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NOTE

BACTERIAL INFLUENCE ON ALKENONES IN LIVE MICROALGAE[1]

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The microalga *Emiliania huxleyi* produces alkenone lipids that are important proxies for estimating past sea surface temperatures. Field calibrations of this proxy are robust but highly variable results are obtained in culture. Here, we present results suggesting that algal-bacterial interactions may be responsible for some of this variability. Co-cultures of *E. huxleyi* and the bacterium *Phaeobacter inhibens* resulted in a 2.5-fold decrease in algal alkenone-containing lipid bodies. In addition levels of unsaturated alkenones increase in co-cultures. These changes result in an increase in the reconstructed growth temperature of up to 2°C relative to axenic algal cultures.

Key index words: **alkenone; biomarker; co-culture; *Emiliania huxleyi*; *Phaeobacter inhibens*; Roseobacter clade; SST**

***Abbreviations*: CFU, colony forming units; Et, ethyl; Me, methyl; SST, sea surface temperature**

The microalga *Emiliania huxleyi* is the dominant oceanic coccolithophore and the main producer of the long chain (C_{37}) polyunsaturated alkenones, a key ocean temperature biomarker (Okada and Honjo 1973, 1975, Okada and McIntyre 1977, 1979, Boon et al. 1978, De Leeuw et al. 1980, Volkman et al. 1980a,b, Marlowe et al. 1990). The ratio of the di- to di- plus tri-unsaturated C_{37} methyl alkenones has been defined as the $U^{K'}_{37}$ index and was shown to vary linearly with the sea surface temperature (SST) in which the algae grow (Prahl and Wakeham 1987, Prahl et al. 1988).

Analysis of environmental samples including seawater, sediment traps and core tops have demonstrated the robustness of alkenones as a paleo-SST proxy (Prahl and Wakeham 1987, Sikes et al. 1991, 1997, Conte et al. 1992, Conte and Eglinton 1993, Sikes and Volkman 1993, Prahl et al. 1995, Rosell-Melé et al. 1995, Madureira et al. 1997). However, much variation is seen in the relationship between temperature and alkenone unsaturation in laboratory cultures of *E. huxleyi* (Prahl and Wakeham 1987, Prahl et al. 1988, 1995, Conte et al. 1995, 1998, Epstein et al. 1998, 2001, Popp et al. 1998, Laws et al. 2001). The difference between field studies and laboratory experiments has suggested that paleotemperature reconstructions based on $U^{K'}_{37}$ contain uncertainties that are related to factors other than temperature (Conte and Eglinton 1993, Sikes and Volkman 1993, Prahl et al. 1995, 2003, Sikes et al. 1997, 2005, Conte et al. 1998, Epstein et al. 1998).

Both mutualistic and antagonistic relations have been documented in various algal-bacterial interactions demonstrating that algal physiology is influenced by bacteria (Ashen et al. 1999, Brinkhoff et al. 2004, Miller and Belas 2004, Seyedsayamdost et al. 2011a,b, Sule and Belas 2013, Wang et al. 2014, Amin et al. 2015). We wanted to determine if bacterial influences could account for some of the observed irregularities in $U^{K'}_{37}$ obtained in culture experiments. To this end, we examined alkenones in axenic algal cultures and in cultures of the algae with the Roseobacter *Phaeobacter inhibens*. We chose a Roseobacter because *E. huxleyi* blooms contain bacterial populations, at times dominated by Roseobacters (Gonzalez et al. 2000), and *P. inhibens* was previously found in the bacterial assemblage associated with *E. huxleyi* (Green et al. 2015).

To determine if *P. inhibens* affected *E. huxleyi* alkenones, we grew them in co-culture. Axenic *E. huxleyi*

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algae (strain CCMP3266) were cultivated in L1-Si medium (see Appendix S1 in the Supporting Information; Fig. 1a). *P. inhibens* DSM17395 cannot grow in this medium but grows well in the rich medium 1/2YTSS (see Appendix S1; Fig. 1b). To co-cultivate algae and bacteria, a bacterial inoculum of *P. inhibens* was introduced into a pre-established axenic algal culture. In the resulting co-culture both algae and bacteria grew. Algae grew from 1×10^6 to 2.5×10^6 cells $\cdot$ mL^{-1} and bacteria grew from 1×10^2 to 1×10^6 colony -forming units (CFU) $\cdot$ mL^{-1} by 14 d in co-culture, with the bacteria attached onto the algal cells (Fig. 1c). Many members of the Roseobacter clade, including *P. inhibens*, are adapted to surface attachment (Dang and Lovell 2002, Bruhn et al. 2006, Frank et al. 2015).

