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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Investigation of the structure and hardness properties of *Anodonta anatina* mussel shells

Anodonta anatina midye kabuklarının yapısı ve sertlik özelliklerinin araştırılması

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Abstract: In this study, the shell structure of the freshwater mussel *Anodonta anatina* (Linnaeus, 1758) which has a widespread population in Gölbaşı Lake (Hatay) and is not economically exploited, was microscopically examined at a morphological level. It was determined that the shells of *Anodonta anatina*, which are not under significant fishing pressure, are mostly found discarded along the shores of the lake. This mussel species is important as a composite biological material with multifunctional roles in freshwater ecology. Considering the potential use of freshwater mussel shells as a biological material, an assessment of the shell structure, physical properties, mechanical strength, shell microstructure, and morphological characteristics of *A. anatina* was conducted. When cross-sections of the shell taken from the umbo, middle periostracum, and the region close to the pallial edge were examined in the dorsal-ventral direction, it was determined that the periostracum layer in the umbo region had a more prismatic and polygonal structure. The interior of the shell was found to consist of a shiny nacreous layer. In nacreous shell sections, it was observed that the nacreous layer contained more distinct layers near the pallial edge. Vickers microhardness tests were performed on individual shells, and it was found that the hardness value of the inner layer was the highest (625.5 ±172.7 HV), while the outer layer had a lower hardness value (531.5 ±110.7 HV). Based on XRF data, it was shown that the seashell powder is mainly composed of calcium oxide (98.8% wt., CaO) as a biological material.

Keywords: Biocomposite, bivalve, microhardness, morphological properties, nacre

Öz: Bu çalışmada, Gölbaşı Gölü'nde (Hatay) yaygın bir popülasyonu bulunan ve ekonomik olarak değerlendirilmeyen tatlı su midyesi *Anodonta anatina*'nın (Linnaeus, 1758) kabuk yapısı, morfolojik olarak mikro düzeyde incelenmiştir. Midye avcılığı bakımından üzerinde av baskısı olmayan *A. anatina'nın* kabukları çoğunlukla gölün kıyısında âtıl olarak bulunduğu tespit edilmiştir. Bu midye türü, tatlı su ekolojisi açısından, çok işlevli rollerle ilişkili kompozit bir biyolojik malzeme olma yönünde önem taşımaktadır. Biyolojik bir materyal olarak tatlı su midye kabuğunun olası kullanımı göz önüne alındığında, model tür olarak *A. anatina*'nın kabuk yapısı, fiziksel özellikleri, mekanik dayanımları, kabuk mikro yapıları ve morfolojik özelliklerinin değerlendirmesi yapılmıştır. Kabuğun dorsal-ventral yönde umbo, orta periostrakum ve pallial kenara yakın bölgeden alınan enine kesitleri incelendiğinde, umbo bölgesindeki periostrakum tabakanın daha prizmatik ve poligonal olduğu tespit edilmiştir. Kabuğun iç kısımlarının parlak sedef tabakasından oluştuğu ve sedefli kabuk kesitlerinde, sedefli tabakanın pallial kenara yakın bölgede daha belirgin sedef katmanları içerdiği belirlenmiştir. Midye kabukları üzerinde, Vickers mikrosertlik testleri yapılmış, iç tabakanın sertlik değerinin en yüksek (625,5 ±172,7 HV) ve dış tabakanın sertlik değerinin iç tabakaya göre daha düşük (531,5 ±110,7 HV) olduğu belirlenmiştir. *A. anatina* kabuk tozlarının kimyasal bileşimi, X-ışını floresans spektroskopisi ile kalsinasyon işleminden sonra analiz edilmiştir. XRF verilerine dayanarak, biyolojik bir malzeme olan kabuk tozlarının ana bileşiminin kalsiyum oksitten (CaO, ağırlıkça %98,8) oluştuğu belirlenmiştir.

