Research Article

Obligate mixotrophy of the pigmented dinoflagellate *Polykrikos lebourae* (Dinophyceae, Dinoflagellata)

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The marine sand-dwelling dinoflagellate *Polykrikos lebourae* possesses obvious gold-brown pigmented plastids as well as taeniocyst-nematocyst complex structures. Despite of the presence of the visible plastids, previous attempts to establish this species in culture all failed and thus the unavailability of cultures of this species has posed a major obstacle to further detailed exploration of ecophysiology of the dinoflagellate. Here, we isolated *P. lebourae* from sandy sediment of an intertidal flat on Korean western coast, successfully established it in culture, and have been maintaining the stock culture over the past 3 years. Using this stock culture, we explored phagotrophy and potential prey resources of *P. lebourae*, growth and grazing responses of *P. lebourae* to different prey organisms, the effect of prey concentration on growth and grazing rates and gross growth efficiency (GGE) of *P. lebourae* when fed three different prey organisms, and the growth kinetics of *P. lebourae* under different light regimes. *P. lebourae* captured prey cells using a tow filament and then phagocytized them through the posterior end. The dinoflagellate was capable of ingesting a broad range of prey species varying in size, but not all prey species tested in this study supported its sustained growth. GGE of *P. lebourae* was extremely high at low prey concentration and moderate or low at high prey concentrations, indicating that *P. lebourae* grows heterotrophically at high prey concentrations but its growth seems to be more dependent on a certain growth factor or photosynthesis of plastids derived from the prey. In the presence of prey in excess, *P. lebourae* grew well at moderate light intensity of 40 μmol photons m^{-2} s^{-1}, but did not grow at dim and high (10 or 120 μmol photons m^{-2} s^{-1}) light intensities. Our results suggest that the benthic dinoflagellate *P. lebourae* is an obligate mixotroph, requiring both prey and light for sustained growth and survival.

Key Words: benthic cryptophytes; gross growth efficiency; growth response; obligate mixotrophy; *Polykrikos lebourae*; sand-dwelling dinoflagellate

INTRODUCTION

Polykrikoid dinoflagellates have distinctive morphological features compared to other dinoflagellates as they form multinucleated pseudocolonies. The polykrikoid dinoflagellates divide into two genera, *Pheopolykrikos* Chatton and *Polykrikos* Bütschli, with the type species being *Pheopolykrikos beauchamphii* and *Polykrikos schwartzii*, respectively. At present, they include 8 species (i.e., *Ph. beauchamphii* in the genus *Pheopolykrikos* and *P. geminatum*, *P. hartmannii*, *P. herdmanae*, *P. kofoidii*, *P. lebourae*, *P. schwartzii*, and *P. tanit* in the genus *Polykrikos*). Among these species, classification of one species, *P. hartmanii* has been controversial, transferring

back and forth between the two genera over the past 80 years (Zimmermann 1930, Matsuoka and Fukuyo 1986, Hoppenrath et al. 2010) because there was no consistency about taxonomic keys (e.g., ratio of the numbers of zooids and nuclei of pseudocolonies, photosynthesis, a single-celled life cycle stage) to classify the two genera.

Polykrikoid species are all known to be marine planktonic dinoflagellates, except for the benthic species *Polykrikos lebourae* Herdman, which resides in intertidal sandy sediments. Pseudocolonies of *P. lebourae* consist of eight zooids and two nuclei, although reduced pseudocolonies comprised of four or five zooids with one nucleus have been observed (Hoppenrath and Leander 2007*b*). Pseudocolonies of *P. lebourae* are laterally flattened and lacking zooid borders although each zooid has its own a pair of flagella and cingulum. Descriptions of *P. lebourae* have included both heterotrophic and photosynthetic forms (Herdman 1923, Balech 1956, Dragesco 1965). The non-pigmented, heterotrophic form of *P. lebourae* is now classified as a different species, namely *P. herdmanae* Hoppenrath et Leander, because the molecular phylogeny placed it into a separate lineage from the photosynthetic form (Hoppenrath and Leander 2007*b*). *Polykrikos lebourae* contains both plastids and taeniocyst-nematocyst complex structures like *P. hartmannii*, but the plastid of *P. lebourae* exhibits unusual ultrastructure, having only two enveloping membranes, thylakoids in stacks of two, and central pyrenoids devoid of thylakoids. By contrast, *P. hartmannii* possesses typical plastids in peridinin-containing dinoflagellates having three stacks of thylakoids and enveloping by three outer membranes (Hoppenrath and Leander 2007*b*). Also, molecular phylogenetic relationships demonstrated that *P. lebourae* is closely related to several heterotrophic *Polykrikos* species rather than to other phototrophic species within polykrikoids, suggesting that the plastids of *P. lebourae* may be either acquired via an endosymbiotic replacement event following the reduction or loss of the peridinin-containing plastids, or be retained via kleptoplastidy (Hoppenrath and Leander 2007*a*, Tang et al. 2013). Despite the presence of plastids, however, previous attempt to establish *P. lebourae* in culture failed (e.g., Hoppenrath and Leander 2007*b*) and thus the unavailability of cultures of this species has posed a major obstacle to further detailed exploration of ecophysiology and evolution of plastid of the dinoflagellate.

Here, we isolated *P. lebourae* from sandy sediment of an intertidal flat on Korean western coast, successfully established it in culture by supplying a benthic cryptophyte, *Rhodomonas* sp., as prey, and have maintained stock culture over the past 3 years. Using this stock culture, we explored 1) phagotrophy and potential prey resources of *P. lebourae*, 2) growth and grazing responses of *P. lebourae* to different prey organisms, 3) the effect of prey concentration on the growth and grazing rates and gross growth efficiency of *P. lebourae* when fed three different prey organisms, and 4) the growth kinetics of *P. lebourae* at different light intensities.

MATERIALS AND METHODS

Sampling and culture set-up

Sediment samples were taken from Dongho beach of Korean western coast during low tide in November 2011. A trowel was gently scrapped over the surface of the sandy sediment and used to scoop sediment and ambient seawater into plastic bag. The samples were transported to the lab and observed at 100× to 400× magnification using an inverted microscope (IX-51; Olympus, Tokyo, Japan). The benthic dinoflagellate *P. lebourae* was isolated using a capillary pipette and grown in 30 psu f/2-Si medium (Guillard 1975) at 20°C under 14 : 10 of light : dark cycle of cool-white fluorescent light at 40 μmol photons m^{-2} s^{-1}, with the cryptophyte *Rhodomonas* sp. 2 (rCR04) provided as prey. Five benthic cryptophytes, *Chroomonas* sp. 1 (gCR07), *Chroomonas* sp. 2 (gCR09), *Rhodomonas* sp. 1 (rCR02), *Rhodomonas* sp. 2 (rCR04), and *Rhodomonas* sp. 3 (rCR05) and four benthic dinoflagellates, *Amphidinium* sp. (bdAmp01), *Heterocapsa* sp. (bdHet01), *Thecadinium kofoidii* (bdTK01), and *Prorocentrum fukuyoi* (bdPF03) were isolated using the same method described for *P. lebourae* and grown under the same culture condition as described above.

Microscopy and micrographs

Live specimens were observed using a bright field microscope (Axio imager A2; Carl Zeiss Inc., Hallbergmoos, Germany) equipped with differential interference contrast optics. Light micrographs were taken at 1,000× magnification using a photomicrographic system (AxioCam HRc; Carl Zeiss Inc.) coupled to the microscope.

