Nacre characterization of black lip pearl oyster *Pinctada margaritifera* from North Sulawesi

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Abstract. Mollusk's shell is a crystal composite of calcium carbonate ($CaCO_3$), calcite and aragonite, embedded into layers. Nacre of bivalves (genus *Pinctada*) is the most interesting aragonite microstructure to be studied. This study aims to analyze the microstructure, biomineral composition of the nacre, and the mineral of *Pinctada margaritifera* nacre powder. Shell samples were collected from 3 locations, Bahoi, North Minahasa Regency, Arakan, South Minahasa, and Talengen, Sangihe group of islands Regency, the Province of North Sulawesi. The body organ removal from the shell was done in the Laboratory of Faculty of Fisheries and Marine Sciences, Sam Ratulangi University Manado. The shell ventral margin of 5 mm thick was analyzed under a Scanning Electron Microscope (SEM). The sample was then blended into powder for ICP-OES tests. Results showed that the nacre grew layer by layer. The nacre tablet was coated by mortal or adhesive layer to bind one tablet to another. The microstructure of *P. margaritifera* nacre is polygonal shaped and composed as a tidy brick wall with a thickness of 0.61-1.11 µm and mean thickness of 0.72 µm. The biomineral composition consisted of Ca, Mo, Na, S, Al, Mg, Zn, Fe, Se, Co, Mn, Ni, P, Cr. The mineral concentrations of the nacre powder was 44.333 mg kg^{-1} for Ca, 1.510 mg kg^{-1} for Na, 372 mg kg^{-1} for P, 31 mg kg^{-1} for Mg, 2.1 mg kg^{-1} for Mn, and 2 mg kg^{-1} for Fe. The nacre powder contained Ca, Na, P, Mg, Mn, and Fe.
Key Words: biomineral, microstructure, powder, SEM, tablet.

Introduction. Mollusks are a great expert in shell formation composed of the mostly varied microstructures among invertebrates (Checa 2018). Shells are the composite of calcium carbonate ($CaCO_3$) crystals, calcite, and aragonite, deposited to be a layer where the forming crystals are arranged in a configuration called a microstructure (Checa & Rodriguez-Navarno 2005). Nacre is the most interesting aragonite microstructure of the bivalve, gastropod, and cephalopod (Nudelman 2015).

Many researches have recently been focused on the nacre layer of bivalves (Checa 2000; Rousseau et al 2009), biomineralization (Dhami et al 2013; Addadi & Weiner 2014), and microstructure of nacre (Lopes-Lima et al 2010; Zouari et al 2011). Not only organic matrix components, but also the mineral phase actively functions in the nacre microstructure formation of Pteriidae (Checa et al 2006).

Black-lip pearl oysters *Pinctada margaritifera* (Linnaeus, 1758), Pteriidae, are the Indo-Pacific species widely distributed in the Red Sea, Arabian Sea, Persian Gulf, India, Srilanka, South Japan, Australia, New Caledonia, Polinesia, Micronesia, Papua New Guinea, Hawai Cocos Keeling Islands, Madagaskar, and Southeast Asia including Indonesia (Southgate & Lucas 2008). This species is found to be abundant in North Sulawesi waters with a size range of 4.39 to 13.95 cm (dorsal-ventral) and a mean length of 8.72 cm (Kalesaran et al 2018). Shells are basic patterns of biologically controlled mineralization. The shiny layer covers the internal shell and its optic feature is a unique combination that makes it be attractive in jewelry and pearl industries (Marin et al 2013). *P. margaritifera* is an economic species in pearl industries, but information on the microstructure and the biomineral of *P. margaritifera* nacre from North Sulawesi waters is very limited. This study aims to analyze the microstructure, biomineral element composition of the nacre, and the mineral concentration of *P. margaritifera* nacre powder.

Material and Method. Samples of *P. margaritifera* were collected from September 2022 until January 2023 from 3 localities, namely Bahoi waters, North Minahasa Regency (site 1), Arakan waters, South Minahasa (site 2), and Talengen waters, Sangihe Group of Islands Regency (site 3), North Sulawesi Province (Figure 1). They were taken using a knife, brushed to remove the dirts, and cleaned in running water. Preparations for body organ removals from the shell were done in the Fish Health, Environmental, and Toxicological Laboratory, Faculty of Fisheries and Marine Sciences, Sam Ratulangi University, Manado.

