Geochemistry Geophysics Geosystems G3

AN ELECTRONIC JOURNAL OF THE EARTH SCIENCES

Published by AGU and the Geochemical Society

Technical Brief
Volume 10, Number 3
3 March 2009
Q03001, doi:10.1029/2008GC002228
ISSN: 1525-2027

Isolating coccoliths from sediment for geochemical analysis

Paul R. Halloran
Department of Earth Sciences, University of Oxford, Parks Road, Oxford OX1 3PR, UK

Now at Hadley Centre, Met Office, FitzRoy Road, Exeter EX1 3PB, UK (paul.halloran@metoffice.gov.uk)

Nigel Rust
Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Rosalind E. M. Rickaby
Department of Earth Sciences, University of Oxford, Parks Road, Oxford OX1 3PR, UK

[1] Trace element analysis of open-marine sedimentary carbonates provides a wealth of paleoclimate data. At present, the majority of this data is obtained from foraminifera tests. Complications regarding the variability of conditions experienced by foraminifera throughout test formation and the influence of diagenetic processes on sample chemistry limit the value of foraminifera samples in certain situations. Coccoliths, the calcium carbonate plates produced by coccolithophores, represent a second major pelagic open-marine carbonate source with the potential to provide a wide range of valuable trace element proxy data but which have, until now, been unavailable for analysis of many trace elements because of clay contamination. Here we describe a novel technique, which utilizes fast sorting flow cytometry, to enable the production of clay-free sedimentary coccolith samples.

Components: 4653 words, 4 figures, 1 table.

Keywords: coccolith; coccolithophore; clay; sorting; trace element; geochemistry.

Index Terms: 0473 Biogeosciences: Paleoclimatology and paleoceanography (3344, 4900); 1065 Geochemistry: Major and trace element geochemistry; 1051 Geochemistry: Sedimentary geochemistry.

Received 4 September 2008; **Revised** 14 January 2009; **Accepted** 23 January 2009; **Published** 3 March 2009.

Halloran, P. R., N. Rust, and R. E. M. Rickaby (2009), Isolating coccoliths from sediment for geochemical analysis, *Geochem. Geophys. Geosyst.*, *10*, Q03001, doi:10.1029/2008GC002228.

1. Introduction

[2] Through understanding past climate we can elucidate the processes which must be captured in numerical models to accurately predict future climate change. The surface ocean governs air-sea gas exchange and drives atmospheric circulation, and consequently, reconstructing past surface ocean conditions is critical when exploring the climate of the past. The trace element compositions of the various calcium carbonate structures produced by surface-dwelling pelagic organisms are known to vary with a wide range of environmental parameters, therefore, analysis of the trace element chemistry of biological carbonates recovered from ocean sediment cores can provide valuable paleoclimate data. Presently, the majority of open-ocean geochemical records are produced from analysis of foraminifera tests. Foraminifera account for around half of the total pelagic calcium carbonate production, with the ma-

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jority of the remaining calcium carbonate being produced coccolithophores [*Iglesias-Rodriguez et al.*, 2002; *Schiebel*, 2002]. Despite an individual coccolith typically weighing at least a factor of 10^6 less than an individual foraminifera, the concentration of coccolithophores, each producing multiple coccoliths, commonly exceed 10^6 cells per liter in the open ocean, with the number of coccoliths per gram of sediment potentially orders of magnitude higher.

[3] Two major challenges must be addressed before coccolith geochemistry can begin to provide a similar breadth of paleoenvironmental data to that produced routinely from foraminifera; separation of coccoliths produced by individual species, and measurement of coccolith chemistry without interference from clay-bound trace elements. In recent years, a number of methods have been developed to tackle the first of these problems [e.g., *Minoletti et al.*, 2001, 2009; *Stoll et al.*, 2007a; *Stoll and Ziveri*, 2002], yet none have been proposed to tackle the second; cleaning samples for clay contamination.

[4] Here we describe and demonstrate a novel technique, which utilizes differences in the optical properties of calcite and clay, to facilitate sorting by flow cytometry. The degree of cleaning is assessed both visually and chemically, validating this procedure as an important step toward obtaining sedimentary coccolith trace element geochemistry.

