain the observed growth rates in high and low food concentrations as functions of irradiance, with photosynthesis as the only carbon source. This was done by replacing the observed growth rate from Expt 2 in the equation for GE_{photo}.

(3) *Both pathways for carbon.* For the alternative that carbon enters *K. armiger* through both pathways, the mixotrophic growth efficiency (GE_{mixo}) was based on ingestion and growth rates from Expt 2 and the range of photosynthetic capacity similar to strictly phototrophic cells (Expt 1) and was calculated as:

$$GE_{mixo} = 100\% \cdot \frac{\mu}{IR + \text{C-fix}_{treat}} \quad (7)$$

where the subscript (C-fix_{treat}) refers to either short-term or long-term prey-starved cultures and μ refers to the observed growth rate. This range of phototrophic capacity is assumed to reflect situations where the photosynthetic machinery is stimulated (short-term prey-starved) or down-regulated due to feeding (long-term prey-starved).

To evaluate the potential limiting nutrient gain through phagotrophy, we assumed a similar ratio between carbon to limiting nutrient in the predator and the prey and considered nutrient growth efficiencies, which are similar to carbon-based phagotrophic growth efficiencies. Thus,

$$GE_{nut} = GE_{phago} \quad (8)$$

Statistics

We used linear and non-linear regression models to test the statistical significance of treatment effects on the measured variables (growth and ingestion rates, cellular carbon fixation rates, biovolume and chl *a* content). To determine differences between specific treatment means, we used Tukey's HSD tests. Results were considered significant at $p < 0.05$. To test whether growth and ingestion rates were significantly different from zero, 95% confidence intervals of the mean (n = 3, df = 2) of a small sample (*t*-distributed) were used. The analyses were conducted using R statistical software, and non-linear regressions were done using the 'drc' package. Growth rates were fitted to 3 parameters, while ingestion rates were fitted to a common 2-parameter Michaelis-Menten equation:

$$\mu(I) = c + \frac{d - c}{1 + e / I} \quad (9)$$

$$IR(I) = \frac{d}{1 + e / I} \quad (10)$$

where c is the growth rate when irradiance (I) is 0, d is the maximum rate, and e is the half saturation constant. The non-linear fits were used for addressing carbon budgets as a function of light intensity.

RESULTS

Expt 1: photosynthetic capacity

Without prey, the growth of *Karlodinium armiger* was low even under high irradiances, and cell concentrations increased or decreased only slightly (Fig. 1a–e). When food was still present in the fed cultures, cell concentrations of *K. armiger* increased and growth rates were higher (from ~0.04 to 0.6 d^{-1}) under all irradiances (Fig. 1f–j; Tukey's HSD tests, $p < 0.05$). At the lowest irradiance (7 µmol photons m^{-2} s^{-1}), the prey concentrations initially decreased from ~2000 to 1000 cells ml^{-1} (first 13 d of incubations), while cell concentrations of *K. armiger* remained constant (Fig. 1f). We added additional prey to investigate whether *K. armiger* was able to increase the growth rate by feeding under the low irradiance. At prey concentrations from ca. 5000 cells ml^{-1}, the cell concentration of *K. armiger* increased slightly, with a growth rate of 0.04 ± 0.01 d^{-1}. More prey was added after 26 d to reach a prey concentration >10 000 cells ml^{-1}, but this did not further increase the growth rate (Fig. 1f). At higher irradiances (20–250 µmol photons m^{-2} s^{-1}), prey and *K. armiger* cell concentrations increased initially as a function of time (for ~5–10 d), but when the predator reached a certain cell concentration, the prey concentrations rapidly decreased (Fig. 1g–j). After 18 to 22 d of incubation under 20, 85 and 250 µmol photons m^{-2} s^{-1}, the prey populations were depleted (Fig. 1g,i,j).

At the time of measurement of the physiological properties (arrows in Fig. 1) of both short- and long-term prey-starved cells, growth rates were not different from 0 and did not increase as a function of irradiance (Fig. 2a). However, despite this lack of strict phototrophic growth (Fig. 2a), the cellular carbon fixation rates increased as a function of irradiance (Fig. 2b) in both treatments. Cellular carbon fixation rates were 2.5 to 3.4 times higher in the short-term than in long-term prey-starved cultures (Fig. 2b). Apparently, light saturation for photosynthesis was not achieved for phototrophic *K. armiger* at the highest irradiance (Fig. 2b).

