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Calcifying Coccolithophore: An Evolutionary Advantage Against Extracellular Oxidative Damage

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The evolutionary advantages afforded by phytoplankton calcification remain enigmatic. In this work, fluoroelectrochemical experiments reveal that the presence of a $CaCO_3$ shell of a naturally calcifying coccolithophore, *Coccolithus braarudii*, offers protection against extracellular oxidants as measured by the time required for the switch-off in their chlorophyll signal, compared to the deshelled equivalents, suggesting the shift toward calcification offers some advantages for survival in the surface of radical-rich seawater.

1. Introduction

Reactive oxygen species (ROS) are ubiquitous in open ocean waters. The formation pathways of ROS in seawater, and their propagation reactions after formation, are complex.[1,2] ROS arise from photoreactions near the sun-lit surface and are leaked by bio-organisms under stress or metabolism imbalance. Ocean water contains photoactive molecules such as chromophoric dissolved organic matter, nitrate, or nitrite that can be electronically excited by sunlight forming H_2O_2 and $OH^{\cdot}$. Hydroxyl radicals ($OH^{\cdot}$) react at a mass-transport limit with dissolved organic matter, carbonate anions, chloride, and bromide, present at various concentrations in seawater, and propagate to form other radicals, for example, $DOM^{\cdot}$ (dissolved organic matter), $CO_3^{\cdot-}$, $Br^{\cdot}$, and $Cl^{\cdot}$.[1,2] H_2O_2 is a strong oxidant. Moreover, due to the high bond-breaking kinetic barrier in the absence of free transition metal ions,[3] H_2O_2 has a long retention time in seawater of the order of hours to days.[2] Its concentration is related to that of other ROS such as $OH^{\cdot}$.[2] The concentration of H_2O_2 in seawater fluctuates dynamically as a function of depth, latitude and longitude readings.[2] Surface marine water typically contains 20×10^{-9}–800×10^{-9} M of H_2O_2.[4–6] Causes of the dynamic fluctuation of H_2O_2 in surface waters include the result of rainwater[7,8] and or biological activities in regions such as bloom-forming seaweed *Ulva*.[9] Rainwater over the South and Central Atlantic Ocean, May-June 1996, contained $26(\pm 22) \times 10^{-6}$ M of H_2O_2,[4] and, in separate studies, post rain measurements report after concentrations of H_2O_2 ranging from 0.1×10^{-6}–0.2×10^{-6} M.[8,10]

Some of the most successful autotrophs in our oceans are the coccolithophores, marine phytoplankton that calcify. Coccolithophores can bloom over 100 000 km^2 in the open surface water, shedding liths that cause a blue–green hue shift in the surface water visible via satellite imagery.[11] They are estimated to sequester more than 1 billion tonnes of $CaCO_3$ to the deep per annum, playing a significant role in the ocean carbon cycle[12] and mineralizing CO_2 at a rate directly comparable to anthropogenic release of CO_2.[13] Calcification, however, has a huge cellular energy expenditure and is estimated to cost approximately $1/3^{rd}$ of the photosynthetic budget of the phytoplankton.[14] The origin of haptophytes dates back to ≈1.2 billion years ago[15] whereas the shift of haptophytes to precipitate liths, by self-encrusting with plates of calcite, coccoliths, took place only ≈209 million years ago, "shortly" after the Permian–Triassic mass extinction event.[16] The rich diversity of coccolith size and morphology between species remains a puzzle from both ecological and evolutionary standpoints and there is no single, unified understanding.[14] Plausible emerging thoughts include protection against grazing, photodamage, viral/bacterial attack, and a potential carbon concentration mechanism.[14] Here we question, does the presence of a biogenic calcite shell provide an evolutionary advantage against extracellular oxidative damage? As noted, ROS and other radical species are ubiquitous in oceans with a maximum H_2O_2 concentration reported as high as 2×10^{-6} M following certain events. Abiotic ROS and H_2O_2 are potent and detrimental to marine life and in particular, marine phytoplankton.[2] In an isolated culture of pure *Prochlorococcus*, a genus of common marine cyanobacteria that is sensitive to ROS due to its lack of catalase and other ROS protective mechanisms, H_2O_2 at concentrations as low as 0.8×10^{-6} M is lethal for its survival.[17] In the natural environment, *Prochlorococcus* lives in