2. Rationale

[5] Foraminiferal test trace element measurements provide a range of valuable paleoclimate information, from calcification temperatures using Mg/Ca ratios, to seawater pH using B/Ca ratios [e.g., *Rickaby and Halloran*, 2005; *Yu and Elderfield*, 2007]. A full understanding of the environmental implications of this data is often prevented by the complexity of the signal. It is becoming evident that planktonic foraminifera contain within them, not just a single piece of geochemical information, but rather a story about the life cycle, depth changes, diet and environmental conditions experienced during their days to weeks of growth [e.g., *Eggins et al.*, 2003]. Conversely, coccoliths, precipitated over minutes to hours, have the potential to provide high-resolution, almost instantaneous, information about the conditions at the time of coccolith formation. Furthermore, the large number (millions) of coccoliths present even in low-mass samples allow the production of significantly more statistically representative data than is possible, using small (one to ten test) foraminifera samples [*Boyle*, 1995], as are increasingly being utilized in high-resolution studies. Combined with the stratigraphic and geographic coverage possible from coccoliths belonging to single species, these factors highlight the value of further investigating the paleoenvironmental potential of coccolithophore calcite.

[6] To improve our reconstructions of nutrient-controlled growth rates from the established coccolith Sr/Ca growth rate proxy [*Rickaby et al.*, 2002; *Stoll and Ziveri*, 2002], methods have been developed to obtain monospecific coccolith samples. *Minoletti et al.* [2001] describe a technique which utilizes repeated ultrasonic suspension and decanting, centrifuging and decanting, and filtering steps to narrow the range of species in a sample, and have now demonstrated an extension of this technique by which samples containing coccoliths produced by a single species can be obtained [*Minoletti et al.*, 2009]. In parallel to this, a method has been developed to hand pick the coccoliths belonging to individual species using a sharpened tungsten needle, allowing the trace element analysis of a group of specimens stimulated with a focused ion beam [*Stoll et al.*, 2007a, 2007b, 2007c]. Samples produced by these two methods are ideal for Sr/Ca analysis because Sr is present in high concentrations in coccoliths, but only in negligible concentrations in clays [*Emiliani*, 1955; *Rickaby et al.*, 2002]. Consequently, to extend these analyses to other potentially valuable trace elements, it is necessary to clean samples for clay contamination. Unfortunately, because the Stoke's Law parameters (size range, density, and broad morphology) of coccoliths (diameter $\approx$ 1–15 μm, density = 2.7 μm) overlap with those of clay grains (diameter $\approx$ 0.02–8 μm, density $\approx$ 2.6–2.9 μm [*Terzaghi et al.*, 1996]), a high degree of separation by sieving or settling is not possible. A novel approach to the problem of coccolith-clay sorting, which does not rely on the physical properties of the particles, is therefore required.

[7] Many microseparation techniques exist within the physical and biological sciences. Of these, flow cytometry is perhaps the most flexible, and the function for which it is designed, cell sorting, most similar in nature to the problem of coccolith-clay separation. Flow cytometry is a technique well established in the biomedical sciences, in which a suspension of micron-sized particles (typically living cells) is hydrodynamically focused into a narrow linear stream by a sheath of flowing saline liquid, to allow individual sequential particle examination,

and subsequently sorting at high speed, as will be described. The flow cytometer sheath fluid focuses the sample stream through a laser beam such that the particles held in suspension intersect the beam one at a time. Opposite the laser lies a series of charged couple devices or photo multiplier tubes, organized to detect the degree of radial scatter of light incident on the particle in a number of wavelength bands. In addition to the light sensors facing the laser and collecting forward scattered light, lie a set of detectors measuring side scattered light (perpendicular to the laser beam). The signals produced by the detectors are sent to a computer where they are queried against a set of predefined criteria. If the criteria are found to be satisfied, a sort occurs. To facilitate the sort, the nozzle of the chamber from which the fluid stream exits, is vibrated at a high frequency (Hz to kHz), causing the stream to break into individual droplets. If a droplet is known to contain a particle fitting the selection criteria, a charge (positive or negative) is passed to the droplet as it separates from the nozzle, allowing it to be directed by charged plates into one of a number of collection vessels.

3. Flow Cytometry: Method

[8] Separating coccoliths from clays using flow cytometry requires that either the two particle types physically scatter the incoming light in a characteristic and contrasting way, or that the particle types can be distinguished by their nonmorphologically mediated optical characteristics. At present, flow cytometers typically distinguish particles based either on the intensity and angle of light scatter from the particle, or the wavelength and intensity of fluorescence emitted from a particle. We found that light scatter alone would not allow distinction between coccolith and clay particles. Subsequently it was necessary to utilize diagnostic optical characteristics to which the flow cytometer could be made sensitive.

