Morphology of *Emiliania huxleyi* coccoliths on the North West European shelf - is there an influence of carbonate chemistry?

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Abstract

Within the context of the UK Ocean Acidification project, *Emiliania huxleyi* (type A) coccolith morphology was examined from samples collected during cruise D366. In particular, a morphometric study of coccolith size and degree of calcification was made on scanning electron microscope images of samples from shipboard CO_2 perturbation experiments and from a set of environmental samples with significant variation in calcite saturation state ($\Omega_{calcite}$). One bioassay in particular (E4 from the southern North Sea) yielded unambiguous results - in this bioassay exponential growth from a low level occurred with no artificial stimulation and coccosphere numbers increased ten-fold during the experiment. The samples with elevated CO_2 saw significantly reduced coccolithophore growth. However, coccolithophore morphology was not significantly affected by the changing CO_2 conditions even under the highest levels of perturbation (1000 μatm). Environmental samples similarly showed no correlation of coccolithophore morphology with calcite saturation state. Some variation in coccolith size and degree of calcification does occur but this seems to be predominantly due to genotypic differentiation between populations on the shelf and in the open ocean.

1 Introduction

Coccolithophores are one of the most abundant and widespread groups of calcifying plankton and so have attracted extensive study in terms of their likely response to ocean acidification. Early experimental work with laboratory cultures and large-scale semi-enclosed field cultures, mesocosms, suggested that there was a clear reduction in calcification rates with increasing pCO_2 (Riebesell et al., 2000; Riebesell, 2004; Zondervan et al., 2002; Engel et al., 2005). They did, however, note that other effects such as growth rate and cell size changes could confuse this response, that the response was often muted and that it was important to look at changes in the ratio of calcification to photosynthetic carbon fixation and at calcification rates per cell.

Building on these initial indications of a distinct influence of carbonate chemistry on coccolithophores, several ecological studies suggested that variations in carbonate saturation state might influence aspects of the distribution of modern coccolithophores, such as timing of blooms (Merico et al., 2006) and absence of coccolithophores from parts of the Antarctic Ocean (Cubillos et al., 2007) and from the Baltic Sea (Tyrrell et al., 2008). Most strikingly it has been suggested that coccolith mass in *Emiliania huxleyi* and closely related species is controlled by saturation state in both the modern ocean and the Late Quaternary fossil record (Beaufort et al., 2011). This work indeed suggested progressive effects across carbonate saturation states from $\Omega_{Calcite}$ 2 to 9.

Other work, however, has suggested that coccolithophores show a much more complex response to carbonate saturation. Laboratory culture work has shown that species other than *E. huxleyi* can show very different responses with some species showing negligible response to elevated pCO_2 (Langer et al., 2006). Moreover, it has been shown that even within *E. huxleyi* the response of different laboratory strains is highly variable (Langer et al., 2009), and at least one strain shows almost no calcification response to strongly elevated pCO_2 conditions, or even increased calcification (Iglesias-Rodríguez et al., 2008; Jones et al., 2013).

Conflicting results have also been found from field and geological evidence. Two studies of high-resolution sediment records from the past 200 years have provided evidence for increased mass of coccolithophores over this time period, despite the rise in atmospheric CO_2, or possibly even as a, counter-intuitive, response to it (Iglesias-Rodríguez et al., 2008; Grelaud et al., 2009). Study of coccolithophores in the Bay of Biscay has shown that the winter decline in carbonate saturation is paralleled by an increase rather than a decrease in degree of calcification of *E. huxleyi* coccoliths, as recorded by the relative abundance of normally calcified and over-calcified morphotypes (Smith et al., 2012). Finally Berger et al. (2014) have shown that coccolith mass during the Holocene varied significantly even though CO_2 concentrations are thought to have been stable.

Within the context of the UK Ocean Acidification programme (http://www.oceanacidification.org.uk/) we have participated in a project aimed at investigating the likely effects of ocean acidification in the surface ocean via cruise-based research with a mix of field sampling across waters with naturally variable carbonate chemistry conditions and large-scale shipboard incubation experiments - bioassays. The first cruise within this project was carried out in June-July 2011, cruise D366 of the *RRS Discovery* around the North West European continental shelf. This included sampling of a diverse range of regions in terms of stratification, nutrients, water depth, coccolithophore abundance, carbonate chemistry and other parameters.

Coccolithophores are an abundant and diverse component of the North Atlantic phytoplankton community (e.g. Okada & McIntyre 1979; Jordan 1988; Dandonneau 2006; McGrane 2007) but on the shelf they are generally subordinate to other phytoplankton and *Emiliania huxleyi* is usually the predominant and often the only species (e.g. Houghton 1988, 1993; Charalampopoulou et al. 2011).

Blooms of *Emiliania huxleyi* are regular summer features in the area particularly along the shelf break and in the seasonally stratified parts of the North Sea (e.g. Holligan et al. 1993; Wal et al. 1995; Buitenhuis et al. 1996; Harlay et al. 2010). The widespread abundance of *Emiliania huxleyi* in the area and the limited occurrence of other species meant it was the inevitable focus of our study. It is also a good species to study for detecting the effects of ocean acidification, since the open architecture of *E. huxleyi* coccoliths means that it can vary greatly in degree of calcification, i.e. in the amount of calcite that is incorporated within a coccolith of a given size. Nonetheless, there are a series of potential taxonomic complications which could lead to results being complicated by genotypic variation. First there is a similar sized *Gephyrocapsa* species, *G. muellerae,* which can occur in the area, especially in the offshore oceanic waters. Second, there are two major morphotype groups of *Emiliania huxleyi*, type A and B (Young et al. 1991, 2003) with type B being distinctly less calcified, and both are known to occur in the study area (e.g. van Bleijswijk et al. 1991), although the A type is usually most common. Third, genotypic variation occurs within each group as evidenced by both morphological work (e.g. Young et al. 2003) and molecular genetic work (Hagino et al. 2011, Bendif et al. 2014). Notably both Hagino et al. (2011) and Bendif et al. (2014) distinguish warm and cool water clades within the global *E. huxleyi* population with overlapping occurrence in the NW European shelf area. So, a scanning electron microscope (SEM) based technique was adopted to allow consistent identification of taxa, accurate size measurement, and study of degree of calcification independent of size.

2 Material

A very large dataset of samples was collected for the project including samples from 65 CTD stations (4 to 6 depths at each), 190 underway samples (single samples from the ship's uncontaminated sea water sampling system with an intake at 5m water depth), and 5 bioassay experiments. For detailed morphometric work we concentrated on the bioassay experiments, to study the response of *Emiliania huxleyi* to changing carbonate chemistry, and on selected CTD stations which were also studied for in situ calcification rates (Poulton et al. this vol.).

The bioassay experiments were major shipboard culture experiments, full details of which are given in Richier et al. (this vol.). In brief, , for each experiment at a different location, a whole CTD rosette of 24 x 20 litre OTE (Ocean Tech Equipment) bottles was collected and were divided into 72 x 4.5 litre bottles which were treated with appropriate combinations of equimolar of HCl and bicarbonate in order to adjust the pH and CO_2 to target levels equivalent to 500, 750 and 1000 μatm CO_2, as well as a control set in which pH was not adjusted - "ambient" conditions. The cultures were incubated in a container lab on the ship with light and temperature regulated to match those of the sample locality (see Richier et al. this vol). The objective of these experiments was to observe the reaction of the total *in situ* plankton assemblage to CO_2 change under as close to natural conditions as possible, and so zooplankton were not removed and nutrients were not added. Sampling was carried out of the at the time of initial water collection and at two time points; 48 h and 96 h after the start of the experiment. At each time point samples were collected from the 4 CO_2 conditions with three replicate samples for each condition, resulting in a set of 12 samples per time point.