The cell size of *K. armiger* increased slightly as a function of irradiance from ~1200 to 1400 $µm^3$ in the long-term starved cultures (Fig. 2c) (Linear Model,

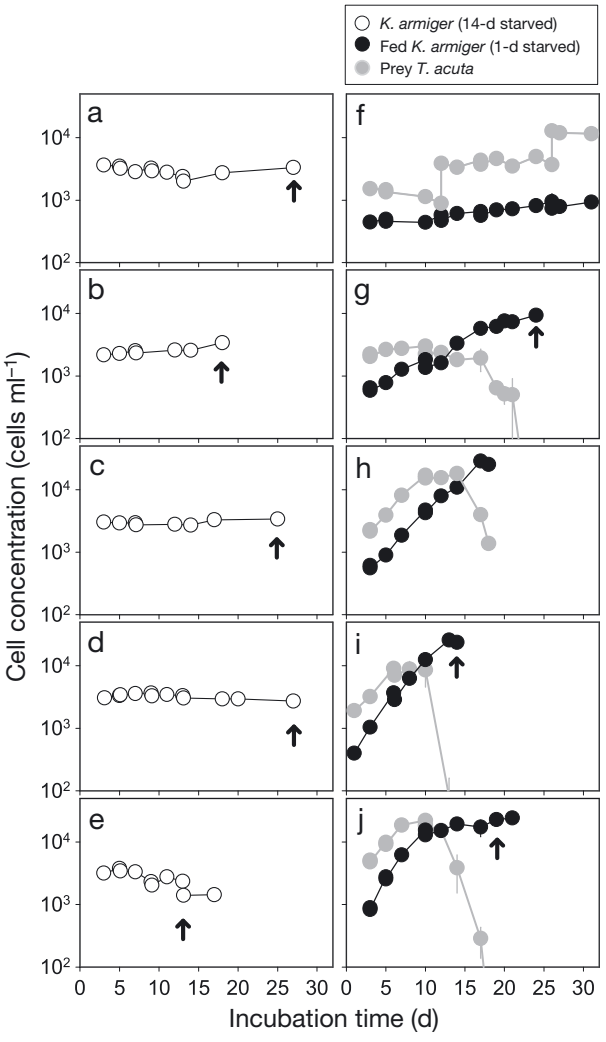

Fig. 1. Cell concentrations of (a–e) phototrophic *Karlodinium armiger* (white) and (f–j) *K. armiger* (black) fed *Teleaulax acuta* (grey) as a function of time under 5 irradiances: (a,f) 7, (b,g) 20, (c,h) 70, (d,i) 85 and (e,j) 250 µmol photons m^{-2} s^{-1}. Data points are means of 3 replicate cultures and error bars are SE. Arrows indicate time of measurements of carbon fixation, chlorophyll content and cell size for comparison between long-term starved cells (a–e) and short-term starved cells (g, i and j).

$p < 0.05$). The cell size was significantly lower in the short-term starved phototrophic cultures (1100 $µm^3$) than in the long-term starved cultures (1400 $µm^3$) at irradiances >70 µmol photons m^{-2} s^{-1}, but similar at 20 µmol photons m^{-2} s^{-1} (Tukey's HSD tests, $p < 0.05$). This is likely due to recent cell division in the short-term starved cells (see Berge et al. 2008a).

The very low phototrophic growth rates (<0.05 d^{-1}) and the relatively high rates of carbon fixation resulted in very low phototrophic growth efficiencies under all irradiances (3–12%; Fig. 2d). For compari-