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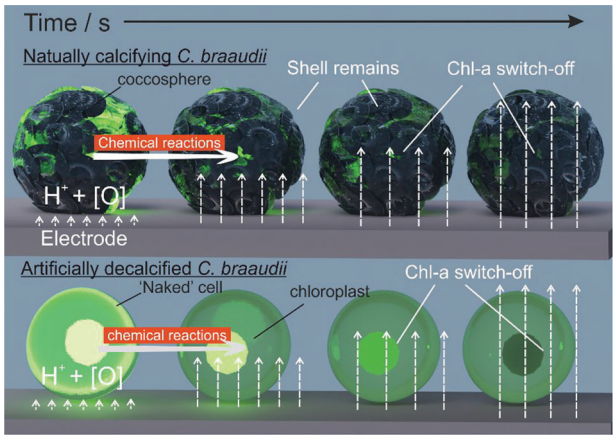

Figure 1. Schematic diagram showing the (electro)chemical processes at the electrode surface. Naturally calcifying *C. braarudii* cells (top) and, in a separate experiment, those artificially decalcified prior to start (bottom), are immobilized onto the surface of a glassy carbon electrode (diameter = 3 mm). The current ramped linearly from zero at a rate of 10 μA s^{-1} while simultaneously recording the chl-a fluorescence signal of the biological cell. [O] represents electrogenerated radicals.

symbiosis with catalase-containing extant microbes at the sunlit ocean surface.[17] Did coccolithophores adopt a different evolutionary strategy by encrusting themselves with calcium carbonate to protect themselves from oxidative damage? In this paper we consider the specific case of *Coccolithus pelagicus subsp. braarudii*.

Recently, advances in fluoroelectrochemical analysis allow the oxidative resilience of phytoplankton to be rapidly quantified at a single cellular level for species classification.[18,19] The nature of the electrogenerated oxidants is controlled by the potential applied to the electrode and the composition of the culture medium (artificial seawater). Importantly, it was shown that the number of moles (charge) of oxidants required to react leading to chl-a switch-off of green algae cells were independent of the chemical identity of the electro-generated oxidant(s) among hypobromous acid, chlorine, or water-derived oxidants.[20] The addition of strong acid, however, did not result in the catastrophic drop in cellular chlorophyll-a (chl-a) intensity within a timescale that is relevant to the electrochemical experiments.[13] By exposing the phytoplankton cell to oxidants generated controllably via a galvanostat, the amount of oxidant (moles) required to be delivered to the cell for their chlorophyll-a (chl-a) fluorescence signal to switch-off (cellular death) can be calculated via the charge passed.

2. Results and Discussion

In the following, as illustrated in **Figure 1**, a culture of a naturally calcifying coccolithophore, *C. braarudii*, with, and without, its biogenic calcite shell is exposed to oxidants generated via a linear current ramp galvanostatic technique while their chl-a fluorescence signal is monitored as a function of time. During the linear ramp in current, the interfacial potential is driven anodically to oxidize Br^-, Cl^- and H_2O in the culture medium present at natural seawater concentrations to initially form $Br^{\cdot}$, $Cl^{\cdot}$ and $OH^{\cdot}$.[18] These radicals propagate away from the electrode to encounter and react with the *C. braarudii* cells either with its calcite shell or after decalcifying.

The calcite shell on *C. braarudii* was artificially removed by immersion into a culture medium undersaturated with respect to $CaCO_3$ (for details see Experimental Section). Note that Ca^{2+} and HCO_3^-/CO_3^{2-} ions contribute less than 5% of the overall ionic strength of the culture medium. The deshelling of individual *C. braarudii* coccospheres was observed to occur fully within 2 h. This timescale is approximately in agreement with the auto-dissolution of single micron-sized $CaCO_3$ particulates, pristine synthetic particles[21] and *C. braarudii* coccoliths,[22] in deionized water undersaturated with respect to calcium carbonate. **Figure 2**a,b shows reflective microscopy images of representative *C. braarudii* cells before and after the removal of its calcite shell, respectively. A histogram summarizing the "particle" size is shown in Figure 2c. The mean radius of calcified cells is 10.6 (±0.8) μm and this is reduced to 6.9 (±0.5) μm *after* the removal of calcite shells. It was observed elsewhere in the literature that artificially decalcified *C. braarudii* cells, when placed back in its culture medium, continue to secrete coccoliths at a rate that is similar to unaltered calcified cells.[23] This suggests that the decalcification process of the calcite shell is purely chemical with minimal physiological perturbation to the underlying biological cell. Further details on the deshelling procedure, electrochemical, and fluorescence monitoring synchronization can be found in the Experimental Section.