The locations of the 5 bioassay experiments and the 15 CTD stations used for detailed morphological work are indicated on the map (Fig. 1) and the key environmental conditions in them are detailed in Table 1.

3 Methods

3.1 Sample collection

For coccolithophore research, samples were processed by filtration of seawater onto membrane filters, using 25 mm diameter filters and typically filtering 250 mL onto each filter. Two filters were collected from every sample, one polycarbonate filter for Scanning Electron Microscopy (typically Whatman Nuclepore or Cyclopore 0.8 µm pore size filters) and one cellulosic filter for light microscopy (typically Whatman WCN cellulose nitrate 0.8 µm pore size filters). After filtration the filters were oven dried (50-60^{o}C, 8-10 h) and stored in Petri-slides. Light microscope slides were made up immediately on ship using Norland Optical Adhesive No 74. For electron microscopy, portions of the filters were mounted on aluminium SEM stubs using photographic film.

Protocols for measurements of environmental parameters are given in Ribas-Ribas et al. (this vol.) and for protocols for measurement of *in-situ* calcification rates are given by Poulton et al. (this vol.).

3.2 Microscopy

Light microscopy examination was carried out using cross polarised light illumination with x100 oil immersion objective on Leitz Ortholux and Olympus BX 51 microscopes. This was used for coccolithophore cell counts.

Electron microscopy was primarily carried out using a Leo 1450VP, Carl Zeiss microscope at NOC Southampton. This microscope is equipped with an automated imaging system (SmartSEM software), and matrices of 10 x 11 images were taken from each sample at x 5000 magnification. These images were used for morphometric work and for counts of the numbers of loose coccoliths.

3.3 Morphometric measurements

Morphometric work was undertaken using the public domain program Fiji (Schindelin et al. 2012), a distribution of ImageJ (Schneider et al. 2012). A set of macro routines was written to facilitate this. In a first step the images from each sample were scanned through and all flat-lying *E. huxleyi* coccoliths seen in distal view were collected as standard size sub-images, until 60 images had been collected or the entire set of images scanned. Type A and type B coccoliths were then separated based on coccolith morphology (Young et al. 1991). In practice type B coccoliths were absent from most samples and never formed more than 10% of the assemblages in the samples studied in detail. Detailed morphometric results hence are reported for type A coccoliths only. For each coccolith image the length and width were measured by dragging an ellipse around the coccolith perimeter. Positions on the outer and inner edge of the tube were then fixed manually at points were they were clearly visible and from these the rim width was calculated (Fig. 2). The calculation is based on the observation that coccolith geometry closely approximates to a set of co-axial parallel ellipses (Young et al. 1996). A routine was also developed to automatically count the number of rays (elements) and measure their width. However, ray number, along with most other parameters was found to be very strongly correlated with coccolith length ($r=0.92$, 150 measurements) and so this did not yield useful data. Ray width did appear to be variable but the image resolution was not high enough to reliably record this.

Tube width does vary significantly between *E. huxleyi* coccoliths, from lightly calcified coccoliths in which the central area is broad and the tube is narrow, to heavily-calcified coccoliths in which the central area is almost closed (Fig. 2). To obtain a size independent parameter to measure this degree of calcification variation we used relative tube width = 2 x tube width / coccolith width (Fig. 2). Since this is a ratio it is dimensionless and should be size-independent. For the total set of 1488 coccoliths measured there was a weak negative correlation between coccolith length and relative tube thickness, $r = -0.17$ ($p<0.01$): i.e. there is a weak tendency for the degree of calcification (size-normalised calcite content) to decrease with increasing coccolith size. Due to the large sample size this correlation is statistically significant ($p<0.01$), however when correlation coefficients are calculated for individual samples there is no consistent pattern, with correlation coefficients varying from +0.32 to -0.27.

Malformation frequencies have sometimes been used in culture work to record the effect of growth conditions. However, as is usual with natural populations, significant malformation was not seen in any samples, so malformation frequency was not a useful character for this study.

Coccolith mass has also often been used in studies of the impact of ocean acidification on coccolithophores. Young & Ziveri (2000) showed that the mass (m) of coccoliths could be estimated as $m = 2.7 \times k_s \times l^3$ where l is coccolith length and k_s a shape dependant constant. For normally calcified *E. huxleyi* coccoliths they derived a value of $k_s = 0.02$, if length is given in microns and mass in picogrammes. The profile this is based on (Fig. 3 of Young & Ziveri 2000) has a relative tube thickness of 0.3. Other aspects of degree of calcification, such as ray width appear to broadly co-vary with relative tube width (Figure 2) so we would predict that coccolith mass would be roughly proportional to relative tube width, i.e. it can be used as an estimate of k_s and specifically that $k_s = 0.07 \times rtw$.

4 Results

4.1 Bioassays

Of the five bioassays, three (E1, E4 and E5) had significant abundances of *Emiliania huxleyi*. E2 was from the Irish Sea and had only trace abundances (<2 cells ml^{-1}) of *E. huxleyi*, along with rare *Coccolithus pelagicus* ssp. *braarudii*. E3 from the Bay of Biscay had slightly higher abundances of *E. huxleyi*, accompanied by *Gephyrocapsa muellerae* and *Syracosphaera marginaporata*, but too few detached *E. huxleyi* coccoliths were present for analysis of changes between culture conditions.

Results from the three remaining bioassays are summarised in Table 3 and Figure 3. This gives results on four parameters (*E. huxleyi* coccosphere numbers, loose *E. huxleyi* coccolith numbers, coccolith length and relative tube width) from each of the three bioassays. To facilitate comparison between the bioassays common scales are used in each row of plots of a single parameter. Within each sub-graph the results are shown from the initial conditions, i.e. the seawater which was introduced into the bottles (initial sample), and from the four different experimental conditions from each of the sampling time points, after 48 h and 96 h. The x axis represents time but the experimental conditions are separated slightly along this axis in order to show the data more clearly. For cell and coccolith counts, data from each of the three replicates are given. For coccolith size and relative tube thickness the mean and standard deviation are given from measurement of ca. 60 specimens per condition, but from only one replicate.

Bioassay E1 was located off western Scotland, to the south of the island of Mingulay (Fig. 1). There was a high initial *E. huxleyi* population, ca. 300,000 cells per litre, but this included many obviously dead cells (empty coccospheres) and there was a large number of loose coccoliths (>100 $x10^6$ coccoliths L^{-1}). These suggest a mature population, possibly the stationary phase of a weak bloom. During the bioassay the cell numbers and loose coccolith numbers were stationary or declined, but there was no consistent difference between the different CO_2 conditions.

Significant calcification was recorded through the experiments (Table 3; average 0.2 µg C l^{-1} d^{-1}, equivalent to 4 coccoliths per cell per day) so coccolith production was continuing, but was compensated for by losses due to grazing, and possibly dissolution in some microenvironments. So there should have been at least a moderate turnover of coccoliths during the duration of the experiment. Nonetheless there was no detectable morphological change through the course of the experiment or between culture conditions.

Bioassay E5 was rather similar - it was located in the mid North Sea on the fringe of a major bloom feature, which had been visible in satellite images for more than one month (Kreuger-Hadfield et al. 2014). We repeatedly sampled this bloom and it was clear that it was a late phase bloom with often very high ratios of loose coccoliths to cells (>100 loose coccoliths/coccosphere) and many large clumps of coccoliths without clear coccospheres. There was an increase in cell numbers over the first 48 h but this was not continued over the second 48 h. Loose coccolith numbers remained stationary. Again calcification rates indicate that coccolith production was continuing (Table 3; average 0.23 µg C l^{-1} d^{-1}, equivalent to 5 coccoliths per cell per day) and therefore that new coccoliths were being produced, but coccolith morphology did not change through the experiment or between conditions.