Next, we examined whether the presence of bacteria influences algal alkenones. Alkenones were suggested to be part of the algal membrane (Sawada and Shiraiwa 2004) and they can be found stored in intracellular lipid bodies (Epstein et al. 2001, Eltgroth et al. 2005). We thus tested if the presence of bacteria affected algal lipid bodies. We used Nile red to visualize algal lipid bodies (Cooksey et al. 1987) in the presence and absence of bacteria over a period of 14 d (Fig. 2a). Quantification of Nile red-stained cells revealed that in early stages ~5% of algae in both axenic culture and in co-culture harbor lipid bodies (Fig. 2b). In axenic algal cultures the percent of algae with lipid bodies steadily increased over time, with almost 25% of the population stained by day 14 (Fig. 2b). In contrast, after 14 d, only around 10% of the algae in co-culture had visible lipid bodies (Fig. 2b). These observations demonstrate a quantifiable bacterial influence on the alkenone-rich algal lipid bodies.

To determine if alkenone distribution was altered by the presence of bacteria, we analyzed the profile of unsaturated alkenones in axenic algal cultures and co-cultures. We extracted total alkenones from 7- and 14-d-old cultures and determined their alkenone profile (see Appendix S1). A pure *P. inhibens* bacterial culture was processed identically as a negative control and yielded no measurable alkenones. Although all algal populations were grown under otherwise identical conditions, we found that in the presence of bacteria, algae were enriched with unsaturated alkenones (Fig. 3, a and b and Fig. S1 in the Supporting Information). The largest enrichment was observed for Me $C_{37:2}$ alkenones which exhibited over 60% increase in the presence of bacteria at day 7 of incubation (Table 1 and Fig. 3a).

The alkenone composition was determined and the $U^{K'}_{37}$ and growth temperature were calculated (see Appendix S1, Table 1 and Table S1 in the Supporting Information). The resulting temperature estimates deviated substantially from the growth temperature of our experiments. Although all cultures were grown at 18°C, the calculated temperatures were 8.2°C and 10.0°C at day 7 of incubation for the pure algal cultures and the co-cultures, respectively. Large differences between calculated temperatures and actual growth temperatures have been reported for culture experiments (e.g., Conte et al. 1995, Popp et al. 1998, select studies summarized in Table S2 in the Supporting Information). Together with our data, these studies underscore the problem of reproducing the original $U^{K'}_{37}$ calibration in cultures (Prahl and Wakeham 1987).

Our data show that the 14-d-old cultures, in which both algal and bacterial populations were denser, yielded even lower $U^{K'}_{37}$ and consequently lower calculated temperature values relative to the 7-d-old cultures (Table 1 and Fig. 3). These observations are in agreement with previous work by Conte et al.

