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NOTE

DETECTION OF PHAGOTROPHY IN THE MARINE PHYTOPLANKTON GROUP OF THE COCCOLITHOPHORES (CALCIHAPTOPHYCIDAE, HAPTOPHYTA) DURING NUTRIENT-REPLETE AND PHOSPHATE-LIMITED GROWTH[1]

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Mixotrophic algae that combine photoautotrophy with phagotrophy in a single cell are prevalent in marine ecosystems. Here, we assessed the ability of food ingestion in coccolithophores, an important group of calcifying haptophytes inhabiting the oceans. We tested four species from different coccolithophore lineages (*Emiliania huxleyi*, *Calcidiscus leptoporus*, *Coccolithus braarudii*, and *Calyptrosphaera sphaeroidea*). For both *E. huxleyi* and *C. leptoporus* we included different life phases (haploid and diploid). For *C. braarudii* we only tested diploid hetero-coccolithophore cells, while for *C. sphaeroidea* we only tested haploid holococcolithophore cells. Phagotrophy was assessed using fluorescently labeled bacteria (FLB) as model prey item, under nutrient-replete and phosphate-limited conditions. We detected by microscopy ingestion of FLB by all species, except the diploid *C. braarudii* strain. However, a previous study detected ingestion by haploid cells of *C. braarudii*. These overall results indicate that mixotrophy and the ability to ingest prey is widespread in coccolithophores. Yet, in all tested species the ingestion of FLB was low (<1% of the population contained prey at all time points over 2 days), namely for *E. huxleyi* and the diploid cells from *C. leptoporus* where detection of ingestion was sporadic. Moreover, no clear differences were detected between life phases in *E. huxleyi* and *C. leptoporus* under equal circumstances, or between replete and limited growth conditions.

Key index words: **bacterivory; coccolithophores; life cycle; mixotrophy; phytoplankton**

***Abbreviations*: DAPI, 4′,6-Diamidino-2-Phenylindole, Dihydrochloride; DTAF, 5-([4,6 dichlorotriazin-2-yl]-amino)-fluorescein; FLB, fluorescently labeled bacteria; HET, heterococcolithophore; HOL, holococcolithophore**

Increasing evidence indicates that most phytoplankton are mixotrophic, possessing both the capability for photoautotrophy but also, to varying extents, for ingesting prey items through phagotrophy (Sanders 1991, Jones 1997, Stoecker 1998, Flynn and Mitra 2009, Stoecker et al. 2017). This dual trophic strategy appears to be particularly beneficial when light or inorganic nutrients are limiting (Mitra et al. 2016). Moreover, recent studies indicate that mixotrophy is widespread and can be responsible for the largest fraction of total bacterivory in the oceans (Unrein et al. 2007, 2014, Zubkov and Tarran 2008, Hartmann et al. 2012, Sanders and Gast 2012). Thus, the functional role of phytoplankton influencing the structure of the marine food web and the flow of carbon to higher trophic levels and the deep ocean is more complex than usually depicted (Worden et al. 2015, Mitra et al. 2016, Stoecker et al. 2017). Mixotrophy appears to be important in several microalgal lineages, namely dinoflagellates, cryptophytes, chrysophytes, and haptophytes that are widely abundant in marine ecosystems (Zubkov and Tarran 2008, Unrein et al. 2014, Leles et al. 2017). However, the prevalence of mixotrophy within some of these lineages has been less explored. In this context, we examined the occurrence of mixotrophy (i.e., combined photoautotrophy and phagotrophy) in coccolithophores that are a distinct subgroup of haptophytes (Calcihaptophycidae, de Vargas et al. 2007), which typically produce composite exoskeletons composed of calcite scales called coccoliths, and thus play a central role in the global carbon cycle (Rost and Riebesell 2004, Thierstein and Young 2013). Additionally, coccolithophores, like other haptophytes, display complex sexual haplodiplontic and heteromorphic life cycles (Billard 1994, Houdan et al. 2004, Cros and Estrada 2013, Frada et al. 2019). Diploid cells often produce coccoliths made of interlocking calcite crystals with modified shapes and are denominated as heterococcolithophores (HET). In contrast, haploid cells often possess lighter coccoliths composed of