A low abundance population of *Braarudosphaera bigelowii* occurred in this bioassay and increased in abundance in the low CO_2 treatments, these results will be described in a separate paper.

Bioassay E4 from the southern North Sea was rather different. The initial sample had very low cell numbers. Coccolith to cell numbers were moderately high and it is possible that some of the coccoliths may have been old specimens in suspension.

Despite this unpromising start the populations grew markedly during the experiment and some distinct morphological change occurred. Calcification rates were also significant during the experiment (Table 3; average 0.18 µg C l^{-1} d^{-1}, equivalent to 9 coccoliths per cell per day). To show these better the cell and coccolith abundance data is re-plotted in Figure 4 with axes adjusted to show the data optimally. Similarly in Figure 5 the coccolith size and relative tube thickness data are re-plotted as frequency histograms.

The coccolith cell numbers increased from ca. 20,000 cells L^{-1} in the inoculum to >100,000 cells L^{-1} 96 h. With only three sampling points it is not possible to tell if exponential growth occurred throughout the sampling period but the plot on logarithmic axes (Fig. 4A) suggests that it did. Loose coccolith numbers also increased especially in the 96 h samples. In terms of cell numbers there is no difference between the inoculum and the two intermediate CO_2 treatments but the high CO_2 treatment shows consistently lower cell numbers (Fig 4A). The experiment duration was too short to determine if this was as an acclimation effect. Loose coccolith numbers are also somewhat lower in the high CO_2 treatment.

In terms of coccolith morphology there is a clear trend of increasing coccolith length and decreasing relative tube thickness through the course of the experiment. There is weak evidence of increasing size and decreasing tube thickness with increasing CO_2 concentrations, but most of this variation is within the error margin of the mean values and the pattern is not clearly shown in the raw data (see histogram plots in Fig. 5).

4.2 Field samples

Although the morphological parameters of coccolith length and relative tube width show only slight variation within bioassays, they do show marked variation between bioassays (Fig. 3). This might be due either to genotypic variation between the populations in the different bioassays or to an ecophenotypic response to ecological conditions. To investigate these possibilities data from further environmental samples can be examined. Figures 6 and 7 are histogram plots of coccolith length and relative tube width from the 18 environmental samples and from the initial sample of the bioassays (for bioassay E3 and E4 these were supplemented by coccoliths from the ambient and low CO_2 treatment samples after 48 h).

The plots reveal similar within sample variability to that seen in the bioassay samples. In Figure 8 the mean values of these parameters per sample are plotted against a range of environmental parameters - calcite saturation state, temperature, salinity and nutrient concentrations and in Table 2 correlation coefficients are given. The correlation coefficients are all low, below the 5% level of statistical significance for the sample sizes, and the plots do not show any evidence of an underlying non-linear relationship. So it appears that the morphological variability is not directly related to these environmental variables. It did, however, appear that the North Sea samples tended to have larger coccoliths, so the data was also plotted on a map (Fig. 9). This shows a rather distinct biogeographic pattern to the coccolith length data.

The oceanic sites to the southwest, west and northwest of the study area are typified by smaller coccoliths, with mean lengths of 2.8-3.1 µm. In contrast, the more neritic samples from the southern North Sea, Irish Sea and English Channel all have larger average coccolith lengths, typically 3.3-3.4 µm. The Mingulay location off western Scotland is an interesting anomaly, this site was sampled at both the beginning of cruise (Bioassay 1) and at the end of the cruise (underway sample, U323) with rather different results. This could, however, be due to different conditions at the two sampling times, with advected oceanic water during the initial sampling and neritic water during the later sampling.

Relative tube thickness does not show a consistent biogeographic pattern (Fig. 9) or vary significantly between the two groups of samples (Fig. 10A). This was slightly surprising since the subjective impression had been that the neritic samples were characterised by less heavily calcified coccoliths. However, as noted above, the sign of the correlation coefficient between coccolith length and relative tube width varies between samples - i.e. in some samples degree of calcification (calcite content) increases with size and in others it decreases. In Figure 10B the correlation coefficients are plotted against mean coccolith length and this cross-plot clearly separates the ocean samples from the neritic ones. This indicates that there is a weak tendency for an increase in calcification with size in the oceanic populations, but a decrease in calcification with size in the neritic populations. This should mean that the difference between the populations will be most apparent in the larger coccoliths. To test this the populations in each sample were sorted by size, the largest 25% (upper quartile) selected and means of coccolith length and relative tube width for these sub-samples were calculated (Fig. 10C). This shows an improved separation of the oceanic and neritic samples and so suggests that there is a distinct difference in the relationship between size and degree of calcification between these two groups.

5 Discussion

The best test for the presence of measurable effects of seawater carbonate chemistry on *Emiliania huxleyi* was provided by Bioassay E4 from the Southern North Sea. In this experiment strong coccolithophore growth occurred from a low base level, possibly because of incubating a light-limited initial community in a deep mixed layer in higher irradiance conditions, or because a water mixing event prior to our sampling had fertilised the water, or because of a fortuitous absence of relevant predators and competing phytoplankton. This strong growth meant that the effect of CO_2 addition on the growth could be studied and also that the vast majority of coccoliths present at the end of the experiment must have been produced during the experiment, and so under the adjusted carbonate chemistry conditions. In addition the basic methodology of the bioassays, of studying natural populations with minimal possible manipulation of conditions, made this a robust experiment. In this bioassay there is a clear inhibition of coccolithophore growth at the highest CO_2 conditions, suggesting that elevated CO_2 levels are detrimental to the growth of *Emiliania huxleyi.* However this might be a short term acclimation effect and similar effects of inhibition of growth rates at high CO_2 treatments were shown by other phytoplankton during the experiments (Richier et al. this volume). Nonetheless, even in this experiment there is no clear or strong effect of CO_2 levels on coccolith morphology (Fig. 4, 5).

Coccolith size does increase with time through the experiment in Bioassay E4, but this occurs in all culture conditions. We would normally expect cell and coccolith size to decrease during exponential growth (e.g. Gibbs et al. 2013), so the size increase is somewhat surprising. Possibly in this case there is selection occurring between smaller and larger *E. huxleyi* strains. The parallel decrease in calcification (relative tube width) may also be due to this or may reflect the tendency in the North Sea *E. huxleyi* populations for the larger coccoliths to be less heavily calcified, i.e. an example of allometric growth. The striking result is thus that even though the populations are all actively growing and the carbonate chemistry levels are having a clear effect on growth rates the variation in carbonate chemistry does not have a significant effect on either coccolith size or degree of calcification (as measured by relative tube width).

Weaker tests of the effect of carbonate chemistry are provided by the other two bioassays with significant populations of *E. huxleyi* (E1 and E5) and by the environmental samples. Neither of these showed any effect of carbonate chemistry on coccolith morphology, or cell numbers. These are weaker tests than bioassay 4, since in the case of bioassay 1 and 5 there was no net population growth, so a large proportion of the coccoliths at the end of the experiment would have been present at the start of the experiment. This would have diluted any effects on coccolith morphology and so makes it less certain that no morphological change occurred. In the case of the environmental samples, the range of carbonate chemistry conditions was rather muted - $\Omega_{calcite}$ varied from 3.5 to 5. Nonetheless, following hypotheses which predict a strong effect of carbonate chemistry on coccolith morphology we would have expected a clear signal even under these conditions. Hence, these results can be taken as evidence that any effect of *in situ* carbonate chemistry on *E. huxleyi* size and degree of calcification is low.

