The presence of irregular layers on the nacre of the high- and low-quality *Pinctada fucata martensii* pearls

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Abstract. Only 60% of the pearls harvested from the Japanese Akoya pearl culture are valuable, and only as low as 10% of them are considered high-quality. Furthermore, many pearls are considered valueless due to the irregular layer deposits, which further cause blemishes and protuberances. Therefore, the importance of studying the pearl's irregular layer is similar to, if not more than, studying the neatly stacked brick-and-mortar nacreous layers. We report the irregular layer on the low-quality pearls to be significantly thicker than that of the high- ($p < 0.001$), even though their total nacre thickness is not ($p > 0.05$), which causes the portion of the irregular layer to total nacre thickness on the low- to be much larger than that of the high- ($p < 0.001$). Furthermore, a significant amount of radially- and tangentially-growing organic structures were observed in the irregular layers of the low-, while the high-quality pearls are generally composed of the aragonite crystals. Consequently, a high portion of the irregular layer to nacre and a vast amount of dark organic matter in the low-quality pearls render its darker color, aggravating its low value.

Key Words: biomineralization, irregular layer, nacre, pearl, *Pinctada fucata martensii*.

Introduction. Species of the *Pinctada* (Roding 1798) genus could deposit $CaCO_3$ (calcium carbonate) in the form of aragonite and calcite crystals by the epithelium of the mantle tissue facing the shells (Garcia Gasca et al 1994; Du et al 2002; Fougerouse et al 2008). These crystals made up the shells, which have three layers consisting of 1) the outermost layer made of organic matter or called periostracum; 2) the second layer made of calcite crystal or better known as the prismatic layer; 3) the third layer made of aragonite crystal or known as the nacreous layer (Fougerouse et al 2008). Thus, not only are the positions of the layers consistent, but the structure of each layer is also unique and different from each other.

When a foreign material enters the cavity of the oyster and somehow injures the vulnerable mantle tissue, aragonite and calcite crystals will be deposited to envelop the materials and form the natural pearls (Wada 1991; Taylor & Strack 2008). The deposition occurs in a soft tissue called "the pearl sacs" that is proliferated from the accidentally excised mantle tissue during the injury. Thus, despite the similarity to that of the shells, calcite is likely to be deposited first followed by aragonite on the natural pearl formation, hence the name "the reversed shells". Such a view was started by Kawakami (1952), which is also confirmed by Taylor & Strack (2008). However, recent studies from Cuif et al (2011, 2020) and Dauphin et al (2013) considered this view inappropriate as pearl formation is complex and involves many forms of $CaCO_3$ polymorphs.

The concept of the natural pearl formation was adopted by human intervention for producing the cultured pearl (Dauphin et al 2013), initiated by Mikimoto Kokichi in the late 19th century in Ago Bay, Mie Prefecture, Japan, and developed by Mise and Nishikawa not too long after (Taylor & Strack 2008). *Saibo* – a piece of the pallial part of the mantle tissue – is purposedly excised from the donor oysters that usually show superior soft and hard tissue growth and are specially prepared to be sacrificed. This tissue piece was then implanted into the recipient oysters along with a rounded nucleus, which subsequently proliferated into a pearl sac. This process is critical in cultured pearl formation as it affects the nacre crystal deposited on the nucleus's surface. It is composed of three essential steps consisting of 1) wound repair after grafting operation; 2) pearl sac creation; and 3) the initial pearl formation (Nakahara & Machii 1956; Machii & Nakahara 1957; Nakahara 1957; Nagai 2013).

Soon after forming, the pearl sac should deposit the thin brick-and-mortar aragonite crystal tablets. The oysters should maintain the deposition process steady for producing a perfectly round high-quality pearl expected by the industry. Our previous study has proven that to produce a white-colored *Pinctada fucata martensii* (Dunker 1880) pearl with a solid pink interference color, which is one of the highest-valued pearls in the Akoya pearl market, the oyster should be able to deposit considerably thick aragonite crystal tablets around the surface of the nucleus, and significantly thin tablets in the middle of the nacreous layer, then keep its thickness stable to the surface of nacre. The oysters should also deposit thin (the thickness) and large (the surface area) nacre tablets before the pearl is harvested to produce a strong interference color pearl (Muhammad et al 2021a). However, this is by far only lasts on the theoretical expectancy. It is almost impossible to program the oysters to naturally deposit the nacre tablets following the theoretical orders to satisfy the market demands. In the Japanese pearl industry, only 60% of pearls harvested are valuable, and only as little as 10% would be considered high quality (Shirai 1981). This report reinforced our previous study that in 4 batches of experimental groups of recipient oysters, 70% of the pearls harvested were considered of low quality (Muhammad et al 2021b). In French Polynesia, where 90% of the *P. margaritifera* pearls are produced, most of the pearls collected after a two-year culturing period are not spherical and show some irregularity on the nacre (Cuif et al 2020).