[9] Two optical characteristics with the potential to allow distinction between these particle types are fluorescence and birefringence. All natural calcite is fluorescent to some degree in the red-wavelength band when stimulated with an UV light source [*Habermann et al.*, 1998]. To test whether this fluorescence could be used as a diagnostic property, measurements were made to assess the natural fluorescence of coccolith calcite and clay across a range of wavelengths, which indicate that although both clays and coccoliths exhibit fluorescence in the red wavelength band, the shapes of the fluorescence intensity curves contrast (Figure 1). This can be exploited by using a dichroic mirror to split the light with wavelengths above and below 580 nm, to allow analysis of the relative intensity of these two signals and therefore particle distinction. Theoretically, coccoliths will show a lower (<580 nm)/(>580 nm) light intensity ratio than clay grains, when stimulated by UV light source. The data presented in Figure 1 were obtained from a particle suspension held in a fluorometer. Because fluorometer analysis is highly dependent on the concentration of the sample in suspension, and the measurement of fluorescence in flow cytometry is dependent on the size of particle examined and the particle's orientation with respect to the incident laser beam, it was decided to use the relative fluorescence in different wavelength bands, rather than absolute fluorescence intensities when designing the sorting procedure.

[10] The second criteria we have chosen to allow rigorous particle distinction is birefringence. Assuming an equal mineral thickness, the retardation of the slow ray in calcite is almost two orders of magnitude higher than that occurring in kaolinite or chlorite clay [*Deer et al.*, 1992]. To utilize the difference in birefringent properties when examining samples using a flow cytometer, a laser beam focused through the samples stream can be split into two separate light paths, and polarizing filters used to detect the degree of wave rotation. The ratio between the light intensities recorded by the light detectors inline with the two polarizing filters gives a first-order indication of the degree of birefringence of the particles. The low birefringence of clay will result in very limited rotation of the incident ray orientation, so both detectors will measure similar intensities, whereas the high birefringence of calcite will cause significant rotation of the incident beam and the two light detectors will measure very different intensities.

[11] To confirm that the theoretically described criteria allow discrimination between coccoliths and clay grains in the experimental environment three different samples were tested: (1) pure cultured coccoliths (*Coccolithus pelagicus* cleaned for organic material by heating for 1 h in a 50:50 solution of H_2O_2 and NaOH, then leaving to cool in this solution overnight before centrifuging for 10 min at 6000 r.p.m., removing supernatant, then rinsing three times in buffered H_2O, to remove residual H_2O_2 and prevent contamination of the flow cytometer); (2) pure illite (soaked in HNO_3 to ensure that no $CaCO_3$ is present); and (3) organically cleaned (as above) marine sediment (from the North Atlantic