Growth and grazing of *Polykrikos* with different prey organisms

Growth and grazing responses of *Polykrikos lebourae* were determined for nine different prey organisms:

five benthic cryptophytes, *Chroomonas* sp. 1 (gCR07), *Chroomonas* sp. 2 (gCR09), *Rhodomonas* sp. 1 (rCR02), *Rhodomonas* sp. 2 (rCR04), and *Rhodomonas* sp. 3 (rCR05), and four benthic dinoflagellates, *Amphidinium* sp. (bdAmp01), *Heterocapsa* sp. (bdHet01), *Thecadinium kofoidii* (bdTK01), and *Prorocentrum fukuyoi* (bdPF03). Stock cultures of prey species were diluted with f/2-Si medium to achieve the ratio of 1 : 30 of predator and prey based on biovolume, expect for *P. fukuyoi* adjusted to the ratio of 1 : 10. The diluted cultures of the cryptophytes and the dinoflagellates were distributed into a total of 24 sets of 48-well plate. Each set of the plate had three wells containing prey and predator mixtures as treatments and six wells containing either prey only or predator only as controls. Every two plates including treatments and controls for the nine prey species were fixed at each sampling day. Stock culture of *Polykrikos* used for this experiment was starved for two weeks prior to the set-up of the experiment. Three *Polykrikos* cells were individually added to the treatment wells including each prey organism. When the prey *Amphidinium* was depleted in the treatment cultures at the day 10, the prey cells were freshly supplied once again to achieve the final concentration of the ratio of prey and predator of 10 to 1. All treatments and controls were placed on the shelves under the same culture condition as described above. Each well plate including prey and predator mixture and predator only was fixed by adding 3 μL of Lugol's solution (final conc. 1%) into each well at 2-3 days intervals for first 10 days of the experiment period and thereafter at 5-7 days intervals for the remaining period. Abundances of the organisms were enumerated using Sedgwick-Rafter chambers at 100-200× magnification under a microscope (Olympus BX-50; Olympus). Cells present in optical transects of each chamber were summed until the entire chamber was examined, or until over 200 cells were counted. Growth rates of *Polykrikos* (μ) with different prey organisms were calculated using the following exponential growth equations:

$$\mu = \frac{lnN_1 - lnN_0}{t_1 - t_0} \qquad (1)$$

, where N_1 and N_0 are cell concentrations at t_1 and time t_0, respectively, and $t_1 - t_0$ is the time interval between samplings. Ingestion rate (*I*, in cells *Polykrikos*$^{-1}$ d^{-1}) of *P. lebourae* was calculated according to Kim et al. (2008): ingestion rates of *P. lebourae* were estimated from changes in prey cell numbers in treatments compared with prey densities in controls. Gross growth efficiency (GGE, %) was defined as predator carbon produced per prey carbon

ingested (Kim et al. 2008). GGE was calculated as follows:

$$GGE = \frac{C_{po}(e^\mu - 1)}{1 \times C_{pe}} \times 100 \qquad (2)$$

, where μ is growth rate (d^{-1}) of *Polykrikos*, *I* is ingestion rate (prey cells *Polykrikos*$^{-1}$ d^{-1}), C_{po} and C_{pe} indicate carbon contents of *Polykrikos* and each prey, respectively. Cell volume was determined by measuring cell length, width, and depth using a Zeiss Axio Imager A2 microscope equipped with an AxioCam HRc (Carl Zeiss Inc.) photomicrographic system at 400× magnification. Biovolume was calculated using the geometric formulas of $\pi/6 \times width^2 \times length$ and $\pi/6 \times width \times depth \times length$ for the cryptophytes and the dinoflagellates, respectively (Vadrucci et al. 2007). Carbon content was estimated from the cell volume using conversion factors of 220 fg C μm^3 for cryptophytes (Børsheim and Bratbak 1987) and pg C cell^{-1} = 0.760 × (volume, μm^3)$^{0.819}$ for dinoflagellates, respectively (Menden-Deuer and Lessard 2000).

Growth and grazing of *Polykrikos lebourae* with different prey concentration

To investigate the effect of prey concentration on growth and grazing responses of *P. lebourae*, we chose three prey organisms led to relatively high growth rates of the predator based on the result of the previous experiment: the cryptophyte *Rhodomonas* sp. 2 (rCR04) and the dinoflagellates *Amphidinium* sp. and *Heterocapsa* sp. The initial concentrations (cells mL^{-1}) of each predator/prey mixture were 5/25, 5/50, 5/100, 5/250, 5/500, 5/1,500, and 5/3,500. In addition, triplicate controls containing only prey and only predator were also run at the same concentrations as ones in the mixtures of predator and prey. Data on growth rates were fitted to a modified Michaelis-Menten model, which includes a positive y-axis intercept. The modified equation was applied following Kim et al. (2008) and the parameters were estimated using the software SigmaPlot (version 10.0; MMIV Systat software Inc., San Jose, CA, USA) and the equation:

$$\mu = \frac{\mu_{max}(x - x')}{K_m + (x - x')} \qquad (3)$$

, where μ is growth rate of *Polykrikos*, μ_{max} is maximum growth rate, *x* is prey concentration (cells mL^{-1}), *x'* is compensation point of prey concentration where growth is 0 (μ = 0), and K_m is prey concentration sustaining 1/2 μ_{max}.

Data on ingestion rates were fitted a Michaelis-Menten model and the equation:

Table 1. List of the source, biovolume, and carbon content of prey organisms and growth (μ) and grazing rates (*I*) and gross growth efficiency (GGE) of *Polykrikos lebourae* during exponential growth period supplied each prey organism

Prey species (strain)	Source[a]	Biovolume[b] (μm^3)	Carbon content (ng C cell^{-1})	Exp. growth period[c] (d)	μ (d^{-1})	*I* (cells Polykrikos^{-1} d^{-1})	*I* (ng C Polykrikos^{-1} d^{-1})	GGE (%)
Cryptophytes								
Chroomonas sp. 1 (gCR07)	Mohang	400 ± 38	0.09 ± 0.01	18	0.08 ± 0.01	9.19 ± 4.97	0.81 ± 0.44	51 ± 11
Chroomonas sp. 2 (gCR09)	Mohang	112 ± 10	0.02 ± 0.00	21	0.05 ± 0.01	34.14 ± 1.34	0.84 ± 0.03	46 ± 10
Rhodomonas sp. 1 (rCR02)	Dongho	228 ± 18	0.05 ± 0.00	42	0.09 ± 0.01	1.55 ± 2.41	0.08 ± 0.12	280 ± 72
Rhodomonas sp. 2 (rCR04)	Mohang	521 ± 25	0.11 ± 0.01	42	0.16 ± 0.01	3.86 ± 1.15	0.44 ± 0.13	340 ± 112
Rhodomonas sp. 3 (rCR05)	Dongho	1,244 ± 139	0.27 ± 0.03	24	0.09 ± 0.00	0.76 ± 0.19	0.22 ± 0.06	329 ± 69
Dinoflagellates								
Amphidinium sp. (bdAmp01)	Mohang	11,591 ± 896	1.62 ± 0.20	10	0.17 ± 0.02	0.83 ± 0.02	1.34 ± 0.03	97 ± 2
Heterocapsa sp. (bdHet01)	Mohang	3,613 ± 161	0.62 ± 0.05	28	0.17 ± 0.00	0.29 ± 0.06	0.18 ± 0.04	842 ± 116
Prorocentrum fukuyoi (bdPF03)	Mohang	22,003 ± 791	2.74 ± 0.18	18	0.06 ± 0.00	0.08 ± 0.06	0.22 ± 0.17	90 ± 11
Thecadinium kofoidii (bdTK01)	Mohang	12,247 ± 487	1.69 ± 0.12	10	0.09 ± 0.00	0.05 ± 0.11	0.08 ± 0.19	428 ± 192

Data represent mean ± standard error for triplicates.
[a] Collection site in western beaches of Korea.
[b] Cell biovolume was calculated by measuring 30 cells for each species. Values indicate as mean ± standard error.
[c] Days of exponential growth phase.

$$ I = \frac{I_{max} \times x}{K_s + x} \tag{4} $$

, where *I* is ingestion rate of *Polykrikos*, I_{max} is maximum ingestion rate, *x* is prey concentration (cells mL^{-1}), K_s is prey concentration sustaining 1/2 I_{max}.

