UNDERSTANDING THE ORIGINS OF BIOADHESION IN MARINE ORGANISMS

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ABBREVIATIONS

RNA	Ribonucleic acid
SEM	Scanning Electron Microscopy
EDX	Energy-dispersive X-ray
EDTA	Ethylenediaminetetraacetic acid
DOPA	Dyhydroxyphenyl Alanine

ABSTRACT

Tibabuzo Perdomo, Andrés Mauricio Ph.D., Purdue University, August 2019. Understanding the Origins of Bioadhesion in Marine Organisms. Major Professor: Jonathan J. Wilker.

Curiosity is a powerful tool, and combined with the ability to observe the natural world, grants humankind an unique opportunity, the opportunity to wonder why. Why do things exist?, why do they do the things they do?, why is this even possible?

Research in our lab is focused on the basic understanding and potential application of biological materials, in particular, biological adhesives produced by marine organisms such as oysters. Oysters produce a cement-like material that is able to withstand the dynamic conditions found in coastal environments. The focus of this dissertation is to lay the basis of the characterization of new biological materials by observing and analyzing its physical properties, to measure the performance of the material in natural conditions and finally to identify the basic components that give the material the properties that we observe. The end goal of this project is to understand the properties of this material so we are able to develop a synthetic system that is able to imitate, as close as possible, what we find in nature. These results, and more importantly, the new questions that emerge from this research, provide a first look at the adhesive system of oysters leading the way to new discoveries in the future.

1. INTRODUCTION

We live in an extraordinary world. When we stop for a moment and actually see our surroundings we can be amazed at everything nature has to offer. Mountains, oceans, lakes, forests, all of them shaped over millions of years by different events, and with them, the organisms that dwell within. From simple microorganisms to the biggest of animals, all of them have a story to tell, a story older than humankind itself. Organisms have adapted to a wide arrange of conditions, giving rise to interesting traits and behaviors that we also use to our advantage.

There are multiple examples on how we have turned to nature to seek traits and adapt them to our daily lives. In architecture, for example, we have imitated the skeleton of glass sponges to create buildings [1], looked at shells to create earthquake resistant buildings [2], borrowed the schematics for ventilation systems from termite mounds [3], and even creating bricks using bacteria. [4]

One of the traits that we are most interested is the ability of marine organisms to generate adhesives. Various organisms use these adhesives for many purposes. Sandcastle worms use it to build their habitat [5], cadissfly use it to protect their larvae. [6] Mussels, barnacles and oysters use it to attach themselves to substrates for reproduction and protection. [7] It is the feature of being able to attach to surfaces underwater what is more appealing to our research due to its potential for application in different industries. From all the creatures under the sea that have this ability our laboratory works on two of them, mussels and oysters. However, from the two, oysters have been the least studied.

There is a great amount of information on oysters; there are two books that cover everything we know about their biology [8], one of them exclusively on the species

we are working with. [9] Nevertheless, the information on the adhesive they produce is scarce. The earliest, and almost only work, on oyster adhesion dates back to the thesis of Christian Tomaszewski in which he describes that the periostracum of the oyster (a thin organic layer covering the shell) and subsequent tanning due to phenol oxidases were responsible of the attachment to the substrate. [10] Since then, new research on the topic has appeared on closely related species to the eastern oyster that has helped us understand a little bit more about its origins. This dissertation will expand on that knowledge to try and answer the questions; "What does this material look like?" "How strong is it?" and "What is it made of?"

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2. PHYSICAL CHARACTERISTICS OF THE ADHESIVE FOUND IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*)

2.1 Introduction

Homo sapiens are visual creatures and, since the dawn of humanity, we have taken advantage of this evolutionary trait. [1] It has helped us forage for food and be aware of dangers, but it has also helped us ignite our curiosity. Most, if not all, of the greatest accomplishments in the history of humankind has been the result of this. When our curiosity is triggered, we become more engaged, think more in depth and find creative solutions to challenges that we address day by day. [2]

In the field of science this is no different. It is due to our curious minds that we have studied seemingly simple organisms such as garden peas [3], fruit flies [4], worms [5] and sea urchins [6,7]. We have realized over time that the knowledge we have gained from these organisms have started a biotechnological revolution. For example, understanding how protein secretion works on yeast, allowed the development of recombinant human insulin [8]. Genetic crosses on fruit flies have increased our understanding on the role of certain genes in human disease [9–12]. Work on the round worm Caenorhabditis elegans facilitated the discovery of interference RNA (RNAi) [13] leading up to the first RNAi therapy approved by the U.S. Food and Drug Administration (FDA) [14]. Perhaps most surprising of all, how studies of the defense mechanism bacteria have against bacteriophages has resulted in a groundbreaking technology for gene editing [15].

Now more than ever there is pressure to focus our research on application rather than discovery. This way of thinking disregards the fact that many of the examples

mentioned previously were sparked by curiosity, and built on the foundation of basic biology.

One organism that has awakened curiosity in our research is the Eastern Oyster *Crassostrea virginica*. This bivalve is a great example of a research paradox, it has been studied for a long time and yet there is still more to discover. Oysters have been part of our lives since the earliest civilizations. In particular, oyster cultivation is probably one of the oldest forms of aquaculture in human history [16,17]. Nowadays, from July to October of 2018, 6.2 M pounds were cultivated and exported, generating around 20.2 M dollars in revenue in the US alone [18].

Besides being used as a source of food, oyster reefs also provide important ecosystemic services, such as creating habitats for other species [19,20], filtering organic matter from the water column and regulating the growth of harmful microrganisms [21]. Due to the importance of oysters in the economy and our environment, scientists and aquaculturists have gathered a vast amount of information on the eastern oyster. Most of this knowledge is focused on methods to improve cultivation of oysters [22], preventing diseases affecting production [23,24] and their role as indicator species [25,26]. However, one of the most fundamental aspects of its biology has not been studied as much. How do they settle underwater and create reefs.

Information on the initial settlement and cementation of the eastern oyster can be traced back to only two sources. Prytherch, who in 1934, described in detail how the pediveliger stage of the oyster larvae looks for a suitable substrate and settles [27]. Almost 50 years later Tomaszewski described and tried to characterize the adhesive produced by the oyster larvae [28]. Since 1981 there have been attempts at characterizing this adhesive material in other oyster species [29–33]. However, no attempts have been tried in the eastern oyster, until now.

In this chapter, my goal is to describe the characteristics of the adhesive found in the oyster *Crassostrea virginica*. This characterization was performed using various microscopy techniques and analytical techniques. All of this with the objective of gathering more information on this elusive, yet interesting, topic.

2.2 Materials and Methods

2.2.1 Culturing of oysters

About 150,000 - 200,000 pediveliger larvae of Eastern oysters (*Crassostrea virginica*) were obtained several times, over multiple spawning seasons and years from the Virginia Institute of Marine Sciences and Louisiana State University. Upon arrival in the laboratory, larvae were acclimated in aged seawater (18 parts per thousand salinity) for 15-30 minutes prior to transferring into an aerated 10 gallon glass aquarium at the same salinity, maintained at room temperature. Beds of plastic (vinyl, Rinzl) and glass (Thermo Scientific Gold Seal) microscope slides were placed at the bottom of the aquarium for larval settlement. Oysters where fed daily with an algae mixture (Shellfish Diet 1800) and water changes performed every three days to ensure growth into spat. After 48 hours, most larvae were in the crawling-settling stage.

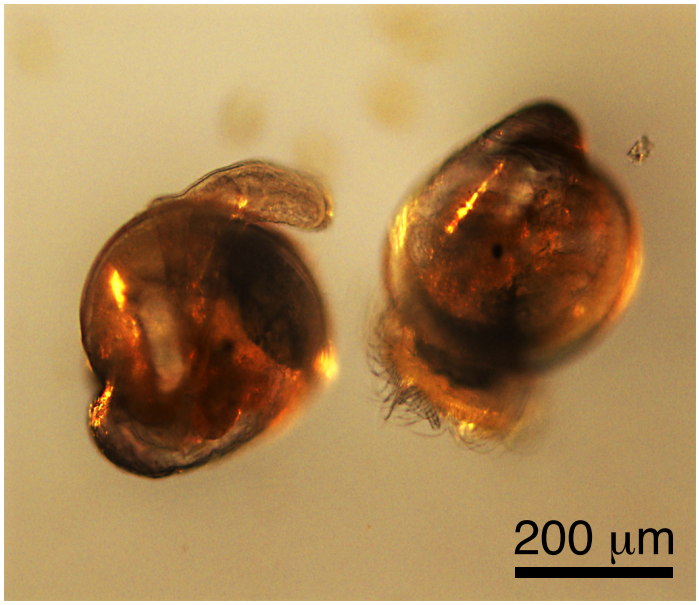

Figure 2.1. Oyster larvae. The image shows two oyster larvae in the pediveliger stage. The larva on the left is scouting the substrate with its foot. The larva on the left is still in the free swimming stage.

2.2.2 Instrumentation

Optical and Fluorescence Microscopy

Optical and fluorescence microscopies were carried out on an Olympus BX51 with USH-102DH and USHIO lamps as well as an Olympus DP71 CCD camera. Wavelengths for the filters used were: Blue fluorescence ($\lambda_{\text{excitation}}$ = 310-390 nm, $\lambda_{\text{emission}}$ = 420+ nm). Green fluorescence ($\lambda_{\text{excitation}}$ = 450-490 nm, $\lambda_{\text{emission}}$ = 520+ nm). Red fluorescence ($\lambda_{\text{excitation}}$ = 510-550 nm, $\lambda_{\text{emission}}$ = 590+ nm).

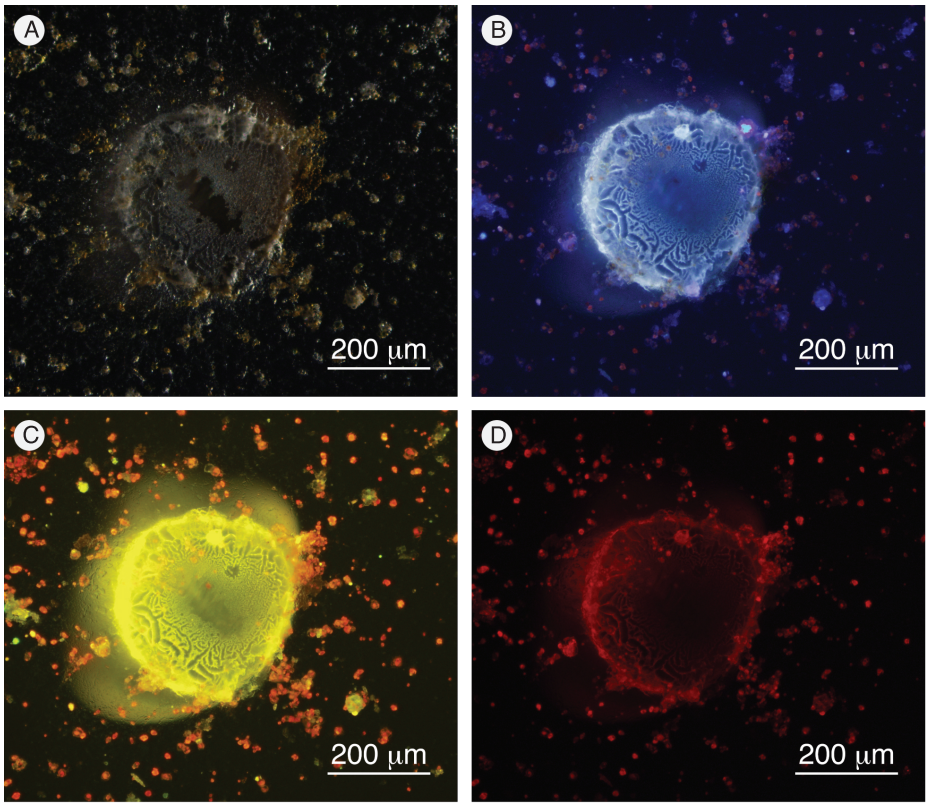

Figure 2.2. Fluorescence microscopy of settled oyster larvae. Images of a recently settled oyster larva were captured under white light (A), blue (B), green (C) and red (D) fluorescence filters. The images show autofluorescence of the material used by the larvae to attach the substrate.

Scanning Electron Microscopy

An FEI Quanta 3D FEG dual-beam scanning electron microscope (SEM) as well as an FEI Nova NanoSEM, both with Everhart-Thornley and through-the-lens detectors (TLD), were used. Typical parameters included 5-20 kV accelerating voltages and 4.5-10 μm working distances. Oysters were covered with platinum via a sputter coater prior to imaging.

