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Mixotrophic uptake of organic compounds by coccolithophores

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Abstract

Osmotrophy is one of the main modes of mixotrophic acquisition of carbon by phytoplankton, but historically it has been under-investigated and its physiological and ecological relevance remains poorly understood. Here, we investigate osmotrophy in coccolithophores. Coccolithophores are one of the major contributors to the ocean biomass inhabiting both euphotic and subeuphotic depths in the marine environment. Coccolithophores demonstrate the potential to utilize a wide array of organic compounds in darkness. In experiments with BioLog Ecoplates, we screened a wide array of organic compounds as potential carbon sources, and observed that the major types of organic compounds taken up by coccolithophores were primarily carbohydrates along with a few amino acids and polymers. Furthermore, in subsequent radiotracer experiments, the uptake rates of ^{14}C-labeled dissolved organic carbon compounds in the dark were low relative to the maximal rates of photosynthetic carbon fixation in the light. The time course of uptake for some compounds suggests constitutive capacity for their transport, while for others the transport appears to be activated. Nonetheless, the collective slow uptake rate of a large array of organic compounds found in seawater, might be the only way that osmotrophy could fuel significant coccolithophore growth in the deep euphotic and subeuphotic zones in the sea.

Mixotrophy is a physiological feature of combining autotrophy and heterotrophy within a single organism (Worden et al. 2015; Caron 2016). In recent years, research on algal mixotrophy has accelerated (Stoecker et al. 2017; Leles et al. 2018; Mitra 2018) and photosynthesis is no longer considered the sole mode of algal cell maintenance and growth (Tuchman et al. 2006; Caron 2016). Mixotrophy occurs through three mechanisms: absorbotrophic (aka osmotrophic), biotrophic (aka symbiotic), and necrotrophic (aka predatory) (Selosse et al. 2017). Osmotrophs are mixotrophs that absorb dissolved organic compounds, which they use for their nutrition and/or carbon sources. Two other mechanisms, characterized as nonconstitutive mixotrophs (symbiotic) and constitutive mixotrophs (predatory), occur in different levels of complexity (Mitra et al. 2016).

Historically, osmotrophy has been under-investigated and its physiological and ecological relevance remains poorly understood (Tuchman et al. 2006; Selosse et al. 2017). In part, this is due to the vast array of organic compounds (Glibert and Legrand 2006) present in the ocean, many of which are unknown, of differing lability, and/or highly variable in time and space. Organic compounds within the dissolved pool generally include amines, amino acids, nucleic acids, carbohydrates, polypeptides, polyphenolic substances, polysaccharides, and polymers. The standard technique for determining rates of uptake of such compounds involves using tracer techniques (both stable- and radio-isotopes), but this is limited to selected commercially available compounds. However, new tools have emerged that can detect the uptake and assimilation of large suites of individual compounds by cells. One such technique is the BioLog plate method. This is a redox system in which 96-well plates are filled with organic substrate and a colorless tetrazolium dye, which is reduced to a violet formazin color when the substrate is oxidized by bacteria or microalgae (Stefanowicz 2006). The plates have been used mostly to screen bacterial metabolisms (Sala et al. 2010; Yang et al. 2011). Moreover, they have also been used to screen freshwater diatoms for heterotrophic uptake of organic compounds (Tuchman et al. 2006), plus multiple other applications, like investigating the potential of *Chlorella* sp. to pretreat saline antibiotic wastewater (Shi et al. 2018).

Coccolithophores are predominantly marine, unicellular algae generally thought of as photoautotrophs and covered with "coccoliths," scales made of calcium carbonate. Taxonomically, coccolithophores fall into two orders, Isochrysidales and Coccolithales, within the Haptophyta phylum (Adl et al. 2019). Observations suggest that globally, haptophytes contribute more biomass than the ubiquitous picophytoplankton *Prochlorococcus*

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and *Synechococcus* (Liu et al. 2009). Haptophytes uniquely possess a haptonema, a filament-like organelle that appears to be used for adhesion to other particles or substrata (Inouye and Kawachi 1994), for prey capture (Kawachi et al. 1991), and/or a sensory organelle (Inouye and Kawachi 1994). Coccolithophores have an alternating life cycle of diploid and haploid phases. These life cycle phases can reproduce asexually through binary fission, until they fuse during syngamy, and transition between phases. Coccolith origin and morphology varies between life cycle phases. Heterococcolithophores are diploid cells that produce elaborate coccoliths, heterococcoliths, in intracellular compartments. Holococcolithophores are haploid cells that produce simple coccoliths, holococcoliths, on the cell surface (Edvardsen et al. 2016). Houdan et al. (2006) hypothesized that life cycle phases of coccolithophores could also have different modes of nutrition by which they increase the span of their ecological niches.

Dissolved organic uptake by haptophytes remains poorly understood, despite the fact that it was first described almost eight decades ago (Conrad 1941). Pintner and Provasoli (1963) demonstrated that *Syracosphaera* uses lactate as a carbon source to allow faster recovery of growth from darkness. Furthermore, cell yields of *Chrysochromulina breviturita* were increased by glycine and L-valine (5 mmol L^{-1}) and yeast extract (0.5 g L^{-1}) (Wehr et al. 1985). Enhanced survival of axenic cultures of *Chrysochromulina kappa* was observed for 6 months at low light with media containing 10.8 mmol L^{-1} glycerol (Pintner and Provasoli 1968). This concentration of glycerol doubled the growth of *C. kappa* under dim light to be as high as cells grown at high light with no glycerol, but such a high concentration would unlikely be observed in the marine environment. In polar waters, where low light levels may not be sufficient for photosynthesis, species of genus *Chrysochromulina* probably use mixotrophy to allow for growth (Marchant and Thomsen 1994). Thus, generally uptake of dissolved organic carbon (DOC) compounds would allow for faster recovery of growth following extended darkness.

One of the most thorough works documenting DOC-mixotrophy in coccolithophores was a doctoral dissertation of Blankley (1971). He examined *Emiliania huxleyi* and *Cricosphaera carterae* (current taxonomic name: *Chrysotila carterae* [T. Braarud & E. Fagerland] Andersen, Kim, Tittley & Yoon) for their ability to grow on various DOC sources in darkness. He maintained axenic cultures in media supplemented with unusually high concentrations (500 mmol L^{-1}) of glycerol. He tested 21 different strains of these two species, and found that they could grow through 5–20 transfers for 20–49 d in darkness with identical cell morphology to light-grown cultures; but in darkness, they produced fewer coccoliths and had longer generation times, 6.4–8.6 d for *E. huxleyi* and 3.3–4.0 d for *C. carterae*. For media without glycerol, cell growth, chlorophyll production, and calcite formation stopped after 2 d in darkness, relative to light-grown cells. Unfortunately, Blankley died shortly after he completed his Ph.D. and never published his dissertation, so his work remained poorly known. We have followed up Blankley's experiments using modern screening techniques that are much more sensitive for measuring the assimilation of a whole variety of organic molecules.

