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PRODUCTION AND CELLULAR LOCALIZATION OF NEUTRAL LONG-CHAIN LIPIDS IN THE HAPTOPHYTE ALGAE *ISOCHRYSIS GALBANA* AND *EMILIANIA HUXLEYI*[1]

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***Isochrysis galbana* Parke, *Emiliania huxleyi* (Lohm.) Hay and Mohler, and some related prymnesiophyte algae produce as neutral lipids a set of polyunsaturated long-chain (C_{37-39}) alkenones, alkenoates, and alkenes (PULCA). These biomarkers are widely used for paleothermometry, but the biosynthesis and cellular location of these unique lipids remain largely unknown. By staining with the fluorescent lipophilic dye Nile Red, we found that *I. galbana* and *E. huxleyi*, like many other algae, package their neutral lipid into cytoplasmic vesicles or lipid bodies. We found that these lipid bodies increase in abundance under nutrient limitation and disappear under prolonged darkness and show that this pattern correlates well with the concentration of PULCA as measured by TLC. In addition, we show that lipid vesicles purified by sucrose density gradient centrifugation consist predominantly of PULCA. We also found significant pools of neutral lipid associated with chloroplasts, and PULCA component profiles in lipid vesicles and chloroplasts are similar. Examination of cell ultrastructure shows conspicuous cytoplasmic and chloroplast lipid bodies, and we suggest that PULCA may be synthesized in chloroplasts and then exported to cytoplasmic lipid bodies for storage and eventual metabolism. Our results connect and extend prior observations of lipid bodies and membrane-unbound PULCA in *I. galbana* and *E. huxleyi*, as well as the behavior of PULCA during nutrient and light stress.**

Key index words: **alkenoates; alkenones; alkenes; *E. huxleyi*; haptophytes; *I. galbana*; lipid bodies; neutral lipids; Nile Red; PULCA; TLC; ultrastructure**

Abbreviations: **ER, endoplasmic reticulum; LB, lipid body; NR, Nile Red; PULCA, polyunsaturated long-chain alkenes, alkenones, and alkenoates; TAG, triacylglycerol**

Some prymnesiophyte taxa of the order Isochrysaldes (*Isochrysis, Emiliania, Gephyrocapsa, Chrysotila*) produce only small amounts of triacylglycerol (TAG) as their neutral lipid (Marlowe et al. 1984a,b). They produce instead a suite of polyunsaturated long-chain (C_{37-39}) alkenes, alkenones, and alkenoates (Sukenik and Wahnon 1991, Patterson et al. 1994, Fábregas et al. 1998), abbreviated here as PULCA (Fig. 1). These compounds are unlike the *cis*-polyunsaturated fatty acids typical of eukaryotic membrane constituents in that they have two to four unusual *trans*-alkene bonds occurring at 7-carbon intervals (Fig. 1a), although this has been confirmed only for the C_{37} alkenones (de Leeuw et al. 1980, Rechka and Maxwell 1988a,b) and C_{37-38} alkenes (Riley et al. 1998). No saturated isomers have been reported, but Rontani et al. (2001, 2004a) detected monounsaturated alkenones in all taxa. Another set of C_{31-33} alkenes produced by these taxa has been determined to have *cis* geometry (Riley et al. 1998) and are likely biosynthetically distinct.

At colder growth temperatures, PULCA are more highly polyunsaturated (Conte et al. 1995, 1998), and the proportion of diunsaturated isomers of the C_{37} methyl alkenones (the "unsaturation index" $U^{K'}_{37}$) was proposed as a growth temperature proxy (Brassell et al. 1986, Prahl and Wakeham 1987). Because these compounds were first discovered in marine coccolith-bearing sediments (de Leeuw et al. 1980, Volkman et al. 1980a,b), sedimentary core-top $U^{K'}_{37}$ has been widely adopted by geochemists as a proxy for surface water paleotemperature (Müller et al. 1998). However, the calibration of unsaturation index depends on strain genetics (Conte et al. 1998) and environmental factors such as nutrient limitation or light availability (Epstein et al. 1998, 2001, Yamamoto et al. 2000, Versteegh et al. 2001, Prahl et al. 2003), and the utility of this tool remains limited by our lack of understanding of the biosynthesis of these compounds.

The cellular location of the PULCA has long been a puzzle. Early reports (Brassell et al. 1986, Prahl et al. 1988) assumed they were membrane lipids and suggested the increased degree of PULCA unsaturation at colder growth temperatures played a role in maintaining membrane fluidity. One ultrastructural study (van der Wal et al. 1985) hypothesized that an unusual "intermediate" layer of the plasma membrane might be the location of these compounds. In contrast, Conte and Eglinton (1993) stated that alkenones were not detected in the membranes of lysed cells, although they provided no data in support of that assertion. However, a recent study by Sawada and Shiraiwa (2004) examined isolated cell membrane fractions of *E. huxleyi* and found that alkenones and alkenoates

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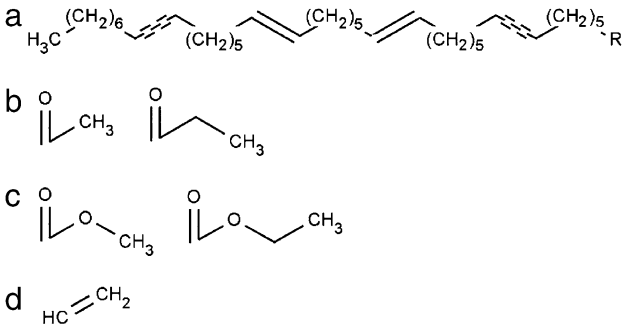

FIG. 1. PULCA structures. (a) C_{35}-alkene-based PULCA skeleton. Diunsaturated isomers at C_{14} and C_{21} are shown, with sites of additional *trans*-desaturation at C_7 and C_{28} denoted as interrupted double bonds. Longer C_{36} and C_{37} analogs occur with additional carbons at the R group. (b–d) Terminal R groups include C_{37} methyl/C_{38} ethyl alkenones (b), C_{36} methyl or ethyl alkenoates (c), and C_{37} alkenes (d).

associated with internal organelles, such the endoplasmic reticulum (ER) and coccolith-producing compartment, and suggested these PULCA components are membrane-unbound lipids. Additional PULCA were also found in the Golgi and plasma membranes and in chloroplast thylakoids. But unlike the ER and coccolith-producing compartment, these fractions were dominated by fatty acids typical of membrane lipids.

