Title: Extracellular calcification of *Braarudosphaera bigelowii* deduced from electron microscopic observations of cell surface structure and elemental composition of pentaliths

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Abstract

We have performed morphological and crystallographic studies of *B. bigelowii* using various light and electron microscopy techniques. LM study revealed that *B. bigelowii* has a haptonema, and uses it for adhesion to external substrates. TEM study of pentaliths indicates that the well-known lamina substructure is formed in turn of consistently oriented elongated grains of fine-scale calcite having perfectly identical crystallographic orientation. Cytological study shows that the pentaliths of *B. bigelowii* are surrounded by organic structure consists of a pentalith-substrate and thin layers. The pentalith-substrate underlies the proximal surface of the pentaliths and extends between the sides of the individual pentaliths, it also extends between the five segments forming a pentalith. Thin organic layers, which apparently originate from ridges of pentalith-substrate, cover the distal surface of the trapezoidal segments. The close association between the pentalith-substrate, organic layers, and pentaliths lead us to the hypothesis that the *B. bigelowii* calcifies their pentaliths extracellularly, between the
pentalith-substrate and organic layers. Relatively high Mg contents observed from pentaliths supports our hypothesis of extracellular calcification of *B. bigelowii*.

**Key Words**

*Braarudosphaera bigelowii*, calcification, coccolith, coccolithophore, haptonema, haptophyte, nannolith

1. **Introduction**

The family *Braarudosphaeraceae* comprises unicellular coastal phytoplankton and belongs to the Class Prymnesiophyceae, Division Haptophyta (Takano et al., 2006). They are characterized by very distinctive calcareous scales called pentaliths which have pentameral symmetry and are formed of 5 segments with a laminar sub-structure, (e.g. Perch-Nielsen, 1985a, b). The family first appeared in the Early Cretaceous, and *Braarudosphaera bigelowii*, the extant type species of the family, appeared in the Late Cretaceous (e.g. Bown, 1998). Fossils of *B. bigelowii* were usually very rare or absent
68 in marine sediments, however, exceptionally became dominant in specific
time-intervals; in the early Danian immediately after the K/Pg mass extinction that
eliminated ca. 90% of calcareous nannofossils, and in the Oligocene diversity minimum
(e.g. Bown et al., 2004). Thus, *B. bigelowii* is an important species for understanding
the calcareous nannofossil assemblages after the extinction events. Extant *B. bigelowii*
has not been successfully cultured yet, and its ecological preferences are still unclear.
However, progresses of various studies on living *B. bigelowii* as well as that of
members of the class Prymnesiophyceae (Division Haptophyta) that includes the family
Braarudosphaeraceae in the last decade have unveiled the nature of the
Braarudosphaeraceae, as below.

78 The Division Haptophyta is predominantly marine unicellular phytoplankton,
characterized by a thread-like organelle, the haptonema, with a unique microtubular
cytoskeletal structure, which is inserted between two flagella (e.g. Green and
Leadbeater, 1994). The Haptophyta consists of two classes Pavlovophyceae and
Prymnesiophyceae. Members of the Pavlovophyceae have two unequal flagella and a
non-coiling rudimentary haptonema, while the members of the Prymnesiophyceae have
two equal/subequal flagella and a variably developed haptonema (e.g. Edvardsen et al., 2011; Green and Hori, 1994). In the class Prymnesiophyceae, *Chrysochlomulina* possesses coiling haptonema (e.g. Edvardsen et al., 2011), and some *Chrysochromulina* species use the haptonema for adhesion to external substrates (Inouye and Kawachi, 1994) and for handling of food (Kawachi et al., 1991).

Members of the Prymnesiophyceae have organic and/or mineralized scales on their cell surface, and those which produce calcareous scales, are called coccolithophores. (de Vargas et al., 2007) proposed the subclass Calcihaptophycidae for coccolithophores, although it has not yet been confirmed whether the lineages with calcified scales have a monophyletic origin, since the position of the Family Braarudosphaeraceae in the Prymnesiophyceae changes dependant on the analyses (Hagino et al., 2013; Takano et al., 2006).

Calcified scales of coccolithophores are roughly classified into three groups; heterococcolith, holococcolith, and nannolith, based on their morphology. Heterococcoliths are formed of a radial array of complex crystal units, while holococcoliths are formed of numerous minute euhedral crystals. Calcareous scales,
which do not clearly conform to either the heterococcolith or holococcolith pattern are referred to as nannoliths, with the understanding that this is likely to be a very mixed group (Young et al., 1999; Young et al., 2003). Pentaliths of the Braarudosphaeraceae consists of five segments each of which behaves optically as a single crystal unit but which have a distinctive laminar sub-structure (e.g. Bown, 1998). The pentaliths do not conform to either the heterococcolith and holococcolith calcification mode, and so are included in the nannolith group (e.g. Young et al., 1999; Young et al., 2003).