Anahtar kelimeler: Biyokompozit, çift kabuklu yumuşakça, mikrosertlik, morfolojik özellikler, sedef

INTRODUCTION

Anodonta anatina (Linnaeus, 1758) is an enigmatic nominal taxon with a limited distribution in the Orontes River drainage in Turkey and Syria (Graf and Cummings, 2007; Lopes-Lima et al., 2021). It was first identified from the former Amik Lake (historically Lake of Antioch), (Linnaeus, 1758) a large but shallow freshwater lake in the Lower Orontes River basin, Hatay Province, Turkey (Graf and Cummings, 2007; Tomilova et al., 2020). The reproductive cycle and glochidia of *A. anatina* have been carefully examined in the literature (Şereflişan et al., 2009a, b). The shell of a freshwater mussel at the larval stage contains an organic matrix and chitin, primarily composed of calcium carbonate (Schönitzer and Weiss, 2007; Weiss and Schönitzer, 2006; Lopes-Lima et al., 2008). In mollusk shells, hard, protective, and solid inorganic mineral formation mechanisms are often referred to as biomineralization. The most common biominerals in mollusk shells are calcium carbonate and calcium phosphate (Istin and Kirschner, 1968; Lowenstam and Weiner, 1989; Pratoomchat et al., 2002; Wegner, 2005; Ziegler et al., 2006). Mollusks produce a highly ordered organomineral composite skeleton to protect and support their soft tissues (Carter, 1991).

Biomaterials in engineering have become increasingly popular due to their unique properties and potential for sustainable development. One such biomaterial is the mineral composition of mollusk shells, which typically consist of calcium carbonate ($CaCO_3$) in the form of calcite and/or

aragonite. These two forms of $CaCO_3$ have distinct morphological and microstructural characteristics, which have been attributed to several different factors. Three estimates have been proposed regarding the orientation of these structures and crystals, including the specific proteins present in the organic matrix of the shell, cellular remodeling, and physicochemical conditions in the microenvironment of biomineralization (Zhang et al., 2019). Understanding the mechanisms behind the formation of these mineral structures is critical for their use in various engineering applications. For example, mimicking the structure and properties of mollusk shells could lead to the development of stronger and more durable materials for use in the biomedical industry, material science, marine science, and other industries. Additionally, the renewable and biodegradable nature of these biomaterials makes them an attractive alternative to traditional, non-renewable materials that are harmful to the environment (Barthelat et al., 2009; Dhanaraj and Suresh, 2018; Lemos et al., 2006).

In this experimental study, the researchers aimed to investigate various properties of the shell of *A. anatina*, a type of mollusk, that could potentially make it a valuable resource for different fields of biomaterial. Specifically, they examined the microstructure, chemical composition, and microhardness of the shell in order to gain a better understanding of its potential uses. Although the properties of mollusk shells have been discussed in previous marine sciences literature, this study marks the first time, to the authors' knowledge, that the structural shell properties of *A. anatina* have been evaluated in such detail. By conducting this study, the researchers hope to provide valuable insights into the potential applications of this particular type of mollusk shell and to inspire further research into the unique properties of natural materials like this.

MATERIALS AND METHODS

Preparation of freshwater mussel shell samples

A. anatina shells were collected from Gölbaşı Lake between April and May of 2022. Lake Gölbaşı is located in Turkey's Eastern Mediterranean region, 50 kilometers north of Antakya. The mesotrophic nature of Gölbaşı Lake was determined through a study conducted using the Brachionus/Trichocerca index (Türkmen et al., 2006). The lake has a surface area of approximately 12,000 decares, with 4,000 decares consisting of wet-reed fields. During summer, the maximum depth of the lake reaches 4 meters, while in winter, it can reach up to 6 meters (Şereflişan, 2003). Four stations were designated in the lake for mussel collection. Among the mussels collected, mature individuals whose shells were not damaged were preferred. The location and coordinate information of the collection stations was provided in Figure 1. *A. anatina* was found to inhabit the muddy substratum of the lake. The collection was performed manually by SCUBA diving at depths ranging from 0 to 3 meters. After collection and identification (Graf and Cummings, 2007; Kinzelbach, 1989), the soft tissues were carefully removed using a scalpel. The remaining shells were cleaned under running water, disinfected with ethanol, and then sun-dried for three days. In addition, any fresh remains adhering to the shells were carefully removed before using them in the experiments. The shells were measured for total length to the nearest 0.01mm using a digital vernier caliper and weighed to the nearest 0.01g. The shell length of the mussels is the distance from the anterior edge of the valve to the posterior edge, the shell width is the distance from the dorsal edge of the valve to the ventral edge, and the shell height was measured as the umbo height between the two valves of the mussel held horizontally (Figure 2).