In this article, we investigated osmotrophy in coccolithophores. The focus of this work was to test the hypothesis that coccolithophores show the ability to assimilate a wide array of organic molecules. We surveyed different coccolithophore species in their ability to sequester and utilize naturally occurring organic compounds. Once we confirmed compound assimilation, we then more thoroughly tested compounds that previously showed the strongest positives, to confirm that coccolithophores took up and metabolized these compounds at environmentally realistic concentrations. For that, we used radiotracer experiments and preformed time course uptake experiments. These results provided an overview of coccolithophore potential to assimilate diverse organic substrates from the sea.

Material and methods

Algal culture strains and growth media

We studied 25 strains of 12 different coccolithophore species: *Emiliania huxleyi*, *Gephyrocapsa oceanica*, *Calcidiscus leptoporus*, *Cruciplacolithus neohelis*, *Ochrosphaera neapolitana*, *Ochrosphaera* sp., *Ochrosphaera verrucosa*, *Chrysotila placolithoides*, *Chrysotila carterae*, *Chrysotila scherffelii*, *Chrysotila elongata*, and *Chrysotila* sp. Strains were obtained from the National Center for Marine Algae and Microbiota (NCMA, former Culture Collection of Marine Phytoplankton [CCMP]) (Table 1). Ploidy of strains was noted from NCMA strain information, when provided (Table 1). We subjected all strains to axenic screening by microscopic examination and a sterility test. We used a general-purpose test medium to detect the presence of bacteria and fungi in marine cultures (Hallegraeff et al. 2003). Almost all strains we used were axenic, except CCMP374, CCMP2051, CCMP2054, CCMP3392, CCMP594, and CCMP299 (Table 1). We maintained all cultures in L1 enriched seawater medium (Hallegraeff et al. 2003). To avoid introducing naturally occurring organic compounds, all culture media was based on artificial seawater (ASW) with reduced calcium (Keller et al. 1987). Cultures were maintained at 16°C or 22°C (*see* Table 1) with a 14/10 light/dark illumination cycle, with light intensity of 400 μmol photons m^{-2} s^{-1} from fluorescent lights.

Screening for uptake of various DOC compounds with BioLog plates

For determining the metabolic potential for heterotrophic utilization of a large array of organics by coccolithophores, we used the BioLog Eco-plates (BioLog, Haywood, CA, U.S.A.). BioLog Eco-plates contained 96-wells, prefilled with: (1) a colorless tetrazolium dye that reduces to a violet formazin if the substrate is oxidized (Stefanowicz 2006), (2) triplicates of 31 different organic compounds (Table 2) in equimolar

Table 1. List of coccolithophore strains used in the BioLog Ecoplates experiment. Table lists the strain code, species name and abbreviation used for Table 2, ploidy with N marking haploid holoccoliths, and 2N diploid heterococcoliths, × marks the unknown lifecycle phase. In addition, information on condition, maintenance, and the collection site of each culture are listed.

Strain code	Species	Abbreviation	Ploidy	Axenic	Maintained at:	Collection site
CCMP1949	*Emiliania huxleyi*	E.hux	N	●	16°C	Gulf of Maine
CCMP2090	*Emiliania huxleyi*	E.hux	N	●	16°C	South Pacific
CCMP3266	*Emiliania huxleyi*	E.hux	2N	●	16°C	Tasman Sea
CCMP3268	*Emiliania huxleyi*	E.hux	N	●	16°C	Tasman Sea
CCMP370	*Emiliania huxleyi*	E.hux	N	●	16°C	North Sea
CCMP372	*Emiliania huxleyi*	E.hux	N	●	22°C	Sargasso Sea
CCMP373	*Emiliania huxleyi*	E.hux	N	●	22°C	Sargasso Sea
CCMP374	*Emiliania huxleyi*	E.hux	N	-	22°C	Gulf of Maine
CCMP379	*Emiliania huxleyi*	E.hux	N	●	16°C	English Channel
CCMP2051	*Gephyrocapsa oceanica*	G.oce	2N	-	22°C	Collection site unknown
CCMP2054	*Gephyrocapsa oceanica*	G.oce	N	-	22°C	Collection site unknown
CCMP3392	*Calcidiscus leptoporus*	C.lep	N	-	16°C	Cape of Good Hope
CCMP298	*Cruciplacolithus neohelis*	C.neo	2N	●	16°C	California Bight
CCMP2002	*Ochrosphaera neopolitana*	O.neo	2N	●	22°C	Keala Kekua Bay, Island of Hawaii
CCMP593	*Ochrosphaera neapolitana*	O.neo	×	●	16°C	Collection site unknown
CCMP2029	*Ochrosphaera* sp.	O.sp	2N	●	22°C	Nosy Iranja Island, Madagascar
CCMP594	*Ochrosphaera verrucosa*	O.ver	2N	-	22°C	Gulf of California
CCMP299	*Chrysotila placolithoides*	C.pla	×	-	16°C	Collection site unknown
CCMP3337	*Chrysotila carterae*	C.car	2N	●	16°C	Nantucket Sound
CCMP3338	*Chrysotila carterae*	C.car	2N	●	16°C	Nantucket Sound
CCMP647	*Chrysotila carterae*	C.car	2N	●	16°C	Salton Sea
CCMP649	*Chrysotila scherffelii*	C.sch	×	●	16°C	North Atlantic
CCMP874	*Chrysotila elongata*	C.elon	2N	●	16°C	Collection site unknown
CCMP300	*Chrysotila* sp.	C.sp	×	●	16°C	Collection site unknown
CCMP875	*Chrysotila* sp.	C.sp	×	●	16°C	Collection site unknown

quantities, and (3) triplicate water blanks as a control for airborne bacterial contamination. We used a single coccolithophore strain in each plate. We started each experiment by inoculating a 96-well plate with 100 μL of log-phase cell suspension from each log phase culture. We carried out all inoculations under subdued light and all incubations in complete darkness. At time-zero (T0), 24 h, 48 h, and 72 h, we measured the reduced violet tetrazolium dye absorption on a FilterMax F5 multimode plate reader (Molecular Devices, LLC, San Jose, CA, U.S.A.) for absorption at 595 nm. Preliminary experiments ran through 96 h, and showed that most results were evident by 48 h. We interpreted the increasing optical density at 595 nm as heterotrophic metabolism of the organic compound by the coccolithophore cultures.

The potential utilization of each organic compound was expressed as the average compound color development (ACCD). We averaged the absorption observed at five points across the center of each well at each time point and subtracted the average water control well (cells A1, A5, and A9). We then calculated the mean absorbance of replicate wells for each compound. The ACCD was computed as the percentage of the absorbance of each compound over the sum of absorbance of all compounds on a plate. Moreover, we performed a paired sample *t*-test to look for statistically significant ($p < 0.05$) changes in the mean absorbance between "time-zero (T0)" and 72 h.

