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 14C-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 brevituritta were increased by glycerine 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 Crisocolintha cartae (current taxonomic name: Chrysothila 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, Ochrospora neapolitana, Ochrospora sp., Ochrospora veurcosa, Chrysothila placolithoides, Chrysothila carterae, Chrysotila scherffelii, Chrysothila elongata, and Chrysothila 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 (Hallegraef 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 (Hallegraef 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, Hayward, 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
of the absorbance of each compound over the sum of each compound. The ACCD was computed as the percentage
We then calculated the mean absorbance of replicate wells for tracted the average water control well (cells A1, A5, and A9).
across the center of each well at each time point and sub-
expression as the average compound color development
(pierce et al. 2014). It is critical to lower the Ca++ concentration from 10 mmol L
-1 in order to eliminate false positives (pierce et al. 2014). The BioLog Company suggests doing this with
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). 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

| 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         | North 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 leptopus | C.lep |   | -     | 16°C         | Cape of Good Hope |
| CCMP298     | Cruciplacolithus neoheilis | C.neo | 2N    | ●      | 16°C         | California Bight |
| CCMP2002    | Ochrosphaera neopelitana | O.neo | 2N    | ●      | 22°C         | Keala Kekua Bay, Island of Hawaii |
| CCMP593     | Ochrosphaera neopelitana | 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     | Chrysothila placolithoides | C.pla | ×     |  -    | 16°C         | Collection site unknown |
| CCMP3337    | Chrysothila carterae | C.car | 2N    | ●      | 16°C         | Nantucket Sound |
| CCMP3338    | Chrysothila carterae | C.car | 2N    | ●      | 16°C         | Nantucket Sound |
| CCMP647     | Chrysothila carterae | C.car | 2N    | ●      | 16°C         | Salton Sea      |
| CCMP649     | Chrysothila scherfeli | C.sch | ×     | ●      | 16°C         | North Atlantic  |
| CCMP874     | Chrysothila elongata | C.elon | 2N    | ●      | 16°C         | Collection site unknown |
| CCMP300     | Chrysothila sp. | C.sp  | ×     | ●      | 16°C         | Collection site unknown |
| CCMP875     | Chrysothila sp. | C.sp  | ×     | ●      | 16°C         | Collection site unknown |

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 holococcoliths, and 2N diploid heterococcoliths, × marks the unknown lifecycle phase. In addition, information on condition, maintenance, and the collection site of each culture are listed.
| Isochrysidales | Coccolithophores |
|---------------|------------------|
| **Compound**  | **Strain**       |
| 1949          | 2009             |
| 2366          | 370              |
| 372           | 373              |
| 374*          | 379              |
| 2051*         | 2054*            |
| 3392*         | 399*             |
| 593           | 2929             |
| 647           | 300              |
| 849           | 300              |
| 875           |                  |
| **Species**   | **Ploidy**       |
| E.hux         | N                |
| E.hux         | N                |
| E.hux         | N                |
| E.hux         | N                |
| E.hux         | N                |
| G.oce         | N                |
| G.oce         | N                |
| G.lep         | N                |
| G.neo         | N                |
| G.neo         | N                |
| O.neo         | N                |
| O.neo         | N                |
| O.sp          | N                |
| C.pla         | N                |
| C.car         | N                |
| C.car         | N                |
| C.car         | N                |
| C.chl         | N                |
| C.elon        | N                |
| C.sp          | N                |
| **Amine**     |                  |
| PEA           | ●● ●             |
| Putrescine    | ●● ●             |
| **Amino acid**|                  |
| L-Arg         | ●● ●             |
| L-Asp         | ●● ●             |
| L-Phe         | ●● ●             |
| L-Thr         | ●● ●             |
| L-Ser         | ●● ●             |
| Gly-L-GlutA   | ●● ●             |
| **Carbohydrates**|            |
| β-M-D-Glu     | ●● ●             |
| D-Man         | ●● ●             |
| α-Cyclo       | ●● ●             |
| N-A-D-Galacto | ●● ●             |
| D-Cellob      | ●● ●             |
| D-Lacto       | ●● ●             |
| D-GA-γ-Lactn  | ●● ●             |
| D-Gal-Acid    | ●● ●             |
| 2-H-BenzA     | ●● ●             |
| 4-H-BenzA     | ●● ●             |
| γ-H-ButyA     | ●● ●             |
| D-GluA        | ●● ●             |
| ItacidA       | ●● ●             |
| α-K-ButyA     | ●● ●             |
| D-MalA        | ●● ●             |
| **Ester**     |                  |
| Pyr-Meth-E    | ●● ●             |
| Glu-1-P       | ●● ●             |
| D,L-α-Gly-P   | ●● ●             |
| **Polymer**   |                  |
| Tween 40      | ●● ●             |
| Tween 80      | ●● ●             |
| Glycogen      | ●● ●             |
| **Total**     | 17               |

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; l-Ery: l-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.
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.

