X-ray nanotomography of coccolithophores reveals that coccolith mass and segment number correlate with grid size

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Coccolithophores of the Noëlaerhabdaceae family are covered by imbricated coccoliths, each composed of multiple calcite crystals radially distributed around the periphery of a grid. The factors that determine coccolith size remain obscure. Here, we used synchrotron-based three-dimensional Coherent X-ray Diffraction Imaging to study coccoliths of 7 species of Gephyrocapsa, Emiliania and Reticulofenestra with a resolution close to 30 nm. Segmentation of 45 coccoliths revealed remarkable size, mass and segment number variations, even within single coccospheres. In particular, we observed that coccolith mass correlates with grid perimeter which scales linearly with crystal number. Our results indirectly support the idea that coccolith mass is determined in the coccolith vesicle by the size of the organic base plate scale (OBPS) around which R-unit nucleation occurs every 110–120 nm. The curvation of coccoliths allows inference of a positive correlation between cell nucleus, OBPS and coccolith sizes.
Coccolithophores are unicellular marine planktonic algae that have significantly impacted global biogeochemical cycles since their appearance around 220 million years ago, both via their ability to produce organic carbon (photosynthesis) and inorganic carbon (calcification). Coccolithophores produce calcareous scales (coccoliths) inside the cell that are subsequently extruded to form an exoskeleton, termed a coccosphere. Despite their minute (micrometer-scale) size, coccoliths are biogeochemically relevant because coccolithophores are one of the most abundant phytoplankton groups in the marine environment, to such an extent that these unicellular algae are considered being the main extant calcifying organisms with a coccolith production of ~10^{26} year^{-1}.

Therefore understanding how environmental factors influence the degree of calcification of coccoliths is of significant interest. In particular the main current focus concerns the potential impact of ocean acidification. At present, the ocean absorbs about one-third of atmospheric CO₂ emissions, resulting in a shift in seawater carbonate chemistry: while dissolved CO₂ and HCO₃⁻ concentrations increase, pH, CO₃²⁻ concentration and calcium carbonate saturation states (Ω) decrease. This global ocean change affects a variety of marine life forms. In particular, ocean acidification has been reported to decrease the degree of biogenic calcification of corals, echinoderms, and foraminifers. However, despite their crucial role in ocean, the response of coccolithophore calcification to ocean acidification is far from being well understood. One of the reasons for this is the technical difficulty of measuring the mass of individual coccoliths which weighs <400 pg, with coccoliths produced by members of the most widespread extant coccolithophore family, the Noë-laerhabdaceae (including the genera *Gephyrocapsa*, *Emiliania*, and *Reticulofenestra*), being particularly light (i.e., mass ranging from 1 to 30 pg).

Average coccolith and coccosphere mass can be obtained in bulk samples from measurements of total calcite mass and total cell concentration, but measuring the mass of individual coccoliths is more challenging. Mass can be estimated from in-plane and out-of-plane 2D images or from resonance frequency difference as demonstrated for a quite large (~12 µm) coccolith of *Coccolithus pelagicus*. However, the only technique to date able to determine the mass of large quantities of individual coccoliths is polarized light microscopy (PLM) which deduces the in-plane thickness of individual coccoliths from the brightness observed in circular or cross polarization.

Three-dimensional X-ray coherent diffraction imaging (3D-CXDI) is a novel synchrotron technique based on Fourier transformation of a numerically-phased coherent scattering pattern oversampled in the far-field which provides the 3D electron density distribution of isolated objects. The spatial resolution of 3D-CXDI is currently <100 nm, i.e., intermediate between optical and electron microscopy, hence the technique is well suited to image coccospheres of 1–7 µm size and to determine the mass of individual coccoliths. Moreover, 3D-CXDI has the key advantage compared to PLM of being independent of the c-axis orientation of calcite crystals. In addition, 3D-CXDI can access not only the thickness of coccoliths but all morphological features of these calcareous plates even within a single coccosphere.

