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Microstructural Alterations of the Inner Shell Surface of *Anodonta cygnea* induced by Demineralizing Solutions

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Abstract: The shell of a wide variety of freshwater bivalve species consists of an outer prismatic layer and an inner nacreous layer, both composed predominantly of calcium carbonate ($CaCO_3$) crystals embedded within an organic matrix that typically represents up to 5% of the shell dry weight. Depending on the species, calcium carbonate occurs mainly as aragonite, although calcite may also be present in specific shell regions. The highly ordered hierarchical arrangement of mineral crystals and organic macromolecules confer exceptional mechanical strength, fracture resistance, and structural integrity while maintaining a relatively lightweight biomineralized structure.

The aim of this study was to characterize the microstructural alterations induced by acidic and alkaline demineralizing solutions in the inner shell of *Anodonta cygnea*, thereby improving the understanding of the shell's hierarchical organization. After exposure to different demineralizing solutions for 1 h, biomineral microstructure of shell fragments was examined using scanning electron microscopy (SEM). In the control samples, the shell exhibited an outer prismatic layer composed of columnar prisms surrounded by an alveolar organic matrix, and an inner nacreous layer consisting of densely packed, flat aragonite biomineral. Acidic solutions induced preferential dissolution of the aragonite prisms, whereas the organic matrix remained largely intact. Within the nacreous layer, the acidic treatment appeared to preferentially disrupt the intercrystalline organic matrix, promoting crystal detachment and the subsequent dissolution of the aragonite crystals. Basic solutions exhibited a weaker demineralizing effect on the aragonite prisms than acidic solutions. In the nacreous layer, partial disintegration of the superficial layers was observed, suggesting that alkaline treatment altered both the intercrystalline and intracrystalline organic matrices, thereby compromising the structural cohesion of the aragonite crystals.

The analysis of the shell microstructure following exposure to acidic and alkaline solutions provides insight into the layer-specific structural alterations resulting from the interaction between the mineral and organic components of the shell. Although primarily of fundamental biological interest, these findings may also have potential relevance for biomaterials research and, to a lesser extent, for biomedical and bioengineering applications, where controlled biomineral dissolution and hierarchical composite structures are of increasing interest.

Keywords: Scanning electron microscopy, *Anodonta cygnea*, Shell microstructure, Freshwater bivalve, Shell demineralization

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Introduction

Bivalves are mollusks whose soft body is enclosed within a rigid external shell composed of two articulated valves. Shell formation occurs through biomineralization, a biologically controlled process responsible for producing a highly organized composite structure that provides mechanical support, protection against predation, and resistance to environmental stressors (Marin and Luquet, 2004). In most bivalve species, the shell consists of three distinct layers: an outer organic periostracum, which protects the underlying calcareous layers from chemical dissolution; an outer prismatic layer; and an inner nacreous layer (Checa, 2000; Marin and Luquet, 2004). Although the relative thickness, organi-zation, and mineral composition of these layers vary considerably among species, bivalve shells are generally composed of 95–99% calcium carbonate and 1–5% organic matrix. This organic matrix consists primarily of proteins, glyco-proteins, polysaccharides, lipids and amino acids, which play essential roles in regulating crystal nucleation, growth, orientation, and the overall mechanical properties of the shell (Miyashita *et al.*, 2000; Duplat *et al.*, 2006). These macromolecules are distributed within both the intercrystalline and intracrystalline compartments of the nacreous layer and may occur as either soluble or insoluble fractions following shell demineralization (Michenfelder *et al.*, 2003). Microstructural analysis of the shell provides valuable insights into how acidic and alkaline solutions affect shell integrity at the micrometre scale. The shell's resistance to these chemical agents is largely determined by the composition and organization of the organic matrix and its interaction with the mineral phase. This organic–mineral composite confers remarkable mechanical strength and toughness to the shell (Michenfelder *et al.*, 2003) and contributes to the characteristic iridescence of the nacreous layer (Suzuki *et al.*, 2016). Shell matrix proteins (SMPs), secreted by the calcifying mantle, play a key role in biomineralization by regulating calcium carbonate crystal formation and polymorph selection (Marin *et al.*, 2007b). Depending on this biological regulation, molluscan shells may be composed of either of the two main calcium carbonate polymorphs, aragonite or calcite (Weiner, 1983; Checa *et al.*, 2007). However, in the order Unionida, which includes freshwater mussels such as *Anodonta cygnea*, the shell is composed exclusively of aragonite (Marie *et al.*, 2007).