The physiological role of PULCA appears to resemble the role of other neutral lipids, which often serve as energy reserves. Pond and Harris (1996) showed that in *Emiliania huxleyi*, PULCA are present in all growth phases but cellular pools increase in stationary phase, as is typical for storage lipids (Henderson and Sargent 1989, Hodgson et al. 1991). Epstein et al. (1998) and Prahl et al. (2003) observed increased PULCA accumulation under N or P limitation, up to 10%–20% of cell C in stationary phase, although this varied considerably among strains. Epstein et al. (2001) and Prahl et al. (2003) also showed that under energy-depleted growth conditions (prolonged darkness), PULCA pools decrease due to catabolism. Other eukaryotes that store TAGs often compartmentalize these into lipid vesicles or lipid bodies (LBs) (Murphy 2001). These droplets can be visualized with the fluorescent stain Nile Red (NR) (Cooksey et al. 1987, Brzezinski et al. 1993, Dempster and Sommerfeld 1998, Kimura et al. 2004), which selectively stains neutral lipids. Oleaginous fungi (Kamisaka et al. 1999) and algae (Schneider and Roessler 1994) accumulate very high amounts of neutral lipid and have been studied for oil production and as aquaculture food stocks. Among haptophytes, there is little known about LBs, but Liu and Lin (2001) observed lipid droplet formation and accumulation in *Isochrysis galbana*, especially as cells entered the stationary phase.

In this study, we present data on the cellular location of the long-chain neutral lipids in the haptophytes *I. galbana* and *E. huxleyi*. We identified LBs within these algae, showed their behavior is consistent with a role in energy storage, and determined the composition of purified LBs to be PULCA.

MATERIALS AND METHODS

Cultures. *Emiliania huxleyi* and *I. galbana* cultures (Table 1) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) or were a gift from Suzanne Strom, Western Washington University. *Emiliania huxleyi* CCMP 1742 is a synonym for strain 55a, the calibration standard for alkenone paleothermometry (Prahl and Wakeham 1987). *Emiliania huxleyi* CCMP 1516 is a calcifying strain chosen by the DOE, U.S. Dept. of Energy for genome sequencing (B. Read and T. Wahlund, personal communication). Cultures were grown in 100–200 mL volumes of f/2 or f/20 media under 80 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ cool-white illumination at 16° C in a 16:8-h light:dark cycle. In some experiments, we grew cells with either nitrate or phosphate reduced to 10% f/2 levels to specifically induce N or P limitation. For cells incubated in complete darkness, the temperature and other conditions remained the same. Cell numbers were determined by hemocytometer or by Coulter Counter (Coulter Electronics, Ltd., Luton, U.K.) using a 70-μm orifice.

NR staining, microscopy, and spectrofluorometry. Cells fixed with glutaraldehyde (final concentration 0.5%) were stained in 1-mL aliquots with 6 μL of an NR stock solution (500 μg $\cdot$ mL^{-1} in acetone) and counterstained with one drop of 15 μg $\cdot$ mL^{-1} DAPI. We viewed wet mounts with an Olympus BX51 microscope (Melville, NY, USA) and a Pixera Penguin 600ES charged coupled device camera (Los Gatos, CA, USA) for bright-field, Nomarski DIC, or fluorescence using blue excitation (455 nm). A Schott glass bandpass output filter centered at 495 nm (Oriel optics no. 51720, Stratford, CT, USA) was added to mask chl emission. Spectrofluorimetry was performed on 3 mL stained fixed cells with an Ocean Optics (Dunedin, FL, USA) USB2000 spectrofluorometer driven by a Sutter Instruments (Novato, CA, USA) Lambda-LS Xe light source with 492 ± 10 nm band pass filter (Edmund Optics #NT46–042, Barrington, NJ, USA).

Cell fractionations. Cells were pelleted in a clinical centrifuge, and the pellets were repeatedly subjected to osmotic lysis with a protease inhibitor cocktail containing 1 mM

TABLE 1. CCMP algal cultures used in this study.

CCMP strain	Synonyms	Origin	Latitude	Growth (° C)	Calcification
Emiliania huxleyi					
1742	VAN556, 55a, NEPCC55a	N.E. Pacific	50 N	8–25	No
1516	CCMP2090	S. Pacific	2 S	14–22	Under low nutrients
370	(451B, F451)	Norway	59 N	11–16	No
374	(89E, CCMP1949)	Gulf of Maine	42 N	11–22	No
Isochrysis galbana					
1323	ISO, Strain "I", NEPCC2	Irish Sea	54 N	3–28	No

EDTA, 1 mM PMSF, 1 mM benzamidine, and 10 μg · mL^{-1} leupeptin and pepstatin A (Sigma-Aldrich, St. Louis, MO, USA). Cell debris and unlysed cells were removed by low-speed centrifugation (3000 rpm, 5 min), and the remaining cell-free homogenate was collected. Two to 3 mL of the cell-free homogenate was layered over a discontinuous sucrose gradient consisting of 5 mL 60%, 5 mL 45%, 5 mL 30%, and 5 mL 15% sucrose. The gradients were ultracentrifuged at 30,000 *g* for 16 h at 4° C. Buoyant LB fractions were collected from the surface by pipette. Chloroplast fractions were collected from the 45%–60% sucrose interface and were resuspended in small volume of water before lipid extraction.

Lipid extractions. Cells or cell fractions were collected by filtration onto GF/F (Whatman, Florham Park, NJ, USA) filters, by centrifugation, or by addition of aqueous samples directly to the extraction solvents. Lipids were extracted using a modified Bligh and Dyer procedure (Bligh and Dyer 1959). Briefly, filters or pellets were immediately placed in 10:5:4 $CHCl_3/MeOH/H_2O$ and vortexed and then incubated for 2–24 h. Chloroform and water were added to a final concentration of 10:10:9 $CHCl_3/MeOH/H_2O$ to produce two phases, which were separated by centrifugation in a clinical centrifuge. The organic layer was removed, and the remaining aqueous layer was reextracted with chloroform. The collected extracts were evaporated to dryness under N_2 at 50° C. Samples were resuspended in 2:1 $CHCl_3$/MeOH, capped under N_2, and stored at −20° C until analysis.