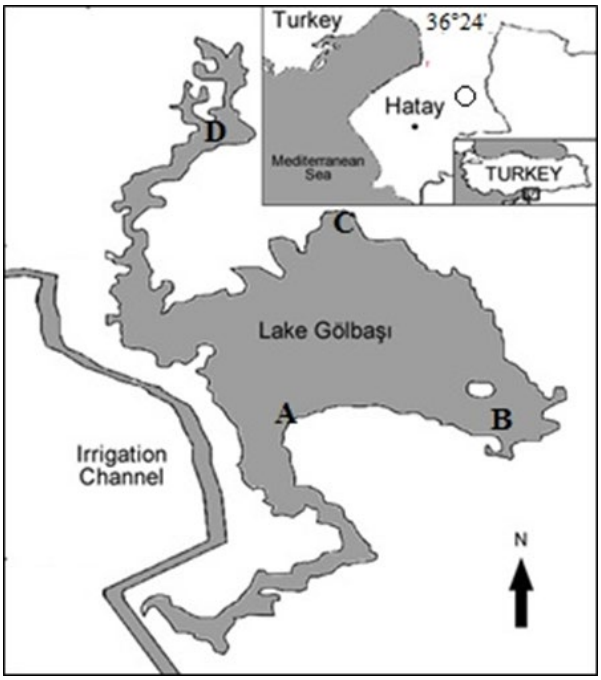

Figure 1. Collection area of *A. anatina* and coordinates of four stations determined in the study area. A: 36°30'15" N - 36°29'14" E, B: 36°30'10" N - 36°29'43" E, C: 36°30'43" N - 36°29'20" E, D: 36°30'55" N - 36°28'53" N

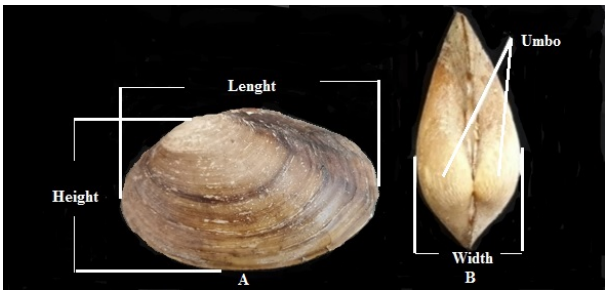

Figure 2. Schematic image of body length, height (A) and width (B) measurement of *A. anatina*

In order to achieve the characterization of the shells, 4 shells of *A. anatina* were randomly selected from the collections. The mean length, height, width, live weight, and total shell weight of the mussels were determined as 10.08±0.22 cm, 5.98±0.19 cm, 3.02±0.15 cm, 58.29±0.23 g, and 34.15±0.50 g respectively. Selected areas were marked and prepared for the experiments related to shell microstructure and mechanical characterization (Lee et al.,

2008; Liang et al., 2016). All experiments were performed in triplicate.

Vickers microhardness (HV) experiments

In order to investigate the Vickers microhardness of shells, the shells with dimensions of 65mm × 15mm × 1mm were cut from the umbo to toward the posterior end of the body using a precision cut-off machine (Brilliant 220–ATM GmbH) with diamond disc and cooling water under a fixed cutting condition of n = 4000 rpm and f_z =5 mm/z. The cut shells were sliced transversely again. To achieve homogeneity in the loading direction and to facilitate the microhardness test, the specimens were fixed with epoxy resin (Epoxy Cure, Buehler). After grinding off the epoxy resin on the surface of the sample, it was carefully polished using abrasive papers to measure the microhardness of the outer layer. The same modified procedure was applied to the inner layer of the shells (Liang et al., 2016). The Vickers microhardness test was performed on a Shimadzu HMV-2000 model 3212 at 0.1 kg-f load and 15 s dwell time according to ASTM C1327-08. Thirty-two indents were made per region (outer/inner surface) for each mussel shell, and the data are presented as mean ±standard deviation (SD). The distribution of the Vickers microhardness values for the outer layer and inner layer was characterized and discussed (Özer and Öksüz, 2019; Yang et al., 2011).