Several natural and artificial methods for demineralizing molluscan shells have been described in the literature. Although these methods are primarily used to isolate the shell organic matrix for biochemical and biotechno-logical applications (Brzeski, 1987; Marin and Luquet, 2005; Mahmoud *et al.*, 2007; Ehrlich *et al.*, 2009), they also provide a valuable experimental tool for investigating the structural organization of different shell layers and the contribution of their organic and mineral components to shell architecture. Demineral-ization occurs naturally through the action of bacteria, fungi, and algae (Carriker and Smith, 1969; Ehrlich *et al.*, 2009), but it can also be induced artificially using dilute hydrochloric acid, which rapidly dissolves calcium carbonate (Percot *et al.*, 2003; Mahmoud *et al.*, 2007). In most protocols, acid demineralization is

followed by alkaline extraction to remove proteins and further characterize the organic matrix (Isa *et al.*, 2012; Ameh *et al.*, 2015). Alternative demineralization methods can be selected according to the intended application and the desired shell-derived products. These include mineral acids, such as sulfuric and hydrochloric acids, chelating agents such as ethylene-diaminetetraacetic acid (EDTA), and organic acids including acetic and lactic acids (Ehrlich *et al.*, 2009; Ameh *et al.*, 2014; Ameh *et al.*, 2015). EDTA removes calcium carbonate by chelating Ca^{2+} ions, thereby preserving and releasing the organic matrix into solution. In contrast, acids such as hydrochloric and acetic acids dissolve the mineral phase, yielding acid-soluble and acid-insoluble organic matrix fractions (Ma *et al.*, 2005). Sodium hydroxide has been used to separate the prismatic layer from the nacreous layer (Ma *et al.*, 2005), whereas sodium hypochlorite has been employed to isolate calcitic prismatic crystals (Marin *et al.*, 2007a) and remove the periostracum (Freer, 2009).

Studies of the shell organic matrix, calcium carbonate biomineralization, and shell microstructure have broad relevance to materials science and biomedicine. Shell matrix proteins (SMPs), in particular, have attracted considerable interest because of their ability to regulate crystal formation and inspire the development of biomimetic materials. Their potential applications include nanocrystal manipulation, the synthesis of advanced biomaterials for biotechnology and nanotechnology, and the development of bone grafts, implants, and regenerative materials (Rousseau *et al.*, 2003; Marin and Luquet, 2005; Marin *et al.*, 2007b; Vecchio *et al.*, 2007). In addition, improved knowledge of shell biomineralization may benefit pearl production and other aquaculture industries with economic importance (Marin *et al.*, 2007b).

The present study aimed to improve the understanding of the microstructural organization of the prismatic and nacreous layers of the *Anodonta cygnea* shell by examining the effects of controlled partial demineralization. Acidic and alkaline solutions at concentrations selected from previous ultrastructural studies were applied directly to the inner shell surface to induce the selective dissolution of calcium carbonate and/or the organic matrix. *Anodonta cygnea* was chosen because its shell ultrastructure and biomineralization processes are well characterized.

Materials and Methods

According to previous studies (Lopes-Lima *et al.*, 2010; Lopes *et al.*, 2014), *Anodonta cygnea* specimens were collected from Mira Lagoon (Praia de Mira), northern Portugal (40°27′14″ N, 8°47′80″ W), and maintained in tanks at room temperature for 48 h. The soft tissues were used for other studies, whereas only the shells were used in the present investigation.

After rinsing with deionized water, the shells were sectioned from the ventral margin into consecutive fragments approximately 2 cm in length, exposing both the nacreous layer and, at the shell edge, the prismatic layer (Fig. 1). During 1 h, the inner surface of control fragments was immersed in deionized water, while the remaining fragments of experimental samples were exposed to 50% hydrochloric acid (extreme demineralization conditions) (Dolez and Love, 2002), 10% acetic acid (Ma *et al.*, 2005), 0.5 M EDTA (Michenfelder *et al.*, 2003), 1 M sodium hydroxide (Percot *et al.*, 2003), or 1% sodium hypochlorite (Marie *et al.*, 2011).

Following treatment, shell fragments were examined from the shell edge towards the pallial line using scanning electron microscopy (SEM; FEI Quanta 400 FEG ESEM/EDAX Genesis X4M). Prior to imaging, specimens were sputter-coated with a gold–palladium layer (JFC-1100 Fine Coat Ion Sputter) and mounted on aluminum stubs.

