



*Supplement of*

## **Early Eocene evolutionary trajectories within the *Toweius* genus: insights from a newly identified species in the equatorial Atlantic**

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## S1. Estimation of coccolith thickness

Coccolith thickness (and derived mass) estimates in this study were determined using the bidirectional circular polarization (BCP) approach as described by Beaufort et al. (2021). This method provides theoretical absolute thickness of calcite particles that is independent of any calibration and yields consistent results across different camera settings (e.g., at different exposure times; Figures S1 and S2). The microscope setup employs two circular polarization states (right- and left-handed orientation; Chroma Technology), a 16-bit black and white digital camera (Hamamatsu ORCA-Flash 4.0, C11440), and a monochromatic green light filter (ZET561/10x, Chroma Technology). Images were acquired with fixed aperture and field settings (set at 10) and 200 ms exposure time. The ratio between right and left circularly polarized images (CP/CPI) is linearly correlated to thickness, allowing for coccolith thickness to be determined following the equation (Beaufort et al., 2021):

$$d = \frac{\lambda}{\pi\Delta n} \arctan\left(\sqrt{\frac{CP}{CPI}}\right) \quad (S1)$$

where  $d$  is the estimated thickness,  $\lambda$  is the wavelength of the incident light (monochromatic green light;  $\lambda = 561$  nm),  $\Delta n$  is the birefringence of calcite ( $\Delta n = 0.172$ ), and CP and CPI represent the light intensity measured with a right and a left circular polarizer, respectively (Figure S1).

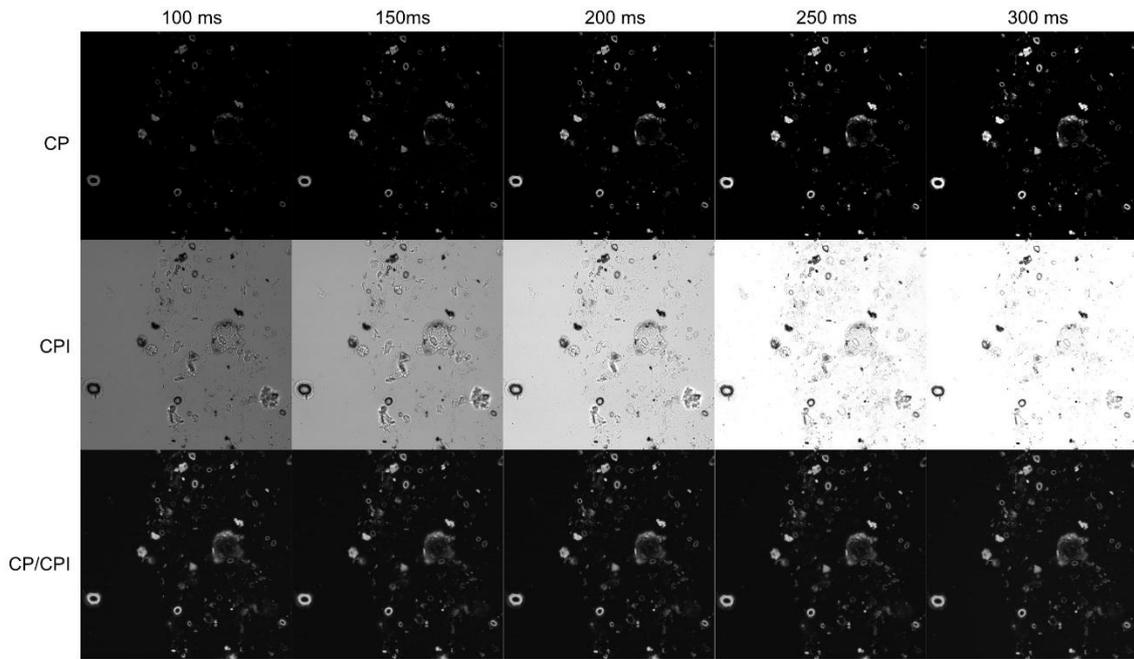
Alternatively, coccolith thickness can be derived directly from a composite image (the CP/CPI ratio; Figure S1) by multiplying the mean gray-level value with the theoretically maximum measurable thickness (1.63  $\mu\text{m}$  for a 16-bit camera and green monochromatic light) and dividing by 256. Based on our microscope setup, we obtain a background (empty slide) thickness of 0.11  $\mu\text{m}$  and a maximum measurable (maximum gray-level) thickness of 1.40  $\mu\text{m}$  which is about 0.86% of the theoretically measurable maximum thickness of 1.63  $\mu\text{m}$  (Figure S2). The reduced measurable thickness range (0.11–1.40  $\mu\text{m}$ ) observed in this study relative to the theoretical range (0–1.63  $\mu\text{m}$ ) is primarily attributed to the limited grayscale achievable with circular polarizers compared to linear polarizers (Beaufort et al., 2021).