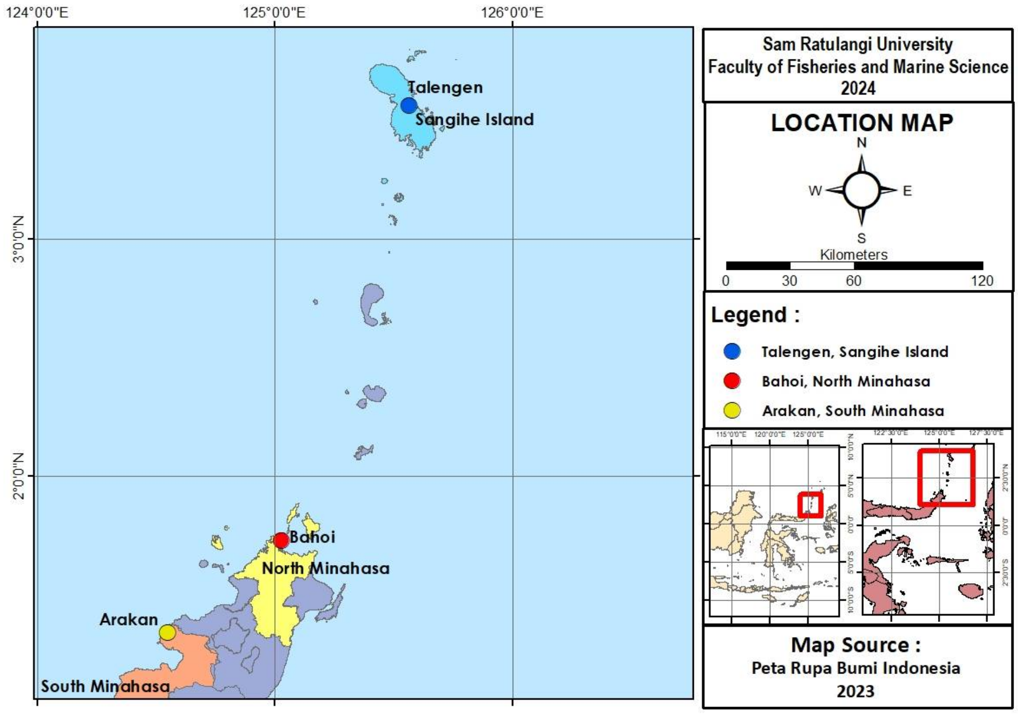


Figure 1. Sampling localities.

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS). Morphometric measurements were carried out for shell length (DVM) using a digital Vernier caliper. The samples were selected into 3 size categories, large (> 100 mm), medium (80-100 mm), and small (< 80 mm). The shell was cut using a small plier about 3-5 mm long at the ventral margin for SEM and EDS analyses.

Inductively Coupled Plasma (ICP-OES). The clean shell was grinded on the outer layer of the shell to obtain a strikingly colored shell layer (Figure 2). The nacre pieces were heated and refined into powder. The sample powder of 50-80 g was placed in a plastic bag and taken in the Water Laboratory Nusantara (WLN) of Manado to analyze calcium (Ca), iron (Fe), magnesium (Mg), mangan (Mn), sodium (Na), and phosphorous (P), using an iCAP 6000 Series-typed ICP-OES.

