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Effects of biosynthesis and physiology on relative abundances and isotopic compositions of alkenones

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[1] **Abstract:** The number of double bonds in long-chain, unsaturated ketones (alkenones) produced by some members of the Haptophyceae is correlated with the ambient temperature at the time of synthesis. For these same organisms the depletion of carbon-13 in biosynthetic products relative to dissolved inorganic carbon is related directly to the specific growth rate and inversely to the concentration of dissolved carbon dioxide. This report summarizes issues relating to the physiology, metabolism, and biochemistry of alkenone producers and how they affect the abundances and isotopic compositions of alkenones. These considerations show that an understanding of cellular responses to parameters governing uptake of inorganic carbon (C_i), isotopic fractionation, growth under diverse nutrient conditions, and genetic variability, both in the field and in culture, is necessary for developing a conceptual understanding of the biological significance of the ε_P and $U^{K'}_{37}$ indices. *Emiliana huxleyi* is the best known alkenone producer and can serve as a model organism for these studies. This report identifies knowledge gaps and appropriate objectives for both field- and laboratory-based research.

Keywords: Alkenones; biosynthesis; physiology; haptophytes; carbon-13; sea surface temperature.

Index terms: Biogeochemical processes; instruments and techniques; paleoceanography.

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1. Introduction

[2] Only a few taxa of the coccolithophorid phytoplankton, among them the widely dispersed *Emiliania huxleyi*, synthesize distinctive, long-chain ketones (alkenones [*Volkman et al.*, 1980]). The alkenones, typically 36 to 39 carbons in length, occur as mono-, di-, and triunsaturated species, with the degree of saturation dependent on ambient temperature at the time of synthesis [*Brassell et al.*, 1986]. Alkenones are not only preserved in sediments over long periods of time but also tend to retain the degree of saturation fixed at the time of synthesis at the sea surface. Since the degree of saturation is correlated with growth temperature, fossil alkenones are being used as a proxy thermometer for ancient sea surface temperatures.

2. Cellular and Genetic Aspects

[3] Very little is known about the biosynthesis of alkenones and its regulation, about the locations and roles of these products within cells, or about the degradation and turnover of these lipids. If available, such information could provide an improved basis for estimation or reconstruction of environmental conditions that led to induction of the biosynthesis and accumulation of alkenones in coccolithophorid algae.

[4] Since alkenone unsaturation varies as a function of growth temperature, it has been speculated that alkenones somehow regulate membrane fluidity [*Conte et al.*, 1998]. In cyanobacteria the amount of fatty acid desaturase messenger ribonucleic acid (mRNA) is regulated by growth temperature. In higher plants, polyunsaturated lipids are important at low temperatures and are particularly critical for photosynthesis at all temperatures. If the principal role of alkenones were regulation of membrane fluidity, the alkenone content of the membrane should constitute a significant proportion of the total alkenones within the cell. The coextraction of alkenones along with the cellular membrane fraction is not enough to suggest that alkenones are key components of membranes if the membrane-associated alkenones represent only a small portion of the total in the cell. Careful fractionation of cellular components and contents in order to determine distributions within cells is required. Rates of alkenone biosynthesis as a function of growth temperature should also provide useful insights, since a higher rate of synthesis and/or desaturation might be expected at lower temperatures regardless of other growth parameters.

[5] Alkenones might also be serving as storage molecules [*Epstein et al.*, 2001]. If this role were confirmed, the accumulation of alkenone stores might correlate with stationary-phase populations, for example. In this context, information concerning the effect of light on the $U_{37}^{K'}$ index and accumulation of alkenones under culture conditions would also be very instructive. Clearly, more work is needed.

3. Isotopic Fractionation

[6] The carbon isotopic compositions of phytoplankton depend on the isotopic composition of the dissolved inorganic carbon (DIC), which serves as a carbon source, on the species or form of DIC that is assimilated by the organism (i.e., dissolved CO_2 or bicarbonate), on any isotope effects associated with the uptake of DIC (e.g., isotope effects related to the transport of DIC into the cell and to the leakage of DIC from the cell), on isotope effects associated with fixation of carbon (i.e., those of rubisco and of other enzymes catalyzing the incorporation of carbon in biomass), and on cellular carbon budgets (i.e., the fraction of assimilated carbon which is fixed into organic matter versus that which leaks from the cell or which is incorporated in intracellular carbonate minerals). The isotopic fractionation imposed by phytoplank-