The mass of coccoliths varies considerably between and within species. Amongst the main environmental variables, carbonate chemistry, alkalinity, and salinity, as well as phosphate level and trace metal concentration of seawater may affect the calcification of coccoliths. However, during their formation coccoliths are not in direct contact with seawater but are rather formed inside the cell, generally one at a time, in a distinct compartment called the coccolith vesicle (CV). The proximal side of the CV is closely apposed to the nuclear membrane and the distal side is intimately associated with a reticular body (RB) in *E. huxleyi* and *G. oceanica* and probably also in *R. parvula*. Coccolithogenesis within the CV begins with the formation of a protococcolith ring at the periphery of an underlying organic base-plane scale (OBPS) and only afterwards the OBPS stabilizes and takes on a form similar to that of the peripheral grid, which is constructed by the intersection between the tube and the grid (called also “central area”). By assuming that the peripheral grid perimeter is an ellipse, the measurement of the major axis a₆ and the minor axis b₆ of the grid allows determination of p from p = πr(√(a₆² + b₆²) / 2 - (a₆ - b₆)²) / 8. By using X-ray tomography to image coccolithophores in three dimensions, our work highlights that the mass m and the segment number n of noë-laerhabdacean coccoliths correlate with grid perimeter p according to m ∼ p³ and n ∼ p. In particular, the proportionality between n and p means that the width of the segments is a constant close to 110–120 nm, whatever the coccolithophore species. As the grid size of mature coccoliths is related to the OBPS size around which nucleation and growth of the coccoliths took place, we propose that the outer perimeter of the OBPS fixes the CaCO₃ nucleation site number (with one site every 110–120 nm) and as a consequence the segment number. We therefore speculate that the large variability in mass and segment number of coccoliths in a single coccosphere may originate from the variability in size of the OBPS during the cell growth/division cycle.

**Results**

**X-ray nanotomography of coccolithophores.** Details about the principle of CXDI, the real resolution and the analysis methods are given in Supplementary Note 1 and Supplementary Figs. 1–5. Comparisons between SEM and 3D-CXDI images validated the accuracy of 3D-CXDI reconstructions as shown for *G. oceanica* in Fig. 1a, b. Additional 3D-CXDI reconstructions for other noë-laerhabdacean species are displayed in Fig. 1c. The coccospheres had external diameters ranging from 4.1 µm for *G. ericsonii* to 7.3 µm for *G. oceanica* and contained between 10 and 24 coccoliths per coccosphere. As reported for other coccolithophore species, the larger the coccosphere, the longer the coccolith major axis (Supplementary Fig. 6). *E. huxleyi* RCC1212 was the only culture strain that had a coccosphere with two layers of coccoliths (Supplementary Fig. 7).

**From coccospheres to coccoliths.** Individual coccoliths were extracted from 3D matrices of the coccospheres using manual segmentation of the 3D array via the Imegal software (Supplementary Fig. 3). Top distal views show the elliptical shape of coccoliths with shield eccentricities ranging from 0.48 to 0.65, whereas central area eccentricities range from 0.66 to 0.81 (Fig. 2a, Supplementary Fig. 8, Supplementary Table 1). These eccentricities may originate from the elliptical shape of the OBPS on/around which the nucleation of the coccolith took place. In addition, cross-sections show that both shields of the coccoliths are out-of-plane inclined by about α ~ 30 ± 5° along the major axis and α ~ 25 ± 5° along the minor axis (Fig. 2b, c). These inclinations likely correlate to the curvature of the nuclear membrane to which the coccolith vesicle is apposed during intracellular formation of the coccolith. Whereas the major axis of the coccolith varies from 2 to 6 µm between species, the constant value of the inclination of the shield α suggests a positive correlation between the size of the cell nucleus and coccolith size. When coccospheres are mechanically deformed on contact with the Si₃N₄ support, some coccoliths may exhibit an increase or a decrease of the out-of-plane inclination which in this case is an...
experimental artifact (Supplementary Fig. 9). The top view thicknesses of the coccoliths are shown in Fig. 2d. They are calculated by multiplying the cubic root of one voxel (i.e., 28.8 or 32.5 nm) by the number of voxels along the direction normal to the plane of the coccolith having an intensity greater than the isovalue. The isovalue is the voxel intensity below which a voxel is considered as empty and above which it is considered as filled by calcium carbonate. For comparison, the top view images of individual coccoliths obtained by PLM are shown in Fig. 2e. Both the X-ray and optical images clearly show that thickness is maximal in the tube region of coccoliths. As the tube region is in direct contact with the central area, this observation highlights in a qualitative way the link between the size of the central area and the mass of the coccoliths.