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numerous euhedral calcite crystallites and are denominated as holococcolithophores (HOL), but in some species like *Emiliania huxleyi* haploid cells are noncalcified (Houdan et al. 2004, Frada et al. 2019). Broadly, HET are frequently associated with nutrient rich conditions, while HOL are frequently found in higher light, nutrient poor conditions (Cros and Estrada 2013, Šupraha et al. 2016, Frada et al. 2019). Overall, given the affiliation to the haptophytes it is expected that mixotrophy is inherent to coccolithophores. Yet, reports on coccolithophore mixotrophy are confined to single observations of particle ingestion (synthetic micro-beads) by a haploid (1n) *Coccolithus braarudii* cell (Houdan et al. 2006) and a diploid (2n) *E. huxleyi* cell (Rokitta et al. 2011). Thus, in order to further validate and expand the current understanding of mixotrophy in coccolithophores and to test the variability between life cycle phases that generally occupy distinct niches, we monitored in culture four species (including for two species both life cycle phases): 2n (HET) and 1n noncalcified *E. huxleyi* (RCC 1216/RCC1217), 2n (HET) and 1n (HOL) *Calcidiscus leptoporus* (RCC 1131/RCC 1130), 2n (HET) *C. braarudii* (RCC 3779) and the 1n (HOL) *Calyptrosphaera sphaeroidea* (RCC 1178). The cultures were nonaxenic, containing variable densities of cohabiting bacteria (Table S1 in the Supporting Information). Cells were grown at 18°C, 80 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-2} of irradiance, and 14:10 h light:-dark photoperiod in both nutrient-replete (K/5; Keller et al. 1987) and phosphate-limited (K/5-P) media to test the effect of P limitation that usually enhances phagotrophy in other mixotrophs (Smalley et al. 2003, Carvalho and Graneli 2010). P limitation was ensured by testing cells displaying for at least 24 h stationary growth in K/5-P (Fig. S1 in the Supporting Information). For comparative purposes, the cells in K/5 were diluted with fresh medium to the same densities as K/5-P cultures prior to feeding assays (Table S1). Phagotrophy was tested with fluorescently labeled bacteria (FLB) as model prey item (FLB: coccolithophore ratio ~20:1) prepared with a *Brevundimonas diminuta* (Spanish Type Culture Collection, Burjassot, Valencia, Spain) heat-killed and stained with 100 pg $\cdot$ mL^{-1} of the green fluorochrome DTAF (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA; Sherr et al. 1987). This bacterial strain was selected as prey item because it was shown to be grazed by haptophytes (Unrein et al. 2007, 2014). Cells and FLB, as well as total living bacteria were enumerated by flow cytometry (Attune NxT, Thermo Fisher Scientific, Waltham, MA, USA; Unrein et al. 2007, Marie et al. 2014) at t0, t1 h, t2 h, t4 h, t24 h, and t48 h. Samples were also collected and fixed with 2% glutaraldehyde (Sigma) and 0.1% Poloxamer 188 solution (Sigma) to prevent aggregation, filtered onto 0.8/1.2 μm polycarbonate membranes (Nuclepore and Isopore accordingly, Whatman, Merck KGaA, Darmstadt, Germany) and used to count the fraction of coccolithophores with ingested FLB by epifluorescence microscopy (Eclipse Ti-S, Nikon Instruments Inc., Melville, NY, USA). Uptake counts at t0 was considered artifactual and subtracted from the following time points. Additional samples of *C. leptoporus* and *C. sphaeroidea* were fixed with 1% paraformaldehyde, counterstained with DAPI (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and used for confocal microscopy (FV-1200; Olympus, Tokyo, Japan).