The irregularity, or in this study henceforth called irregular layer, is defined as a structure deposited by the pearl sac in the form of organic matter, calcite crystal, or the overly thick aragonite crystal due to the lack of organic matrix, which is significantly distinguishable with the brick-and-mortar structure of nacre layers. Previous studies also mentioned this layer as a non-nacreous structure (NNS) (Sato & Komaru 2019; Cuif et al 2020). Furthermore, some studies have mentioned that this layer is formed when the pearl sac proliferation is disrupted, causing the granular hemocytes and cell debris to be included with the bead nucleus, which further induces the protuberances, blemishes, or flaws (Tsujii 1960; Aoki 1961, 1966; Nagai 2013). Hence, we suggested that the importance of studying this structure is similar to, if not more than, studying the neatly stacked brick-and-mortar structure of nacre for pearl aquaculturists and researchers as an attempt to produce a higher number of valuable pearls, as mentioned by Aoki (1966). Furthermore, our previous study has discussed the difference of the irregular layer in the valuable pearls and valueless pearls, which are composed of the aragonite and calcite crystals, respectively (Sato & Komaru 2019). However, we did not study the thickness of the irregularity on the respective pearls quantitatively. Therefore, in this study, we compared the irregular layer thickness by scanning electron microscopy (SEM) on the low- and high-quality pearls judged by a pearl expert quantitatively and an in-depth discussion of the structural differences between the two quality groups.

Material and Method

Oysters and pearls. In June 2018, 400 Japanese oysters were selected as recipient oysters and subsequently implanted with a rounded nucleus and *saibo* from Japanese oyster donors. The recipient oysters were then cultured in Ago Bay (Figure 1). Finally, in December 2018, pearl samples were harvested and then visually graded by a pearl expert who assessed their quality according to their color, shape, and flaws. As a result, 15 high- (henceforth will be called grade H) and 15 low-quality (henceforth will be called grade L) pearls were selected for this study (Figure 2).