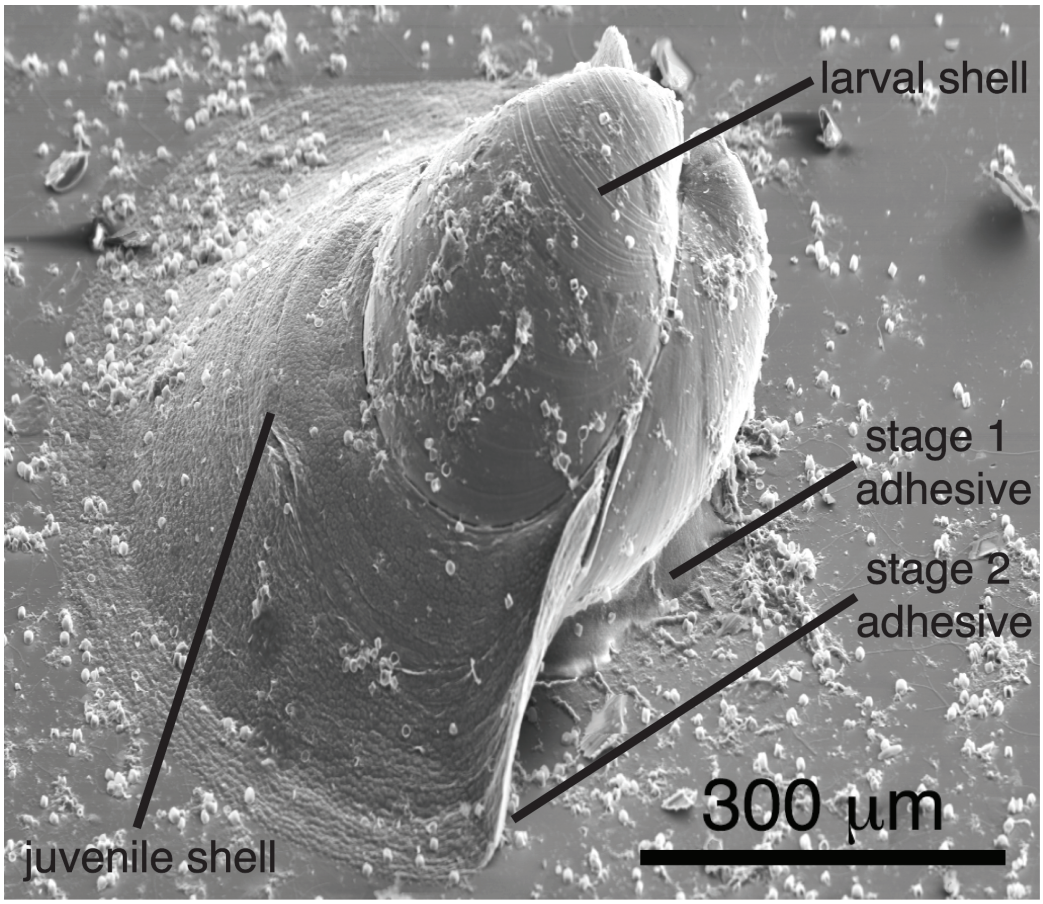

Figure 2.3. Scanning Electron Microscopy of a juvenile oyster secreting a secondary adhesive. In this image we can observe how a material coming from inside the shell of the oyster is laid down. Towards the leading edge of this material we start to see crystallization of $CaCO_3$ columns.

Energy Dispersive X-ray (EDX) Spectroscopy

Energy dispersive X-ray (EDX) spectroscopy was accomplished with an Oxford INCA Xstream-2 on a Quanta 3D FEG microscope. For EDX parameters, 20 kV, 50 μm objective aperture, and 100 seconds of collection time were used most often. Oxford AZtecEnergy EDS software was employed for data analyses.

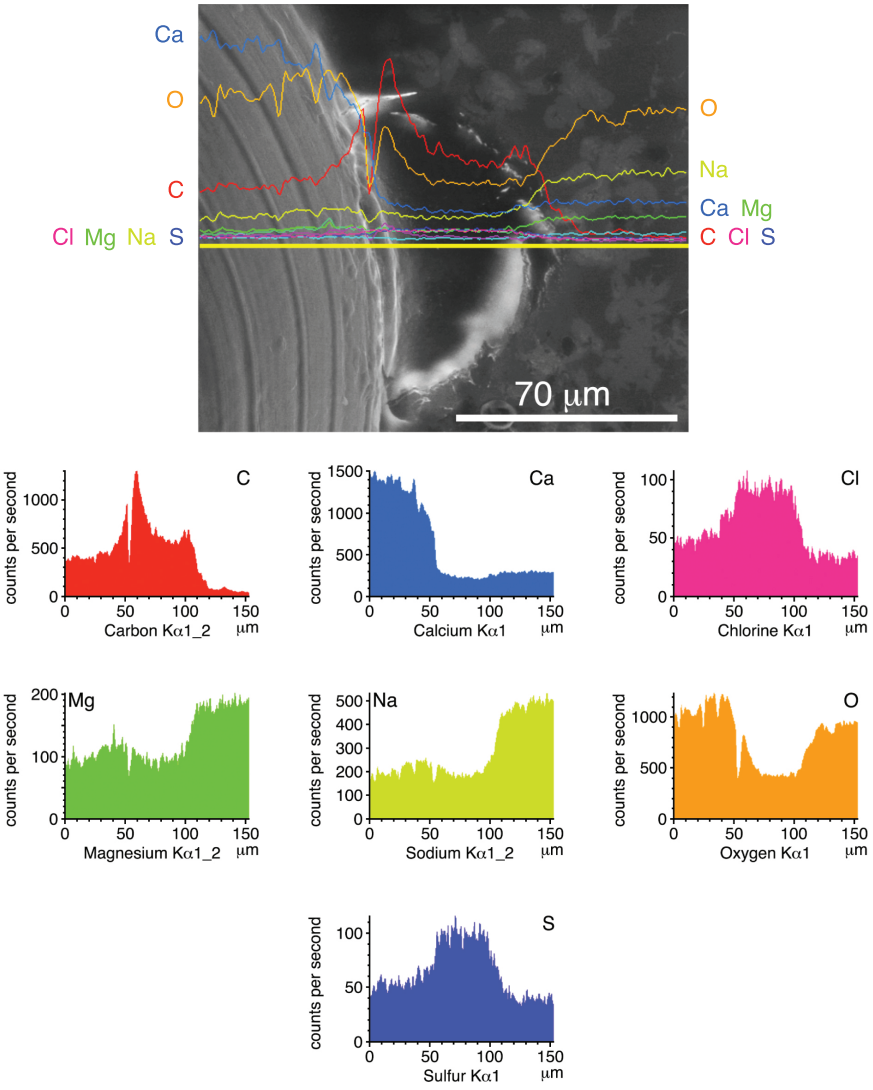

Figure 2.4. Energy dispersive X-ray (EDX) spectroscopy of the larval adhesive. Elemental analysis of the larval shell and the initial adhesive is shown.

2.2.3 Sample preparation

Video collection

Forty eight hours after seeding the eyed larvae in the aquarium, glass slides containing larvae were collected and transferred to a petri dish with salt water. Using the bright field objective at 2X magnification, the glass slide was scanned to look for larvae in the settling stage. Once a larvae was spotted the video recording started until the larvae was settled.

Samples for Scanning Electron Microscopy

Samples for Scanning Electron Microscopy had to be detached and prepared in a different way than those used for fluorescence microscopy. Oyster spat that were settled in plastic microscope slides were detached, placed upside down on a SEM mount and fixed with carbon tape. Then the samples were coated with platinum with the help of a sputter coater under vacuum (Cressington Sputter Coater 208).

Cement extraction

Clusters of oysters were separated at the interface between shells, in which the cement resides. This adhesive material was then removed by mechanical means, using a chisel, or by chemical means, using EDTA or Acetic acid 10%. Samples removed by mechanical means were used for observation while the rest of the samples were stored for protein extraction.

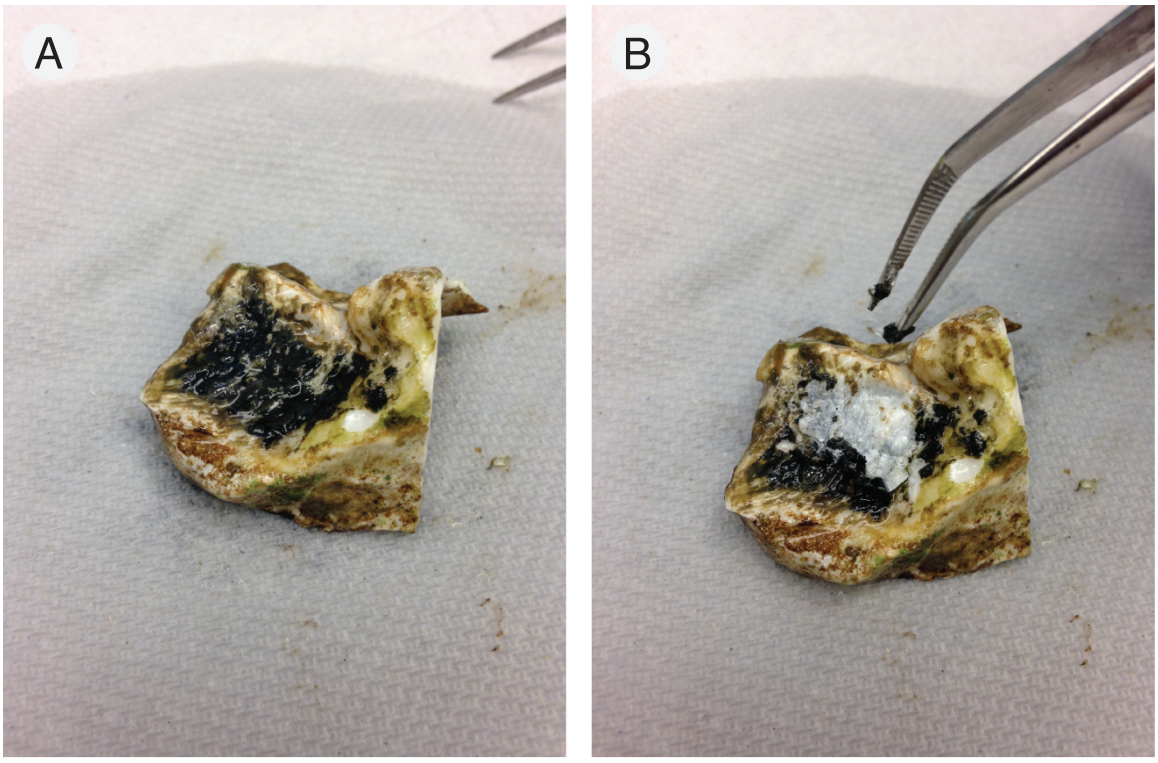

Figure 2.5. Chemical extraction of oyster cement. Cross sections of oyster clusters were separated and the cement layer was removed using acetic acid 10% or EDTA.

2.3 Results

2.3.1 Optical microscopy

Oyster larvae in the crawling-settling stage were observed 48 hours after transferring the eyed larvae into the aquarium. While most of the oysters were in the free swimming (veliger state), a few of them were ready to settle (Fig 2.1). After scouting several slides it was possible to record for the first time, to the best of my knowledge, how oyster larvae attach to substrates.

During the first few seconds, the larva lays its foot on the substrate and starts to expand and contract while the rest of the oyster body moves. Once it has found a suitable spot, the foot is extended and is followed by an upward movement of the shell. This ends a couple of seconds later when the body of the larva rests again in the substrate staying completely still and slowly retracting its foot. The full length video can be found in the supporting information section of the article published by our research group [34].

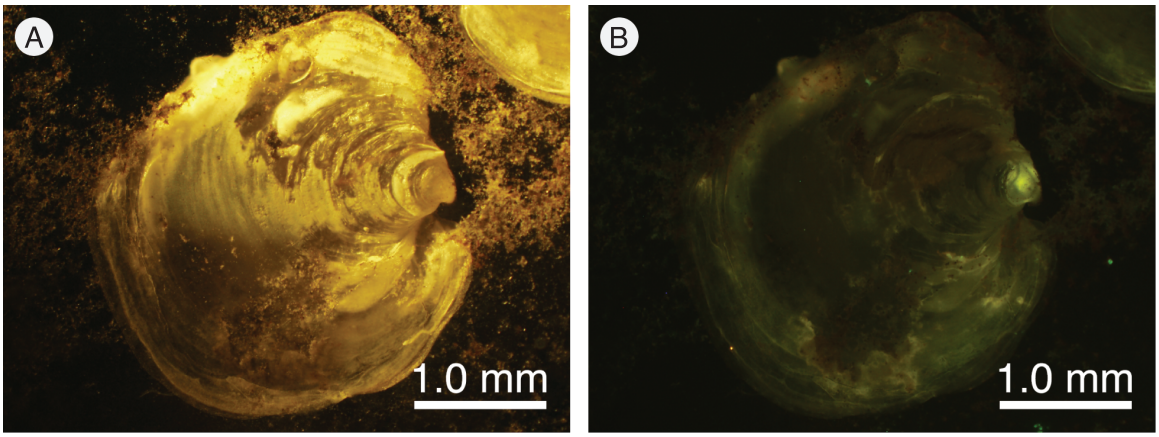

Figure 2.6. Optical and fluorescence microscopy of a 5 month old oyster spat. This image shows the underside of an oyster grown for 5 months. Here we can observe the beginning of mineralization and the shift in autofluorescence towards the edge of the shell.

2.3.2 Fluorescence microscopy

Oyster spat

Previous research performed in our laboratory, discovered the presence of auto fluorescence by looking at the cement layer on cross-sections of oyster clusters [35]. The use of glass and plastic microscope slides allowed us to see through the substrates into the adhesive material itself (Fig 2.2). One day after settling, auto fluorescence could be observed in the oyster spat. The adhesive material seemed to cover the entire area of the larval shell. This adhesive presented a particular fibrous pattern towards the leading edge of the oyster spat. This pattern is slightly more visible under the blue fluorescence filter (Fig 2.2B). As samples continued growing the auto fluorescence started to disappear toward the center of the animal and became more visible towards the leading edge of the growing oyster (Fig 2.6).

Scrapped cement

The adhesive material was collected from adult oyster samples in order to observe if auto fluorescence was still present. Samples, extracted with acetic acid or EDTA, were easily removed as a thin film (Fig 2.5) and observed under the microscope. Images show that the material still fluoresced under blue ($\lambda_{\text{excitation}}$ = 310-390 nm, $\lambda_{\text{emission}}$ = 420+ nm) and green filters ($\lambda_{\text{excitation}}$ = 450-490 nm, $\lambda_{\text{emission}}$ = 520+ nm), but not under the red filter ($\lambda_{\text{excitation}}$ = 510-550 nm, $\lambda_{\text{emission}}$ = 590+ nm). The intensity of the fluorescence seems to be diminished compared to that of the larval adhesive evidenced by the need of longer exposure times to capture the image. Interestingly, within the adhesive sample and visible under all filters, algae could be observed emitting red fluorescence (Fig 2.7).

