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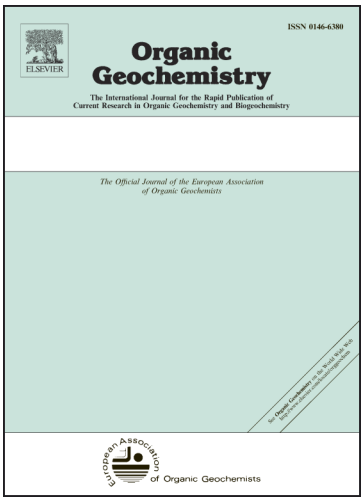

Note

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Hydrogen isotope fractionation response to salinity and alkalinity in a calcifying strain of *Emiliania huxleyi*

Gabriella M. Weiss[1], Anne Roepert[2], Jack J. Middelburg[2], Stefan Schouten[1,2], Jaap S. Sinninghe Damsté[1,2], Marcel T.J. van der Meer[1]

[1]Department of Marine Microbiology and Biogeochemistry, NIOZ, Royal Netherlands Institute for Sea Research and Utrecht University, Den Burg, The Netherlands

[2]Department of Earth Sciences, Faculty of Geosciences, Utrecht University, Utrecht, The Netherlands

Correspondence to: Gabriella M. Weiss (gabriella.weiss@nioz.nl)

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Abstract

Hydrogen isotope ratios of long-chain alkenones (δ^2H_{C37}) correlate with water isotope ratios and salinity, albeit with varying degrees of biological fractionation between alkenones and water. These differences in fractionation are the result of environmental and species related effects, which in some cases have consequences for the magnitude of the δ^2H_{C37} response per unit increase in salinity. Earlier culture experiments have focused on constraining hydrogen isotope fractionation factor α in non-calcifying strains of *Emiliania huxleyi*. Here we studied isotopic fractionation in a calcifying strain of *E. huxleyi* and show that although absolute fractionation is different, the response to changes

in salinity and alkalinity is similar to those of non-calcifying species. This suggests that calcification does not alter the δ^2H_{C37} response to salinity significantly.

Introduction

Haptophyte algae are one of the most abundant phytoplankton groups in the modern ocean (Monteiro et al., 2016). Certain species of haptophytes create tiny plates of calcium carbonate called coccoliths. These calcifying haptophyte algae are extremely important for the global carbon cycle, and are believed to have contributed most of the precipitated marine calcium carbonate across the Cenozoic (Monteiro et al., 2016). Particular groups of haptophytes also synthesize long-chain alkenones (Volkman et al., 1980; De Leeuw et al., 1980), which are methyl and ethyl ketones typically with a chain length between 35 and 40 carbon atoms (Longo et al., 2013). Hydrogen isotope ratios of long-chain alkenones (δ^2H_{C37}) correlate significantly with salinity in cultures, and this relationship appears to be largely related to a salinity response of biological hydrogen isotope fractionation (α) between alkenone and water δ^2H ratios (Schouten et al., 2006, M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017). Haptophytes, including *Emiliania huxleyi*, generally produce coccoliths in the natural environment, but can also be found in non-calcifying or naked forms believed to be caused by mutations (Paasche, 2002). Additionally, calcifying haptophytes have diploid (calcifying) and haploid (non-calcifying) life stages that are not only morphologically distinct, but also have different

responses to environmental conditions (Fiorini et al., 2010). However, so far, the majority of previous cultures have focused on the δ^2H_{C37} ratios and α_{C37} values of non-calcifying haptophyte strains. Here we present data for a calcifying strain of *E. huxleyi* to identify potential impacts of coccolithophorid calcification on α_{C37} in relation to salinity and alkalinity.