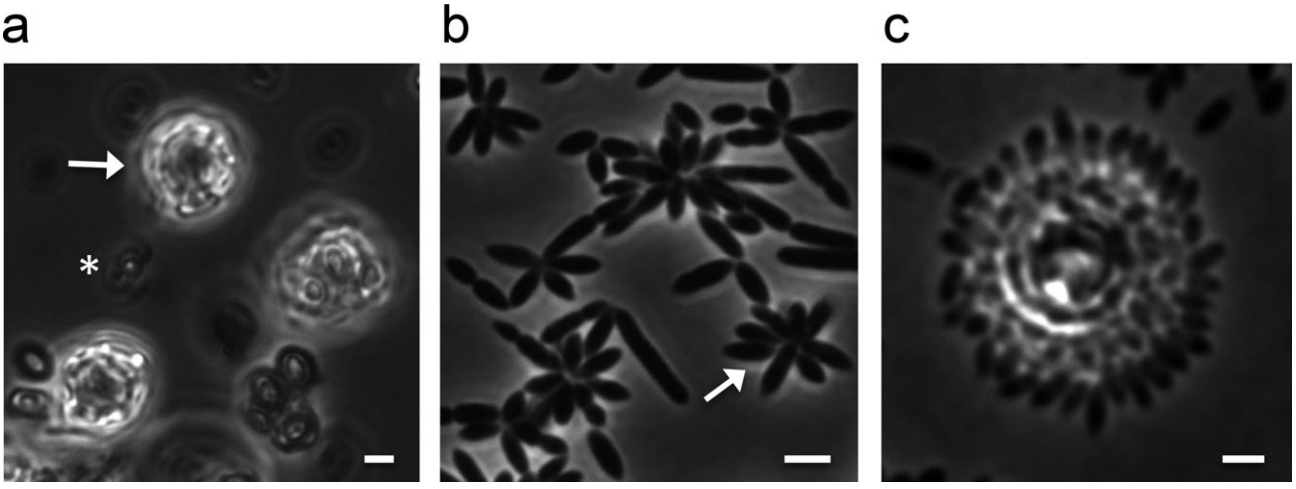

FIG. 1. Algal-bacterial co-cultures. Phase contrast microscopy of (a) Calcified cells of *Emiliania huxleyi* (CCMP3266) axenic algal culture. Arrow indicates an algal cell and asterisk a shed coccolith. (b) A pure culture of *Phaeobacter inhibens* (DSM17395) bacteria. These bacteria tend to form multi-cellular structures called rosettes. Arrow indicates a rosette. (c) Algal-bacterial co-culture showing numerous *P. inhibens* bacteria surrounding an *E. huxleyi* algal cell that no longer bears coccoliths. Scale bars correspond to 1 μm.

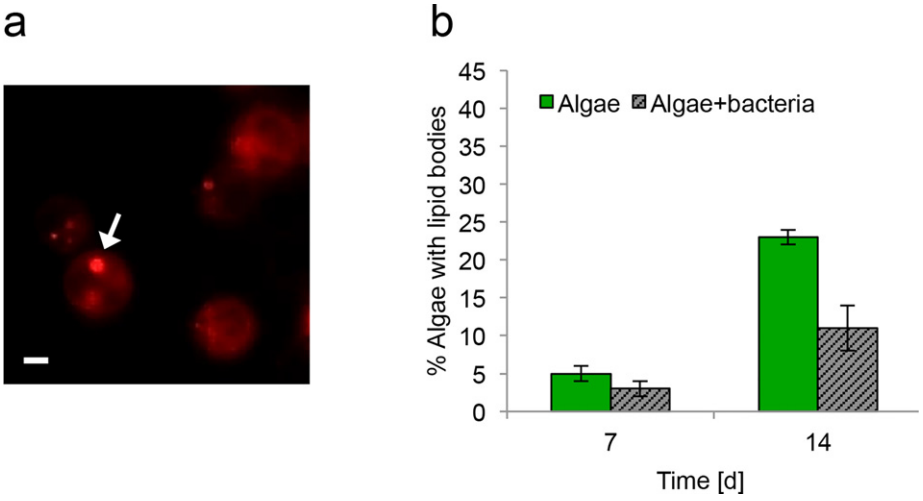

FIG. 2. Algal lipid bodies are influenced by the presence of bacteria. (a) Fluorescent image of algae stained with Nile red. Lipid bodies are seen as intracellular droplets. Arrow points to a lipid body. Scale bar corresponds to 1 μm. (b) Quantification of Nile red-stained algal cells containing lipid bodies in algal axenic culture (green bars) and algal-bacterial co-culture (gray bars) (see Appendix S1). For each time point $n > 300$ cells. Two biological replicates were examined and error bars represent the standard deviation among analyzed fields.