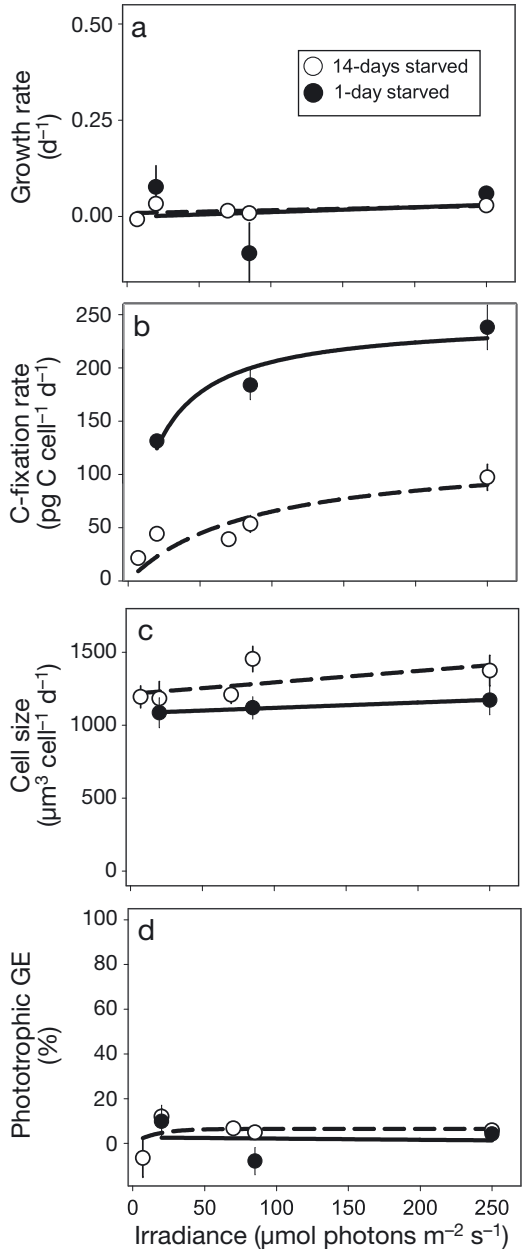

Fig. 2. (a) Growth rates, (b) photosynthetic rates, (c) cell size and (d) phototrophic growth efficiency (GE) as functions of irradiance in 1 d prey-starved *Karlodinium armiger* (black symbols, solid line) and 14 d prey-starved *K. armiger* (white symbols, dashed line). Points are means of 3 replicates and bars are SE. Lines are linear or Michaelis-Menten fits

son, in the typical phototrophic organism used as prey, *Teleaulax acuta*, the phototrophic growth efficiency increased as a function of irradiance to a maximum level of ~85%.

The cellular chl *a* content was approximately 30 to 40% higher in short-term compared to long-term

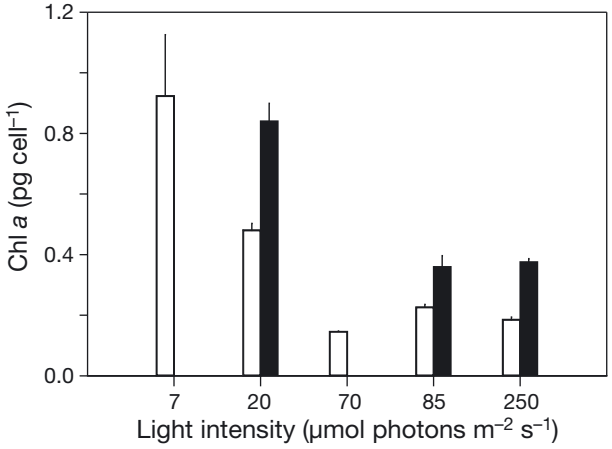

Fig. 3. Cellular chl *a* content as a function of irradiance in 1 d prey-starved *Karlodinium armiger* cells (black bars) and 14 d prey-starved cells (white bars). Data represent means of 3 replicates and bars are SE

starved *K. armiger* (Tukey's HSD tests, $p < 0.05$) at similar irradiances (Fig. 3). The chl *a* content decreased as a function of irradiance (Fig. 3) in both treatments.

Expt 2: ingestion and growth rate in prey-limited and prey-saturated mixotrophic cells

When *K. armiger* was fed plenty of prey (prey-saturation), maximum ingestion rate at the lowest irradiance (7 µmol photons m^{-2} s^{-1}) was only ~0.6 cells cell^{-1} d^{-1} (Fig. 4a). The ingestion rate at prey saturation increased as a function of irradiance and reached a maximum level of ~4 cells cell^{-1} d^{-1} (corresponding to ~1.5 d^{-1} in specific rate [pg C pg C^{-1} d^{-1}]; Fig. 4a). At prey saturation, the growth rate increased from 0.04 d^{-1} at 7 µmol photons m^{-2} s^{-1} to a maximum growth rate of ~0.60 d^{-1} at irradiances above 70 µmol photons m^{-2} s^{-1} (Fig. 4b). Growth rate and ingestion rate saturated between 20 and 70 µmol photons m^{-2} s^{-1} (Fig. 4b) under food saturation.

Despite the fact that *K. armiger* ingested ~5 times less prey at food-limitation than at food-saturation, the growth rates were similar in the 2 prey concentration treatments at irradiances above 70 µmol photons m^{-2} s^{-1} (Fig. 4a,b; Tukey's HSD tests, $p > 0.05$). Below 20 µmol photons m^{-2} s^{-1}, the growth rate was approximately 2 times higher at prey-saturation than at prey-limitation ($p < 0.05$), but similar at 7 µmol photons m^{-2} s^{-1} (Tukey's HSD tests, $p < 0.05$; Fig. 4a,b).