ton ($\equiv \varepsilon_P$) is usually expressed in terms of the isotopic difference between dissolved CO_2 and biomass. With the exception of $^{13}\delta_{DIC}$ itself (which establishes the starting point) all of the steps just listed contribute to ε_P. When values of ε_P are estimated from comparisons of $^{13}\delta_{DIC}$ and $^{13}\delta_{alkenone}$, it is necessary to know in addition the temperature and speciation of DIC (which affect the isotopic composition of CO_2 relative to DIC) and the isotopic difference between the alkenones and total biomass. That difference is controlled by isotope effects on the biosynthetic pathway leading from photosynthate (i.e., C_3 and C_6 carbohydrates produced by the Calvin-Benson cycle) to alkenones and on carbon budgets at all branch points along that pathway. This net biosynthetic fractionation, the isotopic difference between biomass and alkenones, is usually assigned the symbol $\Delta\delta$. A systematic discussion of the isotopic compositions of alkenones and of their significance can be based on a stepwise consideration of these factors.

[7] Potentially, carbon isotopic discrimination could occur at any point in a pathway where the active site of an enzyme or transporter is sensitive to isotopic mass differences. Unfortunately, very little is known regarding the specific enzymes and transporters of the haptophytes. The potential for fractionation begins with initial movement of inorganic carbon, either CO_2 or HCO_3^-, across the cell membrane. Active uptake of HCO_3^- by means of an ion transporter (or antiporter) might have the potential for significant isotope discrimination, whereas isotope effects associated with diffusion of CO_2 across the cell membrane are much smaller. Furthermore, coccolithophores have multiple, compartment-specific, carbonic anhydrase isoenzymes. Each mediates interconversion of CO_2 and HCO_3^- in either the periplasmic or chloroplastic compartments. In higher plants with C_3 metabolism, ribulose bisphosphate carboxylase-oxygenase (rubisco) is the primary carboxylase and leads to substantial depletion of ^{13}C. In contrast, the depletion is much smaller if the first step in carbon fixation is catalyzed by phosphoenol-pyruvate carboxylase (PEP carboxylase). PEP carboxylase utilizes HCO_3^- rather that CO_2 as its source of inorganic carbon and has a much smaller carbon kinetic isotope effect than that associated with rubisco [*O'Leary*, 1981]. Although a recent report [*Reinfelder et al.*, 2000] suggests that PEP carboxylase can be important to carbon fixation in a marine diatom, there is no parallel evidence for the coccolithophores.

[8] A better understanding of the biosynthesis of alkenones would also be welcome. In general, *n*-alkyl carbon skeletons with up to 16 carbon atoms and C_{20} and C_{40} polyisoprenoids are plastidic products *Kleinig*, 1989; *Ohlrogge and Jaworski*, 1997], whereas C_{15} and C_{30} polyisoprenoids (including the precursors of sterols) and extended *n*-alkyl chains are of cytosolic origin [*Kleinig*, 1989]. Moreover, for the haptophytes, different pathways of isoprenoid biosynthesis are expected to prevail in the plastids and in the cytosol [*Lichtenthaler*, 1999]. The hydrogen isotopic consequences of these distinctions have recently been documented [*Sessions et al.*, 1999], and there is evidence that transfer of C_2 units between plastids and the cytosol is probably associated with significant isotopic fractionations [*Monson and Hayes*, 1982]. Characteristics of coccolithophore desaturases are unknown. Comparisons of the isotopic compositions of lipids cannot therefore be interpreted securely unless the sites of synthesis (and probable pools of acetate and other biomonomers utilized) are known.

4. Ecophysiological Aspects

[9] The cosmopolitan nature of the coccolithophorids and their known genetic variability suggest their capacity for growth under diverse

conditions. Within the class Prymnesiophyceae, there are more than 200 coccolithophorid species belonging to about 70 different genera [*Van den Hoek et al.*, 1995]. Species diversity is negatively correlated with latitude, with only a few species (e.g., *Coccolithus pelagicus* and *Emiliania huxleyi*) having been reported from subpolar regions [*Thomsen et al.*, 1994]. Distinct geographical assemblages have been identified in both the Atlantic and Pacific oceans [*McIntyre and Bé*, 1967; *Okada and Honjo*, 1973; *Okada and McIntyre*, 1979] and in the Pacific Ocean. Assemblages found exclusively or primarily at depths greater than 100 m have been reported by *Okada and Honjo* [1973], *Fryxell et al.* [1979], *Reid* [1980], and *Boysen* [1991]. Even strains from the same species can differ remarkably in their physiology, for example, in their ability to acquire inorganic carbon [*Elzenga et al.*, 2000].