We detected FLB ingestion by most coccolithophores used in this study and respective life phases over the 48 h time course, with the exception of HET cells of *Coccolithus braarudii* (Fig. 1). Yet, the fraction of cells with FLB was low and in some strains only recorded sporadically, namely in both life phases of *Emiliania huxleyi* although the tested cell densities and thus cell: FLB contact rates were the highest (Table S1). Higher fractions of ingestion were detected in 1n *Calcidiscus leptoporus* and in *Calyptrosphaera sphaeroidea* where up to ~1% of the cells detected over 48 h consistently contained FLB (Fig. 2). Further confirmation of FLB ingestion in these species was performed by confocal microscopy, clearly highlighting the presence of FLB within the cells' cytoplasm (Fig. S2 in the Supporting Information). However, no significant differences were detected between nutrient regimes, with the exception of *C. sphaeroidea* where at t48 h cells in K/5-P contained slightly more FLB than in K/5 (~0.5% and ~0.2%, respectively, $P < 0.05$, $n = 3$, *t*-test; Fig. 2A). Given higher ingestions in 1n *C. leptoporus* and *C. sphaeroidea*, we attempted to determine the FLB removal rates by assessing the decline of FLB in the media over time. However, the decay of FLB when grown without coccolithophores was often higher than in the presence of coccolithophore cells. We could not technically solve this problem, which hampered accurate calculations of ingestion rates by coccolithophores. Yet, assuming that within each experiment in the presence of algal cells the decay of FLB was identical, we used regression analysis of FLB over time to comparatively determine the relative prey removal rates (FLB $\cdot$ h^{-1}) and the relative prey removal rates per coccolithophore cell (FLB $\cdot$ $cell^{-1}$). Total prey removal rates in *C. sphaeroidea* in K/5, where cell density was also 3× higher than in K/5-P, was twice as high than in K/5-P. However, no statistically significant differences were detected on a per cell basis (Fig. S3, A and B in the Supporting Information). In 1n *C. leptoporus* total prey removal was comparable in both media, but here prey removal rates per cell were significantly higher (2× to 4x) in K/5-P (Fig. S3, C and D). Comparisons between 1n and 2n *C. leptoporus* were also performed. However, in this case the densities of 2n cultures were considerably lower than 1n cultures. Thus, comparative experiments were performed at relatively low cell densities, compatible to the achievable

concentrations of 2n cultures. However, at such low coccolithophore densities ingestion of FLB was only rarely detected likely due to very low cell: FLB contact rates. No further analyses were undertaken in this case. Finally, we note that overall in all experiments in K/5 the cells grew considerably better than in K/5-P as expected given nutrient limiting conditions, and that no differences were detected in both nutrient regimes in terms of growth in the presence of FLB relative to nonfed controls for all species (Fig. S4 in the Supporting Information).

In summary, our results show that coccolithophores can ingest bacterial prey, and thus are constitutive mixotrophs (i.e., they are unicellular algae with the innate ability to photosynthesize that can consume other organisms; Sanders and Porter 1988, Mitra et al. 2016). As already mentioned above, ingestion of particles was previously detected in 1n *Coccolithus braarudii* (Houdan et al. 2006) and 2n cells of *Emiliania huxleyi* (Rokitta et al. 2011). Additionally, ingestion of particles was also recently examined and detected in *Isochrysis galbana*, a noncalcified Calcihaptophyte that is closely related to *E. huxleyi* (Anderson et al. 2018). Moreover, it has been shown that polar coccolithophores from the family Pappospheraceae appear to lack chloroplasts (Marchant and Thomsen 1994), and likely rely on phagotrophy for acquisition of nutrient resources. Thus, altogether these and our results strongly indicate that the ability to phagocytize prey is likely a synapomorphy of the Calcihaptophycidae inherited from noncalcified haptophytes that are prevalent mixotrophs in marine ecosystems (Liu et al. 2009, Carvalho and Graneli 2010, Unrein et al. 2014). Nonetheless, phagotrophy by coccolithophores appears to be considerably lower compared to other haptophytes like *Prymnesium* and *Chrysochromulina* that present rates of ingestion of 0.2–2 prey $\cdot$ cell^{-1} $\cdot$ h^{-1} and the fraction of cell feeding is considerably higher even in the presence of cohabiting bacteria (Legrand 2001, Hansen and Hjorth 2002). Still, variations could be detected between tested coccolithophore species indicating a variability between species in the utilization of