Conversely, there does seem to be evidence of a morphological contrast between oceanic and neritic *E. huxleyi* populations in this area. The neritic populations tend to be larger (Fig. 9A) and to show a decrease in calcification with size in contrast to the oceanic populations which tend to be smaller and show an increase in degree of calcification with size (Fig. 10B, C). This contrast also parallels change in the coccolithophore assemblages. The neritic sites tend to be dominated by *E. huxleyi* with rare *Acanthoica quattrospina, Braarudosphaera bigelowii* and *Coccolithus pelagicus* whilst the oceanic assemblages are more diverse and include *Gephyrocapsa muellerae* and *Syracosphaera* spp. This consistent separation is not obviously related to any short term environmental parameter, including carbonate chemistry (Fig. 8), but does reflect the generally observed rule that there is a strong contrast between neritic and oceanic phytoplankton (e.g. Murray & Hjort 1912, Longhurst 2006) even if the controls on this are less well-established. So the strongest control on *in-situ E. huxleyi* morphology within this region appears to be a genotypic contrast between the coastal and oceanic populations, with no obvious effect of carbonate chemistry.

Intriguingly, recent molecular genetic work on *E. huxleyi* using rapidly evolving mitochondrial genes has highlighted a major subdivision of type A *E. huxleyi* into groups, termed Clades I and Clade II (Hagino et al. 2011, Bendif et al. 2014). These have been characterised as broadly warm water and cool water groups but strains of both genotypes occurred in the current study area (Hagino et al. 2011) with the boundary between them approximating the shelf break. Hence it is possible that the coastal and oceanic populations characterised here may correspond to the clades I and II of Hagino et al. (2011).

6 Conclusion

The only unambiguous effect of changing carbonate chemistry that was observed was decrease in growth rate of *Emiliania huxleyi* in Bioassay Experiment 4. This was an ideal experiment for coccolith morphology work since substantial *E. huxleyi* populations (>100,00 cells l^{-1}) grew from low initial levels (ca. 20,000 cells l^{-1}) with no alteration of the environmental conditions other than carbonate chemistry. Nonetheless even in this experiment no effect of carbonate chemistry on coccolith size or degree of calcification was observed. This reinforces the emerging consensus from recent culture experiments that whilst the net effect of ocean acidification on *Emiliania huxleyi* is likely to be detrimental the magnitude of this effect is likely to be low, to be variable between strains and to be reduced by adaptation and strain-selection. Patterns of variation in coccolith size and degree of calcification can be seen in the data but are not readily explained by ocean chemistry and more probably reflects genotypic variation. This reinforces the conclusions of Smith et al. (2012) and Berger et al. (2014) that degree of calcification of coccoliths, even within a single species, may be most strongly driven by factors other than carbonate chemistry

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Table 1 Environmental conditions for the samples used for detailed morphometric investigation; including sampling date, location, depth and physico-chemical metadata, also summary statistics from the morphometrics. NB for bioassays coccolith measurements were included from the following samples E1 - initial sample plus 48hours ambient conditions; E3 & E4 initial sample plus 48 hours ambient and low CO_2 conditions; E5 initial sample only. Carbonate chemistry is from Ribas Ribas et al. (this vol.).

d - depth; N - number of specimens measured; length - average coccolith length; sd l - standard deviation of length; rtw - relative tube width; sd rtw standard variation of rtw; Ω - $\Omega_{calcite}$; temp. - temperature; sal. - salinity; NO_x - nitrate + nitrite; PO_4 - phosphate.

samples	date	Lat.	Long.	d	N	length	sd l	rtw	sd rtw	Ω	temp	sal.	NO_x	PO_4
				m		μm	μm				°C	psu	μM	μM
CTD-15	11-Jun	52.14	-11.71	5	76	3.08	0.39	0.26	0.06	3.90	12.01	35.55	4.27	0.24
CTD-19	13-Jun	51.61	-5.72	5	64	3.33	0.32	0.23	0.08	4.19	13.35	35.16	0.07	-0.01
CTD-24	15-Jun	50.03	-4.38	3	72	3.49	0.41	0.22	0.04	4.19	13.84	35.33	0.01	-0.01
CTD-29	19-Jun	46.50	-7.21	5	62	2.84	0.35	0.22	0.05	4.36	15.03	35.75	0.88	0.08
CTD-32	21-Jun	46.18	-7.23	5	22	2.81	0.23	0.18	0.03	4.33	15.31	35.78	0.61	0.06
CTD-34	22-Jun	48.00	-7.19	5	58	3.09	0.32	0.24	0.04	4.30	14.54	35.62	0.23	0.02
CTD-38	24-Jun	50.03	-4.36	3	107	3.48	0.35	0.22	0.04	4.26	13.97	35.32	0.29	0.03
CTD-43	26-Jun	52.99	2.50	2	83	3.39	0.39	0.26	0.06	3.60	14.57	34.08	0.74	0.13
CTD-45	27-Jun	54.31	7.31	2	64	3.41	0.42	0.25	0.06	3.90	14.06	33.23	4.26	0.03
CTD-54	29-Jun	57.76	4.59	5	69	3.40	0.26	0.19	0.05	4.05	13.22	34.81	0.26	-0.01
CTD-65	02-Jul	56.49	3.61	12	80	3.46	0.36	0.19	0.03	3.76	6.30	34.98	0.19	0.02
CTD-67	02-Jul	59.68	4.13	4	62	3.19	0.31	0.19	0.04	3.68	13.34	30.68	0.18	-0.05
CTD-70	04-Jul	60.00	-2.66	2	57	3.46	0.47	0.20	0.07	4.75	13.05	35.20	0.25	0.02
CTD-71	05-Jul	59.99	-5.98	5	61	2.94	0.32	0.23	0.04	4.04	11.65	35.32	4.73	0.32
U323	07-Jul	56.83	-7.39	10	61	3.37	0.32	0.19	0.03	4.09	12.85	34.67	14.50	1.09
Bioassay E1	08-Jul	56.79	-7.41	6	157	2.97	0.30	0.31	0.08	3.74	11.27	34.80	1.14	0.09
Bioassay E3	21-Jun	46.20	-7.22	10	89	2.89	0.34	0.22	0.06	4.05	15.31	35.77	0.56	0.06
Bioassay E4	26-Jun	52.99	2.50	10	177	3.48	0.45	0.24	0.07	3.67	14.57	34.05	0.73	0.13
Bioassay E5	02-Jul	56.50	3.66	10	66	3.41	0.33	0.19	0.04	3.88	13.86	34.99	0.18	0.05

Table 2 Correlation coefficients for the samples listed in table 1 matrix of Pearson correlation coefficients for coccolith morphology parameters and key physico-chemical environmental parameters. n=19 so the 95% confidence level is 0.389 and the 99% confidence level is 0.444. The only significant correlations are of salinity with Ωcalcite and nitrate+nitrite with phosphate. Neither coccolith size nor degree of calcification show significant correlation with any individual environmental variable in this data set.

	Coccolith length	Relative tube width	Ω calcite	Temp.	Salinity	NOx	PO4
Coccolith length	1	-0.218	-0.136	-0.210	-0.273	0.009	-0.004
Relative tube width	-0.218	1	-0.296	0.034	0.043	-0.021	-0.062
Ω calcite	-0.136	-0.296	1	0.282	**0.546**	-0.043	-0.031
Temperature	-0.210	0.034	0.282	1	0.022	-0.099	-0.088
Salinity	-0.273	0.043	**0.546**	0.022	1	-0.028	-0.030
Nitrate+Nitrite	0.009	-0.021	-0.043	-0.099	-0.028	1	**0.956**
Phosphate	-0.004	-0.062	-0.031	-0.088	0.080	**0.956**	1

Table 3

Calculation of average coccolith production rates per cell during the course of the experiments for the Bioassays with significant *E. huxleyi* populations. Inorganic carbon fixation was measured radiometrically as described in Poulton et al. (2014), conversion to coccoliths assumes a coccolith weight of 2pg and hence an inorganic carbon quota of 0.24pg.