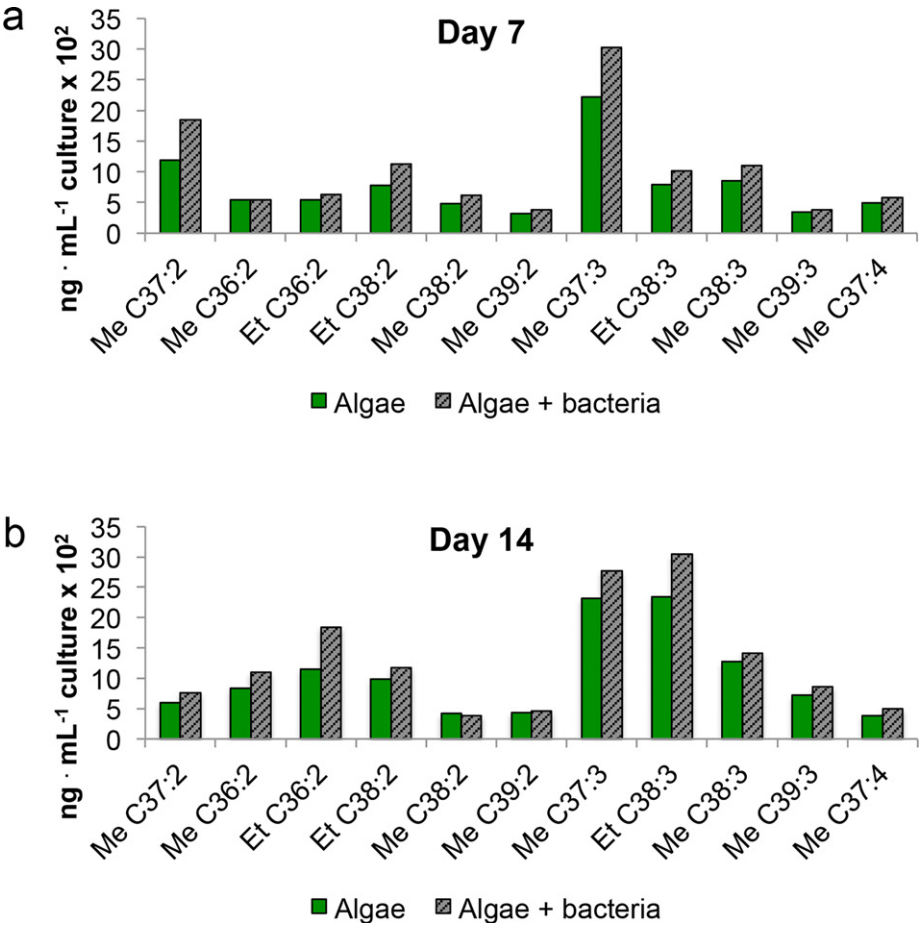

FIG. 3. Bacteria influence algal alkenones. Abundance of algal unsaturated alkenones in the presence (gray bars) and absence (green bars) of bacteria at days 7 (a) and 14 (b). Shown is the abundance of methyl (Me) and ethyl (Et) alkenones (and alkenoates, in the case of $C_{36:2}$ molecules) with chains of 36–39 carbons containing two, three, and four double bonds (designated 2, 3, and 4, respectively). In the presence of bacteria all unsaturated forms of alkenones demonstrate higher abundance than in algal pure cultures. For each data set, two biological replicates were tested and yielded similar results, shown is one representative replicate (data for a second replicate are shown in Fig. S1).

(1998) in which the majority of stationary phase cultures yielded lower $U^{K'}_{37}$ in comparison to values obtained for exponential phase cultures. Chivall and colleagues also documented a marked impact of growth phase on alkenone distribution (Chivall et al. 2014). At both time points examined in our study, the $U^{K'}_{37}$ values for the co-cultures were higher than the pure algal cultures. The presence of bacteria consequently brought the calculated $U^{K'}_{37}$ temperature closer to the actual growth temperature. Of note, algal-bacterial interactions can influence the density of both populations (e.g., Wang et al. 2014); therefore, all data in the current study were normalized in order to account for

TABLE 1. Calculation of $U^{K'}_{37}$ and the growth temperature of algal cultures and co-cultures.

