The Characterization of *Haliotis Rufescens* (Red Abalone): Mesolayer Growth

by

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Abstract

The structure of an abalone's shell, as well as its self-assembly process, has been the focus of a considerable amount of research over the years due to the exceptional properties it possesses. The self-assembly process creates highly ordered hierarchal structures and has become a popular alternative in the synthesizing of materials. The abalone undergoes a self-assembly process called biomineralization, which gives the abalone's shell unique structural properties that are fascinating to researchers, and especially intriguing to those trying to replicate the structure. In depth analyses have been performed on an important portion within the shell, called the nacre or otherwise known as "Mother of Pearl". Investigations into the nacre portion of the shell could help researchers in imitating this structure to produce materials with specific characteristics needed for applications in industry.

The nacre is made up of aragonite tablets, which is a form of calcium carbonate. These aragonite tablets are stacked in a columnar-like structure, and connected by a thin organic layer in a 'brick' and 'mortar' type structure. Within the nacre, another structure occurs periodically, and is easily distinguishable in the structure of the abalone found in the ocean. This structure is called the mesolayer, and it has its own unique structure that differs from the nacre tablet structure. The mesolayer can be divided into three distinct layers, with each having their own unique structure: the columnar-like layer, organic layer, and prismatic layer. Even though extensive research has been done on the nacre's tablet structure, the mesolayer has been given little consideration.

The nacre grows in in a confined spaced created by the outer portion of the shell and a part of the abalone's soft-tissue, which is called the mantle. Within the space between the shell and the mantle, the abalone secretes a colloidal mucus, and the nacre grows within that in an environment that is isolated from the surrounding elements. In order to examine the biomineralization process the abalone undergoes, substrates were implanted between the shell and the mantle using the Flat Pearl Method. This technique helped to better understand how the saltwater environment affected the nacre tablet growth, as well as the mesolayer growth.

The abalones used in this research were purchased from an abalone farm in California, and integrated in to tanks filled with artificial saltwater, which is made to replicate the oceanic environment. To ensure the nacre and mesolayer growth would occur, the parameters of the saltwater and feeding were meticulously monitored to guarantee the environment was suitable for the abalone. The parameters tested for each tank were: temperature, pH, salinity, concentrations of Mg^{2+} and Ca^{2+}, nitrate, nitrite, and alkalinity. The temperature was controlled to allow the formation of the mesolayer. In this research, many experiments were focused on the increase of temperature, since the multiple mesolayers created in the wild abalone are thought to mainly be due to seasonal temperature changes. After the substrates were implanted into the abalone, each tank started at an initial temperature for a few weeks, then the temperature was increased for a period of time, and finally brought back down to the initial temperature for the remaining of the testing cycle. Various temperatures and periods of time were used to try to distinguish how and when the mesolayer forms within the nacre.

Even though an abundant amount of research has focused on the nacre structure, there are still many qualities that have yet to be revealed, and much deliberation on the nacre growth and how its exceptional properties influence the structure of the nacre. The research completed in this thesis aspired to expand the understanding of how the outside environment affects the nacre tablet growth, as well as the mesolayer.

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List of Abbreviations

ACC	Amorphous Calcium Carbonate
BSE	Backscattered Electron
MS	Millisiemens
OM	Optical Microscope
PPM	Parts per Million
PPT	Parts per Thousand
SE	Secondary Electron
SEM	Scanning Electron Microscope

1. Introduction

The *Haliotis Rufescens*, or Red Abalone, has been studied extensively due to the unique microstructure it has within its shell. The nacre is the main focus of most studies, since it is assumed that it is the source of its unique mechanical properties.

In this thesis, various sections of the Red Abalone shell are researched and examined to better understand the properties it possesses. Various methods of research were used to promote the nacre growth during the biomineralization process. The investigation into the abalone's distinctive structure could offer new techniques to expand and improve the structure of other composite materials.

The main focus of this thesis is on a layer within the nacre itself, which is called the mesolayer. This layer is considered an interruption during the self-assembly nacre growth process. Since not much research has been done on this layer, it is not clear how the mesolayer affects the mechanical properties in the nacre. Thus, the research completed for this portion of the nacre is concentrated on trying to control the growth of this interruption to better understand the extent to which it impacts the nacreous layer. The following methods were applied:

- Change in various environmental factors, such as Temperature and feeding, while recording changes in pH, Calcium, Magnesium, salinity, Nitrates, Nitrites, and Alkalinity.
- Various periods of time for changes in environmental factors.

The Red Abalone chosen for this research were acquired from a farm in California. The live abalones were selected based on the relatively large size and availability. The large size allows for testing to be performed with less difficulty.

In this section, essential points of this research will be discussed, such as self-assembly and biomineralization processes, Red Abalone nacre and mesolayer growth, experimental methods, and the motivation behind the research.

1.1. Self-Assembly and Biomineralization

Abalones are mollusks that are classified into the *Haliotis* genus. The *Haliotis Rufescens* (Red Abalone) are indigenous from the Baja California Peninsula to northern California. [1] They go through a self-assembly process that is particularly interesting in the field of Materials Science.

Self-assembly has become a popular method to consider as an alternative in synthesizing materials, since material production has a negative impact on the environment due to the chemicals and production conditions needed during processing. The conditions for self-assembly are not harsh and do not require high levels of heat or pressure during application. [2]

Self-assembly is an independent process, where the structure is formed without the need for human intervention. It is used in many systems, such as sensors, self-healing structures, computer networks, and smart materials. It is also a realistic approach to creating nanostructures; therefore it is a crucial part of nanotechnology. [3]

Self-assembly is extensively used in biological systems. [2] Biomineralization is a self-assembly process that has evolved over time and is utilized by many species in the wild. Additionally,

biomineralization has a direct impact on their survival in the natural environment. It is a process where a mineral formation is formed from supersaturated ion solutions. This is vital in the development of biological hard tissues, specifically calcified shells. The wide range of shapes and the formation of the structure for each species are dependent on the organic matrix. The organic matrix of the biomineral controls the process of selective precipitation of the inorganic mineral. [4, 5]

Calcium is the most broadly distributed element in biominerals. This is especially the case in the 'hard parts' of the structure. Calcium carbonate based biominerals are located in a considerable number of organisms. The shells of mollusks are included in this group, and calcium carbonate is thought to be responsible for the exceptional properties the shell displays. [4]

Calcium Carbonate has been studied a significant amount due to its ability to be applied to many fields in industry. In many applications where cracking is problematic on the micro-scale, sealants and patching may not help, so the formation of calcium carbonate through biomineralization would a big step in remedying these complications. In the natural environment, most species are capable of precipitating calcium carbonate, as long as the essential substrates are accessible. Although, the metabolic reactions for generating calcium carbonate might vary from species to species to a certain degree. [6]

The growth of the abalone nacre is a prevalent example in the importance of the biomineralization process. The abalone incorporates components from its surrounding ecosystem to create exceedingly organized structures. Researches are interested in this area due to the remarkable mechanical properties, and it has become a quite popular biomineral to attempt to imitate. [7]

1.2. Sections and Shape of *Haliotis Rufescens* (Red Abalone) Shell

The abalone shell is comprised of two sections: an outer layer that faces the ocean, and an inner layer that lays against the abalone's soft body tissue **(Figure 1)**. The outer layer is rough, while the inside layer is smooth and iridescent. This inner portion of the shell, called the nacre, is what gives the abalone the name "Mother of Pearl".

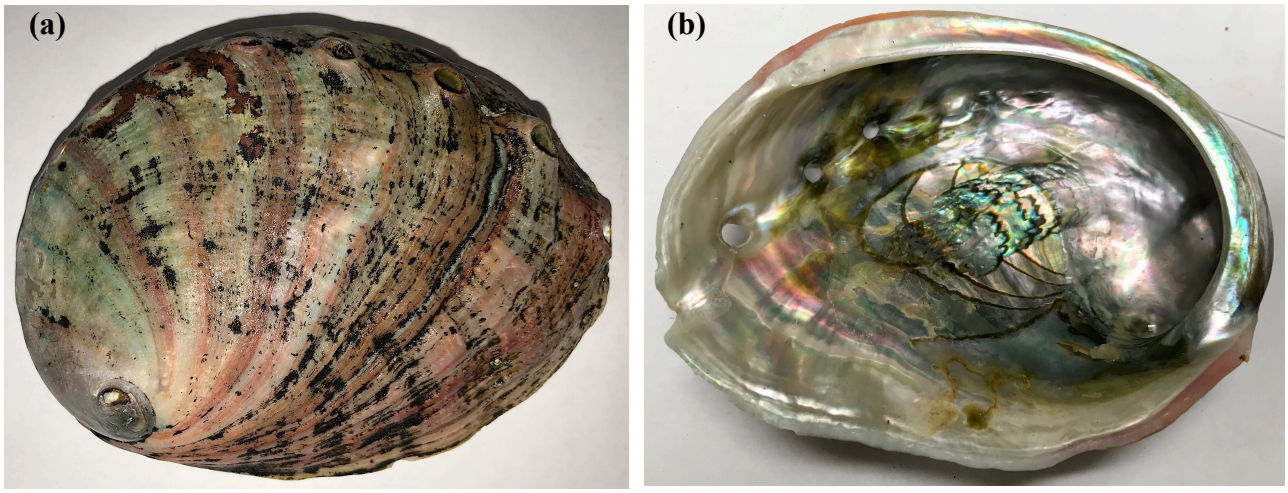

Figure 1. *Photos taken of red abalone shell. Image (a) displays the outside portion of the shell that faces the seawater and has a rough surface. Image (b) illustrates the inside portion of the shell that lies against the soft tissue of the abalone's body. This image displays its iridescent color, as well as its smoothness.*

When the shell is cut and small sections are removed, it is possible to see the boundary between the outer layer and the inner layer. The boundary between the two is distinct, as they are different colors and visible to the naked eye. The outer layer is a pinkish color, while the nacre is an opaque white color **(Figure 2)**. Even though it is easily visible to the naked eye, the difference

between the two layers of the shell can easily be discerned using an optical microscope **(Figure 3)**.

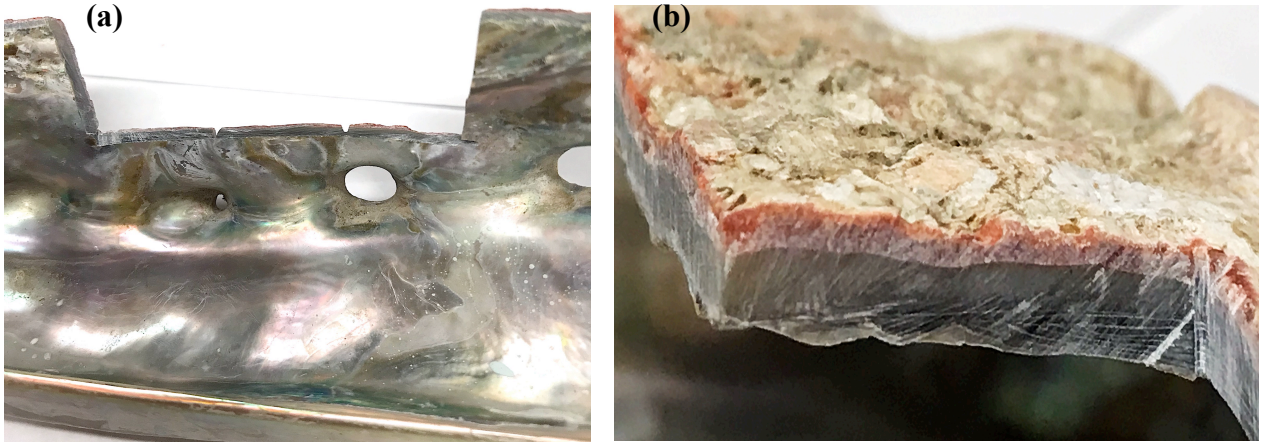

Figure 2. *Photos taken of a cross-sectioned portion of the shell. Image (a) illustrates a cross-section cut into the shell to show the boundary between the outer layer of the shell and the nacre. Image (b) illustrates the difference in color for the layers of the shell. The outer layer that faces the ocean is pink, while the inner nacreous layer is opaque white.*

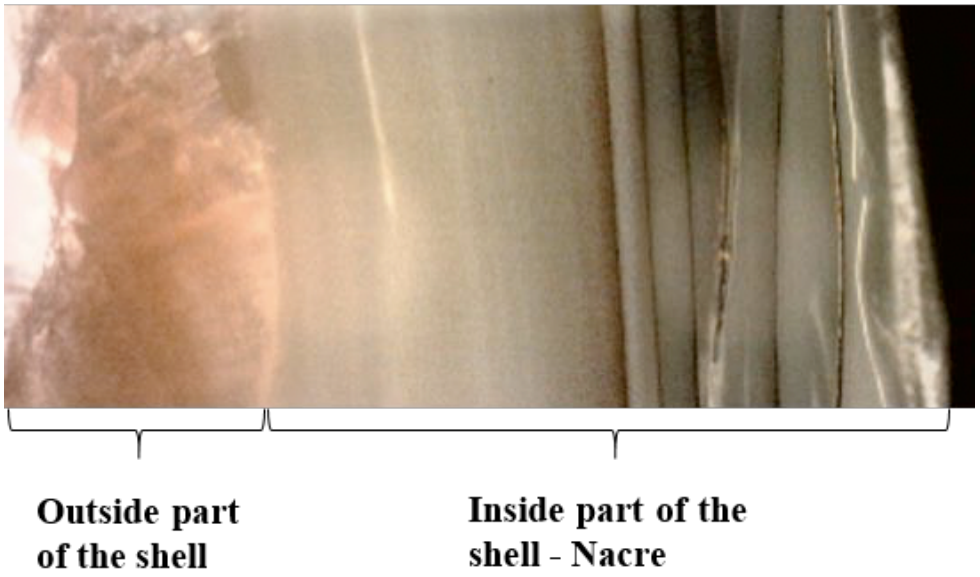

Figure 3. *This image is taken of a cross-sectioned red abalone shell using an optical microscope. The boundary between the two layers is well defined, so the outer layer is easily differentiated from the inner nacreous layer. Reprinted from Zhang, Anqi. "The Investigation of Haliotis Rufescens (Red Abalone): Mesolayer Growth & Mechanical Behavior." (2017). [19]*

As mentioned above, the shell forms through a self-assembly process, which gives the abalone its spiral shape **(Figure 4(a))**. The spirals on the shell are similar to that of rings on a tree. Located along the dorsal side of the shell, a row of circular apertures is created, and is used for the secretion of waste **(Figure 4(b))**. As the shell grows larger through biomineralization, new holes are created and the previous are filled. [8]

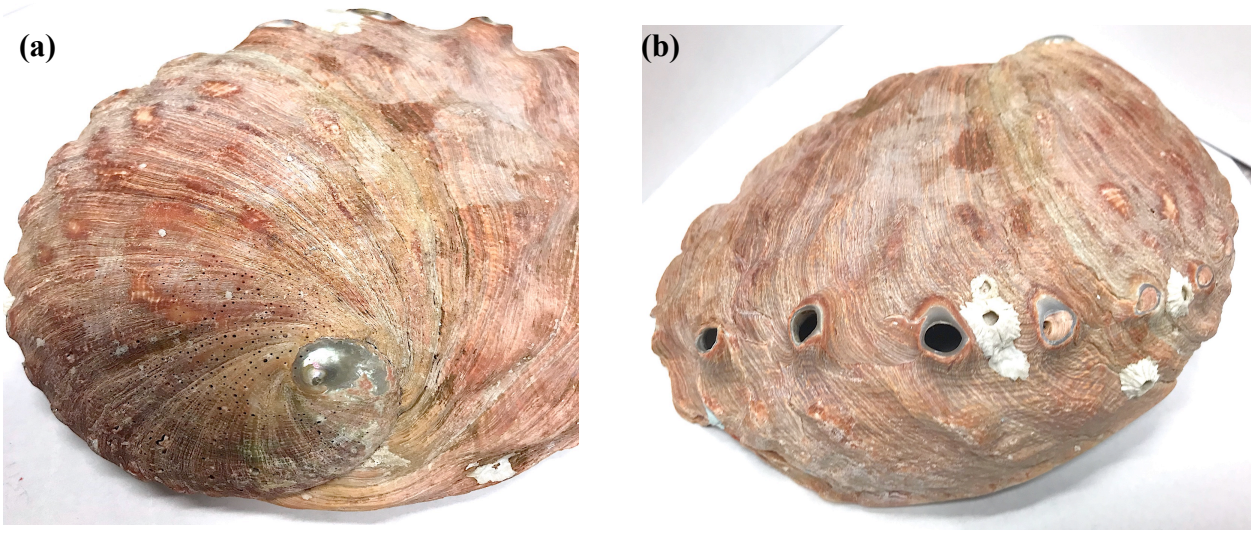

Figure 4. *Photos taken of the California red abalone shell. Image (a) shows the spiral shape of the shell obtained by its self-assembly process. Image (b) shows the circular apertures created during the self-assembly process for secretion of waste.*

1.3. Red Abalone Nacre

As mentioned in the previous section, the inner layer of the shell is called the nacre. It grows in the colloidal mucus secreted by the abalone between the outer layer of the shell and the soft tissue of the body, which is called the mantle. The mantle is closely attached to the shell; therefore the space between the mantle and the shell creates an insulated environment. This allows the nacre to grow in the mucus secreted by the abalone without any interference from the outside seawater environment. Extensive research has been done on this portion of the shell due to its exceptional properties. Being lightweight and yet, having high strength, as well as being crack-resistant, are just a few of the properties that entice researchers to study the nacre formation in the shells. [8]

1.3.1. Nacre Composition

The Haliotis Rufescens is a calcium carbonate based shell. The outer layer of the shell is comprised of calcite, but the nacre is made up of aragonite. [8,9] Both calcite and aragonite are forms of calcium carbonate with particularly similar crystal structures. These are both common mineral formations found in organisms. [10]

The nacre of *Haliotis Rufescens* is made up of more than 95 wt.% of calcium carbonate (aragonite), and less than 5 wt.% of organic material. [11] The organic layer consists of many macromolecules and proteins, such as silk fibroins, although β-Chitin has long been suspected as being the organic compound in the nacre. [13] The β-Chitin fibers are thought of as highly ordered planar arrays that are coated with soluble acidic macromolecules. These coated fibers control the morphology and crystallization. [14]