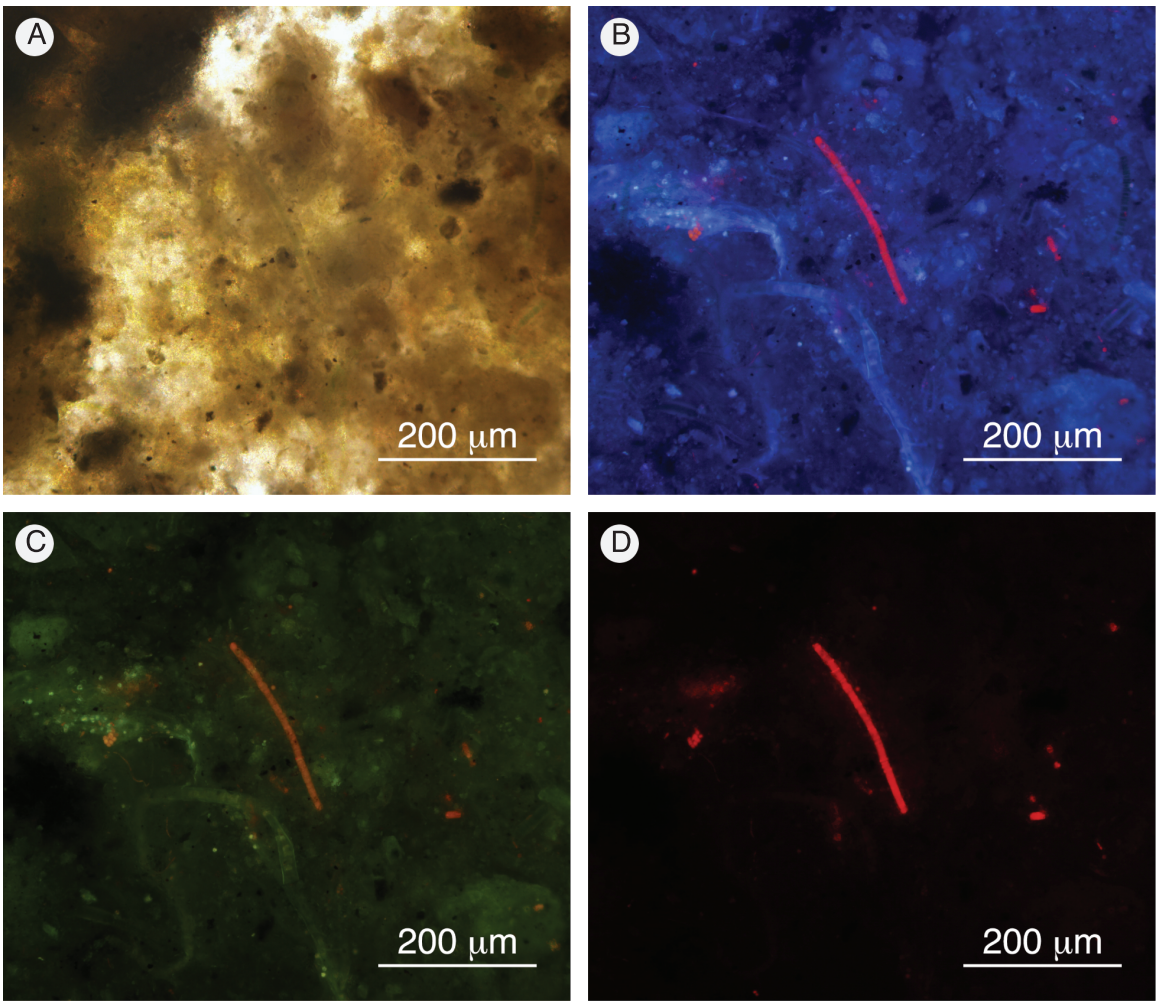

Figure 2.7. Optical and fluorescent microscopy of oyster cement. Extracted cement was analyzed under white light (A), blue (B), green (C) and red (D) fluorescence filters. Auto fluorescence of the material was still present under blue and green filters. The images also show the presence of green algae trapped within the cement.

2.3.3 Scanning Electron Microscopy (SEM)

To take a closer look at the adhesive material SEM was used. Images were captured on the ventral side of the animal (Fig 2.8), where the adhesive was laid, and on the dorsal side of the animal (Fig 2.10), where radial growth occurs. At the center of the larval shell we can observe a difference in the topology at the interface where shell ends and the adhesive starts (Fig 2.9A, 2.8B). Upon closer inspection, the adhesive has the consistency of a fibrous material similar to collagen or actin (Fig 2.8, 2.9). Moving toward the edge of the larval shell a groove forms and then a smoother material starts to appear. At the dorsal side of the oyster spat, towards the leading edge of the shell, columns of calcium carbonate start to appear (Fig 2.10C). These columns are bound by fibers of the adhesive (Fig 2.10D) and this same phenomenon appeared on all the samples analyzed.

This second adhesive starts to appear once the oyster has settled. At first, the material is secreted from the inside of the shell (Fig 2.3), when the adhesive is laid down on the substrate small calcium carbonate crystals start to form. Over time these columns start to grow forming the structures described previously at the leading edge of the shell (Fig 2.10D).

2.3.4 Energy dispersive X-ray (EDX) spectroscopy

To identify the composition of the adhesive materials found on the oyster spat EDX was used. This technique allows us to obtain qualitative and quantitative data on the elemental composition of the samples. Performing a line scan with the instrument showed that the fibers had higher carbon content in contrast to the oxygen and calcium found in the shell (Fig 2.4).

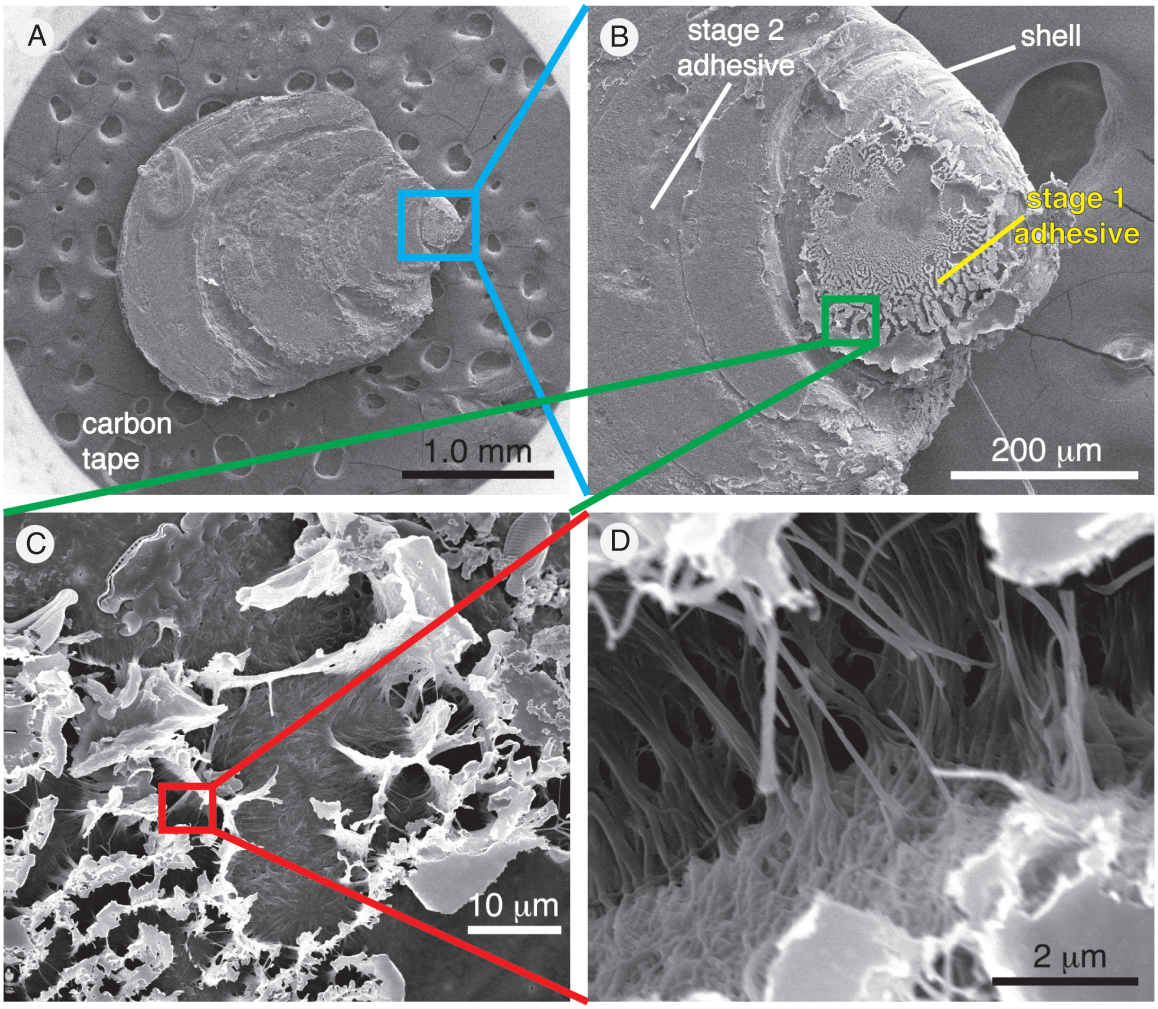

Figure 2.8. Scanning Electron Microscopy of the ventral side of an oyster spat. Here a close up of the adhesive material produced by the oyster larva is shown. There are two different materials that start to appear. An initial fibrous material found directly below the larval shell and a smoother material that starts to appear at the edge of the larval shell.

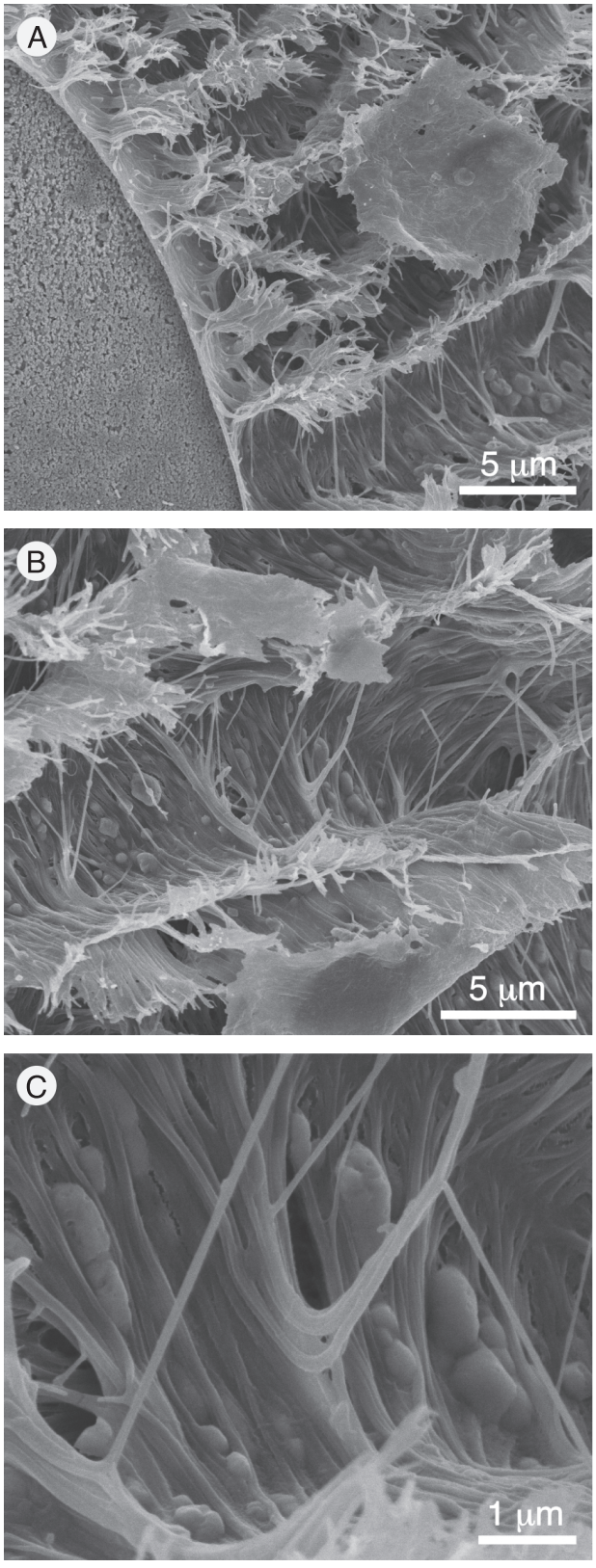

Figure 2.9. Closeup of the stage 1 adhesive. Here we see the contrast between the organized aragonite layer (A) and the fibrous stage 1 adhesive (B,C).

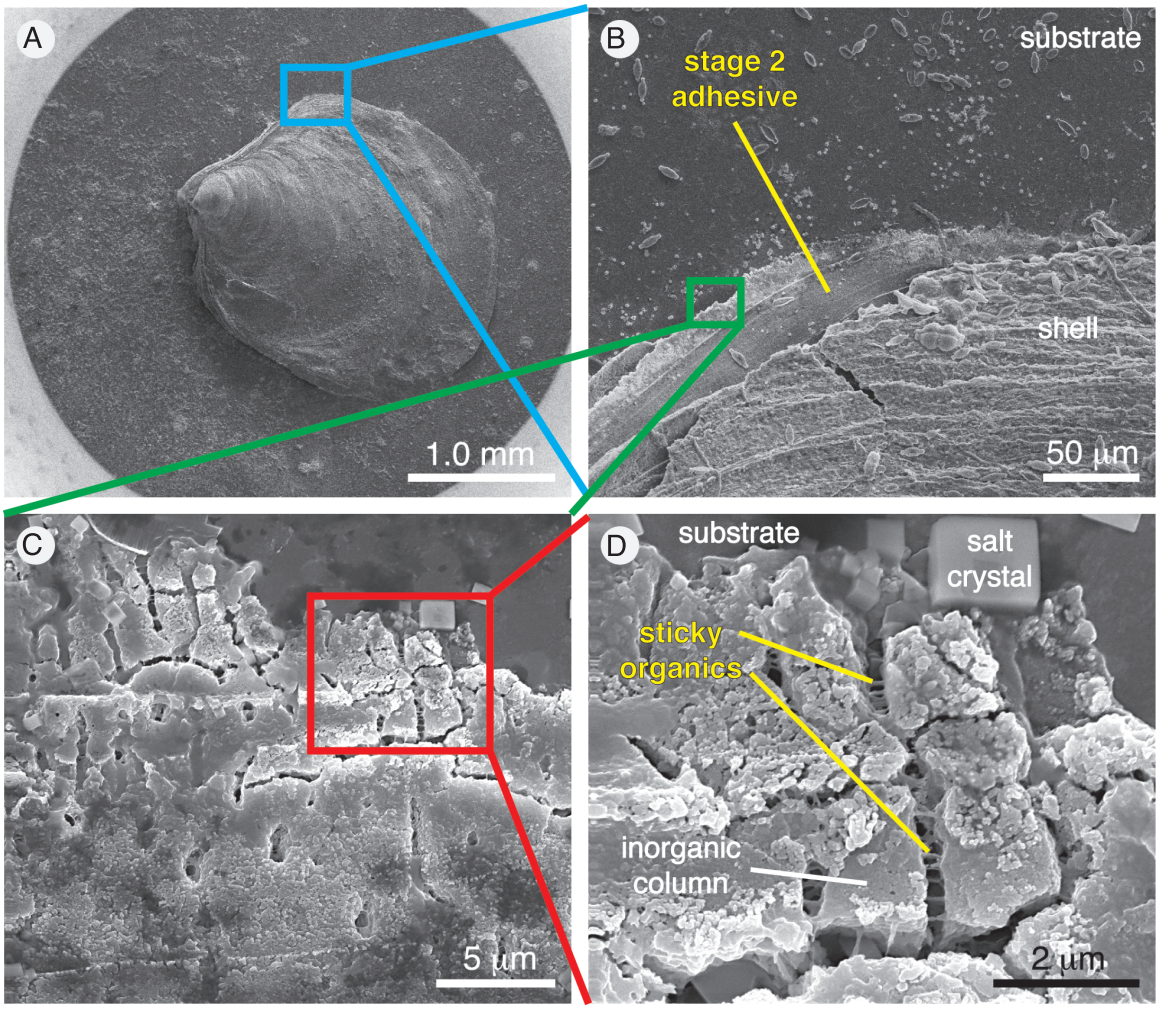

Figure 2.10. Scanning Electron Microscopy of the dorsal side of an oyster spat. Here a close up of the leading edge of the oyster shell is shown. Upon closer inspection we can observe the formation of inorganic columns being held together by a fibrous material.