## S2. Additional notes on biometric parameter estimation

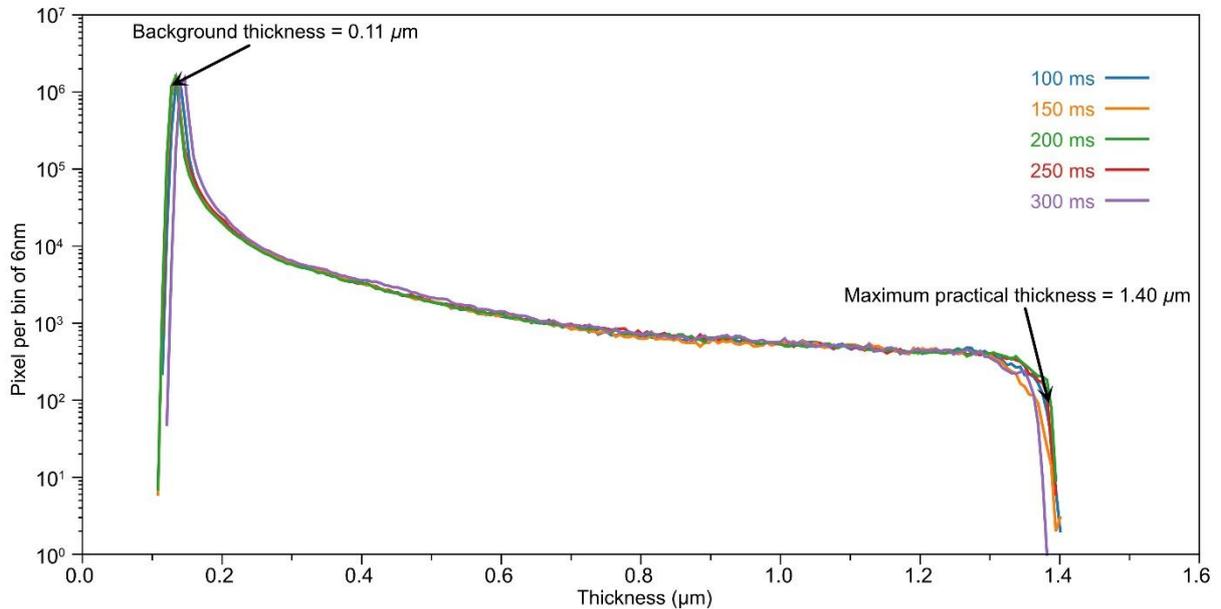
Initial biometric data acquisition with SYRACO was done manually, and prioritized the tight discrimination (segmentation) of coccolith outlines and central area diameter. This was achieved by applying variable gray-level thresholds (with the lowest set at GL=22 for *Toweius* type I and GL=27 for *Toweius* type II), particularly for small, faintly birefringent specimens and for delineating backgrounds in cloudy samples (hereafter called ‘primary dataset’). To validate the relative morphological differences between *Toweius* types I and II, a subsequent round of biometric data acquisition was performed using a fixed threshold value of GL=25 for both groups (hereafter called ‘validation dataset’).

The validation dataset yielded overall lower mean thickness and mass, but larger coccolith size estimates (i.e., length and area) (Figure S3). Offsets between the primary and validation datasets are primarily attributed to the application of different threshold values (Figure S4). Averaging near-background gray-level values led to overall lower estimates of mean thickness and mass. Similarly, the larger coccolith size estimates in the validation dataset are due to the inclusion of near-background pixels in the fitted ellipse around the coccolith margin, which contributes towards increased coccolith length and area measurement. Nevertheless, results from both approaches show a clear separation between *Toweius* types I and II populations