Chitin has a low reactivity and high level of insolubility, and is comparative to cellulose due to these properties. It is a mucopolysaccharide, which amply occurs in nature, and is usually found to consist of 2-acetamido-2-deoxy-β-d-glucose through a β (1→4) linkage. These are natural polymers that have exceptional properties, like biocompatibility, biodegradability, absorption, and non-toxicity. Chitin is the supplementary material for many organisms. Some examples would be crustaceans, insects, etc. [15]

1.3.2. Nacre Structure and Growth

The nacreous layer of the shell forms a hierarchal structure on the nano-scale during self-assembly. This structuring is the component that gives the abalone its exceptional properties. [12] The organization of these hierarchal structures is responsible for the extraordinary mechanical properties of many biological organisms. [17]

The structural levels appear on differing scales, and can be used to analyze the physical properties of materials. This type of structure can improve physical properties, such as strength and toughness, but it can also create strange properties, like a negative Poisson's Ratio. Hierarchal structures can occur in nature or can be man-made. The man-made structures are of great use in building and engineering. The World Trade Center is an example of a first order hierarchal structure. Many materials can exhibit this structure, such as polymers, composites, and biological materials. There are also numerous examples of his hierarchal structure in nature; some examples would be wood, bone, tendon, and bamboo. [18]

There are two different types of nacre, which are columnar and sheet structures respectively. For the columnar nacre, tablets are stacked vertically across each layer. Whereas the tablets in sheet

nacre are distributed at the interface of the underlying tablet, which makes the structure arrangement far more random compared to the columnar nacre. Abalones have the more organized columnar nacre. [25]

The *Haliotis Rufescens* nacre is made up of numerous aragonite tablets, which are separated by a thin organic layer **(Figure 5)**. The micro-scale aragonite tablets, nano-scale organic layer, and the macro-scale periodic growth interruptions form the hierarchal structure. The tablets and organic layer are stacked in columnar layers, and are described similarly to a 'brick' and 'mortar' structure. The 'brick' relates to the aragonite tablets, and the 'mortar' corresponds to the organic layer. The aragonite tablets range from 0.4-0.6 μm in thickness, and 8-10 μm in width. The organic layer is much smaller, and ranges from 20-50 nm in thickness. [11,12,16] The nacre is iridescent due to the aragonite tablets thickness range between 0.4-0.6 μm, which is analogous to the wavelength for visible light. [9]

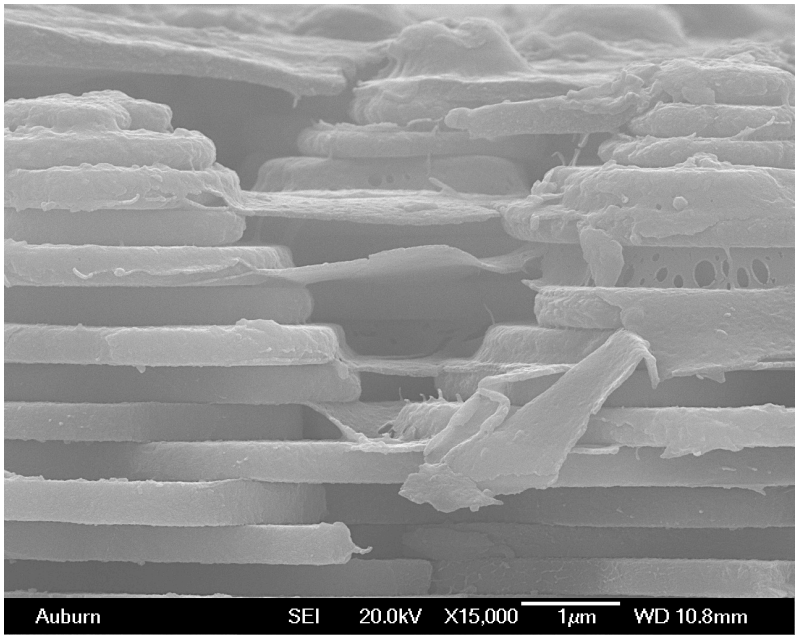

***Figure 5**. This image was taken using the SEM. It illustrates the stacked aragonite tablets that are separated by the thin organic layer.*

The SEM can be used to characterize the tablet structure of the abalone. By imaging samples using the SEM, it is possible to see the different stages of growth the nacre undergoes. During its early development stage, the nacre structure presents the "Christmas Tree" type morphology **(Figure 6)**. However, as the nacre matures, the stacked aragonite tablets develop into the columnar configuration **(Figure 7)**.

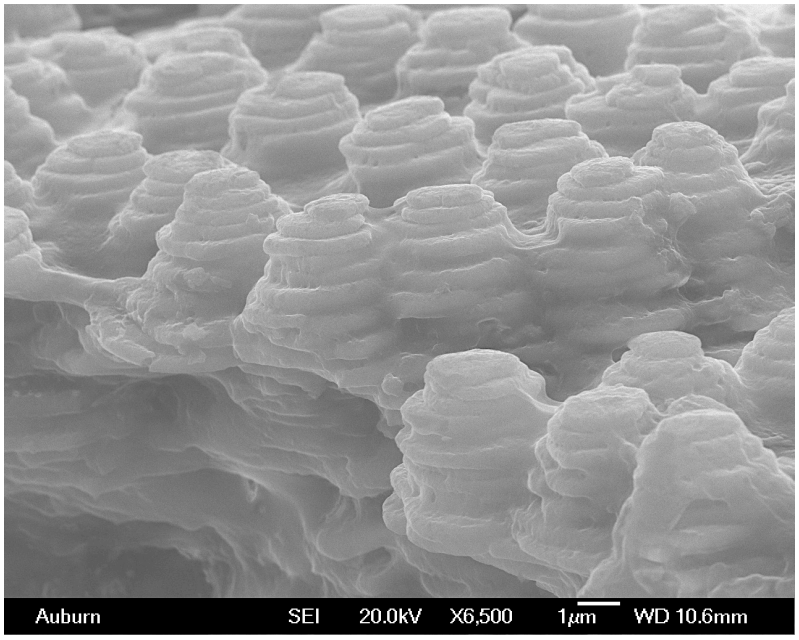

***Figure 6**. SEM images taken of the abalone nacre growth in its developmental stages, which displays the 'Christmas Tree' morphology.*

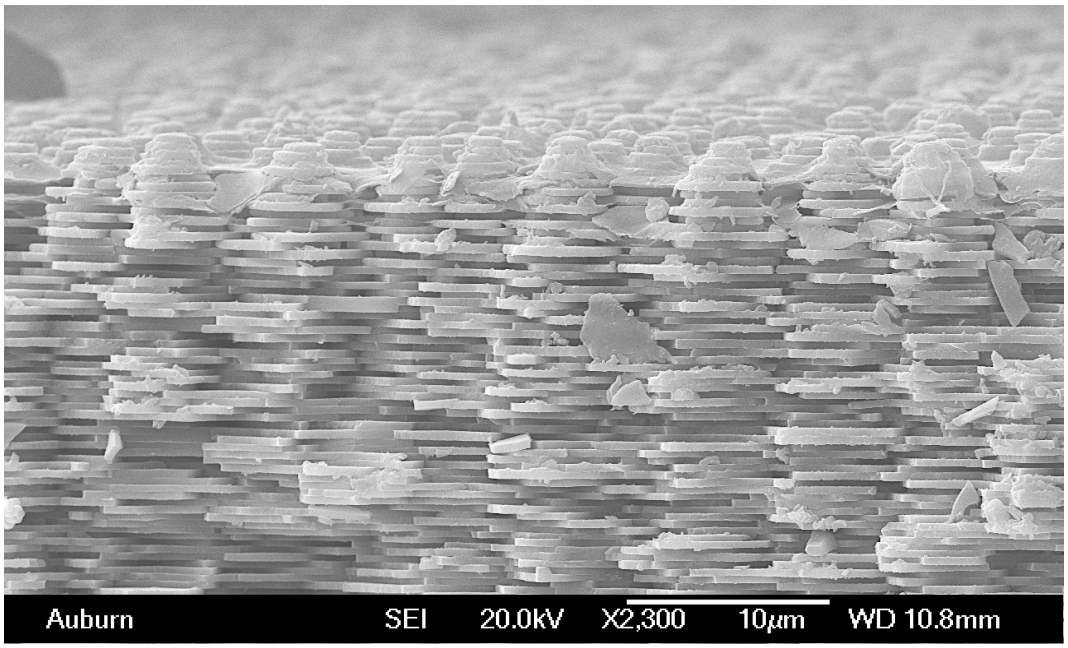

Figure 7*. SEM images taken of the nacre growth once it has matured. This image displays the columnar-like stacked aragonite tiles.*

The crystallization of calcium carbonate is an important process in the growth of the abalone nacre, since amorphous calcium carbonate (ACC) is essential for growth. There are three forms of calcium carbonate: calcite, aragonite, and vaterite. ACC can quickly convert to all three forms of calcium carbonate, unless it is kinetically stabilized. Calcite is the most stable, aragonite is metastable, and vaterite is the least stable of the three. The aragonite is not preferred in the crystallization process due to its difficulty in nucleation. However, it could nucleate in a particular medium, such as the mucus secreted by the abalone. Since aragonite is the main component of the abalone's nacre formation, the mucus in which it grows is the most imperative part of the biomineralization process. [12, 20]

The organic element of the nacre formation controls the morphology of the crystallization, as well as the growth pattern of calcium carbonate. β-Chitin and the silk protein act as a substrate in

calcium carbonate crystallization, which produces calcite for the outer layer of the shell and aragonite for the inner nacreous layer of the shell. If the protein is absent from the substrate, aragonite will not form. Instead, the calcium carbonate will form into vaterite. This confirms the importance of the proteins and β-Chitin acting together as the substrate. [21]

1.3.3. Mechanical Properties

Throughout time, abalones have been able to survive threats from their environment due to their resilience and ability to evolve. Even though the body of the abalone is made up of a soft tissue, its shell protects it from danger. The mechanical properties of the shell are remarkable and have been of great interest to researches for some time. In much of the previous research, the main focus has been on the internal nacre portion of the abalone shell.

The nacre of the abalone shell is known for its considerably high toughness and significant inelasticity. The toughness of the nacre can reach up to 1.5 kJ/m^2. This toughness value is typically twice the toughness for high-tech ceramic materials, 1000 times the toughness of aragonite, and 3000 times the toughness for calcite. Along with toughness, the nacre presents exceptional values for strain in shear and in tension. In shear, the inelastic strain can stretch up to 15%. Furthermore, in tension, the inelastic strain can reach at least 1%. It has been broadly recognized that the hierarchal microstructure of the abalone nacre is essentially responsible for its significantly high toughness and inelasticity. [22]

The nacreous layer is also known for its high strength and high impact resistance, which are both excellent protective qualities. The nacre's 'brick' and 'mortar' structural make-up allows for it to be one of the preeminent natural body armors. [23] This naturally generated armor allows for the

shell to shield the body of the abalone during an outside attack. Also, during an aggressive incident, this natural armor allows for the integrity of the shell to stay intact. [22]

As mentioned above, the nacre's hierarchal microstructure make up is believed to be responsible for the excellent mechanical properties it exhibits. This hierarchy in the nacreous layer also initiates numerous energy absorbing mechanisms. These mechanisms are: tension deformation of tablets, flaw insensitivity, and crack propagation and deflection along the interface. [23]

Studies have shown that crack deflection in the nacre structure can enhance the energy dissipation of the shell when exposed to impact loads. After analyzing the effects of the impact load, it was stressed that the organic material in the nacre is crucial to energy dissipation when subjected to dynamic loads. However, when the nacreous layer is undergoing static loads, the interfacial strength between the tablets and organic material is a main factor in reaching both high toughness and strength. Under a static load, the tension failure in the tablets occurs at the same time as the shear failure in the organic matrix. Yet, when the nacre is subjected to dynamic impact loads, the interfacial strength significantly affects the behavior of the tablet layers. The separation, or sliding, of the tablet layers during impact, as well as the damage within each of those layers, is considerably influenced by the interfacial strength. If the tablet separation and intralayer tablet damage concurrently occur during impact loads, and have a suitable interfacial strength, then maximum energy dissipation and ideal impact resistance can be accomplished. [23]

The separation of the tablets in the nacreous layer is an essential mechanism for increasing strength and toughness. The separation predominantly appears at the organic layer between two tablets **(Figure 8)**. The tablets slide apart instead of fracturing through the tablets. [24]

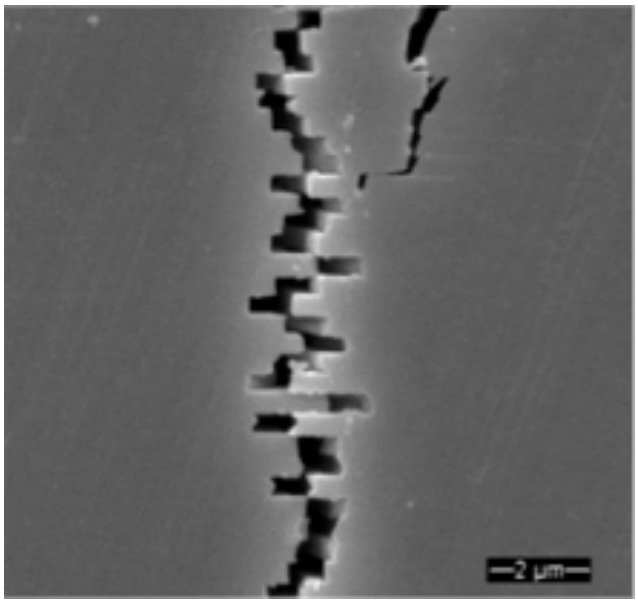

***Figure 8**. The separation of Red Abalone tablets after being subjected to a load during tensile testing. Reprinted from Lin, Albert Yu Min, Marc André Meyers, and Kenneth S. Vecchio. "Mechanical properties and structure of Strombus gigas, Tridacna gigas, and Haliotis rufescens sea shells: a comparative study." Materials Science and Engineering: C26.8 (2006): 1380-1389. [24]*

The superior mechanical properties of the abalone nacre make it a great model for the structure of composites and materials used in infrastructures. Structures created based on the abalone nacre would have high impact resistance and a resilience to blast loads. [22] The nacre would also be a great alternative for materials used in tissue engineering, bone repair, and other regenerative medical applications. [25]

1.4. Mesolayer

The hierarchal structure built of numerous aragonite tablets is not the only structure found in the nacreous layer of the abalone shell. The additional structure that appears within the nacre is called the mesolayer. The mesolayer can be seen with the naked eye. As shown in **Figure 3**, there are distinctive black lines noticeable in the nacre. Furthermore, when photos are taken of the inner layer of the shell, it is apparent which area of the nacre has a mesolayer and which area does not **(Figure 9)**. In the bottom left sample picture, nacre without a mesolayer is shown, and an arrow is pointing toward its corresponding location on the shell. In the bottom right picture, nacre with a mesolayer is shown, and an arrow is pointing to its corresponding location on the shell as well. The darker curved lines throughout the nacre on the bottom right picture indicate that there is mesolayer beneath those lines. The nacre without the mesolayer is yellow, while the nacre with the mesolayer shows the iridescent color with the darker lines throughout. There has not been much research focused on this particular structure in the nacre, so it is unknown how the mesolayer affects the mechanical properties of the nacre or the nacre structure as a whole.

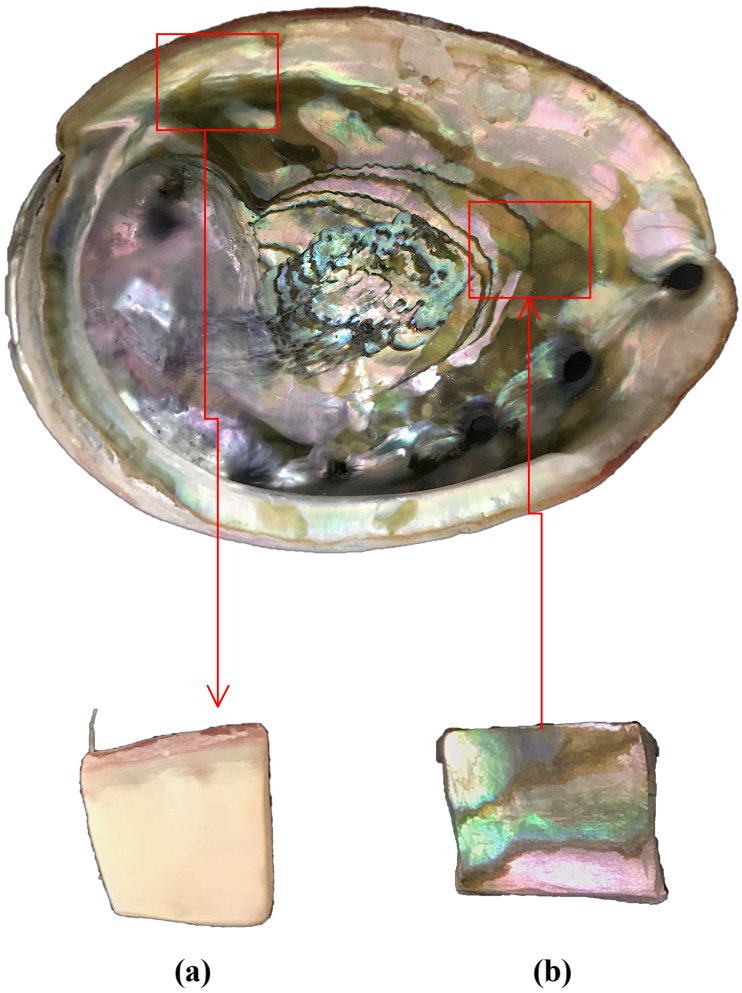

Figure 9. *Photos taken of nacre sample (a) without mesolayer and (b) with mesolayer and arrows pointing to their corresponding locations on the shell.*

The mesolayer, also known as growth interruptions or growth bands, is an important part of the hierarchal structure of the nacre, which is not frequently studied. This growth interruption appears due to fluctuations in temperature cycles and disruptions in seasonal feeding patterns.