Results

Microstructural analysis of the control shell fragment of *Anodonta cygnea* revealed the transition between the prismatic and nacreous layers (Fig. 2-A1). The prismatic layer appeared

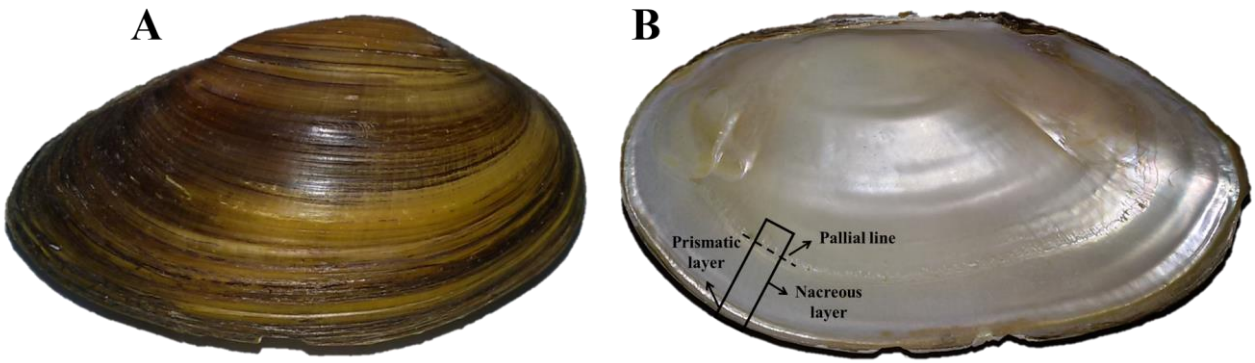

Fig. 1: *Anodonta cygnea* shell approximately 18 cm in length. **(A)** Exterior view of the shell showing the periostracum; **(B)** Interior view of the shell illustrating the cut fragments used for SEM observation of the prismatic and nacreous layers.

largely intact and consisted of columnar, convergent aragonite prisms embedded within an alveolar organic matrix (Fig. 2-A1). In the adjacent nacreous layer, two to four superficial lamellae of aragonite tablets can be observed (Figs. 2-B1, 3-A, B). These tablets, corresponding to the outermost nacreous layer, exhibiting the typical morphology of well-preserved aragonite crystals, including a characteristic approximately rhombic shape (Fig. 3-A) and hexagonal arrangements (Fig. 3-B).

Exposure of shell fragments to acetic acid resulted in partial dissolution of the aragonite prisms within the prismatic layer, whereas the surrounding organic alveolar framework remained largely unaffected (Fig. 2-A2). So, the selective removal of the mineral phase exposed the underlying organic scaffold, clearly revealing the columnar arrangement of the prisms and the honeycomb-like architecture characteristic of the prismatic layer. In the nacreous layer, partial dissolution of the aragonite tablets still exposed to acetic acid showed the underlying succession of crystal lamellae involved in shell growth and biomineralization (Fig. 2-B2).

Following hydrochloric acid treatment, a complete dissolution of the aragonite prisms in the prismatic layer occurs exposing the three-dimensional organic alveolar network that originally surrounded the mineralized prisms (Fig. 2-A3). This treatment still provided a clear visualization of the organic framework that serves as the structural template for prism formation. Similar to the acetic acid treatment, exposure to hydrochloric acid preserved the organic alveolar structure surrounding the aragonitic prisms. However, owing to its greater acidity, hydrochloric acid induced more extensive dissolution of the nacreous layer (Fig. 2 – B3), exposing deeper regions characterized by eroded aragonitic tablets and the formation of pronounced cavities within the nacreous microstructure. At higher magnification (Fig. 2 – B3.1), dissolution of the crystals can be observed extending into their interior, indicating a more pronounced degradation of the aragonitic tablets. In the lateral view of the shell fragment exposed to the 0.5 M EDTA solution (Fig. 2 – A4), the alveolar organic matrix surrounding the partially dissolved prisms remains clearly visible. The columnar arrangement associated with prism formation is also evident. Compared with the acidic treatments, EDTA at 0.5 M appeared to induce less extensive dissolution of the nacreous layer, with no exposure of the deeper nacreous regions (Fig. 2 – B4). Nevertheless, the treatment clearly promoted the separation and disaggregation of the calcium carbonate crystals, indicating a distinct mode of