Several limitations to the BioLog Eco-plates should be noted (Stefanowicz 2006). One limitation is that the technique assumes that the uptake of each substrate is independent from any other (i.e., two substrates are not used in a synergistic fashion). The technique also assumes the concentrations of the substrates in each well of the microtiter plate are optimal for the organism in question, rather than too high (i.e., inhibitory) or low (limiting) based on the organism's specific uptake kinetic parameters. Therefore, we could not make any inferences on the uptake kinetics of DOC utilization using the microtiter plates. Furthermore, ions in seawater (specifically Ca^{++}) can cause false positives in BioLog Eco-plates (Pierce et al. 2014). It is critical to lower the Ca^{++} concentration from 10 mmol L^{-1} (normal concentration in seawater) to ~ 2.5 mmol L^{-1} in order to eliminate false positives (Pierce et al. 2014). The BioLog Company suggests doing this with chelators. An alternative method was suggested by Tuchman et al. (2006) in which the cultures were centrifuged and

Table 2. Uptake of coccolithophore strains to BioLog Ecoplates compounds tested in the experiment. X marks significant *t*-tested uptake after 72 h ($p < 0.05$), ● marks > 2% ACCD. Abbreviations: (1) compounds PEA: Phenylethyl-amine; Putrescine; L-Arg: L-arginine; L-Asp: L-asparagine; L-Phe: L-phenylalanine; L-Ser: L-serine; L-Thr: L-threonine; Gly-L-GlutA: Glycyl-L-glutamic acid; β-M-D-Glu: β-methyl-D-glucoside; D-Xyl: D-xylose; I-Ery: I-erythritol; D-Man: D-mannitol; α-Cyclo: α-cyclodextrin; N-A-D-Galacto: N-acetyl-D-galactosamine; D-Cellob: D-cellobiose; α-D-Lact: α-D-lactose; D-GA-γ-Lactn: D-galactonic acid-γ-lactone; D-Gal-Acid: D-galacturonic acid; 2-H-BenzA: 2-hydroxy benzoic acid; 4-H-BenzA: 4-hydroxy benzoic acid; γ-H-ButyA: γ-hydroxy butyric acid; D-GluA: D-glucosaminic acid; ItacidA: itaconic acid; α-K-ButyA: α-keto-butyric acid; D-MalA: D-malic acid; Pyr-Meth-E: pyruvic acid methyl ester; Glu-1-P: glucose-1-phosphate; D,L-α-Gly-P: D,L-α-glycerol-phosphate; Tween 40; Tween 80; Glycogen. (2) Strains (for more information, see Table 1). Strain with bold numbers and * mark are nonaxenic.

		Isochrysidales												**Coccolithales**												
Compound	Strain	1949	2090	3266	3268	370	372	373	**374***	379	**2051***	**2054***	**3392***	298	2002	593	2029	**594***	**299***	3337	3338	647	649	874	300	875
	Ploidy	N	N	2N	N	N	N	N	N	N	2N	N	N	2N	2N	×	2N	2N	×	2N	2N	2N	×	2N	×	×
	Species	E.hux	E.hux	E.hux	E.hux	E.hux	E.hux	E.hux	E.hux	E.hux	G.oce	G.oce	C.lep	C.neo	O.neo	O.neo	O.sp	O.ver	C.pla	C.car	C.car	C.car	C.sch	C.elon	C.sp	C.sp
Amine	PEA			●	●						●					X										
	Putrescine														●											
Amino acid	L-Arg		●	X				X	X		●			X						X	●			X	X	
	L-Asp		●												●	●		●	●						●	●
	L-Phe			●																						
	L-Ser								●					●	●	X								●	●	
	L-Thr	●	●	●			●	●	X					X	●	●		●		X	●					●
	Gly-L-GlutA	●	●	●			●		●				●	●	●	●		●							●	
Carbohydrates	β-M-D-Glu	●	●	●	●	●	●	X	X	●	●	●	●	X	●		●	●	●	X	●	●	●	●	●	●
	D-Xyl	●	●	●	●	●	●	X	●	●	●			X	●	●			●	X	●	●		X	X	●
	i-Ery	●	●	●	●		X	X	X	●	●	●	●	X	●	●	●	●	●	X		●	●	X	X	●
	D-Man	●	●	●				●	X	●		●		X	●	X		●	●	X	●		●	X	X	●
	α-Cyclo	●					●		●				●	●	●			●								
	N-A-D-Galacto	●	●		●	X	●	●	X		●	●	●	X	●		●	●	●	●	●		●	●	●	●
	D-Cellob	●	●		●		●	●	●		●		●	X	●	●		●	●	●	●			X	●	●
	α-D-Lact	●	●	●	●			●	●				●	●	●	●		●	●	●	●	●		X	●	●
Carboxylic acid	D-GA-γ-Lactn			●										●		●										
	D-Gal-Acid	●							●					●		X		●	●						●	
	2-H-BenzA												●			●						●				●
	4-H-BenzA			●																						
	γ-H-ButyA	●	●	●		●	●	●	●				●		●			●	●		●			●	●	●
	D-GluA		●	●			●		X					X	●				●	X	●	●	●	X	●	●
	ItacidA	●	●	●	●	●	●	●	●		●	●	●	●	●	●	●		●	●	●	●	●	●	●	●
	α-K-ButyA	●		●			●		●		●			●			●			●	●	●		●	●	
	D-MalA			●																						
Ester	Pyr-Meth-E																	●								
P-H	Glu-1-P																●				●			●	●	●
	D,L-α-Gly-P			●																						
Polymer	Tween 40	●	●	●	●	●	●	●			●	●	●	●			●	●		●	●	●	●	X	●	●
	Tween 80	●			●	●	●	●			●	●	●					●	●	●	●	●	●	X	●	●
	Glycogen	●														X	●	●	●							
	Total	17	15	19	10	7	14	13	17	4	11	7	12	18	16	15	8	16	14	14	15	10	8	16	19	16

pelleted to separate them from the nutrient-rich media, which might contain other growth-inducing substrates. Then the pellet should be resuspended in nutrient-free saline solution. We followed these latter recommendations and resuspended the coccolithophores in ASW with 2.5 mmol L^{-1} calcium.

^{14}C-labeled uptake experiments

After determining the metabolic potential, we investigated the uptake of specific dissolved organic compounds, which showed high potential for osmotrophy. We selected five ^{14}C-labeled-DOC compounds based on results of the BioLog Eco-plates survey as well as commercial availability of radiotracer-labeled compounds. The selected compounds included sugar alcohols (glycerol and mannitol), carbohydrate (xylose), and amino acid (arginine). Additionally, we selected acetate due to its biochemical importance and availability in marine ecosystems (Wu et al. 1997; Ho et al. 2002). Specific activities of the radiotracers were: glycerol: 160 μCi μmol^{-1}; mannitol: 57 μCi μmol^{-1}; xylose: 200 μCi μmol^{-1}; arginine: 338 μCi μmol^{-1}; and acetate: 52 μCi μmol^{-1} (acetic acid sodium salt) (PerkinElmer, Waltham, MA, U.S.A.). As a reference uptake compound, we used ^{14}C-bicarbonate (56 μCi μmol^{-1}) (MP Biomedicals, LLC, Santa Ana, CA, U.S.A.) incubations in photosaturated light conditions. We performed radiolabel uptake experiments on axenic coccolithophore strains, CCMP289 *C. neohelis* and CCMP3337 *C. carterae* (NCMA lists the strain as *Pleurochrysis carterae*). We maintained the cultures in media and light conditions as described above, and at 22°C (CCMP289) and at 16°C (CCMP3337).

