<div align="center">NOTE</div>

AN EFFICIENT METHOD FOR THE PLATING OF HAPLOID AND DIPLOID *EMILIANIA HUXLEYI* ON SOLID MEDIUM[1]

Alastair W. Skeffington, Annett Grimm, Steffi Schönefeld, Kerstin Petersen, and André Scheffel[2] (iD)

Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, D-14476 Potsdam-Golm, Germany

   ***Emiliania huxleyi* is a globally important coccolithophore and one of the most successful eukaryotic organisms in the modern oceans. Despite a large body of work on this organism, including the sequencing of its genome, the tools required for forward and reverse functional genetic studies are still undeveloped. Here we present an optimized method for the clonal isolation of *E. huxleyi* by plating on solid medium. We demonstrate the utility of this method for a variety of strains including haploid, calcifying-diploid, and noncalcifying diploid strains. We show that, in contrast to previous studies, no changes in cell ploidy status occur when the cells are plated. Our method will greatly aid attempts to elucidate the genetic basis of the remarkable physiology of *E. huxleyi* by forward and reverse genetic approaches.**

   Key index words: **Coccolithophore; diploid; *Emiliania*; genetics; haploid; isolation clonal strains; microalgae; plating**

   Abbreviations: **C-cell, calcifying cell (diploid phase in *Emiliania*); DMSP, dimethylsulfoniumpropionate; FACS, fluorescence-activated cell sorting; RNase A, ribonuclease A; S-cells, swarmer cell (motile, haploid phase in *Emiliania*); S-phase, synthesis-phase**

Coccolithophores are the dominant group of calcifying marine phytoplankton, forming blooms covering large tracts of ocean which, through calcification and photosynthesis, have profound effects on marine biogeochemical cycles and eukaryotic and prokaryotic community structure (Monteiro et al. 2016, Braeckman et al. 2018, Laber et al. 2018). They are also prolific producers of the climate-altering compound DMSP (Malin and Steinke 2004). The intricate nature of the calcified scales that cover coccolithophore cells makes these scales interesting models in the context of nanotechnology and bioinspired materials chemistry (Skeffington and Scheffel 2018). *Emiliania huxleyi* is one of the most successful eukaryotes in the modern oceans and has emerged as an experimental model for coccolithophore biology, a status greatly enhanced since the sequencing of its genome (Read et al. 2013).

   Given the importance of coccolithophores, molecular and genetic tools for theses algae are relatively underdeveloped. There is not yet a transformation system for *Emiliania huxleyi*, and the application of classical genetic techniques is hindered by the lack of reliable methods to isolate mutants and to induce meiosis or syngamy. For the development of such tools, an efficient method for the plating of *E. huxleyi* cells on solid media is highly desirable. This would allow selection of cells with selective agents after transformation and permit the progeny of independent transformation events to be identified. The isolation of independent mutants generated via mutagenesis would also be possible. Furthermore, the technique could be used to generate genetically uniform cultures much more easily than by single-cell picking, and to easily maintain strains over a period of months without laborious regular transfers to new medium or costly cryo-preservation.

   There is, to our knowledge, only one previous report of the growth of *Emiliania huxleyi* on solid medium. In this study the authors plated the cells simply by spreading them on F/50 medium solidified with 1.5% agar (Laguna et al. 2001). They found that after a few days, the calcified cells disappeared to be replaced by small (0.5×0.25 μm) rod like cells, which the authors interpreted to be the motile, haploid swarmer-cell (S-cell) phase of the *Emiliania huxleyi* lifecycle. However, doubt has been cast on this interpretation (Billard and Inouye 2004) since previous studies on the morphology of *E. huxleyi* S-cells have shown them to be rounded in shape and about 3 μm in diameter with easily discernible flagella (Klaveness 1972, Green et al. 1996). In addition, there have been no further reports of the successful application of this method.

   In this work, we aimed to develop a robust, high efficiency protocol for the growth of *Emiliania huxleyi* on solid medium in which cell ploidy remains

---

stable. Simply spreading diploid *E. huxleyi* cells on 1.5% agar plates containing modified Aquil medium (composition as described in Morel et al. (1979) but with modified concentrations of nitrate [0.2 mM] and phosphate [0.01 µM]) did not support cell growth or colony formation (Fig. 1), although we and others found a similar method works well for other coccolithophore species such as *Chrysotila carterae* (formerly *Pleurochrysis carterae*; Marsh and Dickinson 1997, Endo et al. 2016) and *Cruciplacolithus neohelis* (Lakeman and Cattolico 2007). Agar plates of F/50 medium, as used in Laguna et al. (2001), also did not support cell growth in our hands.