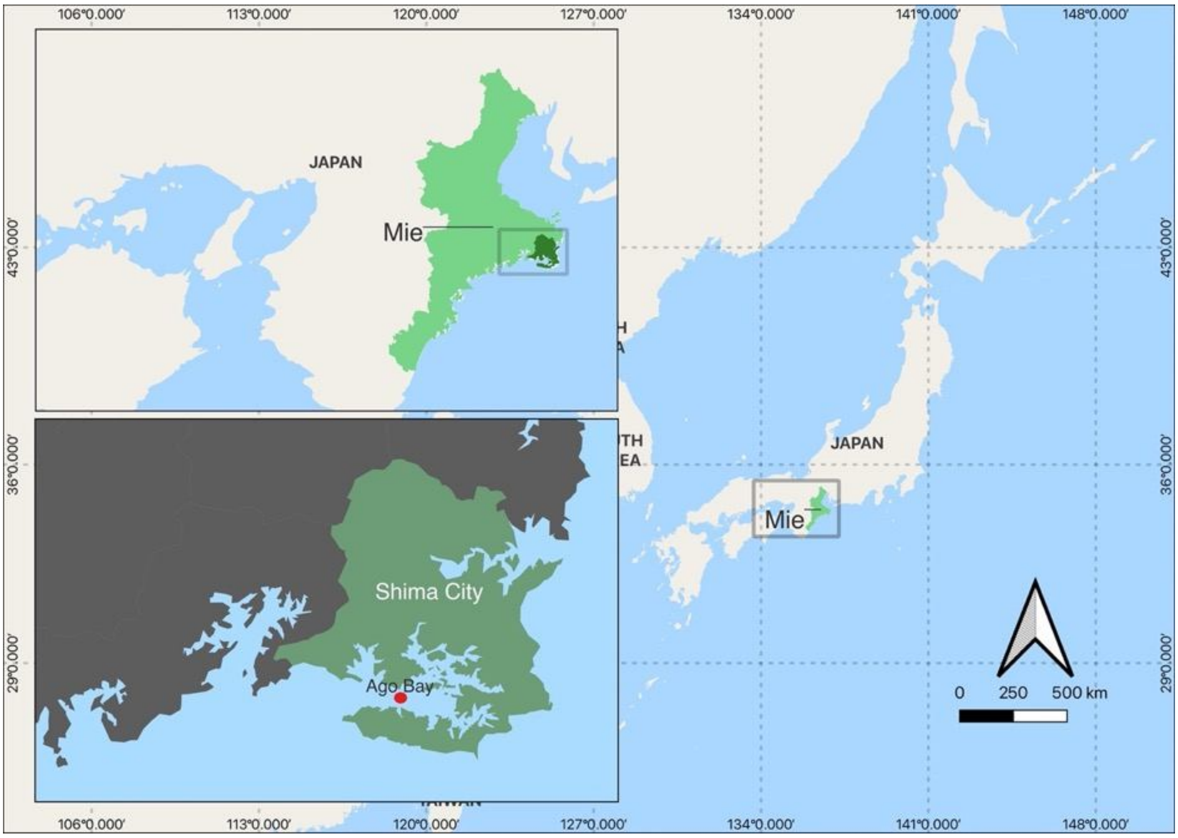

Figure 1. Map of Ago Bay, Shima City, Mie Prefecture Japan. This map was made by QGIS open-source software with shapefile available from https://www.naturalearthdata.com and https://www.gsi.go.jp/.

Figure 2. Pearl samples were used for the irregular layer study. A) 15 grade L pearls used for this study, which shows blemishes, protuberances and generally has darker body color; B) 15 grade H pearls used for this study, which have white body color and visible luster and interference color.

Cross-sectional sample preparations. Pearls were sectioned to measure nacre tablet thickness following our protocol that involved embedding, polishing, and etching (Muhammad et al 2021a).

Nacre irregular layer observation. A tabletop TM-1000 SEM (Hitachi, Japan) was used to observe and estimate the total nacre and irregular layer thickness. Four pictures from 4 sampling sites (Figure 3A) were captured, from which the total nacre and irregular layer thickness were located and measured using ImageJ open-source software (National Institute of Health, United States) and calibrated using digital scales on each SEM image following software protocols (Figure 3).

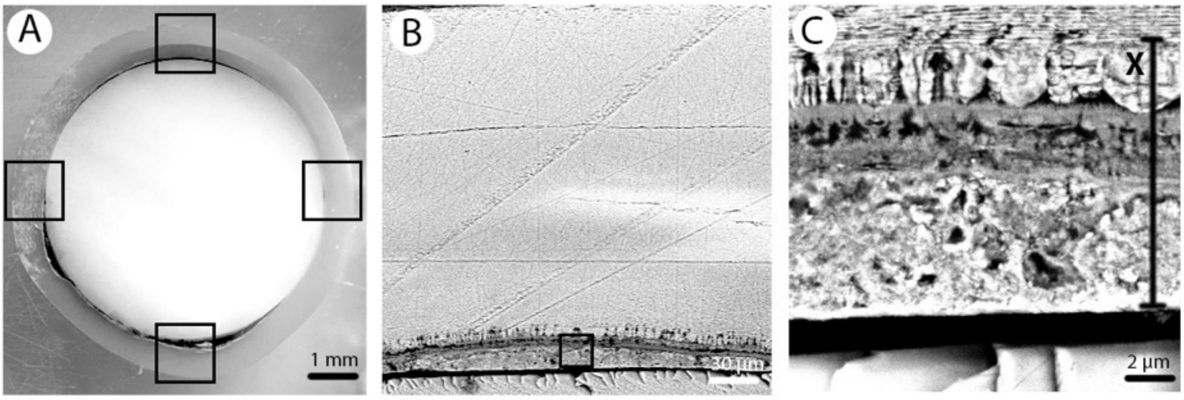

Figure 3. A pearl from the second experiment captured by the stereo microscope and SEM. A) four sampling sites on each pearl; B) pearl with the irregular layer on the SEM with 600 × magnification; C) the irregular layer on B in the rectangle area with 2.500 × magnification; X) the irregular layer thickness measured by ImageJ.

Statistical analysis. Mann-Whitney U test was conducted to compare the total nacre thickness, irregular layer thickness, and the percentage of the irregular layer from the total thickness of nacre between grade H and L pearls. The analysis and data visualization used open-source software, RStudio, following the codes available from Albert & Rizzo (2012) and Chang (2013).