Shell microstructure analysis

Light microscopy (LM) investigations were conducted using a Nikon ECLIPSE L150 instrument to observe the microstructures of the freshwater mussel shells. Freshwater mussel shells selected for morphological analysis were gently broken into pieces, and smooth surfaces were chosen to capture images of the inner, outer, and cross-section surfaces. Each shell fragment was embedded separately in epoxy resin and marked on the surface of the surrounding epoxy resin block. Subsequently, resin blocks were carefully grinded with emery paper from 400# to 1500# and then ultra-polished using aluminum oxide (5µm) and polycrystalline diamond paste (0.25µm). To access the successive inner layers of shells, a dilute chemical etching agent, ethylene diaminetetraacetic acid (EDTA) was used at room temperature for 5 minutes. The samples were cleaned in an ultrasonic bath with anhydrous ethanol for 30 minutes at 35°C and then sun-dried for microstructural observations (Meng et al., 2018; Yang et al., 2011).

X-ray fluorescence analysis (XRF)

Energy dispersive X-ray fluorescence spectroscopy (XRF, Thermo Scientific™ Niton™ XL3t, USA) was used to evaluate the chemical composition of the shell powders. Freshly prepared dry shells from four different, undamaged, mature, and healthy specimens were ground into powder using a mortar and pestle for XRF analysis. The crushed mussel shells were then calcined at 900°C for 3 hours, with a heating rate of 5°C/min, to determine the amount of calcium oxide (CaO) present (Gao et al., 2019; Moustafa et al., 2015).

Statistical analyzes

The collected data were presented as means ± standard deviations of the mean SD based on at least thirty-two independent measurements. Data were statistically analyzed using a Bivariate Correlate test was used to determine the degree of correlation between the variable of external shell hardness and internal shell variables of hardness. The strength of the correlation was measured by using a correlation coefficient (Pearson's Correlation r) at a statistical significance level of 0.05 using SPSS 20.0 (Newyork, USA) software.

RESULTS

Microstructure observation and characterization

The optical microscope photographs of *A. anatina* mussel shells showed three distinct layers in the transverse section: the outer periostracum layer, the prismatic layer, and the inner nacreous layer. The optical microscope photographs presented in Figure 3 of the *A. anatina* mussel shells show that the outermost layer, known as the periostracum, consist of a proteic sclerous double layer that cover the external surface of the shells (Checa, 2000). Below the periostracum, there is a layer without a definite shape, which is comprised of calcite ($CaCO_3$). Next, comes the aragonite sheet nacreous layer, which makes up nearly the entire thickness of the shell, and finally, the aragonite prismatic layer (Chakraborty et al., 2020). Polishing and chemical acid etching revealed the inner vacuolar periostracum, which was frequently continuous with the following inner mineralized layers. Optical microscope photos of the inner vesicular layers of the shells periostracum revealed prominent vacuoles (Carter, 1991; Nakamura-Filho et al., 2014; Vaughn, 2017). The microstructure described can be readily observed in Figure 4 and Figure 5 through the optical microscope photographs, displaying distinct features and characteristics.

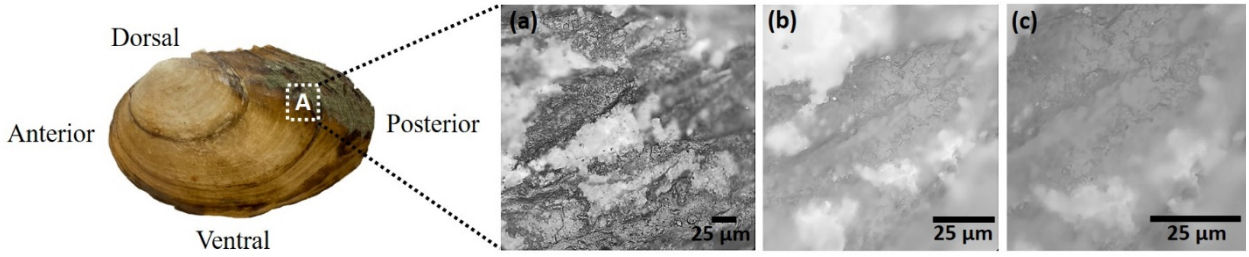

Figure 3. General and microstructural photographs of the periostracum of *A. anatina* at various magnifications in the dorsal-ventral direction. (A) Middle periostracum, (a) 20x, (b) 50x, (c) 100x