The mesolayer is an organic layer that separates larger portions of the stacked tablets in the nacre. In comparison to the more textured tablets, the mesolayer is a soft, viscoelastic layer. The growth interruptions appear in the nacre approximately every 300 μm, and are roughly 20 μm

thick. Prior to and following the growth interruption, the morphology of the calcium carbonate changes. It goes through a transition stage that leads to stable tablet growth after the interruption. These growth bands appear periodically throughout the nacre, and the interruptions are comparable to tree rings **(Figure 10)**. [16, 24, 26]

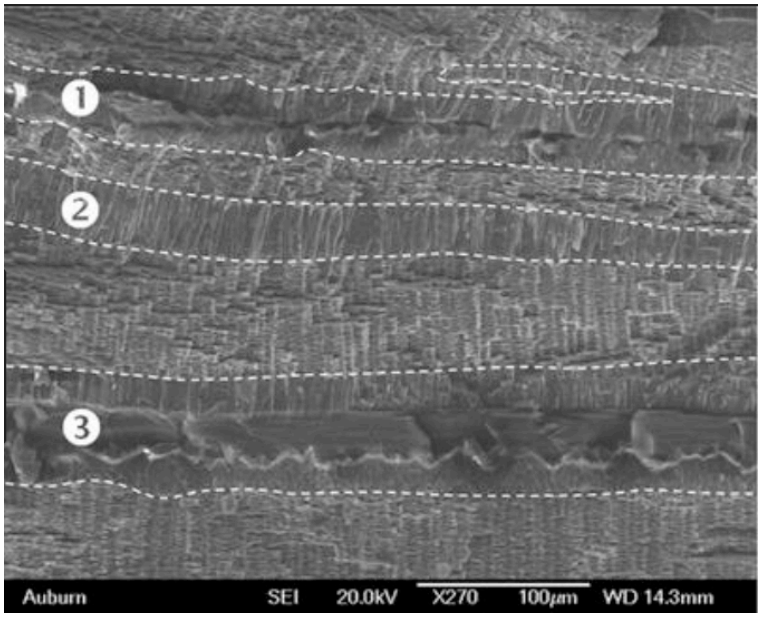

Figure 10. *SEM images of three periodic growth interruptions labeled as (1), (2), and (3) on the image. Reprinted from Sullivan, Marianne, and Barton C. Prorok. "New Insight into the Toughening Mechanisms of Nacre." Mechanics of Biological Systems and Materials, Volume 4. Springer, Cham, 2014. 93-98. [27]*

Using the SEM, the mesolayer can be easily characterized on the micro-scale. This characterization shows that the mesolayer can be separated into three sub-layers: the prismatic layer, the organic layer, and the columnar-like layer **(Figure 11)**. It is clear in the SEM images that the mesolayer has a different structure than the tablets in the nacre. Each layer of the growth interruption has a different structural morphology, which is distinct to each sub-layer. In the

little research that has been done regarding the mesolayer, most was based on the Flat Pearl Method, which will be discussed in the section "**1.6 Flat Pearl Method**".

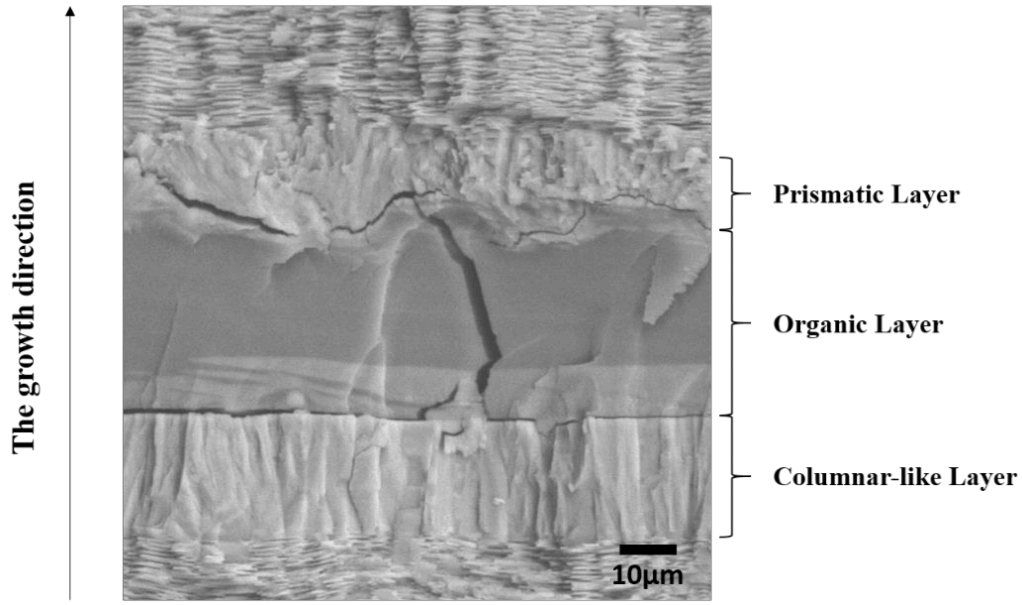

Figure 11*. An SEM image of the three sub-layers of the mesolayer: the prismatic layer, organic layer, and columnar-like layer. Reprinted from Zhang, Anqi. "The Investigation of Haliotis Rufescens (Red Abalone): Mesolayer Growth & Mechanical Behavior." (2017). [19]*

1.5. Seawater Environment and Calcium Carbonate Formation

Abalones are indigenous to the ocean. Therefore, it is important to study the seawater environment they live in. Since the natural environment affects their physiological behavior, this will be essential when examining the nacre growth in abalone.

The ocean is able to regulate itself without any outside intervention. To imitate their natural environment in our lab, a few factors were used: sea salt, live rock, and seaweed. These are important so that the tanks can self-adjust similar to the ocean environment. Many factors affect

the parameters of the seawater in nature and in the mimicked tank environment. Also, algae, bacteria, and some possible chemical reactions will affect the parameters in the tank environment. However, there are many more factors that will affect the parameters in the natural environment that do not occur in the tanks.

From observations made in our lab, there are relationships between some of the parameters. It was observed that when the pH in the tank decreases, the temperature increases. Also, feeding has an affect on pH as well. When feeding the abalone with dried seaweed, the pH will marginally decrease for a few days and then bounce back its previous level. Temperature also has an affect on the abalone's food consumption. When the temperature is too high, around 20 °C, the abalone will slowly stop consuming the provided food. If the temperature is kept at that level for too long, it may even cause some abalones to perish.

The relationships between the parameters in the natural seawater environment have been largely investigated. Parameters such as temperature, pH, and concentrations of Mg^{2+} and Ca^{2+} can be affected by factors such as seasonal cycle changes, ocean acidification, partial pressure of CO_2, sulfate concentrations, sea-floor spreading rates, etc. For example, studies show that the rise in the partial pressure of CO_2 in the seawater has caused a decrease in pH. The dropping pH values cause the acidity in the ocean to rise, which damages shell construction in organisms. However, among these factors, temperature is the parameter that influences pH and concentrations of Mg^{2+} and Ca^{2+}. The Mg^{2+}/Ca^{2+} ratios are important in controlling the calcium carbonate formation of aragonite and calcite. Therefore, the temperature is expected to impact the formation of calcium carbonate in the shell-creating organisms. [28-32]

1.6. Flat Pearl Method

The Flat Pearl Method is a well-accepted method to use for the nacre growth in abalone shells. [16, 33] For this approach, multiple substrates are glued to the shell in the insulated space between the inside layer of the shell and the mantle **(Figure 12)**. The mantle is gently maneuvered down so that no harm comes to the abalone during implantation, and the research can be completed without killing the abalone. The abalone can also contribute to numerous experiments using this method.



Figure 12*. Photo taken of glass slides implanted as substrates in abalone based on the Flat Pearl Method.*

The Flat Pearl Method allows for the nacre growth to be observed over a period of weeks. Within days the mantle covers the substrate, and allows for nacre growth. The substrates are then removed at the designated time, and then the SEM can be used to analyze the nacre growth on the substrates. For this research two different substrates were used. First, circular nacre

substrates, with a 0.8 mm thickness, were used. When no growth was observed, 7mm round glass slides were used for the substrates, with a 0.13-0.16 mm thickness.

1.7. Research Motivation

The research done for this thesis is intended to further progress the investigation of the growth in the nacre and the mesolayer structures of the Red Abalone.

The abalone nacre inspires numerous innovative studies. The nacre's structure and the mechanical properties it possesses are what draw researchers interest in mimicking this structure for numerous applications, such as composites or protective materials. The abalone's biomineralization process is of particular interest. In examining this process, researchers believe that the nacreous structure can be imitated and used to create a structure synthesis similar to that of nacre.

For many years, the structure of the nacre has been studied, but not much exploration has been done on the mesolayer. This structure, which occurs within the nacre itself, has a different structure than the nacre's aragonite tablet structure and appears periodically. Thus, the mesolayer development during the biomineralization process presents a unique opportunity to better understand how changes in the external environment affect the abalones self-assembly process. Researches have studied how the affects of temperature change, availability of food, etc. in the environment relate to the nacre growth using the Flat Pearl Method. However, this has not often been applied to study the mesolayer growth within the nacre in depth.

In this research, the relationship between the change in temperature and the mesolayer growth formation is examined. Also, parameters such as pH, and Mg^{2+} and Ca^{2+} concentrations were

recorded daily and observed in relation to the temperature in the seawater tank environment. Since not much has been done in investigating the mesolayer growth, it is not clear what affect the mesolayer has on the mechanical properties of the nacre. Thus, the research completed for the mesolayer is focused on trying to control the growth interruption formation to better understand the extent to which it impacts the nacre and its properties.

Investigating the mesolayer could present many advantages in furthering nacre-like structures for other material applications. This structure could be paramount in designing and developing many composite materials using the nacre-like structure model.

2. Experiment Instruments

The photos taken of these instruments will be included in "**Appendix I**". Only the descriptions of these devices will be included in this section.

2.1. Optical Microscope (OM)

The Optical Microscope used in this research was the Mitutoyo Corporation made OM (Serial No. 300113). It has a digital camera that is used to take photos of the samples. The digital camera is a ZEISS, AxioCam MRc. The digital camera and the eyepiece display different magnifications. So, when the computer software is used to capture photos of the samples, the total magnification should de determined based on the magnification that is displayed by the camera.

The optical microscope is useful for observing and imaging structures of samples in the macro-scale. Details and structures on samples that are not visible to the naked eye can be seen on images taken using the OM.

2.2. Scanning Electron Microscope (SEM)

In this research, the JEOL JSM 7000F SEM was used. The high resolution of the scanning electron microscope is essential for this research. This device is mainly used to characterize the structure of the aragonite tablets in the nacre, the structure of the mesolayer, as well as the 'Christmas Tree-Like' formations formed in the seawater environment.

For the SEM, the working distance, beam current, Electron Alignment, Focus, probe current, and Working Distance must all be considered to attain a high-resolution image of the samples. The ideal Working Distance for the glass substrates was 10 mm. The voltage varies depending on the

properties of the sample, but mostly 20 kV was used. Caution must be used when utilizing the tilt function to better see the sample specimen. If not careful, the tilt could be too high and cause the sample holder to slam into the detectors. This is why the IR camera must be open during the tilt and the rise of the sample holder.

Both Energy Dispersive Spectrometry (EDS) and Backscattered Electron (BSE) were used to analyze the composition of the samples. Secondary Electron (SE) function was used to examine the structure of the samples.

2.3. Electron Dispersive Spectrometry (EDS)

The EDS detector is installed in the same vacuum chamber as the SEM, and the OXFORD Instruments X-Max 20mm^2 detector was used for this research. The EDS helps to determine the composition of the sample. It shows specific types of elements in the sample, while BSE only shows the difference in elements along an area of the sample surface.

2.4. Instruments Used In Red Abalone Lab

The *Haliotis Rufescens*, Red Abalones, in our lab are held in an artificial seawater tank environment that is meant to imitate their natural environment. The abalones were purchased from the Cayucos Abalone Farm in California. In each tank, there are 3-4 large Red Abalones.

The tanks are 38 gallons, and filled with a saltwater mixture made of Instant Ocean Sea Salt and deionized water that's made in the lab. The salinity of the tank saltwater is kept around the salinity of ocean seawater, which is approximately 35 ppt.

Chillers installed in the tank control the temperature. The AquaEuro USA Max-Chill Titanium Aquarium Chillers (1/13HP) are used. Each tank has an external filter attached to it that filters

the water 24 hours a day, which is called the Tetra Whisper 60 filter, along with Tetra Whisper Bio-Bag Large Carbon Filer Cartridges. The Uniclife Aquarium Air Pump, with a 4-Watt 4-LPM adjustable flow rate, delivers oxygen to the tanks.

Each day, parameters of the tank are measured and recorded. These parameters are: temperature, pH, Mg^{2+} concentration, Ca^{2+} concentration, salinity, Nitrate, Nitrite, and Alkalinity. The temperature and pH were measured using the Hanna® pHep+ Pocket-sized pH Meter. The concentrations of Mg^{2+} and Ca^{2+} were measured with the Salifert® Magnesium Profi Test Kit and the Salifert® Calcium Profi Test Kit, respectively. The salinity was measured using the American Marine Inc. Pinpoint® Salinity Monitor. The Nitrate was tested using the Salifert® Nitrate Profi Test Kit, and the Nitrite was tested using the API® Nitrite Test Kit. Lastly, the Alkalinity was measured using the Tetra EasyStrips Aquarium Test Strips.

The levels for Mg^{2+}, Ca^{2+}, pH, salinity, Nitrate, Nitrite, and Alkalinity strive to be maintained within the range for natural saltwater. These acceptable ranges for each are listed in **Table 1** below. These are the ranges listed on the test kits and instruments used in the lab.

	Saltwater Ranges
Magnesium (Mg^{2+})	1300-1500 ppm
Calcium (Ca^{2+})	415-450 ppm
Salinity	~50.3 mS = ~33.0 ppt
pH	7.8-8.4
Nitrate (NO_3^-)	0-80 ppm
Nitrite (NO_2^-)	0-3.0 ppm
Alkalinity	120-300 ppm

Table 1*. Acceptable ranges of saltwater values for Mg^{2+}, Ca^{2+}, pH, salinity, Nitrate, Nitrite, and Alkalinity.*

3. Results and Discussion

3.1 Discussion on Experiments Completed Using Different Substrates

For this research, two different types of substrates were used for implantation. The first substrate was natural nacre of two different thicknesses. The second type of substrates utilized was glass slides.

3.1.1. Natural Nacre Substrates

The thicknesses of the natural nacre substrates range between 1.2 mm to 1.5 mm and 0.5 mm to 0.8 mm, respectively. The first nacre substrate used was the thicker option at around 1.5 mm in thickness (**Figure 13**). In the **Figure 13** below, a top view of the substrate (**a**) and a side view of the substrate (**b**) are shown.

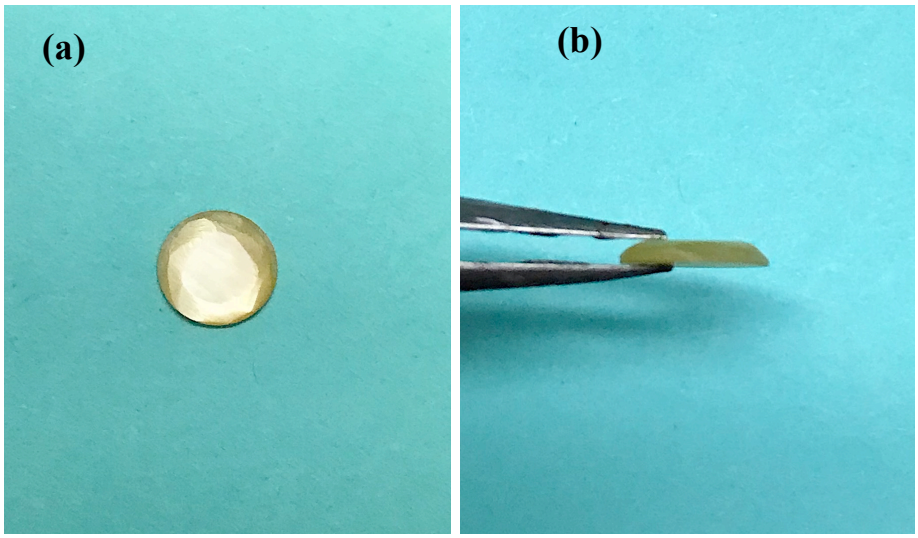

Figure 13*. The natural nacre substrate is shown from (a) the top view and (b) the side view.*

However, this substrate would have been too thick for the mantle to cover during the allotted testing time, and the abalone would have rejected the implantation. Therefore, before implanting the substrate, a Dremel 8100 rotary tool was used to grind down the edges of the sample to create a slope around the entire substrate. This gives the substrate a convex shape on the top portion of the substrate, which will be covered by the mantle, and a flat bottom surface that is attached to the shell during implantation (**Figure 14**). Once the substrate was grinded down, it was polished to rid the surface of scratches and create a smooth surface for growth.

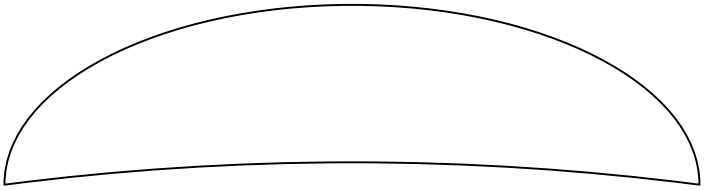

Figure 14*. A diagram of the substrate's convex shape once the edges are grinded down using the Dremel 8100 rotary tool.*

As mentioned in section "**1.6. Flat Pearl Method**", the implantation of the substrates is done using the Flat Pearl Method. The implantation of the thicker nacre substrate is shown below in **Figure 15**. In the image, this is the first substrate implanted out of two total. Only two slides were implanted while using the natural nacre substrates due to the possibility that using more could cause the abalone unnecessary stress and trigger the rejection of the substrates all together.