Haptophytes, including coccolithophores, reproduce asexually by binary fission in both the diploid and haploid phases. Morphology of coccolith drastically changes in their life cycle (e.g. Young et al., 2003). Members of the Coccolithales, Syracosphaerales, and Zygodiscales produce heterococcoliths and holococcoliths in their diploid and haploid phases, respectively. Members of the Noëlaerhabdaceae (Isochrysidales) produce heterococcoliths in the diploid phase, but do not calciy in the haploid phases (e.g. Houdan et al., 2004; Young et al., 2003). Morphological change of B. bigelowii accompanying with alternation of life cycle has been partly revealed by molecular phylogenetic study. A sequence from a non-calciying motile cell culture strain, which
was originally identified as *Chrysochromulina parkeae* (Medlin et al., 2008), fell within
the *B. bigelowii* clade in a molecular phylogenetic tree based on 18S rDNA sequences
(Hagino et al., 2013; Thompson et al., 2012). As a result and following cytological
study, *C. parkeae* was determined to be an alternate life-cycle phase of *B. bigelowii*, and
*B. bigelowii* has priority over *C. parkeae* in taxonomy (Hagino et al., 2013).

Previous studies have revealed that the sites for calcification of heterococcoliths and
holococcoliths differ from each other. Calcification of heterococcoliths occurs
intracellularly, in the Golgi cisternae or in a special vacuolar system of the endoplasmic
reticulum directly connected to the nuclear membrane, and subsequently extruded onto
the cell surface (e.g. Drescher et al., 2012; Westbroek et al., 1989). The mechanism of
calcification of holococcoliths has not been determined enough yet, although it is
thought that calcification occurs extracellularly, and the outermost membrane or
‘envelope’ plays some role in calcification (Rowson et al., 1986). The morphology of
pentaliths greatly differs from that of both heterococcoliths and holococcoliths,
therefore, it is difficult to infer the site and mechanism of calcification of pentalith from
its morphology. Indeed the site and mechanism of calcification of pentaliths is an
interesting unsolved question, in particular it is unknown whether the pentaliths form intracellularly and are transported to the cell-surface or whether they form in situ, and so extracellularly.

A possible approach to this is to use coccolith chemistry. (Cros et al., 2013) compared the elemental composition of heterococcoliths and holococcoliths using energy dispersive spectroscopy (EDS) equipped to secondary electron microscope (SEM). They showed that holococcoliths differ from heterococcoliths in their Mg/Ca ratio, and suggested that this is likely caused by the difference in calcification mechanism. At this moment, there is no information on elemental compositions of pentaliths of the Braarudosphaeraceae.

We have not successfully grown *B. bigelowii* in culture yet, and have not observed process of calcification of pentaliths in laboratory. However, we have undertaken SEM and transmission electron microscope (TEM) studies, which reveal a unique cell surface structure on *B. bigelowii* that is likely related to calcification of pentaliths. In this study, we will discuss the formation of pentaliths of *B. bigelowii* based on the cell surface
structure morphology, crystallographic texture and elemental composition of the pentaliths.

1. Introduction ver. 2; started with explanation of the Haptophytes.

The Division Haptophyta is predominantly marine unicellular phytoplankton, characterized by a thread-like organelle, the haptonema, with a unique microtubular cytoskeletal structure, which is inserted between two flagella (e.g. Green and Leadbeater, 1994). The Haptophyta consists of two classes Pavlovophyceae and Prymnesiophyceae. Members of the Pavlovophyceae have two unequal flagella and a non-coiling rudimentary haptonema, while the members of the Prymnesiophyceae have two equal/subequal flagella and a variably developed haptonema (e.g. Edvardsen et al., 2011; Green and Hori, 1994). In the class Prymnesiophyceae, Chrysochlomulina possesses coiling haptonema (e.g. Edvardsen et al., 2011), and some Chrysochromulina species use the haptonema for adhesion to external substrates (Inouye and Kawachi, 1994) and for handling of food (Kawachi et al., 1991).
Members of the Prymnesiophyceae have organic and/or mineralized scales on their cell surface. Some lineages of the Prymnesiophyceae, which produce calcareous scales, are called as coccolithophores collectively. (de Vargas et al., 2007) proposed the subclass Calcihaptophycidae for coccolithophores, although it has not yet been confirmed whether the lineages with calcified scales are monophyletic origin or not, since the position of the Family Braarudosphaeraceae in the Prymnesiophyceae changes dependant on the analyses (Hagino et al., 2013; Takano et al., 2006).