Growth kinetics of *Polykrikos lebourae* with different light intensity

This experiment was conducted to investigate the effect of light intensities on growth and ingestion rates of *P. lebourae*. Irradiance was measured with a radiometer (Model QSL-2101; Biospherical Instruments, San Diego, CA, USA). Stock culture of *P. lebourae* that had been maintained at 40 μmol photons m^{-2} s^{-1} was distributed to a total of 18 plates (96-well in each plate). Three *Polykrikos* cells were distributed to each well in triplicate treatments including prey *Rhodomonas* sp. 2 (rCR04) to achieve a predator/prey ratio of 1/30 which is assumed to represent prey saturation. One third of the 18 sets of well plates was incubated at each of the following irradiances: 10, 40, and 120 μmol photons m^{-2} s^{-1} for about 40 days. Monocultures with only *Rhodomonas* sp. 2 or *Polykrikos* served as controls for each light intensity. Prey and predator cells in treatments and controls were fixed using Lugol's solution at every 1-4 days during the experiment period and counted for cell abundance as described above.

Statistical analyses

Data were presented as mean ± standard error (SE) of triple replicates unless otherwise stated. All statistical analyses were performed using SPSS Statistics version 21 (IBM Co., Armonk, NY, USA). Significant differences were assessed by one-way ANOVA and Turkey's honestly significant difference *post hoc* test at the 95% significance level (p < 0.05).

RESULTS

Growth and grazing responses of *Polykrikos lebourae* to different prey organisms

The biovolume of the benthic prey used in this study covered a broad range, being smallest in the cryptophyte *Chroomonas* sp. 2 (112 μm^3) and largest in the dinoflagellate *Prorocentrum fukuyoi* (22,003 μm^3) (Table 1). Despite the large difference in biovolume of prey, *P. lebourae*
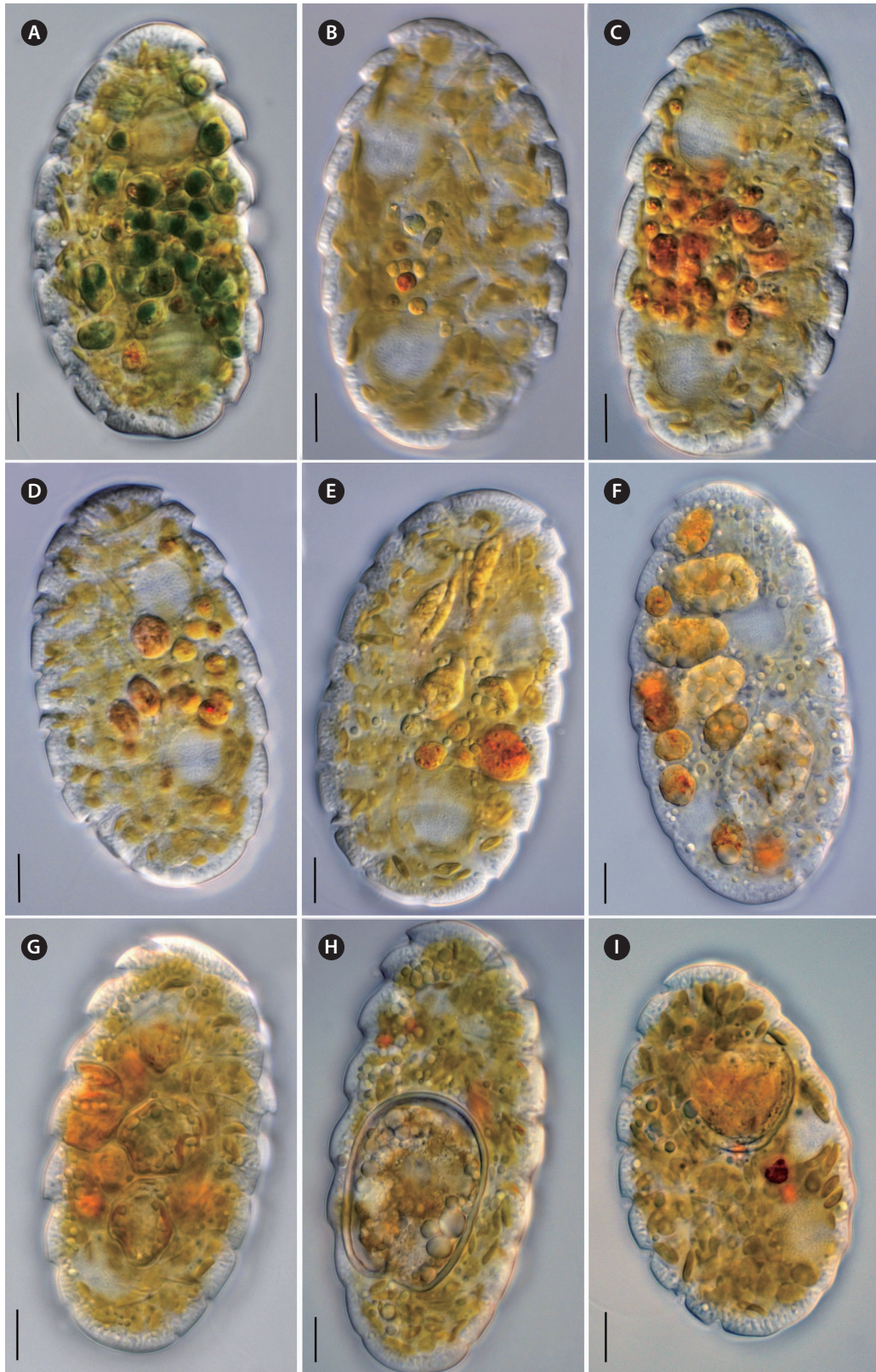
Fig. 1. *Polykrikos lebourae* ingested each prey organism tested in this study. *Polykrikos* fed *Chroomonas* sp. 1 (gCR07) (A), *Chroomonas* sp. 2 (gCR09) (B), *Rhodomonas* sp. 1 (rCR02) (C), *Rhodomonas* sp. 2 (rCR04) (D), *Rhodomonas* sp. 3 (rCR05) (E), *Amphidinium* sp. (bdAmp01) (F), *Heterocapsa* sp. (bdHet01) (G), *Prorocentrum fukuyoi* (bdPF03) (H), and *Thecadinium kofoidii* (bdTK01) (I) as prey. Scale bars represent: A-I, 20 μm.
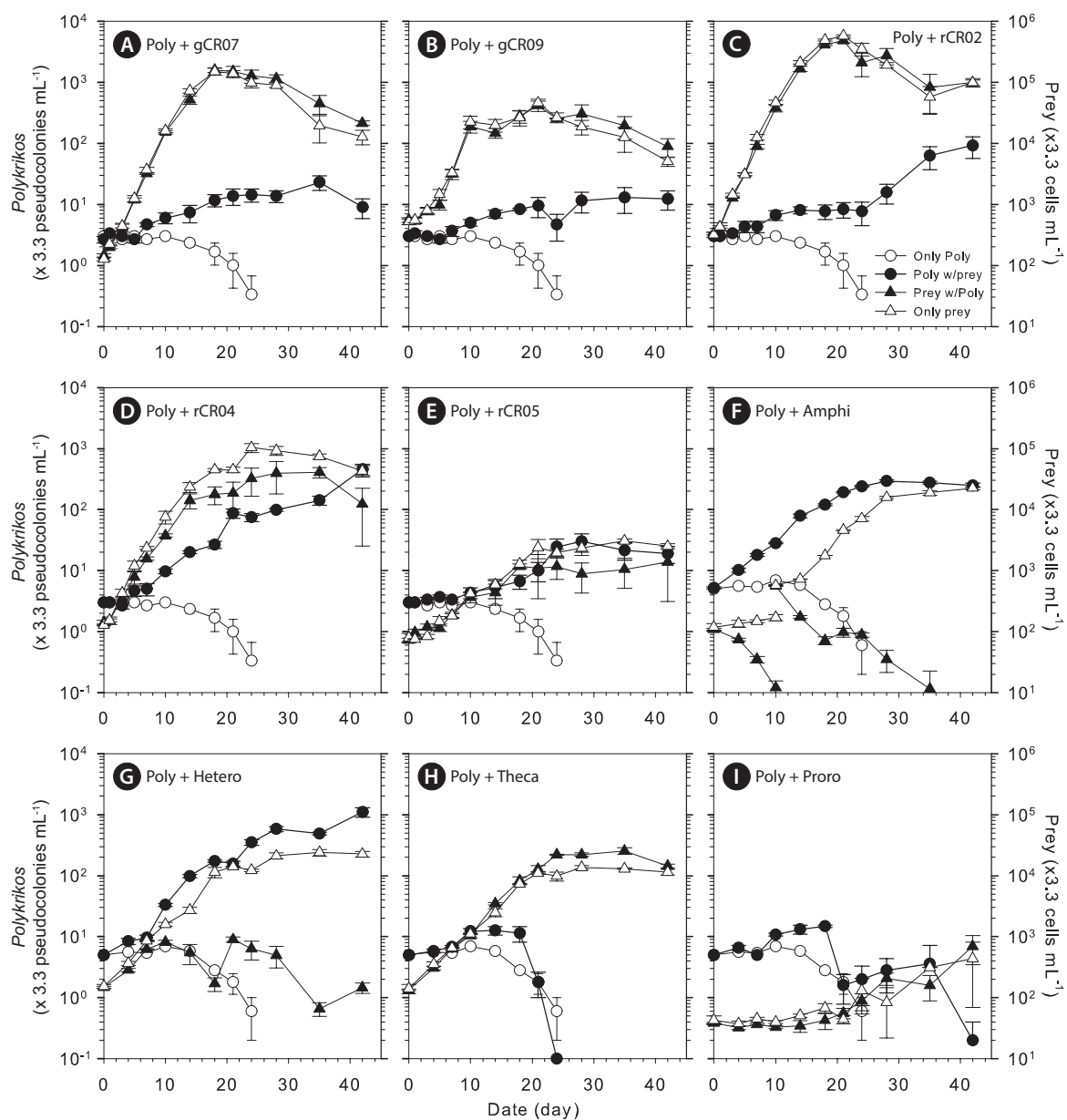
Fig. 2. Growth of *Polykrikos lebourae* when supplied 9 different prey organisms. Circles and triangles indicate *Polykrikos* and each prey organism, respectively. Closed symbols indicate the mixed cultures of predator and prey, and open symbols indicate monocultures of predator or prey. *Chroomonas* sp. 1 (gCR07) (A), *Chroomonas* sp. 2 (gCR09) (B), *Rhodomonas* sp. 1 (rCR02) (C), *Rhodomonas* sp. 2 (rCR04) (D), *Rhodomonas* sp. 3 (rCR05) (E), *Amphidinium* sp. (F), *Heterocapsa* sp. (G), *Thecadinium kofoidii* (H), and *Prorocentrum fukuyoi* (I) as prey. Symbols and error bars represent mean and standard errors of triplicate cultures.