Lipid analysis. Total lipids were separated by TLC using a double development system (Olsen and Henderson 1989) on silica HPTLC plates (60 μm particle size, Camag Scientific, Wilmington, NC, USA). The development of polar lipids was performed with 25:25:25:10:9 Me acetate/isopropanol/$CHCl_3$/MeOH/KCl (0.9% aqueous solution). For nonpolar lipids, a solvent containing a 80:20:2 mixture of hexane/diethyl ether/glacial acetic acid was used. Plates were scanned at 600 dpi on a flatbed scanner to detect pigments. Lipids were detected by spraying with 3% (w/v) cupric acetate in 8% (v/v) H_3PO_4 and charring for 10 min at 160° C. Standards included phosphatidylcholine, phosphatidylglycerol, digalactosyl diacylglyceride, cholesterol, a TAG mixture, and a fatty acid methyl ester (FAME) mixture (Sigma-Aldrich). Detection limit was about 5 μg for individual lipid components. Plates were scanned again, and TIFF images were analyzed for densitometry with Kodak 1D image analysis software (New Haven, CT, USA). For densitometry, calibrations standard curves (5–25 μg) included phosphatidylcholine (phospholipids), digalactosyl diacylglyceride (glycolipids), and TAGs (PULCA). GC analysis of PULCA fractions was performed as described in Prahl et al. (2003).

TEM. Cells pelleted for TEM were fixed in 0.5× Karnovsky's fixative (pH 7.4) for 30 min and rinsed twice for 5 min each in 0.1 M Sorensen's phosphate buffer. For standard staining, cells were postfixed in 1% OsO_4 for 30 min and rinsed in 0.1 M Sorensen's phosphate buffer. For osmium thiocarbohydrazide-osmium staining (Willingham and Rutherford 1984, Guyton and Klemp 1988), cells were postfixed with a saturated aqueous solution of thiosemicarbazide, followed by a 5-min buffer rinse, a 20-min postfix in 1% OsO_4, and a 5-min buffer rinse. All samples were dehydrated in a series of 70, 90, and three 100% acetone solutions, each for 10 min. Pellets were infiltrated with a 50:50 mixture of Epon/acetone overnight and then embedded in 100% Epon resin and polymerized for 48 h at 65° C. Sections were cut on an LKB ultramicrotome and collected on 300 mesh Cu grids. Sections were stained in 2% uranyl acetate for 5 min, rinsed three times for 15 s each in distilled water, then stained in Fahmy lead for 5 min, followed by a rinse for 15 s in 20 mM NaOH, then three 15-s rinses in distilled water. Sections were viewed and photographed on an Hitachi H-300 transmission electron microscope (Schaumberg, IL, USA), and negatives were digitized with a Microtek scanner (Carson, CA, USA) at 3600 dpi.

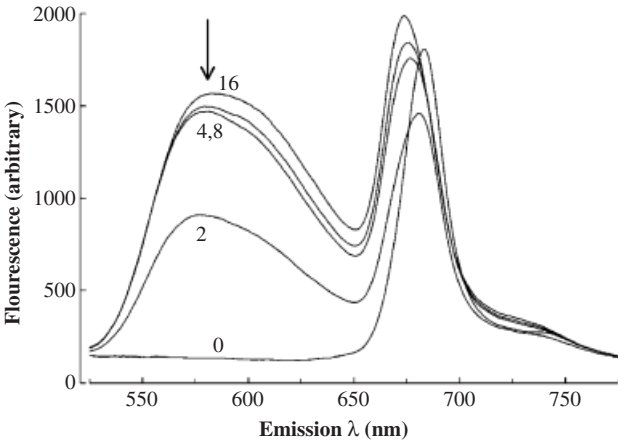

FIG. 2. Fluorescence emission spectra of *Pyrenomonas salina* stained with 0, 2, 4, 8, or 16 μg · mL^{-1} NR. Excitation at 492 nm. Chlorophyll peak is at 675 nm, whereas NR emission from neutral lipid (TAG) at 580 nm (arrow) increases up to 4 μg · mL^{-1}.

RESULTS

LBs observed by epifluorescence and LM. Preliminary tests with NR showed optimal staining at 4 μg · mL^{-1}, with emission at 580–625 nm (Fig. 2). Under NR staining, *Isochrysis* and *Emiliania* cells in late exponential phase showed red-staining cell membranes with large yellow-staining lipid vesicles (Fig. 3a). When a Schott glass bandpass filter was added to the emission path to block red fluorescence, some yellow staining was also visible in chloroplasts and sometimes around the cell membrane (Fig. 3b). Lipid bodies varied in number and size among cells, but most cells had multiple LBs, typically located next to the chloroplasts or at the periphery of cells. Nomarski DIC images of cells without NR staining showed LBs visible as refractive inclusions (Fig. 3c), and with bright-field illumination, LBs often showed a blue coloration (Fig. 3d).

LB and PULCA behavior during nutrient and light stress. We next examined what happens to these bodies during times of lipid anabolism or catabolism. *Emiliania huxleyi* 1742 cells that reached stationary phase due to N or P limitation showed a qualitative increase in LBs under NR staining (Fig. 4a, day 10). The TLC showed major increases of PULCA pools in stationary phase (Fig. 4b, days 4–10), and neutral lipid accounted for nearly all the increased lipid over this time (Fig. 4c). In contrast, when *E. huxleyi* cells were placed into the dark for 72 h, NR-stained cells showed nearly complete loss of NR signal at 580–625 nm, with only partial loss of chl fluorescence (Fig. 5). The TLC analysis showed that in the dark, PULCA stores were markedly reduced, although there were changes in polar lipids as well (Fig. 6a).