Calcified scales of coccolithophores are roughly classified into three groups; heterococcolith, holococcolith, and nannolith, based on their morphology.

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ratio, and suggested that this is likely caused by the difference in calcification mechanism. The family Braarudosphaeraceae is a unicellular coastal phytoplankton and belongs to the Class Prymnesiophyceae, Division Haptophyta (Takano et al., 2006). They are characterized by five-fold symmetric calcareous scales with laminar structure called pentalith (e.g. Perch-Nielsen, 1985a, b). Pentaliths of the Braarudosphaeraceae consists of five segments each of which behaves optically as a single crystal unit but which have a distinctive laminar sub-structure (e.g. Bown, 1998). The pentaliths do not conform to either the heterococcolith and holococcolith in structure, and so are included in the nannolith group (e.g. Young et al., 1999; Young et al., 2003).

Extant *Braarudosphaera bigelowii*, the type species of the family, have twelve regular pentagonal pentalith each consisting of five trapezoidal segments on their cell surface (Fig. 1). *B. bigelowii* have never been maintained in culture despite many attempts, but its morphological change accompanying with alternation of life cycle has been partly revealed by molecular phylogenetic study. A sequence from a non-calcifying motile cell culture strain, which was originally identified as *Chrysochomulina parkeae* (Medlin et
al., 2008), fell within the *B. bigelowii* clade in a molecular phylogenetic tree based on 18S rDNA sequences (Hagino et al., 2013; Thompson et al., 2012). As a result and following cytological study, *C. parkeae* was determined to be an alternate life-cycle phase of *B. bigelowii*, and *B. bigelowii* has priority over *C. parkeae* in taxonomy (Hagino et al., 2013).

Morphology of pentalith greatly differs from that of heterococcoliths and holococcoliths, therefore, it is difficult to assume the site and mechanism of calcification of pentalith from its morphology. Mechanism of calcification of pentalith is an interesting unsolved question. We have not successfully grown *B. bigelowii* in culture yet, and not observed process of calcification of pentaliths in laboratory. However, we have undertaken SEM and transmission electron microscope (TEM) studies, which reveal a unique cell surface structure on *B. bigelowii* that is likely related to calcification of pentaliths. In this study, we will discuss the formation of pentaliths of *B. bigelowii* based on the cell surface structure morphology, crystallographic texture and elemental composition of the pentaliths.
2. Materials and Methods

2-1. Morphological studies

Sea surface water samples were collected from Tomari Port and offshore Tomari Port, Tottori Prefecture, Japan, on 232 occasions during studies on living coccolithophores from July 2008 through June 2014 (Fig. 1). Detailed information on the samples was given in (Hagino et al., 2015). One or two litre sea-surface water samples were collected using a bucket, prefILTERED through a 50-μm plankton net (Sefar Inc. Din-110), and filtered onto Millipore HAWP04700 and/or Whatman 7060–4710 filters. Twelve filter samples, which were known to contain common Braarudosphaera bigelowii from previous study (Hagino et al., 2015), were selected for morphological studies on pentaliths of B. bigelowii (Table 1). Small pieces of each filter sample were cut out, and fixed onto an SEM stub using double-sided carbon tape. Samples were coated with gold (Sanyu SC701 MKII), and then examined with an SEM (JEOL JSM 7001F).

Three cells of B. bigelowii; US15.2-sc11, Furu-sc2, and Furu-SEM1, which were collected from Usuka and Furue Bays, Nagasaki Prefecture, Japan during previous molecular phylogenetic study (Hagino et al., 2009), were used for light and scanning
electron microscopic observations of *B. bigelowii* in this study (Table 2). Sea surface water samples were concentrated using a plankton net with 5 µm openings. The cells were isolated using a capillary micropipette in an inverted light microscope (Olympus CKX41), and then photographed by a camera (Olympus DP50) equipped to an upright microscope (Olympus BX50). The side length of pentalith of each isolate was measured on LM images to allow classification of morphotypes of *B. bigelowii*. After the LM study, the specimen Furu-SEM1 was fixed with 4% osmium tetroxide for 1 minute, and adhered to poly-L-lysine-coated glass plates, according to the procedure of (Tsutsui et al., 1976). The cell was rinsed with ion-exchanged water for three times, kept in ion-exchanged water for two days, dehydrated in an ethanol series (30, 50, 70, 90, 95 and 100%), and then dried using a critical point dryer (Hitachi HCP-2). The cell was coated with gold (Sanyu SC701 MKII), and was examined with SEM (JEOL JSM 7001F).