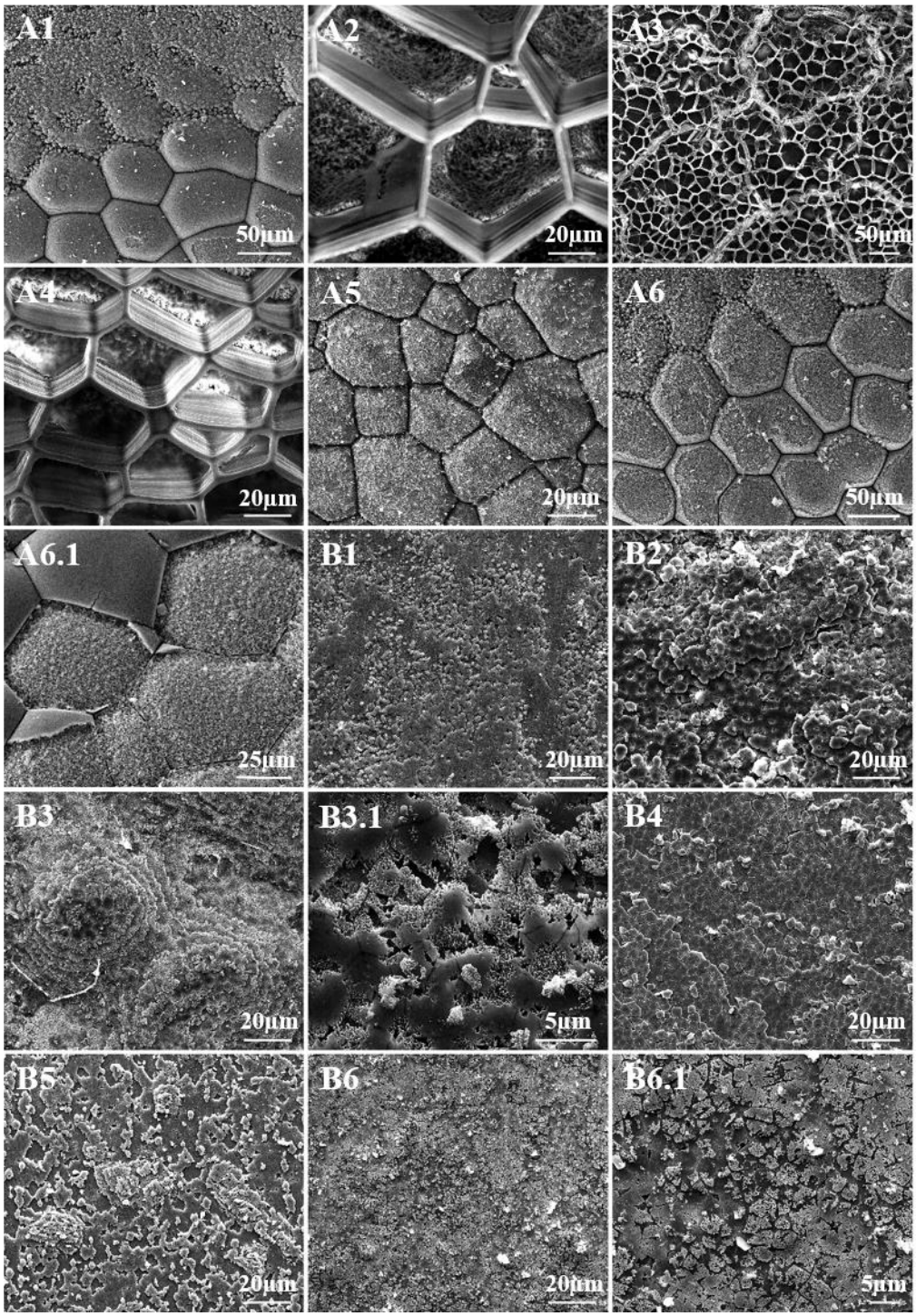

Fig. 2: SEM observations of the microstructure of the inner shell fragments of Anodonta cygnea after 1h of exposure to different solutions showing the prismatic **(A)** and nacreous layers **(B)**. The solutions used were 1-water (control), 2-acetic acid, 3-hydrochloric acid, 4-EDTA, 5-sodium hydroxide, and 6-sodium hypochlorite. Images A6.1, B3.1 and B6.1 are magnifications of the respective images A6, B3 and B6.