Similar results were obtained from samples prepared 5 months after settlement. The only observed difference between young and old oyster spat was the detection of small amounts of sulfur in the adhesive fibers of older oysters (Fig 2.13).

2.3.5 Additional information

Two materials were used as a substrate for the settlement of oyster larvae, glass and plastic. Both substrates were placed in the aquarium at the exact same conditions. However, after weeks of growth, a notable difference started to appear between the two materials. A higher proportion of the larvae preferred to settle in the glass substrate rather than the plastic substrate (Fig 2.11). It also seemed that oyster spat that settled on plastic grew larger than those in glass, but with the samples analyzed there was no statistical difference in the area size (Fig 2.12).

Another interesting observation came from oyster spat that were detached from the substrates due to hydrodynamic forces. Unlike mussels, oysters settle once and stay that way creating reefs. However, oyster spat that were removed from their substrates were able to re-settle to the base and the sides of the aquarium (Fig 2.14).

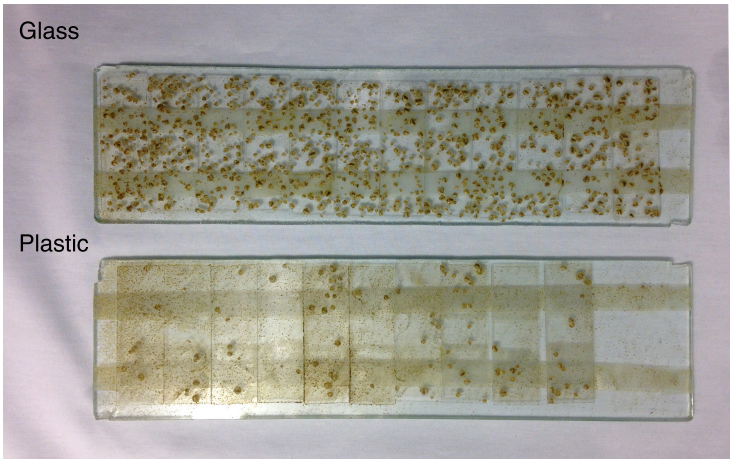

Figure 2.11. Oyster larvae grown in different substrates. Oysters were grown in glass (up) and plastic (down) microscope slides. There is a significant difference in the preference of the substrate.

2.4 Discussion

Even simple and common creatures have something to teach us. Such is the case of larvae from the eastern oyster, *Crassostrea virginica*. While doing research on this animal, I had the opportunity to witness its initial stages of settlement and cementation. The process itself has been reported previously for the eastern oyster and other bivalves. Some reports attribute the adhesive properties to proteins and polysaccharides excreted by the larvae [29–32]. There are other works proposing that the formation of inorganic crystals of calcium carbonate is the main mechanism of cementation [36–39]. However, we now have recorded evidence of the settling behavior of the eastern oyster, which follows the process described by Prytherch [27]. Our results show that the way oysters attach to substrates might be a combination of both the protein and the mineralization hypotheses.

Two adhesives to settle

Fluorescence and Scanning Electron microscopy showed two distinct adhesives that appear at different time points in settlement. A stage 1 adhesive secreted by the larva prior to settlement that is fast curing. This allows the oyster to be able to fix itself to substrates quickly and allow time for a more permanent adhesive to take place. The stage 2 adhesive comes from inside the oyster shell and is shown to promote mineralization and shell formation. The time observed between the initial attachment and the secretion of the stage 2 adhesive is approximately one week.

Tracking the growth of the settled oysters, we could observe auto fluorescence coming from the stage 1 adhesive. The same happened at the leading edge of the shell where the stage 2 adhesive starts to be produced. This auto fluorescence is indicative of cyclic aromatic compounds present within the adhesive material [40–42].

It has been proposed for many glue laying marine organisms that the presence of Dihydroxyphenyl Alanine (DOPA) allows their adhesives to be able to stick underwater. DOPA is a modified amino acid that is involved in many physiologically

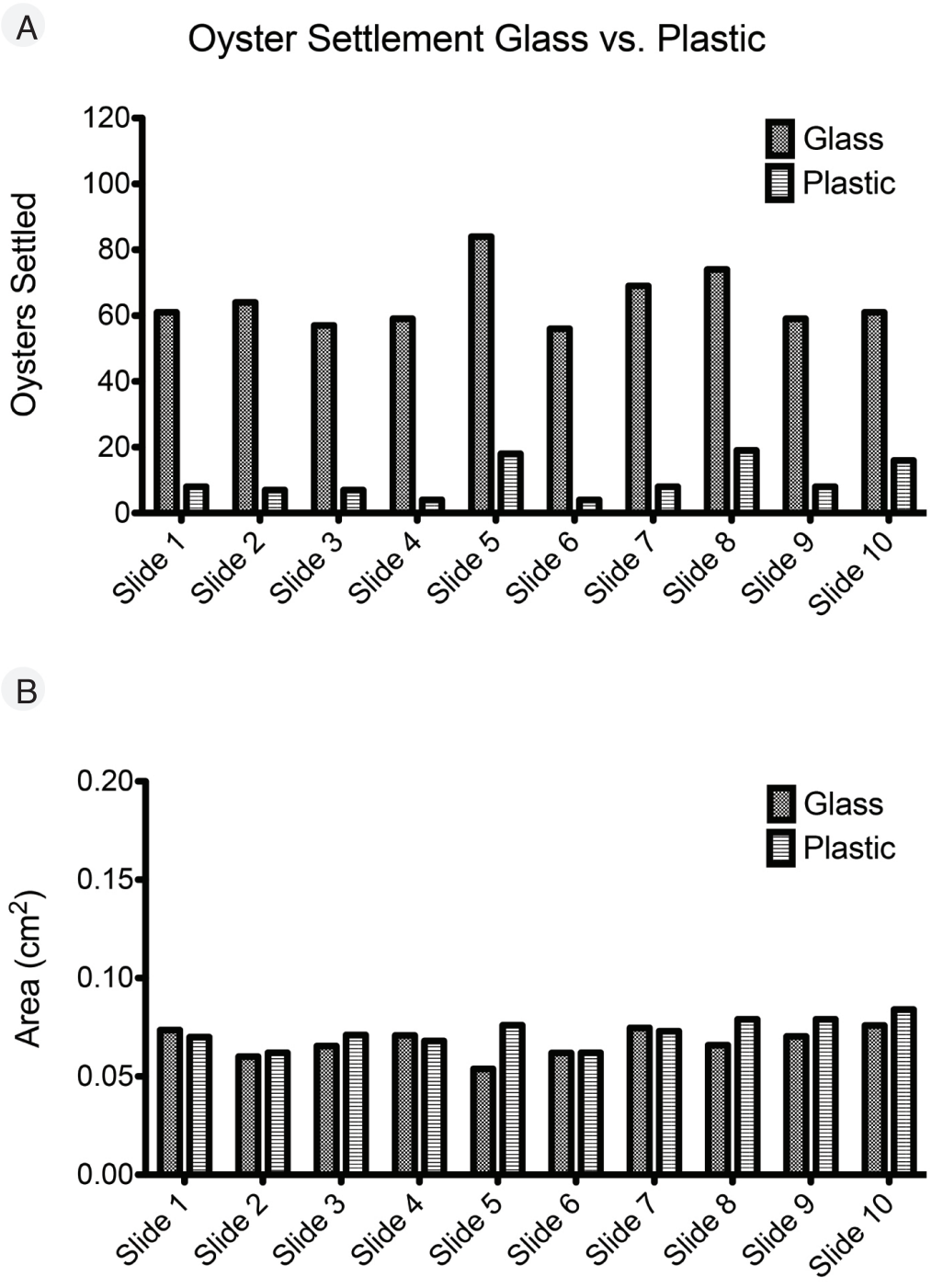

Figure 2.12. Comparisons between the substrates. In this graph we observe the difference of oysters that settled in each substrate (A) as well as the average area of the oysters that settled per slide (B).

relevant pathways and the treatment of Parkinsons disease [43]. However, in the context of adhesion, oxidative crosslinking of proteins with a high percentage of DOPA is believed to be the mechanism behind underwater adhesion [44].

The first studies of marine bioadhesion started with mussels [45–47] and expanded to other organisms where many DOPA containing proteins have been characterized [48] supporting this hypothesis. However, from an evolutionary point of view, it seems unlikely that many organisms from different branches of the tree of life converged to the same answer of using DOPA as means to settle underwater. While it is undeniable that this amino acid is a key player in the adhesive properties of the material, there might be other factors involved as well, especially considering how different the natural history of each organism is compared to one another. While the objective of this chapter is not to discuss DOPA and catechol chemistry in relation to its adhesive properties, this review by Javier Saiz-Poseu [49] gives a comprehensive look at what is known so far.

Another characteristic that separates stage 1 and stage 2 adhesives in the oyster is the mineralization of the stage 2 adhesive. This can be seen both in the optical and fluorescence microscopy as well as the SEM images. In figure 2.2 we can observe the stage 1 adhesive and the pattern it forms, then in figure 2.6 we see how the oyster starts to grow radially outside the larval shell and how this stage 2 adhesive is starting to mineralize. Evidence of this is the shift of color from a transparent material to a chalk white color in some of the sections of the oyster. The SEM images confirm this observation. When we zoom into the larval shell, we see the fiber-like structures of the stage 1 adhesive in contrast to the smooth mineralized surface of the stage 2 adhesive. This clearly indicates a change in the composition of both adhesives that serve two different functions.

The elemental analysis of the cement also showed the differences between the adhesive and the shell in the stage 1 adhesive (Fig 2.4) and the stage 2 adhesive (Fig 2.13). On the first stage we can clearly observe the increase of carbon content and the drop in calcium immediately after the line scan reaches the adhesive. It is also

worth mentioning that there is a sudden increase in the content of sulfur. Compared to the change of the spectral counts in the other elements this might seem minimal, the same is observed in the stage 2 adhesive with an increased count. The presence of sulfur in the adhesive sheds light into the proteic nature of the adhesive. It might also indicate the contribution of methionine or cysteine to the adhesive system in addition to DOPA [50]. While EDX is not the most suitable technique to address this question, a preliminary proteomic characterization will be discussed later in chapter four.

Adult cement vs. larval cement

What we believe to be the adhesive material in the adult oyster was extracted using 10% acetic acid (Fig 2.5). In contrast to the larval oyster grown in the lab, the material varied in color. Different samples extracted from the same clutch of oysters had a grey, red or green color. Under fluorescence microscopy the material still auto-fluoresced but with less intensity than the larval adhesive.

Another difference was the amount of organic sediment found on the material of the adult oyster. This makes sense due to oysters being filter-feeder organisms and the samples being collected in their natural habitat. Among the organic sediments trapped on the material we could find green algae. This was evident under fluorescence microscopy due to the characteristic red emission of chlorophyll [51, 52].