Bioassay	E1	E4	E5
Cells (x10^3 l^{-1})			
Minimum	68	14	61
Maximum	306	150	272
Average	205	85	195
Inorganic carbon fixation (µg C l^{-1} d^{-1})			
Minimum	0.03	0.09	0.05
Maximum	1.2	0.63	0.74
Average	0.2	0.18	0.23
Inorganic carbon fixation per cell (pg C $cell^{-1}$ d^{-1})			
Average	1.0	2.1	1.2
Coccolith production (liths $cell^{-1}$ d^{-1})	4	9	5

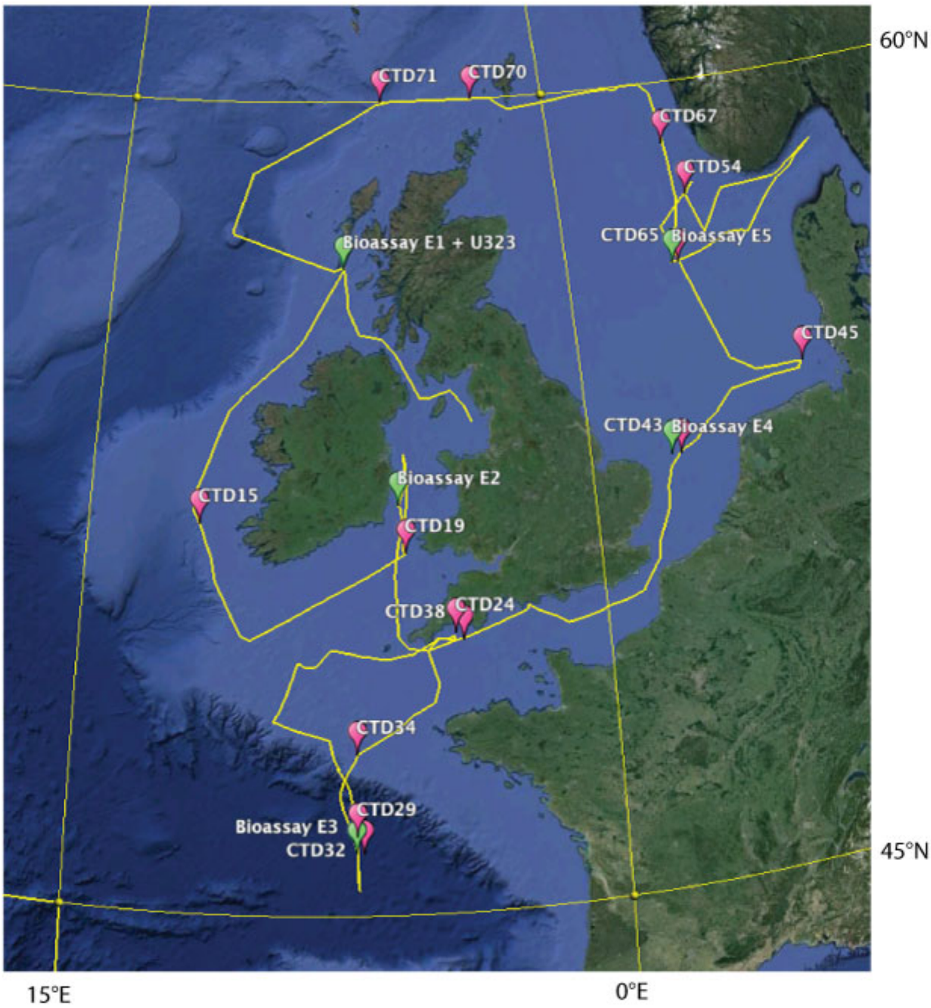

Figure 1. Track of cruise D366 and location of samples studied in detail here. Cartography, Google Earth.

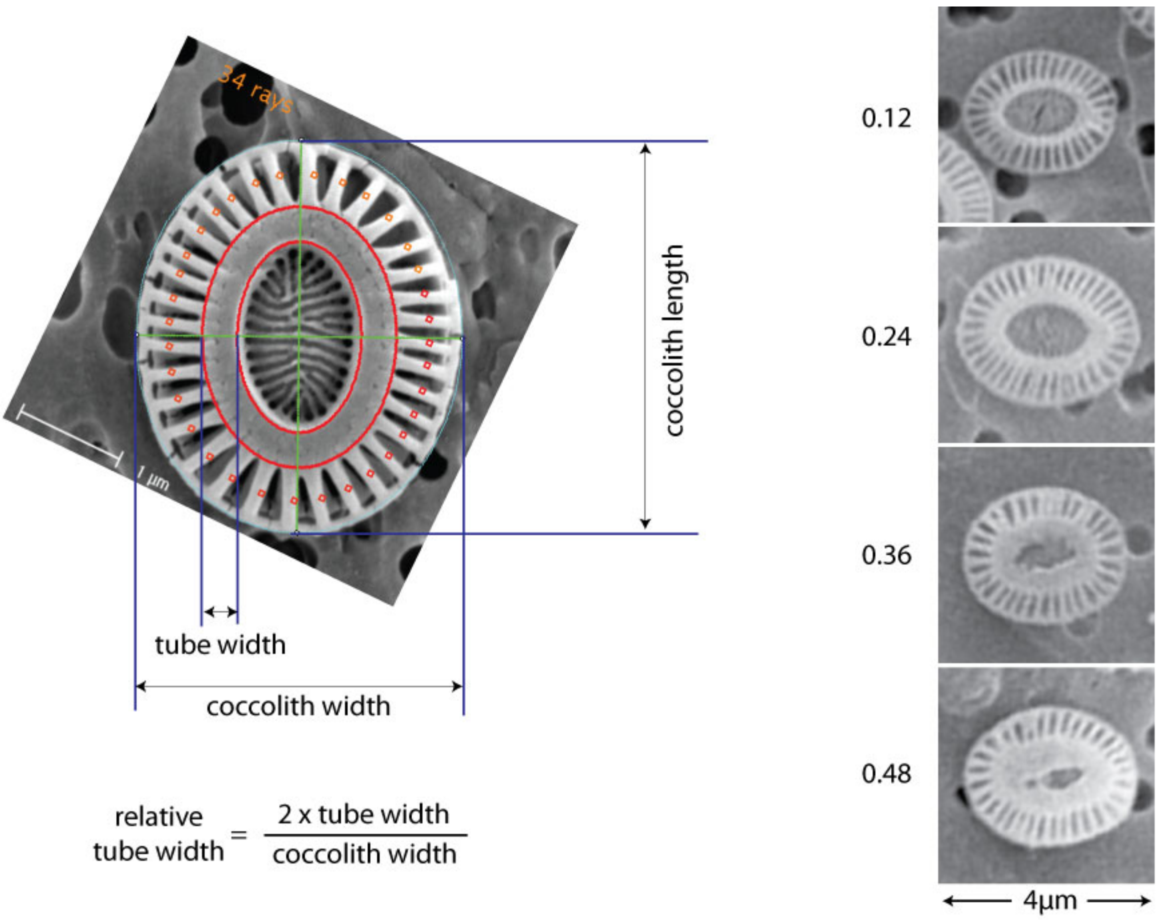

Figure 2. Morphometric parameters measured. Scanning electron micrograph (SEM) of an *Emiliania huxleyi* coccolith in distal view. Right hand panel - four *E. huxleyi* type A coccolith specimens from this study illustrating variation in relative tube width from 0.12 to 0.48 - this parameter is used here as an index of calcification.