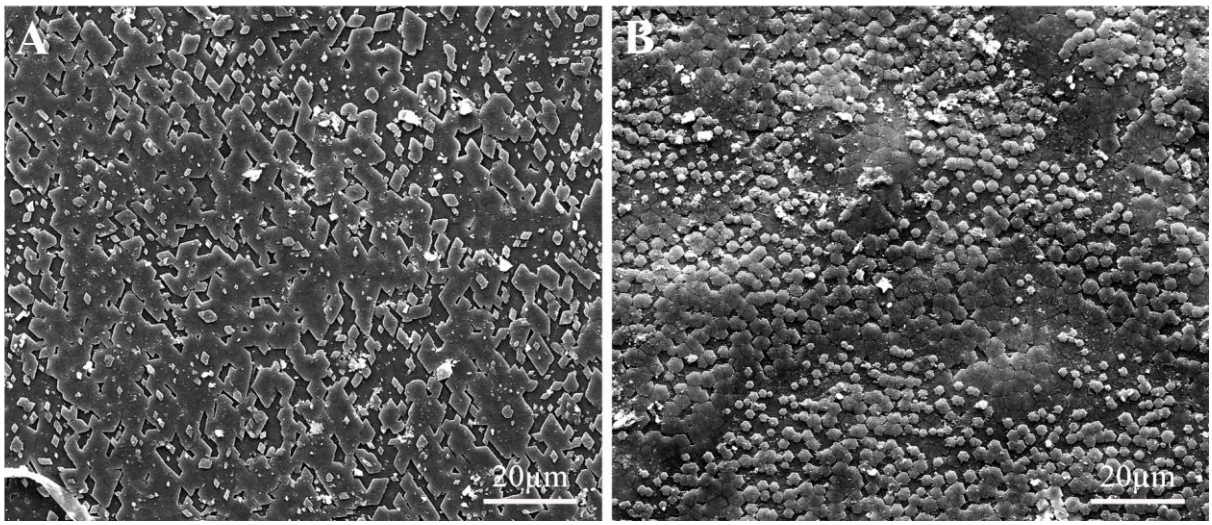

Fig. 3: SEM observation of different crystal sizes and shapes ("crystal habits") in the nacreous layer microstructure of *Anodonta cygnea* exposed to water (control). **(A)** exhibits an approximately rhombic shape and **(B)** exhibits hexagonal shape.

interaction with the mineral phase compared with the direct dissolution observed following acid exposure. Following exposure to the basic solution of sodium hydroxide, superficial erosion of the aragonitic prisms was observed, accompanied by the deposition of sodium-containing crystals, as indicated by the energy-dispersive X-ray spectroscopy (EDS) profile (data not shown) (Fig. 2 – A5). In the nacreous layer (Fig. 2 – B5), corrosion of the superficial aragonite crystal layers was also evident.

Exposure to sodium hypochlorite produced more pronounced alterations in the prismatic layer (Figure 2 – A6), characterized by an almost complete removal of the mineral-associated organic matrix surrounding the aragonitic prisms. This alteration is more clearly visible at higher magnification (Fig. 2 – A6.1). In the nacreous layer (Figure 2 – B6), separation and corrosion of the superficial aragonite crystal layers were observed, with these alterations becoming more evident at higher magnification (Fig. 2 – B6.1).

Discussion

The inner shell of *Anodonta cygnea* comprises a prismatic layer characterized by columnar, convergent aragonitic prisms surrounded by an alveolar organic matrix, and a nacreous layer composed of multiple aragonitic tablets exhibiting variable microstructural features. The control shell fragments examined in the present study displayed the expected microstructural morphology described for these shell layers in previous studies (Machado *et al.*, 1991; Checa, 2000; Marin and Luquet, 2004, 2005; Marie *et al.*, 2007; Lopes-Lima *et al.*, 2010; Lopes *et al.*, 2014).

The variation in crystal size and morphology, or crystal habit, observed within the nacreous layer (Fig. 3) represents a normal feature of shell microstructure and has been reported to vary seasonally in *A. cygnea* (Machado *et al.*, 1991). While the polymorphic form of calcium carbonate is primarily associated with the interaction between the mineral phase and components of the organic matrix, the external morphology and habit of the crystals may also be influenced by environmental and physiological factors. According to Machado *et al.* (1991), crystals formed during spring and summer tend to exhibit more rhombic and irregular geometries, respectively, whereas those formed during autumn and winter generally display more regular

morphologies. In particular, a predominantly hexagonal crystal habit has been associated with winter shell growth.