It is interesting that most, if not all, the organic sediment present in the adult oyster is absent in the oyster larvae grown in the lab. This means that the material we observe in between the adult oyster shells might not be the adhesive material produced by the animal. My hypothesis is that this material is the sediment present in the surface of the substrate where the larval oysters attach. Once the larvae settle, the material they produce is spread out on the substrate entrapping the organic sediment in between the oysters. This sediment might not be the adhesive itself but it might be acting as a mortar that enhances bulk adhesion [53, 54]. While more experiments and characterization are needed to support this claim, we can see that

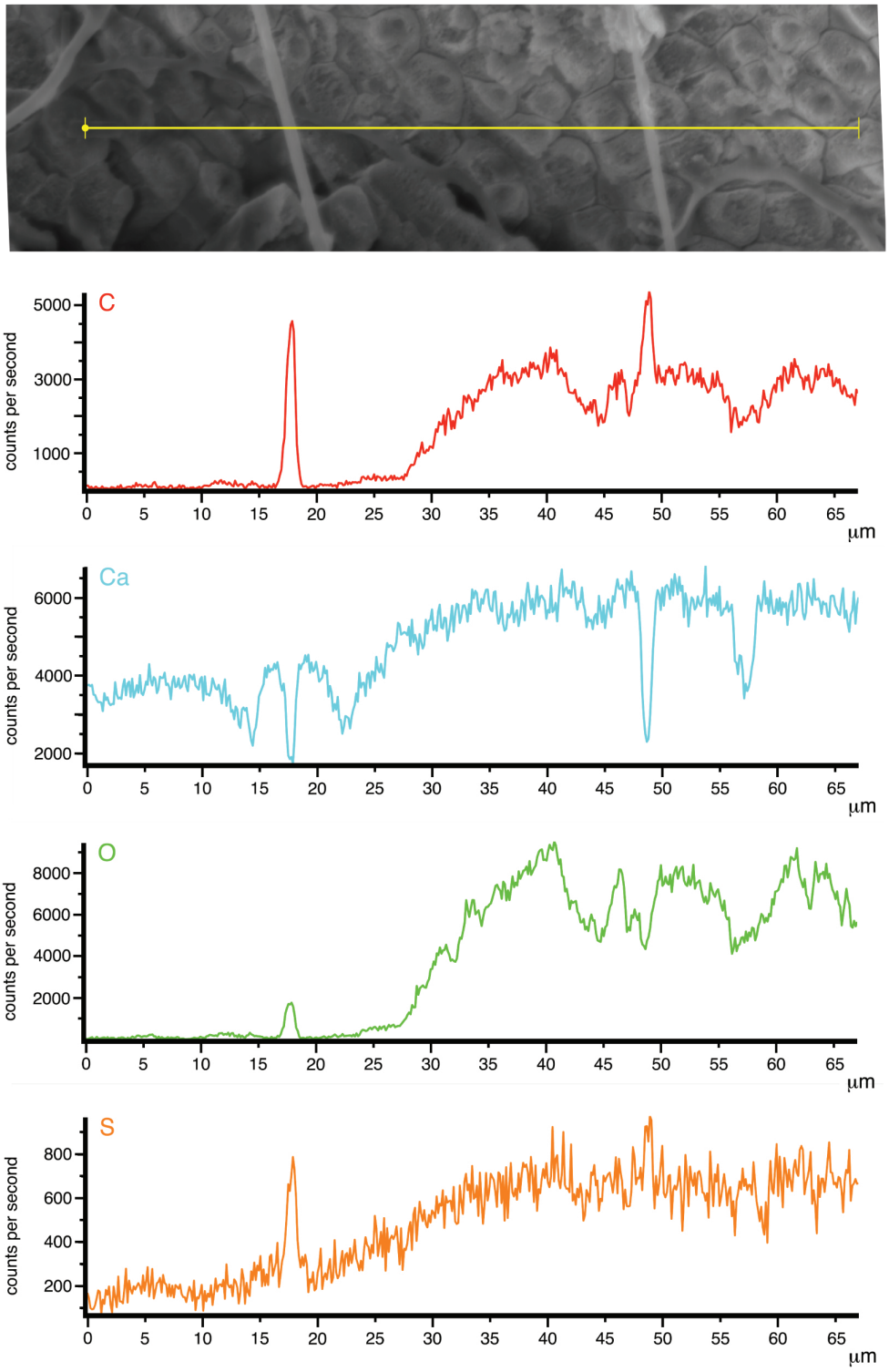

Figure 2.13. Energy dispersive X-ray (EDX) spectroscopy of the larval adhesive. Elemental analysis of a section of a 5 month old oyster is shown. A section of the oyster that contained both shell and adhesive was analyzed to show the difference between the materials.

this material has not mineralized over a long period of time unlike the oysters grown in the aquaria (Fig 2.14).

Observations of the behavior of larval oysters

During the time spent cultivating and maintaining the larval oysters some interesting behaviors could be observed.

There is a vast array of mechanisms by which glue laying organisms attach to substrates [48]. Most of them do it with a specialized organ for this function; in marine animals this organ is the foot [33, 47, 55]. In mussels this organ is present at all stages of life, giving them the ability to re-settle on another substrate. Oysters have a foot as well during the larval stage that is absorbed within the shell after settlement [27]. This gives the impression that oysters have only one opportunity to find a suitable substrate. However, while doing our experiments, I found that many of the oysters that were detached from the substrates were able to re-settle at the sides of the aquarium (Fig 2.14). Since the foot had already retracted inside the shell, the most logical explanation is that the stage 2 adhesive was used for this purpose. The benefit of having a redundant system with two adhesives gives oysters an advantage in terms of survival [56]. If the first substrate was not suitable and the animal was detached then the stage 2 can be used after the animal lands on a different substrate.

As mentioned in the results oyster larvae settled more on the glass microscope slides rather than the plastic ones. A possible explanation of this might be the difference in the surface energy of the substrates [57–59]. In the past in our lab we have seen evidence of this on mussels [60]. While testing different formulations of antifouling coatings we saw how the animals scouted the substrate with the foot, if the surface was not optimal for settling (i.e. the substrate with the anti-fouling coating), the mussel did not lay any adhesive or moved until it found a better place to settle. An unpublished video of this behavior can be found in the computers in our laboratory. Unlike mussels, oysters have only one opportunity to lie that stage 1

adhesive, this means that the animal must be very selective to increase its chances of survival.

A difference that was observed between the substrates was the growth of the animals. At a simple glance it seemed that the oysters that were able to grow in the plastic slides grew bigger than those that grew on the glass slide. At first I thought this could be explained by competition on resources given that the oysters growing on the plastic slides had more access to food. However, after measuring and comparing the area of the animals that grew in each substrate, I found that there were no significant differences. The method used for this analysis will be explained further in the next chapter.

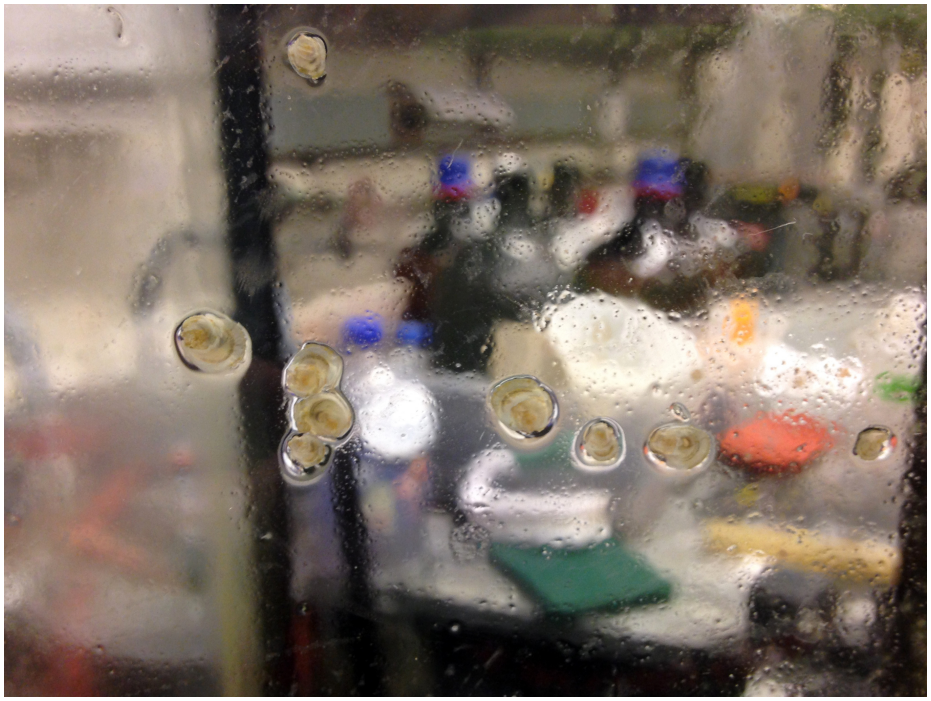

Figure 2.14. Oysters re-settling at the bottom of the aquarium. This image shows evidence of oysters that were detached from the substrates re-settling in another substrate.

2.5 Future Directions

The work presented in this chapter is just a small step towards the characterization of this adhesive system. However many questions still remain unanswered. Some of these I will mention briefly here and will try to answer in the following chapters.

What is the material made of? What is the difference between the stage 1 and stage 2 adhesive? What are the mechanisms involved in adhesion and mineralization?

We have an idea that the material is most likely protein. However, there are clear differences between the two stages, one is more fibrous than the other and one mineralizes while the other does not. It would be interesting to see the differences in composition between the two stages and the motifs involved in adhesion and mineralization.

What are algae doing in the adhesive of the oyster in nature?

Algae was found trapped inside the cement and seems to be alive. Fluorescence indicates the presence of chlorophyll which is in line with them using photosynthesis, then how are they surviving in between two oyster shells with no direct access to sunlight? Is there any kind of symbiosis happening here? If there is how does this work? Are the algae or bacteria associated providing some kind of scaffold?

Can we use transcriptomics to narrow down the candidate genes that produce the adhesive?

We know that there are two distinct adhesives. We also know that reattachment is driven only by the stage 2 adhesive because the foot is no longer available in this life stage of the oyster. If this is true, that might suggest that the genes involved

in generating that stage 1 adhesive are active in earlier life stages rather than later stages, due to the lack of the organ delivering the material.

The more we look at this project the more questions may arise, which is great to write future grants.

2.6 Acknowledgements

The work presented here is the culmination of almost 10 years of work in which many people were involved. This could not have been possible without the collaboration of Stephen Taylor, Erik Alberts, Debra Sherman, Chia-Ping Huang, Chris Gilpin, Laurie Mueller and Robert Seiler.

2.7 Disclaimer

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3. HOW STRONGLY DO OYSTERS STICK? MEASURING STRENGHT OF ADHESION IN JUVENILE AND ADULT OYSTERS

3.1 Introduction

The study of biological materials is an area of research that has caught traction in recent years. [1–4] Now more than ever we are turning back to observing nature, that had millions of years to experiment, to look for solutions for our daily lives challenges. [5] An example of this can be found in our search for novel materials with unique properties such as fracture resistance [6, 7], deformability [8], binding to different kinds of surfaces [9, 10] and more. From these properties adhesion is what we find most interesting because of its wide range of applications. From medical devices [11] to furniture [12], from cosmetics [13] to airplanes [14], adhesives are present in our daily lives, whether we see them or not.

In nature there are organisms whose survival depends on the adhesive they produce. Be it to attach to a suitable substrate [15] or to capture prey. [16, 17] It is the interplay between their chemical and physical characteristics that influence the materials strength, stiffness, toughness and flexibility. [18] Taking all of this into consideration, it is clear that elucidating the composition and mechanism of action of these biological materials will be of utmost importance in order to develop new synthetic, strong and environmentally friendly adhesives. [19]

One might think that due to the relevance and potential application of these materials in our lives, someone must have probably studied them by now. However, only a handful of glue laying organisms have been studied so far. [20] Mussels [21], barna-

cles [22] and tubeworms [23] represent a majority of the literature, partly motivated by their role in biofouling. [24] Most recently, examples of non-traditional organisms like tunicates [25] and starfish [26] have appeared as well. In the case of our organism of interest, the eastern oyster, only a few studies can be found in regards of the adhesive material they produce. [15, 27, 28] Moreover, the work has focused mainly on the adult animals while larvae and juvenile stages, where adhesive production plays an important role, are not as studied.

Therefore, the work presented in this chapter will focus on the development of a method to be able to answer. How strongly do oysters stick? For larvae and juveniles of other organisms experimental procedures like force gauge [29,30], rotating disk [31] or water jets [32] have been used. Christie and collaborators [33] used this approach to measure the adhesion strength of algae and was later used for other organisms. [34–36] These studies used the equations developed by Rajaratnam and Beltaos to calculate the maximum shear stress produced by the water jet when it comes into contact with the surface. [37] This will be the starting point to measure adhesion in juvenile oysters. For the adult oysters, a method was designed in order to use the Instron testing system in our laboratory. It is worth mentioning that, even though the systems developed still need some work, they are a good first attempt at tackling this question.

3.2 Materials and Methods

3.2.1 Sample collection

Larval oysters

As mentioned in the previous chapter, about 150,000 - 200,000 pediveliger larvae of Eastern oysters (*Crassostrea virginica*) were obtained from the Virginia Institute of Marine Sciences and Louisiana State University. Upon arrival in the laboratory, larvae were acclimated in aged seawater (18 parts per thousand salinity) for 15-30 minutes prior to transferring into an aerated 10 gallon glass aquarium at the same salinity,

maintained at room temperature. Beds of plastic (vinyl, Rinzl) and glass (Thermo Scientific Gold Seal) microscope slides were placed at the bottom of the aquarium for larval settlement. Oysters where fed daily with an algae mixture (Shellfish Diet 1800) and water changes performed every three days to ensure growth into spat.

Adult oysters

Intact clusters of oysters were collected at the North Inlet estuary near the Baruch Marine Field Laboratory in Georgetown, South Carolina. These clusters were maintained in the laboratory in a 150 gallon aquarium with artificial seawater at 28 parts per thousand salinity at 18°C until the experiments were performed. These clusters were separated further with chisel and hammer until pairs of bonded oysters were left. Then the samples were cut with a tile saw in order to have samples of 1 cm width and where the shell-cement-shell interface was present (Fig 3.1).