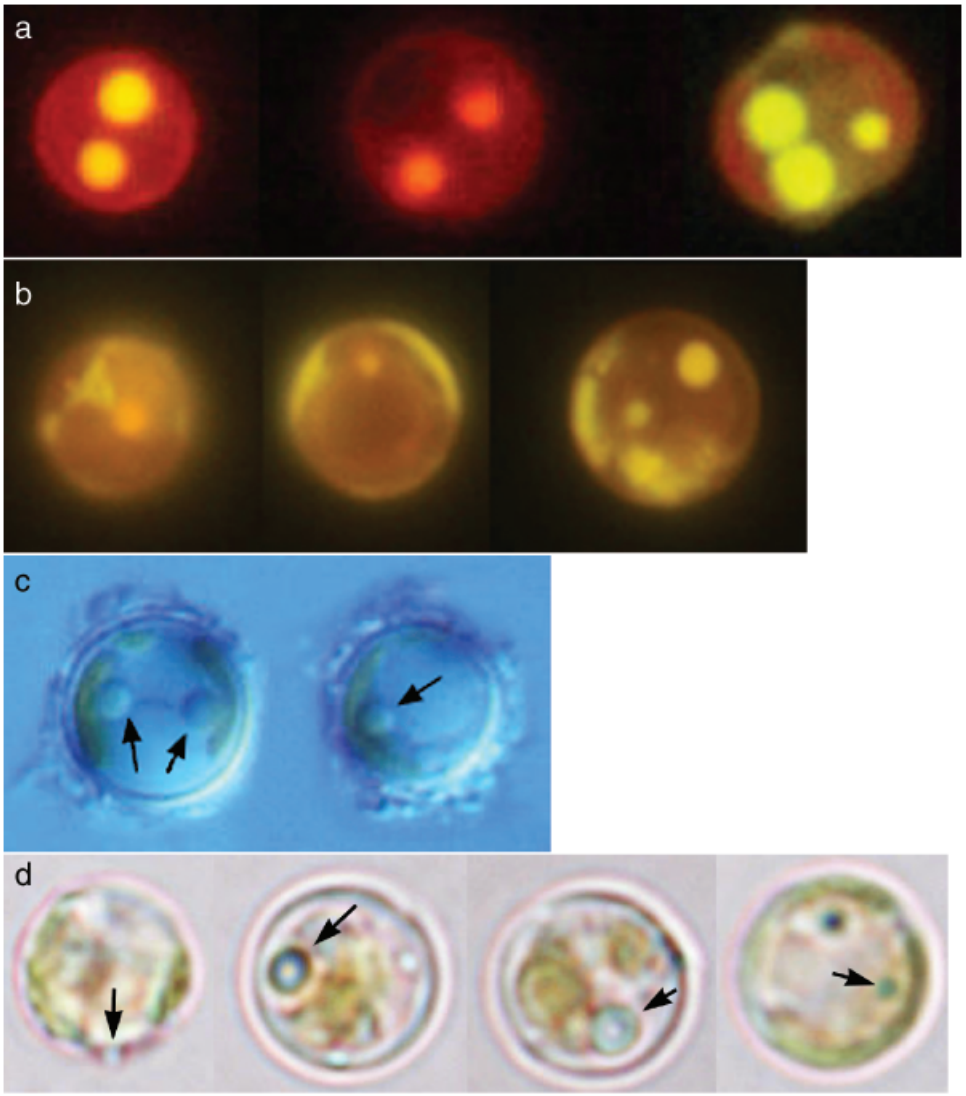

FIG. 3. Light micrographs of LBs. (a, b) NR-stained epifluorescence. (a) *Isochrysis galbana* without emission bandpass filter. Neutral lipid appears yellow, polar lipids and chl, red. (c) *Emiliania huxleyi* 1742 with emission filter to remove red fluorescence. (c) Nomarski DIC images of *E. huxleyi* 1516. Ragged coccosphere is visible on cell exterior. Spherical lipid vesicles (arrows) are seen associated with chloroplasts. (d) Bright-field image of *E. huxleyi* 1742. Note blue coloration of LBs (arrows). Cell diameters 3–5 μm.

Isochrysis cells in prolonged darkness exhibited a small decrease in PULCA, with relatively little change in polar lipids (Fig. 6b). However, even after 7 days in darkness, we did not observe large NR fluorescence reductions in *I. galbana*. Rather, we observed that neutral lipid seemed to move from chloroplasts to cytoplasmic LBs (Fig. 6, c and d). This pattern was also characteristic of lighted but nutrient-limited *Isochrysis* (Fig. 3a), although total PULCA pools increased in the light.

PULCA in subcellular fractionations. After sucrose density ultracentrifugation, NR staining of the buoyant fraction of stationary phase cell lysates revealed numerous LBs (Fig. 7a), as did the chloroplast fraction (not shown). The TLC analysis of the buoyant fraction revealed that LBs are comprised primarily of neutral alkenones and alkenes, with some phosphatidylcholine and a sterol or carotenoid (Fig. 7b, lane 2). The latter might account for the pigmentation of LBs under bright-field microscopy (Fig. 3d). The TLC also revealed a significant amount of neutral lipids in the chloroplast fraction (Fig. 7b, lane 3). *Isochrysis galbana* and *E. huxleyi* cultures produced similar results, but *E. huxleyi* lipids associated with LB were more difficult to recover after centrifugation, even when cells had notable lipid vesicles (not shown). The GC analysis of cell fractions showed similar profiles of PULCA components (Fig. 8). This pattern was observed for several *E. huxleyi* strains as well as *I. galbana*, although component profiles were strain- or species-specific (Table 2).

LB ultrastructure. The transmission electron micrographs of stationary phase *E. huxleyi* cells showed large dark-staining lipid bodies in the cytoplasm (Fig. 9). As with light micrographs (Fig. 3), TEM showed lipid bodies typically associated with chloroplasts, usually appressed to the chloroplast membrane (arrows, Fig. 10, a and b). Chloroplasts contained smaller dark-staining bodies adjacent to thylakoid membranes (arrows, Fig. 10, c and d). Osmium thiocarbohydrazide-osmium staining produced very dark LBs, whereas standard osmium