Figure 15*. An image taken of the thicker nacre substrate during the implantation process.*

During implantation, it was difficult to adhere the substrates to the shell where there were ridges, due to the thickness of the substrate. Even though the glue had dried and adhered the substrate to the shell, some of the substrates had fallen off by the time the substrates were removed. It was assumed this was due the mantle not being able to cover the substrate, so it was exposed to the saltwater only during the allotted testing time.

Once the testing period was completed, the substrates were removed and observed using the SEM. It was confirmed that the first testing period using the thicker nacre substrates yielded little to no nacre growth (**Figure 16**). As mentioned previously, the nacre substrates grow in a stacked tablet formation, with an organic compound between these tablets. Shown in **Figure 16** are SEM images of an overall view of a fractured tablet sample (a) and a view of the top portion of the

sample where the growth should have occurred (b). In **Figure 16 (a)**, a red dotted line is placed where the substrate sample begins and ends. Below the substrate is the tape used to hold the sample in place during imaging, and above the substrate is where nacre growth should have occurred. **Figure 16 (b)** shows the only tablet growth that occurred during the entire testing cycle, which was very little and not in the usual tablet 'brick' and 'mortar' structure. Since there was hardly any tablet growth for the nacre, then absolutely no mesolayer growth occurred.

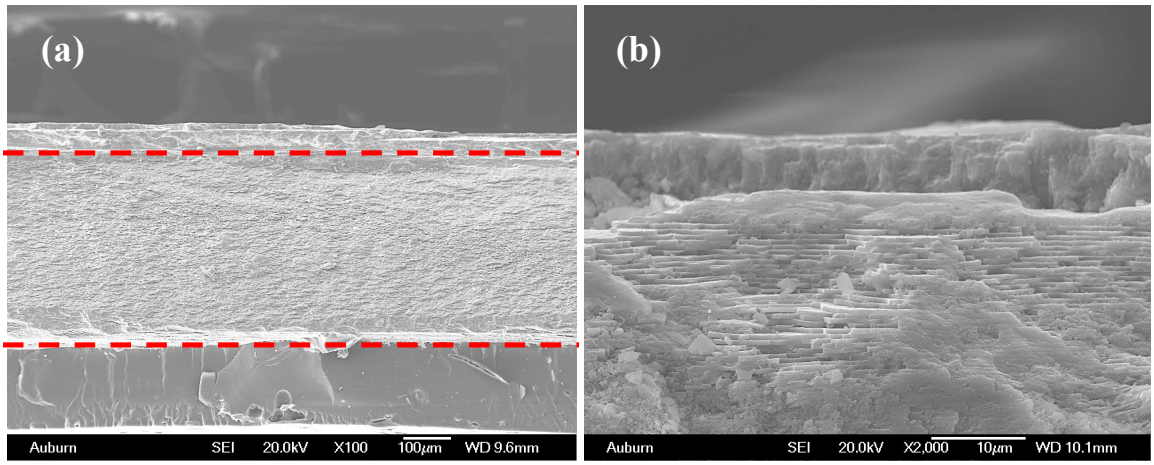

***Figure 16**. SEM images taken of (a) an overall view of a fractured tablet sample and (b) a view of the top portion of the sample where the growth should have occurred.*

Therefore, the first nacre substrate was too thick for the mantle to cover; so a smaller version of the nacre substrate was used. The thickness of the second natural nacre substrate was approximately 0.8 mm (**Figure 17**). In **Figure 17** below, the thinner substrate is shown from the top view (a) and from the side view (b). Just by looking with the naked eye, it can be seen that the second nacre substrate used for implantation is much smaller than the first.

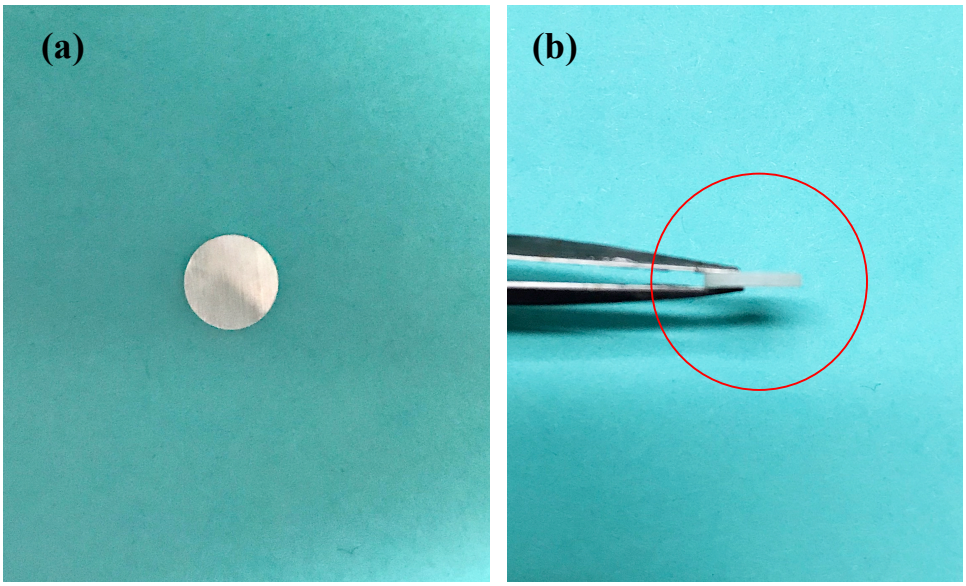

Figure 17*. The thinner natural nacre substrate is shown from (a) the top view and (b) the side view.*

Since this substrate is thinner than the first nacre substrate used, the edges were not grinded down using the Dremel 8100 rotary tool. Due to the thinner edges, it was assumed that the top half of the substrate would not need the sloped, convex shape for the mantle to be able to cover it. Additionally, the surface of this substrate was not grinded or polished, so it was implanted as is. In **Figure 18** below, an image taken during initial implantation is shown.

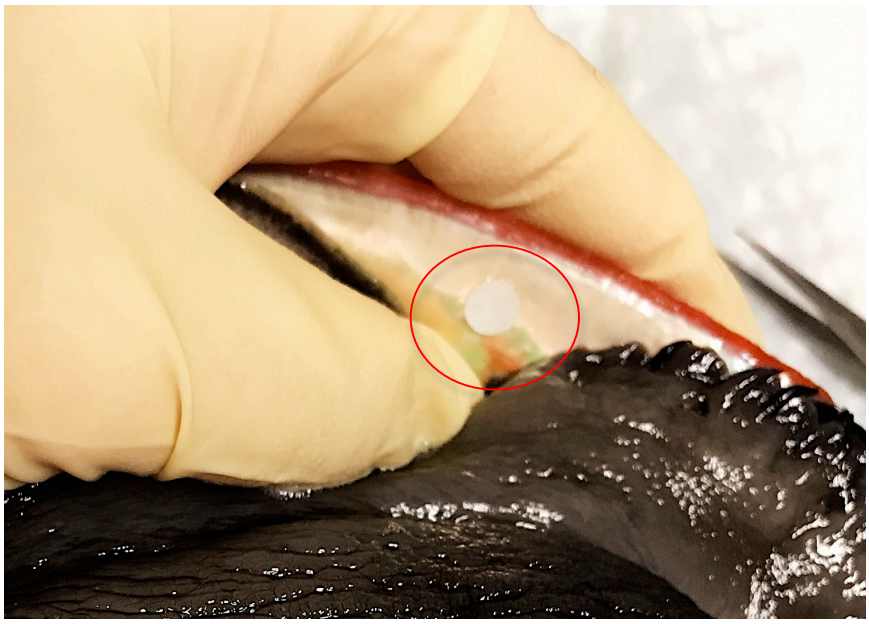

***Figure 18**. An image taken of the thinner nacre substrate during the implantation process.*

When the substrates were removed, the mantle was barely covered. So, during the removal of the substrates, the mantle did not have to be pulled down very far for the substrate to become completely visible (**Figure 19**). Therefore, it was assumed that it took the abalone longer than necessary to cover the substrate, which would have caused the substrates to be exposed to the saltwater throughout most of the allotted testing time. When observed using the SEM, there was either little to no growth or there was some tablet growth with no hint of a mesolayer, which depended on whether or not the abalone was able to entirely cover the mantle during the testing cycle (**Figure 20**). In **Figure 20**, the red dashed line on the SEM image shows where the initial growth started on the substrate. After the columnar-like initial growth, the slight stacked tablet growth is shown. Where the tablet growth occurred, there was no mesolayer within it. This is due to the mantle not being covered long enough during the temperature change cycle for it to appear in the nacre growth, since the mesolayer is directly related to the cycle change.

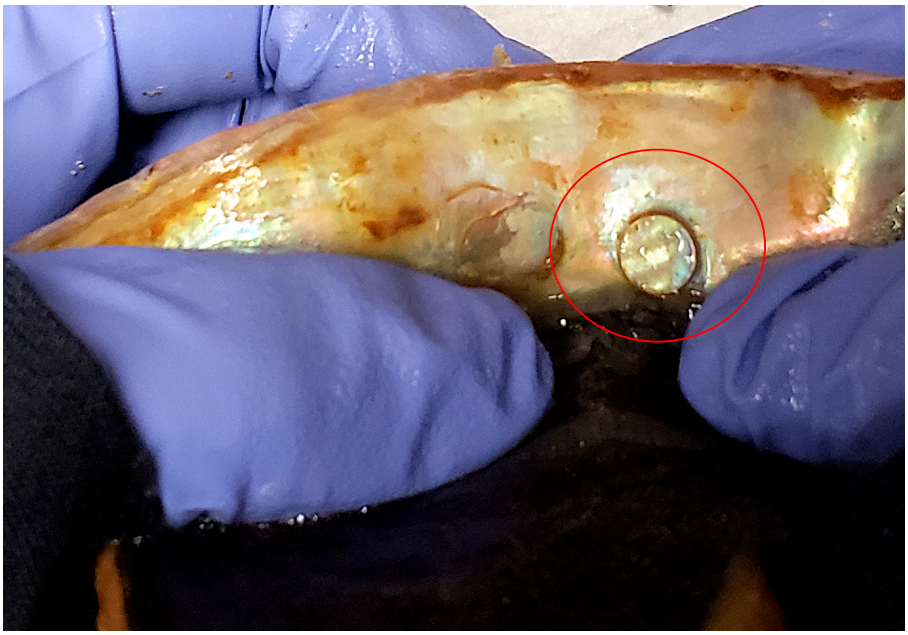

Figure 19. *Image of the thinner nacre substate taken before it is removed once the testing period was complete.*

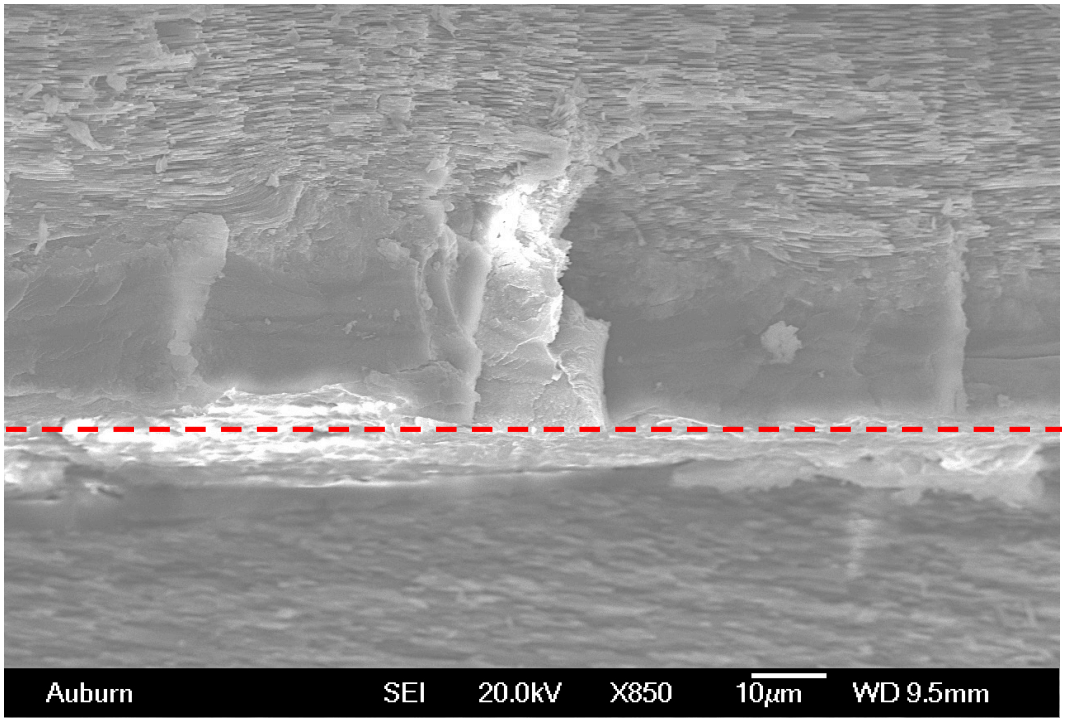

***Figure 20**. An SEM image taken of the tablet growth on the thinner nacre substrate sample with little nacre tablet growth.*

Even though the second natural nacre substrates were much thinner than the first, in most cases it was still too thick for the mantle to completely cover and produce growth. Thus, the substrate must be much thinner so the mantle covers the substrate entirely throughout the testing cycle, and the thickness of the natural nacre substrates would need to be grinded down to a much smaller size for growth to occur. Due to the lack of nacre growth using these substrates in this research, a different type of substrate was chosen for implantation going forward. However, in prior research done in this lab, before these five experiments commenced, a very thin natural calcite substrate, which was taken from the shell of a deceased abalone in the lab, was used with much success. This further substantiates that a natural nacre substrate would work well if the correct thickness was used.

3.1.2. Glass Slide Substrates

Since there was little to no tablet growth observed with the prior natural nacre substrates, a glass slide was chosen for implantation instead, since there was much success with these slides in earlier research done in this lab. The glass slides are a 7 mm, #1 thickness, which is a thickness ranging between 0.13 mm to 0.16 mm (**Figure 21**). In comparison to the natural nacre substrates previously used with thickness ranges of 1.2 mm to 1.5 mm and 0.5 mm to 0.8 mm, these slides are exceedingly thinner. The nacre substrates are 5 to 12 times larger than the thickness of the glass slides. In **Figure 21**, a top view (a) and a side view (b) is shown of the glass slides.

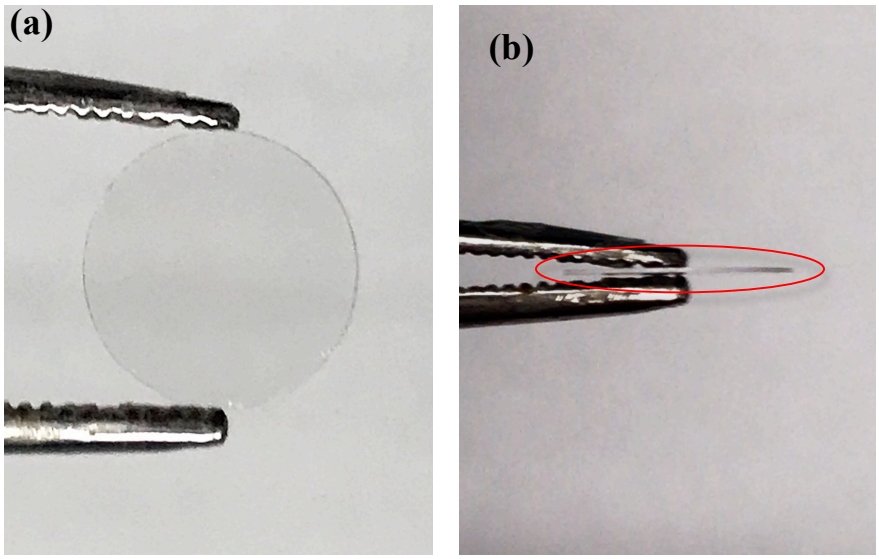

Figure 21. *Images taken of the glass slides from (a) the top view and (b) the side view.*

It was assumed that the substantial decrease in thickness of the slides used for implantation would allow for the mantle to fully cover the substrate fairly quickly after implantation. This would permit growth that reflects the changing temperature cycle, and allow for a mesolayer to form within the nacre. Also, more slides could be used during implantation (**Figure 22**). Since the glass slides are so much thinner than the nacre substrates, there was less of a chance the abalone would be harmed or reject the slides. **Figure 22** shows the implantation of the glass slides onto one of the abalone, which are circled with a red line.

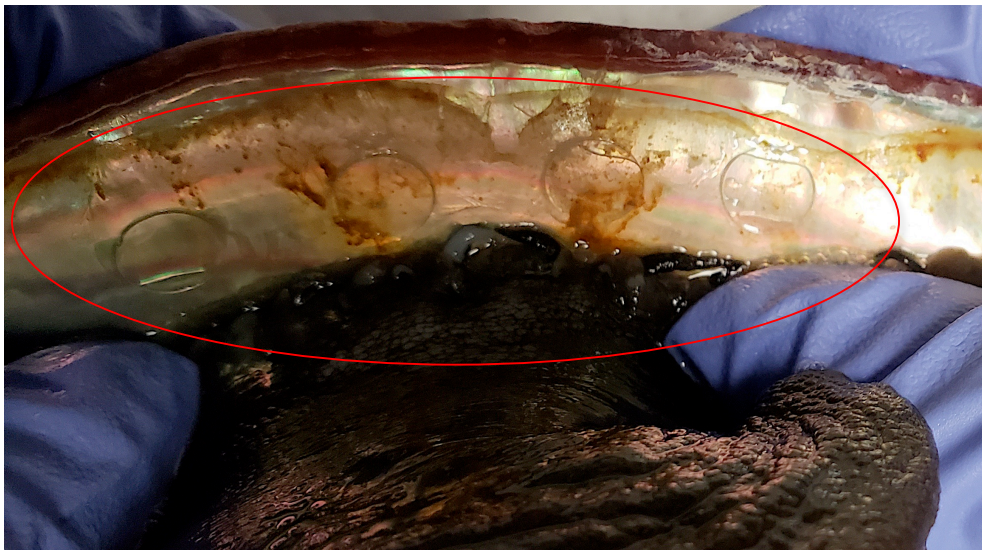

Figure 22. *Image taken during the the implantation of the glass slides.*

Once the testing cycle was completed, it was observed during the removal of the slides that the mantle had convered the slides entirely, which was due to the slides being significantly thinner than the nacre substrates. Upon pulling the mantle down to remove the slides, it was visibly noticeable that there was some growth on the slides (**Figure 23**). In **Figure 23**, an image taken of the slides before being removed from the abalone shell is shown, and circled in red.