Results and Discussion. Mollusk shells are composed of calcium carbonate ($CaCO_3$) crystals, calcite and aragonite, embedded within an organic framework (Checa & Rodriguez-Navarno 2005). The interaction between calcium carbonate and organic matrix forms the shell microstructure. According to Strag et al (2020), the investigations of the mollusk shells revealed a variety of microstructures depending on the species. The nacre layer was horizontally observed under an electron microscope. The SEM image that demonstrated the *P. margaritifera* nacre at different shell lengths and localities showed a regular and tidy nacre layer surface (Figure 2A). The nacre grows together as layer by layer. The nacre tablet is covered by a mortal layer or adhesive to tie a tablet to the other one. This finding reconfirms Rousseau et al (2005) that Voronoi model is the growth model of nacre tablet of *P. margaritifera*. It is a layered structure of the nacre tablet surrounded by the organic matrix (Figure 2B). In *P. fucata*, the SEM image shows that the nacre layer is composed of polygonal aragonite tablets interspersed with a thin matrix sheet between organs and accumulated parallel to the shell surface (Muller 2011). Our observations showed that the shape of the nacre tablets changed as the shell length increased. In small-sized shells, the nacre tablet is elliptically shaped and grows polygonally. The microstructure of nacre tablet of *P. margaritifera* seems to be filled with the organic matrix around the tablet (Figure 2C). Several researchers have studied the organic matrix of the nacre under a SEM (Levi-Kalisman et al 2001; Kroger et al 2021). It confirms the present finding that the organic matrix coating the nacre tablet plays an important role in the nacre and microstructure formation.

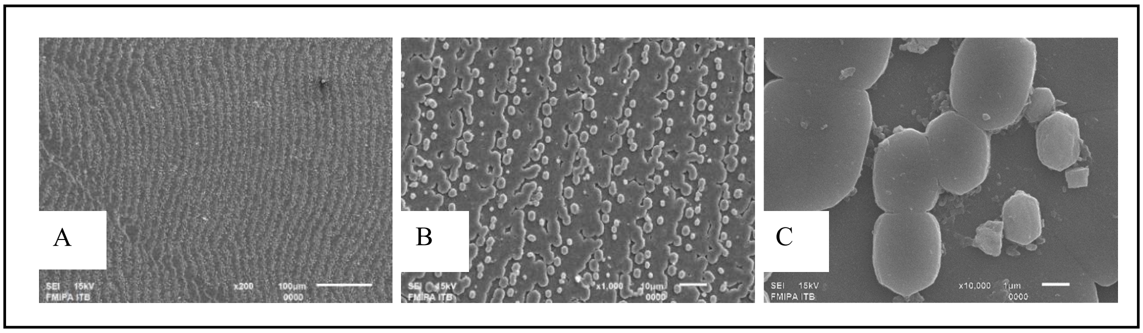
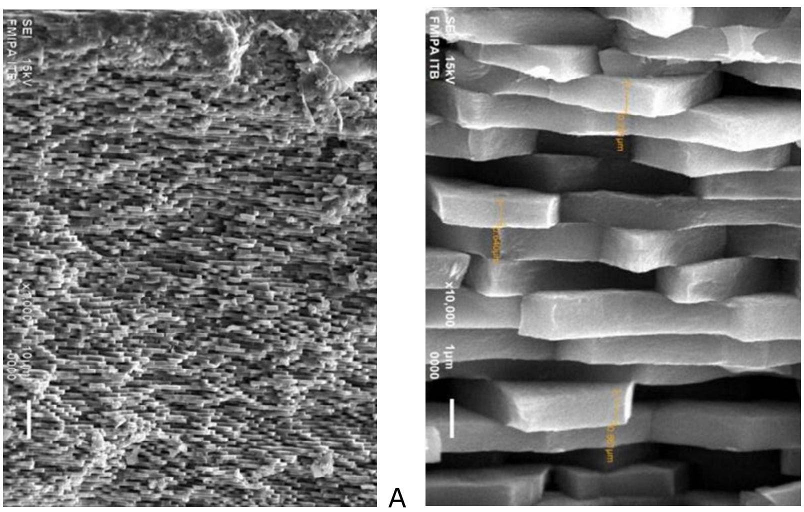


Figure 2. The nacre layer surface of *P. margaritifera.* (A) Bar scale of 100 μm; (B) Bar scale of 10 μm; (C) Bar scale of 1 μm.