Results. Despite there were no significant differences in the pearl total thickness of the grade H (273.408±12.922 µm) and grade L (339.734±20.390 µm) pearls ($p > 0.05$, Figure 4A), the significant differences of the irregular layer thickness between the two quality groups were apparent ($p < 0.001$, Figure 4B), amounted 2.705±0.546 µm and 73.689±9.395 µm for the grade H and grade L pearls, respectively. The ratio of the irregular layer compared to the nacre thickness of the pearls was also analyzed. Grade H pearls showed a significantly low ratio of irregular layers to the nacre thickness (0.9±0.1%) compared to grade L pearls (24.4±3%) ($p < 0.001$, Figure 4C).

The fluorescence microscopy and SEM observation on the grade L pearls have revealed that most of them deposit a significant amount of radially (Figures 5C, 5D) and tangentially growing (Figures 5K, 5L) organic structure on the nucleus surface. The radially growing organic structure glows under the UV-1A filter and resembles the prismatic layer of the shells. These organic structures are deposited with the calcite crystals (Figures 5D, 5H) or aragonite crystals (Figure 5L).

Despite the fact that the grade H pearls do not show any dark pigmentation and protuberances (Figures 6A, 6D), the fluorescence microscopy and SEM observation revealed that some of them still show irregular layers on their nucleus surface. However, unlike grade L pearls, which deposit a significant amount of organic structure on their irregular layer areas, most of the grade H pearls' irregular layer consists of calcite and aragonite crystals (Figures 6F). Moreover, the nacre layer deposited afterward is thick and regular enough to cover the irregular layer on the nucleus surface, making the protuberance invisible from the pearl surface. Nevertheless, pearls with no irregular layers are dominant in numbers in the grade H pearls' population. The aragonite crystal is deposited around the nucleus surface in the form of the concentric mineralizing sheet (CMS), which is slightly thicker than the subsequent nacre layers. Hence, a clear brick-and-mortar view can be observed right after the nucleus surface (Figure 6C).