Materials and Methods

Media Conditions

Batch cultures of a calcifying strain of *E. huxleyi,* RCC2050, isolated from the Mediterranean Sea, were grown in media created from filtered North Sea water with added vitamins and trace metals following the K medium recipe from Roscoff Culture Collection. From a stock of filtered North Sea water, salinities above and below 34 were produced by adding NaCl and ultra-pure water, respectively. $KHCO_3$ and K_2CO_3 were added to change the alkalinity of the media. In the final media, nitrogen and phosphate were at K/10, but vitamin and trace metal amounts were at K/2 concentrations (following Keller et al., 1987). Alkalinity was measured spectophotometrically using an automated spectrophotometric alkalinity system (ASAS) as described in Liu et al. (2015). Temperature and salinity were measured using a VWR CO310 portable conductivity, salinity and temperature instrument, and pH was measured using a Metrohm pH meter.

Experiments

Before each experiment, cells were acclimated for 4 generations. Experiments were conducted in triplicate in 500 mL of media at six different conditions (Table 1). Light intensity was kept between 170 – 200 μ mol photons m^{-2} s^{-1} with a 16:8 light:dark cycle at a temperature of 15 °C. Cells were counted using an Accuri C6 flow cytometer. Cell densities were kept at or below 100,000 cells / mL in both acclimation phase and final experiment to avoid major changes to the alkalinity of the media. All cultures were checked for continued calcification using phase contrast light microscopy. Growth rates were ascertained by determining the slope of the linear fit of the natural logarithm of cell density in the exponential part of the growth curve. Cells were harvested by filtration over pre-combusted GF75 0.3 μm GF/F filters. Filters were freeze-dried and biomass was extracted ultrasonically using dichloromethane : methanol 2:1 (*v : v*). Extracts were further separated into three fractions following methods described in Weiss et al. (2017).

Isotope Measurements

Hydrogen isotope ratios of the culture media were measured on TC/EA/irMS following Weiss et al. (2017). Hydrogen isotope ratios of long-chain alkenones were measured on GC/TC/irMS using an RTX-200 60 m GC column with the following GC temperature program: 70 ˚C to 250 ˚C at 18 ˚C /min, 250 to 320 ˚C at 1.5 ˚C / min., and kept at 320 ˚C for 25 min with a flow rate of 1.5 mL / min. H_3^+ correction was made at the start of each day (2.929 – 3.165 ppm mV^{-1}) and an n-alkane mix (Mix B) supplied by A. Schimmelmann (Indiana University)

was measured prior to sample analysis. Samples were run only when average and standard deviation for the Mix B standard were within 5 ‰. ^{2}H monitoring gas of predetermined isotopic composition was introduced into the ion source at the start and finish of each analytical run. Squalane (-164 ± 3 ‰) was co-injected with each sample to ensure machine stability and fits with the predetermined value of -170 ± 4 ‰. Error bars for $\delta^{2}H_{C37}$ ratios are the result of duplicate measurements and thus represent reproducibility. We report both the individual $\delta^{2}H_{C37:3}$ and $\delta^{2}H_{C37:2}$ as well as integrated $\delta^{2}H_{C37}$ ratios (Table 1), but use the integrated values for comparison with previously published results.

Results & Discussion

Hydrogen isotope ratios of alkenones of the calcifying *E. huxleyi* strain RCC2050 grown at salinities of 32 to 40 and alkalinites of 2043 to 3579 µmol kg^{-1} (Table 1) span from -248 ‰ to -216 ‰. There is a strong positive linear correlation between α_{C37} – salinity ($r = 0.77$, $p < 0.005$; Fig. 1a), but there is no significant relationship between α_{C37} – alkalinity ($r = 0.18$, $p > 0.05$). The latter is in agreement with previous results (Weiss et al., 2017). Furthermore, no relationship between growth rate (varying between 0.45 and 0.69 divisions per d^{-1}) and alkalinity is observed. The linear correlation between α_{C37} – salinity is in line with previous results for non-calcifying strains of *E. huxleyi* (Fig. 1a). The magnitude of this response is statistically similar across all experiments: ranging between 0.001 – 0.003 change in α_{C37} per unit salinity (Schouten et al.,