3.2.2 Data collection

Larval adhesion measurements

For the larval adhesion measurements a commercially available water jet was used (WaterPik Aquarius® Water Flosser). This model was used because it provided the pressure settings needed to test the strength of attachment of the larvae to the substrate since most other models or alternatives had higher pressures that made the measurements difficult to perform. The experimental setup can be seen in figure 3.2. The water jet was fixed at a 90°angle with respect to the sample at a constant height of 1.5 cm. The sample was placed on a Teflon rail designed for this experiment to be able to move the microscope slide back and forth. This whole system was placed over an orbital shaker at 25 rpm to ensure an even distribution of the pressure. Each sample was tested starting at the lowest pressure setting and increasing it until all oyster spat in the microscope slide were detached. If no changes were noticed under

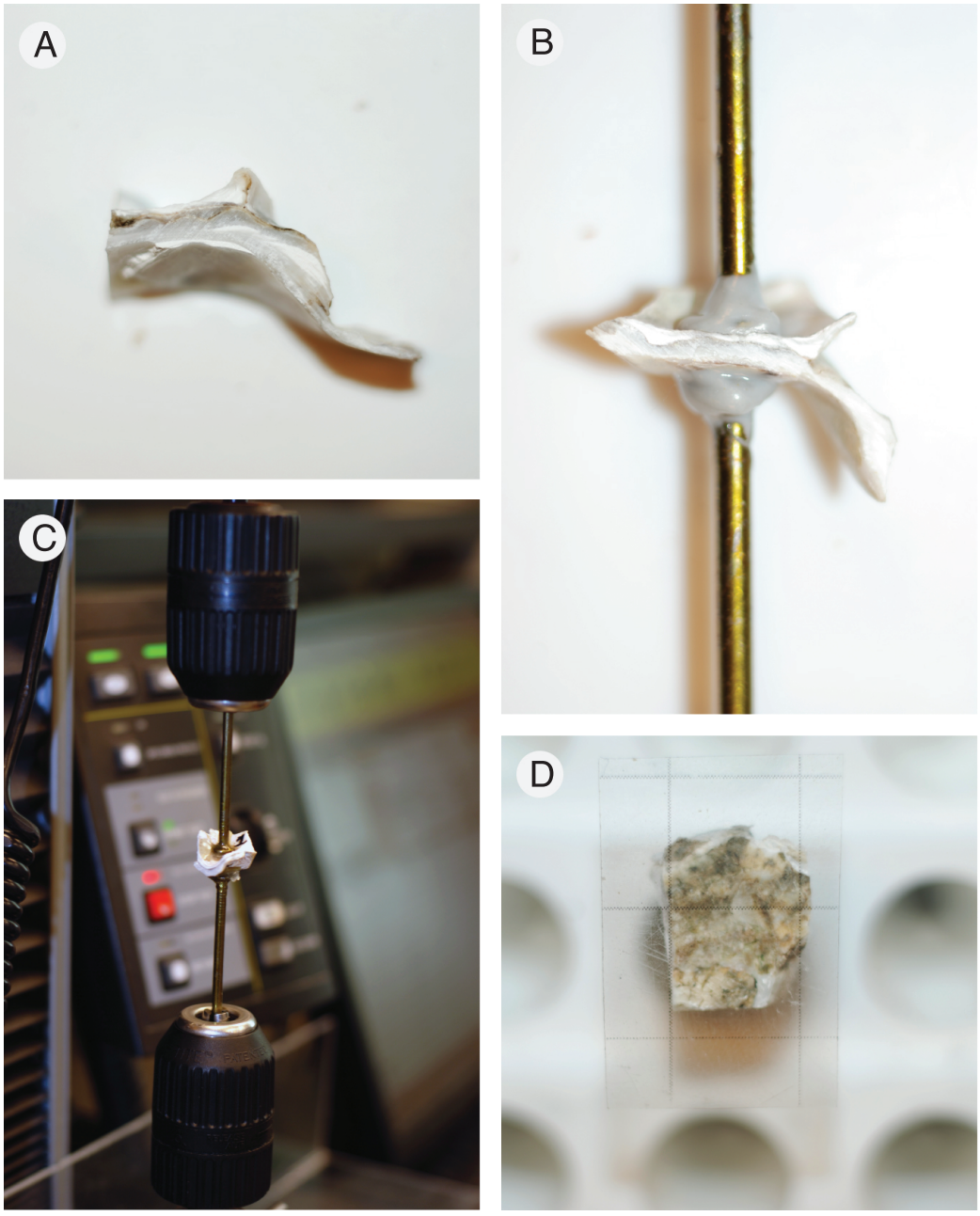

Figure 3.1. Adult oyster sample preparation. To measure adhesion strength on bonded adult oysters samples were prepared as shown. A cross section of 1 cm (A) was bonded to nails as support (B) and then measured in the instron (C). Samples were later photographed and their areas of contact measured (D).

a pressure setting after a minute then the next pressure setting was used. Digital images were collected before and after the measurements at each pressure setting to be able to identify how many oysters detached. ImageJ software was used to analyze the images and calculate the surface area of the oyster spat.

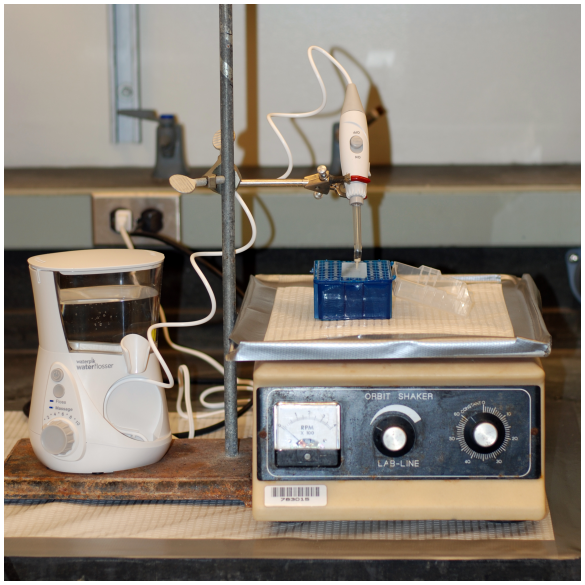

Figure 3.2. Water jet experimental setup. Final iteration of the water jet pressure testing system to measure oyster spat adhesion.

3.2.3 Water jet calibration

In order to obtain consistent results the water jet was calibrated to ensure that each pressure setting exerted a constant force. This process was performed according to the work of Cassé and collaborators [34]. A fixed duration of 15 seconds and six calibration points were collected to calculate the flow rate at each pressure setting. The nozzle diameter (0.5 cm), necessary for the hydrodynamic calculations was measured with the use of ImageJ.

3.2.4 ImageJ workflow

To measure the area of multiple samples in a very efficient manner the following workflow on ImageJ was performed. As shown on figure 3.3, first the selected image was calibrated with the internal measure reference grid of 1 cm (A). Then, contrast and brightness settings were maxed out in a way that the edge of each oyster was clearly visible (B). This image was the converted to grayscale (C) and a threshold was used to highlight all the structures in the image, this creates a white and black version of the image (D). Once you have this you can go to the analyze menu and select analyze particles. Here you can adjust the settings to count the particles in the image and how the results are going to be displayed. Once this is done you will have a copy of the black and white image with the outline of the particle and a number (E), as well as a table with the area of each particle (F).

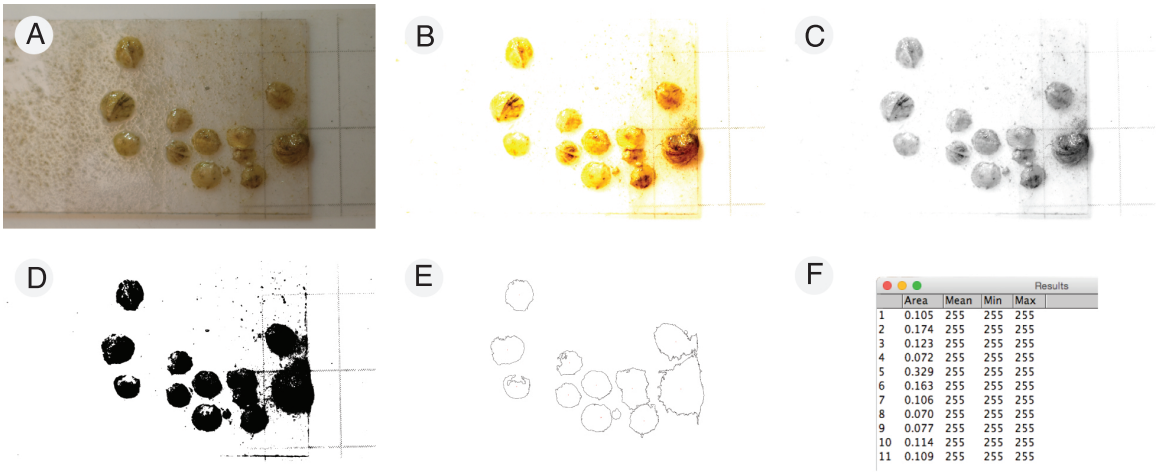

Figure 3.3. Particle analysis. A step by step representation of the workflow used to measure the areas of multiple oyster samples.

3.2.5 Adult adhesion measurements

Samples obtained from the adult clusters were prepared by adhering to each side of the shell a nail glued with epoxy (Figure 3.1). The nails were aligned with one another to ensure that force measurements were as evenly distributed as possible. Then the samples were cured overnight at room temperature and measured the next day. Force measurements were collected on an Instron 5544 materials testing system. After the measurements were completed, digital images of the bonded interface were captured. The ImageJ software was used to determine the area of contact of the cement.

3.3 Results

3.3.1 Water jet calibration

The commercial water jet was calibrated to identify several hydrodynamic parameters that were useful to measure the strength of attachment of the oyster spat. The following parameters were determined for each of the pressure settings.

$$Fr = \frac{\text{volume of water jetted}}{\text{jetting time}}$$

$$U = \frac{Fr}{\text{area of the nozzle}}$$

$$Ip = \frac{U^2}{2}$$

$$\tau = \frac{0.32Ip}{\text{distance nozzle-surface}/(\text{nozzle diameter})^2}$$

Were Fr is defined as the flow rate, U is defined as the average velocity of the jet and Ip is defined as the impact pressure. After measuring each parameter for the

different pressure settings it was found that the impact pressure had a linear relationship with the increasing pressure settings ($R^2 = 0.92$). In order to compare the adhesion strengths of the oysters to other values reported in literature the maximum shear stress (τ) was calculated for each of the pressure settings (Fig 3.4).

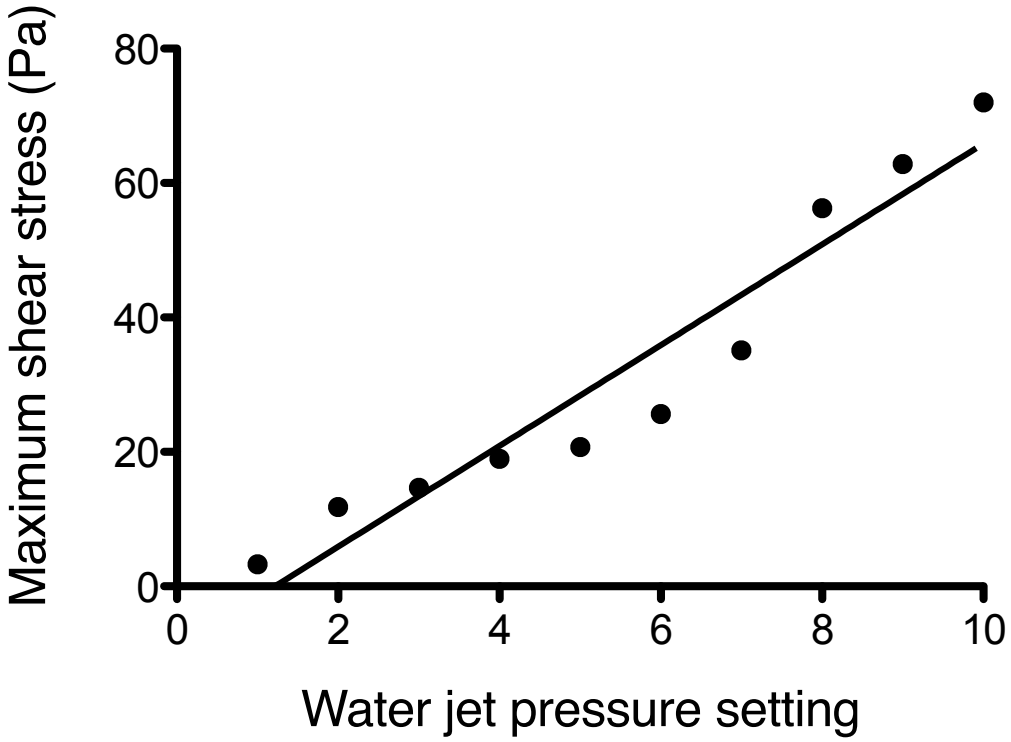

Figure 3.4. Water jet calibration. Maximum shear stress in Pa calculated for each pressure setting on the water jet.

3.3.2 Strength of adhesion measurements

Proof of concept

To test our experimental setup, samples collected and grown in plastic and glass microscope slides a year prior (2016) were used (Fig 2.11). Using this approach it was found that most of the oyster spat grown on glass slides were detached at the first pressure level whereas the oysters grown in plastic slides were detached at the second pressure level. This corresponds to a pressure of 4.1 Pa in glass and 9.44 Pa in plastic microscope slides (Fig 3.5).

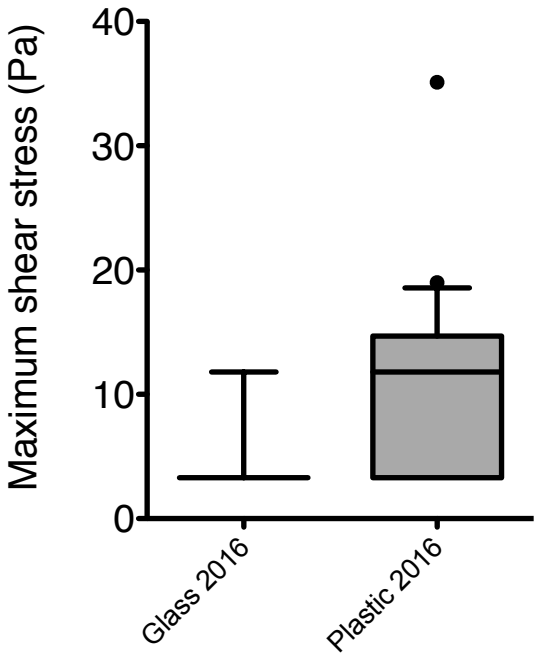

Figure 3.5. Substrate comparison glass vs. plastic (2016). Comparison of strength of adhesion between oysters grown on glass and plastic surfaces.

Measurement of fresh samples (2017)

Once the feasibility of the method was tested, live samples of oysters that were growing in our laboratory were used after 1 and 2 months of growth. It is worth noting that each slide contained several samples. Most of the 1-month-old oysters in the slide detached at pressure setting 6 while the 2-month-old samples detached at pressure setting 3 on the glass microscope slide. This corresponds to a force of 33.6 Pa and 17.6 Pa respectively. In the case of the plastic slides, for both the 1 and 2-month-old spat, part of the oysters were detached at pressure level 6 (33.6 Pa). However, part of the oysters remained even after the highest pressure setting was used (Pressure level 10, $\tau = 72$ Pa).