5. DIC Uptake

[10] The response of phytoplanktonic growth rates to changes in CO_2 supply is largely determined by the mechanism of C_i uptake. Recent work indicates that the transport routes for carbon ions and for CO_2 are important. Several studies indicate that both CO_2 and HCO_3^- can be utilized by *E. huxleyi* [e.g., *Nimer and Merrett*, 1992; *Buitenhuis et al.*, 1999; *Elzenga et al.*, 2000]. Uptake of CO_2 can involve passive diffusion or active transport across the plasma membrane into the cell. Uptake of HCO_3^- by diffusion is restricted by the low solubility of charged molecules in membrane lipids and by the inside-negative electric potential across the plasma membrane. Consequently, HCO_3^- is either taken up actively or is converted from HCO_3^- to CO_2 extracellularly in the presence of carbonic anhydrase (CA), with CO_2 entering the cell [*Sültemeyer*, 1998]. In the case of direct uptake of HCO_3^-, rates of cellular growth are expected to be unaffected by concentrations of external CO_2. In contrast, CA-mediated utilization of HCO_3^- accelerates formation of CO_2 from HCO_3^- but has no effect on equilibrium concentrations of CO_2 at the cell surface. Therefore the presence of extracellular CA does not prevent phytoplankton from being affected by changes in extracellular [CO_2(aq)].

[11] Using the so-called isotopic disequilibrium method, *Elzenga et al.* [2000] were able to show that two strains of *E. huxleyi* transported CO_2 rather than bicarbonate across the plasma membrane. However, one strain was able to use bicarbonate by converting it to CO_2 with an external carbonic anhydrase. The other strain lacked this ability. On the basis of results obtained by *Thompson and Calvert* [1995], *Laws et al.*, 1998] concluded that *E. huxleyi* uses HCO_3^- as its primary carbon source at low growth rates (i.e., low photon flux densities, PFD) and changes to active uptake of CO_2 at high growth rates (i.e., high PFD).

[12] Bicarbonate is the main carbon source for calcification [*Sikes et al.*, 1980]. This is also indicated by $\delta^{13}C$ of the coccolith calcite, which is close to that of the total dissolved inorganic carbon (and hence HCO_3^-). It has been suggested that flows of carbon to calcification and photosynthetic fixation can be coupled [*Nimer and Merrett*, 1992; *Brownlee et al.*, 1995; *Buitenhuis et al.*, 1999]. In that case, the rate of calcification should have an effect on carbon isotope fractionation. With increasing calcification relative to photosynthetic carbon fixation, the impact of calcification-mediated HCO_3^- utilization on carbon isotope fractionation should increase. This, however, was not indicated in a recent study by B. Rost et al. (Effects of CO_2 concentration, growth rate, and light conditions on carbon isotope fractionation in the coccolithophorid *Emiliania huxleyi*, submitted to *Limnology Oceanography*, 2000, hereinafter referred to as B. Rost et al., submitted manuscript,

2000), in which isotope fractionation was independent of the relative contribution of calcification to overall cellular carbon fixation. Hence these data do not provide evidence for a direct link between calcification and photosynthetic carbon fixation. *Quiroga and González* [1993] and *Israel and González* [1996] have proposed that protons generated when bicarbonate is used to form calcite in the coccolith vesicle are the link between calcification and photosynthesis in *Pleurochrysis*, another haptophyte that calcifies at high rates.