The most robust method we tested was based on starch embedding (Fig. 1), adapted and optimized from a method previously used for *Chlamydomonas reinhardtii* (Shimogawara et al. 1998). In the optimized method, plates are made by mixing 3% (w/v) agar (Sigma-Aldrich: A7921) melted in distilled water with double concentrated modified Aquil medium. Note that the agar does not completely dissolve if autoclaved directly in Aquil medium. Dry wheat starch (Sigma-Aldrich: S5127) is autoclaved and then resuspended at a concentration of 0.5 g $\cdot$ mL^{-1} in sterile modified Aquil medium diluted with sterile deionized water to a two-thirds concentration. Working in sterile conditions, *Emiliania huxleyi* cells are suspended in 20 µL of modified Aquil medium, mixed with 1.2 mL of the starch suspension and spread evenly on standard 8.5 cm diameter petri dishes. The plate is then allowed to dry just long enough that no surface liquid can be seen. Note that over-drying can compromise cell growth. Plates are sealed with plastic wrap (we find that microporous tape results in over-drying of the plates on incubation) and then incubated at 18°C to 20°C under continuous light at a fluence rate of 50 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}.

In developing this method, a number of parameters were varied to ensure good performance. Corn starch (Sigma-Aldrich: S9679) was compared with wheat starch for the embedding of strain AWI1516 (a calcifying strain from the Alfred Wegener Institute, originally derived from CCMP1516 from the National Centre for Marine Algae and Microbiota as sequenced by Read et al. (2013)), but resulted in a much reduced colony-forming efficiency (colonies formed per cells plated). Other substances composed of fine particles were also tested as alternatives to starch. An acid-cleaned quartz sand (100–360 µm particles, Carl Roth: 4309.2) supported growth but colony formation was poor. Note that, due their large size, the sand particles immediately settled on the agar surface, making it difficult to distribute them homogeneously. Medium remaining in the channels and spaces between the sand particles allowed for cell division and after three weeks of incubation many cells could be seen in these channels and around individual sand particles. Areas on the agar surface devoid of sand particles, however, were free of cells demonstrating the growth supporting effect is restricted to a few hundred micrometers around the particles. Hydrous magnesium silicate particles of an average diameter of 10 µm (Talc, Sigma-Aldrich: 243604) did not support colony formation. Diatomaceous earth (Sigma-Aldrich: 06858 and 85665) in contrast, supported colony formation efficiently. These results suggest that the chemical nature of the support particles play and the particle properties (such as size, shape, charge, or hygroscopicity) play a role in promoting colony formation. In further experiments, growth in continuous light was compared with growth under a 12:12 h light:dark regime, but no significant difference in colony numbers was observed. We also found that colony formation was unaffected when certain antibiotics
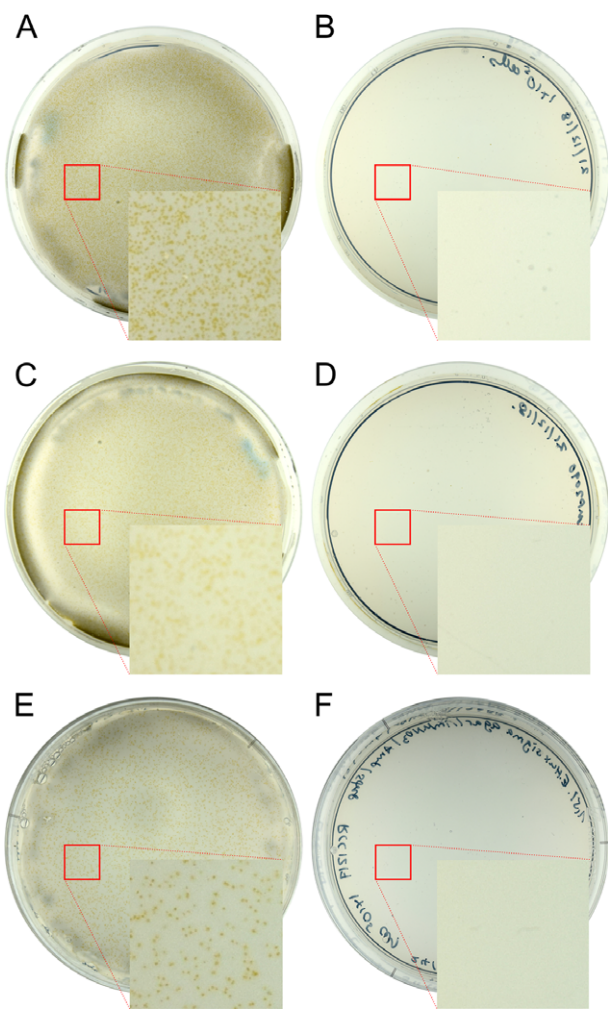