was capable of ingesting all 9 potential prey organisms tested in this study (Fig. 1). The dinoflagellate captured prey cells using a tow filament and then phagocytized them through the posterior end. Sustainable growth of *P. lebourae*, however, was supported only with addition of the cryptophytes, *Rhodomonas* sp. 1 and *Rhodomonas*

sp. 2, and the dinoflagellates, *Amphidinium* sp. and *Heterocapsa* sp. (Fig. 2C, D, F & G). When offered other prey, cell abundance of *P. lebourae* slightly increased by 24 days at growth rates of less than 0.1 d^{-1} and reached shortly to stationary phase or dramatically decreased although the prey cells still remained replete (Table 1, Fig. 2A, B, E,
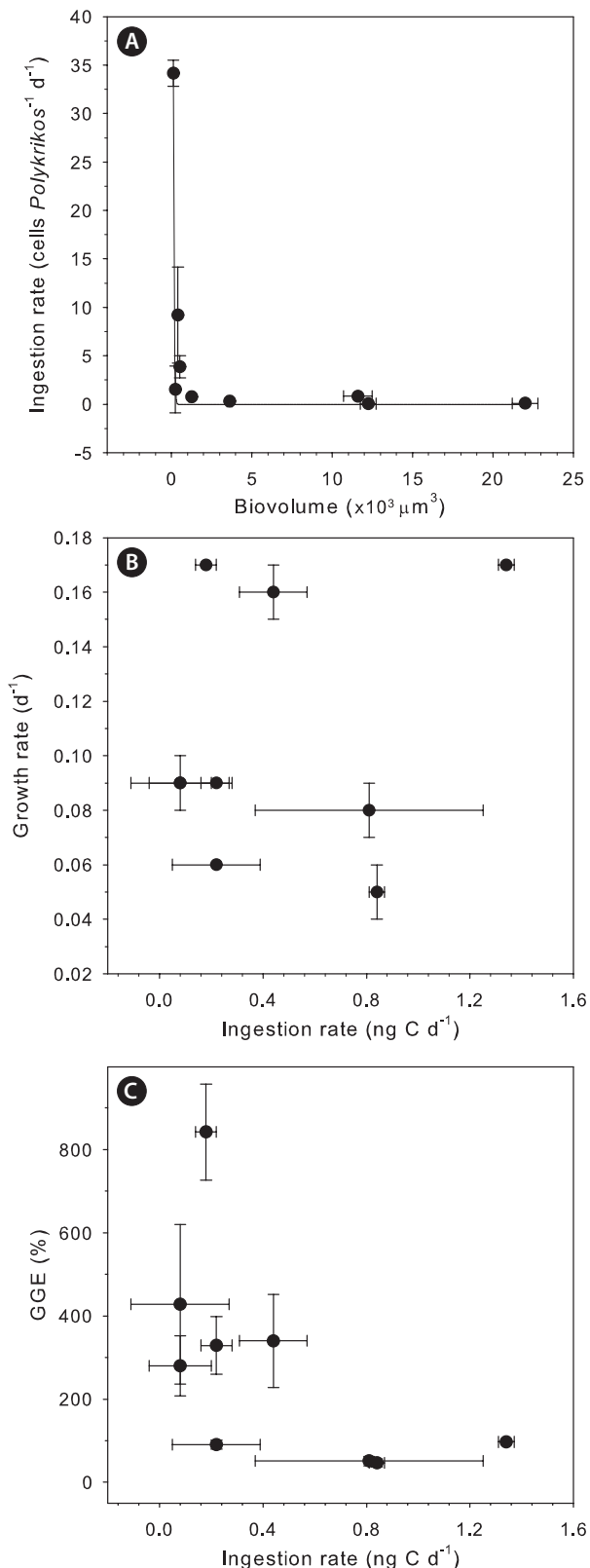
Fig. 3. *Polykrikos lebourae*. Gross growth efficiency (GGE) on 9 different prey species vs. prey biovolumes (A), growth rates vs. ingestion rates (B), GGE vs. ingestion rates (C).

H & I). The growth rates of *Polykrikos* were relatively higher in the presence of *Amphidinium* sp., *Heterocapsa* sp., and *Rhodomonas* sp. 2 than other prey species with mean growth rates of 0.17, 0.17, and 0.16 d^{-1}, respectively, during exponential growth period of the predator (Table 1). By comparison, cell abundances of *Polykrikos* in monocultures remained constant up to 10 days and thereafter gradually decreased (Fig. 2).