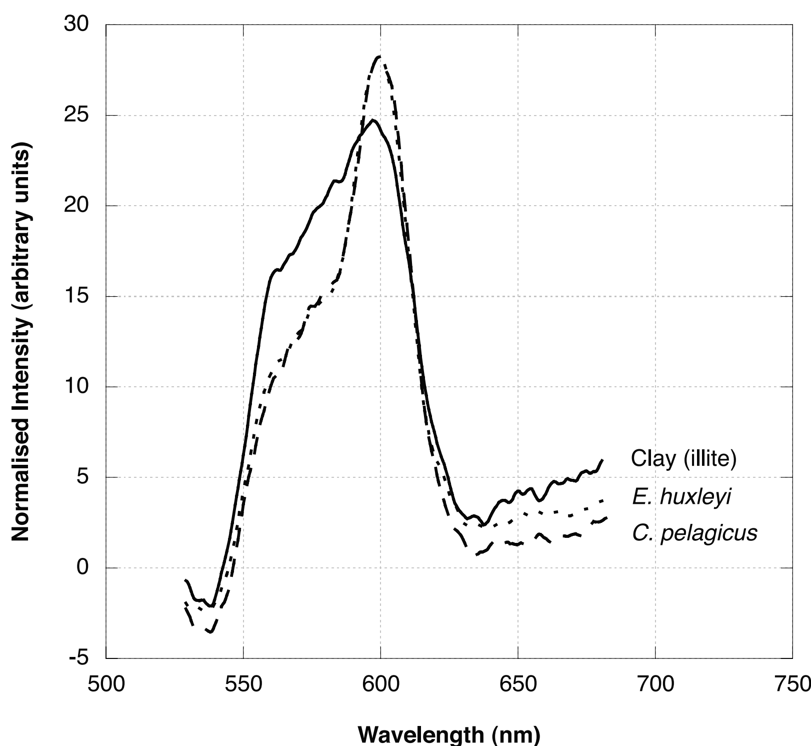

Figure 1. Fluorescence of clay (solid line), *Emiliania huxleyi* (fine-dashed line), and *Coccolithus pelagicus* (long-dashed line) between 525 and 675 nm when stimulated with a 400 nm light source. Measurements were made using a fluorimeter, and therefore fluorescence intensity is a function of the particle fluorescence and the particle concentration. For this reason the intensity has been normalized to a mean value.

core MD04-2829CQ). Samples were suspended in buffered H_2O and filtered through an 8 μm laser etched polycarbonate membrane using bubble agitation to exclude noncoccolith $CaCO_3$. The <8 μm fraction was then processed using a Cytomation MoFlow flow cytometer, with both a 488 nm and an ultra violate laser (Coherent I 90). Within the flow cytometer, forward scatter from the 488 nm light source was both detected on a photodiode, and split through two orthogonal polarizing filters before detection on photomultiplier tubes. Sidescatter from the 488 nm laser was recorded, and orthogonal light from the UV laser examined for fluorescence. Fluorescence was scrutinized by splitting the side scatter signal at a 580 nm long-pass dichroic mirror, and detecting the >580 nm fluorescence, and 565 ± 20 nm fluorescence on two separate photomultipliers. The pulse widths of signals were tightly gated to eliminate the collection of multiple-particle containing droplets.

[12] Using the configuration described the birefringence and fluorescence in the cultured coccolith, clay, and marine sediment samples was examined. Figure 2 presents the light intensities recorded by the flow cytometer at the detectors inline with the two polarizing filters, for a clay and a cultured coccolith sample (each plotted point represents measurements made on a single particle). As described theoretically, the low birefringence of the clay particles results in an approximately equal light intensity measured by the detectors following the two polarizing filters, whereas the coccolith sample produces a range of light intensity ratios, from approximately equal, to strongly skewed values. On the basis of these observations, data pertaining to particles falling in the region above the black line in Figure 2 are considered to represent $CaCO_3$ particles.

[13] Upon satisfying the birefringence criteria, particle fluorescence is examined. The main section of Figure 3 shows the measured fluorescence intensities of these particles. We proposed from theory that the region with higher fluorescence >580 nm relative to fluorescence < 580 nm should be the region containing the most $CaCO_3$ particles. However, when optically examining samples sorted from all regions in fluorescence-fluorescence space (i.e., with a variety of >580: <580 nm ratios), coccoliths were observed. Chemical analyses were therefore made on samples systematically sorted

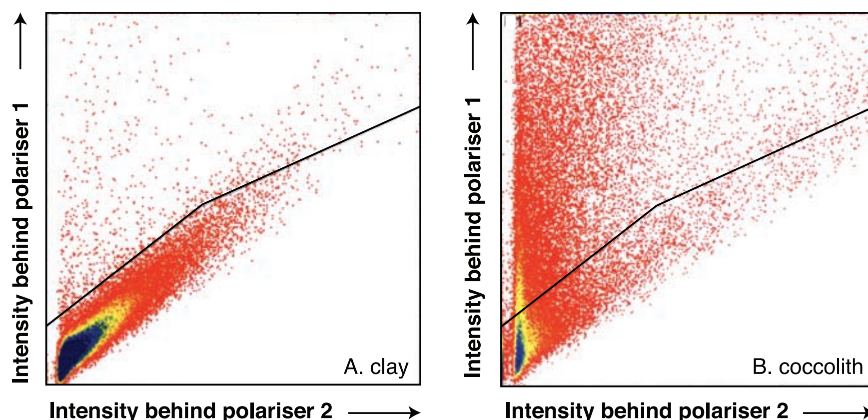

Figure 2. Flow cytometry intensity plots representing the degree of birefringence produced by each (a) clay and (b) cultured *C. pelagicus* particle. The *x* and *y* axes record the intensity of light passing through the polarizing filters arranged at contrasting angles. The contrast between Figures 2a and 2b represents the different birefringence of the clay and $CaCO_3$ particles and therefore can be used as a separation criteria. Particles falling below the black line are therefore discarded. It should be noted that this selection criteria results in a systematic loss of coccoliths, the orientation (at the time of examination) or morphology of which results in a "clay-like" birefringence signal.