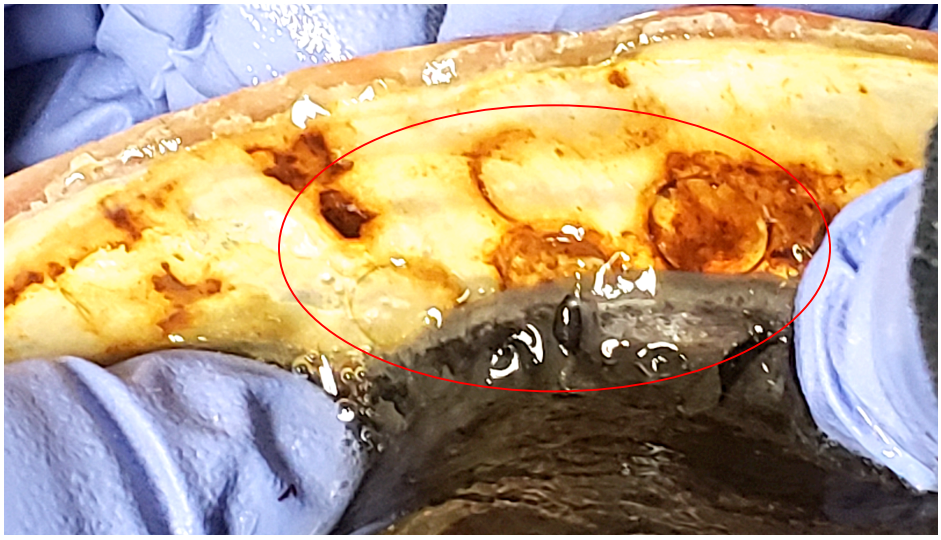

Figure 23. *Image taken of the glass slides before it is removed once the testing period was complete.*

Once the slides were removed and fractured, they were imaged using the SEM. Since the mantle was able to completely cover the slides during the testing cycle, growth occurred on the substrate. On most of the samples, a large 'brick' and 'mortar' stacked tablet structure was seen (**Figure 24-25**). The SEM image in **Figure 24** shows the overall tablet growth on the glass slide without the mesolayer. While the SEM image in **Figure 25** shows a zoomed in view of the same nacre tablet growth on the glass slide without a mesolayer.

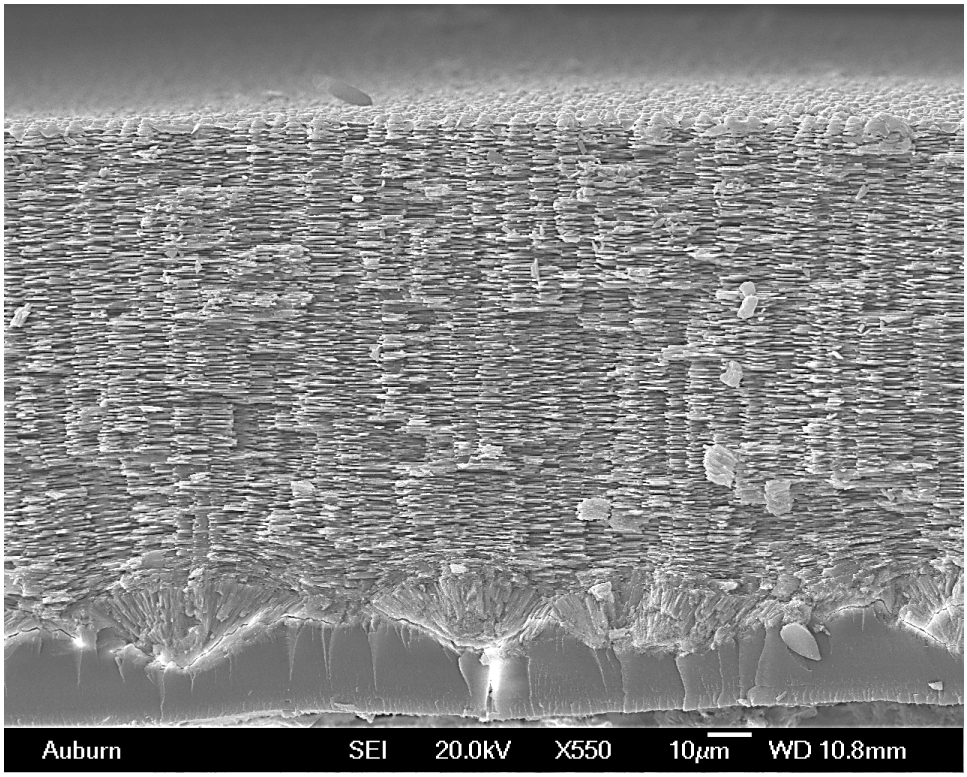

Figure 24*. SEM image taken of overall nacre tablet growth on glass slide without a mesolayer present.*

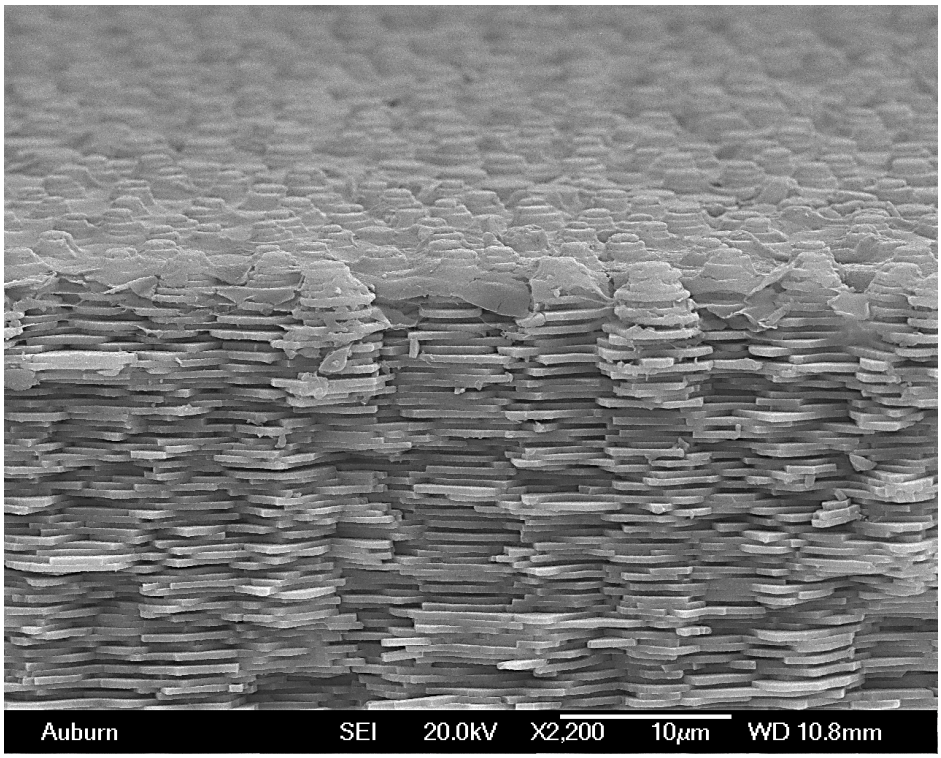

Figure 25*. Magnified SEM image taken to see a closer view of the nacre tablet growth without the presence of a mesolayer on the glass slide.*

However, there were a few samples that showed a slight interrupetion in the nacre tablet growth on the galss slides (**Figure 26**). This is a small mesolayer growth, and is outlined in the red dashed line on the SEM image. The mesolayer in this image did not completely form in the usual structutre of the mesolayer. It only displayed the columnar structure of the mesolayer, without showing the prismatic and organic layers.

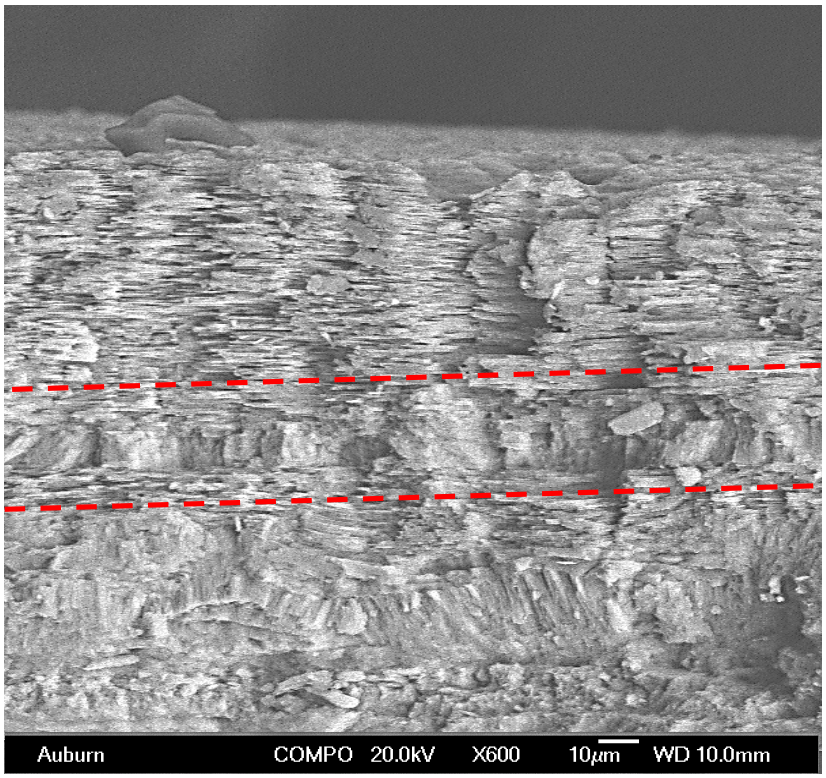

Figure 26*. An SEM image taken of the slight interruption in the nacre tablet growth on the glass slide, showing the presence of the mesolayer.*

Since there was nacre tablet growth, and a growth interruption present in some of the samples, the glass slides had more success than the natural nacre substrates that were previously used. This is due to the thickness being so much smaller in the glass slides than in the nacre substrates. This substantial decrease in thickness allowed the mantle to cover the slides quickly, and the abalone did not reject these after implantation. The glass slides were exposed to the elements in the saltwater for a much shorter period of time than the nacre substrates as well. While some of the glass slides had more nacre growth than others, as well as some having a growth interruption while others did not, this is not caused by the mantle's ability to fully cover the slides. Since it was proven that a thinner substrate allows for nacre growth, then this is due to the location in

which the slides were implanted onto the abalone shell for the testing cycle. This will be discussed in the next section.

The glass slides worked much better in this research for promoting nacre growth, but the natural nacre samples have potential to have much success as well. Since there was minor nacre tablet growth in the nacre substrates, there is the possibility that this substrate will work well once the thickness is grinded down to a much more acceptable size, similar to the thickness of the glass slides. The glass slides are much more brittle than the natural nacre substrates, and more difficult to work with. The samples sometimes shatter or crack during implantation and removal, making it difficult to have viable samples for imaging. The nacre substrates would be a much more reliable option to use, since they are easier to work with during the hands on phase of the process and are not as brittle as the glass slides. Because of varied success, there is no real conclusion to which substrate is better. It appears that the location of implantation is more important than the type of substrate.

3.2. Location of Substrates on Shell

As mentioned in the previous section, it was found that the thinner substrates allow for nacre growth to occur. Although, of the substrates that are implanted, some have more nacre growth than others, and some have a growth interruption while others do not. This is due to the location of the substrates implanted along the growth edge of the shell.

While the abalone is in the saltwater tank and suctioned to its walls, the abalone pushes its mouth out when feeding. In **Figure 27**, the image is taken of an abalone in one of the saltwater tanks, and the important areas near the mouth and growth edge are labeled. In **Figure 28**, the image is

taken of an abalone removed from the tank with its mantle better exposed. It demonstrates that the portion of the mantle near the mouth and head area is closer to the edge of the shell; and as the mantle moves along the shell beyond that front head area, the further down the mantle is from the edge of the shell.

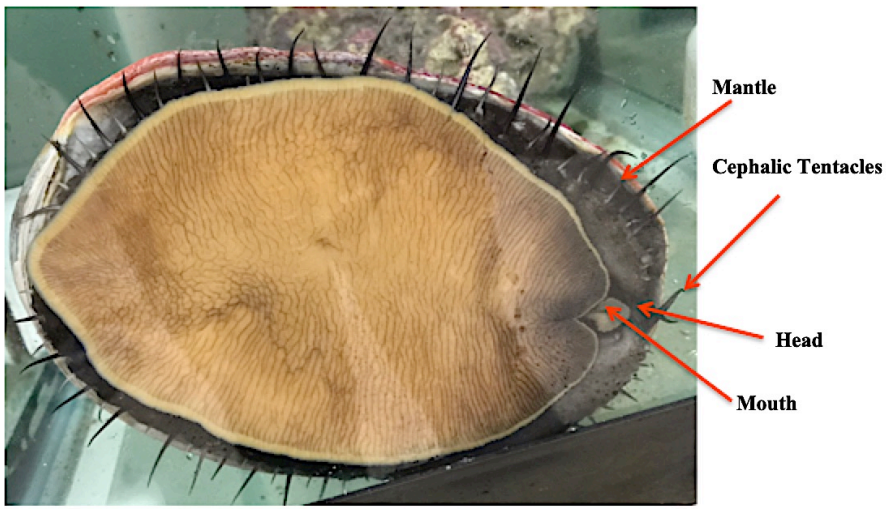

Figure 27*. An image taken of an abalone in one of the saltwater tanks with the important areas near the mouth and growth edge are labeled.*

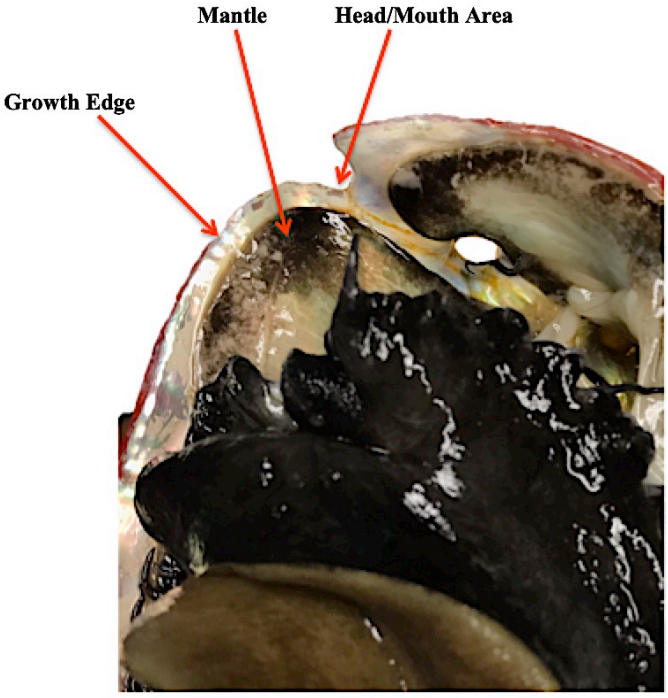

***Figure 28**. An image taken of an abalone removed from the saltwater tank with its mantle better exposed.*

When the abalone uses its mouth to reach for food, it causes the mantle to move closer to the edge of the shell and allows the substrates closest to the mouth to be covered first. The mantle coverage is vital since the substrates must be completely covered to prompt growth. Therefore, the closer the substrates are placed to the where the head tentacles and mouth are located on the shell, along the growth edge, the more growth that will occur. Thus indicating the significance of the location in which the substrates are placed during implantation, and what an important role it plays in producing growth. In **Figure 29** below, a schematic is shown of the locations in which to place the substrates during implantation.

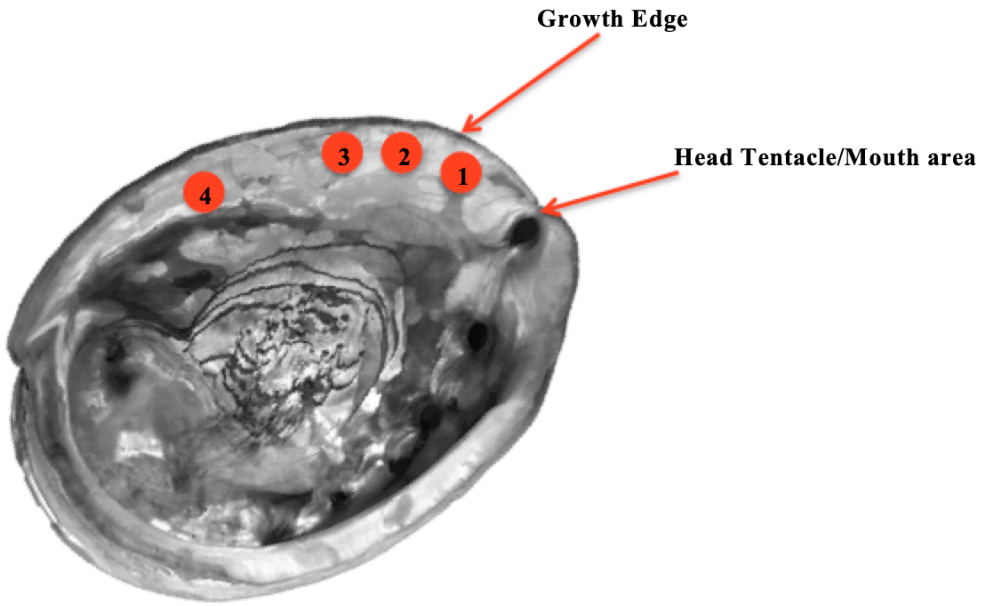

Figure 29. *Schematic of the locations to implant the substrates along the growth edge of the abalone shell.*

In the schematic, the red circles represent the substrates that are implanted on the shell. As mentioned previously, the shell grows in a spiral structure, and the growth edge is along the posterior side of the shell, opposite the circular apertures. The growth edge is where the nacre growth occurs, so following this edge and implanting the slides along it should produce growth on the substrates.