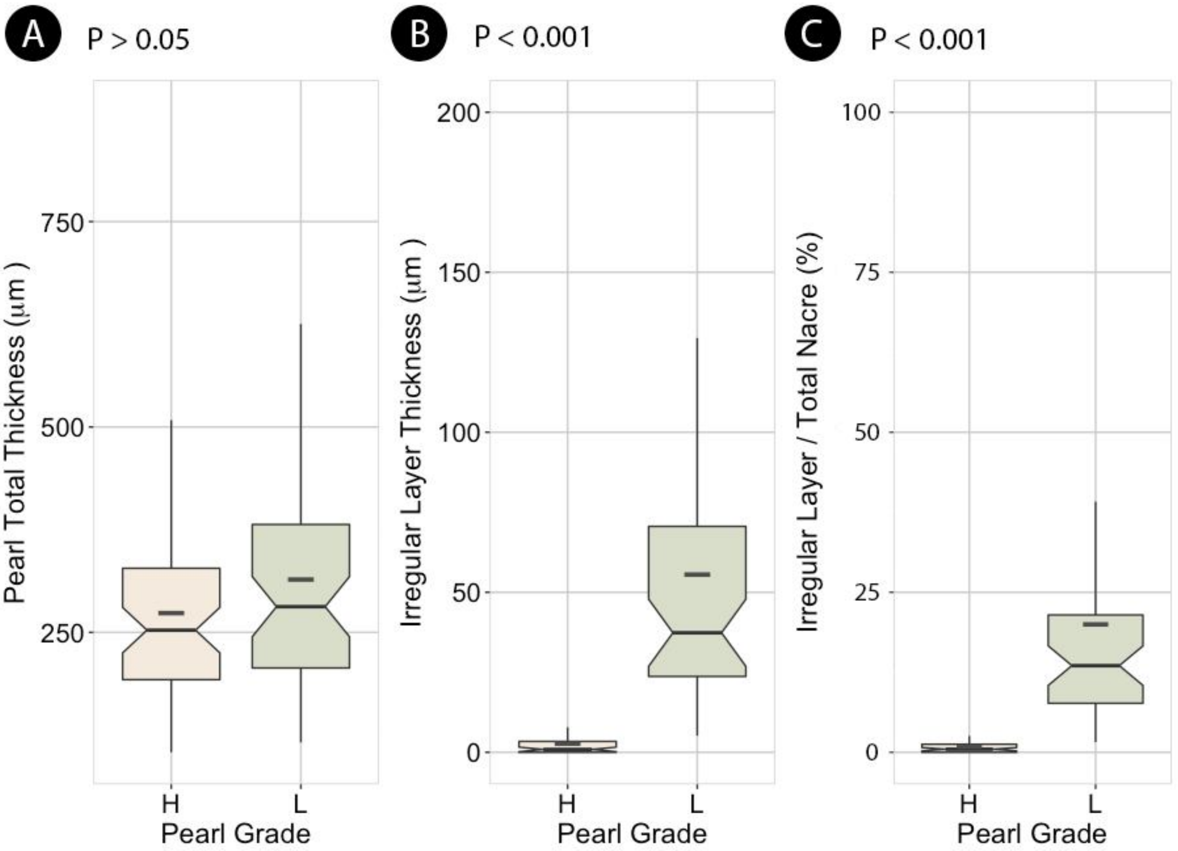

Figure 4. Pearl total thickness (A), irregular layer thickness (B), and the percentage of the irregular layer compared to the nacre thickness on the high- and the low-quality pearls (C). P-value shows the significance of the Mann-Whitney U test between the groups of samples.

Discussion. This study reports that the irregular layer is present on the grade L and some of the grade H pearl groups. The differences between them are the type of the irregular layer, the thickness, and the portion to nacre total thickness. The type of irregular layer crucially determines the pearl quality as it affects the color that appears as the light reflected by the basal part of the nacreous layer. Our previous study discussed this, which concluded that the irregular layer on the valuable pearls is exclusively composed of aragonite (Sato & Komaru 2019). However, we did not address the amount of organic structure in the irregular layer, which renders the darker color on the grade L pearls. This study complemented what we have been discussed in Sato & Komaru (2019) that in addition to the calcite crystal, the irregular layer of the grade L pearls also has aragonite crystal and, most notably, a significant amount of radially (Figure 5D) or tangentially (Figure 5L) growing organic structure. Wada (1999) has reported that this organic structure is what the pearl farmer called impurities on the nucleus's surface and is the causative behind the pearl body color as the organic structure tends to be rich with dark pigment. Therefore, the low-quality Akoya pearls with prominent protuberances and an uneven structure are likely to have darker body color (Figures 5A, 5E, 5I) than their higher-quality counterparts (Figures 6A, 6D).

A significant amount of organic structure is likely to be formed due to the proliferating process of the pearl sac from the *saibo*. If the pearl sac proliferation process is disrupted, it causes the organic substances such as hemolymph, inflammatory cells, germ cells, and bacteria to be included with the bead nucleus (Tsujii 1960; Aoki 1961, 1966; Norton et al 2000; Nagai 2013). Another study reported that this is related to the histological changes the oysters went through as an inflammatory reaction towards bacterial infections (Ogimura et al 2012). Therefore, the process before (pre-operative), during (operative), and after (post-operative) the implantation of the nucleus and *saibo* is significant (Taylor & Strack 2008; Nagai 2013) and has been attracted the attention of many studies.