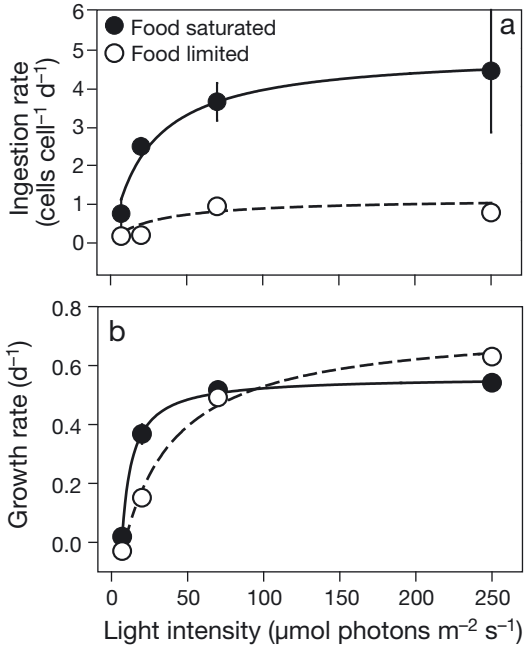

Fig. 4. (a) Ingestion rates and (b) growth rates of *Karlodinium armiger* in food-saturated (black symbols, solid line) and food-limiting conditions (white symbols, dashed line) as functions of irradiance. The prey organism was the phototrophic cryptophyte *Teleaulax acuta*, and nutrient concentrations were high (standard f/2 medium). Data points are means of 3 replicate cultures and bars are SE. Lines are Michaelis-Menten fits

Potential sources of carbon during mixotrophic growth

We converted the cellular data on photosynthesis (Expt 1) and ingestion (Expt 2) to carbon-specific values (pg C pg C^{-1} d^{-1}) in order assess the 3 alternative carbon sources for growth in *K. armiger* through either (1) phagotrophy, (2) phototrophy or (3) both during mixotrophic conditions (Fig. 5a,b).

(1) Carbon for growth from food only

At prey-saturation, growth efficiencies of food (GE_{phago}) approaching ~30% would explain the observed growth rates if phagotrophy was the only source of carbon (Fig. 5c,d). At prey limitation, however, growth efficiencies approaching 200% would be required to explain the observed growth rates, and phagotrophy alone could not provide enough carbon to explain the observed growth rates (Fig. 5d). This shows that photosynthesis was an important additional carbon source for growth at low prey concentrations.

(2) Carbon for growth from photosynthesis only

The phototrophic capacity of short-term prey-starved cells suggests that photosynthesis alone had the potential to fix enough carbon to support the observed growth rates during mixotrophic growth (Fig. 5c,d). If the photosynthetic machinery performs at the lowest level observed in long-term starved cultures, phototrophic growth efficiencies (GE_{photo}) exceeding 100% would be required to support the observed growth rates (grey field in Fig. 5c,d). On the other hand, if the photosynthetic machinery performs well and is stimulated by feeding, more realistic phototrophic growth efficiencies approaching 40% would explain the observed growth rates in both high and low prey concentration (Fig. 5a–d). This shows that if photosynthesis is performing well, carbon fixation may cover the carbon needs of *K. armiger,* while phagotrophy may only be used to obtain essential nutrients.

(3) Carbon from both photosynthesis and phagotrophy

Changes in photosynthetic capacity have little effect on total mixotrophic growth efficiency (µ/[C-fix + IR]) under food saturation (Fig. 5g). GE_{mixo} was only 9 to 11% lower under similar irradiances if the photosynthetic capacity was stimulated compared to depressed (Fig. 5g). However, changes in the phototrophic capacity have a large potential effect on total mixotrophic growth efficiency in prey-limited conditions (Fig. 5h). These findings suggest a stimulation of photosynthesis at low prey concentrations. If both pathways served equal roles in carbon acquisition under mixotrophic conditions, from 18 to 35% (food-saturation) and 50 to 80% (food-limitation) of the total carbon gains were due to photosynthesis (Fig. 5g,h), depending on the photosynthetic capacity (up or down regulated).

To summarize our main results, Expt 1 showed that the low phototrophic growth rates of *K. armiger* in the light could not be explained by low carbon-fixation rates and a photoheterotrophic (energy generation without carbon fixation) role of the plastids. The photosynthetic potential of *K. armiger* is similar to the typical phototroph, *T. acuta*, but depends on prey starvation. Chl *a* and photosynthesis was higher in 1 d than in 14 d prey-starved cultures, indicating nutrient limitation as the cause of slow growth in the light without food in cultures. Expt 2 confirmed previous observations, that light is essential for both growth and ingestion in this species (Berge et al.