For the survey of arginine and xylose net uptake in darkness, we prepared two 70 mL master samples (concentration of 1×10^5 cells L^{-1}) of CCMP289 and CCMP3337 cultures in log phase growth. We measured cell concentrations using a hemocytometer on an American Optical Microscope (Spencer Lens Company, Buffalo, NY, U.S.A.) with polarization optics. We added unlabeled arginine or xylose to each strain's master sample up to a 20 μmol L^{-1} final concentration. From each master sample, 10 mL were then removed into separate borosilicate vials that were kept in the dark for subsequent cell counts over the duration of the experiment. To the remaining 60 mL culture samples containing unlabeled arginine or xylose, we added ^{14}C-arginine or ^{14}C-xylose, to a final concentration (labeled and unlabeled) of 20.25 μmol L^{-1} and 20.83 μmol L^{-1}, respectively. We withdrew 45 mL of the 60 mL sample and divided that into three 15 mL replicate vials. We transferred the remaining 15 mL into a fourth vial with buffered formalin as a formalin-killed control. Due to logistical issues in sample manipulation, the actual time of addition of the first ^{14}C-labeled compound was 10 ± 5 min after addition of formalin to the labeled control. We then subsampled and filtered all 16 vials (12 samples [triplicates of the two ^{14}C-labeled compounds × two strains] and four formalin samples [two compounds × two strains]). After the first time point, we placed samples in the dark incubators at 22°C for CCMP289 and 16°C for CCMP3337. Subsampling for each time course experiment was performed at 3, 6, 24, and 48 h. For subsampling, we performed filtration of each 2 mL of culture subsamples onto each 0.4 μm pore size, 25 mm diameter polycarbonate filter. Following filtration, filters were carefully rinsed three times with ASW (including a careful rim rinse) to remove any ^{14}C-labeled, dissolved compound left on the filter. Each filter was then placed in the bottom of a clean scintillation vial, and scintillation cocktail was added (Balch et al. 2000). The radioactivity was measured using a Tri-Carb 3110TR liquid scintillation analyzer (PerkinElmer, Waltham, MA, U.S.A.). We calculated the ^{14}C-labeled-compound net uptake rates following the equations of Parsons et al. (1984).

$$v = ([R_n - R_f] \times W)/(R \times T)$$

where v is the net uptake rate (mol L^{-1} h^{-1}), R_n is the sample count (dpm) at time T, R_f is the formalin-killed control count (dpm), and W (mol L^{-1}) is the concentration of available compound in the sample. R is the total activity (dpm) of the added compound to a sample and T (h) is the number of hours of incubation.

We also examined the net uptake of ^{14}C-arginine and ^{14}C-xylose uptake in illuminated cultures over 24 h. We added these ^{14}C-labeled compounds to axenic cultures (CCMP289 or CCMP3337) to a final concentration of 0.37 μmol L^{-1} for ^{14}C-arginine and 1 μmol L^{-1} for ^{14}C-xylose. We sampled at T15 min and T24 h, stopping the incubation by filtration, and measured the net ^{14}C uptake as described above.

Furthermore, we examined the net uptake of ^{14}C-acetate, ^{14}C-glycerol, and ^{14}C-mannitol in darkness over 24 h and compared it with ^{14}C-bicarbonate net uptake (in light). Prior to addition of radiolabeled compounds, axenic cultures (CCMP289 or CCMP3337) were divided into separate vials and 5 mL of log-phase culture were removed for the enumeration of cell concentration. To correct for any effects due to EtOH solvent in the ^{14}C-acetate, in one 5 mL sample we added only 0.0125 mL of EtOH as a control. We added ^{14}C- labeled compounds to each separate vial to a final concentration of 4.81 μmol L^{-1} of ^{14}C-acetate, 1.49 μmol L^{-1} of ^{14}C-glycerol, 4.18 μmol L^{-1} of ^{14}C-mannitol, and for comparison we used ^{14}C-bicarbonate to a final concentration of 2.6 mmol L^{-1} of labeled and unlabeled form. Triplicate samples for uptake measurements were filtered after 15 min and 24 h of darkness.

Results

Screening of coccolithophores for the assimilation of organic compounds

In our BioLog Eco-plate survey of the metabolism of organic compounds by coccolithophores, we interpreted ACCD higher than 2% as indication of utilization potential (Sala et al. 2005). Isochrysidales, on average, utilized 49% of compounds in BioLog Eco-plates, while Coccolithales, on

average, utilized 59% of compounds. The average number of potentially utilized compounds by coccolithophores was 13, ranging from 4 to 19 (Table 2). ACCD higher than 2% was noted for all compounds at least once, and carbohydrates and polymers were the most potentially utilized compounds. Based on ACCD in the BioLog Eco-plates, the most commonly utilized carbohydrates were: β-Methyl-D-Glucoside, D-Xylose, i-Erythritol, and N-Acetyl-D-Galactosamine.

Based on more stringent criteria (a paired sample *t*-test at an alpha level of < 0.05) for absorbance over time, changes were noted for 10 strains (Table 2). Of the strains from the order Isochrysidales, five significantly utilized organics of which four were haploid strains and one was diploid. A nonaxenic *E. huxleyi* strain CCMP374, from the Gulf of Maine (Table 1), significantly utilized seven compounds (Table 2), four of which were carbohydrates. Axenic *E. huxleyi* strain CCMP373 from the Sargasso Sea (Table 1) significantly metabolized up to four compounds, three of which were carbohydrates (Table 2). Three more strains of *E. huxleyi* significantly utilized organic compounds, one per strain. Five strains from the order Coccolithales, three diploid and two of undetermined ploidy, showed significant uptake of organic compounds and all high performers were axenic (Fig. 1). *C. neohelis* (CCMP298) significantly metabolized nine compounds, of which six were carbohydrates, *C. elongate* (CCMP874) also significantly utilized nine compounds, with five of them being carbohydrates, and *C. carterae* (CCMP337) significantly used seven compounds, four of which were carbohydrates. The carbohydrate i-Erythritol and amino acid L-Arginine were the most significantly utilized compounds (Fig. 1), taken up by seven different strains. Furthermore, of the carbohydrates, six strains were able to significantly take up D-Mannitol, five D-Xylose, and four β-Methyl-D-Glucoside. The D-Glucosaminic Acid, which is a carboxylic acid, was significantly taken up by four strains (Table 2).