The organic matrix plays a key role in limitng the nacre thickness and the shell structure. It is composed of biomacromolecules that have multifunctions. On one hand, some proteins are absorbed in the matrix surface to provide nucleation sites for the next crystal layer. On the other hand, some proteins on the matrix inhibit and terminate the crystal growth to ensure a uniform thickness of the nacre layer (Xie et al 2011). Therefore, the organic matrix (interlamellar) plays an important role in the nacre biomineralization (Muller 2011). The shell of *P. margaritifera* has very strong colors depending on the thickness of several peripheral layers of the nacre and the regularity of the tablet on the shell surface. Mollusk metabolism and growth are affected by their environment that eventually influences the shell size and shape. Hence, this environmental modification is crucial for the biomiralization of *P. margaritifera* shell (Rousseau & Rollion-Bard 2012).

The microstructure is composed like a brick wall, tidily arranged, with varied thicknesses (Figure 3A). The nacre tablets exhibited different thicknesses with shell size (Figure 3B).



A B

Figure 3. Nacre microstructure of *P. margaritifera*. (A) Bar scale of 10μm and (B) Bar scale of 1μm.

The thickness of *P. margaritifera* nacre tablets at location 1 ranged from 0.61 to 1.11 μm, at location 2 ranged from 0.63 to 0.75 μm, and at location 3 ranged from 0.62 to 0.67 μm. The nacre thickness of *P. margaritifera* tablet of North Sulawesi varies between 0.61-1.11 μm with an average of 0.72 μm. Several previous studies found different tablet thickness in *P. margaritifera,* such as 0.5-1 μm (Rousseau et al 2009), 0.72-0.91 μm (Debruyne 2014), 0.36-0.90 μm (Lumenta et al 2019) and 0.94±0.10 μm (Deng et al 2022).

The EDS analysis on the sample gave information on the element compositions of *P. margaritifera* shell as follows: carbon (C), oxygen (O), sodium (Na), magnesium (Mg), alumunium (Al), phosphorous (P), calcium (Ca), chromium (Cr), manganese (Mn), ferrum (Fe), cobalt (Co), sulfur (S), nickel (Ni), Zinc (Zn), selenium (Se), and molybdenum (Mo). The element compositions of all samples varied. There were 16 biomineral elements in site 1 (Bahoi waters), C, O, Na, Mg, Al, P, Ca, Mn, Fe, Co, S, Ni, Se, Zn, Cr, and Mo (Figure 4).

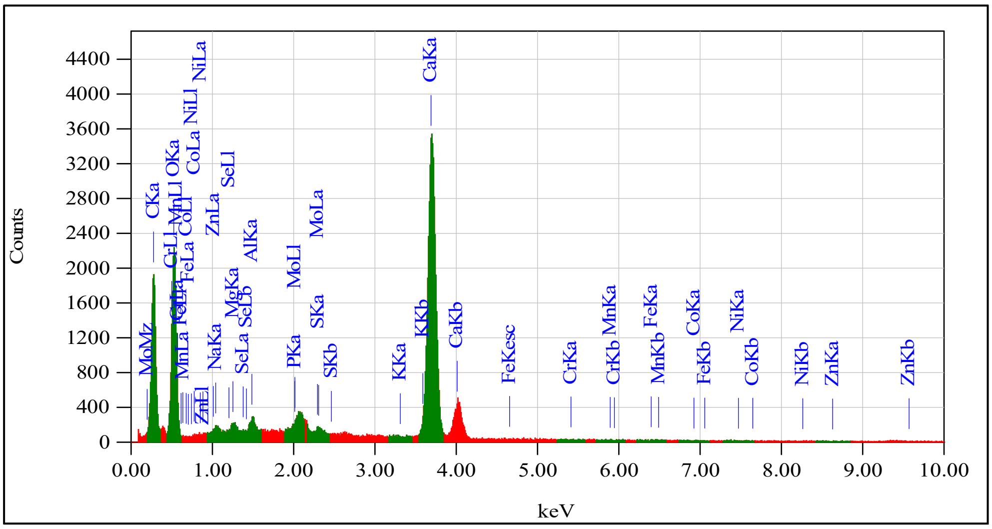


Figure 4. The EDS analysis of *P. margaritifera* nacre from Bahoi waters (site 1). The EDS spectrum shows that the peaks are the X-rays emitted when the electrons return to the K electron shell (Kα and Kβ). The letters K and L refer to the *n* values possessed by these electrons, the K electron is closest to the nucleus and is followed by L, while α and β indicate the size of the transition (Ka = Kα, Kb = Kβ, La = Lα, Lb = Ll = Lβ).