**Mass of coccoliths on single coccuspheres.** For a quantitative approach, the volume of individual extracted coccoliths was determined from 3D-CXDI and converted to mass by assuming that the density of calcite is 2.71 g/cm³. The uncertainties in volume measurements are detailed in the supplementary information (Supplementary Note 1 and Supplementary Figs. 4 and 5) and the results are summarized in Fig. 3 (black dots). The mass of coccoliths significantly varied in single coccuspheres. For instance, the coccusphere of *G. oceanica* analyzed by 3D-CXDI was composed of 10 coccoliths, the mass of which varied more than threefold ($m = 7.2–23.1$ pg). A similar variability was observed for *E. huxleyi* RCC1212 ($m = 1.5–5.1$ pg). 3D-CXDI data were compared to mass estimates obtained by PLM on a coccolith population originating from a large number of coccuspheres obtained from the same cultures (colored dots in Fig. 3). The coccolith mass variability obtained by PLM was only slightly greater than that obtained by 3D-CXDI. This shows that the distribution of coccolith masses within a coccusphere is indicative of the distribution of masses within a species.

**Role of organic base plate scale (OBPS) size.** To investigate the origin of the high coccolith mass variability, a first positive correlation was found between $m$ and $p$ obeying the formula

$$m = k_p \times p^6$$

(1)
with $m$ the mass of a coccolith in pg, $p$ the peripheral grid perimeter of a coccolith in μm. The coefficients are $k_p = 4.92 \times 10^{-2} \pm 2.17 \times 10^{-2}$ and $\beta = 3.175 \pm 0.251$ (with 95% confidence bounds) (Fig. 4a). $k_p$ may be called the coccolith mass index. Remarkably, all studied species have the same index. Indeed, relation (1) means that the mass of a coccolith $m$ is directly linked to the perimeter $p$ of the grid, i.e., when $p$ is known the mass of the coccolith can be estimated from Eq. (1). As observed in Fig. 2d, this reflects the fact that an important part of the mass of a coccolith is located in the tube region. By looking in more detail, we observed also that the length of the proximal rim $L$ scales linearly with $p$ within species, but with a different constant of proportionality for different species (Fig. 4b). The highest $L$ over $p$ ratio is obtained for *E. huxleyi* RCC1212 followed by *E. huxleyi* RCC1216, whereas *G. oceanica* is characterized by a low $L$ over $p$ ratio. Thus, CaCO$_3$ biomineralization takes place by favoring the length of the rim $L$ and the thickness of the tube $t$ scales more or less linearly with $p$. For the sake of clarity, the parameters $a$, $b$, $a_p$, $b_p$, $\alpha$, $p$, $L$, $t$, and $w$ are schematized in Supplementary Fig. 10. Our findings show also that $L$ and $t$ are positively correlated as reported by O’Dea et al.$^{49}$. It is worth noting that by assuming $\beta = 3$, the parameter $k_p = 6.67 \times 10^{-2}$ ($\pm 0.4 \times 10^{-2}$ with 95% confidence bounds) is obtained. In addition, the peripheral grid perimeter $p$ of the coccoliths scales linearly with the number $n$ of calcite segments in all measured coccoliths (Fig. 4d) as

$$p = w \times n$$  \hspace{1cm} (2)