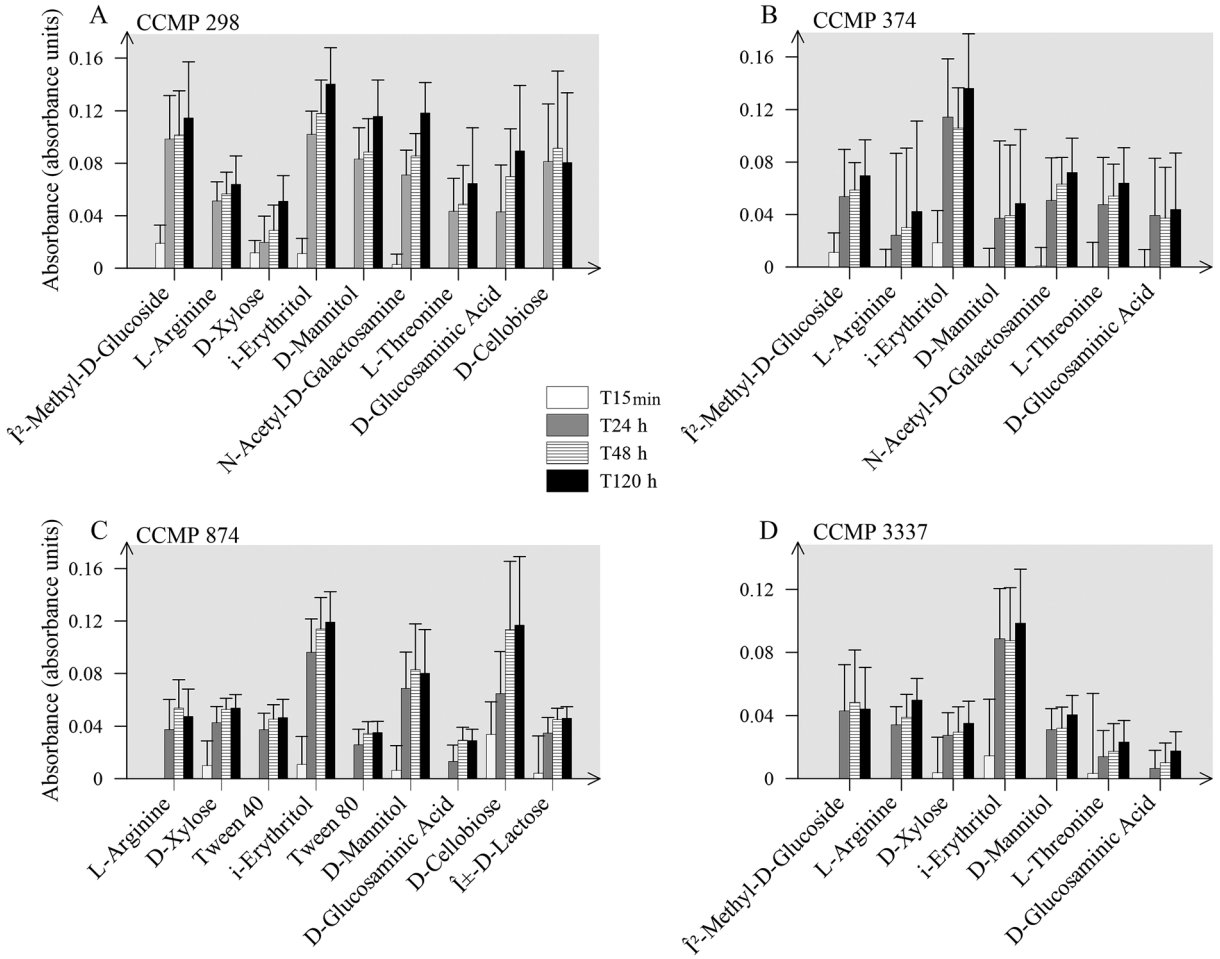

Fig. 1. Uptake of organic compounds showing significant utilization ($p < 0.005$) by four top strains as estimated using BioLog Ecoplates. Histograms show the absorbance at 15 min, 24h, 48h and 120h. Absorbance was corrected for blanks. Values shown are means of five individual measurements from replicate wells (optimally a total N of 15). Error bars represent one standard deviation (which includes error propagation calculations for subtracting mean blank values from sample values, each with their own respective error term).

Net uptake rate measurements of selected organic compounds

Of the compounds that showed significant uptake in the above BioLog plate experiments, i-Erythritol, β-Methyl-D-Glucoside, and D-Glucosaminic Acid were not available in radiotracer form. Thus, we focused on ^{14}C-mannitol, -xylose, -arginine, -glycerol, and -acetate. We added glycerol, even if it was not significantly taken up by any strain in the BioLog Eco-plates, due to the previous experiments performed by Blankley (1971); and acetate because of its ecological significance as a fundamental, simple molecule used in many biochemical reaction pathways (Ho et al. 2002).

We depicted time course experiments of ^{14}C-labeled arginine and xylose in darkness as net uptake in Fig. 2. In general, the net uptake was highest in the beginning, and dropped down toward the end of a time course. The strain CCMP3337 had the highest net uptake of ^{14}C-arginine at T15 min, 4.9×10^{-4} pmol cell^{-1} h^{-1}, and the lowest after 48 h, 1.9×10^{-6} pmol cell^{-1} h^{-1} (Fig. 2A). We did not record net uptake of ^{14}C-arginine for the strain CCMP298. Maximal net uptake of ^{14}C-xylose by CCMP3337 reached 1.4×10^{-3} pmol cell^{-1} h^{-1} at T15 min and it dropped down to 1.1×10^{-4} pmol cell^{-1} h^{-1} after 48 h (Fig. 2B). Maximal net uptake of ^{14}C-xylose by CCMP298 was 2.1×10^{-4} pmol cell^{-1} h^{-1} at 3 h, and it dropped to 5.7×10^{-6} pmol cell^{-1} h^{-}1 after 48 h (Fig. 2C).

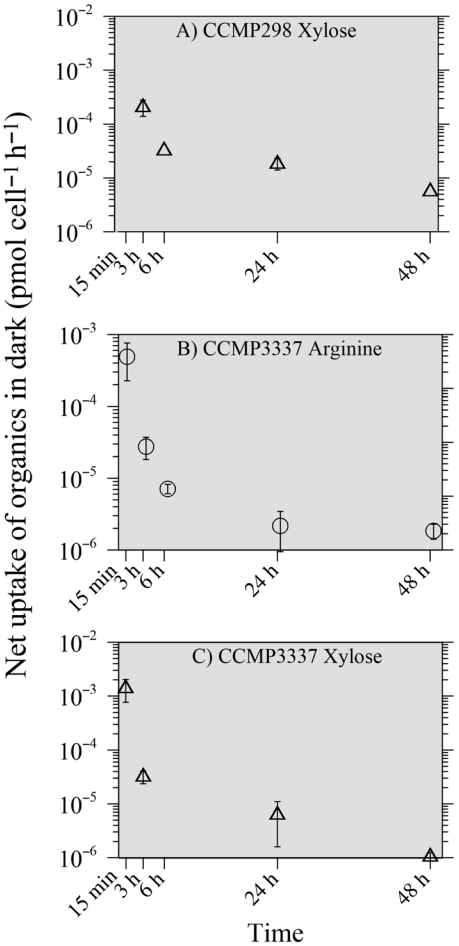

Fig. 2. Scatter plots showing net uptake of ^{14}C-labeled organic compounds over 48 h in darkness by two strains of coccolithophores: (**A**) arginine by *Chrysotila carterae* (CCMP3337), (**B**) xylose by the same species, and (**C**) xylose by *Cruciplacolithus neohelis* (CCMP289). All values were corrected for blanks. Missing values for CCMP3337 for 6 h and CCMP298 for 15 min were negative (not statistically different from zero) and thus could not be shown on a logarithmic axis. Error bars represent one standard deviation (which includes error propagation calculations for subtracting mean blank values from sample values, each with their own respective error term).