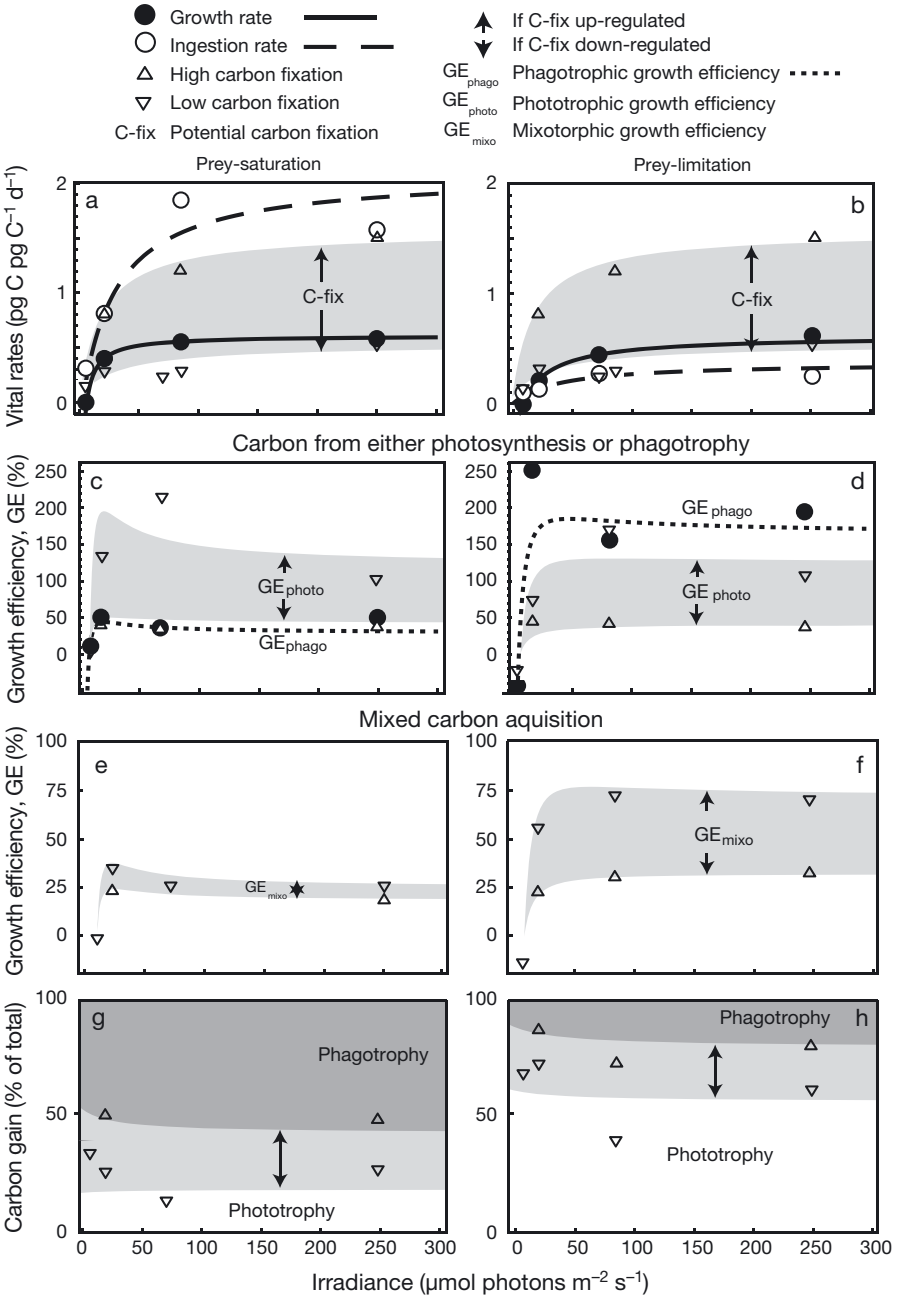

Fig. 5. Evaluation of potential carbon sources during mixotrophic growth at high and low prey concentrations. (a,b) Observed vital rates in (a) prey-saturated and (b) *Karlodinium armiger* cells fed low prey concentrations (prey-limited) as functions of irradiance; growth rate (black circles, solid line), ingestion rate (white circles, dashed line) and photosynthetic rates of the short term prey starved (triangle up) and long term prey starved cells (triangle down). The grey area in (a) and (b) thus represents the potential scope of phototrohpy depending on starvation state (short term, arrow up, long term, arrow down) observed in Expt 1, while ingestion (open circles) and growth rates (closed circles) were observed in Expt 2. C-fix refers to scope of phototrophy. (c,d) Potential phototrophic (grey area) (GE_{photo}) and phagotrophic growth efficiencies (dotted line) (GE_{phago}), based on either carbon sources alone, in (c) prey-saturated and (d) cells fed low prey concentrations. In (c) and (d), triangles refer to phototrophic growth efficiencies required to explain the observed growth rates if the level of photosynthesis was up-regulated to the level observed in short term prey starved (triangle up) or down-regulated to the level observed in long term prey starved (triangle down). (e,f) Total mixotrophic growth efficiency (GE_{mixo}), based on both carbon sources in combination, as a function of irradiance in (e) prey-saturated and (f) cells fed low prey concentrations. (g,h) The potential relative contributions of phototrophy and phagotrophy to the total carbon intake in (g) prey-saturated and (h) cells fed low prey concentrations with a mixed nutritional configuration with both carbon sources in combination. Grey area represents phagotrophy; white area represents contribution from phototrophy. Light grey area represents the scope of contributions depending on the state of the photosynthetic machinery (up- or down-regulated)

2008a). During mixotrophic growth, photosynthesis is the main carbon source for growth under prey-limitation, while phagotrophy dominates carbon acquisition in well-fed cells.