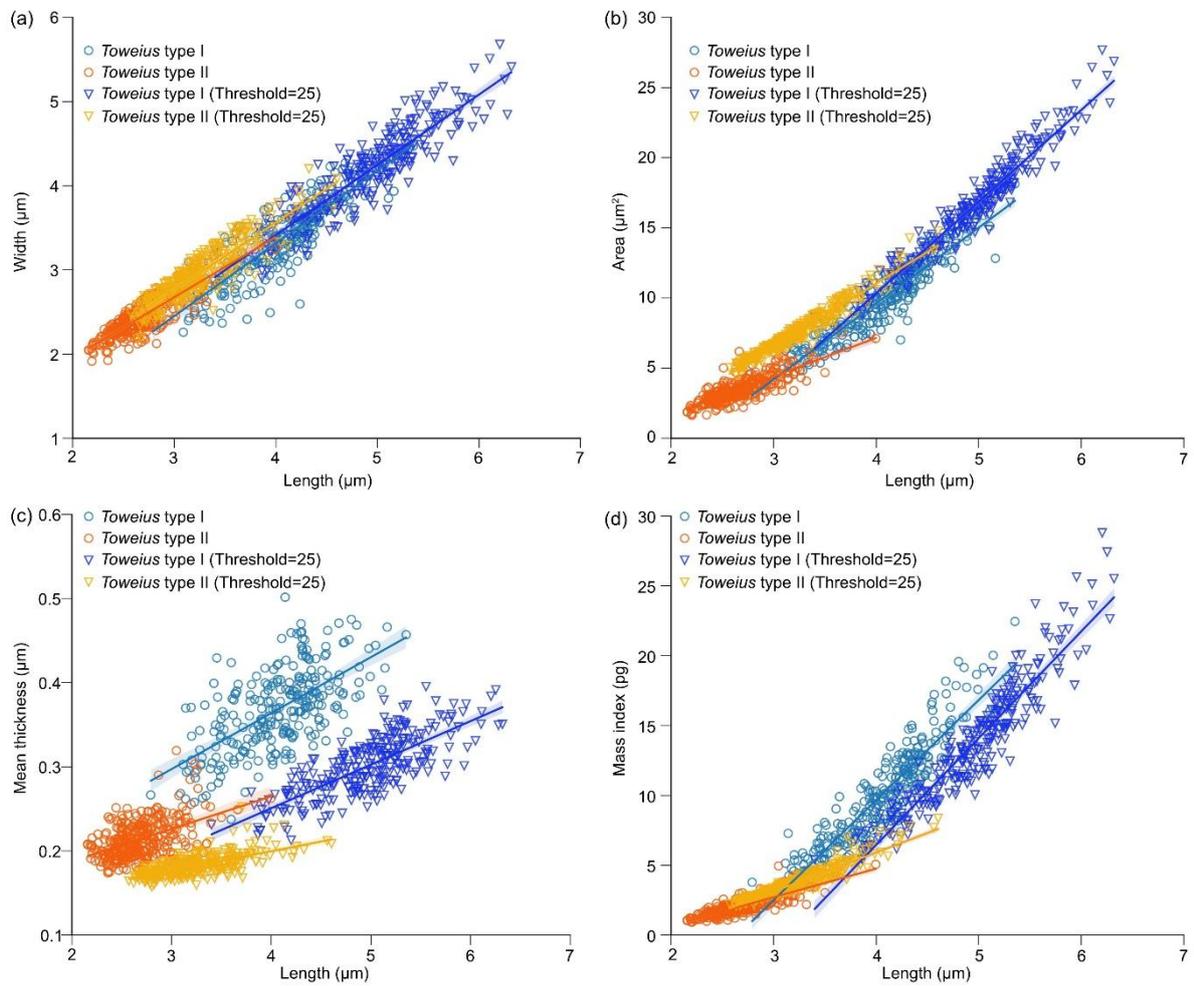
across all measured parameters (Figure S3) and support the analysis and conclusions presented in the main text.



**Figure S1.** Images of the same field of view (FOV) captured at different exposure times (columns: 100, 150, 200, 250, and 300 ms) under right circular polarization (first row; CP; 16-bit images), left circular polarization (second row; inverted circular polarization, CPI; 16-bit images), and as 8-bit composite images (third row; the CP/CPI ratio). The resulting composite images (CP/CPI) show highly similar gray-level across the range of exposure times (compare Figure S2).