Furthermore, the EDS analysis also described 14 biomineral elements in site 2 (Arakan waters), C, O, Na, Mg, Al, S, Ca, Cr, Mn, Fe, Co, Ni, Zn, and Mo (Figure 5).

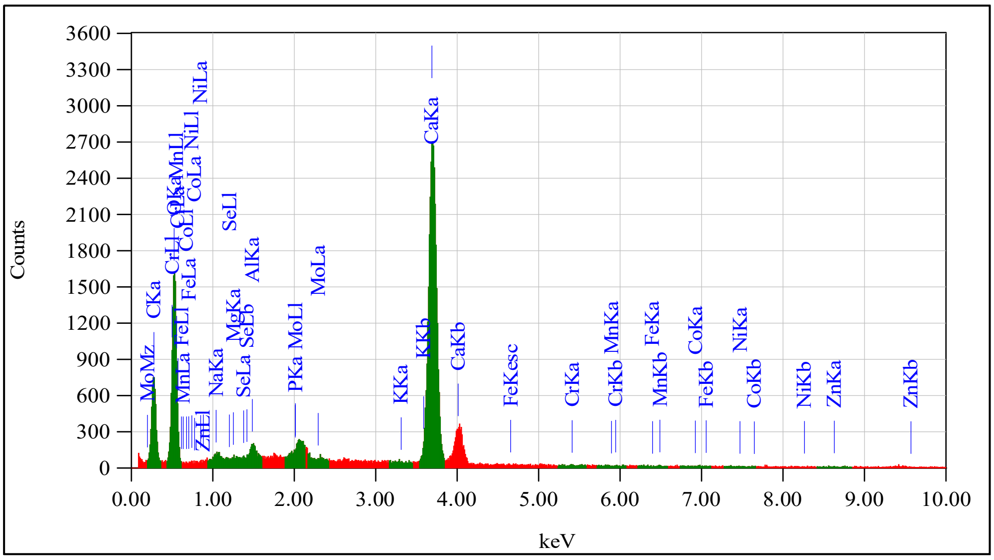


Figure 5. The EDS analysis of *P. margaritifera* nacre from Arakan waters (site 2).

In site 3 (Talengen waters), the present study described 15 biomineral elements, Ca, C, O, Na, Al, Mg, Se, Mn, Co, Fe, Co, Ni, P, Cr, and Zn (Figure 6).

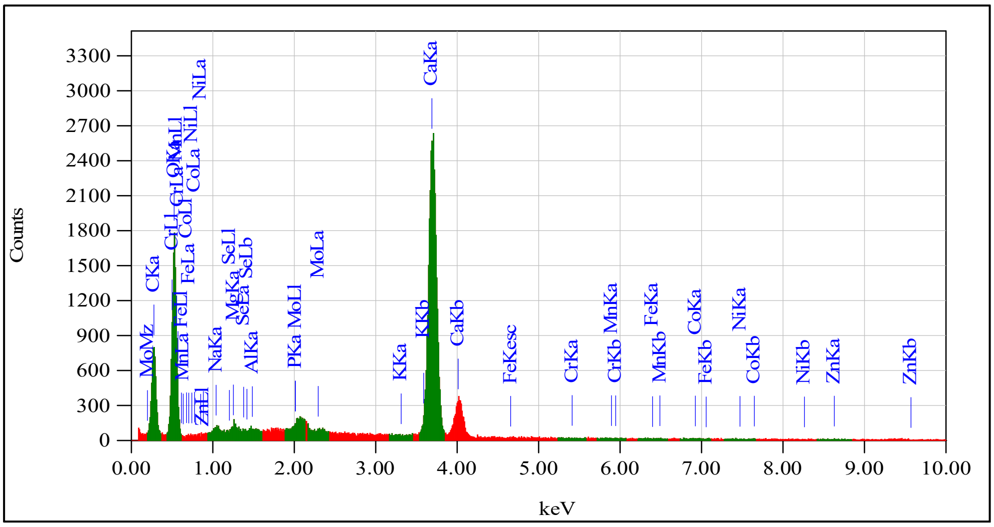


Figure 6. The EDS analysis of *P. margaritifera* nacre from Talengen waters (site 3).