FIG. 1. A comparison of the growth of *Emiliania huxleyi* on plates, with (A, C and E) and without (B, D and F) starch. (A and B) AWI1516, (B and C) CCMP2090, (E and F) RCC1217. In each case, 1.0×10^5 cells were plated per plate, which were then incubated for two weeks in continuous light of 50 µE at 20°C.

(100 µg · mL^{-1} streptomycin and ampicillin) were included in the agar.

The number of cells plated had a strong effect on the colony-forming efficiency (Fig. 2A, ANOVA, $F_{1,27} = 14.52$, $P = 0.0007$), although the effect was predominantly observed at low cell densities and was much less significant when 2,000 or more cells were plated (ANOVA, $F_{1,10} = 0.48$, $P = 0.51$). We tried resuspending the starch in medium supplemented with soil extract (Fig. 2A), which occasionally improves the growth of algae in liquid culture, and this resulted in a 28% increase in colony formation, although the effect was barely significant (Welch's unequal variances t-test, two tailed, $t_{4.8} = -2.51$, $P = 0.05$). In addition, we hypothesized that the high salt concentration resulting from the drying of the Aquil medium on the plate might negatively affect colony formation. Resuspending the starch in 66% concentrated medium instead of 100% concentrated medium led to a dramatic
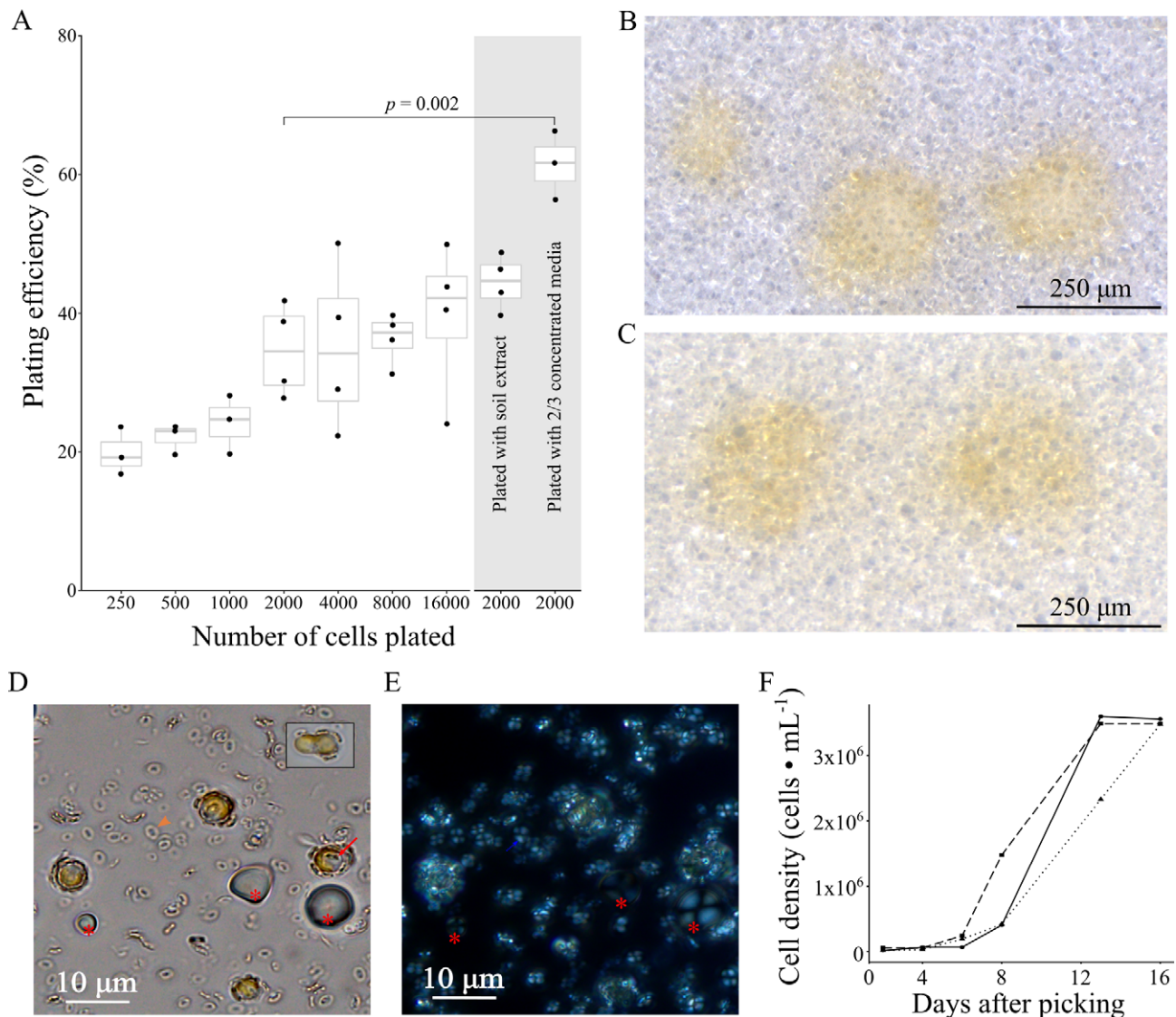