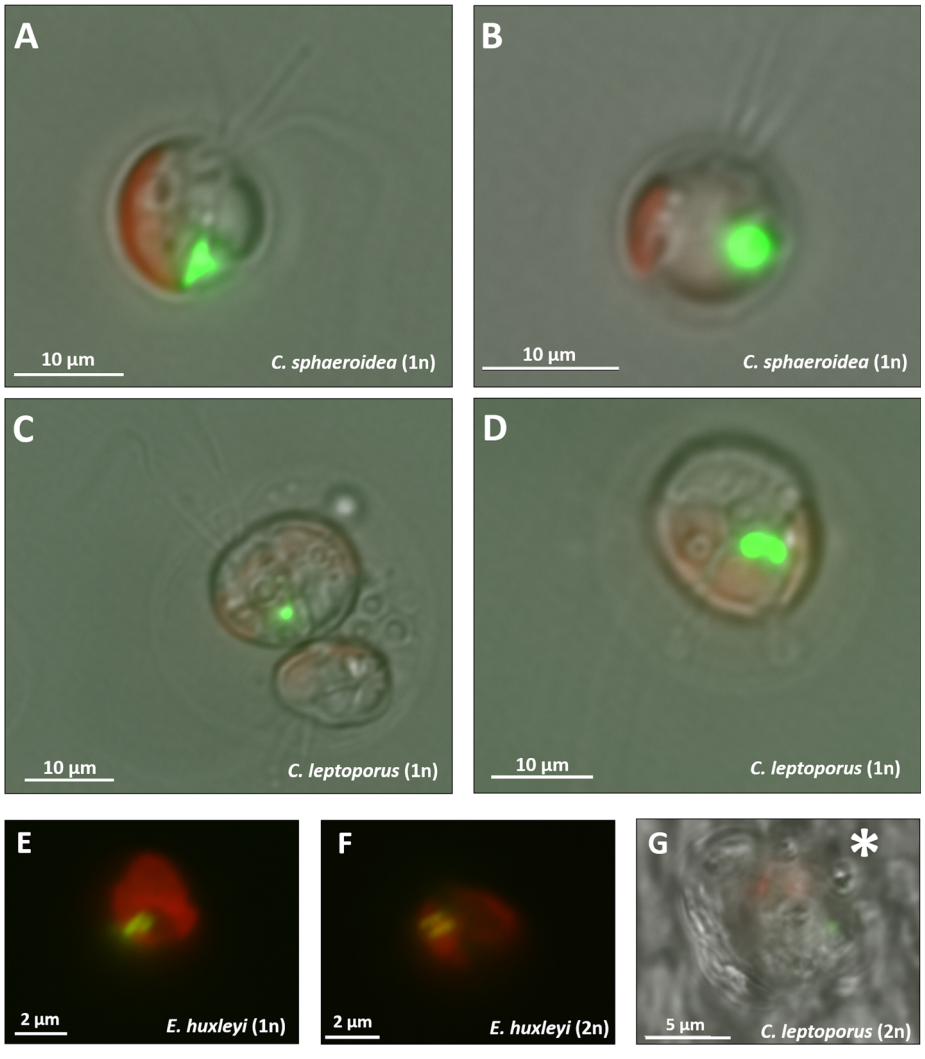

FIG. 1. Detection of ingestion of FLB by coccolithophores by epifluorescence microscopy. (A, B) *Calyptrosphaera sphaeroidea* (1n); (C, D) *Calcidiscus leptoporus* (1n), (E) *Emiliania huxleyi* (1n); (F) *E. huxleyi* (2n); (G) *C. leptoporus* (2n). The images are composed of layered pictures acquired with three channels: visible light showing the cell membrane or coccoliths (marked with asterisk, G) acquired by phase contrast, green fluorescence of the DTAF-stained bacteria (EX:494 