On the schematic in **Figure 29**, the further right the substrate placement is, the more nacre growth, and chance of a mesolayer, the substrate will have. The growth will decrease, as the substrates are placed farther away from the head tentacle and mouth area on the shell. For example, placement of substrate 1 will have more growth than the substrate in placement 3. Since the growth edge is closer the front of the shell, this is where the nacre grows. Nacre does

not form on the rear of the posterior side of the shell. So, as seen in the schematic, if a substrate were placed in location 4, there would be no growth.

This was proven in the results recorded from the parameters in the saltwater tank, as well as the corresponding SEM images taken of the fractured substrates after removal. If the implanted substrates were too far from the front head and mouth area, there would either be no growth or small nacre growth without any presence of a mesolayer. In the first rounds of testing, the substrates were adhered to the shell in locations corresponding to 2 and 3 on the **Figure 29** schematic. These implantations are shown in **Figure 15** and **Figure 19** in the previous section. The resulting SEM images showed there was either little growth or no growth at all, which are shown in **Figure 16** and **Figure 20**. However, in later testing cycles, the implantation was moved closer to the mouth area and is shown in **Figure 22**. This placement of the substrates yielded growth and even had a small growth interruption. These SEM images are shown in **Figures 24-26** in the previous section.

Distance from the edge of the shell also plays an important role in the placement of the substrates during implantation. For this research, when the substrates were placed approximately 1 cm from the edge of the shell, growth occurred. If the substrate is too close to the edge of the shell, then it will take longer for the mantle to completely cover the slide. This will hinder the formation of nacre tablet growth on the substrate. Likewise, if the substrates are implanted too far down from the growth edge, the substrates might end up being outside of the area that produces new growth. Contrasting from if the mantle is to close to the edge, the mantle will cover the slides, but the substrates may be placed in a location where new growth will not occur. In **Figure 30** below, a

schematic is shown of the areas where new growth appears boxed in red, and a schematic of the distance the substrates are placed from the outer edge of the shell during implantation

.

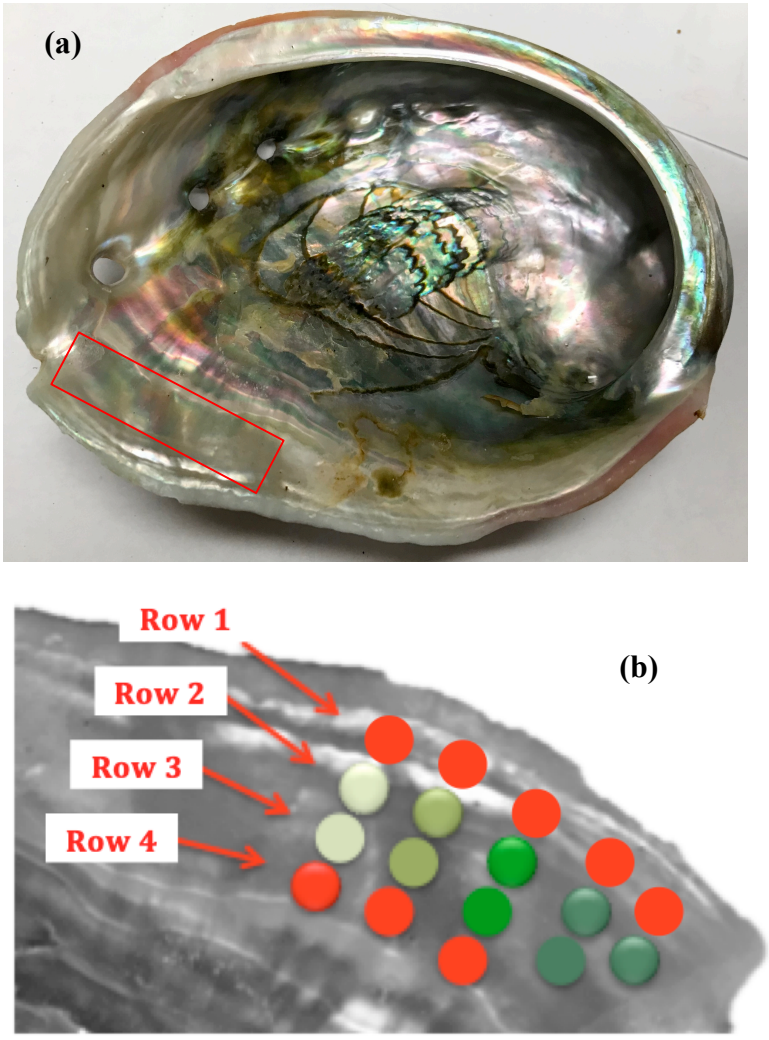

Figure 30*. (a) An image taken of the inner side of the abalone shell, with the growth zone identified in within the red box, and (b) schematic shown of distance of implanted substrates from the edge of the shell.*

The area where new growth appears is boxed in red in **Figure 30 (a).** If the substrate is placed below this area, no growth will occur. In **Figure 30 (b)**, the schematic shows four rows of

substrates Rows 1 and 4, substrates highlighted in red, will not have any nacre growth, since row 4 is too far out of the growth zone, and row 1 is too close to the edge for the mantle to cover during experimentation. Rows 2 and 3, substrates highlighted in green, have a higher probability of producing growth because the mantle will be able to cover the substrates and they are in the growth zone. As the green color becomes lighter, the probability of growth decreases. The resulting growth during experimentation if the substrate was placed too low during implantation was negligible, and only the substrate itself was seen during imaging. There was also thin layer of mucus, which is contained between the mantle and the shell, lying on top of the substrate. **Figure 31** is an SEM image taken of the fractured substrate sample when the substrate was implanted too far from the growth zone. As shown in the image, only the substrate itself was detected when imaging with the SEM in secondary electron mode. Location is one of the most important elements of implantation. If the substrates are not in the correct place, no growth will occur.

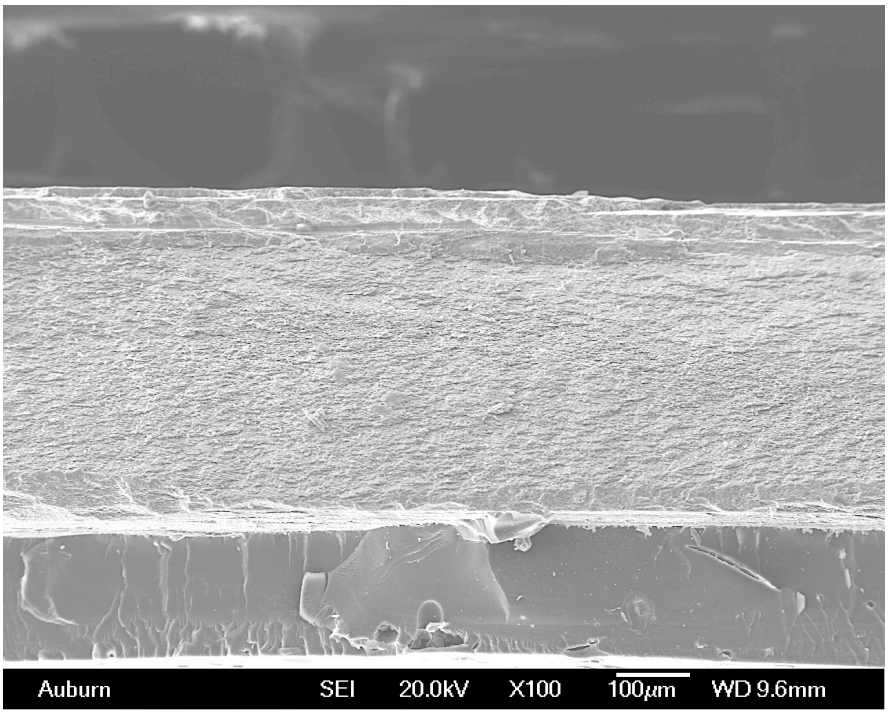

***Figure 31**. An SEM image taken of the fractured substrate sample in secondary electron mode when the substrate was implanted too far from the growth zone.*

3.3. The Relation Between Environmental Factors and Mesolayer Growth

As previously mentioned in section "**1.4 Mesolayer**", a growth interruption within the nacre tablet structure is periodically repeated. This is created due to the changes in the temperature from season to season in their natural environment. In this research, the change in temperature in the natural environment was mimicked on a smaller time scale inside each tank. If there were no change in temperature, there would be no chance for a mesolayer to form. Also, the mesolayer and the nacre tablets have different structures. Before and after the mesolayer occurs, the morphology of calcium carbonate changes, which causes the mesolayer to undergo a transition period during the mesolayer formation, and leads to stable tablet growth after the interruption. The structure of the mesolayer formation starts in the columnar-like layer, and then the organic layer is formed, followed by the prismatic layer, which leads straight to more tablet growth.

Environmental factors such as temperature, pH, and concentrations of Mg^{2+} and Ca^{2+} all have the potential to affect the formation of the calcium carbonate shell and the mesolayer growth. Of these parameters, the concentrations of Mg^{2+} and Ca^{2+} are dependent on temperature, while the pH is affected by feeding patterns. Similarly, the Calcium source for the abalone can emerge from the seawater itself or the food provided. [19] The temperature is the controlled parameter, while the other testing parameters are the variables. In **Table 2** below, there is a layout of all of the various temperature change experiments that were applied during different testing cycles, as well as the specific timeframe for each testing cycle and for the temperature change periods.

	Tank Numbers	Initial Temperature	Temperature Increase	Final Temperature
Experiment 1	1, 3, 4	13°C 3 weeks	17°C 3 weeks	13°C 3 weeks
	5	13°C 3 weeks	17°C 3 weeks, 2 times	13°C 3 weeks, 2 times
	6	13°C 3 weeks	17°C 6 weeks	13°C 3 weeks
Experiment 2	2	13°C 30 days	None 10-15 days of not feeding	13°C 30 days
	3	13°C 30 days	21°C 10-15 days of not feeding	13°C 30 days
	5,7	13°C 30 days	21°C 10-15 days of not feeding	21°C 30 days
Experiment 3	1,6	13°C 3 weeks	17°C 3 weeks	13°C 3 weeks
	9	13°C 3 weeks	17°C 3 weeks, 2 times	13°C 3 weeks, 2 times
	10	13°C 3 weeks	17°C 6 weeks	13°C 3 weeks
Experiment 4	2,7	12°C 3 weeks	16°C 3 weeks	12°C 3 weeks
	3	12°C 3 weeks	16°C 2 weeks	12°C 3 weeks
Experiment 5	10	13°C 4 weeks	12°C 3 weeks	13°C 2 weeks

Table 2. *A layout of the various temperature changes applied during different testing cycles, with some being repeated throughout multiple tanks during a single testing period.*

3.3.1. Insertion of Live Rock Into Saltwater Tanks

Prior to the five experiments completed in this research, the tanks in the saltwater lab were set up with the same equipment, but there was one significant difference: sand. The bottom of each tank was filled with sand, and that was where algae and bacteria thrived. However, when the new

research started, the tanks were cleaned out and all traces of sand removed to provide a fresh start.

For the first two experiments, there was no sand or anything else in the tank, just the abalone and pump. However, it was soon realized that there was nowhere for the algae or microbial, such as bacteria, to cultivate and grow. The sand also acted as a sort of filter system that kept the nitrate, nitrite, and alkalinity levels in the correct ranges. Without the presence of the sand, the nitrate and nitrite levels were fluctuating greatly, until the nitrates were too high and unsafe for the abalone to live in the environment. If the nitrate level gets too high and into the unsafe zone, the abalone could be harmed or even die. This also caused the other parameters to fluctuate greatly and the values were unpredictable, since this behavior was not typical. To help stabilize the saltwater, about twenty percent of the water would be pumped out and replaced with fresh saltwater on a regular basis. However, this was not applicable during the testing cycle experiments to ensure that introducing new saltwater to the tank environments did not skew the results. Once the substrates were implanted for the experiments mentioned above, the parameters were tested everyday and did not show the trend expected (**Figure 32**). After the substrates were removed and imaged in the SEM, they garnered no results. In **Figure 32**, graphs are shown of the recorded data from the parameters per day from one of the tanks in the first of the increasing temperature experiments. The graph shows how the recorded values are not steady, and shift from one day to the next. Instead of the pH being steady, and then decreasing when the temperature is increased, it fluctuates in the same range for the entirety of the testing cycle. The concentration of Mg^{2+} also varies greatly, following no specific trend. The concentration of Ca^{2+} does not keep to the usual trend of decreasing when the temperature increases. It does have an elevation when the temperature is increased, but it quickly drops back down and fluctuates while

steadily decreasing until the testing cycle is completed. Since the parameters were so unpredictable, the experiments including temperature increase and withholding food garnered no results. So, it is unclear how withholding food for a short period of time would have affected the nacre and mesolayer growth.

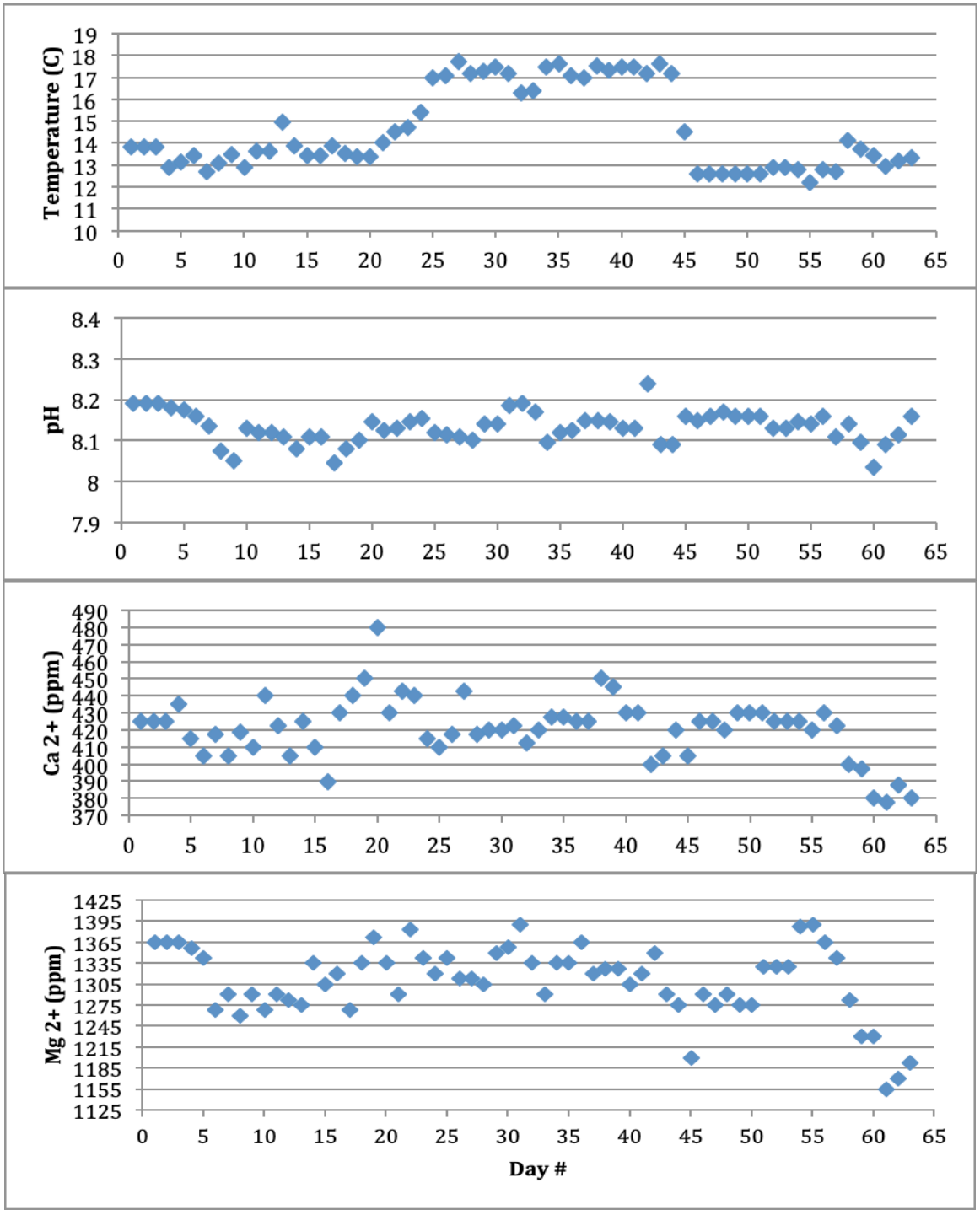

Figure 32. *Graphs that illustrate the Increasing Temperature Experiments for the first testing cycle without Live Rock, and the resulting fluctuaitng paramters from day to day.*

The water levels in the tank affect the abalone, especially the constant fluctuating parameters seen in **Figure 32** above, so the environment was not ideal or healthy enough to promote any growth. To help stabilize the parameters in the saltwater, Live Rock was introduced into the tanks just before the third cycle of experiments. Live Rock is a biological filter, which helps with the nitrification process, made up of dead corals and other organisms that are also made up of calcium carbonate, which is what the majority of coral reefs in the ocean are made of. It allows the algae and microbial to cultivate, while keeping parameters, such as pH, balanced within the tank. Also, the addition of the Live Rock kept the nitrate and nitrite levels in a safe range throughout the testing cycles, since changing the saltwater during the increasing temperature experiments could not be performed (**Figure 33**). The graphs shown in **Figure 33** correspond to the graphs of the parameters shown in **Figure 35** in the following sub-section for the fourth experiment, and show the nitrate and nitrite at safe levels throughout the testing cycle.

