Morphological characterization of biominerals from five multicellular marine algae species

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Silica biominerals are deposited as amorphous solid structures in plant cells and tissues, providing rigidity to different plant parts and assisting in defence. The shape and size of phytoliths are well established and serve as a useful tool in taxonomic analyses. For the first time we extracted and studied silica biominerals of five marine macroalgae, which are known to be deposited as amorphous solid structures. Our research was performed via light microscopy, scanning electron microscopy, and X-ray diffraction analysis (XRD). More than nine different morphotypes of phytoliths ranging from ≥ 10 to ≥ 350 μm in size were found. Some of them were phytoliths made of silica while others showed characteristics of different minerals of calcium. In our study, the “honeycomb” formations were only recorded in Laurencia tropica Yamada and pyramid tabular ones were found only in Tichocarpus crinitus (S.G. Gmelin) Ruprecht. The XRD analysis showed that they consisted of virgilithe and gypsum substance, respectively. Silica phytoliths are intrinsic parts of the algae and their morphological characterization can provide the basis for palaeo-reconstruction and taxonomic investigation of brown and red algae in palaeontological studies of fossils where all organic matter has decayed.

Силевые биоминералы являются полезным инструментом в таксономическом анализе. Впервые мы извлекли и изучили биоминералы для пяти видов морских макроводорослей, которые мы изучили с помощью световой микроскопии, сканирующей электронной микроскопии и рентгеноструктурного анализа (XRD). Было обнаружено более девяти различных морфотипов фитолитов с размерами от ≥ 10 до ≥ 350 микрометров. Часть из этих фитолитов были из окиси кремния, другие из минералов на основе кальция. Гексагональные «сотообразные» образования были зарегистрированы только у водоросли Laurencia tropica Yamada, а фитолиты пирамидальной формы были обнаружены только у красной водоросли Tichocarpus crinitus (S.G. Gmelin) Ruprecht. Рентгеноструктурный анализ показал, что они состоят из виргилиты и гипса соответственно. Кремневые фитолиты являются неотъемлемыми частями водорослей, и их таксономическая характеристика может служить основой для палеореконструкции и таксономического исследования бурых и красных водорослей в палеонтологических исследованиях окаменелостей, где вся органическая материя уже разложилась.

Ключевые слова: биргенный кремний, морфотипы, фитолиты, таксономический анализ.
Introduction

Algal culture is the only aquaculture industry involved in plant production, i.e., primary production. Algal culture is focused on marine, estuarine and freshwater algae (Romanenko et al., 2017; Çelekli et al., 2019). There is no doubt that the process of biomineralization inherent to terrestrial plants is also present in algae. Biominerals are ubiquitous in all classical kingdoms of life: in the ocean, in inland waters, and on land. Interestingly, different members of kingdom plantae produce a suite of rigid microscopic biominerals of various compositions i.e., silicon dioxide (slica), calcium carbonate, calcite (calcite cocoliths), and calcium oxalate (cylotaxls) with significant quantities of phosphorous, magnesium, aluminium, etc. Biomineralization (silification and calcification) has arisen very early in plant lineages, i.e., red algae (Florideophyceae), green algae (Ulviophyceae and Charophyceae), brown algae (Phaeophyceae), and Prymnesiophyceae (Raven and Giordano, 2009). Calcium carbonate and/or calcium oxalate accumulates in red and brown algae extracellularly and intracellularly, respectively (Rao et al., 2014). Silica also accumulates in some green and brown algae and is thought to be mainly located on intracellular compartment, i.e., in cell-walls (Parker, 1969). Strictly speaking, phytoliths are amorphous silica deposits, while distinctions for other types of biominerals exist; however, for the purpose of this study, the term “phytolith” will be generalized to all types of observed biominerals in target species. Phytoliths can also be deposited within different intracellular and extracellular structures of plants and, being inorganic matter, they remain as discrete microscopically identifiable characteristics are produced in large quantities in different groups within the plant kingdom and hence, phytolith surface ornamentation, length, thickness, shape, frequency, and geometry are the basic identification characteristics observed using several microscopic techniques, such as light, transmission electron microscopy, scanning electron microscopy (SEM), and X-ray diffraction (XRD) analysis (Piperno, 2006).

In this study, we used light microscopy, SEM, and XRD for morphological and compositional categorization of phytoliths in three species of red algae (Mastocarpus stellatus (Stackhouse) Guiry, Tichocarpus crinitus (S.G. Gmelin) Ruprecht, and Laurencia tropica Yamada) and two species of brown algae (Saccharina latissima (L.) C.E. Lane, C. Mayes, Druehl & G.W. Saunders, Fucus evanescens C. Agardh), which are found on the Russian side of the Sea of Japan (Maggs, Stegenga, 1999; Garbary, Tarakhovskaya, 2013). These morphological characterizations will provide the basis for palaeontological studies where the differences in morphology of inorganic remains of algae may prove useful for their taxonomic identification.