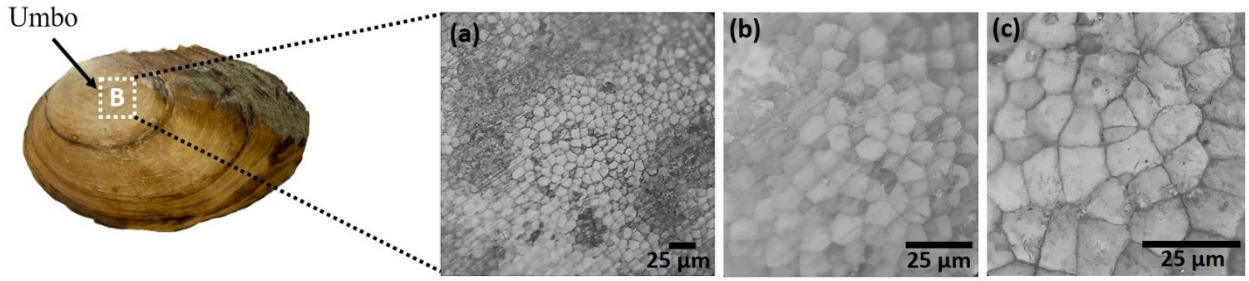

Figure 4. General and microstructural photographs of the periostracum of *A. anatina* at various magnifications in the dorsal-ventral direction. (B) Umbo, (a) 20x, (b) 50x, (c) 100x

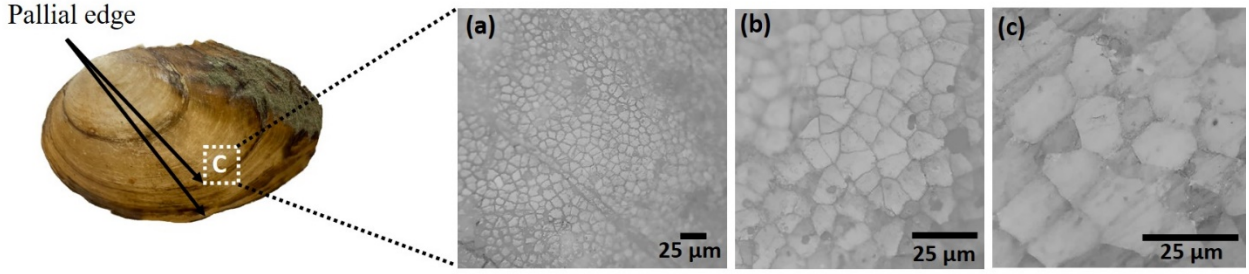

Figure 5. General and microstructural photographs of the periostracum of *A. anatina* at various magnifications in the dorsal-ventral direction. (C) Pallial edge, (a) 20x, (b) 50x, (c) 100x

The optical microscope examination of *A. anatina* mussel shells reveals that all the samples studied exhibit well-preserved nacreous layers. Figure 6 provides microstructural representations of the successive shell layers, with a focus on the mineralized internal layers. It shows that the inner part of the *A. anatina* shell structure is primarily composed of the polymorph aragonite, arranged microstructurally in superimposed sheets. This aragonite sheet nacreous layer covers almost the entire thickness of the shell and is made up of brittle $CaCO_3$ platelets stacked in layers and held together by organic biopolymers (Chakraborty et al., 2020; De Paula and Silveira, 2009).