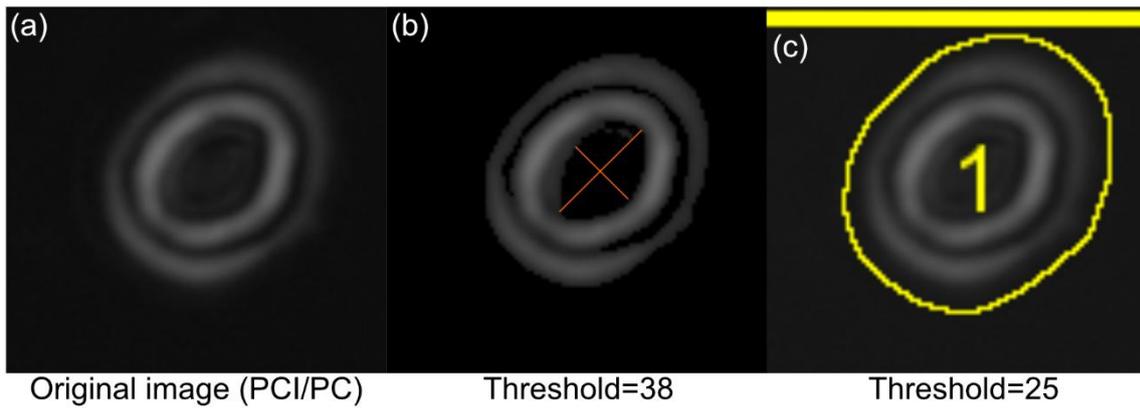


**Figure S2.** Histograms (bins of 6 nm) of calcite thickness estimates derived from CP/CPI composite images at different exposure times for the same field of view shown in Figure S1.

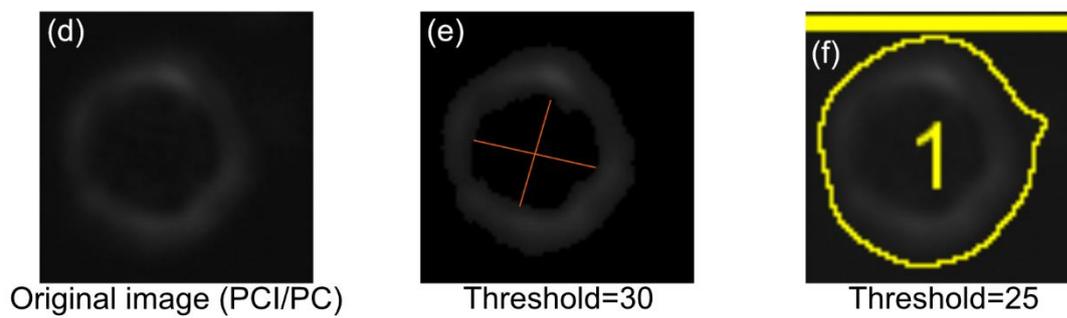


**Figure S3.** Comparison of morphological parameters between the primary (adjusted threshold, GL=22–27) and validation datasets (fixed threshold, GL=25). Cross-plots of *Toweius* type I and II populations showing coccolith length versus (a) coccolith width; (b) area; (c) mean thickness; and (d) mass index. Shading depicts the 95% confidence intervals of linear regression fits for each population and panel ( $R^2 = 0.23\text{--}0.89$ ;  $N = 266$  (type I) and  $N = 333$  (type II)).

*Toweius* type I, SampleID: 1258B\_11R4\_105, ROI: "2"



*Toweius* type II, SampleID: 1258B\_11R4\_105, ROI: "23"



**Figure S4.** Comparison of coccolith segmentation using different thresholds. (a–c) Original and segmented images of *Toweius* type I and (d–f) *Toweius* type II. Central area dimensions in (b) and (e) were obtained through manual segmentation using variable GL thresholds.

### **Dataset S1. Biometry dataset**

This data set is presented in a separate file and contains results of biometric analyses for *Toweius* types I and type II obtained through a manually adjusted SYRACO threshold routine (SI\_Tables S1–S2) and automated ImageJ measurement applied to SYRACO CP/CPI output images using a fixed threshold value set to GL=25 (SI\_Table S3).

Dataset S1 is publicly available at <https://doi.org/10.5281/zenodo.17201433>

### **Table S4**

The table below shows the location of the *Toweius tenuirotondus* holotype (on sample slide 207-1258A-10R-2, 105 cm) as determined on Leica DM6000B and Zeiss Axioscope 40 polarizing light microscopes at the Department of Earth Sciences, Uppsala University. The Leica microscope has a fixed stage that firmly secures a slide along two edges, whereas the sample holder on the rotating Zeiss stage fixes one edge and one corner of a slide, which in our lab introduces slight offsets in the England Finder (EF) coordinates, particularly at high magnification. EF and stage coordinates were therefore identified on both microscopes for reference and easy reproducibility.

<b>Location</b>	<b>Leica (fixed stage)</b>	<b>Zeiss (rotating stage)</b>
England Finder	J42-4 (bordering K42-2)	J43-3
Stage coordinates (mm)	X: 36.23, Y: 12.90	X:34.40, Y: 69.20