Nacre of *P. margaritifera* showed different composition of biomineral elements at each locality. It could result from different chemical features of the environment where they live so it affects the shell formation. It is supported by Joubert et al (2010) that environmental conditions affect the shell growth, such as food supply and water temperature, directly influencing the shell growth of *P. margaritifera*, and modulating the gene expression level of the matrix protein in the mantle and tissue for shell mineralization. According to Deng et al (2022), the outer and inner shells of three pearl oyster species in South China Sea, namely *P. maxima*, *P. margaritifera* and *P. fucata* contain seven elements like C, O, Na, Al, S, Cl, and C, while *Pteria penguin* contains three more elements, namely Mg, Si, and Sr.

This finding is supported by Linard et al (2011) that higher mineral element content is related with the living environment of the oyster. Seawater composition is dominated by dissolved ions that make the water characteristics different from one to the other and impact the shell biomineral composition. Trophic levels also impact the ventral and dorsal growth of the oyster shell, in which the food chain influences the thickness of the aragonite tablet/shell nacre tablet. Moreover, the microstructure of the mollusk shell that is biologically and chemically controlled can affect the chemical and mineral compositions of the shell (Checa & Rodriguez-Navarno 2005). Age, culture method, predator composition, abundance, and water brightness can influence the shell growth as well (Gervis & Sims 1992).

The pearl produced by *P. fucata* contains trace metal ions, and the pearl color is produced by the trace elements in their living environment (Zhang et al 2019). The environmental elements can reach the calcification spot through hosts, intestines, or direct absorption by the external epithelial mantle. In the present study, the nacre powder of *P. margaritifera* was analyzed using an Inductively Coupled Plasma (ICP) to estimate the concentration of several essential minerals, macrominerals, such as Ca, Mg, Na, and P, and microminerals, such as Fe and Mn (Table 1).

Calcium is an essential mineral needed by living organisms and has a biophysiological role in the formation of the tissue structure and body organ components. Bivalve shells are biomineral forms composed of calcium carbonate ($CaCO_3$), and a small amount of the organic matrix proteins in the shell directs a particular crystal growth of the shell (Southgate & Lucas 2008). Laboratory tests indicated that calcium content was the highest, followed by sodium, phosphorous, magnesium (macrominerals), and several microminerals. The biological and mineralogical factors regulate the trace element content in the shell. This finding is also supported by Marin et al (2012) that the integration of mineral elements in the shell needs biochemical processes to import Ca from the environment to the skeletogenetic site.

Table 1

Mineral composition of nacre powder of *P. margaritifera*

No	Mineral	Nacre powder of *P. margaritifera*			Mean	Unit
		Site 1	*Site 2*	*Site 3*		
	Macrominerals					
1	Calcium (Ca)	48,000	41,000	44,000	44,333.3	mg wet kg^{-1}
2	Magnesium (Mg)	26	35	34	31.7	mg wet kg^{-1}
3	Sodium (Na)	1,520	1,530	1,480	1,510	mg wet kg^{-1}
4	Phosphorous (P)	393	375	350	372.7	mg wet kg^{-1}
	Microminerals					
5	Iron (Fe)	2	2	2	2	mg wet kg^{-1}
6	Manganese (Mn)	1	2.9	2.5	2.1	mg wet kg^{-1}

The biomineralization in mollusks is basically a cellular process under the environmental control, such as water physical and chemical factors (pH, temperature, ionic composition) including minerals (calcium and bicarbonate) (Lopes-Lima et al 2010). The element concentration in the shell of *P. margaritifera* from Manihi, French Polynesia, is Ca of 396.4 mg g^{-1}, Na of 5.536 mg g^{-1}, Mg of 2.136 mg g^{-1}, Sr of 890.6 ppm, Fe of 67.89 ppm, Al of 45.74 ppm, P of 27.19 ppm, B of 12.17 ppm, Mn of 2.308 ppm, Cu of 1.050 ppm, and Zn of 0.7180 ppm; Ni, Cr, Hg, As, Cd, Pb, and V are below the detection limit of the ICP-AES (Chang et al 2007). The extraordinary feature of the nacre material makes it very interesting for the physicians, dentists, orthopedists, and researchers in nanno-technology. Several studies showed that the nacre of *P. maxima* could induce bone formation (Zouari et al 2011). The nacre of *P. margaritifera* can be used for new product development as a remedy of the dermatitis symptom (Muller 2011). The shell powder of *P. margaritifera* has been commercialized as calcium supplement in the United States, the Netherland, and Japan (Chang et al 2007). The nacre powder of *P. radiata* is a promising natural material for biomedical applications, such as substitute of bone grafting (Zouari et al 2011).