While *P. lebourae* ingested prey ranging in biovolume from 112 to 22,003 µm^3, ingestion rates were inversely correlated with prey biovolume ($r^2 = 0.90$, $p = 0.0001$) (Table 1, Fig. 3A). Growth rate and GGE of *P. lebourae* were not significantly correlated with the ingestion rate ($p > 0.05$) (Fig. 3B & C).

Growth and grazing responses of *Polykrikos lebourae* as a function of prey concentration

When fed *Amphidinium* sp., *Heterocapsa* sp., and *Rhodomonas* sp. 2, the growth rate of *P. lebourae* increased sharply with increasing prey concentrations up to 618, 1,418, and 60 ng C mL^{-1}, respectively, and thereafter saturated (Fig. 4A). When data were fitted to a modified Michaelis-Menten equation, the μ_{max} of *P. lebourae* was 0.36, 0.29 , and 0.19 d^{-1} when fed *Amphidinium* sp., *Heterocapsa* sp., and *Rhodomonas* sp. 2, respectively ($p = 0.0001$), with K_m being 45.9, 423.3, and 12.0 ng C mL^{-1}, respectively (Table 2). Ingestion rate of *P. lebourae* increased with increasing prey concentration, with saturation occurring above 5,700, 3,100, and 225 ng C mL^{-1} for *Amphidinium* sp., *Heterocapsa* sp., and *Rhodomonas* sp. 2, respectively (Fig. 4B). The I_{max} was 23.17 ng C *Polykrikos*$^{-1}$ d^{-1} for *Amphidinium* sp., 3.45 ng C *Polykrikos*$^{-1}$ d^{-1} for *Heterocapsa* sp., and 5.70 ng C *Polykrikos*$^{-1}$ d^{-1} for *Rhodomonas* sp. 2, with K_s of 1,665.0 ng C mL^{-1} for *Amphidinium* sp., 910.0 ng C mL^{-1} for *Heterocapsa* sp., and 114.4 ng C mL^{-1} for *Rhodomonas* sp. 2 (Table 2). When *P. lebourae* fed either *Heterocapsa* sp. or *Rhodomonas* sp. 2, values for GGE ranged from 68 to 520 % across the range of prey concentrations, with a remarkable increase observed at low prey concentrations (Fig. 5). By comparison, when fed *Amphidinium* sp., *P. lebourae* showed much lower GGE ranging from 18 to 121%, with no sharp increase observed at low prey concentrations (Fig. 5).

Growth kinetics of *Polykrikos lebourae* under three different light regimes

Polykrikos lebourae in the presence of the prey *Rhodomonas* sp. 2 displayed different patterns of response

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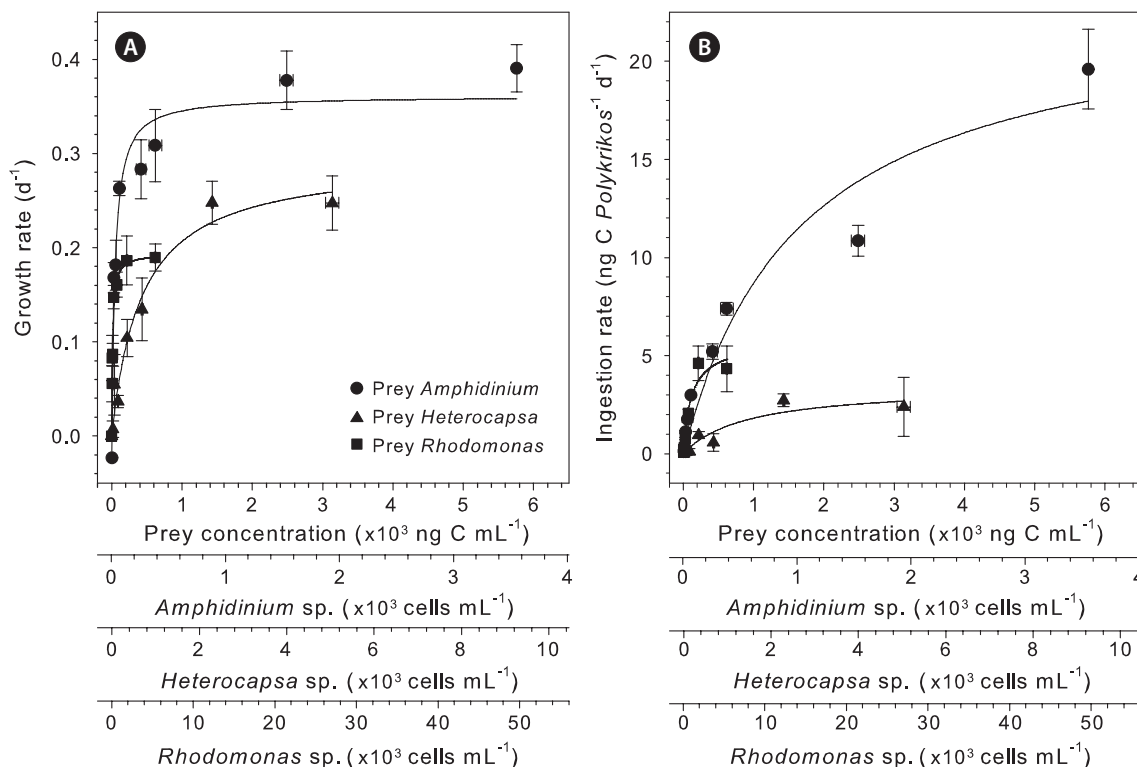
Fig. 4. Growth (A) and ingestion rates (B) of *Polykrikos lebourae lebourae* as a function of prey concentration. Data were fitted to a modified Michaelis-Menten equation for growth and a Michaels-Menten for ingestion. Symbols and error bars represent mean and standard errors of triplicate cultures.
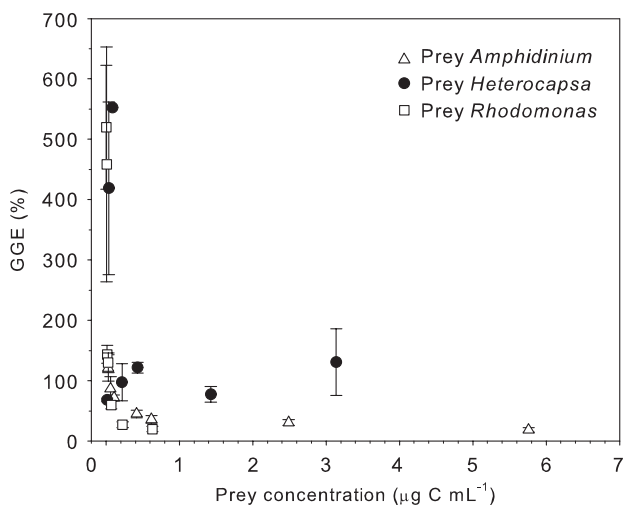
Fig. 5. Gross growth efficiency (GGE) of *Polykrikos lebourae* on prey of either *Amphidinium*, *Heterocapsa*, or *Rhodomonas* vs. prey concentration.