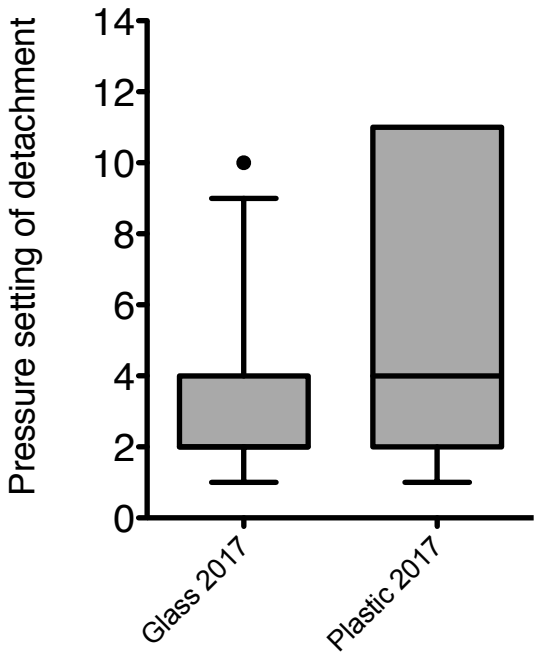

Figure 3.6. Substrate comparison glass vs. plastic (2017). Replicate of the experiment performed in 2016 with new oyster spat samples. The x-axis is shown as pressure settings due to some samples adhering to the surfaces after the highest pressure setting was used.

3.3.3 Parameter comparison

Glass vs. plastic substrates

Samples from both 2016 and 2017 showed higher adhesion strength in plastic substrates than glass substrates. For the 2016 samples the average shear stress at which the oysters were detached was 4.1 ± 2.5 Pa for glass and 9.4 ± 6.6 Pa for plastic. While the standard deviation for the plastic substrates is high, it is worth noting that most of the samples in the plastic substrates were detached at a range of pressures (from 1 to 7) of the water jet (Fig 3.5). In contrast, most of the oysters in the glass slides detached at pressure setting 2. The samples from 2017 were even more interesting because some oysters (9 out of 35) in the plastic substrates were attached even after reaching the highest pressure setting (Fig 3.6).

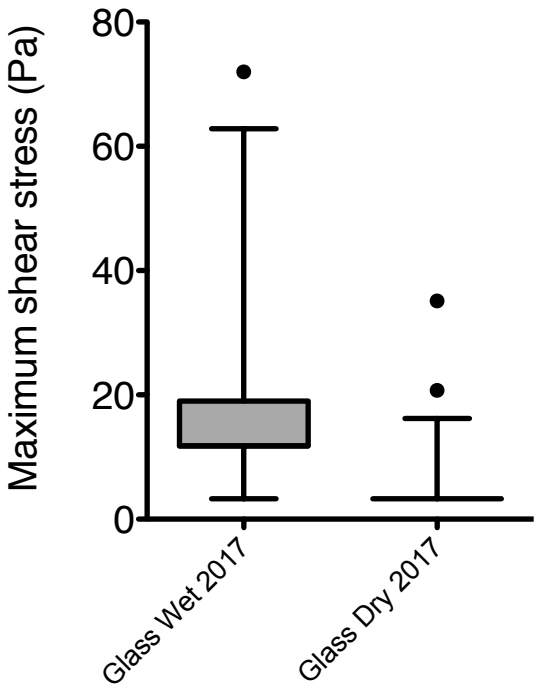

Figure 3.7. Comparison between wet and dry conditions. Comparison of strength of adhesion between samples dried overnight and samples taken directly from the aquarium.

Dry vs. wet measurements

To imitate the dry to wet conditions that oyster reefs are subjected to in nature and to assess its consequences on adhesion strength. A pair of glass substrates was used on each test, one was dried overnight and the other was taken from the aquarium. Adhesion strength measurements under these conditions showed that oysters that were left in the aquarium attached 3 times more strongly than those that were dried overnight (5.4 Pa vs. 16.7 Pa, Fig 3.7).

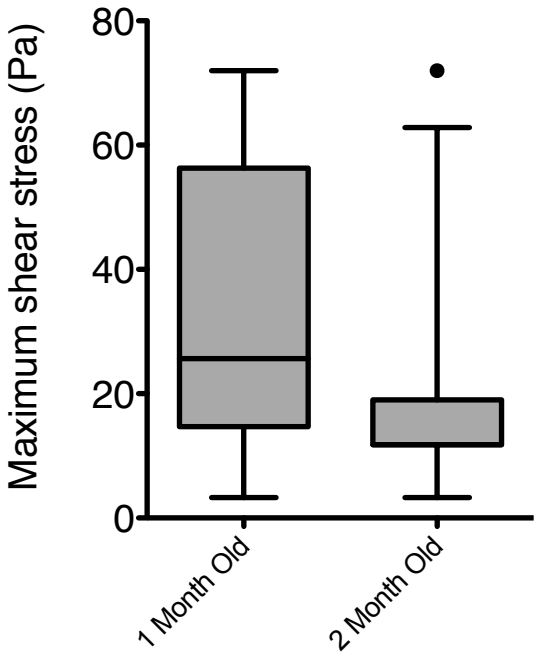

Figure 3.8. Strength of adhesion through time. Maximum shear stress was calculated for oysters after 1 and 2 months of growth in the aquarium.

Growth difference 1 month old spat vs. 2 months old spat

During the course of the experiment oyster samples were allowed to grow to a size that could improve the accuracy of the area measurements. Due to this, a subset of the samples was tested after 1 month of growth while the rest was left in the aquarium to grow for an additional month. Results show that oysters that were 1 month old had twice the adhesion strength than the samples measured after 2 months of growth (33.6 Pa vs. 16.7 Pa, Fig 3.8).

Area vs. strength of adhesion

One of the most logical assumptions while measuring adhesion is that there must be a positive correlation between the area of the attached substrate and its strength. To test this the area of each sample was measured and plotted against the pressure setting the sample detached from the substrate. The average area for the samples are as follows:

Table 3.1.
Oyster spat surface area measurements

Sample	Area (cm^2)
6 months old (2016)	0.0620 ± 0.002
2 months old (2017)	0.0320 ± 0.001
1 month old (2017)	0.0012 ± 0.0003

However, when plotting the results, we find that samples with small surface areas are able to withstand higher pressure settings.

3.3.4 Adult oyster adhesion

Multiple cross sections of bonded oysters with an area close to 1 cm^2 were used to measure for the first time the strength of adhesion of oyster cement. Due to the variability in the shape of the cross section and the testing preparation, samples broke at a different points than the interface were the cement was located. Some samples broke at the edge of the nail that was attached to the shell while others broke at the shell. Since our objective is to measure a clean cohesive break at the cement interface, samples that did not satisfy this criterion were excluded from the analysis. On the first round of testing 7 samples were used and showed an average strength of adhesion of 0.42 MPa $\pm$ 0.013. From these samples a maximum strength of 0.66 MPa and a minimum of 0.26 MPa were found (Fig 3.9).

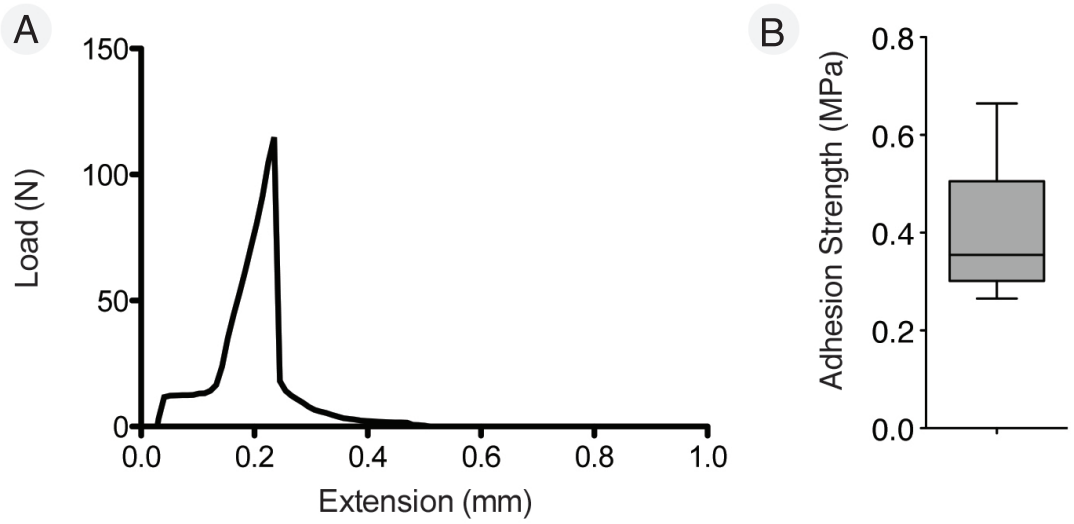

Figure 3.9. Force measurements adult oysters. Data collected from the instron materials testing system shows the maximum load (183 N) needed to separate a cross section of 1 cm^2 of bonded oysters (A). The box plot shows the median value of strength of adhesion while the whiskers show the minimum and maximum values obtained (B).

3.4 Discussion

Experimental setup and proof of concept

Understanding the life cycle and the properties of micro and macro fouling organisms has been of particular interest for the naval and shipping industries. [38] Some of the organisms known to be involved in the fouling problem are mussels, barnacles, microalgae and, to a lesser extent, oysters. [39] While data has been collected for other species, adhesion measurements for the adult and juvenile oysters are lacking. Because of this gap in the information of the strength of fouling of oysters in the wild, our lab designed and tested a method to measure the strength of adhesion of oyster spat and adult oysters based on what has been done on other fouling organisms that have been extensively studied.

Based on the work of Cassé [34] and collaborators and using the resources we had available, we designed an experimental setup with a commercially available water jet and a stage system on top of an orbital shaker to imitate as close as possible the model used by other groups. At first the impact pressure of the different pressure settings in the water jet were calculated. However, the results obtained were not useful to compare them with the reported value for other organisms. In order to make them comparable we had to take into account the hydrodynamic parameters involved in the water jet40. [40] The stream produced by the water jet has three distinct regions, the free jet region, the impingement region and the wall jet region. The free jet region is the stream of water that is ejected from the nozzle that follows a straight trajectory. The impingement region is the point of impact of the water stream and the wall jet region is the stream of water that is displaced radially from the point of impact. [41] In each of these regions different hydrodynamic forces are at play, but the most relevant for our measurements is the shear stress that is equal to zero at the point of impact and reaches a maximum at a radial distance of 0.15 times the distance between the nozzle and the sample (0.225 cm). [37, 41] While this might seem like a simplified version to measure adhesion, the use of the maximum

shear stress as an estimate of adhesion strength has been used before, in particular, for the measurement of micro fouling organisms. [34–36, 40]

Glass vs. plastic substrates

Observation is a key element in any kind of research, especially when it involves a living organism. It was interesting to see that oyster spat showed an apparent difference in growth on both glass and plastic substrates. Initially, my hypothesis was that oysters had a higher probability and needed less material to stick to higher energy surfaces like glass. In contrast, on surfaces with lower energy, like plastic, they had a harder time adhering to the substrate and needed more material and more area to settle. [36,40] The relationship between adhesion and contact area has been studied in several organisms [29,32,34,36,42] but the exact mechanisms on why this happens have not been fully elucidated. However, it is known that both the environment and physical-chemical properties of the material play a role on its performance. [29, 42] From a biological perspective it seems reasonable that a marine glue-laying organism is going to be very selective where and when to place a material that is key for its survival. While the results from this test showed no difference in the area measured for oysters on both substrates, it is clear that there is a difference in the settlement behavior as well as the strength of adhesion.

Dry vs wet measurements

One of the most interesting properties of the adhesive produced by oysters is the ability to endure a constant change between wet and dry environments due to the tides in estuarine ecosystems. Our initial assessment using 2-month old spat showed that there was a significant difference between the adhesion strength in samples taken from the aquarium and those left to dry overnight. Samples in the wet environment were able to withstand higher pressures. From a hydrodynamic perspective it seems reasonable that moisture present in the sample would help maintain the superficial

tension such that the impact of the water jet is slightly diminished upon contact. [40] Another explanation for the change in adhesion might be the physical and chemical changes produced, such as changes in the volume of the material by drying and re-hydrating. [43, 44] While these are interesting explanations to consider more experiments and the characterization of the material are needed to understand the underlying mechanism of adhesion to dry and wet surfaces.

Adult oyster strength of adhesion

In contrast to the larval adhesion measurements, the adult oysters needed less result analysis but more sample preparation. Unlike mussels [9], in order for this to work, the samples had to be carefully chosen. Oyster clusters were separated with a chisel and hammer; only cross sections that presented a shell-cement-shell interface were selected. Another condition for selection was that the shell surface had to be relatively flat to be able to fix the head of the nail to the shell with epoxy. If the surface was to irregular, the nail was difficult to place and almost always resulted in the sample separating at the head of the nail instead of the cement layer. After overcoming most of these obstacles sample testing was straightforward. Usually around 10 samples were prepared, dried overnight and tested. From the samples at least 50% successfully separated at the cement interface. Cross sections that broke at the epoxy layer or the shell were discarded. The results gathered with this method showed us for the first time the adhesion strength of oyster cement in nature.

Initial testing was performed with oyster clusters from previous seasons that were stored in boxes. While the results shown here correspond to fresh samples that were taken directly from the aquarium, it was unexpected to see that the results between the dry samples and the wet samples were within the same range of strength. A plausible explanation for this might be that the adhesive has been shielded from the water in the surroundings; in which case, the strength of adhesion will remain similar regardless of the conditions. [45, 46] However, since this is the first approximation to

measure the strength of adhesion in a natural system, more data and improvements to the method that was developed will be needed.

3.5 Future Directions

The method developed in this chapter still needs improvement but opens up the possibility to ask more questions and discovering more about this natural system.

In this section I will state one of the questions that still linger on my head and some experimental details that might help in future experiments.

Do oyster spat have preference over the substrate they settle? If so, how does this affect their behavior and how strongly they bind?