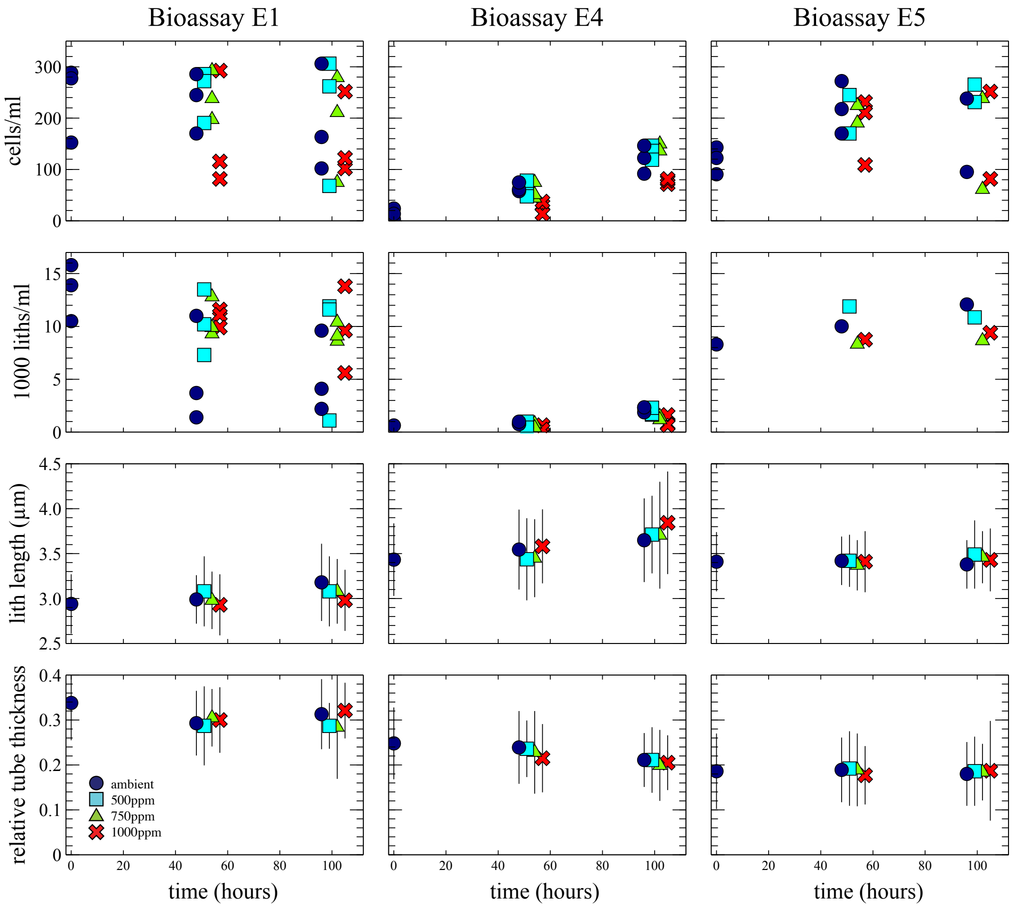

Figure 3. *Emiliania huxleyi* abundances and mophometrics from Bioassays E1, E4 and E5. Top two rows of panels cell abundance and loose coccolith abundance over time. Symbols indicate the culture conditions. There are usually three replicates per time point and culture condition. Sampling was carried out at 48 and 96 hours but samples from separate conditions are moved slightly along the x-axis. Lower two rows of panels coccolith length and relative tube thickness over time. Symbols indicate averages from ca. 60 measurements per sample and vertical bars indicate +/- 1 standard deviation.

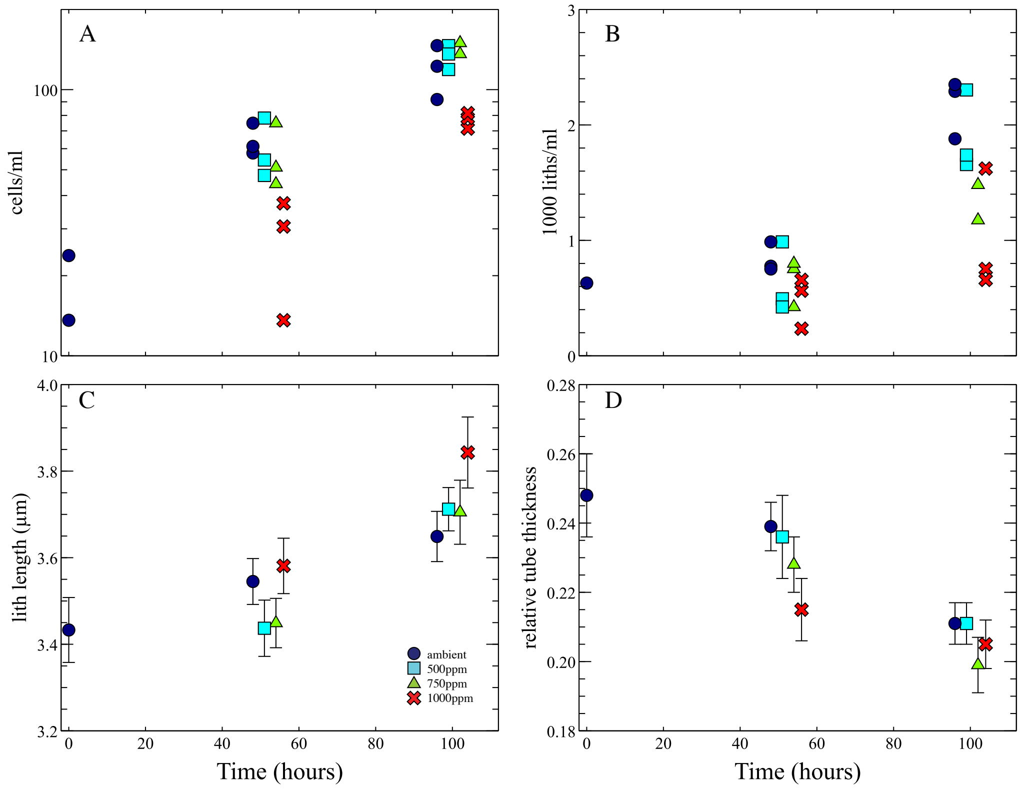

Figure 4. Enlarged plots of *Emiliania huxleyi* abundances (A, B) and morphometrics (C, D) from Bioassay E4, with vertical axes adjusted to emphasise the data. Symbols indicate the culture conditions. Plot A of cell abundance is on a logarithmic scale. On plots C and D the vertical bars are standard errors of the mean, (standard deviation over square root of the sample number), which provides an estimate of possible error in the mean value.