The specimen of *B. bigelowii* used for study of the cell surface by TEM was originally collected for studies on molecular phylogeny and morphology *B. bigelowii* (Hagino et al. 2013) from Tomari Port, Tottori, Japan on June 18, 2011 (Table 1). The specimen
was found from the same seawater sample that yielded the specimen examined in Fig. 3 of (Hagino et al., 2013), and the specimens were prepared for TEM observation together. The methods of preparation for TEM study were fully described in (Hagino et al., 2013).

2-2. Crystallographic and elemental analysis of pentaliths.

Two filter samples collected from offshore of Tomari Port prepared with Whatman 7060–4710 filters, which were known to contain sufficient *B. bigelowii* from previous study (Hagino et al., 2015), were selected for the crystallographic and elemental analyses of pentaliths (Table 1). Plankton preserved on surface of filter samples were transferred onto one of two sides of carbon double-sided tapes, and the double-sided tapes with plankton were placed on brass stubs for TEM and SEM-EDS analyses. The samples for crystallographic analyses were coated with gold (Vacuum Device VS-10), and the samples for elemental analyses were coated with platinum (Sanyu SC701 MC), respectively.
A specimen of *B. bigelowii* with a complete exotheca of twelve pentaliths, and with no evidence of secondary dissolution or falling off of outer layers, was selected for crystallographic analysis under a focused-ion beam (FIB) apparatus (Hitachi SMI4050) (Appendix 1a). A thin-foil section of the pentalith for TEM observation was prepared by FIB after tungsten coating to avoid Ga-ion damage (Appendix 1b). The interlayers between proximal and distal layers of pentalith were thinned to be ca 180 nm in thickness (Appendix 1c). The thin-foil section was not parallel to the plane of pentalith of *B. bigelowii* due to technical difficulty. Under TEM observation (JEOL JEM-ARM200F), micro-morphology of the calcite grains consisting a segment of pentalith was examined by bright field transmission electron image and high-angle annular dark field scanning transmission electron image (HAADF-STEM), and their crystallographic orientations were examined by selected-area electron diffraction (SAED).

Elemental compositions of pentaliths of *B. bigelowii*, coccoliths of *G. oceanica, E. huxleyi*, and *T. adriatica* encountered under an SEM (Hitachi SU1510) was examined using energy-dispersive spectrometer (EDS) (Horiba EMAX X-act) attached to the
SEM. X-ray spectra were acquired at an accelerating voltage of 15 kV for 120 seconds for pentaliths of *B. bigelowii* and heterococcoliths of *G. oceanica* and *T. adriatica*, and for 240 seconds for heterococcoliths of *E. huxleyi*, with 3-8 % of dead time. X-ray intensities in counts of Mg-Kα and Ca-Kα lines in respective spectra were analyzed using the software Horiba EMAX 1.0.

3. Results

3-1. Morphological studies of pentaliths

A total of 57 specimens of *B. bigelowii* were photographed from 12 samples from Tomari Port or offshore Tomari in SEM (Table 1). Side length of pentaliths observed in this study ranged from 5.5-8.0µm, which corresponds to the size range of Intermediate form-B of Hagino et al. (2009). The outermost lamina of each intact pentalith has a smooth surface (topmost pentalith of the Fig. 2a). The inner laminae, which were exposed by detachment of distal laminae, always have fine grooves (Figs 2a and 2b). Direction of the grooves is almost perfectly consistent, as indicated by double-headed arrows on Figs. 2a and 2b. The fine grooves appear to run parallel to the
crystallographic $c$-axis of the calcite crystal units as estimated by previous study (Appendix 2, Kameo and Furukawa, 2007).

The intensity of calcification of the different pentaliths covering a single cell was consistent on all the observed cells, but varied between cells even from the same seawater sample. Pentaliths of lightly calcified specimens are often concave (Figs 2c-d).

A pentalith with incomplete layers was found in the sample collected from st. 1 of Tomari Port in June 20, 2010 (Fig. 2e). In this pentalith, calcification mainly occurred around the outline of the pentalith and along the contact surfaces between the segments (= pentalith-substrate, which is defined in the section 3-3). The central part of four of the five segments was hollow. Each segment was composed of multiple incomplete layers (arrow on Fig. 2e). A specimen without any calcareous pentaliths but with pentagonal impressions on its cell surface (Fig. 2f) was found in a sample (st.1, June 21, 2010), which yielded many well-calcified specimens.

3-2. Light microscopic studies of living *B. bigelowii*
Side length of the pentalith of the isolates Furu-SEM1 (Figs 3a-b), US15.2-sc11 (Fig. 3c), and Furu-sc2 (Figs 3d-e) were c.a. 8.1, 6.5, and 5.5 µm, respectively. Hence, specimen Furu-SEM1 belongs to the large form, whilst the specimens US15.2-sc11 and Furu-sc2 belong to the Intermediate form-B of (Hagino et al., 2009) (Table 2).