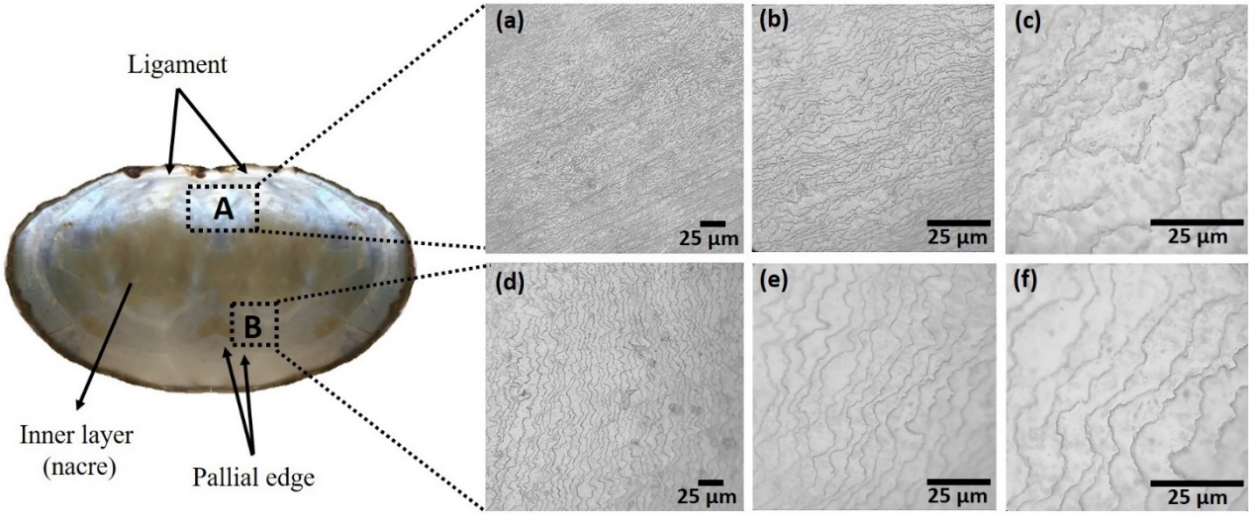

Figure 6. General and microstructural photographs of the inner layer (nacre) of *A. anatina* at various magnifications in the dorsal-ventral direction. (A) Ligament edge, (B) Pallial edge. (a-d) 20x, (b-e) 50x, (c-f) 100x

The shell of the *A. anatina* freshwater mussel contains two prismatic layers, as observed from the cross-section views in Figure 7. The prismatic layer is composed of various columnar crystals and an organic matrix, and a fiber prismatic structure with a common structural nodule is located beneath the periostracum layer. The cloud layer, located below the periostracum layer, is composed mainly of the calcite phase above the aragonite nacreous sheet layer (Carter et al., 2012).

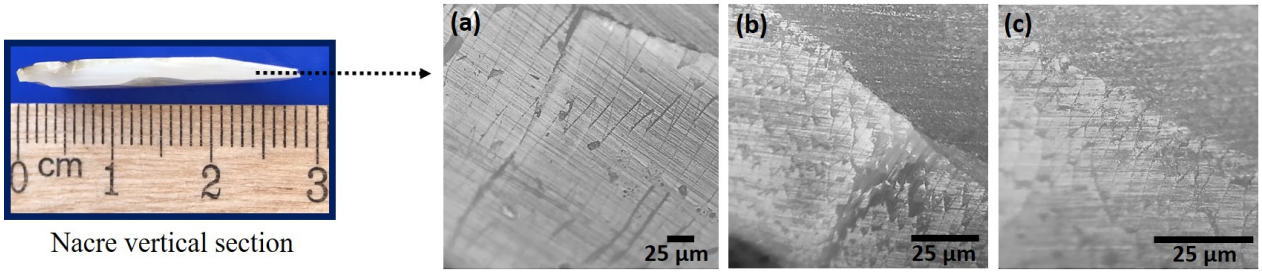

Figure 7. Cross-sectional photographs of the *A. anatina* at various magnifications (a) 20x, (b) 50x, (c) 100x

Microhardness of *A. anatina* shells

Figure 8 presents the relationship between the average microhardness and their corresponding standard deviation of different shell structures at different applied distances (µm). It shows that the Vickers microhardness for the inner and outer surfaces exhibited opposite trends. The experimental studies revealed that the external shell of *A. anatina* has a comparatively lower microhardness than the internal shell from the umbo to the posterior end regions. The mean microhardness values of the external and internal shell samples of *A. anatina* were 531.5 ±110.7 HV and 625.5 ±172.7 HV, respectively.