[13] The isotopic fractionation associated with production of biomass (ε_P) varies systematically both with the concentration of dissolved carbon dioxide and with the specific growth rate (μ). Differing relationships, are, however, found depending on whether (1) rates of growth are limited by supplies of fixed nitrogen or by available light energy, (2) illumination is continuous or cyclic, and (3) chemostats or batch cultures are used. Cultivating *E. huxleyi* in nitrate-limited chemostats under continuous light with photon-flux densities of circa 250 μmol photons m^{-2} s^{-1}, *Bidigare et al.* [1997] investigated the fractionation of carbon-13 by a calcifying strain (B92/11) and by a naked strain (BT 6). B. Rost et al. (submitted manuscript, 2000) examined the same calcifying strain of *E. huxleyi* using nutrient-replete, dilute, batch cultures incubated over a range of photon-flux densities. In the latter experiments, ε_P varied most strongly in response to changes in photon-flux densities and in irradiance cycles. These responses were independent of the associated changes in growth rates and implied active carbon acquisition in *E. huxleyi*. Direct comparison of the data sets indicates higher ε_P values and a considerably steeper slope in plots of ε_P as a function of $\mu/[CO_2(aq)]$ in the study of *Bidigare et al.* [1997]. In a separate study, *Popp et al.* [1998a] showed that ε_P was also related to the ratio of cellular organic carbon content to surface area. Accounting for cell size, however, did not significantly reduce the differences between the ε_P responses observed by *Bidigare et al.* [1997] and by B. Rost et al. (submitted manuscript, 2000).

[14] Similar differences between light-limited batch cultures and nitrate-limited continuous cultures were found by *Riebesell et al.* [2000] in studies of the marine diatom *Phaeodactylum tricornutum*. The contrasting isotopic fractionations were attributed to differences in the regulation of carbon uptake vs. the regulation of carbon fixation. The higher ε_P values in nitrate-limited cultures could, for instance, be caused by proportionately lower uptake of HCO_3^- relative to CO_2. The lower HCO_3^- uptake could be explained by low synthesis of transport proteins under N-limited conditions. If the leakage remains constant, a reduction in uptake of HCO_3^- would consequently require a stronger uptake of CO_2. Consistent with this hypothesis, the ε_P values in N-limited cultures depend on levels of CO_2 more strongly than those in light-controlled cultures. On the other hand, inconsistent ε_P responses could also be caused by differences in the leakage of CO_2 from cells in chemostat and batch cultures. The effect of irradiance on ε_P explains some of the differences between the different approaches, but more information is needed about what other factors control inorganic carbon acquisition and therefore also isotope fractionation in *E. huxleyi*.

[15] These observations indicate that a global and general expression relating ε_P to all of its controlling factors has not yet been found. Accordingly, interpretation of records of ε_P solely in terms of $[CO_2(aq)]$ or in terms of variations in both (but only) μ and $[CO_2(aq)]$ is risky.

6. Modern and Ancient Physiologies

[16] Variations in habitat and in genetic composition increase the difficulty of obtaining laboratory results that will accurately predict

properties of field populations. Both laboratory and field studies that define the parameters and conditions for alkenone production are needed. Although a fundamental understanding of the biology of coccolithophorid alkenone production may not be strictly necessary for the use of alkenones ($U^{K'}_{37}$) for estimates of paleotemperatures, it can only strengthen practical applications. The tremendous potential value that such reconstructions have for studies of paleoecology and for an understanding of the evolution of marine flora and fauna will dramatize the value of coccolithophorids as experimental models for a new generation of biologists.

[17] Values of $\Delta\delta$ are expected to vary if cellular compositions vary. This is because mass balance must prevail. The isotopic composition of the carbon that is fixed determines the overall isotopic composition of the cell. Lipids are depleted in ^{13}C relative to other cellular components. If the relative abundance of lipids within the cell increases, $\Delta\delta$ must decrease (i.e., the depletion of ^{13}C in lipids relative to biomass will decrease). Isotopic differences between lipids and other cellular components (e.g., proteins) will tend to remain constant. Mass balance will be preserved by a compensatory enrichment of ^{13}C in other cellular components. Such concepts are surely valid. The question is whether they have much practical significance. Do cellular compositions of alkenone-producing algae (e.g., ratios of proteins to carbohydrates to lipids) vary widely in nature? Or are the conditions under which alkenone producers can successfully compete and flourish such that cellular compositions do not vary significantly? These questions are discussed in the accompanying review by *Laws et al.* [2000], but further data bearing on alkenone producers in the field would be very welcome.

[18] Clearly, the diversity of factors affecting ε_P and $\Delta\delta$ is large. The variety of possible effects must not be forgotten as isotopic compositions of alkenones are used as a proxy for concentrations of dissolved CO_2 or for rates of growth. Extensive field tests provide the best means of (1) escaping from uncertainties about the extent to which laboratory cultures can duplicate natural responses and (2) determining which factors exert dominant controls over paleochemical isotopic signals.