In the fluoroelectrochemical experiment, the oxidative radicals were generated via a current ramped linearly from zero at a rate of 10 μA s^{-1} while simultaneously recording the chl-a fluorescence signal of the biological cell. **Figure 3**a shows the representative chl-a fluorescence response time series of the two

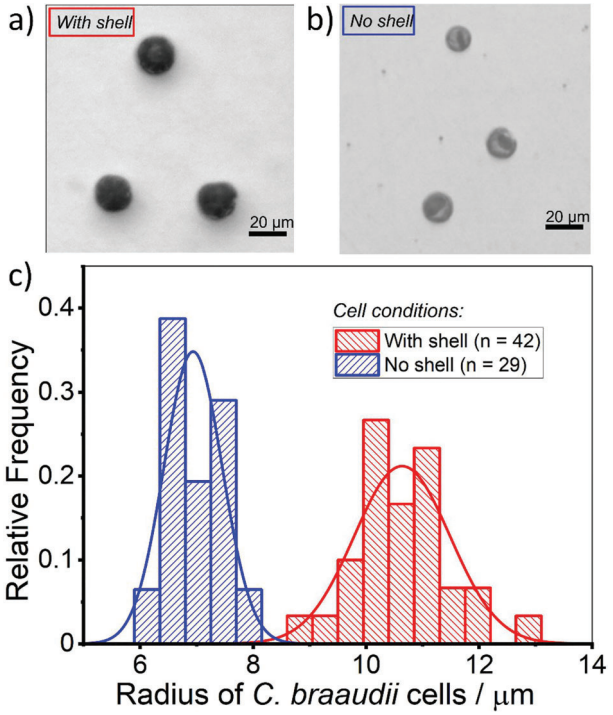

Figure 2. Reflective microscopy image of *Coccolithus braarudii* cells. a) Naturally calcifying, b) artificially decalcified. Scale bars: 20 μm. c) Histogram of cellular radius for naturally calcifying and artificially decalcified cells.

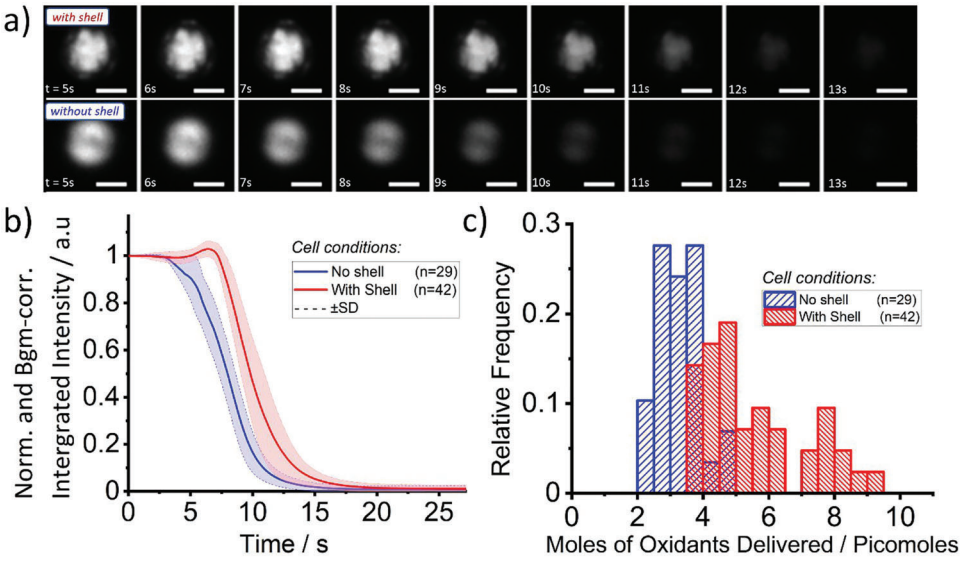

Figure 3. a) Time series chl-a fluorescence images of representative *Coccolithus braarudii* cells with or without their calcite shell during the fluoroelectrochemical experiment. $\lambda_{ex} = 475 \pm 35$ nm and $\lambda_{em} > 590$ nm. Scale bar = 10 µm. b) Average chl-a fluorescence intensity of *C. braarudii* cells with (red) and without (blue) their calcite shell. Sample size: with shell = 42 and deshelled = 29. The shaded region represents the standard deviation of the chl-a signals after normalization and background correction. c) Histogram of the number of moles of oxidants required to be delivered to switch off individual *C. braarudii* cells. The number of moles is calculated using Equation (S1), Supporting Information.