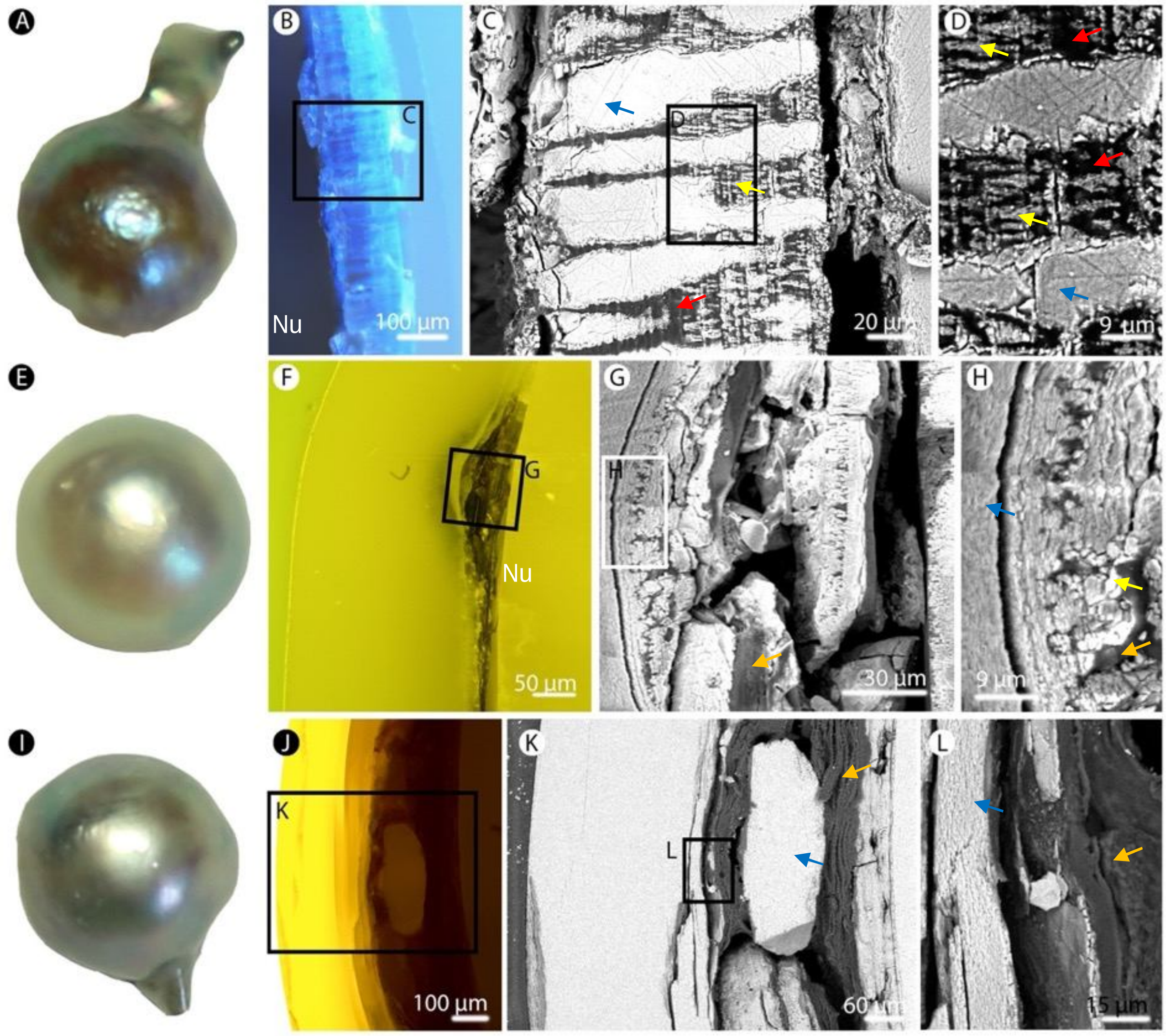

Figure 5. The microstructure of three selected grade L pearls (A, E, I). B) a cross section views of the A pearl under the fluorescence microscopy showing a prismatic-like irregular layer that glows under the ultraviolet UV-1A filter; C) the squared area of B observed on the SEM showing an aragonite-calcite structure with the radially growing organic structure; D) a higher magnification the squared area of C on the SEM pointing out the aragonite, calcite, and the radially growing organic structure; F) a cross section view of the squared area of E on the fluorescence microscopy with B-2A blue excitation filter with 450-490 nm excitation length showing an irregular layer forming an inner protuberance; G) the squared area of F on the SEM showing the aragonite, calcite, and organic structure; H) a higher magnification of the squared area of G pointing out the aragonite and calcite structures; K) a cross-section view of I on the fluorescence microscopy with B-2A blue excitation filter 450-490 nm filter; K) the squared area of J on the SEM showing a massive lump of tangentially growing organic structure surrounding the aragonite crystals; L) a higher magnification of the squared area of K pointing out the tangentially growing organic structure and aragonite crystal; Blue arrow pointing out aragonite crystal; red arrow pointing out the radially growing organic structure; yellow arrow pointing out the calcite crystal; orange arrow pointing out the tangentially growing organic structure; Nu) Nucleus.