The present study found the nacre of *P. margaritifera* as source of macrominerals (Ca, Na, P, and Mg) and several microminerals. This mineral concentration could give information on the powder utilization as alternative source of calcium and other uses.

Conclusions. The observation under the electron microscope demonstrated that the nacre tablet shape of *P. margaritifera* changed with shell growth. In small-sized shells, the nacre tablet is elliptically shaped and grows polygonally. The microstructure was structured as a brick wall, polygonally shaped, and had a thickness of 0.61-1.11 µm. The microstructure of nacre tablet of *P. margaritifera* seems to be filled with the organic matrix around the tablet. The organic matrix surrounding the tablet nacre had an important role in the formation of nacre and microstructure. The biomineral minerals of *P. margaritifera* from North Sulawesi waters are Ca, Mo, Na, S, AL, Mg, Zn, Fe, Se, Co, Mn, Ni, P, and Cr. The composition of biomineral elements in each location is different. This could be caused by differences in the chemical characteristics of the environment in which they live, thus influencing shell formation. The present study also found the nacre of *P. margaritifera* as source of macrominerals (Ca, Na, P, and Mg) and several microminerals. This mineral concentration could give information on the powder utilization as alternative source of calcium and other uses.

Conflict of interest. The authors declare that there is no conflict of interest.

References

Addadi L., Weiner S., 2014 Biomineralization: mineral formation by organisms. Physic Scripta 86(9):098003.