types of *C. braarudii* cells, with or without its calcite shell. Note that although as seen in Figure 2, the size of the calcified *C. braarudii* cells is optically larger than their decalcified equivalents, under fluorescence imaging conditions in Figure 3a, however, only the chlorophyll-containing chloroplasts inside the cells fluoresce (the calcite shell is not fluorescent). Figure 3b plots the average chl-a fluorescence decay of two types of naturally calcified *C. braarudii* cells, either with an intact shell ($n = 42$) or with it artificially removed ($n = 29$) prior to the fluoroelectrochemical experiment. Section S2 (Supporting Information) shows the raw chl-a transients for each of the *C. braarudii* cells under study. As can be seen in the average transients, in the presence of a calcite shell their chl-a signal is distinctively longer lasting before it is completely inhibited (≈15 s) as compared to those without a shell (≈10 s). The magnitude of tens of micro-amps of applied current generates approximately millimolar concentration of oxidants at the electrode interface with the concentration decreasing with distance away from the electrode.[20] Note that the high concentration of ROS generated locally to the electrode provides an accelerated "ageing" of the cells immobilized at the electrode interface leading to a characteristic fluorescence decay (cellular death in tens of seconds). This methodology was chosen, first, for practicality and second, more importantly, to mitigate other physiological events of living *C. braarudii* cells that may mask, or interfere, with the results should the experimental timescale increased to hours or days. Such events include coccolith secretion, cellular mitosis, photo-bleaching of the cellular photosystem and or ROS quenched by seawater far away from the electrode.

Figure 3c is a histogram showing the maximum total moles of oxidants that could have been delivered to the two types of *C. braarudii* cells under the mass-transport limit.[20] The calculation leading to the total moles of oxidants delivered is discussed in Section S1 (Supporting Information). Those with a calcite shell require 5.6 (±1.8) picomoles of oxidants to switch-off their chl-a signal whereas those with their shell predissolved require 3.2 (±1.8) picomoles of oxidants, a much reduced number. Interestingly, the measured 5.6 picomoles of ROS tolerance per calcified *C. braarudii* cells equates to a survival time of 60 h in the sun-lit waters containing 0.2×10^{-6} M ROS, assuming a mass-transport limited reaction (calculations shown in Section S3, Supporting Information). Note that this might be crucial for it to survive in sun-lit surface waters as their lifespan, calculated from cellular division rate, is coincidentally approximately 45–60 h, suggesting a possible protective role for calcite against ROS in their natural environment.[14] A typical *C. braarudii* coccosphere contains around 30–45 picomoles of $CaCO_3$[20] and their shell sizes varied insignificantly during the experiment (see Section S2, Supporting Information). In Section S4 (Supporting Information), the chl-a response of naturally calcifying diploid and naturally noncalcifying haploid cells of *Emiliania huxleyi* originating from the same strain was investigated. For this particular strain, the naturally calcifying diploid cells of *E. huxleyi* exhibit a higher resilience toward the electrogenerated oxidative environment as compared to the naturally noncalcifying haploid equivalent. Please see elsewhere for a detail discussion of the response of different strains of *E. huxleyi*.[24] Note that, in contrast to *C. braarudii* cells, the coccosphere of *E. huxleyi* were found to be fully dissolved by the concomitantly generated acid due to the smaller calcification extent.

3. Conclusion

It can be concluded from the above data that the $CaCO_3$ shell does not quench radicals in seawater but, rather, provides steric hindrance for radicals entering the *C. braarudii* cell providing

a credible evolutionary advantage against extracellular oxidative damage in sun-lit surface waters. This finding is significant as, as discussed above, ROS fluctuates dynamically in seawater and calcification could be advantageous for the survival and blooming of *C. braarudii cells* in radical-filled sun-lit surface waters. While this study suggests some protection from electrochemically produced ROS in the case of *C. braarudii*, this remains to be explored for the much broader range of calcifying phytoplankton.

4. Experimental Section

Chemicals: All chemicals were supplied by Sigma–Aldrich, were of analytical standard and applied without further purification. Ultrapure water (Millipore, resistivity 18.2 MΩ cm at 25 °C) was utilized to make synthetic ocean water.

Phytoplankton Cultures: Naturally calcifying *Coccolithus pelagicus subsp. braarudii* (RCC 1198) and *E. huxleyi* (naturally calcifying RCC 1216 and naturally noncalcifying RCC 1217) strains were supplied by the Roscoff Culture Collection (RCC), France. The culture was maintained by regular batch culturing on a K/2 enriched growth medium modified from the recipe for K medium by Keller et al. with f/2 vitamins.[25] Aquil synthetic ocean water was used instead of natural seawater.[26] The final molarity of each of the medium components in the K/2 recipe is summarized elsewhere.[25,27] Stock cultures of RCC 1198 underwent regular subculturing into fresh growth medium under sterile conditions during the exponential growth phase. The culture was kept under a 14:10 h light–dark cycle with a light intensity of 20–40 µmol m^{-2} s^{-1} at 17 °C, in a PHCbi MLR-352-PE Incubator (PHC Europe B.V.).