Materials and Methods

Extraction of phytoliths

Three species of red algae, M. stellatus, T. crinitus and L. tropica, and two species of brown algae, S. latissima and F. evanescens, were used in the study. Five specimens of each species were collected at depths of 3–6 m from the Sea of Japan (45°01’21.2”N 136°42’23.3”E); they were identified using monographs, floristic studies, and systematic articles (Saunders, Hommersand, 2004; Zuccarello et al., 2005; Belous et al., 2013). Then algae were placed into plastic containers and stored at ~5°C for two days. Phytoliths were extracted using the modified Piperno technique (Piperno, 2006). Approximately 30–50 g of thalli per sample were washed with distilled water twice and burned in covered ceramic-enamelled crucibles in a muffle furnace at +450°C for 4 hrs. The ash was then transferred into glass centrifuge tubes and washed thoroughly with 10 ml of 10% HCl and concentrated nitric acid for 10 minutes, with periodic stirring of the test tube. The samples were then rinsed twice with 10 ml of distilled water and centrifuged for 10 min at 150 g, followed by decanting of water, leaving 0.5 ml mixture in the test tube. A further 200 μl of solution was removed from the test tube bottom with a pipette and the mixture was subjected to microscopy. The remaining solution was used for XRD analysis.

Microscopic investigation

The processed samples were individually placed on a microscope slide. They were examined within one hour on an AxioScope A1 light microscope (Zeiss, Germany) using an AxioCam 3 digital video camera (Zeiss, Germany). The length and width of each particle were measured using the Axio Vision 4.2 program (Zeiss; Oberkochen, Germany). Their morphologies were evaluated by SEM using a Hitachi S-3400N (Hitachi; Tokyo, Japan) with an ultra-dry energy dispersive spectrometer (Thermo Fisher Scientific; Waltham, MA, USA) or with a tabletop SEM TM1000 (Hitachi; Tokyo, Japan). When examined under the S-3400N microscope, the samples were sprayed with platinum; they remained unsprayed when using the TM1000. The definitions of morphotypes as well as the descriptions of phytoliths and cubic, parallellepipedal, tubular oblong, pyramidal, cylindrical polylolate, and of many other defined geometric shapes (Morgan-Edel et al., 2015). While amorphous silica biominerals with unique identifiable characteristics are produced in large quantities in different groups within the plant kingdom and hence, phytolith surface ornamentation, length, thickness, shape, frequency, and geometry are the basic identification characteristics observed using several microscopic techniques, such as light, transmission electron microscopy, scanning electron microscopy (SEM), and X-ray diffraction (XRD) analysis (Piperno, 2006).
other unidentified mineral particles were carried out according to the International Code for Phytolith Nomenclature 1.0 (Madella et al., 2005).

**Mineralogical analysis**

Biomineral particles were characterized by XRD analysis. Solutions containing the washed sediments were placed in plastic vials and left to dry completely for 24 hours at ambient temperature; the dry mass was used for mineralogical analysis. The determination of mineral type was carried out using a Maniple Bench Top X-ray Diffraction Analyzer (Riau; Tokyo, Japan), with 30 kV, 15 mA, and monochrome settings.

**Results and Discussion**

Phytoliths have been recently recognized as proxies to reconstruct ancient environment, flora, and a tool for taxonomy. The low solubility of biominerals makes them a relatively durable component of sedimentary deposits (Schiegl et al., 2004; Cabanes et al., 2011). The role of calcium and silicon phytoliths in defense against biotic and abiotic stress is well recognized and is progressing with improved understanding of various biochemical pathways (Nawaz et al., 2019). However, this development has been mainly witnessed in land plants. Biomineralization of calcium and silicon compounds in algae has not been explored in detail and description of biominerals in many macroalgae is scarce. More than a dozen types of mineral formations were revealed by light microscopy in the investigated macrophytes. Some of them were phytoliths made of silica, while others showed characteristics of different minerals of calcium (Table 1, Fig. 1).