to light intensities (Fig. 6). At the moderate light intensity of 40 μmol photons m^{-2} s^{-1}, *P. lebourae* grew well for the first 17 days with a growth rate of 0.14 ± 0.01 d^{-1} and then reached stationary phase (Fig. 6B). By comparison, at dim light of 10 μmol photons m^{-2} s^{-1} and high light of 120 μmol m^{-2} s^{-1}, the abundance of *P. lebourae* slightly increased with growth rates of 0.03 ± 0.03 and 0.03 ± 0.02 d^{-1} for the first 3 and 6 days, respectively, and thereafter decreased gradually toward the end of the experiment even if prey remained replete in the cultures (Fig. 6A & C). In the absence of prey, *P. lebourae* abundances remained constant at light intensities of 10, 40, and 200 μmol photons m^{-2} s^{-1} for 4, 24, and 16 days, respectively and then rapidly decreased thereafter in all control cultures (Fig. 6G-I). Ingestion rate of *P. lebourae* was estimated as 0.42 ± 0.12, 0.39 ± 0.35, and 0.43 ± 0.10 ng C *Polykrikos*$^{-1}$ d^{-1} at 10, 40, and 200 μmol photons m^{-2} s^{-1}, respectively, during the exponential growth period of each prey (3, 5, and 6 days, respectively) and did not differ significantly among the light regimes (one-way ANOVA, p > 0.05) (Fig. 6).
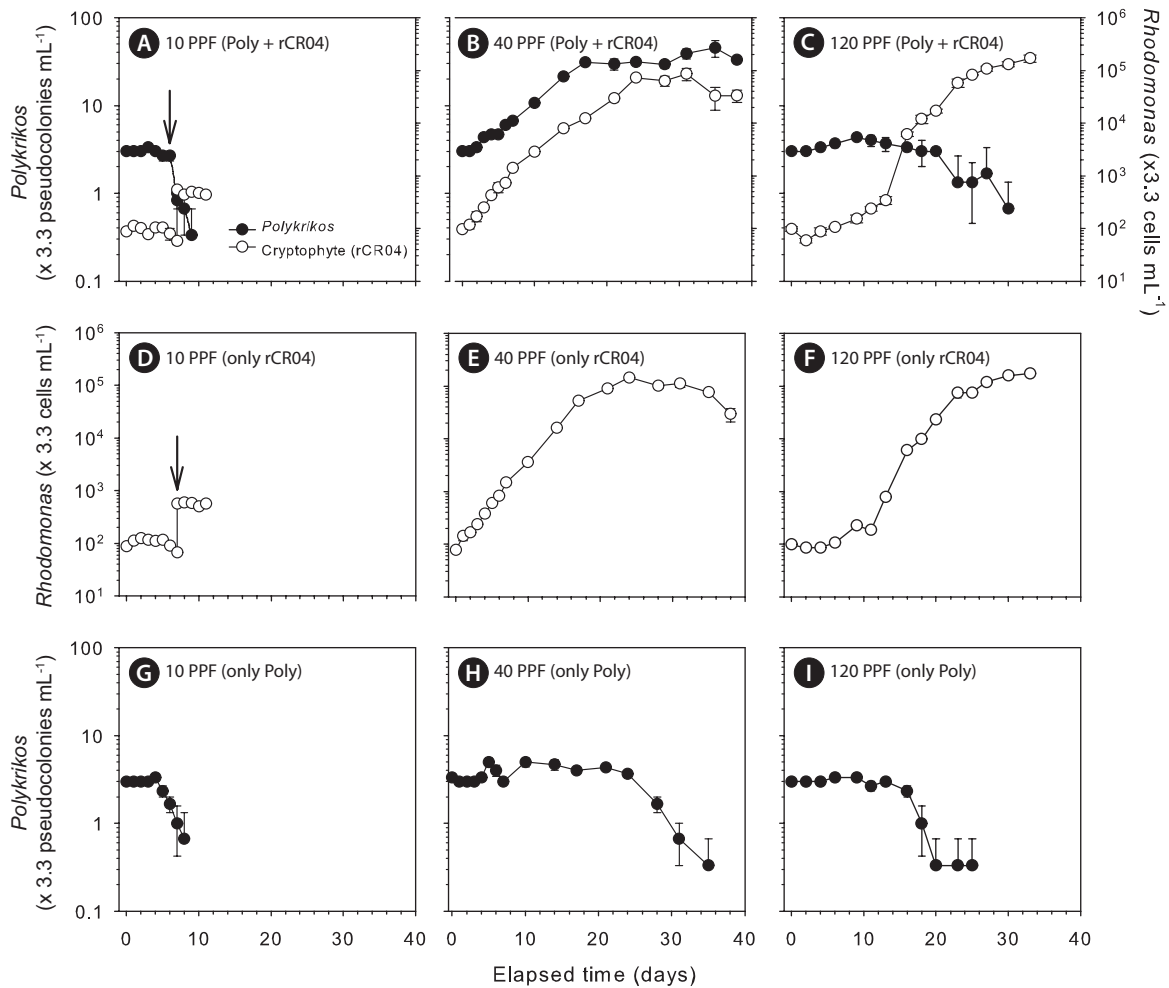
Fig. 6. Growth responses of *Polykrikos lebourae* at low irradiance of 10 μmol photons m^{-2} s^{-1} (A), moderate irradiance of 40 μmol photons m^{-2} s^{-1} (B), and high irradiance of 120 μmol photons m^{-2} s^{-1} (C) when offered *Rhodomonas* sp. 2 as prey. Cultures of prey only (D-F) and predator only (G-I) were also run as controls at each light intensity. Data points are shown as mean and standard error for triplicates. Arrows in (A) & (D) indicate additional supply of prey *Rhodomonas* sp. 2 in the mixed cultures. PPF, photosynthetic photon flux.

Table 2. Parameters fitted to a modified Michaels-Menten equation (Eq. 3) for growth rates and a Michaeles-Menten equation for ingestion rates

	Amphidinium sp.	*Heterocapsa* sp.	*Rhodomonas* sp. 2
Growth			
μ_{max} (d^{-1})	0.36	0.29	0.19
K_m (ng C mL^{-1})	45.9	423.3	12.0
x' (ng C mL^{-1})	2.00	0.00	0.00
r^2	0.96	0.97	0.98
p-value	<0.0001	<0.0001	<0.0001
Grazing			
I_{max} (ng C *Polykrikos*$^{-1}$ d^{-1})	23.17	3.45	5.70
K_s (ng C mL^{-1})	1,665.0	910.0	114.4
r^2	0.93	0.88	0.94
p-value	0.0022	0.0124	0.001

μ_{max} and I_{max}: maximum growth and ingestion rates of *Polykrikos lebourae*, K_m and K_s: prey concentrations sustaining half maximum growth and ingestion rates, x': compensation point of prey concentration where μ is 0.

DISCUSSION

Mixotrophy, i.e., a combination of phagotrophy and phototrophy in a single organism, is widespread among dinoflagellates (e.g., Stoecker 1999, Hansen 2011). Despite the many previous reports on the occurrence of mixotrophy in dinoflagellates, only a few dinoflagellates are known to be obligate mixotrophs, requiring both prey and light for growth and survival. Thus far, *Dinophysis* spp. (Park et al. 2006, Kim et al. 2008), *Esoptrodinium* sp. (Fawcett and Parrow 2014), *Paragymnodinium shiwhaense* (Yoo et al. 2010), and an unidentified Antarctic dinoflagellate (Gast et al. 2007) fit this category. Our results now add a new member, the dinoflagellate *Polykrikos lebourae*, to this list of obligate mixotrophic dinoflagellates.

Prey specificity of obligate mixotrophic dinoflagellates varies greatly from non-specific to highly specific. The obligate mixotrophs, *Dinophysis* spp. and the unidentified Antarctic dinoflagellate appear to require specific prey. The mixotrophic ciliate *Mesodinium rubrum* is a sole prey known thus far for the former species (Park et al. 2006), while the latter was recently reported to specifically feed on the haptophyte *Phaeocystis antarctica* (Sellers et al. 2014). Natural populations of *Dinophysis* spp., however, can contain plastids of multiple algal origins (Kim et al. 2012), indicating the possibility of prey other than the well-known prey ciliate *M. rubrum*. Interestingly, those dinoflagellates with strong prey specificity are obligate mixotrophs that practice the retention of plastids of cryptophyte and haptophyte origins, respectively. The dinoflagellates retain the plastids through selective feeding on the intermediate prey *M. rubrum*, itself also a specific predator for the cryptophyte *Teleaulax* / *Geminigera* genus complex (Yih et al. 2004, Kim et al. 2012) and the haptophyte *P. antarctica* (Gast et al. 2007, Sellers et al. 2014), respectively. Thus far, plastid-retaining, obligately mixotrophic dinoflagellates having broad prey range have not been reported.