Artificial Deshelling: For deshelling, samples of naturally calcified *C. braarudii* cells were dropcasted onto the surface of a glassy carbon working electrode (see below). Residual K/2 culture medium was first removed by tissue paper before immersing the calcified cells with a K/2 medium with Ca^{2+} and CO_3^{2-} omitted. The calcite shell was then left to dissolve for a period of 2 h. Note that the timescale of dissolving the calcite shell was fully consistent with the timescale required to dissolve ≈10 µm sized calcite particles, pristine, or biomineralized, in solution undersaturated with respect to calcium carbonate.[21,22] The underlying biological cell was inferred to be unharmed during the dissolution process as evidenced by the retained chl-a fluorescence signal and, as reported elsewhere in the literature,[23] deshelled coccolithophores resume secretion of new coccoliths to reform the dissolved coccosphere if returned to their regular culture medium. An online supplementary timelapse video attached separately shows the dissolution of calcified *C. braarudii* cells in K/2 culture medium undersaturated with respect to $CaCO_3$. The real-time duration of the video is 2 h, the video caption can be found below.

Supporting Information Video. A timelapse video showing the artificial decalcification process of four naturally calcifying *C. braarudii* cells (appears as black spheres in the video) when placed in K/2 culture medium in the absence of Ca^{2+} and HCO_3^-. The *C. braarudii* cells were initially dropcasted onto a supporting substrate before the reaction chamber was filled with K/2 medium undersaturated with respect to $CaCO_3$. The real-time duration of the video is 2 h and is sped up by approximately 450× to 16 s. The biological cells after complete shell dissolution shows sustained chl-a fluorescence. Scale bar = 20 µm.

Fluoroelectrochemistry Cell Set-Up and Image Analysis: The fluoroelectrochemical cell was designed digitally in Fusion 360 (Autodesk) and was printed using a Form2 3D printer equipped with white resin (Formlabs, USA). A schematic of the opto-electrochemical cell showing the three-electrode setup is reported elsewhere.[28] The 3D printed opto-electrochemical cell (dimensions = 7 × 3 × 1 cm)[28] hosts a graphite counter rod, reference electrode (RE-2BP, saturated calomel electrode (SCE), ALS, Japan) and a glassy carbon working electrode (3 mm diameter, MF-2012, BASi, USA). The working electrode is inserted bottom-up into the opto-electrochemical cell with the surface of the electrode facing the objective lens (20×, NA = 0.5, EC Plan-Neofluar, Carl Zeiss Ltd., Cambridge, UK) of a conventional upright microscope (Zeiss A1 Axio Examiner, Carl Zeiss Ltd., Cambridge, UK). The surface of the working electrode acts as both a supporting substrate and an electrochemical radical generator for the coccolithophores. The chl-a fluorescence excitation light source was provided by a LQ-HXP 120 V lamp. The excitation filter was purchased from Thorlab (FITC 475 ± 35 nm); the dichromic mirror and emission filter were from Zeiss filter set 15 which transmit emission wavelengths above 590 nm.

Prior to the fluoroelectrochemical experiment, the calcified *C. braarudii* cells were dropcasted onto the glassy carbon electrode and the chamber was filled with K/2 culture medium. For the experiment with deshelled plankton, however, since the deshelling process occurs on the glassy carbon electrode (see above), the K/2 culture medium undersaturated with respect to $CaCO_3$ was first removed before filling the reaction chamber with a regular K/2 medium. The timescale for *C. braarudii* cells to secret individual coccoliths (1–2 per hour)[23] is much longer than the timescale of the fluoroelectrochemical experiment (<1 min). At the start of all fluoroelectrochemical experiments, a 40-s equilibration time was applied with the fluorescence light turned on. Then the current, measured at the glassy carbon working electrode, was ramped from 0 to 300 µA at a ramp rate of 10 µA s^{-1}. The means for galvanostatic control and the reasons for its use are described elsewhere.[19,20] The fluorescence intensity of each individual cell was obtained via integration over the area of the cell using ImageJ freeware.[29] After the fluoroelectrochemical measurements were concluded, the size of the underlying biological cell of each calcified *C. braarudii* cell was measured by applying a constant 800 µA to the working electrode for 30 s. During this period of time, sufficient proton was generated via electrolysis of water to completely titrate away the remaining calcite shell to reveal the size of the underlying "naked" biological cell.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Keywords

calcium carbonate, coccolithophore, fluoroelectrochemical measurements, oxidative damage, reactive oxygen species

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