6.1. Research Objectives

[19] Given the current state of knowledge and understanding of the biology of these organisms, the following objectives for laboratory and field research seem appropriate as a guide for further studies:

[20] Rates of production of alkenones relative to the status and condition of producer communities (exponential growth versus stationary phase, levels of specific nutrients) should be examined. Studies by *Popp et al.* [1998a], for example, have shown that nutrient-limited, log-phase growth rates have little effect on the $U^{K'}_{37}$ of two strains of *E. huxleyi*, but *Epstein et al.* [1998a, 1998b] has shown that the transition from log-phase to stationary-phase growth is accompanied by a dramatic change in the $U^{K'}_{37}$ for at least one strain of *E. huxleyi*. Detailed information relating to the role(s) of alkenones in coccolithophorid metabolism as well as the patterns of accumulation and degradation of alkenones should also be useful.

[21] A determination of the parameters that control growth rates of alkenone-producing algae in nature is also important. This information is necessary in making comparisons to laboratory analyses of the effects of nutrient-limited and nutrient-replete growth conditions on carbon isotope fractionation and alkenone unsaturation. These results are required urgently.

[22] Physiological studies of the biosynthesis and turnover of alkenones should be followed by biochemical and molecular examinations of subcellular mechanisms and thresholds for active transport and isotope fractionation of inorganic carbon in the alkenone-producing algae. It has been shown that coccolithophorid species such as *E. huxleyi* are amenable to molecular characterization [*Corstjens et al.*, 1998, 2001]. Thus molecular biological techniques cDNA libraries and cloning, polymerase chain reaction, cDNA microchip arrays, and other such technologies can be used to examine questions relating to diversity and distribution of strains of alkenone-producing algae, genetic expression and regulation of nutrient-uptake transporters, and enzymes related to alkenone biosynthesis. To maximize the utility of models that either predict future responses (of the organism) or reconstruct paleoenvironmental conditions based on the products of ancient physiologies, it is useful to know how changes in such factors as pH, alkalinity, and temperature affect expression of the genes that underlie both modern and ancient physiological responses.

6.2. Recommendations for Experimental Design

[23] Since experiments carried out in the field and the laboratory have not always been complementary, the following recommendations are offered to facilitate experimental design.

[24] Growth rates cannot be compared between strains without careful attention to measurements of cell counts and/or dilution rates. Even fine distinctions of cell health can be achieved by monitoring the gene expression of cell division markers such as the proliferating cell nuclear antigen (pcna), a component of DNA polymerase and a highly conserved gene [*Lin et al.*, 1994].

[25] Defining rates of incorporation of ^{13}C in alkenones and Sr/Ca ratios in coccoliths in modern settings is important for gaining insight into variability of these parameters in fossil samples and core tops. Other measurements and calibrations relating concentrations of phosphate and other nutrients to elemental variations in coccoliths, particularly with respect to ratios of Sr and/or Zn to Ca, are also of interest.

[26] Batch, turbidistat, and continuous cultures should be used to provide complementary lines of evidence. Chemostats are useful for addressing questions related to nutrient-limited growth. Batch cultures and turbidistats are best suited for studies under nutrient-saturated conditions with light or possibly temperature as the growth-rate limiting factor. Since the biochemical composition of phytoplankton at a given growth rate is very dependent on the factor limiting growth [*Shuter*, 1979], it is important to know how growth rate, irradiance, temperature, and light:dark periodicity influence carbon fractionation and parameters such as $U^{K'}_{37}$. Addressing these questions requires a combination of culture methods [e.g., *Riebesell et al.*, 2000]. Furthermore, it is important that experimentalists record and report all pertinent environmental information in laboratory culture experiments, including irradiance, duration of photoperiod, and temperature.

[27] Most parts of the ocean have concentrations of dissolved CO_2 between 10 and 25 mol kg^{-1} and, for alkenone-producing algae, growth rates <0.5 d^{-1} and cell densities of 10^2–10^4 mL^{-1}. Studies should aim to design experiments that provide information pertinent to those conditions in laboratory studies.

[28] In summary, laboratory and field workers should maximize and exploit opportunities for comparison of laboratory, core top, and downcore studies of paleobarometric and paleotem-

perature relationships. Realistic parameters should be employed in laboratory-based experiments in order to provide information that will allow hypotheses to be tested more efficiently and effectively in the field.

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