FIG. 2. Characteristics of *Emiliania huxleyi* plated on solid medium. (A) Plating efficiency of AWI1516 (defined as the percentage of cells plated that form colonies) as a function of the number of cells plated, black circles are raw data, boxplots show the median, 25% and 75% quantiles, whereas the whiskers show the most extreme data point. Differing numbers of cells were always plated in the same volume. (B) Stereo microscope micrographs of colonies formed by AWI1516 and (C) by RCC1217. Individual starch particles are visible, whereas the cells are too small to be resolved. The colonies grow within the starch layer with indistinct margins but do not grow out of it in a vertical direction. Light micrographs of cells from AWI1516 colonies are shown in bright field (D) and under crossed polars (E). Starch granules are marked with an asterisk and display birefringence under polarized light. Coccoliths can be seen surrounding individual cells and free in the medium (e.g., orange arrow head), and also display birefringence. Actively calcifying cell containing an internal coccolith can be seen (red arrow), and dividing cells were also observed (insert top right of (D), at the same scale as the rest of the image). (F) Growth of three cultures started by picking single colonies into one ml of medium. Cell density was measured using hemocytometer slides.

increase in colony-forming efficiency of 77% (Fig. 2A; Welch's unequal variances t-test, two tailed, $t_5 = -6.1$, $P = 0.002$). This result was corroborated with a separate experiment in which we found that resuspending the starch in 75% medium instead of 100% medium led to a 27% increase in colony formation, but resuspension in 50% or 25% medium dramatically reduced colony formation (data not shown). Thus, the optimum medium concentration for starch resuspension probably lies in the range of 60%–70%.

Emiliania huxleyi cells form colonies on the plates (Fig. 2, B and C) that first become visible between seven and 14 d after plating. This is true for the calcifying diploid strains AWI1516 and CCMP371, the noncalcifying diploid strain CCMP2090 and the haploid strain RCC1217. Closer examination of cells within AWI1516 colonies often revealed a large proportion of calcified cells, including cells with internal coccoliths and cells undergoing division (Fig. 2, D and E). Picking colonies to start liquid cultures is a highly efficient process. In one experiment, 30 AWI1516 colonies were each picked to one mL of medium and cell densities were monitored over time using the Cellometer T4 cell counter from Nexcelom Bioscience. After 16 d of growth, one culture had died but all other cultures had reached stationary phase and a cell concentration of at least 1.0×10^6 cell · mL^{-1}. In another experiment, 192 colonies were each picked to one ml of liquid medium in 48-well plates. After 20 d of growth, 82% of the colonies picked had formed dense calcifying cultures, whereas in 18% of the inoculated wells no *Emiliania* cells grew up. Note that very young and therefore small colonies were picked, and that the liquid medium was not checked for the presence of the picked colony immediately after transfer. Thus it is possible that in 18% of the wells no colony was transferred to the liquid media in the first place, explaining the lack of culture development.