with $w = 110–120$ nm corresponding to the tube average tangential width of the calcite segments at the periphery of the grid. For instance, the smallest (Fig. 2, *R. parvula*) and biggest (Fig. 2, *G. oceanica*) coccoliths display $n = 29$ and $n = 61$ segments with peripheral grid perimeters of $p = 3.32 \pm 0.18$ μm and $p = 6.92 \pm 0.19$ μm leading to $w = 115 \pm 6$ nm and $w = 113 \pm 3$ nm, respectively. Each segment of the coccolith is composed of two types of calcite crystals, one with the $c$-axis orientation parallel to the coccolith plane and denoted R-unit ("R" for radial) and the other with the $c$-axis perpendicular to the coccolith plane (V-unit; "V" for vertical)$^{43,47}$. During coccolithogenesis, the proto-coccolith ring at the periphery of the OBPS is composed of alternating V-units and R-units$^{43,47}$. However, in *Emiliania*, *Gephyrocapsa*, and *Reticulofenestra*, mature coccoliths are mainly composed of R-units because V-units do not develop.$^{43}$ Thus, our findings lead us to propose that the periphery of the OBPS controls the mineralization site number $n$, with a R-unit nucleation site every $w$ and also a V-unit nucleation site every $w$. As V-units are not developed, the average width $w$ of the R-unit segments appears to be a constant close to 110–120 nm whatever the species. The extraction of individual segments for *R. parvula* is shown in Supplementary Fig. 11. Combining relations (1) and (2) with $w = 112$ nm and $\beta = 3.175$, it appears that

$$m = k_n \times n^\beta$$  \hspace{1cm} (3)

with $m$ in pg and $k_n = 4.73 \times 10^{-5}$ ($\pm 0.28 \times 10^{-5}$ with 95% confidence bounds) (Fig. 4a). $k_n$ may be called the coccolith mass index.
Among the seven explored species, can be explained by the large amount of CaCO3 of the central mass of the coccolith is poor (see the circle in Fig. 4e). This demonstrates in Fig. 5.

The variability in size and mass results from the intra- and inter-species variabilities. For the two methods coccoliths originate from the OBPS and therefore coccolith mass. We therefore speculate that OBPS size could be determined by the cell nucleus size, which varies significantly through the cell growth/division cycle. Muller and coworkers have shown that calcification in E. huxleyi is largely confined to the G1 (gap 1, assimilation) cell cycle phase21 characterized together with the S and G2 phases by a long growth period resulting from high photosynthetic activity. As the nuclear size is determined by the cytoplasmic volume rather than DNA content53, growth of the cell during interphase may be accompanied by growth of the cell nucleus. Hence, the smallest coccoliths may be formed at the onset of the G1 phase after cell division, when the cell, the cell nucleus and the OBPS are small, whereas the largest coccoliths appear at the end of the G1 phase when the cell, the cell nucleus and the OBPS are larger. This assumption is also corroborated by 3D-CXDI results, which show clearly the correlation between the size of coccoliths and the diameter of the cell after artificially removing the mineralized part to take into account only the organic part (Supplementary Fig. 6B). Even though the positive correlation between cell size and coccolith size was already reported within and between species44,45, further analysis using coherent X-ray diffraction imaging at cryogenic temperature on frozen-hydrated cells54,55 or confocal microscopy on stained cells would be needed to check whether the cell nucleus size, which varies significantly through the cell growth/division cycle, could indeed regulate the size of the OBPS and therefore coccolith mass.

**Methods**

**Culture.** The coccolithophore cultures were obtained from the Roscoff Culture Collection (RCC: http://www.roscoff-culture-collection.org/).