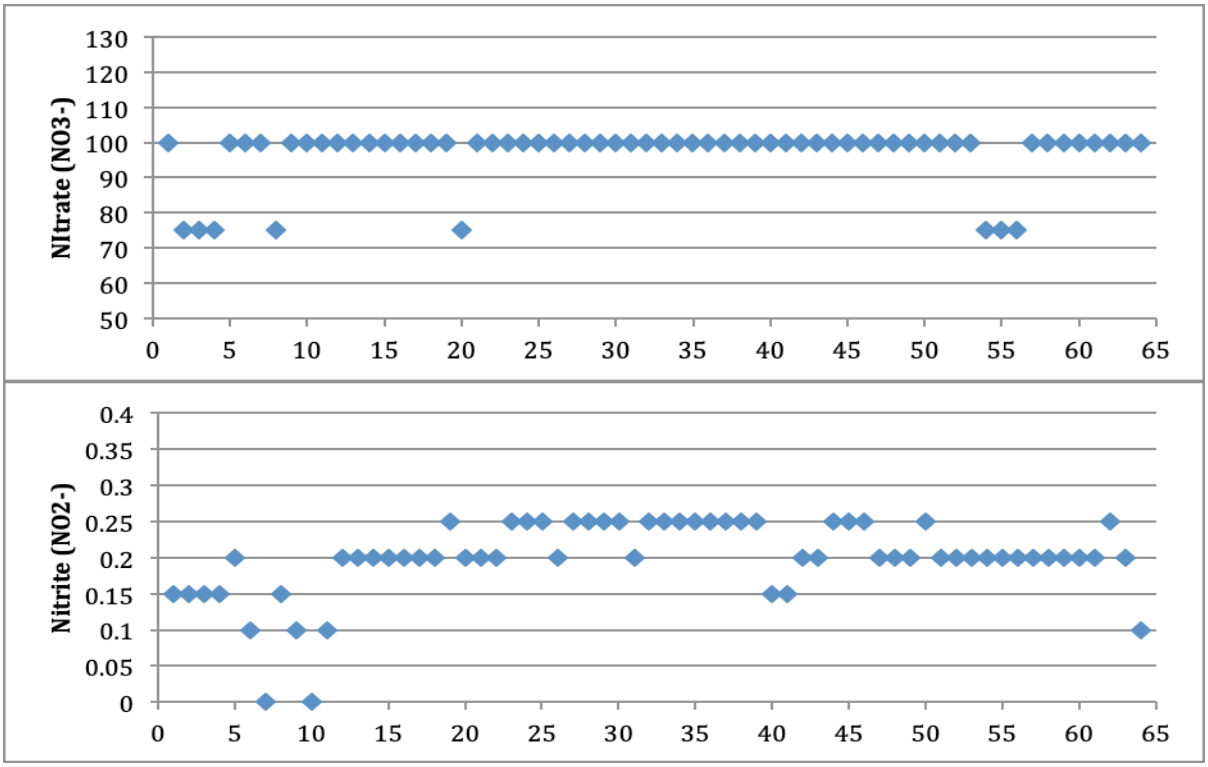

Figure 33. *Graphs illustrating the daily recorded values of the nitrate and nitrite for tank 7 thoughout the fourth experiment for increasing temperature.*

The graphs in **Figure 33** show that the nitrate and nitrite remained in safe ranges, listed in **Table 1**, throughout the testing cycle. The values are fairly constant, but drop down occasionally, and it is not clear why. Even though the Live Rock was added before the third experiment cycle, there were still no viable results obtained from that testing cycle. The Live Rock was not in the tank long enough before testing began to make a significant difference. However, by the time the fourth experiment started, the Live Rock had been in the tanks for three months and the environment had time to acclimate with this new addition. This experiment was the first to show improvements in the parameters recorded each day, as well as much success in promoting growth in the substrates. The parameters were more predictable for this testing cycle, as well as more stable, with little fluctuation compared to the experiments completed without the addition of the Live Rock. This was the first experiment in which a growth interruption could be identified within any tablet growth. There was no presence of a growth interruption, within any of the tablet growth that had been formed, in the experiments concluded up to that point.

3.3.2. Mimic Temperature Change In Nature

To mimic the temperature change in seasonal cycles, experiments were performed with various starting temperatures, periods of changing temperatures, and final temperatures with different time frames for each. The temperature change for each experiment was a few degrees for each tank (**Table 2**).

Within each testing cycle, multiple tanks were utilized. Some of the temperature changes listed in **Table 2** were applied to multiple tanks during the same testing cycle. Other changes were

brought in as factors, such as changing feeding schedules. This was done to try to determine if seasonal feeding changes had any effect on the growth of the mesolayer growth. However, this attempt yielded no results for nacre tablet growth or a growth interruption due to the parameters being so unpredictable. So, it is unclear how withholding food for a short period of time would have affected the nacre and mesolayer growth. Another technique that was applied was using the same temperature changes, but adding a second cycle for increasing and decreasing in temperature, keeping the same timeline as the first phase. This was attempted to try to result in multiple mesolayers, in which to represent seasonal cycles, similar to the wild abalone in the ocean. Again, this testing cycle yielded in no suitable results, due to using substrates that were too thick, substrates implanted in the wrong locations, or the environment in the tank being unsuitable for the abalone without the addition of Live Rock. Additionally, some of the tanks would have the same temperature change cycle, but have a different time schedule for how long the tanks would remain in each stage. Striving to create the mesolayer growth, the time in which the temperature change occurred was varied for comparison with the typical shorter time period for that phase.

Each experiment started with implantation using the Flat Pearl Method, discussed in "**1.6 Flat Pearl Method**". After implanting the nacre substrates or glass slides, the abalones were placed back into the tank and the experiments would begin immediately following the implantation process. Each tank would start at a temperature between 12°C to 13°C for a period of a few weeks. Then, after being at the initial temperature for the allotted time, the temperature would be elevated a few degrees to between 16°C and 19°C for a few weeks time. Even when the temperature change was supposed to be elevated to 21°C, the tanks could never reach this increase in temperature. The highest temperature attained was around 19°C. This was assumed to

be due to the temperature in the research lab being too cool to allow the temperature to increase to 21°C. After the temperature rise period was completed, the temperatures would be brought back down to the initial temperatures, where they would remain for a few weeks until the testing cycles were finished. On the last day of the testing period, the abalones would be taken out of the tanks, and the glass slides or nacre substrates would be removed. Once the samples were dry, they were fractured, and then observed using the SEM. **Figure 34** displays a schematic of what each temperature change looked like, as well as when tablet growth and nacre growth occurred.

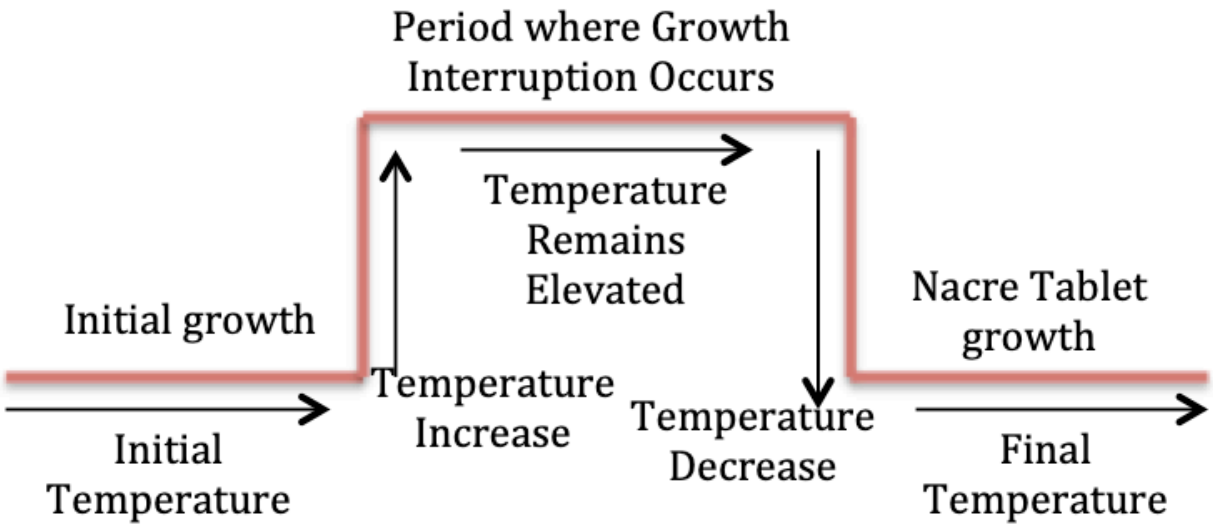

***Figure 34**. Schematic displaying the phases of increasing and decreasing temperature changes during testing cycles, as well as when tablet growth and growth interruptions occur.*

In the fourth experiment, in which a growth interruption occurred in tank 7, the initial temperature was 12°C, and kept at that temperature for three weeks. The temperature was then raised to 16°C for three weeks. After the temperature change phase was completed, the temperature was then dropped back down to 12°C for three final weeks (**Figure 35**). The graphs in **Figure 35** illustrate the temperature change phases, as well as the effect the change has on

each testing parameter for this experiment. The period of increasing temperature is located between the two red dashed lines identified on the graphs.

A second testing cycle implemented during the same experiment, on tank 2, had nacre tablet growth, but no presence of a mesolayer. Like the previous tank 7, the initial temperature was 12°C, and kept there for three weeks. After those initial three weeks, the temperature was increased to 16°C for three weeks. Once the increase in temperature phase was completed, the temperature was decreased back to the initial temperature of 12°C for three weeks (**Figure 36**). The graphs in **Figure 36** displays the temperature change phases, as well as the effect the change has on each testing parameter for this testing cycle. The phase of the testing cycle where the temperature is increased is located between the red dashed lines marked on the graphs.

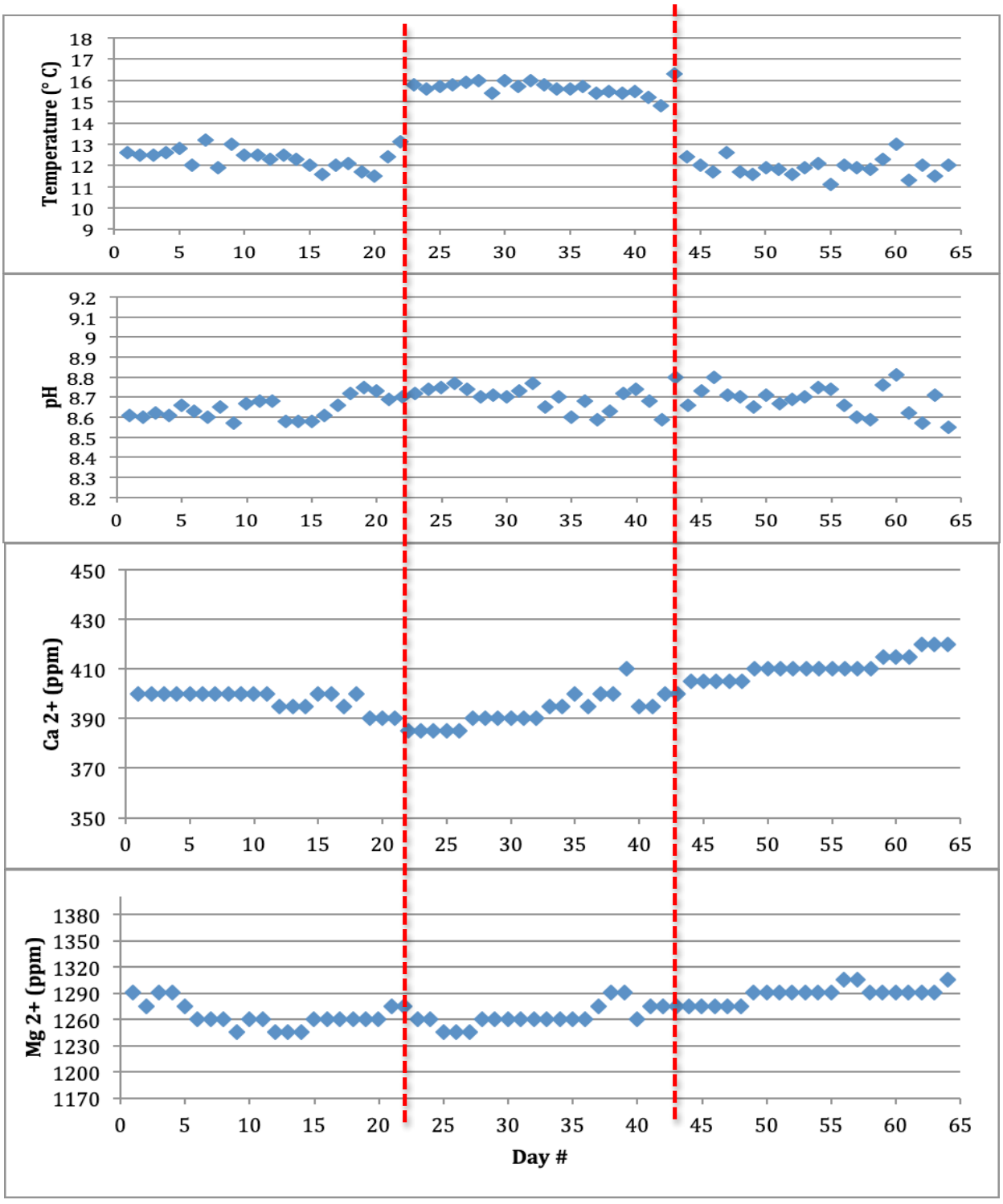

Figure 35. *Graphs that illustrate the Increasing Temperature Experiments for Tank 7, and how the increase temperature affects each testing parameter by the day.*

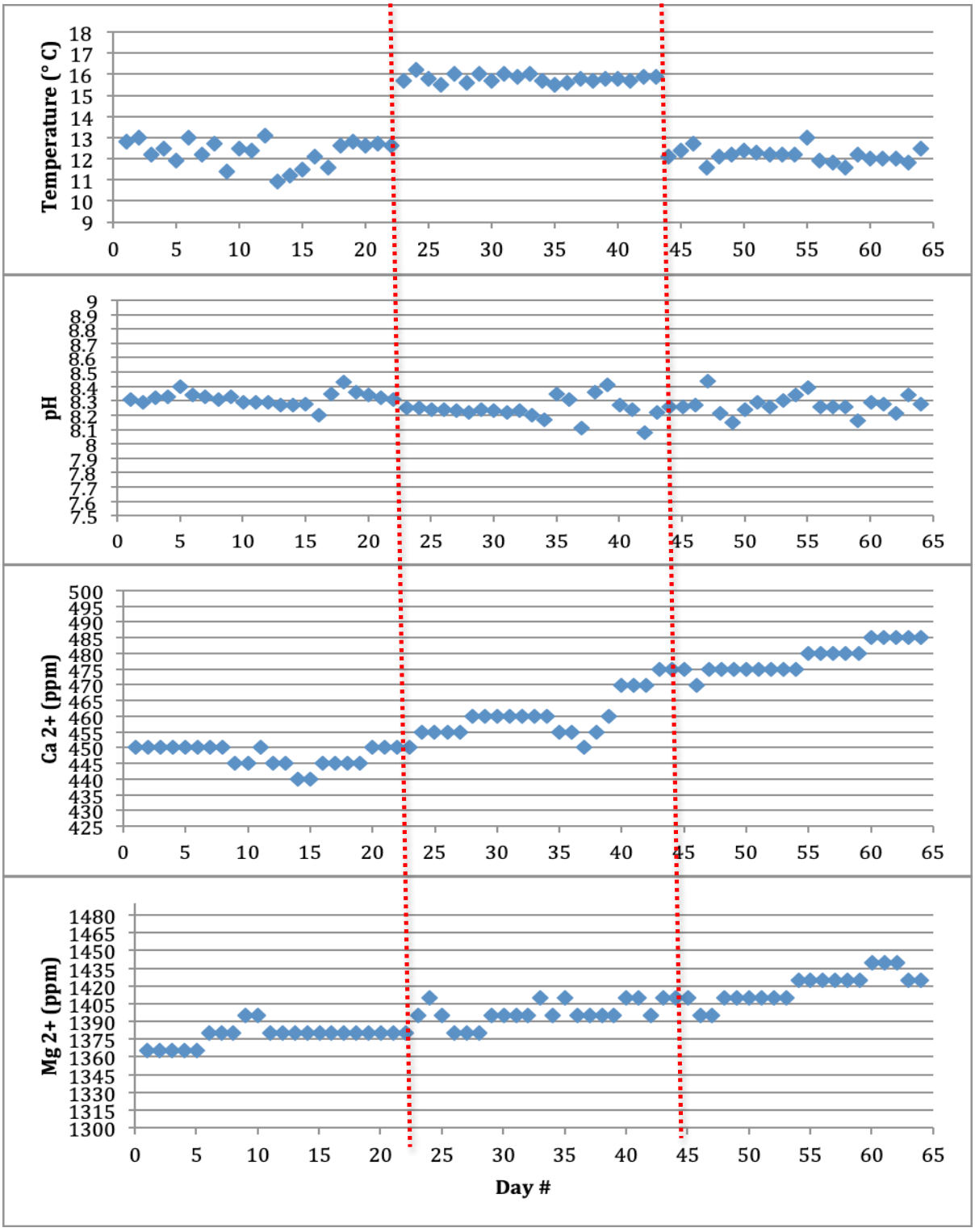

Figure 36. *Graphs illustarting the increase in temperature experiment for Tank 2, and the recorded parameters.*

The parameters for each experiment were tested at the same time every day to avoid as many fluctuations and inaccuracies in the acquired values as possible. As shown in the graphs above, as the temperature is elevated, the pH stays around the same, although a slight gradual decrease is shown, and plateaus once the temperature is brought back down to the lower starting temperature. The concentrations of Mg^{2+} and Ca^{2+} are mostly steady during the initial temperatures, then once the increase in temperature is applied, the two become out of phase until the temperature is dropped down to the original temperature. When the temperature is initially increased, different responses are observed in the concentration of Ca^{2+} and Mg^{2+}. The Ca^{2+} concentration in **Figure 35** for tank 7 shows a slight decrease when the temperature is increased, then slightly increases throughout the temperature change cycle. It then continues to elevate as the temperature is brought back down to its initial temperature for the remaining of the testing cycle. However, the Ca^{2+} concentration in **Figure 36** for tank 2 increases in the beginning of the increasing temperature period, and continually increases with a slight decrease towards the end of the increase in temperature period. It then increases once again and steadily elevates as the temperature is brought back down to its initial temperature for the remaining of the testing cycle. In the graphs, the fluctuations in the temperature are due to the chiller, since it runs on two-hour chilling cycles it is not a constant temperature and will slightly vary even if tested at the same time every day. The same can be said for the pH, since an electronic probe was used that is temperature sensitive, it slightly varied as well.