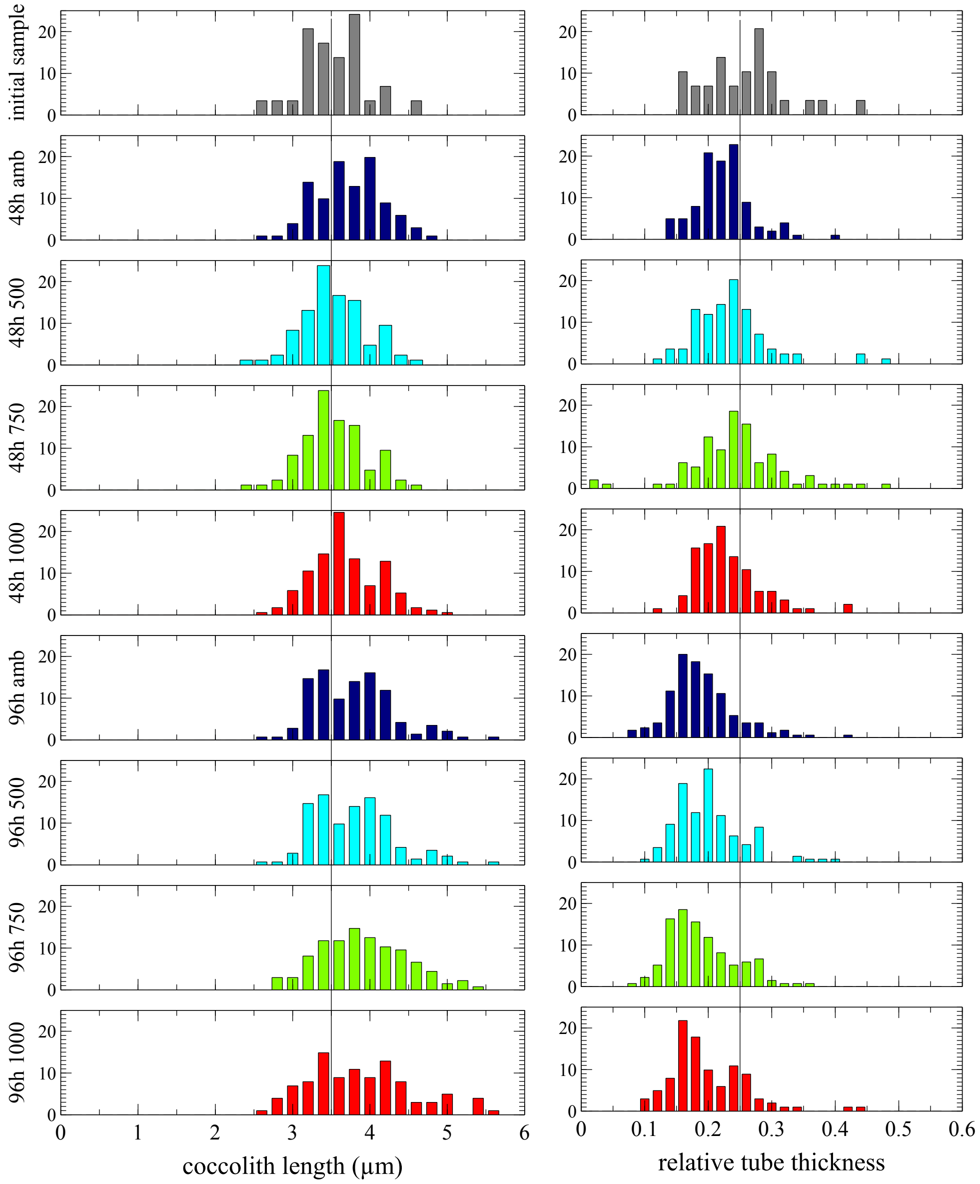

Figure 5. Raw morphometric data from Bioassay E4 plotted as frequency histograms. Vertical scale is percentage abundance. Colour coding is the same as for the symbols on figures 3 and 4. Vertical lines through the data sets represent the mean values in the initial sample; by the end of the experiment (96h) coccolith length had increased significantly and relative tube width decreased significantly but with very little variation between treatments.

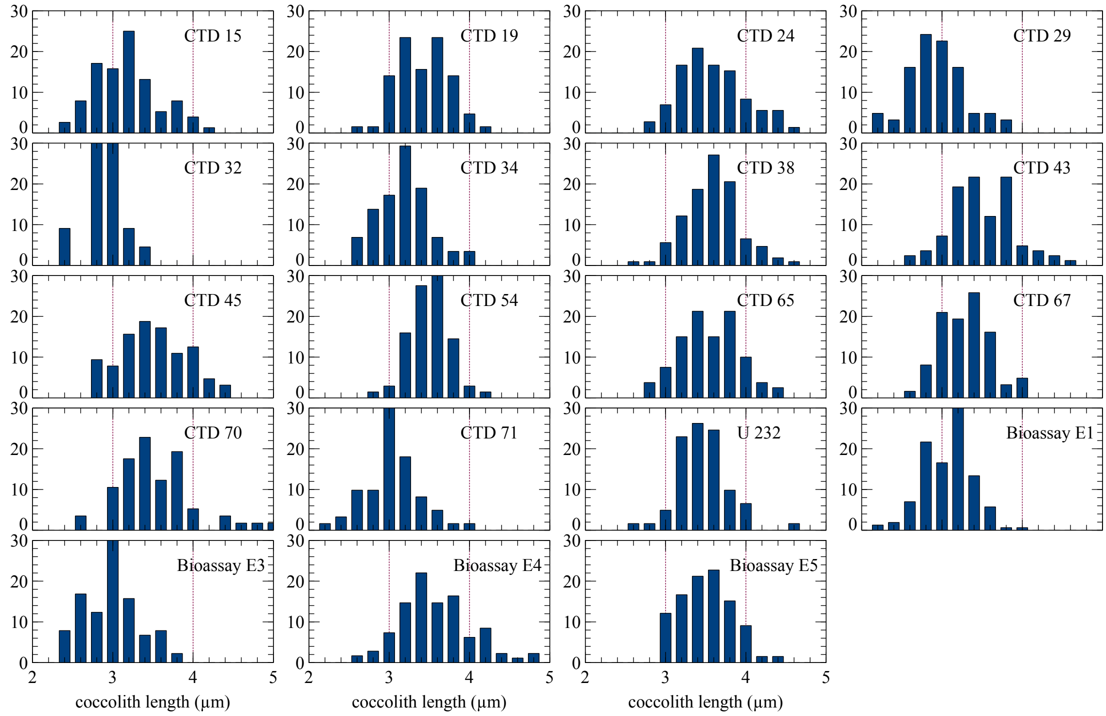

Figure 6 Frequency histograms of coccolith length from near-surface samples from CTD casts and the bioassay initial samples. Vertical axis is percentage abundance, based on measurement of ca. 60 coccoliths per sample. There is clearly significant inter-sample variability between locations.

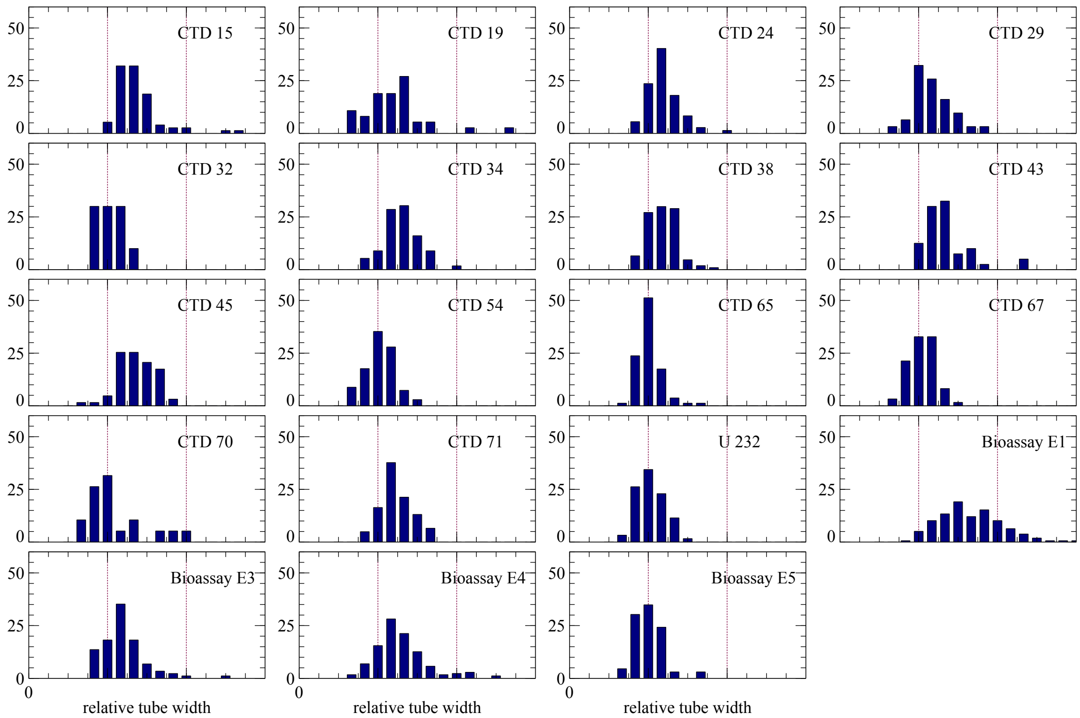

Figure 7. Frequency histograms of relative tube width in near-surface samples from CTD casts and the bioassay initial samples. Vertical axis is percentage abundance, based on measurement of ca. 60 coccoliths per sample. Total variability is fairly low, heavily calcified coccoliths (relative tube width >0.4) are virtually absent, nonetheless there is still considerable variation in degree of calcification between samples.