nm/EM:518 nm), and red fluorescence exciting chlorophyll within the plastids of the algae (EX:649 nm/EM:670 nm).

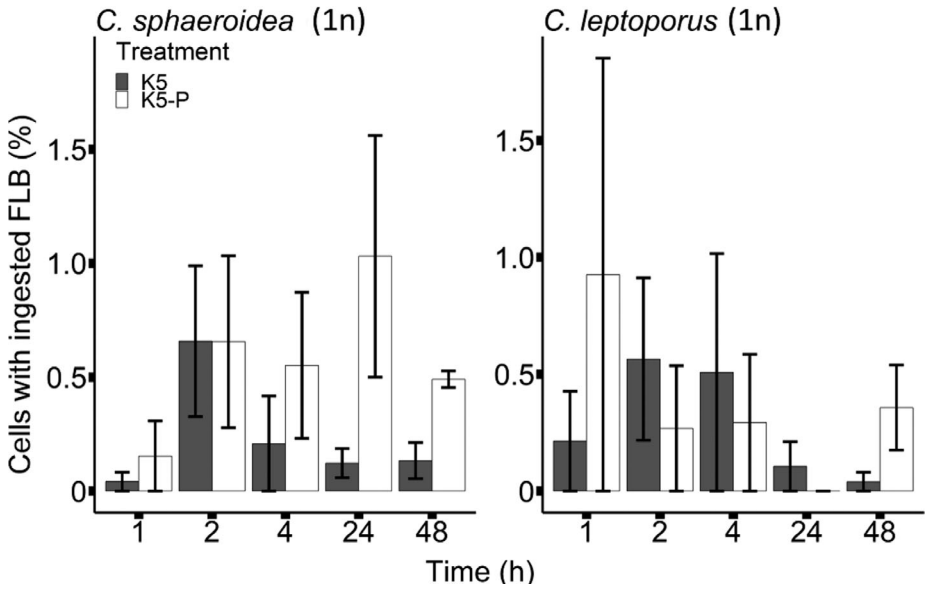

FIG. 2. Fraction of cells (%) containing FLB. Data are shown for cells growing in K/5 and K/5-P. Cell counts were performed by epifluorescence microscopy. (A) *Calyptrosphaera sphaeroidea* (1n); (B) *Calcidiscus leptoporus* (1n). *C. sphaeroidea* (1n) showed significant difference between media at t48 ($P < 0.05$, *t*-test). Error bars denote standard error, $n = 3$.