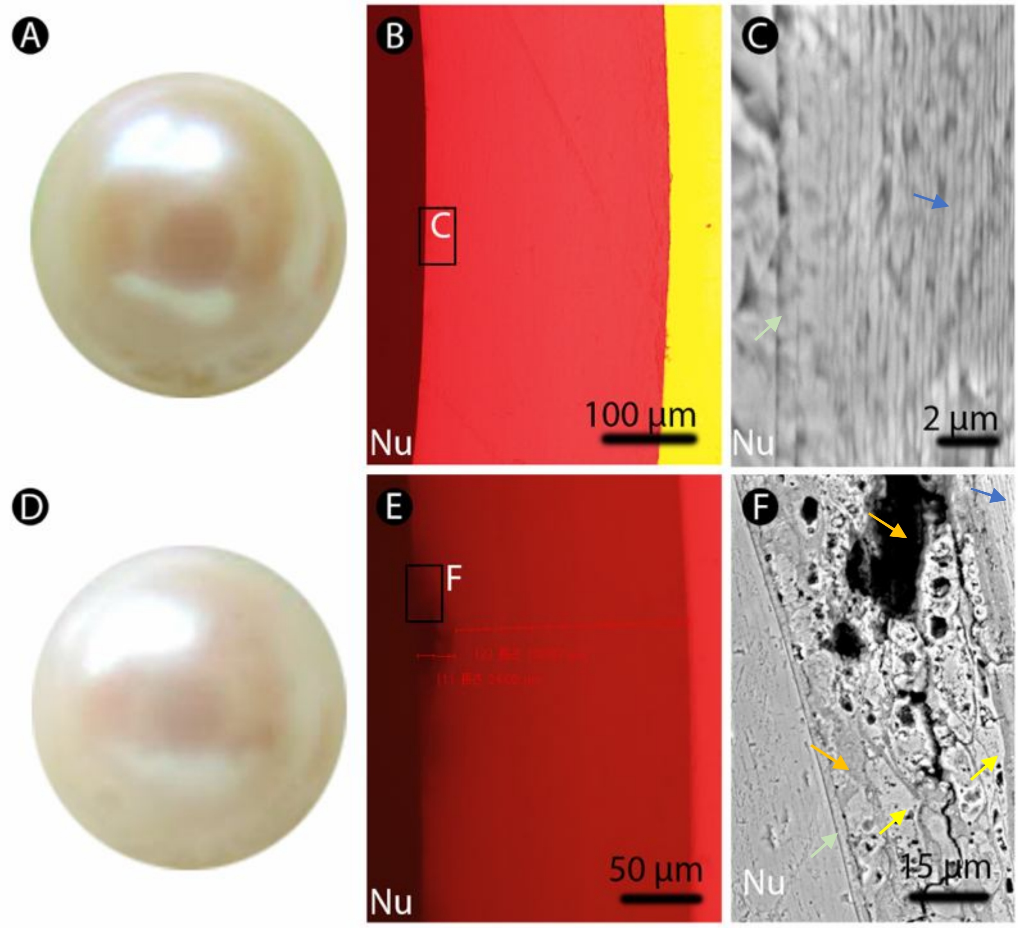

Figure 6. The microstructure of two selected grade H pearls that has no (A) and has (D) irregular layer on its nucleus surface. B) the cross-section view of A on the fluorescence microscopy with a G-2A filter (green excitation wavelength, 510-560 nm); C) the squared area of B on the SEM with 10.000 × magnification pointing out the nacre tablets (blue arrow) and the concentric mineralizing sheets (green arrow); E) the cross-section view of D on the fluorescence microscopy with a G-2A filter (green excitation wavelength, 510-560 nm) showing a small protuberance on the surface of the nucleus; F) a higher magnification of the squared area of E on the SEM pointing out the irregular layer consisting of the organic structure (orange arrows), calcite crystal (yellow arrows), aragonite crystal (blue arrow) and the concentric mineralizing sheet (green arrow); Nu) nucleus.

Our previous study has reported that low-salinity post-operative conditioning lowered the chance of the production of pearls with blemishes by five folds compared to the conventional method (Atsumi et al 2014). Even though we could not prove that this method lowered the hemolymph activity (Sano et al 2017), our later study reported that this method could delay the pearl sac formation process and is positively correlated with a higher chance of a high-quality pearl formation (Atsumi et al 2018).

Furthermore, the irregular layer on the grade L pearl groups was particularly thick. It causes the uneven development of nacre (Cuif et al 2020), which further induces protuberance on the surface of pearls (Figures 5A, 5E, 5I) (Wada 1999; Taylor & Strack 2008). Hence the uneven shape, rendering their invaluable grade in the industry as the shape is one of the grading categories in addition to the surface flaws, size, luster, and color (body- and interference-color) called "the five virtues" (Taylor & Strack 2008).