	Day 7		Day 14	
	Pure algal culture	Co-culture	Pure algal culture	Co-culture
Me $C_{37:2}$ ($ng \cdot mL^{-1}$ culture $\times 10^2$)	11.0	18.5	5.5	8.0
Me $C_{37:3}$ ($ng \cdot mL^{-1}$ culture $\times 10^2$)	23.0	30.0	23.0	27.5
$U^{K'}_{37}$	0.32	0.38	0.19	0.22
Calculated temperature (°C)	8.2	10.0	4.4	5.3

possible differences in cell density (see sections regarding alkenones and lipid bodies in Appendix S1).

Since the cellular function of alkenones is unknown, it is difficult to interpret why a decrease in lipid body content was associated with an increase in alkenone abundance. Interestingly, abundant small-sized bodies (less than 100 nm in diameter) were previously reported to be in association with the chloroplasts in *E. huxleyi* (Eltgroth et al. 2005). In that study, lipid analysis of the chloroplast cell fraction revealed comparable amounts of alkenones in chloroplasts as in lipid bodies (Eltgroth et al. 2005). In light of these observations it is possible that the decrease we observed in Nile red stained lipid bodies is not indicative of the overall status of the cellular alkenone reservoir. It would be fascinating to further explore the bacterial influence on alkenones in different sub-cellular locations.

Bacteria have been shown to play a key role in alkenone geochemistry in early diagenetic processes (Rontani et al. 2013). Previous studies demonstrated the capability of bacteria to degrade alkenones in dead algae under oxic and anoxic conditions (Teece et al. 1998, Rontani et al. 2005). Several studies have shown selective degradation of the more unsaturated alkenones that resulted in a bias toward warmer calculated temperatures (Rontani et al. 2008, Prahl et al. 2010, Zabeti et al. 2010). While these previous studies focused on degradative processes carried out by bacteria after algal death when alkenones have become part of the ocean's detrital organic matter, our data are the first to reveal modifications during biosynthesis. Thus, our observations demonstrate the importance of microbial interactions during the initial production of unsaturated alkenones throughout the life of the algae.

While alkenone unsaturation is a powerful paleoceanographic tool, our observations introduce microbial interactions as a novel factor that may affect the biosynthesis of alkenones and thus has implications for the interpretation of $U^{k'}_{37}$ temperature reconstructions. Several previous studies have elucidated environmental and physiological factors that affect *E. huxleyi*-derived organic biomarkers (Conte et al. 1998, Epstein et al. 1998, 2001, Popp et al. 1998, Yamamoto et al. 2000, Laws et al. 2001, Pan and Sun 2011). Laboratory analyses utilizing the original $U^{k'}_{37}$ calibration (Prahl and Wakeham 1987) result in considerable deviations between the calculated and measured temperature in culture (Conte et al. 1995, 1998, Popp et al. 1998, Pan and Sun 2011, see Table S2). This contrasts with field studies, which show a much tighter fit to the accepted calibration (Prahl and Wakeham 1987, Sikes et al. 1991, 1997, Conte et al. 1992, Conte and Eglinton 1993, Sikes and Volkman 1993, Prahl et al. 1995, Rosell-Melé et al. 1995, Madureira et al. 1997). Our results demonstrate that the deviations in culture may be in part attributed to the presence or absence of bacteria. Therefore, culture-based analyses should assess the bacterial population and maintain a reproducible bacterial assemblage. Of note, differences between model algal strains that have been cultivated in laboratories for many years and their relatives in the wild should also be accounted for prior to comparing laboratory results to environmental samples. In light of our findings, mesocosm experiments in which the natural algal strains and their bacterial assemblage are present might offer a more robust experimental set up. Indeed, a previous mesocosm study conducted by Conte et al. (1994) showed a linear correlation between growth temperature and alkenone unsaturation in temperatures higher than 9°C using the U^{K}_{37} index. Thus, algal-bacterial interactions should be considered a significant influence on algal derived biomarkers.

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Supporting Information

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

Figure S1. Alkenone analysis of a biological replicate.

Table S1. Total Lipid Extract (TLE) weight values of the analyzed samples in Figure 3.

Table S2. Summary of select alkenone studies with *E. huxleyi* cultures.

Appendix S1. Methods.