Light microscopic studies showed that calcified cells of *B. bigelowii* often have a flagellum-like organ (arrows on Fig. 3), which is capable of coiling (arrow on Fig. 3c). *B. bigelowii* often adhered firmly to the surface of slide glass or petridish using this organ (Figs. 3d-e). This behavior of the organ suggests that it is a haptonema. Calcified cells of *B. bigelowii* were non-motile. Two equal flagella were reported from motile non-calcified cells of *B. bigelowii* (= *C. parkeae*) (Green and Leadbeater, 1972), but have never been observed on calcified cells of *B. bigelowii*. In relation to the coccolith formation, numerous calcified *B. bigelowii* cells have been observed during isolation for molecular studies (Hagino et al., 2013; Hagino et al., 2009; Takano et al., 2006) and for attempted culture studies. None of these have ever been observed to possess incomplete pentaliths inside the cells, nor have intracellular pentaliths been recorded in any other study.
3-3. SEM observation of cell surface structure

The specimen Furu-SEM-1 originally had complete 12 pentaliths (Figs 3a-b), however it lost its pentaliths during preservation of the haptonema for SEM observation, and so its cell surface structure was exposed (Fig. 4). SEM observation showed that the sides of the individual pentaliths (white solid arrows on Fig. 4c) and the contact surface between the sides of the trapezoidal segments (black solid arrows on Fig. 4c) are delineated by ridges. This cell surface structure is unlike anything observed on any other coccolithophore, indeed typically coccolithophore cell surfaces are smooth with no trace of the coccoliths. Clearly this distinctive surface is related to the pentaliths and so we will refer to it as the pentalith-substrate.

The ridges on the pentalith-substrate between sides of pentaliths have fine grooves that correspond to laminae forming the pentaliths (dashed black arrow on Fig. 4c). In addition, fine wrinkles occurred on the distal surface of the pentalith-substrate (white dashed double headed arrows on Fig. 4c) with the same orientation as the fine grooves observed on the inner layers of pentaliths in SEM (white solid double-headed arrows on
Figs. 1a and b), and c axis of calcite of the layers (Appendix2, Kameo and Furukawa, 2007). The haptonema emerged from one of the inter-plate ridges of pentalith-substrate (white triangle on Fig. 4b).

3-3. TEM observation of cell structure

Figure 5a shows the general appearance of one of the thin sections obtained from a B. bigelowii cell. The cell is surrounded by thick pentaliths, and the distal surface of pentaliths is covered with a thin black layer (black triangles), that indicates the presence of a thin organic structure covering the pentaliths. The section also shows a spherical body (S. in Fig. 5a) and two chloroplasts (C in Fig. 5a). Figs. 5b and 5c are close up view of Fig 5a, showing details of the organic structure. Relatively thick organic structures were visible at contact surfaces between pentaliths (white solid arrows on Figs. 5b-c) as well as at contact surfaces between trapezoidal segments consisting a coccolith (a black solid arrow on Fig. 5b). These structures correspond to the ridges of pentalith-substrate observed in SEM (black and white arrows on Fig. 4). The organic structure covering distal surface of pentaliths (black triangles on Fig. 5a) consists of
multiple very thin layers (black triangles on Fig. 5c), and those layers were connected to the ridges of pentalith-substrate (a white solid arrow on Fig. 5c). Thus, the trapezoidal segments forming a pentalith are surrounded by an organic structure consisting of the pentalith-substrate and thin distal organic layers.

3-4. Crystallographic study of pentaliths

TEM studies of the thin-foil section prepared from middle part of layers showed (Fig. 6a) that the layers consist of many elongate calcite grains, which were consistently aligned (Figs 6b-c). The directions of elongation were essentially the same as those of the fine grooves observed on pentaliths in SEM (double-headed white arrows on Figs 1a-b) (Plate 2-6 of (Hagino et al., 2009), and of the wrinkles observed on the pentalith-substrate (dashed double-headed white arrows on Fig 4c). The whole area of a segment (Fig. 6c) showed a sharp SAED pattern of calcite along the [21-1] zone axis (Fig. 6d). This result shows all of the elongated grains in a segment have exactly the same crystallographic orientations. The c-axis was not detected from the plane of this thin section.
3-5. Elemental analyses of pentaliths

A total of twenty six pentaliths of *B. bigelowii*, eight heterococcoliths of *Emiliania huxleyi*, five heterococcoliths of *Gephyrocapsa oceanica* and six heterococcoliths of *Tergestiella adriatica* were examined by SEM-EDS (Fig. 7). The integrated counts of Ca-\(\text{K}\alpha\) and Mg-\(\text{K}\alpha\) peaks in each spectrum were obtained. Ca and Mg counts taken from the carbon tape without coccolithophores (background) were mostly lower than 10,000 and 800, respectively. The Ca counts ranging from ca 14,000 to 90,000 in the heterococcoliths, and 40,000 to 80,000 in pentaliths of *B. bigelowii*. The Mg counts of heterococcoliths were usually less than 1,000, although the counts of two coccoliths of *G. oceanica* exceeded 1,000. The Mg counts of pentaliths of *B. bigelowii* ranged from ca. 28,000 to 78,000, and were positively correlated with the Ca counts (\(R = 0.67\)).