Given the previous reports claiming that diploid *Emiliania huxleyi* switches to the haploid phase on plating (Laguna et al. 2001), we wanted to test whether or not this occurs with the starch-embedding plating method. Colonies emerging from plated haploid RCC1217 and diploid calcifying AWI1516 cells were scraped from the plates into one ml fresh medium and partially separated from the starch by straining through a five µm pluriStrainer® filter. The flow-through was centrifuged at 10,000 g for 5 min and the supernatant was removed except for the last 50 µL. A few µL of log-phase *Isochrysis galbana* RCC1353 culture was added to serve as an internal standard. The nuclei were stained through the addition of 200 µL Otto's I buffer supplemented with 10% ethanol followed by, after 2 min, 800 µL Otto's II buffer (Otto 1990) supplemented with 62.5 µg · mL^{-1} RNase A and 25 µg · mL^{-1} propidium iodide. After a 10 min incubation step in the dark, the DNA content was determined by flow
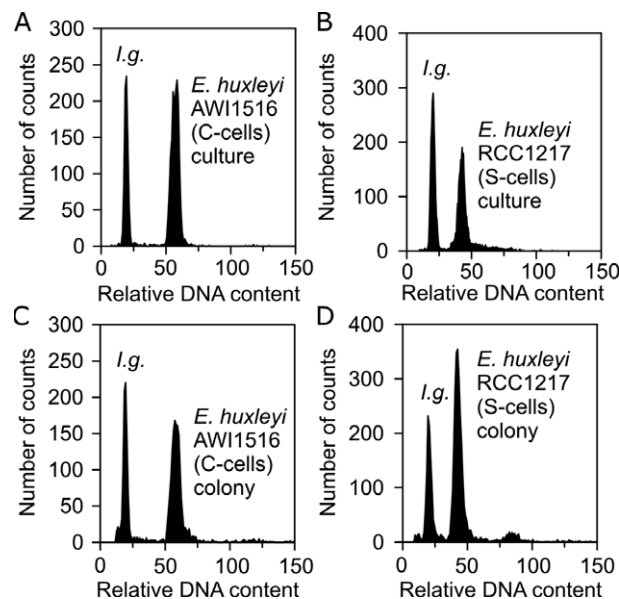

FIG. 3. Ploidy levels of *Emiliania huxleyi* haploid and diploid cells do not change upon plating. The DNA content of C-cells grown in liquid culture (A) and washed from a plate (C), and the DNA content of S-cells (RCC1217) grown in liquid culture (B) and washed from a plate (D), are shown relative to *Isochrysis galbana* RCC1353 (I.g.). (C) and (D) show the results of one of six experiments with virtually identical outcomes. For the six replicate experiments with S-cells (D), the small peak at twice the DNA content of the *E. huxleyi* main peak represents 2.4%–6.8% (average 4.2%) of the RCC1217 counts and for the corresponding experiments with C-cells (C) it represents 3.0%–6.0% (average 4.4%) of the AWI1516 counts. For the three replicates with S-cell suspension cultures (B), this small peak represents 1.2%–2.3% (average 1.4%) of the RCC1217 counts and for the corresponding experiments with C-cells (A) it represents 0.3%–0.6% (average 0.5%) of the AWI1516 counts. In case of the plated S-cells, the counts with twice the amount of DNA content of the main peak may arise from cells that underwent a ploidy change. However, as these counts constitute a similar fraction of the total counts in the C-cell samples the most likely scenario is that the small peaks originate from dividing cells that have completed S-phase but not cytokinesis and/or from aggregates of two cells.

cytometry using the FACSAria II cell sorter (BD Bioscience, San Jose, CA, USA). Propidium iodide fluorescence was measured using a blue laser (488 nm), a 616/23 nm band-pass filter and a 610 LP mirror. Neither AWI1516 nor RCC1217 showed any change in ploidy when grown on plates compared to when grown in liquid culture (Fig. 3). Thus, the starch plating method can be used to grow and select both haploid and diploid strains without ploidy changes, whereas other methods will need to be developed to manipulate the *Emiliania* life cycle.

In summary, we have developed and optimized an efficient starch-embedding based plating system for *Emiliania huxleyi*, in which up to 60% of cells plated may survive to form colonies. In this method, cells are suspended in a 50% (w/v) wheat starch suspension made with two-thirds concentrated modified Aquil medium and then plated on 1.5% agar mixed with modified Aquil medium. The method works

for both haploid and diploid cells. FACS analysis of colonies clearly demonstrated that the cells do not undergo ploidy changes while on the plates, while growing on the solid agar medium described here. The exact role of the starch in maintaining the viability of *E. huxleyi* on plates is unclear, but it seems probably that it creates a microenvironment, which preserves moisture around the cells, particularly since other particulate materials such as diatomaceous earth can also support colony formation. The fact that the number of cells that are plated affects the plating efficiency suggests that the cells themselves further modify this microenvironment. The fact that starch granules of different origin promote colony formation to different extents suggests that the particle properties (size, shape, surface texture) and organic and inorganic impurities of the material also play a role. Note that the corn and wheat starch were tested show differences in the size distribution (found by Coulter-counter analysis) and scattering properties (found by FACS analysis), the latter being related to the surface topology the particles. We expect this method to be useful in the isolation of clonal strains from a mixture of cells, for the storage of strains without the requirement for cryo-preservation (Houdan et al. 2005) or regular subculturing, and most importantly, as a basis for the development of genetic tools for *E. huxleyi*.