Once the glass slides were removed from the abalone in tank 7, and observed using the SEM, tablet growth and a small growth interruption were discovered. The growth interruption was very small, but visible (**Figure 37**). Although, the interruption appeared to only have the columnar-like layer of the mesolayer structure, and did not contain all three layers.

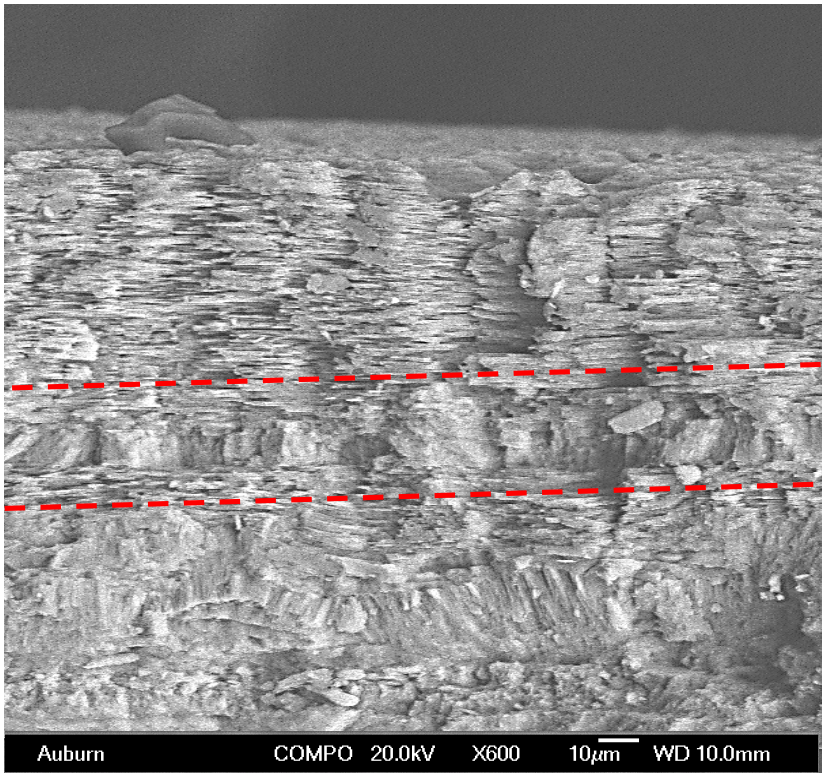

Figure 37*. SEM image taken of the fractured glass slide, from the Increasing Temperature Experiments for tank 7, that resulted in tablet growth, as well as the formation of a slight growth interruption.*

However, when the substrates removed from the abalone in tank 2 were imaged using the SEM, the nacre growth was clearly visible in the 'brick' and 'mortar tablet structure, but no growth interruptions were identified (**Figure 38**). The tablet growth started directly from the substrate into the cone-like structures that become tablets. The nacre growth for tank 2 also presented the 'Christmas Tree' formation at the top of the columnar tablet structure.

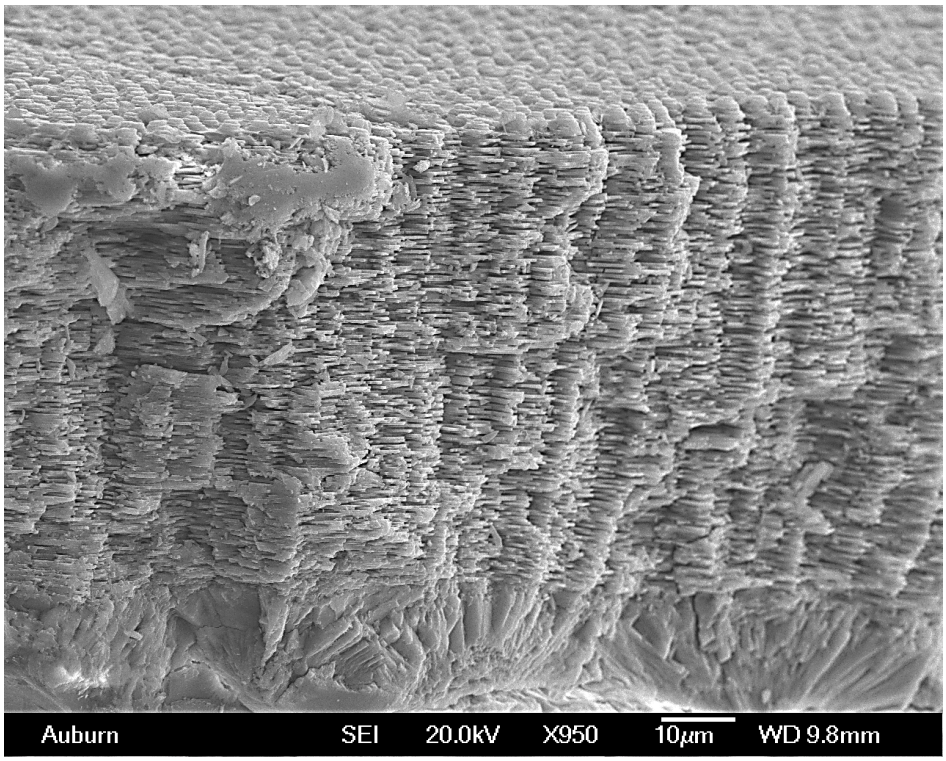

Figure 38*. SEM image taken of the fractured glass slide, from the Increasing Temperature Experiments for tank 2, that resulted in nacre growth with the 'brick' and 'mortar tablet structure clearly evident.*

3.3.3. Conclusions

As discussed in this section, mimicking the temperature change from the abalone's natural oceanic environment is crucial to the formation of a mesolayer within the nacre tablet growth. However, other factors have an impact on the potential for growth on the substrates as well. The thickness of the substrates, and the location in which they are placed during implantation, plays an important role in promoting nacre growth. As shown in sections "**3.1 Discussion on Experiments Completed Using Different Substrates**" and "**3.2 Location of Substrates on Shell**", growth will not occur if the specifications are not met. The thickness of the natural nacre

substrate did not allow for the mantle to completely cover the substrate, which is imperative in creating nacre tablet growth. Since the glass slides were significantly thinner, the mantle was able to cover the slide fairly quick and allowed for tablet growth to form on the slides. However, the glass slides are very brittle and break easily, which makes implantation of these slides difficult and time consuming. The natural nacre substrates would be a better option for implantation, if the substrates were grinded down to a thickness close that of the glass slides. Where those slides are implanted for testing is just as important as the thickness of the substrate being used. The slides have to be adhered to the shell in the growth zone along the growth edge. Growth will not occur if the slide is too close to the edge of the shell or too far down from the edge of the shell. If the slide is placed too close to the edge, the mantle cannot completely cover the substrates in enough time to have any substantial growth. Likewise, if the substrate is placed too far into the shell, it may be placed out of the region of the shell where new nacre growth occurs. There is also more success in nacre growth when the substrates are placed closer to the mouth and head area, as the mantle is able to entirely cover the substrates quicker, since the mouth is pushed out when feeding and moves the mantle closer to the edge.

These two factors are crucial to the formation of tablet growth and growth interruptions, but even if the correct thickness of substrate was used and in an accurate location, no growth will occur if the saltwater environment is not suitable for the abalone. As discussed in section "**3.3 The Relation Between Environmental Factors and Mesolayer Growth**", the abalone cannot thrive in an environment where the parameters in the saltwater are not stable and fluctuate constantly. This was proven when the first two experiments yielded no applicable results, but the fourth experiment succeeded in the formation of nacre growth and presented a growth interruption. This type of growth did not occur until the Live Rock was added to each tank, which stabilized the

saltwater parameters and allowed for algae and other microbial to cultivate within it, making the environment much more suitable for the abalone. Even though there was much more success in producing nacre growth when using the Live Rock, there was still difficulty in generating a mesolayer within that tablet growth. A suggestion would be to go back to filling the tanks with sand, since the research done prior to these experiments had great success in the formation of nacre, as well as the mesolayer. In **Figure 39**, a graph is shown of the parameters from the prior research, and how each parameter should behave. [19]

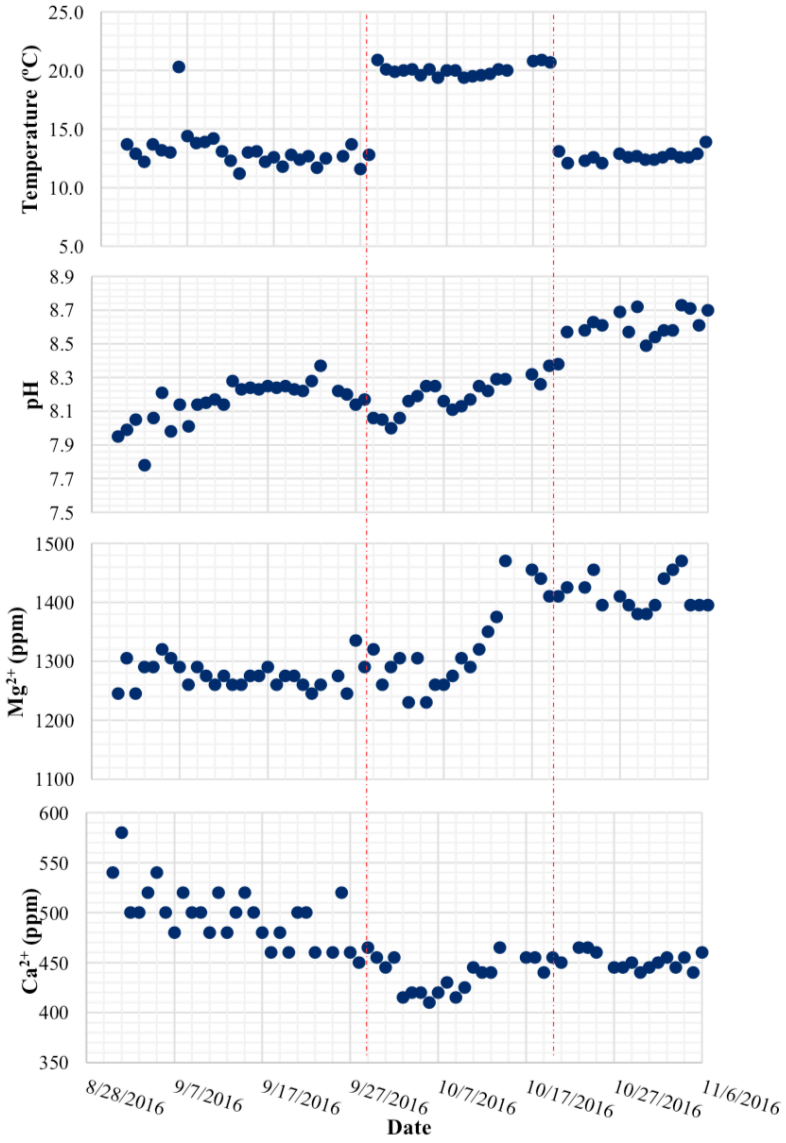

***Figure 39**. Graphs of parameters from prior research completed in the lab, which had sand in the tanks. Reprinted from* Zhang, Anqi. *"The Investigation of Haliotis Rufescens (Red Abalone): Mesolayer Growth & Mechanical Behavior." (2017). [19]*

In **Figure 39**, the graphs show that the calcium decreases while the magnesium increases during the increasing temperature period. It also shows a drop in pH during the increasing temperature change, as well. As shown in **Figure 35** and **Figure 36** in the previous sub-section, the parameters do not experience the typical trends. In the graphs of the parameters that resulted in a growth interruption, **Figure 35**, the Ca^{2+} concentration only slightly decreases during the increase in temperature, and the concentration of Mg^{2+} marginally increases from the initial values during that time. The pH does not decrease during the increase in temperature either. This is the reason there is not much interruption shown for the mesolayer. In the graphs that only resulted in tablet growth, **Figure 36**, the Ca^{2+} concentration increases once the temperature is increased, and the concentration of Mg^{2+} also increases throughout the testing cycle, while the pH stays around the same value. This caused the absence of the growth interruption.

Of all of the experiments completed for increasing the temperature to mimic the oceanic seasonal changes (**Table 2**), the most successful were the ones where the temperature was increased a few degrees. Also, where the temperature started low and was not raised too high during the increasing temperature period. The most ideal testing cycles were the ones that started at 12°C for the initial weeks, then increased to 16°C for a few weeks, then finally brought back down to the 12°C until the testing cycle was completed. The experiments performed on tanks using this timeline and temperature had the most nacre tablet growth, as well as the presence of a growth interruption.

4. Future Work

The experiments performed in this thesis are expected to further the understanding of the nacre structure, as well as the formation of the mesolayer within the nacre growth. Additional work must be completed before the nacre structure can be used in applications in industry.

While analyzing the experiments done for the nacre and mesolayer growth, it was clear that a more in depth investigation is needed in order to understand exactly how the nacre growth relates to the factors within the outside environment. The tanks the abalones are kept in are meant to simulate the natural ocean environment, but there are still many differences. The tanks are much smaller than the wide-open sea area where the abalones are indigenous, and do not have a diverse group of species either. The ocean environment is full of the algae and microbial that helps the abalone to thrive. This is much harder to cultivate in the tanks with an artificial saltwater environment. So, more experimentation focused on how the artificial saltwater environment affects the abalone, could help in better understanding the best conditions to prompt nacre growth.

As for the mesolayer growth, more research needs be done into the increasing temperature experiments, since that is when the mesolayer is formed. The wild abalones in the ocean have multiple mesolayer formations due to changing seasons. So, it is expected that it could be replicated in the tanks as well. This was attempted very early on this research, but had little success. In the future, once the artificial saltwater tank environment is improved, similar experiments could be done using the multiple increasing temperature cycles timeline (**Figure 40**). In **Figure 40**, a diagram of potential temperature change testing cycles to produce multiple mesolayers is shown.

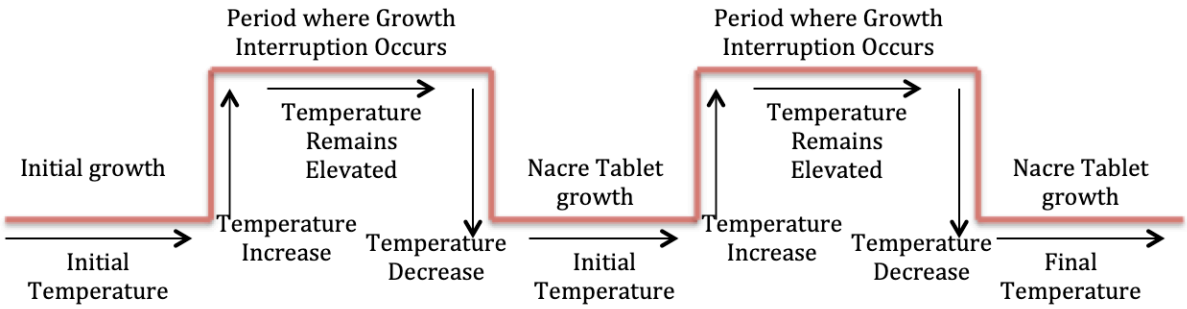

Figure 40. *A timeline for potential experiments to create multiple mesolayers within the nacre, by increasing the temperature multiple times within a testing cycle.*

This timeline for testing cycles could also present an opportunity to advance the knowledge in how to produce mesolayers with differing thicknesses. In later experiments done in this research, the amount of time spent in the increasing temperature change period during the testing cycle was varied. Further investigation needs to be done in this aspect of the mesolayer growth as well. If more research could be done with different times for the increasing temperature period, then there is a possibility that the size, or thickness, of the mesolayer could be controlled. A more in depth characterization of the mesolayer growth could help better comprehend its relationship to the nacre growth, as well as the factors in its saltwater environment, and allow an in depth investigation into the mechanical properties it possesses.

There are still many undiscovered qualities of the nacre and mesolayer structures, as well as the biomineralization process that creates such unique properties in these structures. Further research could lead to researchers using this structure, as well as its biomineralization process, to help in synthesizing composite materials with particular properties and a wide-range of applications.

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Appendix I. Pictures of Main Instruments Used

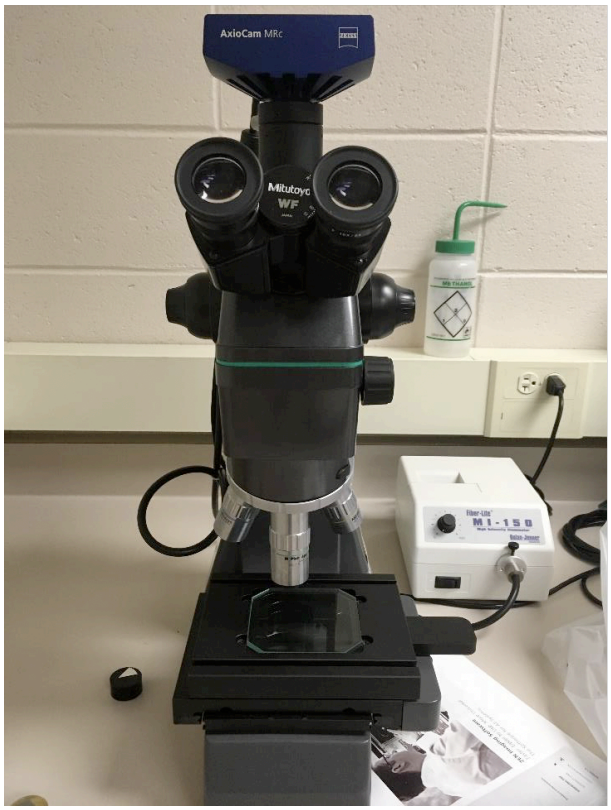

Figure 41. *An image of the Mitutoyo Corporation Optical Microscope*



Figure 42. *The JEOL JSM 7000F SEM used in this research*



Figure 43. *The EDS detector used in this research, which shares the same vacuum chamber with the SEM.*



Figure 44. *The Abalone lab*