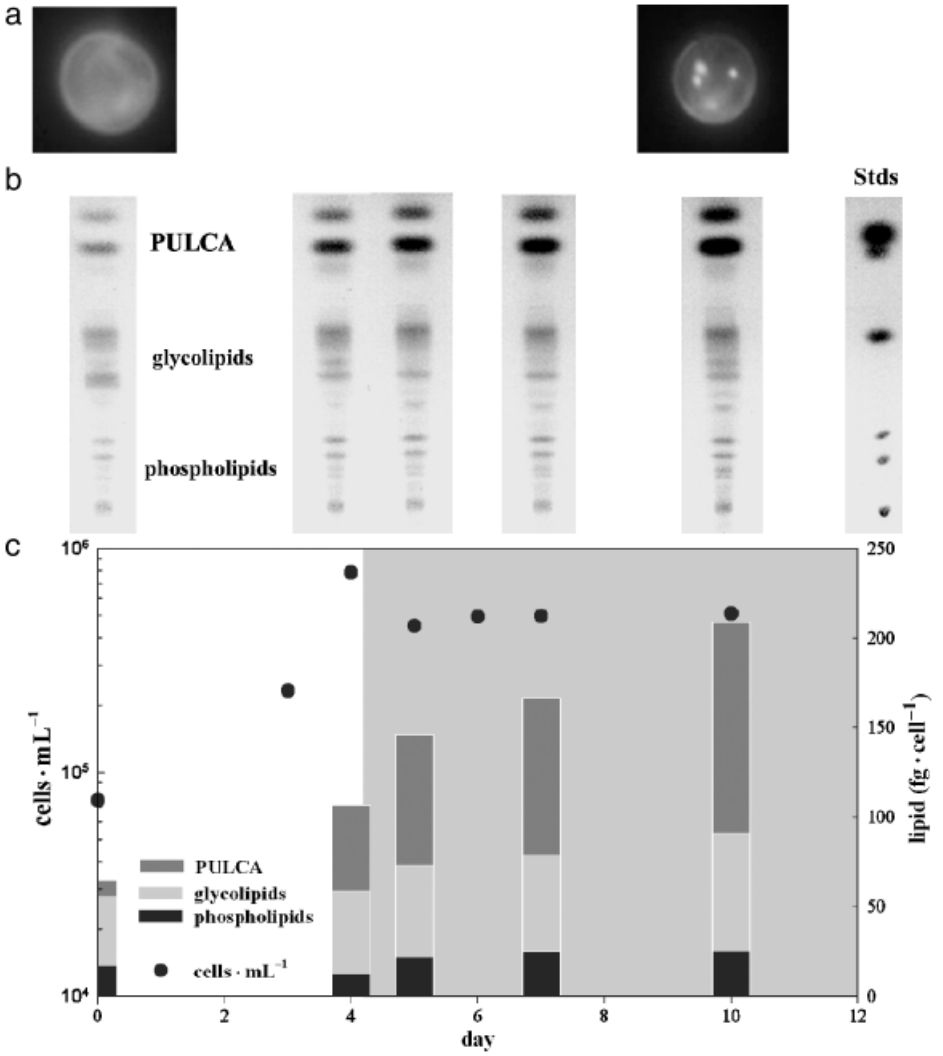

FIG. 4. *Emiliania huxleyi* 1742 LB and PULCA production under nutrient depletion over a 10-day incubation. (a) Examples of NR-stained cells in exponential phase (day 0, left) or late stationary phase (day 10, right). (b) TLC of total lipid extracts on days 0, 4, 5, 7, and 10. Lipid standards consist of (bottom to top) phosphatidylcholine, phosphatidylglycerol, digalactosyl diacylglyceride, cholesterol, TAG, and fatty acid methyl ester. (c) Cell numbers (circles) and major lipid pools (bars), as quantified by TLC densitometry. Stationary phase on days 4–10 is denoted by gray shading.

staining gave more variable results. We saw no evidence of a membrane bounding the bodies. *Isochrysis galbana* cells showed similar features (not shown).

DISCUSSION

Here, we provide evidence that the PULCA-producing taxa *I. galbana* and *E. huxleyi* have conspicuous cytosolic LBs easily visible by LM and EM that consist primarily of NR-staining PULCA neutral lipid. NR labeling showed that LBs increase at stationary phase (P or N limitation) and decrease in the dark, exactly the behavior observed for bulk PULCA (Prahl et al. 2003) and consistent with an energy storage role, as suggested by several workers (Pond and Harris 1996, Epstein et al. 1998, Prahl et al. 2003). Rontani et al. (2004b) recently showed that *Chrysotila lamellosa* can degrade alkenones via epoxidation, adding support to this hypothesis. Cell fractionations show that LBs consist primarily of PULCA, whereas chloroplasts contain a significant pool as well. This idea is supported by electron micrographs that show conspicuous osmophilic LBs in both the cytoplasm and plastids. Although we cannot rule out a pool of PULCA in cell membranes, our results suggest most of this lipid resides in LBs.

LB ultrastructure. Most eukaryotic cells produce LBs consisting of a hydrophobic core of neutral lipids (typically containing TAGs but also sterol esters or wax esters) surrounded by a phospholipid-protein coat (Zweytick et al. 2000, Murphy 2001). Some also contain sterols or carotenoids (Zweytick et al. 2000). Our fractionations suggest that *I. galbana* and *E. huxleyi* have LBs consisting mostly of neutral PULCA but with some sterol or phospholipids, although there is no evidence of a membrane. Light micrographs and lipid separations also suggest some pigment associated with the LBs. In other algae, carotenoids can co-occur with neutral lipids in structures such as chromoplasts (Murphy 2001).

Lipid bodies have not been well studied for haptophytes, although they are common in other algae (Cooksey et al. 1987, Dempster and Sommerfeld 1998). This may be due to the assumption that chrysolaminarin, a $\beta 1 \rightarrow 3$ glucan found in chloroplast

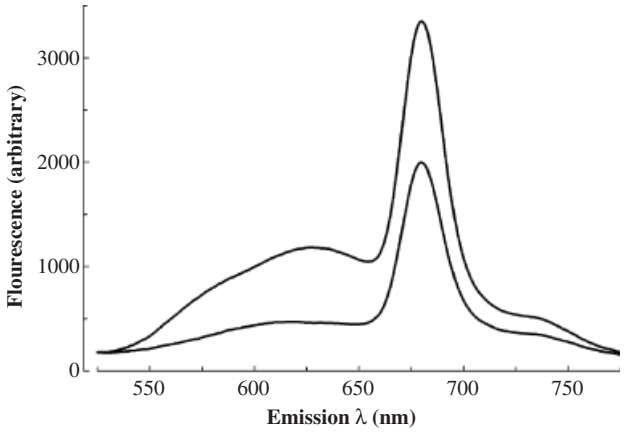

FIG. 5. Spectrofluorometric analysis of NR-stained *Emiliania huxleyi* 1742 cells in light (upper curve) and after 72-h dark incubation (lower curve), showing near total loss of NR peak at 625 nm.

pyrenoids, is the main storage metabolite in coccolithophorids (Pienaar 1994). Authors previously describing the ultrastructure of *E. huxleyi* have failed to identify neutral lipid droplets, although their images show osmophilic bodies that resemble ours strikingly, particularly for cells in nutrient depletion (Wilbur and Watabe 1963 [fig. 19], Klaveness 1972 [fig. 2]). A more recent review of cell ultrastructure (Pienaar 1994) noted that lipid droplets are often located adjacent to the plastid, as we observed, and become more abundant as the cells age, but no specific studies were cited. Liu and Lin (2001) provided the first ultrastructural description of cytosolic LBs in *I. galbana*. They documented increasing LB production in stationary phase and suggested these are synthesized in the chloroplast before being exported to the cytoplasm. Curiously, they did not connect the large LBs to PULCA, speculating instead that those neutral lipids were part of cellular membranes.