The net uptake rates for ^{14}C-labeled arginine and xylose in the light over 24 h for the same two strains are shown in Fig. 3. The net uptake rate for ^{14}C-arginine by CCMP289 at T15 min was 4.3×10^{-6} pmol cell^{-1} h^{-1}, and net uptake rate for ^{14}C-xylose by the same strain at T15 min was 1.0×10^{-6} pmol cell^{-1} h^{-1} (Fig. 3A). After 24 h, net uptake rates for both organic compounds were not significantly different from zero (Fig. 3A). Similar values were noted for the strain CCMP3337 (Fig. 3B). The net uptake rate for ^{14}C-arginine was 6.3×10^{-6} pmol cell^{-1} h^{-1} at T15 min, while the net uptake rate for ^{14}C-xylose was 3.5×10^{-6} pmol cell^{-1} h^{-1} at T15 min (Fig. 3B). After 24 h, net uptake rates for both compounds did not significantly differ from zero.

The net uptake rate for ^{14}C-labeled organics in darkness over 24 h for CCMP289 and CCMP3337 was compared with net uptake of bicarbonate in the light in Fig. 4. For CCMP289, after 24 h, we noted a net uptake rate of 3.4×10^{-6} pmol cell^{-1} h^{-1} for ^{14}C-glycerol and 1.4×10^{-6} pmol cell^{-1} h^{-1} for ^{14}C-mannitol (Fig. 4A). CCMP3337 exhibited similar values of net uptake after 24 h, 1.4×10^{-6} pmol cell^{-1} h^{-1} and 1. 5×10^{-6} pmol cell^{-1} h^{-1} for both ^{14}C-glycerol and ^{14}C-mannitol, respectively (Fig. 4B). In contrast, uptake of ^{14}C-bicarbonate after 24 h in the light was 9×10^{-2} pmol cell^{-1} h^{-1} for CCMP298 (Fig. 4A) and 2×10^{-2} pmol cell^{-}1 h^{-}1 for CCMP3337 (Fig. 4B).

The net uptake of ^{14}C-acetate in darkness over 24 h for species CCMP298 and CCMP3337 was compared with photosynthetic net uptake of ^{14}C-bicarbonate (Fig. 4C,D, respectively). The ^{14}C-acetate net uptake rate per cell dropped from 3.3×10^{-4} pmol cell^{-1} h^{-1} to 9.7×10^{-5} pmol cell^{-1} h^{-1} for CCMP289 after 24 h (Fig. 4C). The cellular net uptake rate for ^{14}C-acetate by CCMP3337 went from 8.8×10^{-5} pmol cell^{-1} h^{-1} to 1.9×10^{-5} pmol cell^{-1} h^{-1} after 24 h (Fig. 4D). Net uptake of ^{14}C-bicarbonate after 24 h was 5×10^{-2} pmol cell^{-1} h^{-1} and 4×10^{-2} pmol cell^{-1} h^{-}1 for CCMP298 (Fig. 4C) and CCMP3337 (Fig. 4D), respectively.

Discussion

Mixotrophy has emerged as a potentially significant mode of nutrition for the plankton and has the potential to change our current modeling efforts of carbon cycling (Chakraborty et al. 2017; Selosse et al. 2017). However, phytoplankton mixotrophy remains a vastly understudied topic in biological oceanography (Mitra 2018), especially osmotrophy, one of the main

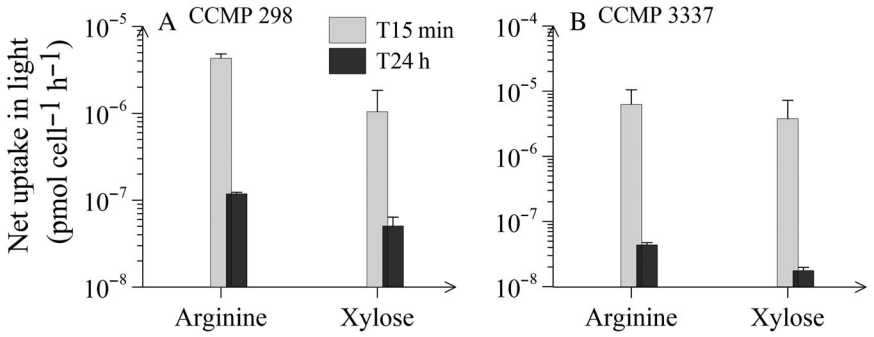

Fig. 3. Histograms showing net uptake of ^{14}C-labeled, arginine and xylose, organic compounds per cell over 24 h in the light for two strains: (**A**) *Cruciplacolithus neohelis* (CCMP289) and (**B**) *Chrysotila carterae* (CCMP3337). Values shown are means of three individual measurements. Error bars represent one standard deviation (which includes error propagation calculations for subtracting mean blank values from sample values, each with their own respective error term).

mixotrophic mechanisms (Selosse et al. 2017). Tuchman et al. (2006) reported that osmotrophy in diatoms enables uptake of a large number of dissolved organic compounds in the darkness, specifically 94% of the tested compounds from BioLog microtiter plates. Diatoms surveyed in Tuchman et al. (2006) were all benthic, likely adapted to light-limited conditions, frequent burials, and high turbidity. This would be a different photic environment from the coccolithophore species used in our experiments. Based on the array of coccolithophore species examined here, we found that coccolithophores possess the potential for heterotrophic uptake and assimilation of a wide variety of carbon compounds present in BioLog Eco plates. Moreover, the potential to utilize organics was found across their classical taxonomic subdivisions, and Coccolithales metabolized on average 56% of the compounds in the darkness, while Isochrysidales metabolized 49%. Coccolithophores inhabit both coastal and oceanic