from regions covering a range of fluorescence ratios, to allow quantitative examination of clay contamination in these different regions. The region highlighted in gray in Figure 3a was shown to contain the lowest Al/Ca and Fe/Ca ratios (Figure 3b) (sample Al/Ca and Fe/Ca are considered to be sensitive indicators of the presence of clay contamination [*Barker et al.*, 2003]), and is therefore considered to produce the purest coccolith samples. As predicted from theory, this region describes particles with a low <580/>580 nm wavelength ratio. To further confirm the purity of the produced samples, specimens have been analyzed optically, and using scanning electron microscopy and the results presented in Figure 3c and the Figure S2 in the auxiliary material.[1] A detailed description of the flow cytometry setup is provided in the auxiliary material.

4. Testing the Geochemistry of Clay-Sorted and Unsorted Samples

[14] Careful validation of this technique has been carried out by optical and geochemical analysis of unsorted and sorted samples from the North Atlantic core MD04-2829CQ (58°56.93′N, 9°34.30′W). Material from MD04-2829CQ was selected because of its relatively shallow depth (1743 m) with respect to the deep calcite compensation depth (CCD) in the North Atlantic, ensuring good preservation of coccoliths. Furthermore, the inclusion of the last glacial-interglacial transition within this core, ensures that samples will contain a range of clay contributions, allowing the robust calculation of correlations between clay and trace element contamination. To test the success of the described sorting procedure, 15 down-core samples were filtered to <8 μm in buffered H_2O and cleaned for organic contamination, then the samples split in half. Half of each sample was sorted using the described flow cytometry procedure, then all samples were centrifuged at 13,000 r.p.m. and rinsed with quartz-distilled Aristar-grade propanol, transferred to new acid cleaned tubes, centrifuged and rinsed in propanol three further times, then given a final (<10 s) rinse in 18 MΩ H_2O as a weak leach, centrifuged, supernatant removed, and dissolved in 0.5 ml Aristar-grade 2% HNO_3. Twenty μl of each dissolved sample was taken into new acid-cleaned centrifuge tubes, spiked with 20 μl of 500 p.p.b. In and diluted to 1 ml with Aristar-grade 2% HNO_3. Sample Ca concentrations could then be calculated from Ca and In measurements performed on samples and standards using a Thermo-Scientific Element Inductively Coupled Plasma Mass spectrometer (ICP-MS) with a CETAC Aridus nebuliser to increase sensitivity. Samples were then diluted to 10 or 2 ppm Ca depending on their initial concentration (i.e., down to 10 ppm if initially above 10 ppm and down to 2 ppm if initially below 10 ppm), to allow sample Ca concentrations to be matched with standard Ca concentrations and minimize errors associated with mismatched element matrices within the ICP-MS plasma when measuring trace element/Ca ratios. Mg, Sr, Cd, Ba, Mn, Zn, U, Fe and Al/Ca analyses were then performed

[1] Auxiliary materials are available in the HTML. doi:10.1029/2008GC002228.