PULCA location and biosynthesis. Neutral lipid synthesis is typically associated with membrane-bound enzymes, and possible sites of biosynthesis include thylakoids, chloroplast membranes, or the ER. Several indirect lines of evidence suggest that PULCA biosynthesis is associated with the chloroplasts. Our TEM preparations usually showed LBs adjacent to chloroplast membranes and often showed incipient LBs in the chloroplasts. Our fractionation experiments demonstrated that chloroplasts always contained a large amount of PULCA, even in stationary phase cultures with abundant cytosolic LBs. The increased production of PULCA under nutrient limitation (Bell and Pond 1996, Pond and Harris 1996, Epstein et al. 1998, Prahl et al. 2003, our

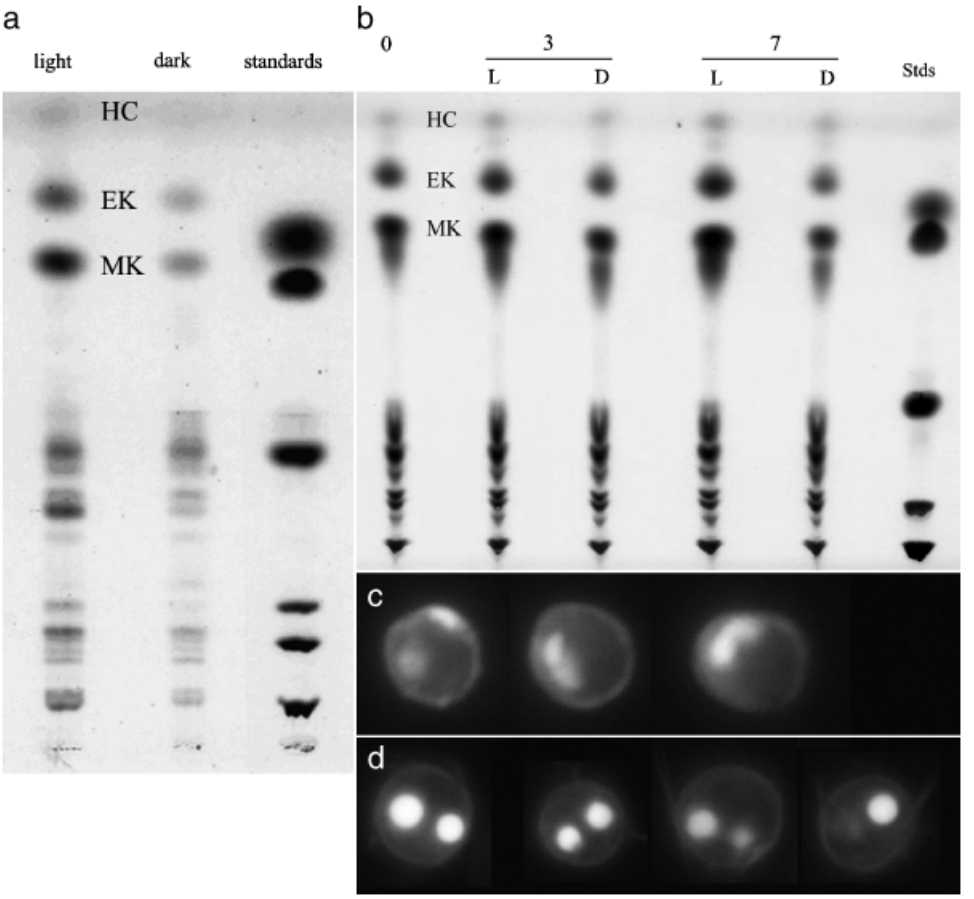

FIG. 6. LB and PULCA modification during prolonged darkness for *Emiliania huxleyi* 1742 (a) and *Isochrysis galbana* (b–d). (a) TLC of *E. huxleyi* 1742 total lipid extracts before (lane 1) and after (lane 2) 3 days of darkness. (b) TLC of *I. galbana* total lipid extracts before (lane 1; day 0) and after 3 days (lanes 2–3) or 7 days (lanes 4–5) in light or dark. HC, hydrocarbons (alkenes); EK, ethyl ketones; MK, methyl ketones. TLC lipid standards consist of (bottom to top) phosphatidylcholine, phosphatidylglycerol, digalactosyl diacylglyceride, cholesterol, TAG, and fatty acid methyl ester. (c, d) NR staining of *I. galbana* cells before (c) and after (d) dark incubation.

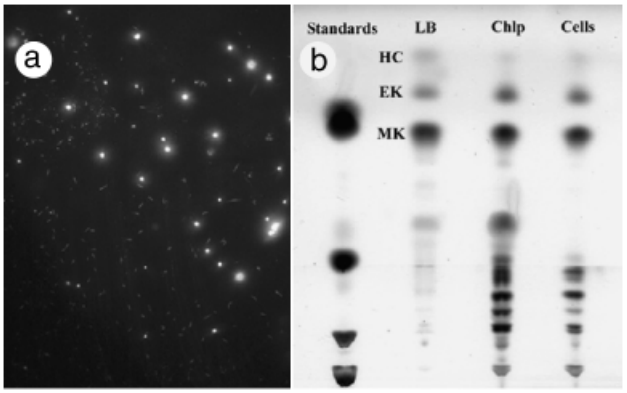

FIG. 7. PULCA distributions in *Isochrysis galbana* cell fractionation experiments. (a) NR-stained buoyant fraction following cell lysis and density centrifugation. Bright fluorescing spots are neutral lipid vesicles; faint NR crystals are also visible. (b) TLC analysis of lipids from *I. galbana*. Lane 1: standard mixture (phosphatidylcholine, phosphatidylglycerol, cholesterol, TAG/fatty acid methyl ester poorly separated); lane 2: buoyant (LB) fraction; lane 3: chloroplasts; lane 4: whole cell lipids. PULCA components: MK, methyl ketones (alkenones); EK, ethyl ketones; HC, hydrocarbons.

observations) also suggests these neutral lipids may function in chloroplasts as a sink for excess reducing power, as hypothesized by Yammoto et al. (2000).