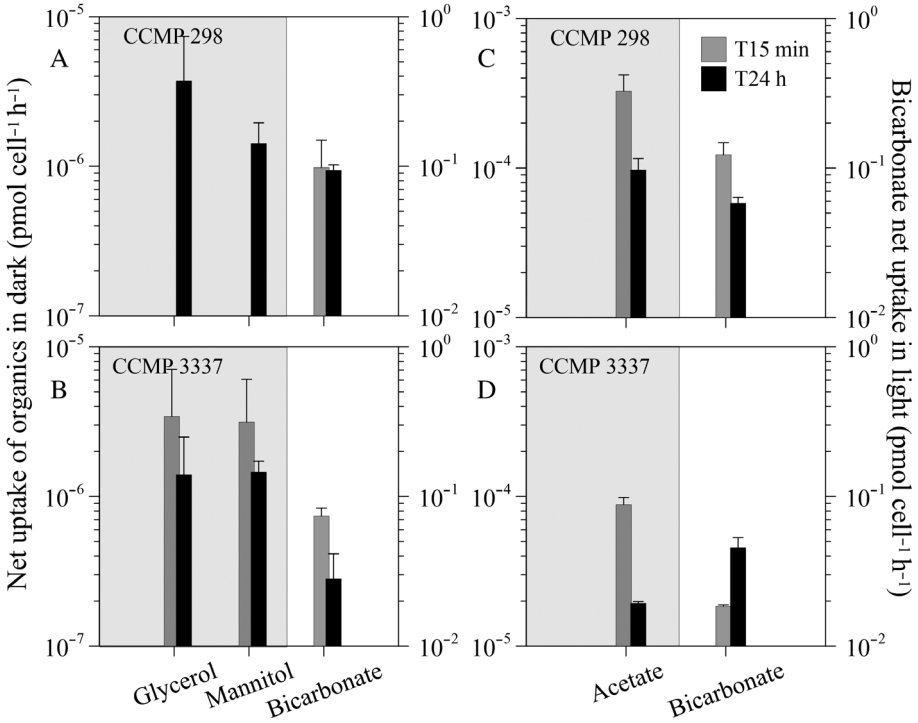

Fig. 4. Histograms show the average net uptake per cell of ^{14}C-labeled organics (in darkness) compared to the net bicarbonate uptake (in illuminated cultures) for two coccolithophore strains, *Cruciplacolithus neohelis* (CCMP289) and *Chrysotila carterae* (CCMP3337). (**A**) Glycerol and mannitol compared with bicarbonate for strain CCMP289, (**B**) glycerol and mannitol compared with bicarbonate for strain CCMP3337, (**C**) acetate compared with bicarbonate for strain CCMP289, and (**D**) acetate compared with bicarbonate for strain CCMP3337. Note that left Y-axis shows values of net uptake of organic in darkness (gray part of each graph), while photosynthetic net bicarbonate uptake values are shown on the right Y-axis (white part of each graph). All values were corrected for blanks. Values shown are means of three individual measurements. Missing values for CCMP298 T15 min glycerol and mannitol were negative (not statistically different from zero) and could not be shown on a logarithmic axis. Error bars represent one standard deviation (which includes error propagation calculations for subtracting mean blank values from sample values, each with their own respective error term).

environments, but they are primarily pelagic (De Vargas et al. 2007). Thus, it is to be expected that coccolithophores exhibit less diverse mechanisms for uptake of organic compounds compared to benthic diatoms.

The successful utilization of an organic compound entails the existence of a transport system for that specific compound and environmental conditions appropriate for activation of the transport system (Amblard et al. 1992; Tuchman et al. 2006). Cellular transport systems for organic compounds are highly specific (Hellebust 1971; Montesinos et al. 1995). One might hypothesize that coccolithophores exhibit higher specificity in the uptake of organic compounds than benthic diatoms, because organic compounds in the pelagic realm are not as diverse (and not at as high concentrations) as in benthic environments. Hence, coccolithophores might not have evolved a diverse set of transport systems for such a wide array of organic compounds as benthic diatoms.

Diversity of organic compounds

Dissolved organic matter in the ocean nonetheless consists of a wide array of compounds. We can find simple biochemicals (amino acids, simple sugars, vitamins, fatty acids), complex biopolymers (proteins, polysaccharides, lignins), and highly complex degradation products (humic substances, black carbon) (Repeta 2015). Researchers are still far from creating a holistic picture of DOC compounds involved in the ocean carbon cycle. Even though the BioLog Eco plates examined only 31 organic compounds, they nonetheless illustrate the great variety of compounds that are potentially metabolized and are an excellent platform for testing hypotheses about algal mixotrophy.

In the BioLog experiments of this study, we observed that the major type of organic compounds taken up by coccolithophores were primarily carbohydrates with a few amino acids and polymers (Table 2). Carbohydrates in general are common constitutes of dissolved organic matter in the ocean (Benner et al. 1997). Skoog and Benner (1997) found no preference by microorganisms for removal of specific sugars from ocean, and showed that carbohydrates were removed in similar molar ratios as they occurred in reactive organic material. They indicated that microorganisms in the ocean preferentially degraded glucose, mainly due to its greater abundance and fundamental role in biogeochemical reactions. Skoog and Benner (1997) suggested investigation of metabolism of other abundant organic compounds would be in order (Skoog and Benner 1997). We found that, among the carbohydrates, β-Methyl-D-Glucoside, D-Xylose, i-Erythritol, and N-Acetyl-D-Galactosamine were utilized most frequently by coccolithophores.

Additionally, we found that the second most potentially utilized organic compounds, after carbohydrates, were amino acids. L-arginine was the most frequently utilized amino acid in BioLog Eco plates. In fact, coccolithophores in that respect showed similarity with the green algae *Chlamydomonas reinhardtii*, which has a highly specific carrier involved in uptake of exogenous arginine (Kirk and Kirk 1978). Kirk and Kirk (1978) were unable to detect signs of carriers of other amino acids, even in cultures maintained on amino acids as a nitrogen source or starved for nitrogen. We did not perform nutrient deficiency experiments, but found that seven strains are able to significantly take up L-arginine, while only three took up L-serine.

It is crucial to note that concentrations of compounds in the BioLog Eco plates were likely higher than in natural environments. Thus, the potential of coccolithophores to utilize specific carbohydrates might be very different at natural concentrations. Nevertheless, Wright and Hobbie (1966) highlighted that in evaluating the importance of the dissolved organics as a carbon source, the study of the rate of uptake is much more important than measurements of the absolute concentration of the dissolved organics available to them.

Net uptake of organic compounds

After demonstrating that coccolithophores exhibit potential uptake of organic compounds, we evaluated the net uptake rate of ^{14}C-labeled organics at a more natural concentration. Fickian diffusion across the cell membrane would indicate that organics are freely exchangeable, while active transport against concentration gradients would indicate the presence of specific transporters requiring cellular metabolic energy. The length of time before significant net uptake was observed varied (e.g., peak uptake of xylose by *C. carterae* after 15 min and by *C. neohelis* after 3 h; Fig. 2B,C, respectively), and could suggest a species-specific activation of uptake. Additionally, *C. carterae* and *C. neohelis* both exhibited a drop in the net uptake rates after 24 h. The net uptake is a measure of both influx and efflux of the ^{14}C-label. Initial net uptake rates are representative of the maximal potential of cells to take up these ^{14}C-labeled compounds, and the drop in the net uptake rates indicates that the influx is approaching the efflux of the label, whether it is attached to the original organic compound or is now attached to another molecule.