4. Discussion

4-1. Ploidy state
This study revealed that calcified cells of *B. bigelowii* have a haptonema, and use it for adhesion to external substrata. The calcified cells are non-motile and do not have flagella, unlike non-calcifying motile cells of *B. bigelowii*, which were originally described as *C. parkeae* as possessing a haptonema and two flagella (Green and Leadbeater, 1994). This is the first known example of haptophytes in which the non-motile cells without flagella possess a haptonema.

Many coccolithophores change their motility and scale morphology in their life cycle. Members of Nöelaerhabdaceae (Isochrysidales) are non-motile and calcifying in diploid phase, and motile and non-calcifying in haploid phase. Members of the Coccolithales are non-motile and calcifying in diploid phase, and motile and calcifying in haploid phase. Members of the Syracosphaerales and Zygodiscales are motile and calcifying in both the diploid and haploid phases (e.g. Houdan et al., 2004; Young et al., 2003). So far as is known, all haploid cells of coccolithophores are motile. The ploidy state of non-motile (calcifying) and motile (non-calcifying) cells of *B. bigelowii* is still unknown due to lack of culture strains, however, comparison of its behavior with that of other coccolithophores suggests that the non-motile (calcifying) and motile
(non-calcifying) stages of *B. bigelowii* likely correspond to diploid and haploid phases, respectively.

### 4-2. Pentalith-substrate

In this study, the presence of pentalith-substrate of the specimen Furu-SEM1 was revealed as a result of dissolution of pentaliths during cleaning of the cell using ion-exchanged water after fixation of the organic structure by osmium tetroxide. In our experience, coccoliths/pentaliths can be dissolved in ion-exchanged water, probably because ion-exchanged water is depleted in ions and the carbon dioxide in the atmosphere easily dissolves in the ion-exchanged water. Another example of dissolution of pentaliths in ion-exchanged water is shown in Appendix 3. The pH of the ion-exchanged water used for cleaning of the specimen Furu-SEM1 is unknown, but probably it was slightly acidic. *(Hochuli, 2000)* reported organic fossils, which closely resemble the pentalith-substrate of *B. bigelowii*, from Oligocene sediments from the North Sea prepared for palynological studies using hydrochloric and hydrofluoric acids. The material forming the pentalith-substrate is unknown, however, observation by
(Hochuli, 2000) indicates that it is probably formed with some resistant non-hydrolyzable biopolymer. At this moment, there are no reports on cell covering formed with resistant non-hydrolyzable biopolymer from the members of the Haptophytes. Therefore, it is difficult to assume the composition of pentalith-substrate at this point.

The morphological similarity between the organic cell covering structure (pentalith-substrate and thin layers) and the pentaliths of B. bigelowii is unusual for coccolithophores. Previous studies showed that diploid cells of typical coccolithophores bearing heterococcoliths have smooth cell membranes, and that there is no relationship between the morphology of the cell membrane and of heterococcoliths (e.g. Drescher et al., 2012; Probert et al., 2007). Haploid cells of typical coccolithophores (e.g. C. pelagicus) have complex cell coverings consisting of the plasmalemma, columnar material, several layers of scales, holococcoliths and an outermost investment called the envelope. The organic ‘envelope’ is considered as delimiting the site for calcification of holococcoliths (Rowson et al., 1986), but again there is no morphological similarity between cell membrane structure and holococcoliths. As we reported above, trapezoidal
segments of pentalith of *B. bigelowii* are surrounded by the pentalith-substrate and multiple very thin organic layers. The site for calcification of the pentaliths has not been confirmed yet due to the lack of in situ observations of calcification, however, the close morphological similarities suggest that the organic pentalith-substrate and thin layers may act as a ‘guide’ for the shaping of pentaliths, and the organic layers covering distal side of trapezoidal segment may correspond to ‘envelope’ of motile cells of *C. pelagicus*.