2006; M'Boule et al., 2014; Sachs et al., 2016; Weiss et al., 2017; this study). However, some differences are observed. First, δ^2H_{C37} ratios from RCC2050 are more depleted and α_{C37} values are lower, implying that calcification might result in more fractionation during alkenone synthesis. Calcification occurs in a closed vesicle (coccolith vesicle) where conditions are tightly regulated (Sviben et al., 2016). H^+ is generated during calcification, and is transported through the cytosol (Taylor et al., 2011; Monteiro et al., 2016). This H^+ might be more abundant in calcifying strains, leading to more fractionation and depleted alkenones. Additionally, if this calcification derived H^+ pool is isotopically depleted as a result of increased concentration in calcifying haptophytes with respect to non-calcifying cells, it might contribute to a more isotopically depleted intracellular pool of H^+ available for biosynthesis of organic compounds (alkenones) in calcifying haptophytes. Alkenones are thought to be synthesized by chain elongation from fatty acids in the cytosol (Rontani et al., 2006), and are thus heavily influenced by cytosolic pools of NADPH. It is possible that these larger fluxes of H^+ into the cytoplasm in calcifying coccolithophores are responsible for the enhanced hydrogen isotope fractionation (lower α_{C37}) observed here. Second, RCC2050 shows a significant positive correlation between α_{C37} and growth rate ($r = 0.55$, $p < 0.05$; Fig. 1b). This relationship is in contrast with results from previous culture experiments which report a negative correlation between α_{C37} and growth rate (Schouten et al., 2006; M'Boule et al., 2014; Sachs and Kawka, 2015; Weiss et al., 2017). One

possibility could be that in calcifying haptophytes, enhanced growth is associated with increased calcification, a phenomenon noted for blooms of coccolithophores during which cells are known to increase calcification and create liths in greater abundance than necessary, resulting in multiple layers of liths in some cases (Paasche, 2002; Monteiro et al., 2016). Increased calcification would generate more H^+ that is then pumped into the cytoplasm. This potentially enhanced H^+ generation at higher growth rates might result in reduced fractionation relative to fractionation at lower growth rates, leading to a relatively more enriched cytosolic pool of H^+ available for synthesis of organic compounds under faster growth, causing this positive correlation. Alternatively, the range of growth rates in this study is rather narrow, and perhaps with a larger range in growth rates or in chemostat cultures, the correlation might be different. Nevertheless, the slope of the linear regression of δ^2H_{C37} – salinity response for RCC2050 (2.7±0.6) is not statistically different from the responses reported for other haptophyte species. Therefore, while fractionation is increased in this calcifying strain, our results suggest that calcification does not appear to significantly affect the δ^2H_{C37} – salinity response. Considering the fact that a majority of alkenone-producing haptophytes in the natural environment are calcifying, this finding is important for the use of δ^2H_{C37} ratios to reconstruct salinity, i.e., sedimentary alkenones produced by both calcifying and non-calcifying haptophytes should not result in significantly different salinity estimates.

Conclusions

New results from a calcifying strain of *E. huxleyi* show that alkalinity does not have an effect on hydrogen isotope ratios and fractionation, similar to findings from non-calcifying strains. Calcification appears to have an effect on hydrogen isotope fractionation of long-chain alkenones, but the α_{C37}-salinity response and δ^2H_{C37} response per unit increase in salinity is similar to that of non-calcifying strains. These findings suggest that application of δ^2H_{C37} ratios to reconstruct salinity should not be significantly impacted by a mixing of calcifying and non-calcifying *E. huxleyi* in the geologic record.

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Figure 1: Hydrogen isotope fractionation between alkenones and water (α_{C37}) versus salinity (a) and growth rate (b). Results are from batch (circles) and continuous (squares) culture experiments of *Emiliania huxleyi*.

Supplementary Figure 1: Scanning electron microscope image showing calcification of the *Emiliania huxleyi* strain RCC2050 investigated in this study.

Table 1: Growth water parameters, hydrogen isotope ratios, and fractionation values for batch cultures of *Emiliania huxleyi* strain RCC2050. For two samples, alkenone concentrations were not sufficient for hydrogen isotope analyses, thus were not measured, indicated by n.d.

Table 2: List of culture conditions for studies investigating growth and salinity effects on hydrogen isotope fractionation of long-chain alkenones in *Emiliania huxleyi*.