Based on the microstructural alterations observed in the present study, the acidic treatments exerted a stronger demineralizing effect on the prismatic layer, resulting in extensive dissolution of the calcium carbonate phase. The partial removal of the mineral component exposed the underlying organic framework, allowed to reveal a well-defined hexagonal alveolar organization associated with the internal columnar arrangement of the prisms. This structural organization is consistent with the model of prism formation described in previous studies (Checa, 2000; Marin and Luquet, 2004, 2005; Freer *et al.*, 2009; Lopes-Lima *et al.*, 2010). In comparison, the alkaline treatments produced less extensive alterations in the prismatic layer, with predominantly superficial erosion of the organic matrix surrounding the prisms. These observations suggest that alkaline solutions may interact preferentially with the organic component associated with the surface of the prismatic layer, although the specific chemical mechanisms underlying this interaction require further investigation.

In the nacreous layer, exposure to acidic solutions resulted in partial separation and erosion of adjacent aragonitic tablets, particularly along their boundaries. This pattern may indicate an interaction between the demineralizing solutions and the organic material located within the intercrystalline spaces. Hydrochloric acid produced the most pronounced alterations in this layer, with deeper dissolution extending into the aragonitic tablets. The internal erosion observed at higher magnification may suggest that the treatment also affected organic components located within the crystal structure, although this interpretation should be considered tentative in the absence of direct chemical characterization of the organic matrix.

Prolonged exposure to acidic solutions would be expected to result in extensive or potentially complete dissolution of the mineral phase, with the soluble organic components remaining associated with the reaction medium and the less-soluble organic fraction being retained as a residual matrix. In contrast, alkaline treatments produced marked disintegration of the superficial nacreous layers. These alterations may reflect interactions between the alkaline solutions and both intercrystalline and intracrystalline organic components, contributing to the separation and disruption of the superficial aragonitic tablets.

In summary, the present study provides insights into the morphological alterations occurring in the inner shell layers of *Anodonta cygnea* following exposure to different demineralizing solutions. In the prismatic layer, acidic treatments produced more pronounced alterations than alkaline treatments, resulting in partial to extensive dissolution of the calcium carbonate phase while largely preserving the underlying alveolar organic framework of the prisms. In contrast, alkaline treatments primarily induced superficial erosion of the organic matrix associated with the aragonitic prisms. In the nacreous layer, acidic treatments appeared to predominantly affect the weaklier organized intercrystalline organic component, resulting in partial separation and dissolution of adjacent aragonitic tablets. Alkaline treatments produced more extensive fragmentation and disintegration of the superficial nacreous structure, potentially reflecting interactions with both intercrystalline and intracrystalline organic components. Overall, the differential responses observed between the prismatic and nacreous layers highlight the distinct organization and chemical susceptibility of their organic–mineral interfaces.

Conclusion

The effects of acid and basic treatments on the organic matrix revealed a marked difference between the nacreous and prismatic layers. In the nacreous layer, residual organic structures were scarcely discernible after exposure to the demineralizing solutions, whereas the organic

matrix within the prismatic layer remained comparatively well preserved. This differential response may be related to the distinct distribution and organization of the organic matrix in the two shell layers. In the nacreous layer, the organic matrix is present in relatively small amounts and is distributed primarily within the interlamellar and intercrystalline spaces, whereas in the prismatic layer, it is more abundant and forms a highly organized alveolar framework surrounding the aragonitic prisms. Consequently, the greater concentration and structural organization of the organic matrix in the prismatic layer may contribute to its greater preservation following chemical treatment.

These findings may contribute to a better understanding of the distinct properties and functional roles of the organic matrix within the prismatic and nacreous layers during shell formation in the freshwater bivalve *A. cygnea*. More broadly, characterizing the microstructural organization and organic–inorganic composition of bivalve shells through their differential responses to specific chemical treatments may provide valuable insights into the interactions between their organic and mineral components. Such knowledge may also contribute to the development of biomimetic materials and the synthesis or modification of novel compounds with potential biomedical and industrial applications (Lertwattanaruk *et al.*, 2012).

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the [1]Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.

Author Contributions

Meireles Diana: methodology, investigation, result collection; *Cunha Sara*: investigation, writing—original draft preparation; *Hinzmann Mariana*: coordination of research tasks, writing—review and editing; *Machado J.* and *Gonçalves J.F.*: Methodology, Conceptualization, project administration and supervision; *Silva R.M.*: Data curation; *Silva R.M.* and *Machado J.*: writing and original draft preparation; *Silva R.M.*, *Machado J.* and *Gonçalves J.F.*: writing, review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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