One possible interpretation is that chloroplasts synthesize PULCA, which are then packaged into LBs and exported to the cytoplasm, as suggested by Liu and Lin (2001) for *I. galbana*. Our observations of *Isochrysis* under both light and nutrient limitation support this hypothesis, as both growth-limiting states caused a net movement of NR-staining neutral lipid from chloroplasts to conspicuous LBs, although total neutral lipid increased in the light but decreased in the dark. Vascular plant chloroplasts often contain lipid bodies (plastoglobuli) adjacent to thylakoids. Although plastoglobuli are generally not thought to be able to move across chloroplast membranes, Guiamét et al. (1999) reported plastoglobuli were exported from senescing soybean chloroplasts to cytosolic sites of catabolism, and other reports suggest lipid bodies may be translocated across membranes, even from cell to cell (Murphy 2001). Our micrographs clearly show that even in stationary phase cells, chloroplast structure was well preserved, with conspicuous thylakoid membranes, and no indication of plastid degradation. This suggests active production of neutral lipids by chloroplasts, rather than conversion or scavenging during senescence.

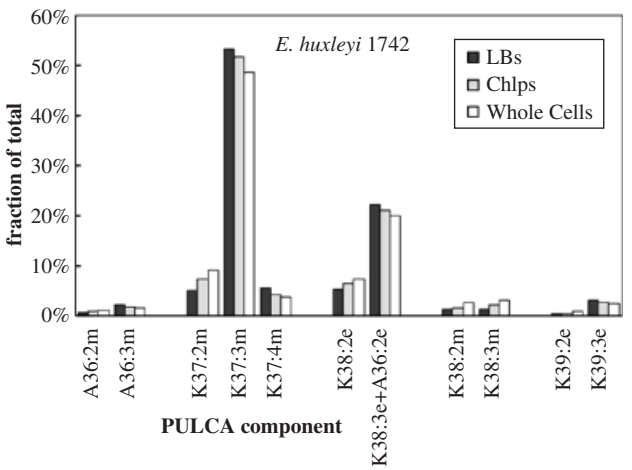

FIG. 8. PULCA components of cell fractions for *Emiliania huxleyi* 1742, shown as a fraction of total PULCA for buoyant lipid bodies, chloroplasts (Chlps), and whole cells. A36:2m, diunsaturated C_{36} methyl alkenoate; K37:2m, C_{37} methyl ketone (alkenone); K38:2m, C_{38} ethyl ketone (alkenone), etc.

However, another possibility is that PUCLA are synthesized in the ER, which then packages them into LBs via budding or translocation of protein-targeted lipids, as for nonphotosynthetic organisms (Murphy and Vance 1999, Zweytick et al. 2000, Murphy 2001). Sawada and Shiraiwa (2004) found alkenones and alkenoates in *E. huxleyi* ER fractions, denoted by the enzyme NADPH:cyt c reductase, and suggested that the alkenones were present as membrane-unbound micelles. Our evidence supports their hypothesis that PULCA are not primarily membrane lipids but strongly suggests they are contained in lipid droplets rather than micelles. Sawada and Shiraiwa (2004) also found only small PULCA pools in the chloroplast thylakoids, in contrast to our results. They used Percoll™ rather than our sucrose gradients, and we suspect that different fractionation techniques led to our different results. In particular, our "chloroplast" fraction, denoted by pigment, probably included other internal membranes such as ER and Golgi as well. Jeffrey and Anderson (2000) showed *E. huxleyi* ER membranes are continuous with the outer membranes of the chloroplast, so we cannot distinguish PULCA produced in the ER from that produced in chloroplasts, and delivered via the ER into cytoplasmic lipid vesicles. Sawada and Shiraiwa (2004) also suggested the coccolith-producing vesicle, denoted by uronic acid, was a major pool of alkenones and alkenoates. However, in our cultures PULCA biosynthesis does not appear linked to calcification.

We found that PULCA components in LBs and chloroplasts appear to be similarly distributed. Sawada and Shiraiwa (2004) also found similar results different *E. huxleyi* cellular pools. However, in our experiments, $U^{K'}_{37}$ predicted a growth temperature far lower than the 16° C culture conditions for *E. huxleyi* 1742. Although this might have been an artifact of separating cell fractions by overnight ultracentrifugation at 4° C, whole cells not subjected to centrifugation also had these unusual signatures. We have observed before that under N- or P-limited stationary phase, cells accumulate predominantly triunsaturated isomers, as reported for *E. huxleyi* (Epstein et al. 1998, Prahl et al. 2003) and *I. galbana* (Versteegh et al. 2001). In particular, the development of these lipids as a tool for paleotemperature determination requires that we un-

TABLE 2. PULCA components (as a percentage of total PULCA) in different cell fractions.

Fraction	*Emiliania huxleyi* 1742			*E. huxleyi* 1516			*Isochrysis galbana*		
	WC	LB	Chlp	WC	LB	Chlp	WC	LB	Chlp
$A_{36:2}$ Me	1	1	1	2	1	2	1	1	1
$A_{36:3}$ Me	2	2	2	1	1	1	2	2	2
$K_{37:2}$ Me	9	5	7	6	5	6	5	3	4
$K_{37:3}$ Me	49	53	52	39	42	40	54	57	58
$K_{37:4}$ Me	4	6	4	0	0	0	3	4	4
$K_{38:2}$ Et	7	5	6	9	8	8	8	5	5
$K_{38:3}$ Et + $A_{36:2}$ Et	20	22	21	29	30	30	24	25	24
$K_{38:2}$ Me	2	1	2	1	1	1	0	0	0
$K_{38:3}$ Me	3	1	2	8	8	8	1	1	1
$K_{39:2}$ Et	1	0	0	1	1	1	0	0	0
$K_{39:3}$ Et	2	3	2	3	3	3	1	2	2

WC, whole cells; LB, lipid bodies; Chlp, chloroplasts. $A_{36:2}$ Me, $C_{36:2}$ methyl alkene; $K_{38:2}$ Et, $C_{38:2}$ ethyl ketone (alkenone), etc.

derstand their biosynthesis and desaturation by factors other than growth temperature. Why these *trans*-unsaturated nonmembrane lipids should be desaturated at colder temperature, if they do not contribute to membrane fluidity, is an unanswered question. Currently, little is known about the genetics of lipid biosynthesis in these algae, but the sequencing of the *E. huxleyi* genome, now in progress (B. Read and T. Wahlund, personal communication) should greatly help to identify genes involved with neutral lipid biosynthesis and transport.