DISCUSSION

Phototrophic growth

The presented data suggest that the plastids of *Karlodinium armiger* fix enough carbon to support much higher growth rates than observed under high irradiances in the laboratory cultures using a standard growth medium (f/2). In stationary phase cultures, carbon-specific photosynthetic rates ranged from 0.13 to 1.5 d^{-1}, and *K. armiger* was also able to photo-acclimate and respond to low irradiances by increasing the cellular photosynthetic pigment content (Fig. 3). These observations suggest an important role of the plastids and their photosynthesis for growth in this species. Why, then, does it grow so poorly in the light, when inorganic nutrients in the standard laboratory growth medium are plentiful?

One possible explanation could be very high rates of respiration compared to photosynthesis (Ryther 1956). Laboratory data of dinoflagellate photosynthesis, growth and respiration suggest that approximately 45% of photosynthates are respired under balanced exponential light- and nutrient-saturated growth (Geider & Osborne 1989). Adolf et al. (2006) measured balanced carbon fixation and growth rates of *K. veneficum* across a range of irradiances and calculated average growth efficiencies of ~56% at high irradiance (150 µmol photons m^{-2} s^{-1}). This is lower than what has been found in the cryptophyte prey species *Storeatula major* and *Teleaulax acuta*, which was 86 and 85%, respectively (Adolf et al. 2006, present study), but it is a typical value for primarily phototrophic dinoflagellates (Falkowski & Raven 1997). We calculated much lower phototrophic growth efficiencies (0–12%) in *K. armiger*. We observed that at low irradiance, a carbon-specific photosynthetic rate of 0.13 d^{-1} supported survival (i.e. growth rate = ~0.0). This may be taken as a rough estimate of the cells' basal maintenance respiration rate. However, at higher irradiances, much higher carbon-specific photosynthetic rates from 0.50 to 1.46 d^{-1} were observed. Taken together, this suggests high rates of carbon excretion or formation of storage material. We observed large amounts of lipid accumulations in stationary-phase *K. armiger* cultures, suggesting that at least some of the carbon is stored.

The very low phototrophic growth efficiencies observed in *K. armiger* are typical for nutrient limited 'phytoplankton' (Ryther 1956). Thus, the poor phototrophic growth of *K. armiger* in nutrient-enriched medium may be due to a very low ability to take up essential inorganic nutrients from the medium used. The fact that we saw negative effects of food starvation on the phototrophic machinery supports the hypothesis of nutrient limitation as the cause for the very low phototrophic growth potential in *K. armiger* (Figs. 2b & 3). Similar observations have been reported for *K. veneficum* (Li et al. 1999) and for the mixotrophic dinoflagellate *Gyrodinium resplendens* (Skovgaard 2000). The degradation of chlorophyll, or chlorosis, is a typical effect on 'phytoplankton' under nutrient limitation (Greene et al. 1991, Geider et al. 1998), and it may serve to reallocate limiting nutrients from the photosynthetic machinery for structural biosynthesis and maintenance. The identity of the growth-limiting substance is at present unknown and may involve any essential macro- or micronutrient or even trace elements.

Effect of light on phagotrophy

Prey ingestion rates of *K. armiger* followed irradiance in a saturating manner (Fig. 4a), which is in contrast to what is typically found for strictly heterotrophic dinoflagellates, which are in general independent of irradiance (Stoecker 1998). Similar findings were reported for *K. veneficum* feeding on the cryptophyte *Storeatula major* at food saturated concentrations (Li et al. 1999). The effects of irradiance on ingestion rates have only been rigorously studied in a few other mixotrophic phytoflagellates: the marine dinoflagellates *Fragilidium subglobosum* and *Dinophysis acuminata* and the freshwater chrysophytes *Poterioochromonas malhamensis*, *Dinobryon cylindricum Ochromonas* sp. and *O. minima.* (Sanders et al. 1990, Caron et al. 1993, Li et al. 1999, Kim et al. 2008, Riisgaard & Hansen 2009). With respect to their light-dependent ingestion rates, *K. armiger* and *K. veneficum* resemble *D. acuminata*, *D. cylindricum* and *O. minima.*

K. armiger ingests prey, but cannot grow and survive when fed in complete darkness or at dim light, even when fed plenty of fresh prey regularly (Berge et al. 2008a). This suggests that food is simply not digested in the dark and that light is directly required for the functioning of the phagotrophic machinery. Several other mixotrophic plastidic dinoflagellates show an obligate dependence on light for survival

and growth (e.g. *K. veneficum*, *D. acuminata, F. subglobosum, Prorocentrum minimum, Heterocapsa triquetra* and *Ceratium furca*; Stoecker et al. 1997, Stoecker 1998). Hansen (2011) reviewed the literature on the role of photosynthesis for the growth of mixotrophic dinoflagellates and was only able to find 6 out of a total of 34 studied species, which could survive in the dark when supplied with food.