While both *Esoptrodinium* sp. (Fawcett and Parrow 2014) and *Paragymnodinium shiwhaense* (Yoo et al. 2010) are also obligate mixotrophs, they are different from the other obligately mixotrophic dinoflagellates mentioned above in that they have their own plastids (i.e., peridinin-type plastids) and have a broad range for prey. The former can feed on a variety of freshwater protists (diatom, chlorophyte, chrysophyte, cryptophyte, dinoflagellate, and euglenoid microalgae) similar in size to itself, but the most suitable prey for promoting active feeding and sustained growth is the cryptophyte *Cryptomonas ovata* (Fawcett and Parrow 2014). Also, *P. shiwhaense* seems to be a prey generalist because it can ingest various protists (prymnesiophyte, cryptophyte, rhaphidophyte, and dinoflagellate), with positive growth supported by most of the different prey (Yoo et al. 2010). The benthic dinoflagellate *P. lebourae* appears to be the same case to *Esoptrodinium* sp. and *P. shiwhaense* rather than to *Dinophysis* spp. and the unidentified Antarctic dinoflagellate, as it 'apparently' has its own plastids (also, see below) as well as has a broad range for prey. *Polykrikos lebourae* appears to have a wider range in prey size than *Esoptrodinium* sp. and *P. shiwhaense*, ingesting prey range from ~5 μm (*Chroomonas* sp. gCR09) to ~30 μm (*Prorocentrum fukuyoi*) in diameter, while *Esoptrodinium* sp. and *P. shiwhaense* mostly ingest prey similar to their own size. The difference in prey size among these dinoflagellates may be associated with feeding mechanism. That is, *P. lebourae* engulfs whole prey cells after capturing them using a tow filament (this study), whereas the other two species ingest prey through a feeding tube or so-called peduncle (Yoo et al. 2010, Fawcett and Parrow 2014).

Despite the fact that *Polykrikos lebourae* can feed on a broad range of prey species varying in size, not all prey species support sustained growth. For example, unlike *Rhodomonas* spp., *Chroomonas* spp. did not support the significant growth of *P. lebourae*, even though ingestion rates of *P. lebourae* on *Chroomonas* spp. (0.81-0.84 ng C *Polykrikos*[-1] d^{-1}) were higher than those on *Rhodomonas* spp. (0.08-0.44 ng C *Polykrikos*[-1] d^{-1}). On the other hand, while GGE (46-51%) of *P. lebourae* on *Chroomonas* spp. were within the range (10-70%) reported for protists (Verity 1985, Caron and Goldman 1990, Skovgaard 1998), those on *Rhodomonas* spp. were greatly high (280-340%). The observed difference in growth response of *P. lebourae* when fed either *Chroomonas* or *Rhodomonas* may be explained by several factors. First, the nutritional value of *Chroomonas* for *P. lebourae* may not be as high as that of *Rhodomonas*. Second, *P. lebourae* might acquire new plastids through feeding on *Rhodomonas*, with active photosynthesis of freshly retained plastids substantially supplementing growth of *P. lebourae*. Unlike the typical peridinin-type plastids which contain thylakoids in stack of three surrounded by a triple membrane envelope (Schnepf and Elbrächter 1999), *P. lebourae* has unusual plastids consisting of thylakoids in stacks of two and enveloping two membranes, although whether these plastids are permanent or are derived from the prey (i.e., stolen plastids; kleptoplastids) is still controversial (Hoppenrath and Leander 2007b). Given that the kleptoplastidic dinoflagellate *Dinophysis caudata* can acquire new plastids of cryptophyte origin through ultrastructural

modification and retention after feeding on the mixotrophic ciliate *Mesodinium rubrum* (Kim et al. 2012), we cannot completely rule out the possibility that *P. lebourae* acquires plastids when feeding on a variety of prey types.

Among the benthic dinoflagellates used as prey in this study, *Amphidinium* and *Heterocapsa* supported significant growth of *P. lebourae*. When fed on these two dinoflagellates, *P. lebourae* grew well at the same growth rate of 0.17 d⁻¹, even though ingestion rate of *P. lebourae* on *Amphidinium* was an order of magnitude greater than that on *Heterocapsa*. Nonetheless, GGE of *P. lebourae* on *Heterocapsa* was much higher (842%) when compared to that on *Amphidinium* (97%). One possible explanation for this pattern is that the nutritional value of *Amphidinium* is much smaller than that of *Heterocapsa*. Indeed, more fecal pellets were observed in cultures of *P. lebourae* fed *Amphidinium* compared to those fed *Heterocapsa* (M. G. P. personal observation), indicating that undigested materials of the prey *Amphidinium* not used by *P. lebourae* are egested and less nutritional. Second, the thecate dinoflagellate *Heterocapsa* may be more resistant to enzymatic digestion in food vacuoles of *P. lebourae* compared to athecate dinoflagellate *Amphidinium*, thereby allowing the functional plastids of *Heterocapsa* to remain photosynthetically active over a relatively longer period.

GGE of protists as a function of prey concentration has been reported for both bacterivorous and plastid-retaining ciliates, in which GGE tends to decrease with increasing prey concentration (Heinbokel 1978, Stoecker and Evans 1985, Jonsson 1986, Schoener and McManus 2012). By comparison, the effect of prey concentration on GGE in dinoflagellates is at present not commonly studied. To our knowledge, the only previous report on the effect of prey concentration on GGE of dinoflagellates was for a kleptoplastidic dinoflagellate *Amylax triacantha*, in which GGE decreased with increasing prey (*Mesodinium rubrum*) concentration, with the highest GGE (81-179%) observed at low prey concentrations (Park et al. 2013). The authors interpreted the extremely high GGE of *A. triacantha* observed at low prey concentrations as being associated with a substantial supplement of photosynthetic products from retained plastids. GGE of *P. lebourae* for three prey species, *Amphidinium* sp., *Heterocapsa* sp., and *Rhodomonas* sp. tested in this study also showed a pattern similar to that of reported for *A. triacantha*, with extremely high GGE at low prey concentrations and moderate or low GGE at high prey concentrations. This suggests that *P. lebourae* grows heterotrophically at

high prey concentrations but its growth seems to be more dependent on a certain growth factor or photosynthesis of plastids derived from the prey. Interestingly, when *Heterocapsa* sp. was offered as prey, GGE of *P. lebourae* almost exceeded 100% over all prey concentrations. As noted above, more resistant characteristics and resultant active photosynthesis or an unknown growth factor from *Heterocapsa* may contribute greatly to the observed high GGE.

Polykrikos lebourae appears to require light for sustained growth, even when a sufficient amount of prey cells was provided. While the cryptophyte *Rhodomonas* sp. grew well at high light intensity (120 µmol photons m⁻² s⁻¹) as well as at moderate light intensity (40 µmol photons m⁻² s⁻¹), *P. lebourae* grew well just at the moderate light intensity (40 µmol photons m⁻² s⁻¹) in the presence of prey, but did not grow at dim light (10 µmol photons m⁻² s⁻¹) and high light (120 µmol photons m⁻² s⁻¹) intensities in prey-replete conditions. Similar growth response on light intensity has also been found in a benthic kleptoplastidic dinoflagellate *Amphidinium poecilochroum* (Jakobsen et al. 2000). Given that *P. lebourae* and its prey *Rhodomonas* live interstitially in sand sediment environment, different growth responses of the predator and prey to light intensity are notable and indicate that the former may be more adapted to a certain moderate light (40 µmol photons m⁻² s⁻¹) environment than the latter. It may be likely that some unknown materials required for growth of *P. lebourae* are produced by the prey at moderate to high light environments. The unknown materials and / or the plastids of *P. lebourae* may be, however, easily photodamaged and could not be repaired inside *P. lebourae* cells under high light (120 µmol photons m⁻² s⁻¹) environment, eventually leading to cell death. At dim light (10 µmol photons m⁻² s⁻¹) environment, production of the unknown materials by prey and / or photosynthesis of *P. lebourae* may not be enough to support to the positive growth of *P. lebourae*.