Why PULCA? Evolutionary and ecological aspects. What might be the fitness benefit of producing lipid bodies containing such unusual neutral lipids? Rontani et al. (1997) found the *trans*-polyunsaturated PULCA are notably resistant to photodegradation and are more stable to singlet oxygen than carotenoids, and Mouzdahir et al. (2001) showed the *trans*-polyunsaturated PULCA C_{37-38} alkenes are more photostable than *cis*-polyunsaturated C_{31-33} alkenes, suggesting the double-bond geometry is a key factor. However, Mouzdahir et al. (2001) further noted that the alkenones' extreme resistance to photodegradation was higher than expected from stereogeometry alone and speculated they were membrane-unbound lipids. Our observations of the packaging of PULCA into lipid bodies support that idea. *Emiliania huxleyi* in

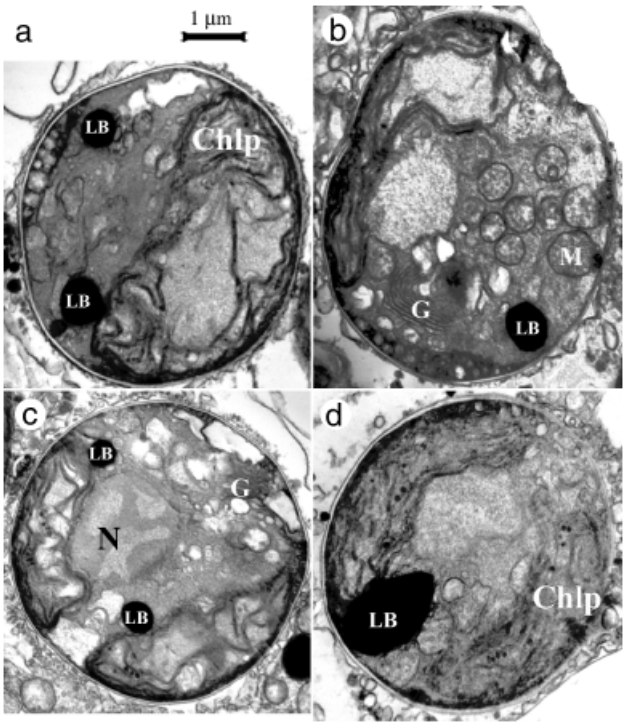

FIG. 9. Ultrastructure of *Emiliania huxleyi* 1516 cells. (a, d) Osmium thiocarbohydrazide-osmium stained sections. (b, c) Standard staining. LB, lipid body; N, nucleus; M, mitochondrion; G, Golgi; Chlp, chloroplast. Magnification, 7000×.

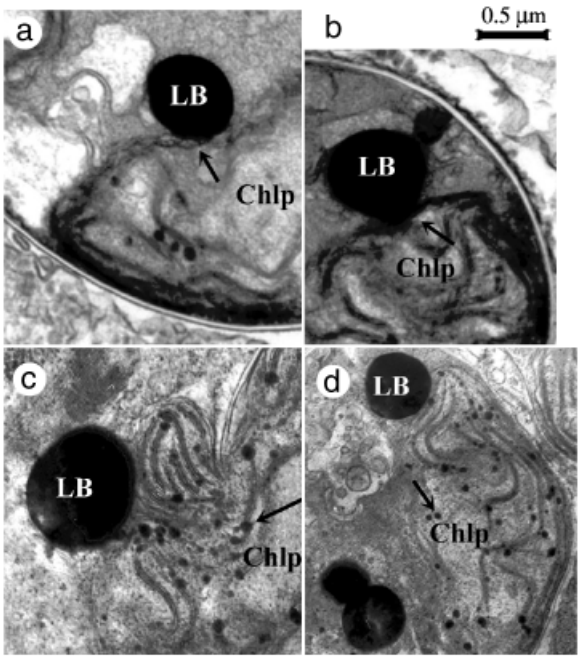

FIG. 10. *Emiliania huxleyi* 1516 ultrastructure (detail). (a, b) Osmium thiocarbohydrazide-osmium stained sections. Arrows show lipid bodies adjacent to chloroplast membrane. (c, d) Standard staining. Arrows show plastid LBs on thylakoid membranes. LB, lipid body; Chlp, chloroplast.

particular thrives under high-light low-nutrient conditions (Nanninga and Tyrrell 1996) and shows little photoinhibition at high irradiances (Paasche 2002, and references therein). Therefore, PULCA might be evolutionarily favored over TAGs because they are a more photostable form of energy storage.

Finally, we suggest these unusual lipids may also have a trophic benefit for their producers. We observed during grazing of *E. huxleyi* by the phagotrophic dinoflagellate *Oxyrrhis marina* that PULCA components were degraded slowly, if at all, by *O. marina* after near-total consumption of prey cells (not shown). Only slight changes in component profiles were recovered after 24 h from predator food vacuoles. Experiments with the ciliate *Helicostomella* showed similar results, though grazing rates were low (S. Strom, personal communication). We speculate that the unusual geometry of these molecules may limit their degradation by grazers, making PULCA in effect "algal Olestra." *Emiliania* is a cosmopolitan taxon that forms notable blooms worldwide, and high-light, low-nutrient blooms may be trophically unsuitable due to accumulation of PULCA. Although *Isochrysis* is a widely used aquaculture food stock, noted for its highly beneficial polyunsaturated fatty acids content (Sukenik and Wahnon 1991), neutral lipid production in haptophytes is highly strain specific (Conte et al. 1998), and the trophic quality of wild strains likely reflects the balance between polyunsaturated *cis*-membrane and *trans*-neutral lipids. A long-standing issue for the use of these compounds in paleothermometry is how $U^{K'}_{37}$ is modified by passage through the food web and sedimentary deposition (Prahl et al. 1993, Teece et al. 1994, 1998). We suggest that grazer modification, like sedimentary diagenesis (Prahl et al. 1993, Teece et al. 1994, 1998), may have minimal impact on $U^{K'}_{37}$ signatures, and future work should focus on genetic and environmental factors affecting biosynthesis.

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