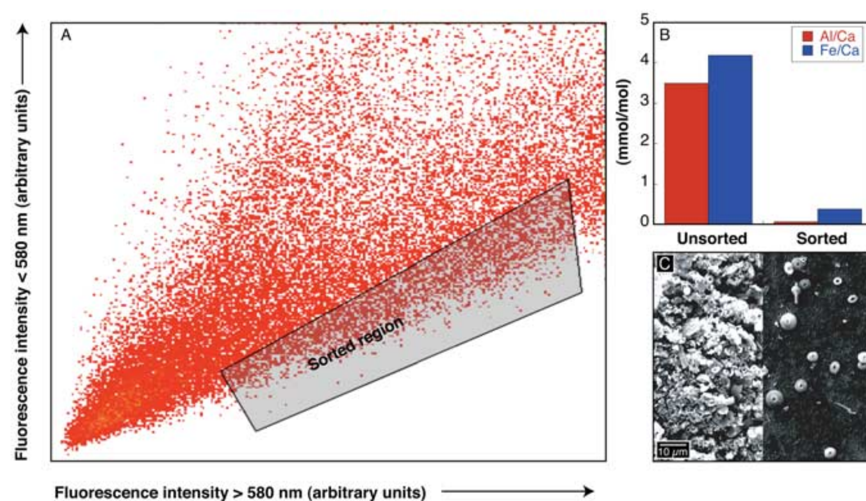

Figure 3. (a) Distribution in (>580 nm fluorescence) – (<580 nm fluorescence) space of organically cleaned marine sediment particles satisfying the birefringence criteria. The grey box represents the region from which sorted samples display the lowest Al/Ca and Fe/Ca. (b) Al/Ca (red) and Fe/Ca (blue) measured in organically cleaned, unsorted, and sorted material from the marine sediment core MD04-2829CQ satisfying the birefringence criteria and fluorescence criteria specified by the grey box in Figure 3a. To illustrate the full range of results, the chemical data presented here are plotted in Figure S2 in the auxiliary material together with the data obtained from all of the other samples sorted from core MD04-2829CQ. (c) Scanning electron microscope images taken from (left) unsorted and (right) sorted samples.

by measuring element intensities in sorted samples, synthetic foraminiferal standards containing Ca, Mg, Sr, Cd, Ba, Mn, Zn, U and multielement standards containing Ca, Fe and Al at 1, 0.1 and 0.1 ppm respectively.

[15] As previously stated, the significance of the correlation between sample Mg/Ca, Mn/Ca and Fe/Ca of Al/Ca chemistry is considered to be indicative of clay contamination [*Barker et al.*, 2003] and therefore comparison of these elemental ratios provides a robust indication of a successful

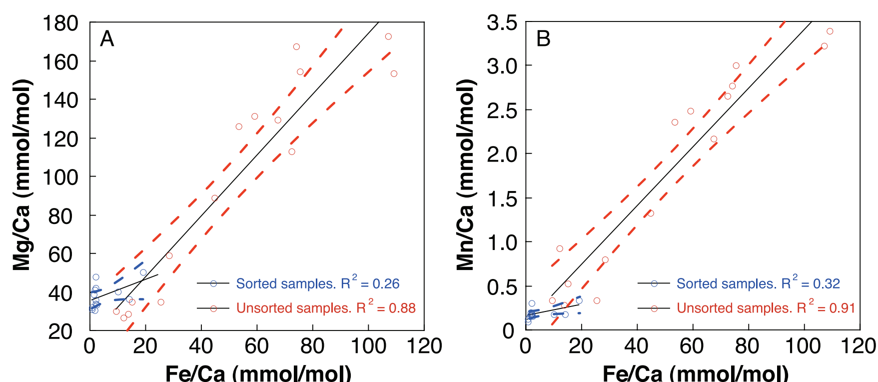

Figure 4. Unsorted <8 μm filtered sedimentary material (red circles) from the top 3 m of core MD04-2829CQ exhibit a strong correlation (R^2 = 0.88 and 0.91, respectively) between (a) Mg/Ca and Fe/Ca and (b) Mn/Ca and Fe/Ca, where the red dashed lines represent the 95% confidence intervals of the least squares linear fit (black line), n = 15 and 15, and the p values = 1.82 e-7 and 4.10 e-8. The strong correlation indicates a significant Mg and Mn contribution from clay contaminants. After sorting, the average Mg/Ca and Mn/Ca drops by up to an order of magnitude, and the significance of the correlations between Mg/Ca and Fe/Ca and Mn/Ca and Fe/Ca decreases, with R^2 = 0.26 and 0.32, n = 13, and p values = 0.075 and 0.044, respectively. Taking p = 0.01 (i.e., a 1 in 100 chance of obtaining the result by chance) as statistical significance, the correlations between Mg/Ca and Fe/Ca and Mn/Ca and Fe/Ca after sorting are not significant. Note that results are only reported for 13 sorted samples. This is due to accidental loss of material during cleaning, highlighted by [Ca] < 0.5 ppm measured in two samples post sorting and cleaning.