phagotrophy. Among these, the 1n *Calcidiscus leptoporus* and *Calyptrosphaera sphaeroidea* that are both flagellate cells like other mixotrophic haptophytes such as *Prymnesium* (Tillmann 1998, Brutemark and Graneli 2011) displayed higher FLB ingestion and could be used in subsequent studies as models for coccolithophore mixotrophy. It is still unclear what are the benefits mixotrophy can provide to coccolithophores. However, by using equation for carbon content based on size, found in Unrein et al. 2014, combined with N:C and P:C ratios for bacteria cells mentioned in Fagerbakke et al. 1996, we estimate a bacterium like *Brevundimonas diminuta* (0.065 μm^3) is composed of ~0.004 pg N $\cdot$ cell^{-1} and ~0.001 pg P $\cdot$ cell^{-1}, which could enable coccolithophores to balance their stoichiometry under limiting conditions, particularly in oligotrophic systems. Further studies are required to test this hypothesis. We also note that we could not find differences between life cycle phases and that ingestion by the dominant bloom-forming *E. huxleyi* was very rare. *E. huxleyi* is highly prolific r-selected species prevailing in nutrient-rich settings (Tyrrell and Merico 2004, Iglesias-Rodriguez et al. 2002). Thus, like diatoms that are also typically r-selected and are one of the few phytoplankton groups where phagotrophy appears to be absent (Lewin and Guillard 1962, Mitra et al. 2016), it may be that *E. huxleyi* essentially operates photoautotrophically. Still, the assessment of mixotrophy in additional strains, namely isolated from oligotrophic settings should be tested. Beyond the approach presented here, additional approached as for example the use of radiolabeled tracers and flow cytometry (Zubkov and Tarran 2008) and gene markers (Burns et al. 2018) could provide interesting avenues of research to evaluate coccolithophore mixotrophy in marine ecosystem. Finally, we highlight that other conditions including nitrogen or particularly light limitation, which have been shown to trigger feeding in other algae (Stoecker et al. 1997, Stoecker and Lavrentyev 2018), or the use of different and/or live prey items should be tested in future studies to further advance and better define the significance of mixotrophy in coccolithophores.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Growth dynamic of coccolithophores in K/5 and K/5-P media: (A) *Calyptrosphaera sphaeroidea* (1n); (B) *Calcidiscus leptoporus* (1n); (C) *C. leptoporus* (1n); (D) *C. leptoporus* (2n); (E) *Emiliania huxleyi* (1n); (F) *E. huxleyi* (2n); (G) *Coccolithus braarudii* (2n). Asterisk denotes that *C. leptoporus* (1n) was grown in low concentration to be compared with its diploid life phase. Filled triangles denote dilution of K/5 culture, that was made in order to keep the culture in exponential

growth phase. Error bars represent standard error, $n = 3$.

Figure S2. Z-stack montages of coccolithophore cells containing FLB by confocal microscope imaging. (A) *Calyptrosphaera sphaeroidea* (1n); (B) *Calcidiscus leptoporus* (1n). Each image represents a slice (ordered), altogether composing one cell from top to bottom. Brightfield (gray) shows cell membrane and vacuoles; Blue color shows DAPI stain of the nucleus; Green color is fluorescence of the prey by DTAF stain. Scale bar = 5 μm.

Figure S3. Ingestion rates of coccolithophores based on FLB removal from the medium in K/5 and K/5-P media. (A) *Calyptrosphaera sphaeroidea* (1n); (C) *Calcidiscus leptoporus* (1n). Ingestion rates per coccolithophore cell in the culture: (B) *C. sphaeroidea* (1n); (D) *C. leptoporus* (1n). Significant difference was found at (A) (*C. sphaeroidea* (1n), $P < 0.05$, *t*-test). Error bars denote standard error, $n = 3$.

Figure S4. Growth dynamics of coccolithophores in K/5 and K/5-P media, after feeding. (A, B) *Calyptrosphaera sphaeroidea* (1n); (C, D) *Calcidiscus leptoporus* (1n). *C. leptoporus* (1n) showed significant difference between treatments at t1 (C, $P < 0.05$, *t*-test). Error bars denote standard error, $n = 3$.

Table S1. Concentrations of coccolithophores, FLB, and cohabiting bacteria in the cultures at t0. Bacterial abundance was measured before the addition of FLB. FLB: Coccolithophore is the ratio (unitless) between the two parameters for each culture. F: T = percentage of FLB from total bacteria (sum of bacteria in culture and FLB). Growth rate (μ) of coccolithophore cells prior to feeding. Numbers in parentheses represent standard error, $n = 3$. In the column "Ploidy," "low*" refers to the experiment performed at lower concentrations in which *Calcidiscus leptoporus* 1n and 2n were compared in terms of ingestion of FLB (see main text for details).