Billard, C. & Inouye, I. 2004. What is new in coccolithophore biology? *In* Thierstein, H. R. & Young, J. R. [Eds.] *Coccolithophores. From Molecular Processes to Global Impact.* Springer, Berlin, pp. 1–30.

Braeckman, U., Janssen, F., Lavik, G., Elvert, M., Marchant, H., Buckner, C., Bienhold, C. & Wenzhofer, F. 2018. Carbon and nitrogen turnover in the Arctic deep sea: in situ benthic community response to diatom and coccolithophorid phytodetritus. *Biogeosciences* 15:6537–57.

Endo, H., Yoshida, M., Uji, T., Saga, N., Inoue, K. & Nagasawa, H. 2016. Stable nuclear transformation system for the coccolithophorid alga *Pleurochrysis carterae. Sci. Rep.* 6:22252.

Green, J. C., Course, P. A. & Tarran, G. A. 1996. The life-cycle of *Emiliania huxleyi*: a brief review and a study of relative ploidy levels analysed by flow cytometry. *J. Mar. Syst.* 9:33–44.

Houdan, A., Véron, B., Claquin, P., Lefebvre, S. & Poncet, J.-M. 2005. Cryopreservation of the coccolithophore, *Emiliania huxleyi* (Haptophyta, Prymnesiophyceae). *J. Appl. Phycol.* 17:413–22.

Klaveness, D. 1972. *Coccolithus huxleyi* (Lohm.) Kamptn II. The flagellate cell, aberrant cell types, vegetative propagation and life cycles. *Eur. J. Phycol.* 7:309.

Laber, C. P., Hunter, J. E., Carvalho, F., Collins, J. R., Hunter, E. J., Schieler, B. M., Boss, E. et al. 2018. Coccolithovirus facilitation of carbon export in the North Atlantic. *Nat. Microbiol.* 3:537–47.

Laguna, R., Romo, J., Read, B. A. & Wahlund, T. M. 2001. Induction of phase variation events in the life cycle of the marine coccolithophorid *Emiliania huxleyi. Appl. Environ. Microbiol.* 67:3824–31.

Lakeman, M. B. & Cattolico, R. A. 2007. Cryptic diversity in phytoplankton cultures is revealed using a simple plating technique. *J. Phycol.* 43:662–74.

Malin, G. & Steinke, M. 2004. Dimethyl sulfide production: what is the contribution of the coccolithophores? *In* Thierstein, H. R. & Young, J. R. [Eds.] *Coccolithophores. From molecular processes to global impact.* Springer, Berlin, pp. 127–64.

Marsh, M. E. & Dickinson, D. P. 1997. Polyanion-mediated mineralization – mineralization in coccolithophore (*Pleurochrysis carterae*) variants which do not express PS2, the most abundant and acidic mineral-associated polyanion in wild-type cells. *Protoplasma* 199:9–17.

Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P., Rickaby, R. E. M., Poulton, A. J., Tyrrell, T. et al. 2016. Why marine phytoplankton calcify. *Sci. Adv.* 2:e1501822.

Morel, F. M. M., Rueter, J. G., Anderson, D. M. & Guillard, R. R. L. 1979. Aquil – chemically defined phytoplankton culture-medium for trace-metal studies. *J. Phycol.* 15:135–41.

Otto, F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. *Methods Cell Biol.* 33:105–10.

Read, B. A., Kegel, J., Klute, M. J., Kuo, A., Lefebvre, S. C., Maumus, F., Mayer, C. et al. 2013. Pan genome of the phytoplankton *Emiliania* underpins its global distribution. *Nature* 499:209–13.

Shimogawara, K., Fujiwara, S., Grossman, A. & Usuda, H. 1998. High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics* 148:1821–8.

Skeffington, A. W. & Scheffel, A. 2018. Exploiting algal mineralization for nanotechnology: bringing coccoliths to the fore. *Curr. Opin. Biotechnol.* 49:57–63.