**Polarized light microscopy.** In order to measure the mass and size of detached coccoliths from cultures, we used a Leica DMR6000 light microscope with high resolution lens (Leica, HCX PL APO 100/1.47) and condenser (Leica P 1.40 Oel) and Chroma circular polarizers. The images were grabbed by high resolution cameras: a Spot Flex camera from Diagnostic Instrument (14-bit depth, 7.4-µm pixel size) for automatic coccolith selection and an ORCA Flash 4 from Hamamatsu (16-bit depth, 6.8-µm pixel size) for manual coccolith selection. Samples were prepared by settling dried coccolithophore cultures following the protocol described in ref. 28. Detached coccoliths from G. oceanica, G. muellerae, and E. huxleyi were selected automatically by a deep-learning software (SYRACO)56. The images were grabbed by high resolution lens (Leica, HCX PL APO 100/1.47) and Chroma circular polarizers. The images were grabbed by high resolution cameras: a Spot Flex camera from Diagnostic Instrument (14-bit depth, 7.4-µm pixel size) for automatic coccolith selection and an ORCA Flash 4 from Hamamatsu (16-bit depth, 6.8-µm pixel size) for manual coccolith selection. Samples were prepared by settling dried coccolithophore cultures following the protocol described in ref. 28. Detached coccoliths from G. oceanica, G. muellerae, and E. huxleyi were selected automatically by a deep-learning software (SYRACO)56. The small coccoliths of G. ericsonii and R. parvula had very dim edges that were easily merged in the background of the images and the size (length and width) of coccoliths were therefore often underestimated with the above protocol. These species we therefore used a camera performing well in low light intensity (ORCA Flash 4) to grab coccoliths individually and manually. The size and mass measurements of the coccoliths were then performed using the protocol described in ref. 28.

**Scanning electron microscopy.** Scanning electron microscopy (SEM, LEO 1530) was performed on the same coccolithophore cells as those studied by 3D-CXDI by locating the position of each specimen. The images were obtained using the secondary electron mode of detection and with an accelerating tension of 20 kV. Specimens were previously metalized with a ~5 nm thick coat of gold. Note that...
3D-CXDI analysis was performed before SEM observations so that the coccoliths could be analyzed in their 3D nanostructure. The coefficient of determination for the linear fit is 95%. The mass of coccoliths as a function of the number of segments and the mass of coccoliths is shown in Fig. 4. The mass of coccoliths is defined as the product of the volume and the density. The volume of each coccolith was calculated by summing the volumes of the individual grid elements. The density of calcite was taken to be 2.71 g cm$^{-3}$.

CXDI measurements and reconstructions. CXDI is an X-ray imaging technique that is well suited for visualizing at few nanometers resolution the 3D nanostructure of biological specimens. We performed the CXDI measurements at the ESRF beamline ID1030. The X-ray beam produced by an undulator source was monochromated by a Si(111) pseudo-channel cut monochromator. Beryllium compound refractive lenses were employed to focus the beam at the sample position. The detector was placed 5.2 m downstream of the sample. The voxel size of the real space images was estimated to be $32.5 \times 32.5 \times 32.5$ nm$^3$.

The intersection at 0.5 threshold was used to estimate the real resolution (see for instance ref. 31). The PRTFs were calculated for each species, and the real resolution of CXDI measurements was estimated using the PRTF and real resolution of CXDI measurements procedure provided in ref. 30. The real space images were obtained by averaging 20 reconstructions. We used 7.0 and 8.1 keV X-rays so that the real space images have the voxel size of $32.5 \times 32.5 \times 32.5$ nm$^3$ for G. oceanica and $28.8 \times 28.8 \times 28.8$ nm$^3$ for E. huxleyi and G. muellerae. The reconstructed real space images suffer from smoothing density variations due to missing data, strongly resembling the “unconstrained modes” reported by Thibault et al. 41. To remedy these density variations, a simple spatial flattening of the electron density was applied to the reconstructions by subtracting in real space a 3D Gaussian function centered at the mass center.

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After subtracting the 3D Gaussian function, voxels with negative density values were set to zero. Examples of the flattening corrections are shown in Supplementary Fig. 1C. Chimera software was used for the visualization of the surface of the 3D volume obtained by CXDI. For each species, the tomographic slice images before the Gaussian subtraction are available in figShare (https://figshare.com/).