**14C-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 14C-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 14C-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 × 10⁵ 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 separated 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 14C-arginine or 14C-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 14C-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 14C-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 rinse) to remove any 14C-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 14C-labeled-compound net uptake rates following the equations of Parsons et al. (1984).

\[
\nu = \frac{(R_o - R_i) \times W}{(R \times T)}
\]

where \(\nu\) is the net uptake rate (mol L\(^{-1}\) h\(^{-1}\)), \(R_o\) is the sample count (dpm) at time \(T\), \(R_i\) is the formalin-killed control count (dpm), and \(W\) (mol L\(^{-1}\)) is the concentration of available compound in the sample. \(\nu\) 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 14C-arginine and 14C-xylose uptake in illuminated cultures over 24 h. We added these 14C-labeled compounds to axenic cultures (CCMP289 or CCMP3337) to a final concentration of 0.37 μmol L\(^{-1}\) for 14C-arginine and 1 μmol L\(^{-1}\) for 14C-xylose. We sampled at T15 min and T24 h, stopping the incubation by filtration, and measured the net 14C uptake as described above.

Furthermore, we examined the net uptake of 14C-acetate, 14C-glycerol, and 14C-mannitol in darkness over 24 h and compared it with 14C-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 14C-acetate, in one 5 mL sample we added only 0.0125 mL of EtOH as a control. We added 14C-labeled compounds to each separate vial to a final concentration of 4.81 μmol L\(^{-1}\) of 14C-acetate, 1.49 μmol L\(^{-1}\) of 14C-glycerol, 4.18 μmol L\(^{-1}\) of 14C-mannitol, and for comparison we used 14C-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 14C-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 14C-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 14C-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 14C-arginine for the strain CCMP298.

Maximal net uptake of 14C-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 14C-xylose by CCMP298 was 2.1 × 10^{-4} pmol cell^{-1} h^{-1} at 3 h, and it dropped to 5.7 × 10^{-5} pmol cell^{-1} h^{-1} after 48 h (Fig. 2C).

The net uptake rates for 14C-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 14C-arginine by CCMP289 at T15 min was 4.3 × 10^{-6} pmol cell^{-1} h^{-1}, and net uptake rate for 14C-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 14C-arginine was 6.3 × 10^{-6} pmol cell^{-1} h^{-1} at T15 min, while the net uptake rate for 14C-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 14C-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 14C-glycerol and 1.4 × 10^{-6} pmol cell^{-1} h^{-1} for 14C-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 14C-glycerol and 14C-mannitol, respectively (Fig. 4B). In contrast, uptake of 14C-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 14C-acetate in darkness over 24 h for species CCMP298 and CCMP3337 was compared with photosynthetic net uptake of 14C-bicarbonate (Fig. 4C,D, respectively). The 14C-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 14C-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 14C-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.

![Fig. 2](image-url) Scatter plots showing net uptake of 14C-labeled organic compounds over 48 h in darkness by two strains of coccolithophores: (A) arginine by *Chrysothila 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).

**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
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. 3. Histograms showing net uptake of 14C-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).

Fig. 4. Histograms show the average net uptake per cell of 14C-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 constituents 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 (Skooq 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 14C-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. cartenea after 15 min and by C. neohealis after 3 h; Fig. 2B,C, respectively), and could suggest a species-specific activation of uptake. Additionally, C. cartenea and C. neohealis 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 14C-label. Initial net uptake rates are representative of the maximal potential of cells to take up these 14C-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 14C-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 14C-CO2 (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 radio-labeled 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 14C uptake experiments. That is, if the 14C-organic compound was catabolized after uptake (e.g., with the 14C-CO2 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 14C-organic compound and the efflux of the 14C breakdown product (such that the net uptake approached zero). Unfortunately, we did not measure the amount of 14C-CO2 remaining in the media (nor the net uptake) approached zero. 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 Provasoni (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 CO2 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 leptopus* 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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