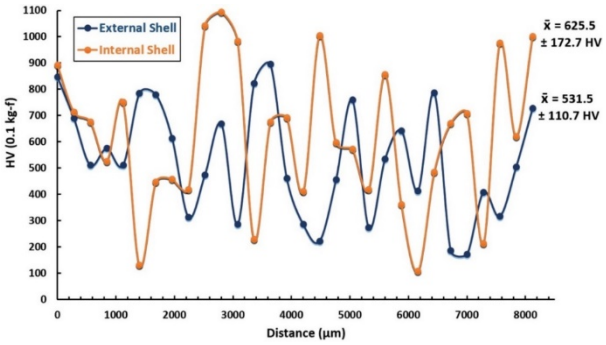

Figure 8. The Vickers microhardness results of the *A. anatina* shell samples

In this study, we conducted an investigation into the relationship between the microhardness values of the external shell and internal shell. To analyze this relationship, we utilized the Bivariate Correlate test, a statistical tool commonly employed to examine the association between two variables (Table 1). The strength of the relationship was quantified using the Pearson correlation coefficient, which yielded a value of r = -0.121. Additionally, we determined the two-tailed significance value, denoted as P, which was calculated to be P = 0.511. To assess the statistical significance of the obtained results, we compared the significance level of 0.025, which represents the predetermined threshold for accepting or rejecting the hypotheses. Upon careful evaluation, we observed that the calculated significance value (P=0.511>0.025) exceeded the significance level (0.025). Moreover, the negative value of the Pearson correlation coefficient (r=-0.121<0) indicated a negative association between the microhardness values of the external and internal shells. Considering these findings, we conclude that there is no significant linear relationship between the microhardness values of the external shell and internal shell in the context of our study.

Table 1. Correlation factors of microhardness values

Correlations		External Shell HV	Internal Shell HV
External Shell HV	Pearson Correlation	1	-,121
	Sig. (2-tailed)		,511
	N	32	32
Internal Shell HV	Pearson Correlation	-,121	1
	Sig. (2-tailed)	,511	
	N	32	32

X-Ray Fluorescence Analysis (XRF)

The elemental/mineral composition of shell powders from the freshwater mussel *A. anatina* was qualitatively determined using XRF analysis (Tomilova et al., 2020). The XRF examination of shell powders revealed that the concentration of calcium oxide (CaO) was 98.8 wt.% after calcination at 900°C for 3 hours as shown in Table 2. In Table 2, as for the other oxides, it predominantly comprised 0.628 wt.% SiO_2, 0.174 wt.% K_2O, 0.132 wt.% MgO, and 0.069 wt.% SrO_2 and the remainder were present in negligible levels (SO_3, Al_2O_3, P_2O_5, Fe_2O_3, and CuO were <0.05 wt.%).

Table 2. XRF analysis of the *A. anatina* shells (wt.%: weight)

Shell composition of *A. anatina*		Results
CaO	[wt.%]	98.8±0.435
SiO_2	[wt.%]	0.628±0.035
K_2O	[wt.%]	0.174±0.031
MgO	[wt.%]	0.132±0.028
SrO	[wt.%]	0.069±0.009
SO_3	[wt.%]	0.047±0.002
Al_2O_3	[wt.%]	0.028±0.003
P_2O_5	[wt.%]	0.08±0.011
Fe_2O_3	[wt.%]	0.032±0.010
CuO	[wt.%]	0.01±0.003

DISCUSSION

The shell microstructure of *A. anatina* has been studied to understand their unique shell structure in this experimental study. The periostracum layer of *A. anatina* serves as a protective layer, shielding the inner layers of the shell from external factors such as erosion, corrosion, and predation. It is the first layer secreted by the folds of the mantle and is made of proteins (Piwoni-Piórewicz et al., 2017). The presence of prominent vacuoles in the inner vesicular layers of the periostracum suggests that this layer is also involved in ion