Table 1. P Values Testing the Statistical Strength of a Linear Correlation Between Element/Ca Ratios and Fe/Ca and Al/Ca

	Fe/Ca p Value: Unsorted	Fe/Ca p Value: Sorted	Al/Ca p Value: Unsorted	Al/Ca p Value: Sorted
Mg/Ca	1.82×10^{-7}	7.55×10^{-2}	6.95×10^{-4}	6.53×10^{-2}
Sr/Ca	5.37×10^{-5}	4.46×10^{-1}	1.82×10^{-2}	4.16×10^{-1}
Cd/Ca	2.73×10^{-1}	6.46×10^{-1}	7.65×10^{-1}	7.33×10^{-1}
Ba/Ca	2.80×10^{-1}	1.76×10^{-1}	1.84×10^{-1}	2.05×10^{-1}
Mn/Ca	4.10×10^{-8}	4.38×10^{-2}	5.02×10^{-4}	3.45×10^{-2}
Zn/Ca	1.82×10^{-1}	8.60×10^{-1}	8.99×10^{-1}	7.78×10^{-1}
U/Ca	7.63×10^{-2}	1.59×10^{-7}	1.54×10^{-2}	3.91×10^{-7}

(or otherwise) sort. We contrast Mg/Ca and Mn/Ca data correlated with Fe/Ca data from sorted and unsorted samples (Figure 4). It can be seen that prior to sorting (red points), a highly statistically significant correlation exists between Mg/Ca and Fe/Ca and Mg/Ca and Al/Ca (p values = 1.82×10^{-7} and 6.95×10^{-4}), and that this correlation no longer exists postsorting (blue points) (p values = 7.55×10^{-2} and 6.53×10^{-2}) (Table 1), demonstrating a high degree of success from the sorting procedure. A similar breakdown of correlation is observed between Mn/Ca:Fe/Ca and Mn/Ca:Al/Ca (Figure 4b), consistent with the presence of Mn in high concentrations in clay minerals [*Deer et al.*, 1992]. The described correlations break down further if the three samples with Fe/Ca or Al/Ca > 5 mmol/mol are removed (Mg/Ca:Fe/Ca p value = 0.026, Mn/Ca:Fe/Ca p value = 0.14, Mg/Ca:Al/Ca p value = 0.27, Mn/Ca:Al/Ca p value = 0.26). Consequently these three samples are considered to still contain a significant degree of clay contamination. It is therefore recommended that samples with Fe/Ca or Al/Ca < 5 mmol/mol should not be considered clay-clean for such analyses. The results we present here clearly demonstrate the successful application of flow cytometry to the sorting of coccoliths from clay-rich marine sediments.

5. Future Challenges

[16] Using the described flow cytometry protocol, 45 min of sorting will typically allow collection of around 250000 positive sorts (coccoliths). Assuming an average coccolith mass to be 10 pg, this corresponds to 2.5 μg of $CaCO_3$ (compared to $\sim$500 μg of $CaCO_3$ in a typical foraminiferal sample). The chemical cleaning procedures applied to foraminiferal samples are associated with a considerable loss ($\sim$50%) of $CaCO_3$ through dissolution [*Barker et al.*, 2003], and as such requires a large initial sample mass. Expensive, long flow cytometry sorts, or methodological improvements to increase sample yield, are therefore required to produce material upon which the various steps involved in the standard foraminiferal cleaning procedure [*Boyle*, 1981] can be performed. Measurement of samples cleaned to varying degrees will indicate if and where further contamination exists, and allow for the first time, confident down-core measurement of multiple-element/Ca ratios in coccoliths. Given larger samples, it will also be valuable to assess the purity of the sorted material with respect to nonclay contaminants, particularly noncoccolith $CaCO_3$, which may possess similar birefringent and fluorescent properties. In addition to sample cleaning, by exploiting the increased fluorescence and birefringence of larger coccoliths, and the chemical dependence of fluorescence intensity and color, with further work, it may be possible to develop techniques to isolate individual species.

6. Conclusions

[17] The methods described and the results presented in this paper represent a significant step toward unlocking new trace element proxies in coccolith calcite. A technique has been developed and demonstrated which can separate, simply and reliably, sedimentary coccoliths from contaminant clays, and begin to provide accurate coccolith trace element data. Future developments are required to assess the cleanliness of the samples with respect to nonclay contaminants, and apply traditional chemical cleaning steps as required to remove these contaminants. Combined with the newly developed techniques to separate single coccolith species from the sorted samples, studies can then begin to examine and exploit the paleoceanographic and climatic information recorded within coccolithophore calcite.

Acknowledgments

[18] We would like to thank Jorijntje Henderiks and one anonymous reviewer for many careful and considered comments. We would also like to thank the Oxford University Museum of Natural History for providing clay samples and

Mark Wallace from the University of Oxford Chemistry Department for providing equipment and expertise to allow the fluorometry analysis. This work was supported by the Natural Environment Research Council (NERC) grant NER/S/S/2004/12772.

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