The thick irregular layer deposit in the grade L pearl group also causes its portion to the total nacre thickness more considerable in percentage than the high-quality pearl group. When there is an incident light hits the pearl (*i*), it will be reflected and diffracted by the surface (*a*) and the translucent inner part (*a'*) of the nacreous layer. Light passes through the basal part of the nacreous layer will be reflected by the nucleus's surface (*b*) and diffused and absorbed by the nacre and the pigment-containing conchiolin (*c*). The combination of *b* and *c* determines the pearl body color (Wada 1999; Muhammad et al

2021a). Thus, although both groups showed no significant differences in pearl nacre total thickness, the quality differences were apparent because the high-organic-structure-contained irregular layer is reflected in the grade L pearl group (Figure 7C). Since the grade H pearl group has a significantly higher nacre portion, even when there is an irregular layer on the surface of the nucleus, the regularly stacked nacreous layer would render its shape to be more even before the harvest (Figures 6D, 6E), and the low-organic-structure-contained irregular layer reduce its chance to reflect a darker body color (Figure 7B).

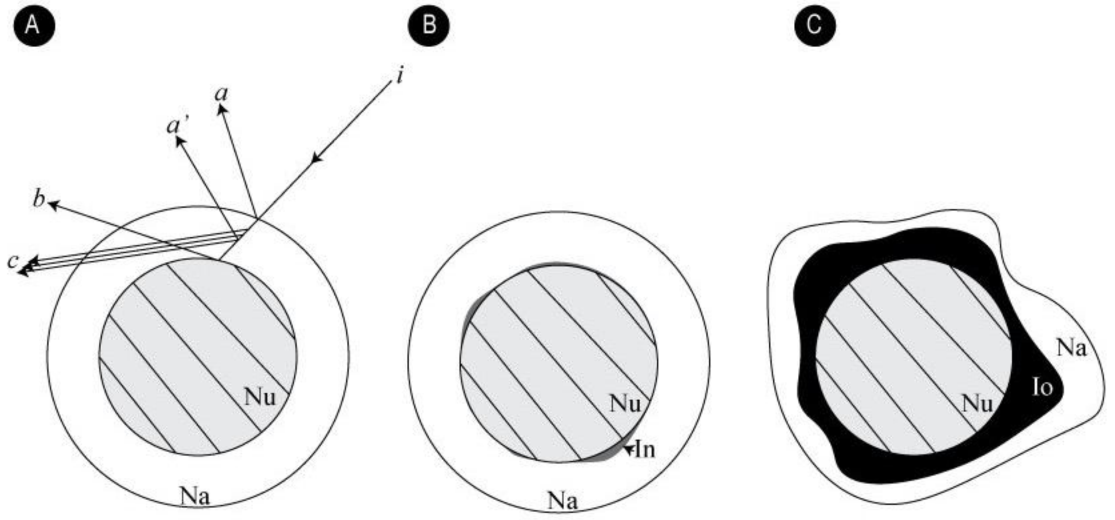

Figure 7. Schematic of light reflection on the surface and inner parts of cultured pearl nacre in grade H pearl with no irregular layer on the nucleus surface (A), grade H pearl with low-organic structure-contained irregular layer on the nucleus surface (B), and grade L pearl with high-organic structure-contained irregular layer. *i*) Incident light; *a*) light reflected and diffracted on nacre surface; *a'*) light reflected and diffracted by aragonite crystal lamellae in the nacreous layer; *b*) light reflected by the surface of the nucleus; *c*) transmitted diffusion light passing through nacre; Nu) nucleus; Na) nacreous layer; In) low-organic structure-contained irregular layer; Io) high-organic structure-contained irregular layer (redrawn and modified from Wada (1999)).

Conclusions. This study has reported that the irregular layer is likely found in the grade L and some of the grade H pearls. Since the total thickness of the nacre in both groups is similar, the thickness of the irregular layer highly determines its portion to the nacre, which further influences the visual look of the pearls. The more significant the portion of the irregular layer to the nacre, the more pronounced the blemishes and protuberances are. Furthermore, the grade L pearl irregular layer tends to contain a significant amount of organic structure compared to the grade H. Consequently, a high portion of the irregular layer to nacre and a significant amount of dark organic matter in the grade L pearls render its darker color, aggravating its low value. Further study related to the proliferation process of the *saibo* to the pearl sac is needed to understand better what triggered the irregular layer formation on the nucleus surface.

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

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