regulation and deposition (Salas et al., 2011). The layer below the periostracum, comprised of calcite, is not well-defined, but it likely plays a role in providing a transition zone between the organic periostracum and the mineralized nacreous layer (Chakraborty et al., 2020). The aragonite sheet nacreous layer, which covers almost the entire thickness of the shell, is responsible for providing the strength and toughness required for the shell to resist mechanical damage (Marin, 2012). The preservation of the nacreous layers observed in all the samples studied highlights the robustness of the shell structure of *A. anatina*. The microstructure of the mineralized internal layers, particularly the aragonite sheet nacreous layer, demonstrates the intricate organization of the shells components. The presence of organic biopolymers plays a significant role in the stability of the aragonite platelets and the overall structural integrity of the shell (Xu and Li, 2011). The specific zones in the layers of the *A. anatina* freshwater mussel shell observed from the cross-section views in Figure 7 provide valuable information regarding the composition and structure of the shell. The prismatic layer, which is mainly composed of columnar crystals and an organic matrix, and the fiber prismatic structure with a common structural nodule located beneath the periostracum layer are noteworthy features. The amorphous and supersaturated cloudy structure in the cloud layer below the periostracum layer promotes shell biomineralization (Carter et al., 2012). Additionally, the overlap between the $CaCO_3$ layer and the contiguous nacre layer, as well as the stacking of the aragonite tablets in parallel, elongated rows in the nacre layer, were observed from the cross-section photos (Frenzel and Harper, 2011). These observations provide valuable insight into the biomineralization process and the structure of the *A. anatina* freshwater mussel shell.

Hardness is a crucial mechanical property of all crustaceans as it helps to protect the live organism within (Lee et al., 2008). Our experimental studies revealed that the external shell of *A. anatina* has a comparatively lower microhardness than the internal shell from the umbo to the posterior end regions. Based on the microhardness values obtained in our study, it can be concluded that there is no statistically significant linear relationship between the microhardness values of the external shell and internal shell (Table 1). When the load was applied perpendicularly to the internal section, the nacreous structure (internal zone, 625.5 ±172.7 HV) exhibited higher hardness values than the prismatic structure (external zone, 531.5 ±110.7 HV) as observed by Leung and Sinha (2009) and Lv et al., (2015). The difference in microhardness values is believed to be due to the internal structure of the shell, where $CaCO_3$ crystals form a harder texture through biomineralization (Marie et al., 2012).

The high concentration of CaO in mussel shells, as revealed by the XRF analysis, makes them an attractive natural source of this important mineral for biomedical applications. The purity of the CaO found in mussel shells is also notable, as the concentrations of other oxides are minimal, which suggests that the shells can be used as a highly pure and natural source of CaO for various applications. These findings could potentially lead to new developments in the use of mussel shells as a sustainable and cost-effective source of highly pure CaO, with implications for a wide range of fields.

CONCLUSION

The microstructures, resistance and material components of shells are crucial for the survival of freshwater mussels. This experimental study investigated the microstructure, phase components and mechanical properties, including microhardness, of *A. anatina's* shell. The shells microstructure had a uniform surface morphology, and the periostracum and ostracum layers were mainly composed of $CaCO_3$ with a calcite density on the crystal basis. Consequently, this surface was more prone to wear than the nacre layer, which contained quadrangular and pentagonal nacre plates. No prismatic nacre crystals were found when examining the periostracum layer. The inner surface of *A. anatina* had a glossy, pearlescent appearance clear nacre plates. A sharp distinction was observed between the periostracum layer and the nacre layer upon examining the cross-sectional surface in the vertical direction. Microhardness measurements were taken from the inner layer to the outer layer in both the periostracum and nacre layers of the shell. The highest microhardness value (625.5 ± 172.7 HV) was found in the inner layer, decreasing to approximately 531.5 ± 110.7 HV as the test moved towards the outer layer. The XRF analysis revealed that mussel shells have a high concentration of CaO, with minimal concentrations of other oxides, making them an attractive and natural source of this mineral. These findings could potentially lead to new developments in using mussel shells as a sustainable and cost-effective source of highly pure CaO, with implications for a wide range of fields. The microstructural information and subsequent analysis of shell microhardness provided an important experimental basis for developing models on *A. anatina.*

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AUTHORSHIP CONTRIBUTIONS

Kerim Emre Öksüz: Conceptualization, methodology, formal analysis, writing - original draft preparation, writing-review and editing, software and visualization. Hülya Şereflişan: Investigation, sample collection and preparation, writing-review and editing.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest or competing interests.

ETHICS APPROVAL

No specific ethical approval was necessary for this study.

DATA AVAILABILITY

Data supporting the findings of the present study are available from the corresponding author upon reasonable request.

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