**4-3. Process of calcification**

A pentalith with incomplete calcareous layers (Fig. 2e) can be considered as in the process of calcification or malformation rather than the result of secondary dissolution of layers, since secondary dissolution of pentalith starts from the margin of pentaliths not from the center of pentaliths (Appendix 3). Presence of multiple incomplete calcified layers along ridges of pentalith-substrate suggests that *B. bigelowii* calcify multiple layers at the same time (arrow on Fig. 2e).
A naked cell without pentaliths but with pentagonal impressions on its cell surface, which resembles the pentalith-substrate of *B. bigelowii*, was observed in this study (Fig. 2f). The sample, which yielded the naked cell, also contained many *B. bigelowii* cells with calcified pentaliths. Therefore, if it is *B. bigelowii*, it should be in the state prior to the start of calcification rather than a cell that lost pentaliths due to secondary dissolution. If the cell is in the precursor state to calcification, presence of twelve impressions of pentaliths on a cell (Fig. 2f) suggest that *B. bigelowii* does calcification of 12 pentaliths on cell surface synchronously.

We have isolated > 500 of cells of *B. bigelowii* through our previous studies and on-going culture studies, but never seen incomplete pentaliths within the cell of *B. bigelowii* (Hagino et al., 2013; Hagino et al., 2009; Takano et al., 2006) (personal observation by KH). The lack of observation of incomplete pentaliths inside the cell supports our hypothesis that *B. bigelowii* calcifies the 12 pentaliths synchronously on its cell surface not inside the cell.

4-3. Mineralogical characteristics of pentaliths
TEM study of a pentalith revealed that the layers in the thin-foil section consist of numerous calcite grains elongated in almost the same direction (Figs 6b-c). Since the thin-foil section was prepared from intermediate part of layers, which is hardly affected by secondary-dissolution, the morphology observed in TEM is a primary structure not the result of dissolution. The direction of the long axis of the grains and their appearance looks to be the same as that of the fine grooves observed from inner layers, which were exposed by loss of the outermost smooth distal layer (double-headed solid arrows in Figs 2a-b) (Fig. 2-6 of Hagino et al., 2009). The similarity in structure observed in both TEM and SEM suggests that the fine grooves observed by loss of outermost layers are also a primary structure. This result raised another question, why does the only outermost distal layer have a smooth surface? The absence of fine grooves/calcite grain structure can be explained by multiple organic layers covering the distal surface of pentalith (black triangles on Fig. 5c) that may conceal the fine structure of distal lamina.

The direction of the long axis of the calcite grains looks to be the same as that of the fine wrinkles observed on the distal surface of the pentalith-substrate (double headed
dashed arrows on Fig. 4c). Similarity in direction of calcite grains and wrinkles of pentalith-substrate suggest a possibility: the pentalith-substrate plays a role on growth of calcite grains.

The examined TEM section should consist of a couple of stacked lamina, since the thin-foil TEM section (c.a. 180 nm) was much thicker than that of a single lamina of the pentalith (<70 nm, Appendix 4). Therefore, calcite grains in all the lamina have perfectly identical crystal orientation. This suggests that *B. bigelowii* strictly controls crystal orientation of calcite grains.

### 4-4. Chemical contents of pentaliths

Heterococcoliths calcified intracellularly contain very low Mg\(^{2+}\) (1/10~1/100) in comparison to foraminiferan tests calcified extracellularly (Stoll et al. 2001). That is consistent with the highly regulated selective ion transport mechanism utilized during calcification (Brownlee and Taylor, 2004; Stoll and Ziveri, 2004). On the other hand, holococcoliths, which are calcified outside the periplast (Rowson et al., 1986), contain higher amount of Mg than heterococcoliths (Cros et al., 2013). Our study revealed that
B. bigelowii almost certainly calcifies pentaliths extracellularly, and always contain a relatively high amount of Mg in the pentalith. Together with the results from (Cros et al., 2013), our study showed that elemental compositions of calcified scales are correlated with the site of calcification, and that higher contents of Mg in the calcified scales indicates extracellular calcification. The phylogenetic positions and the sites of calcification of many other nannolith-bearing species, such as Nannoconus, are still unknown. So, elemental studies of calcareous nannofossils using EDS would be useful to help identify the calcification sites as well as understanding of phylogeny of extinct calcareous nannofossils.

Elemental compositions of foraminiferan tests have been used for reconstruction of the temperature and/or water chemistry in geological ages (e.g. Barker et al., 2005). The high Mg content of pentaliths of B. bigelowii suggests the possibility that pentaliths will record seawater chemistry at the time of the calcification as is the case for foraminiferan tests. The fossil records of the family Braarudosphaeraceae extend back to the Early Cretaceous (140 million years ago) with no change in ultrastructure, (Bown, 1998). So,
we predict that pentaliths of the Braarudosphaeraceae may provide valuable records of
the chemical conditions of seawater in the geological past.