The distribution and morphologies of biominerals varied among the studied species. The representative samples of biomineral formations found in the five algae studied are shown in Fig. 2. Microscopic examination of algal species (*S. latissima*, *F. evanescens*, *M. stellatus*, *T. crinitus*, and *S. latissima*).
L. tropica) revealed various structural types of phytoliths (i.e., cylindric/oblong tabular, globular/ovate tabular, fusiform, hexagonal tabular, pyramid tabular, square tabular, rectangular tabular, and ovate-favose) (Table 1). In our study, the “honeycomb” formations were only recorded in L. tropica and pyramid tabular were found only in T. crinitus (Table 1). The XRD analysis showed that material consisted of virgilite and gypsum substance. (Fig. 3).

Table 1. Morphotypes and size of mineral formations (in μm) found in five macrophyte preparations.

| No. | Morphotype                  | Material          | Mastocarpus stellatus | Tichocarpus crinitus | Laurencia tropica | Fucus evanescens | Saccharina latissima |
|-----|-----------------------------|-------------------|-----------------------|----------------------|------------------|-----------------|---------------------|
| 1   | Cylindrical/oblong tabular  | silica or unknown | L 10–70               | L 25–350             |                  |                 |                     |
| 2   | Globular/ovate tabular      | silica            | L 5–60                | L 5–45               | L 10–15          |                 |                     |
| 3   | Fusiform                    | silica            | W 7–25                | L 15–50              |                  | L 10–25         |                     |
| 4   | Hexagonal tabular           | unknown           | L ~ 10                | L 15–25              |                  |                 |                     |
| 5   | Pyramidal tabular           | silica or unknown | W 10–70               | L 25–70              |                  |                 |                     |
| 6   | Square tabular              | unknown           | L ~ 10                |                      |                  |                 |                     |
| 7   | Rectangular tabular         | unknown           | W 8–10                | L 12–35              | L 30–100         | W 100–130       | W 15–20             |
| 8   | Ovate favose (honeycomb)    | silica            | W 5–70                | L 12–150             |                  |                 |                     |
| 9   | Unclassified                | silica or unknown | L 25–35               | L 15–30              | L 10–40          | L 10–20         |                     |

L. tropica) revealed various structural types of phytoliths (i.e., cylindric/oblong tabular, globular/ovate tabular, fusiform, hexagonal tabular, pyramid tabular, square tabular, rectangular tabular, and ovate-favose) (Table 1). In our study, the “honeycomb” formations were only recorded in L. tropica and pyramid tabular were found only in T. crinitus (Table 1). The XRD analysis showed that material consisted of virgilite and gypsum substance. (Fig. 3).

Fig. 3. A typical X-ray diffraction pattern of a mineral sample obtained after drying preparations of the red alga Mastocarpus stellatus

Рис. 3. Типичная рентгеновская дифферометрия образца минерала, полученная после высушивания препаратов красной водоросли Mastocarpus stellatus
Laurencia tropica (5), Mastocarpus stellatus (4), Fucus evanescens (3), and Saccharina latissima (2). The hexagonal mineral formation in Mastocarpus stellatus and T. crinitis is the first finding of this geometrical shape in algae phytoliths. Cylindrical or oblong, pyramidal tabular, square, rectangular tabular crystals described in Cladophorophyceae (Leliaert and Coppejans, 2004) were also observed in this study. Cylindrical or oblong tabular forms were not found in either two brown algae. Moreover, these crystal types also were not observed in Turbinaria ornata, Sargassum miyabei, or Dictyota dichotoma (Golokhvast et al., 2015), which leads to our hypothesis that cylindrical or oblong tabular phytoliths are only contained in red and green seaweeds.

The size ranges of the phytoliths in the algae examined were different from the phytoliths found in plants. In our algae samples they varied from ≥ 10 to ≤ 350 μm, while relatively smaller phytoliths (6.9–25.2 μm) have been reported in grasses (Piperno, 1984). Morphotypes of phytoliths in two species of marine angiosperms and two macroalgae (Table 1). The observation of phytoliths of fixed geometrical shapes, i.e., hexagonal, rectangular and square shapes, in studied macroalgae is of particular interest. Previously it was known that different plant species accumulated similar three-dimensional crystals of calcium oxalate and calcite as examined in horsetail and creosote bush (Morgan-Edel et al., 2015). The mineralogical content of these crystals revealed that they are mainly composed of calcium and oxygen with inclusions of Al, S, and Fe (Fig. 3).

In conclusion, phytoliths of a regular hexagonal shape have been discovered for the first time in the red algae. Although limited by small sample size, the results of our study suggest that there are differences in phytoliths between studied seaweed species and higher plants. We recognize that our data have limits to understanding the diversity of phytolithology in seaweeds and the comparability in reconstruction of paleo-environments. There is a pressing need of advancing the comparative collection of macroalgae phytolith morphologies.

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