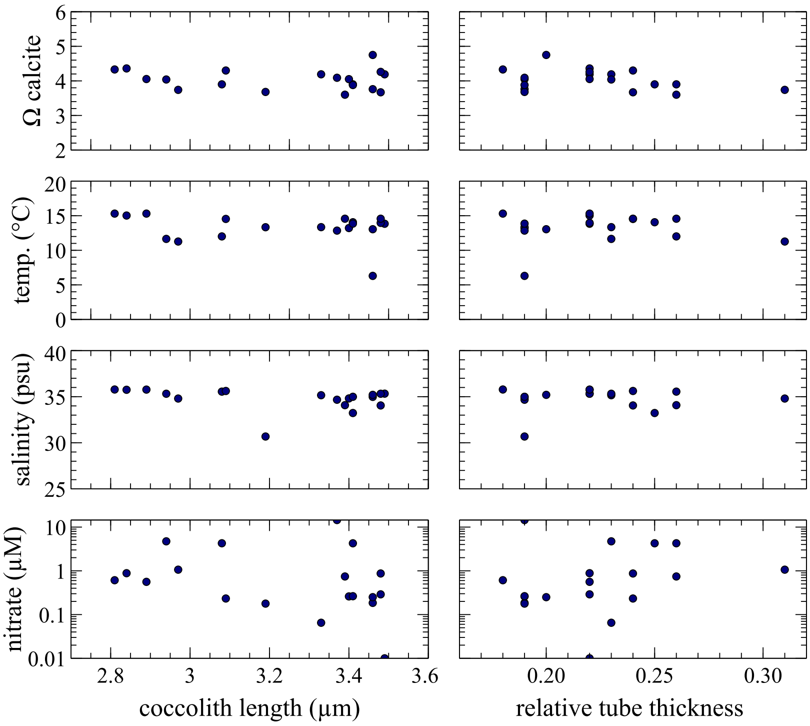

Figure 8. Mean values per sample of coccolith length and relative tube thickness from the 19 CTD and bioassay initial samples, as per the histogram of figs 6 and 7, plotted against carbonate chemistry, temperature, salinity and nutrient concentration. Nitrate and phosphate concentrations are strongly correlated in the sample sets (R= 0.96) so only nitrate (strictly nitrate + nitrite) concentration is plotted, this is shown on a logarithmic axis owing to high variability in absolute values. There is no obvious relationship of the morphological parameters to any of the environmental parameters, and correlation coefficients were below significance levels.

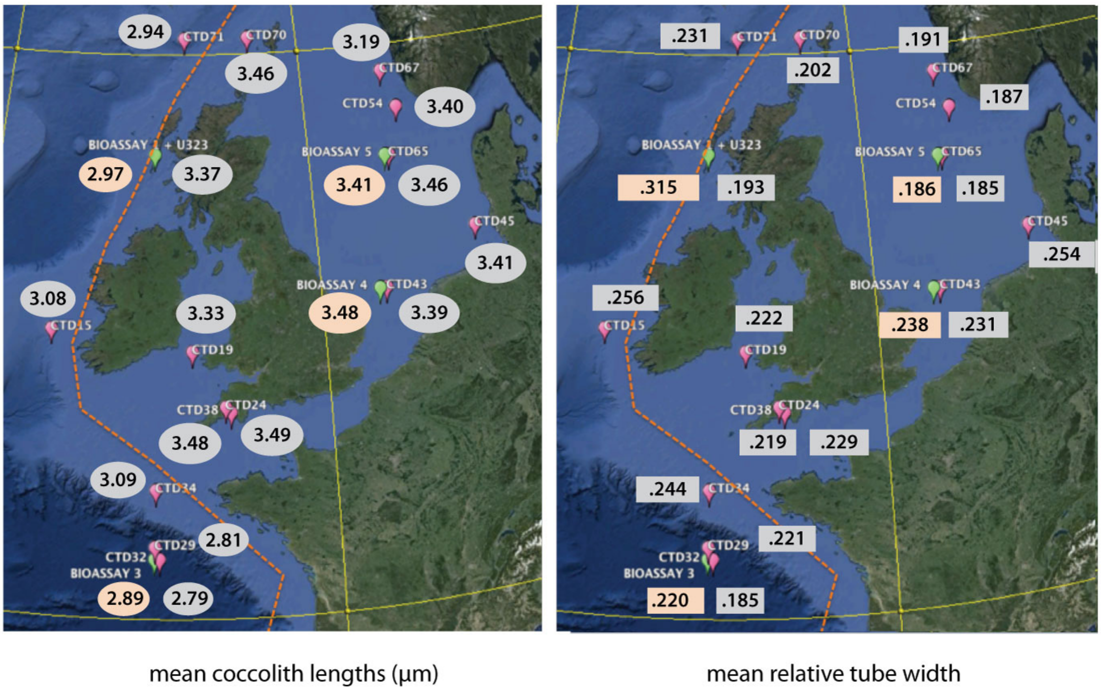

Figure 9 A. Map showing mean coccolith lengths in samples studied in detail - ellipse length and width are scaled to length. B. Map showing mean relative tube widths - rectangle width is scaled to mean relative tube width. This shows contrast in terms of distal shield length between neritic and oceanic samples (as separated by orange dotted line), but there is no obvious pattern to relative tube widths.

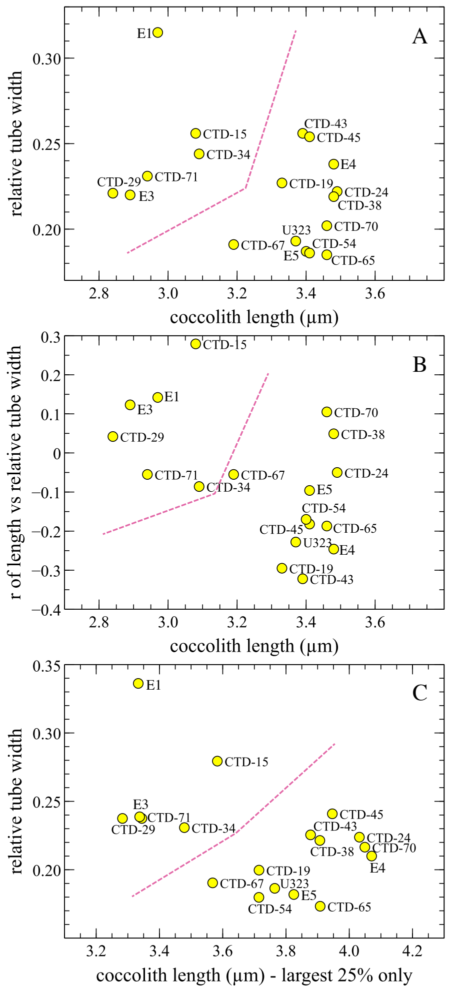

Figure 10. Relationship between relative tube width and coccolith length. A. Relative tube width vs coccolith length, the oceanic samples (to left of pink dotted line) are smaller but poorly separated on relative tube width. B. Correlation coefficient of relative tube width vs coccolith length. C. Mean coccolith length vs relative tube width for the largest 25% of the coccoliths per sample, showing consistent separation of the samples into two sets, corresponding to oceanic vs neritic locations