The mechanisms behind light-dependent ingestion rates are not understood at present. Light-aided digestion has even been reported in some heterotrophic dinoflagellates and ciliates (Skovgaard 1996, Strom 2001, Tarangkoon & Hansen 2011). In mixotrophs, it may reflect a primary role of phagotrophy for nutrient acquisition to support a preference for photosynthetic carbon assimilation. Alternatively or in combination, a strong preference for photosynthates relative to prey-derived carbon for respiration and energy provision may cause light-dependent phagotrophy (Putt 1990, Flöder et al. 2006). Further studies targeting the balance between carbon and nutrient flows through the alternative routes during mixotrophic growth are needed to fully understand the dependence on light for growth and ingestion in mixotrophic phytoflagellates.

Potential sources of carbon for *K. armiger* during mixotrophy

The relative importance of phototrophy and phagotrophy in carbon and nutrient acquisition during mixotrophic growth can be considered by evaluating growth efficiencies necessary to explain the observed growth rates in our feeding experiment. Growth efficiencies of strictly heterotrophic dinoflagellates vary between species and range from 10 to 70% with an overall average between 30 and 40% (Verity 1985, Straile 1997) (Fig. 5c,d). Phototrophic growth efficiencies of plastid-bearing dinoflagellates seem to be slightly higher, between 50 and 60% (Adolf et al. 2006) (Fig. 5c,d). In prey-limited conditions, phagotrophy alone provided only half of the required carbon, and growth efficiencies above 200% would explain the observed growth rates and show that photosynthetically derived carbon was used for growth during mixotrophy (Fig. 5c,d). The importance of phototrophy for growth is further supported by the fact that *K. armiger* had relatively high potential for carbon fixation under strictly phototrophic conditions (up to 1.5 pgC pgC^{-1} d^{-1} in short-term starved cells; Figs. 2b & 5). These photosynthetic rates were able to supply enough carbon to support the observed growth rates during mixotrophy in the feeding experiment. However, if photosynthesis was the only source of carbon, under both prey-saturated and prey-limited conditions, the photosynthetic machinery would have to be stimulated to the maximum level that we observed in the phototrophic cells (Fig. 5c,d). Stimulation through feeding may be important in cells exhibiting low ingestion rates, but it is very unlikely that *K. armiger* up-regulates phototrophy while engaging heavily in feeding.

Depending on the photosynthetic capacity of *K. armiger* in mixotrophic conditions, 60 to 90% or 20 to 50% of the total carbon gains were obtained through phagotrophy at prey saturation or prey limitation, respectively (Fig. 5e,f). Such a cooperative strategy for carbon acquisition seems to exist in other plastidic dinoflagellate species relying heavily on phagotrophy (e.g. *D. acuminata, Paragymnodinium shiwhaense, F. subglobosum* and *F. mexicanum*; Hansen 2011). The fact that growth rates were similar while ingestion rates varied 3 orders of magnitude between food-saturated and food-limited cells suggests a preference for prey-derived carbon in *K. armiger* (Fig. 5e,f). Using ^{14}C-labelled inorganic carbon and ^{14}C-labelled prey cells to track the fate of carbon through phototrophy and phagotrophy under mixotrophic growth conditions, Adolf et al. (2006) found the highest contribution from prey-derived carbon for the growth of mixotrophic cells of *K. veneficum.* They also reported a general preference for photosynthates for respiration in this species.