PRTF and real resolution of CXDI measurements. The real resolution of the average images was estimated using the phase retrieval transfer function (PRTF). The intersection at 0.5 threshold was used to estimate the real resolution (see for instance ref. 31). The PRTFs were calculated for each species, and the real resolution of CXDI measurements was estimated using the PRTF and real resolution of CXDI measurements procedure provided in ref. 30. The real space images were obtained by averaging 20 reconstructions. We used 7.0 and 8.1 keV X-rays so that the real space images have the voxel size of $32.5 \times 32.5 \times 32.5$ nm$^3$ for G. oceanica and $28.8 \times 28.8 \times 28.8$ nm$^3$ for E. huxleyi and G. muellerae. The reconstructed real space images suffer from smoothing density variations due to missing data, strongly resembling the “unconstrained modes” reported by Thibault et al. 41. To remedy these density variations, a simple spatial flattening of the electron density was applied to the reconstructions by subtracting in real space a 3D Gaussian function centered at the mass center. After subtracting the 3D Gaussian function, voxels with negative density values were set to zero. Examples of the flattening corrections are shown in Supplementary Fig. 1C. Chimera software was used for the visualization of the surface of the 3D volume obtained by CXDI. For each species, the tomographic slice images before the Gaussian subtraction are available in figShare (https://figshare.com/).
size of the specimen (scattering volume) and the exposure time. The exposure time was chosen in order that the measured 2D diffraction patterns were fully covered by speckles up to the edge of the detector. As a result, the resolution in this study was mainly limited by the detector size.

**Segmentation of coccoliths from coccospheres.** The methodology is composed of several steps illustrated in Supplementary Fig. 3 in the case of *E. huxleyi* P41, a coccosphere having \( C_{90} = 14 \) coccoliths. In the first step, a coccolith is extracted from the whole coccosphere. The extracted coccolith is then subtracted from the coccosphere leading to a new coccosphere matrix containing \( C_{90} - 1 \) coccoliths. The methodology is repeated several times (14 times in the case of *E. huxleyi* P41) to extract all coccoliths. Coccoliths which are broken or not correctly extracted due to their close contact with neighbors were not taken into account in mass determinations.

**Data availability**

Raw data generated by 3D-CXDI (i.e. reconstructed real space images before the subtraction by the 3D Gaussian functions) that support the findings of this study have been deposited at [figShare.com](https://figshare.com) with the identifiers: [https://doi.org/10.6084/m9.figshare.7407143.v1](https://doi.org/10.6084/m9.figshare.7407143.v1) for *Emiliania huxleyi* RCC1212 in Supplementary Fig.7A, [https://doi.org/10.6084/m9.figshare.7454078.v1](https://doi.org/10.6084/m9.figshare.7454078.v1) for *Emiliania huxleyi* RCC1212 in Fig.1c and Supplementary Fig.7B, [https://doi.org/10.6084/m9.figshare.745072.v1](https://doi.org/10.6084/m9.figshare.745072.v1) for *Emiliania huxleyi* P41 in Fig.1c, [https://doi.org/10.6084/m9.figshare.7415300.v1](https://doi.org/10.6084/m9.figshare.7415300.v1) for *Gephyrocapsa ericiensis* RCC4032 in Fig.1c, [https://doi.org/10.6084/m9.figshare.7413491.v1](https://doi.org/10.6084/m9.figshare.7413491.v1) for *Gephyrocapsa muellerae* RCC3370 in Fig.1c, [https://doi.org/10.6084/m9.figshare.7413377.v1](https://doi.org/10.6084/m9.figshare.7413377.v1) for *Reticulofenestra parvula* RCC4036 in Fig.1c. In addition, the data with the dimensions of the coccoliths are collected in Supplementary Table 1.
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Author contributions
A.G. and T.B. had the idea to measure coccolithophores in three dimensions. L.B. had the idea to compare the mass obtained by 3D-CDXI with that obtained by optical microscopy. I.P. provided coccolithophore strains and culturing expertise. L.B. performed circular-polarized light microscopy. Y.C., F.Z., A.G. and T.B. managed the 3D-CDXI measurements at the synchrotron. T.B., I.P., L.B., and B.S. mounted samples on the membranes. Y.C. and T.B. performed reconstructions. A.G. and T.B. undertook analyses to segment coccoliths from the coccospheres. T.B. wrote the paper with feedback from all authors.

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