Conclusively, the benthic dinoflagellate *Polykrikos lebourae* is an obligate mixotroph, which requires both prey and light for sustained growth and survival. The benthic dinoflagellate *P. lebourae* is a prey generalist with a limited variety of prey supporting positive growth and fits the category of the mixotrophic dinoflagellates, in which food uptake results in a large increase in growth rate (i.e., assigned as type 2 in Hansen 2011). Availability of *P. lebourae* in a culture may provide a better understanding of the status of the plastids and plastid evolution in this dinoflagellate.

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REFERENCES

Balech, E. 1956. E'tude des dinoflagellés du sable de Roscoff. Rev. Algol. 2:29-52.

Børsheim, K. Y. & Bratbak, G. 1987. Cell volume to cell carbon conversion factors for a bacterivorous *Monas* sp. enriched from seawater. Mar. Ecol. Prog. Ser. 36:171-175.

Caron, D. A. & Goldman, J. C. 1990. Protozoan nutrient regeneration. *In* Capriul, G. M. (Ed.) *Ecology of marine protozoa*. Oxford University Press, NY, pp. 283-306.

Dragesco, J. 1965. Étude cytologique de quelques flagellés mésopsammiques. Cah. Biol. Mar. 6:83-115.

Fawcett, R. C. & Parrow, M. W. 2014. Mixotrophy and loss of phototrophy among geographic isolates of freshwater *Esoptrodinium*/*Bernardinium* sp. (Dinophyceae). J. Phycol. 50:55-70.

Gast, R. J., Moran, D. M., Dennett, M. R. & Caron, D. A. 2007. Kleptoplasty in an Antarctic dinoflagellate: caught in evolutionary transition? Environ. Microbiol. 9:39-45.

Guillard, R. R. L.1975. Culture of phytoplankton for feeding marine invertebrates. *In* Smith, W. L. & Chanley, M. H (Eds.) *Culture of Marine Invertebrate Animals*. Plenum Press, NY, pp. 26-60.

Hansen, P. J. 2011. The role of photosynthesis and food uptake for the growth of marine mixotrophic dinoflagellates. J. Eukaryot. Microbiol. 58:203-214.

Heinbokel, J. F. 1978. Studies on the functional role of tintinnids in the southern California Bight. I. Grazing and growth rates in laboratory cultures. Mar. Biol. 47:177-189.

Herdman, E. C. 1923 Notes on dinoflagellates and other organisms causing discolouration of the sand at Port Erin III. Proc. Trans. Liverpool Biol. Soc. 37:58-63.

Hoppenrath, M. & Leander, B. S. 2007*a*. Character evolution in polykrikoid dinoflagellates. J. Phycol. 43:366-377.

Hoppenrath, M. & Leander. B. S. 2007*b*. Morphology and phylogeny of the pseudocolonial dinoflagellates *Polykrikos lebourae* and *Polykrikos herdmanae* n. sp.

Protist 158:209-227.

Hoppenrath, M., Yubuki, N., Bachvaroff, T. R. & Leander, B. S. 2010. Re-classification of *Pheopolykrikos hartmannii* as Polykrikos (Dinophyceae) based partly on the ultrastructure of complex extrusomes. Eur. J. Protistol. 46:29-37.

Jakobsen, H. H., Hansen, P. J. & Larsen, J. 2000. Growth and grazing responses of two chloroplast-retaining dinoflagellates: effect of irradiance and prey species. Mar. Ecol. Prog. Ser. 201:121-128.

Jonsson, P. R. 1986. Particle size selection, feeding rates and growth dynamics of marine planktonic oligotrichous ciliates (Ciliophora: Oligotrichina). Mar. Ecol. Prog. Ser. 33:265-277.

Kim, M., Kim, S., Yih, W. & Park, M. G. 2012. The marine dinoflagellate genus *Dinophysis* can retain plastids of multiple algal origins at the same time. Harmful Algae 13:105-111.

Kim, S., Kang, Y. G., Kim, H. S., Yih, W., Coats, D. W. & Park, M. G. 2008. Growth and grazing responses of the mixotrophic dinoflagellate *Dinophysis acuminata* as functions of light intensity and prey concentration. Aquat. Microb. Ecol. 51:301-310.

Matsuoka, K. & Fukuyo, Y. 1986. Cyst and motile morphology of a colonial dinoflagellate *Pheopolykrikos hartmannii* (Zimmermann) comb. nov. J. Plankton Res. 8:811-818.

Menden-Deuer, S. & Lessard, E. J. 2000. Carbon to volume relationships for dinoflagellaltes, diatoms, and other protist plankton. Limnol. Oceanogr. 45:569-579.

Park, M. G., Kim, M. & Kang, M. 2013. A dinoflagellate *Amylax triacantha* with plastids of the cryptophyte origin: phylogeny, feeding mechanism, and growth and grazing responses. J. Eukaryot. Microbiol. 60:363-376.

Park, M. G., Kim, S., Kim, H. S., Myung, G., Kang, Y. G. & Yih, W. 2006. First successful culture of the marine dinoflagellate *Dinophysis acuminata*. Aquat. Microb. Ecol. 45:101-106.

Schnepf, E. & Elbrächter, M. 1999. Dinophyte chloroplasts and phylogeny: a review. Grana 38:81-97.

Schoener, D. M. & McManus, G. B. 2012. Plastid retention, use, and replacement in a kleptoplastidic ciliate. Aquat. Microb. Ecol. 67:177-187.

Sellers, C. G., Gast, R. J. & Sanders, R. W. 2014. Selective feeding and foreign plastid retention in an Antarctic dinoflagellate. J. Phycol. 50:1081-1088.

Skovgaard, A. 1998. Role of chloroplast retention in a marine dinoflagellate. Aquat. Microb. Ecol. 15:293-301.

Stoecker, D. K. 1999. Mixotrophy among dinoflagellates. J. Eukaryot. Microbiol. 46:397-401.

Stoecker, D. K. & Evans, G. T. 1985. Effects of protozoan her-

bivory and carnivory in a microplankton food web. Mar. Ecol. Prog. Ser. 25:159-167.

Tang, Y. Z., Harke, M. J. & Gobler, C. J. 2013. Morphology, phylogeny, dynamics, and ichthyotoxicity of *Pheopolykrikos hartmannii* (Dinophyceae) isolates and blooms from New York, USA. J. Phycol. 49:1084-1094.

Vadrucci, M. R., Cabrini, M. & Basset, A. 2007. Biovolume determination of phytoplankton guilds in transitional water ecosystems of Mediterranean ecoregion. Transit. Waters Bull. 2:82-102.

Verity, P. 1985. Grazing, respiration, excretion, and growth rates of tintinnids. Limnol. Oceanogr. 30:1268-1282.

Yih, W., Kim, H. S., Jeong, H. J., Myung, G. & Kim, Y. G. 2004. Ingestion of cryptophyte cells by the marine photosynthetic ciliate *Mesodinium rubrum*. Aquat. Microb. Ecol. 36:165-170.

Yoo, Y. D., Jeong, H. J., Kang, N. S., Song, J. Y., Kim, K. Y., Lee, G. & Kim, J. 2010. Feeding by the newly described mixotrophic dinoflagellate *Paragymnodinium shiwhaense*: feeding mechanism, prey species, and effect of prey concentration. J. Eukaryot. Microbiol. 57:145-158.

Zimmermann, W. 1930. Neue und wenig bekannte Kleinalgen von Neapel. Z. Bot. 23:419-442.