5. Summary

1. Non-motile calcified cells of *Braarudosphaera bigelowii* do have a haptonema but
do not possess flagella. *B. bigelowii* uses the haptonema for adhesion to external
substrates.

2. *B. bigelowii* has a pentalith-substrate that closely underlies the calcareous
pentaliths, and multiple organic thin layers that develop from ridges of the
pentalith-substrate extend onto the distal surface of the pentaliths. The close
morphological correspondence suggests that the pentalith-substrate and organic
thin layers act as a ‘guide’ for shaping the pentaliths, and for calcification of 12
pentaliths covering a cell occur on pentalith-substrate at the same time.

3. *Braarudosphaera* pentaliths consistently show higher Mg content than regular
heterococcoliths, closer to the values, which would be expected for equilibrium
calcification from sea-water. This supports our hypothesis that *B. bigelowii* calcify
their pentaliths extracellularly rather than in an intracellular compartment, it also
makes them of potential value for geochemical study.

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Figure Caption

Fig. 1. Location of samples used in this study: (a) Locality of Furue, Usuka, and Tomari
ports. (b) Locality of sampling stations in the Tomari area.
Fig. 2. Scanning electron and light microscopic images of *Braarudosphaera bigelowii* and SEM image of an unknown specimen, resembling *B. bigelowii*.

(a) *B. bigelowii* from st. 3 of Tomari Port (June 27, 2009). (b) *B. bigelowii* from st. 3 of Tomari Port (June 27, 2009). (c) *B. bigelowii* from st. D of Tomari Port (June 15, 2012). (c) Lightly calcified *B. bigelowii* specimen from offshore Tomari (June 17, 2013). (d) Lightly calcified specimen of *B. bigelowii* from st. D of Tomari Port (June 21, 2011). (e) Incomplete pentalith of *B. bigelowii* from offshore Tomari (June 17, 2013). (f) Unidentified cell that has *B. bigelowii*-like cell surface structure from st. 1 of Tomari Port (June 21, 2010). Note. Arrow on (e) indicates position where multiple laminae are visible. Double-headed arrows on (a) and (b) indicate the orientation of the grooves on the laminae.

Fig. 3. Light microscopic images of *B. bigelowii*: (a) and (b) Specimen Furu-SEM1. (c) Specimen US15.2-sc11, (d) and (e) Furu-sc2. White arrows indicate the haptonema.

Fig. 4. SEM images of cell surface structure of the specimen Furu-SEM1. (a) general view of the specimen. (b) close up view of the base of the haptonema (white triangle). (c) Close up view showing the pentalith-substrate. Solid white arrows indicate...
pentalith-substrate between contact surfaces of pentaliths. Solid black arrows indicate extensions of the pentalith-substrate into the contact surface between trapezoidal segments. Dashed black arrow shows horizontal lines on pentalith-substrate that corresponds to laminae of pentalith. Double-headed dashed white arrows show the direction of fine corrugations on the pentalith-substrate structure.

Fig. 5. TEM images of a cytological section through a *B. bigelowii* cell. (a) Complete cytological section of the *B. bigelowii* cell. (b and c) Details of the cross section. C. and S in Fig. 5(a) indicate chloroplast and spheroid body, respectively. Solid white arrows indicate the pentalith-substrate extending between the pentaliths and protruding slightly beyond them. Solid black arrow indicates pentalith-substrate intruding into the contact surface between trapezoidal segments. Black triangles indicate thin organic layers covering the distal surface of the pentalith and connected to the pentalith-substrate.

Fig. 6. Thin-foil cross section of pentaliths of *B. bigelowii* cut using a focused-ion beam (FIB) apparatus. (a) TEM image of the whole section, this comprises a section parallel to the surface of one pentalith (black arrow) and through the sides of two neighboring pentaliths (white arrows). (b) Close up view of a segment of Fig. 6a in TEM. (c)
High-angle annular dark field (HAADF) image of the segment in Fig. 6b, showing a skeletal texture consisting of elongated calcite grains. The contrast is mainly caused by averaged atomic numbers of the sample. Bright area shows elongated calcite grains. The direction of the elongation corresponds to that of fine graves in SEM observation (Figs. 2a and b). (d) Electron diffraction pattern taken from whole area of the segment b along the [21-1] zone axis. The pattern shows all of the elongated grains have exactly same crystallographic orientations.

Fig. 7. Mg and Ca X-ray microanalysis counts for pentaliths of *B. bigelowii*, heterococcoliths of *Emiliania huxleyi*, *Gephyrocapsa oceanica*, and *Tergestiella adriatica*, and background.

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Fig. 1 Hagino et al.
Fig. 7, Hagino et al.

\[ Y = 6.3X + 3.3 \times 10^4 \]

\( R = 0.67 \)