Total mixotrophic growth efficiency in *K. veneficum* based on carbon acquired through both photosynthesis and food uptake under high light and high prey conditions was found to be ~36% (Adolf et al. 2006). To achieve mixotrophic growth efficiencies of a similar level in our study would require an up-regulation of photosynthesis in prey-limited cultures (Fig. 5h). Support for such up-regulation is provided by previous reports of high growth rates (up to 0.5 d^{-1}) in cells engaging little in feeding (very low ingestion rates on sub-optimal prey species; Berge et al. 2008b). On the other hand, in cells heavily engaging in feeding, investments in the photosynthetic machinery seem to be associated with low carbon gains (Fig. 5g). This suggests that *K. armiger* down-regulates phototrophy in prey-saturated conditions. Support for this is provided by previous reports of very high ingestion rates in prey-saturated *K. armiger* cells fed optimal prey (up to 4.0 pgC pgC^{-1} d^{-1}; Berge et al. 2008b). Moreover, laboratory studies

indicate that mixotrophic dinoflagellates in general down-regulate their photosynthetic capacity during food-replete conditions, and display a decreasing relationship between photosynthesis and ingestion rate (e.g. *K. veneficum* and *F. subglobosum*; Hansen et al. 2000, Skovgaard et al. 2000, Adolf et al. 2006, Hansen 2011).

Potential nutrient gains through phagotrophy

We hypothesize that *K. armiger* cannot take up enough inorganic nutrients to support growth in unfed conditions. The very high investment in phagotrophy in this predatory species may compensate for the low investment in inorganic nutrient harvest. Several plastidic dinoflagellates are known to feed in order to obtain nutrients (e.g. the dinoflagellates *Heterocapsa triquetra*, *P. minimum* and *Ceratium* spp.; Stoecker 1998, Smalley et al. 2003). In *K. veneficum*, ingestion rates are elevated in nutrient-limited compared to nutrient-replete cells, suggesting an important role of phagotrophy for nutrient acquisition in this species as well (Li et al. 2000).

Assuming that the predator and prey have similar food quality in terms of the ratio between carbon and the limiting nutrient (Fig. 5b,d), nutrient acquisition through phagotrophy alone could not support the observed growth rates at prey limitation in our feeding experiment. However, significant species-specific differences in C:N:P ratios and trace element ratios exist (Rhee & Gotham 1980, Klausmeier et al. 2004), so differences in food quality between predators and prey species may be common. Our data show that phagotrophy alone can support the observed growth rates in prey limitation if the carbon: limiting nutrient ratio of *K. armiger* was ~2 times higher than that of the prey (Fig. 5c,d). Li et al. (2000a) used an elemental analyser to measure the carbon, nitrogen and phosphorus content of *K. veneficum* and the cryptophyte prey *Storeatula major* under different nutrient conditions. Their prey species, which is closely related to ours, had a similar C:N ratio as *K. veneficum* under nutrient-replete conditions, but 1.5 fold higher C:N in nutrient-limited cells (Li et al. 2000). C:P ratios were nearly twice as high in *K. veneficum* as in *S. major*, even in nutrient-replete conditions. The precise nature of limiting nutrient for *K. armiger* in phototrophic conditions is presently unknown, and trace element ratios might be involved (Stoecker 1998, Mitra et al. 2016). We are presently addressing growth limitation in *K. armiger* in more detail.

Ecological implication

Karlodinium spp. form harmful algal blooms associated with severe fish kills (Nielsen 1996, Garcés et al. 2006, Place et al. 2012). Blooms containing a mixture of *K. veneficum* and *K. armiger* have had devastating effects on the planktonic ecosystem and have killed tons of farmed fish and mussels in Alfacs Bay, Spain (Fernández-Tejedor et al. 2004). To avoid harmful incidences in aquaculture, it is important to understand the environmental conditions favouring the growth of these species. Natural bloom populations of *Karlodinium* spp. have been found to contain high levels of intraspecific variation in the investments in phototrophy and phagotrophy (Garcés et al. 2006, Bachvaroff et al. 2009, Calbet et al. 2011). This indicates that populations are highly adaptable, and suggests that succession in the dominant trophic mode occurs. During developmental ecosystem stages of new production, when inorganic nutrient concentrations are high (e.g. spring bloom or upwelling systems), investments in phototrophy and uptake mechanisms for inorganic nutrients provide the fastest growth rates. On the other hand, phagotrophy may become more important when inorganic nutrients are depleted and bound in biomass, during more mature stages (e.g. stratified summer periods in temperate eutrophic areas and hyper-oligotrophic deep sea surface waters). This suggests a succession from phototrophy to phagotrophy in seasonal latitudes.

K. armiger has been shown to feed on large metazooplankton (Berge et al. 2012), and it may thus affect the food web in several different ways. The negative effects on the entire food web of *Karlodinium* spp. populations may be related to the dominant trophic mode, as it has been shown that toxicity and harmful effects may be directly related to phagotrophy in *K. veneficum* (Sheng et al. 2010). To predict harmful occurrences of these and similar species (Deeds et al. 2002, Berge et al. 2012), considering the relative investments in the phototrophy, phagotrophy and nutrient harvest in populations through time and space may be more rewarding than more species-specific forecasting models. Our results stress that to capture synergic effects of feeding and photosynthesis for growth in mixotrophs, both carbon and nutrient fluxes must be addressed.

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