The drop of net uptake rates of the ^{14}C-labeled organic compounds after 24 h could have resulted from several scenarios, here expressed as three hypotheses: (1) net uptake stopped because of loss of cell viability in darkness, (2) influx of the organic compound into the cells (either actively or passively) occurred until pools were filled, after which influx stopped, and (3) influx of the radio-labeled organic compounds were followed by its catabolism and subsequent breakdown to ^{14}C-CO_2 (or another breakdown product) which effluxed from the cell. It is important to address these hypotheses using both the results of the BioLog experiments as well as the radiolabeled uptake experiments. Regarding hypothesis (1), we know that cells were viable and could metabolize the various organics after 72 h of darkness based on the BioLog results, so this hypothesis is untenable. Hypothesis (2) could not be eliminated based solely on the radiolabel experiments, however, the BioLog experiments again suggested that metabolism of the organic compound continued well after 24 h in darkness, suggesting that influx of the organic compound did not stop; thus, hypothesis (2) is also untenable. Hypothesis (3),

however, is consistent with the results of both the BioLog and ^{14}C uptake experiments. That is, if the ^{14}C-organic compound was catabolized after uptake (e.g., with the ^{14}C-CO_2 respired from the cell), then one could envision an initial influx of the radiolabeled organic (rapid uptake), followed by its metabolism and subsequent respiration. Such that a steady state developed between the influx of the ^{14}C-organic compound and the efflux of the ^{14}C breakdown product (such that the net uptake approached zero). Unfortunately, we did not measure the amount of ^{14}C-CO_2 remaining in the media (nor the headspace of the incubation vessel, for that matter), thus we can only comment that hypothesis (3) is tenable and most consistent with the observations from these experiments. More experimentation is required to accept hypothesis (3). Note, too, that based on the cell concentrations and total uptake observed over 24 h, we can confirm that < 1% of the total substrate (both labeled and unlabeled) appears to have been metabolized in these experiments, hence it appears unlikely that the reduction in net uptake rates was due to substrate limitation.

Blankley (1971) demonstrated that generation times of *E. huxleyi* and *C. carterae* were long, if grown on glycerol in complete darkness. Slower growth rates during prolonged darkness may indicate the stopping of photo-physiological processes and switchover to respiratory metabolism, which might also explain the low uptake rates after the initial surge. Moreover, calculating from the carbon content (Menden-Deuer and Lessard 2000) of 50 pg for *C. carterae* and 30 pg for *C. neohelis*, we assessed the time necessary for a total carbon turnover for just the organic molecule in question. We calculated that growing solely on xylose, and assuming the net incorporation of the carbon into cell biomass only, total carbon turnover would be extremely slow, 0.35 yr for *C. carterae* and 1.4 yr for *C. neohelis*. Clearly, active growth supported by the net uptake of a single organic carbon source, such as xylose, would be exceptionally unlikely for these coccolithophores given that carbon turnover was so slow.

Poulton et al. (2017) suggested that deep-living coccolithophores supplement their growth with mixotrophy, as light levels in the subeuphotic zone are likely to be well below those required to support photosynthesis. We compared the uptake of arginine and xylose in the dark and light conditions. In our light experiments, *C. carterae* and *C. neohelis* exhibited net uptake rates for arginine and xylose in the very low range of fractions of a pmol $cell^{-1}$ h^{-1}. The extremely slow uptake rate in the light conditions compared to higher uptake in dark conditions would indicate that coccolithophores might have the ability to activate uptake of organics as a survival strategy, albeit at extremely slow rates. Such a survival strategy could have an impact on the carbon cycle in deeper areas of the ocean, especially if they could assimilate a wide array of organics, which appears to be the case based on our experiments with BioLog Plates.

We also observed that uptake of glycerol and mannitol over 24 h in darkness was similar for both *C. neohelis* and *C. carterae*. However, for glycerol, there was no effective net uptake of glycerol at micromolar concentrations by *C. neohelis* and *C. carterae* compared to growth on bicarbonate (turnover of ~ 2 d). Moreover, there was no effective growth on mannitol. When grown in a concentration of 10.8 mmol L^{-1} glycerol, Pintner and Provasoli (1968) reported *C. kappa* survival for 6 months with growth kinetics similar to those in light conditions. However, we acknowledge the differences in uptake might exist between species, as the high variability between varieties, forms, types, ecotypes, subspecies, and so on, is a signature feature of coccolithophores (De Vargas and Probert 2004). Indeed, the possibility of organics allowing survival of cells without cell division remains a plausible hypothesis.

Finally, we investigated acetate, one of the most important and fundamental organic compounds found in the ocean (Ho et al. 2002). Acetate proved to be an excellent organic substrate in the darkness, for both *C. neohelis* and *C. carterae*. Similarly, Singh et al. (2014) showed that *C. reinhardtii* cells assimilate acetate at a faster rate during heterotrophic growth than during mixotrophic growth, primarily due to the competing entry of carbon via CO_2 fixation during mixotrophic growth. The mechanism by which acetate is taken up in both coccolithophores and green algae is not well known, but indeed merits more focus in future studies. In our study, it would take a little under a year for the total carbon turnover in *C. neohelis* based solely on net uptake of acetate, compared with ~ 2 d for the photosynthetic assimilation of bicarbonate. Overall, the turnover times for organics that we observed are long, but in the ocean, there is not just one dissolved organic compound present, but many. Hence, it is plausible to state that mixotrophic uptake in coccolithophores could be significant enough to be incorporated into carbon cycling models if they could assimilate a wide array of organics simultaneously.

Differences in organic uptake between life cycle phases

Coccolithophores have a haplo-diploid life cycle, in which both phases are capable of independent asexual reproduction. The different phenotypes of their life cycle phases also indicate a difference in their ecologies. The process of haploidization and reduction of genome size by half probably requires major reorganization in the pattern of gene expression, leading to the creation of two largely different cellular types (De Vargas and Probert 2004). Moreover, the expansion of the ecological niches by different life strategies may be particularly appropriate for survival in ecosystems with fluctuating nutrient availability (Godrijan et al. 2018). We noted a difference between organic uptake by haploid and diploid phases in Isochrysidales and Coccolithales orders. Isochrysidales appear to mostly utilize organic compounds in the haploid phase, while Coccolithales appear to mostly utilize organic compounds in the diploid phase. Houdan et al. (2006) similarly reported that the diploid phase of *Calcidiscus leptoporus* is more mixotrophic. However, they also observed that the haploid motile phase of *Coccolithus braarudii* enhanced its growth rate 40% in the presence of 0.1 mmol L^{-1} sodium acetate, and was more mixotrophic (osmotrophic and/or phagotrophic) than the

diploid phase (Houdan et al. 2006). Thus, our results are consistent with Houdan et al. (2006) suggesting that mixotrophy in coccolithophores varies between the haploid and diploid phases.

Conclusion

We found that (1) Coccolithophores can metabolize a wide array of organics in darkness, (2) the uptake rates are low relative to the photosynthetic potential for carbon fixation, (3) the time course of uptake for some compounds suggests the constitutive capacity for their transport, while for others the transport appears to be activated, (4) nonetheless, the slow uptake rate for individual organic compounds combined over the large array of organic compounds found in seawater, might be the only way that there could be sustained growth of coccolithophores in the deep euphotic and the subeuphotic zones of the sea.

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Conflict of Interest

None declared.

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