Chang F., Li G., Haws M., Niu T., 2007 Element concentrations in shell of *Pinctada margaritifera* from French Polinesia and evaluation for using as a food supplement. Food Chemistry 104(3):1171-1176.
Checa A., 2000 A new model for periostracum and shell formation in Unionidae (Bivalvia, Mollusca). Tissue and Cell 32(5):405-416.
Checa A. G., 2018 Physical and biological determinants of the fabrication of molluscan shell microstructures. Frontiers in Marine Science 5:353.
Checa A. G., Rodriguez-Navarno A. B., 2005 Self-organization of nacre in the shells of Pterioida (Bivalvia: Mollusca). Biomaterials 26(9):1071-1079.
Checa A. G., Okamoto T., Ramirez J., 2006 Organization pattern of nacre in Pteriidae (Bivalvia: Mollusca) explained by crystal competition. Proceeding of the Royal Society B 273:1329-1337.
Debruyne S., 2014 Stacks and sheets: the microstructure of nacreous shell and its merit in the field of archaeology. The Journal of Human Palaeoecology 19(2):153-165.
Deng Z., Sun J., Wei H. J., Zhao W., Chen M., Li Y., Yu G., Wang Y., 2022 Shell colors and microstructures of four pearl oyster species in the South China Sea. Aquaculture Reports 25:101214.
Dhami N. K., Reddy M. S., Mukherjee A., 2013 Biomineralization of calcium carbonates and their engineered applications: a review. Frontiers in Microbiology 4:314.
Gervis M. H., Sims N. A., 1992 The biology and culture of pearl oysters (Bivalvia: Pteriidae). ICLARM Studies and Reviews 21, 49 pp.
Joubert C., Piquemal D., Marie B., Manchon L., Pierrat F., Zanella-Cléon I., Cochennec-Laureau N., Gueguen Y., Montagnani C., 2010 Transcriptome and proteome analysis of *Pinctada margaritifera* calcifying mantle and shell: focus on biomineralization. BMC Genomics 11:613.
Kalesaran O. J., Lumenta C., Mamuaya G., Rompas R., Watung J., 2018 Biometric relationships of the black-lip pearl oyster, *Pinctada margaritifera* from North Sulawesi waters, Indonesia. AACL Bioflux 11(5):1587-1597.
Kroger N., Brunner E., Estrof L., Mari F., 2021 The role of organic matrices in biomineralization. Discover Materials 1:21.
Levi-Kalisman Y., Falini G., Addadi L., Weiner S., 2001 Structure of the nacreous organic matrix of a bivalve mollusk shell examined in the hydrated state using cryo-TEM. Journal of Structural Biology 135(1):8-17.
Linard C., Gueguen Y., Moriceau J., Soyez C., Hui B., Raoux A., Cuif J. P., Cochard J. C., Le Pennec M., Le Moullac G., 2011 Calcein staining of calcified structures in pearl oyster *Pinctada margaritifera* and the effect of food resource level on shell growth. Aquaculture 313(1-4):149-155.
Lopes-Lima M., Rocha A., Goncalves F., Andrade J., Machado J., 2010 Microstructural characterization of inner shell layers in the freshwater bivalve *Anodonta cygnea*. Journal of Shellfish Research 29(4):969-973.
Lumenta C., Monijung R., Rampengan R. M., 2019 Nacre characterization of pearl oyster *Pinctada margaritifera* from Arakan waters, North Sulawesi Indonesia. AACL Bioflux 12(1):81-86.
Marin F., Le Roy N., Marie B., 2012 The formation and mineralization of mollusk shell. Frontiers in Bioscience 4(3):1099-1125.
Marin F., Marie B., Hamada B. S., Ramos-Silva P., Le Roy N., Guichard N., Wolf S., Montagnani C., Joubert C., Piquemal D., Saulnier D, Gueguen Y., 2013 'Shellome': proteins involved in mollusc shell biomineralization -diversity, functions. In: Recent advances in pearl research. Watabe S., Maeyama K., Nagasawa H. (eds), TERRAPUB, pp. 149-166.
Muller W. E. G., 2011 Molecular biomineralization: aquatic organisms forming extraordinary materials. Springer Science & Business Media, 404 pp.
Nudelman F., 2015 Nacre biomineralisation: a review on the mechanisms of crystal nucleation. Seminars in Cell and Developmental Biology 46:2-10.
Rousseau M., Rollion-Bard C., 2012 Influence of the depth on shape and thickness of nacre tablets of *Pinctada margaritifera* pearl oyster, and on oxygen isotopic composition. Minerals 2(1):55-64.

Rousseau M., Lopez E., Coute A., Mascarel G., Smith D., Naslain R., Bourrat X., 2005 Sheet nacre growth mechanism: a Voronoi model. Journal of Structural Biology 149(2):149-157.
Rousseau M., Meibom A., Geze M., Bourrat X., Angellier M., Lopez E., 2009 Dynamics of sheet nacre formation in bivalves. Journal of Structural Biology 165(3):190-195.
Southgate P. C., Lucas J. S., 2008 The pearl oyster. Elservier, 574 pp.
Strag M., Maj L., Bieda M., Petrzak P., Jarzebska A., Gluch J., Topal E., Kutukova K., Clausner A., Heyn W., Berent K., Nalepka K., Zschech E., Checa A., Sztwiertnia K., 2020 Anisotropy of mechanical properties of *Pinctada margaritifera* mollusk shell. Nanomaterials 10(4):634.
Xie L. P., Zhu F. J., Zhou Y. J., Yang C., Zhang R. Q., 2011 Molecular approaches to understand biomineralization of shell nacreous layer. Progress in Molecular and Subcellular Biology 52:331-352.
Zhang R., Xie L., Yan Z., 2019 Biomineralization mechanism of the pearl oyster, *Pinctada fucata*. Springer, 737 pp.
Zouari A. B., Cherif K., Elloumi-Hannachi I., Slimane N., Jaafoura, 2011 Characterization of mineral and organic phase in nacre of the invasive pearl oyster *Pinctada radiata* (Leach, 1814). Cahiers de Biologie Marine 52(3):337-348.

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