Initially the idea of using plastic slides to grow the oysters came as a necessity of needing samples for SEM experiments. This helped us realize that there was a clear preference of the oyster to settle on glass slides rather than plastic. One of the reasons for this preference might be that the animal somehow senses the substrate before laying the adhesive. Stepping away from the behavioral aspect, it would be very interesting to see the preferences and the strength of adhesion of oysters to substrates with different surface energies. Instead of laying a bed of glass and plastic slides, a set of different surfaces could be used to address this question.

Is there a relationship between area and strength of adhesion?

Our initial results show that there is no correlation between the area and the strength of adhesion. However, this need to be explored further. Most of the samples we used were 1 or 2 months old with a relative similar size. It would be great to see the strength of adhesion of bigger samples that are 3 to 7 months old. This might be challenging due to the high mortality rate in oyster spat and the intrinsic difficulty of growing oysters in the middle of Indiana, but it is possible to grow the oysters to a

size of up to 6 to 7 mm diameter. This way we could observe not only the influence of size but also age in strength of adhesion.

Is the strength of adhesion going to be affected by environmental conditions?

One of the most important characteristics of oyster cement that could have great implications in real world applications is the ability of oyster reefs to withstand dry an wet conditions without affecting their structural integrity. As a permutation of all the ideas mentioned above, it would be great to test them both in dry and wet environments. If planned carefully all these questions can be tackled almost simultaneously.

Words of wisdom (kinda, sort of, maybe)

Here are some of the things that I have learned from the oysters while designing and going through the experiments.

- At the beginning when you are setting the spat in the aquarium. It does matter how much of the eyed larvae you put in the aquarium. If you put too much the mortality rate increases and or the slides will be saturated with larvae. If you put too little you are not going to have enough samples in the slide to measure. While this is not very scientific, I found that around 3 to 4 spatulafuls of larvae work for a 10 gallon tank and around 5 - 6 for a 20 gallon tank.
- Growing animals out of their natural environment is hard. In order to make this work you have to make sure that you take care of food, salinity conditions and cleaning of the tank. All of these factors affect oyster growth. The happier the oysters are while growing up the better the results will be.
- There are going to be a massive amount of samples and results. It is crucial to have a system to collect, record and analyze the data in a way that makes sense and that you will remember later. This is true for any kind of research,

especially when you have so many factors to consider. Since this is going to be somewhat repetitive, developing a checklist is recommended (See Atul Gawande's The Checklist Manifesto).

- Take advantage of the amount of oysters that are laying the stage 1 and stage 2 adhesives. Collect and store the footprints for later use (for example proteomics)

3.6 Acknowledgements

None of this work could have been possible without the help of Nicolás Morato Gutiérrez. An amazing person, excellent student and now fellow graduate student here in the chemistry department at Purdue University. I also want to thank Karen Sisler from the Virginia Institute of Marine Sciences for her help providing the oyster larvae.

3.7 Disclaimer

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4. A PROTEOMIC VIEW OF THE ADHESIVE PRODUCED BY THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*)

4.1 Introduction

Nature has been the inspiration of mankind in the development of new tools and technologies as well as a vast array of materials used in our daily life. From the silk in our shirts, the leather and rubber in our shoes to the glue that we use to stick them together almost all of it can be traced back to a biological system [1]. Of these systems, oysters are an interesting example. Through evolution oysters have found a solution to endure the challenges of living in an environment that is constantly changing. One of the solutions is the development of adhesives that are able to maintain their structural integrity on wet and dry conditions [2, 3]. The ability of these adhesives to endure harsh conditions has led us to study the system more in depth.

In the last few years there has been a lot of work dealing with the discovery of new molecules thanks to the advance in molecular biology techniques, such as transcriptomics [4, 5] and proteomics [6], giving us the opportunity to look closer to non-model organisms, like oysters and marine glue laying animals [7]. Being a commercially important organism, the Eastern Oyster (*Crassostrea virginica*), has been studied mainly in the context of breeding, for production in fisheries [8], and for the ecological role that they play filtering the water of estuaries [9]. However, little to almost no attention has been directed toward the study of the biomaterial they produce to attach themselves to a substrate.

Due to the limited amount of information on the adhesive properties of oyster cement and the ever-increasing need for new materials we present in this study a first approach to the characterization and identification of the proteins involved in adhesion in the Eastern Oyster.

4.2 Materials and Methods

4.2.1 Animal and sample collection

Clusters of oysters (*Crassostrea virginica*) were collected from the North Inlet estuary at the Baruch Marine Field Laboratory. Oysters were removed from the reef and shipped on ice overnight to our laboratory. Upon arrival, mud and dirt from oysters was removed and immediately placed in a 150-gallon Rubbermaid™ tank at 18°C with an adjusted salinity of 18 parts per thousand.

For cement sample extraction a clutch of oysters is selected and the interface between the individuals is opened with hammer and chisel (Fig 4.1). Once the individual oysters are separated, a layer of a greenish substance can be observed. Oysters are then rinsed with deionized water to remove contaminants and the cement layer is then scrapped off the shell of the animal and collected in glass vials.

For comparison purposes the shell of the oyster is also collected. Only oysters without any visible cement layer were chosen. After that, the shells were dried and the outer layers were removed with a rotary tool. Then shells were immersed in liquid nitrogen for 10 minutes and later crushed with a hammer and converted to powder with the aid of a food processor. Shell powder was then weighted and stored in a container until used.

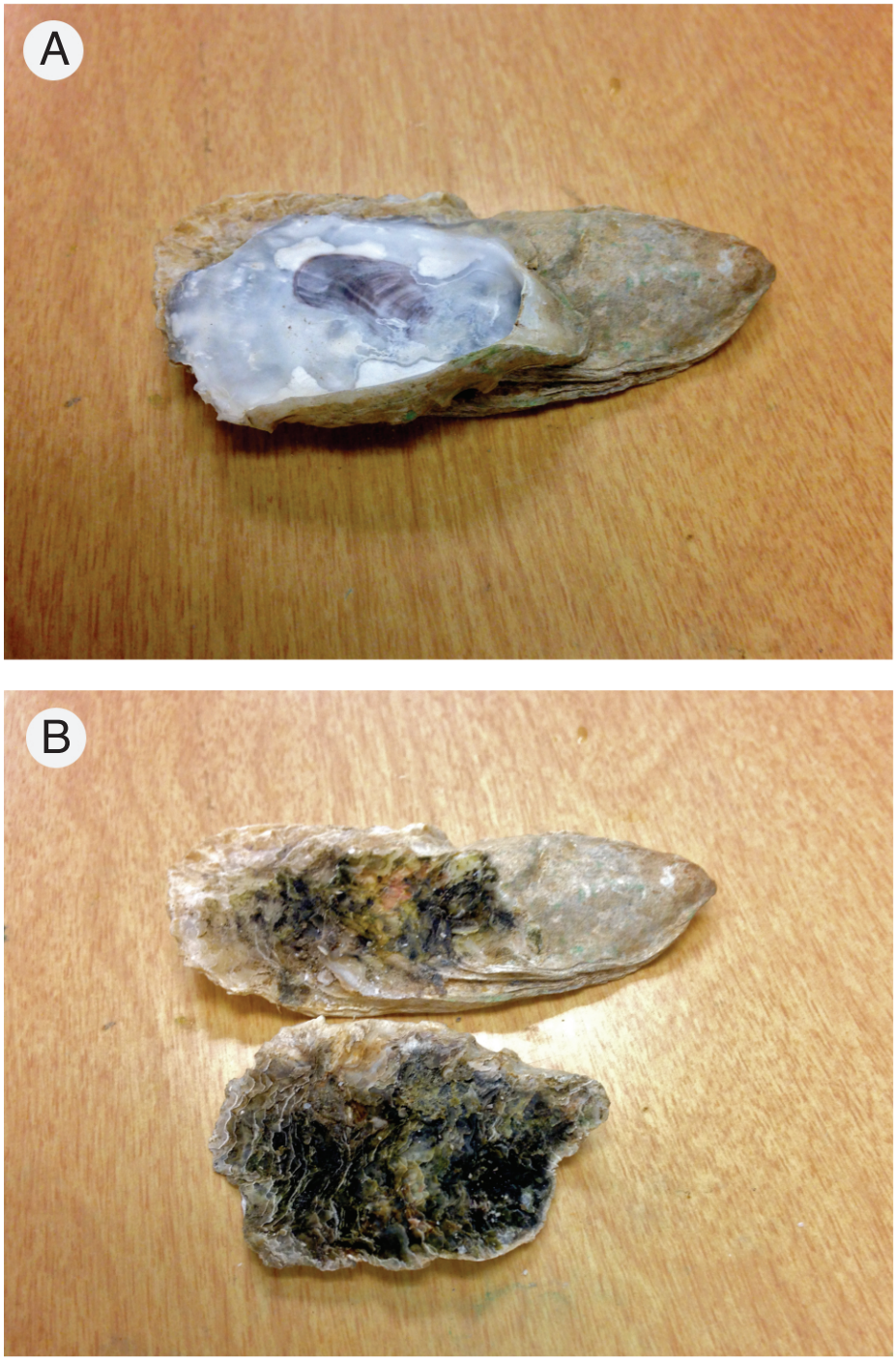

Figure 4.1. Oyster cluster sample. Oyster clusters were separated until a section two bonded shells was obtained (A). After further separation, the green adhesive layer can be found and extracted (B).

4.2.2 Protein extraction

Shell

To remove all the calcium carbonate from the sample a solution of EDTA 17% adjusted to a pH of 8 was used. Approximately 30 g of shell powder were dissolved and stirred overnight at 4°C. After de-calcification the sample was centrifuged at 38.000xg for 30 minutes. The supernatant (Soluble Matrix) was then transferred to a 2 L round bottom to concentrate in a rotary evaporator. The pellet was stored in the -80°C freezer for further extraction of proteins (Insoluble Matrix).

The soluble matrix was concentrated and dialyzed several times to remove the EDTA. For further concentration the sample was transferred to a centricon (Amicon Ultra 15 Millipore centrifugal filters, cut off 10 kDa) and reduced to a volume of 1 mL. Then the sample was transferred to Eppendorff tubes, divided in $\sim$200 μL aliquots and stored at -80°C until needed.

The insoluble matrix was incubated in buffer containing 1.5 M Tris-HCl, 7 M Guanidine-HCl, 20 mM EDTA and 0.5 M DTT (pH 8.5) for 1 hour at 60°C. After this the sample was incubated with 1.5 M iodoacetic acid for 20 minutes at room temperature in a dark room. The sample was then centrifuged and the supernatant collected (Insoluble Matrix 1). One final extraction was performed to the resulting pellet with a buffer solution of 8 M Urea for 1 hour (Insoluble Matrix 2).

Cement

Following a similar approach used to treat barnacle cement [10], approximately 200 mg of cement were incubated in a buffer containing 10 mM sodium phosphate and 6 M Guanidine-HCl adjusted to pH 6. The sample was incubated for 1 hour at constant stirring and then centrifuged. The supernatant was concentrated with a centricon (Amicon Ultra 15 Millipore centrifugal filters, cut off 10 kDa) to a volume

of 1 mL and the pellet was stored at -80°C for further extraction. The insoluble fraction was treated using the same conditions that were used for the shell sample.

4.2.3 SDS-PAGE gel electrophoresis

Soluble and insoluble fractions from both shell and cement were prepared for SDS-PAGE gel electrophoresis. For soluble fractions, 20 μL of sample were incubated together with 20 μL of loading buffer. For isoluble fractions ~20 mg of the pellet were incubated in 40 μL of loading buffer. Samples were heated to 70°C and the loaded into polyacrylamide gels with a 4 - 20% gradient (Genescript ExpressPlus™ PAGE Gels). A Coomassie blue stain solution was used to stain the proteins. If the gels showed no visible bands for the samples then a silver staining procedure was performed.

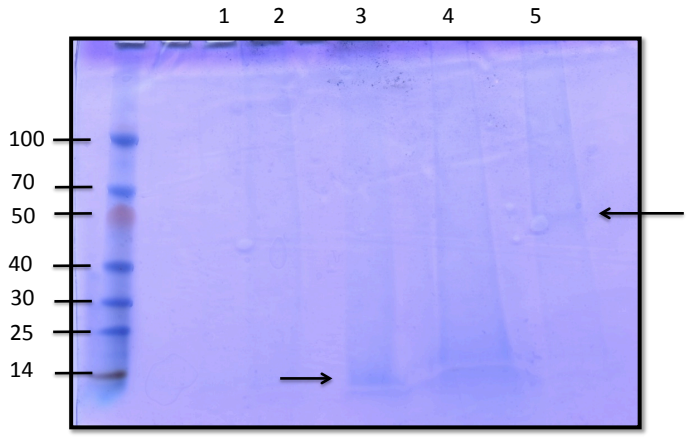

Figure 4.2. Protein gel stained with Coomassie Blue. Proteins from the shell soluble matrix (1), shell insoluble matrix (2), cement (3), cement soluble matrix (4) and the second extraction of the insoluble fraction of shell (5) were separated in a SDS-PAGE gel. Only faint bands could be seen as pointed by the arrows

4.2.4 Mass spectrometry analysis

A total of 8 samples were submited to the Purdue Proteomics Facility at the Bindley Bioscience Center. Samples were further processed, digested with trypsin and analyzed using an Eksigent nano-LC HPLC system connected to a triple quadrupole time-of-flight 5600 mass spectrometer (AB SCIEX, Concord, ON).

Peptide matches from the MS/MS data were compared against the reference proteome for the pacific oyster, *Crasostrea gigas* (Uniprot, proteome ID:UP000005408). [11] using the MASCOT software (Matrix Science, Boston, MA). On the program setup, Ethanolyl (C) was selected as a fixed modification, Acetyl (K) and Oxidation (M) were selected as variable modifications allowing up to 1 missed cleavage from trypsin. Peptide mass tolerance was set to 0.05 Da and the fragment mass tolerance was set to 0.2 Da. The proteins that were identified using these parameters were compiled in an excel file without adjusting the False Discovery Rate (FDR) and setting the FDR at 5%. Other tools from the European Bioinformatic Institute (EBI, http://www.ebi.ac.uk/) and the Swiss Institute of Bioinformatics (SIB, http://www.isb-sib.ch/) were also used to identify protein function and phylogenetic relationships.

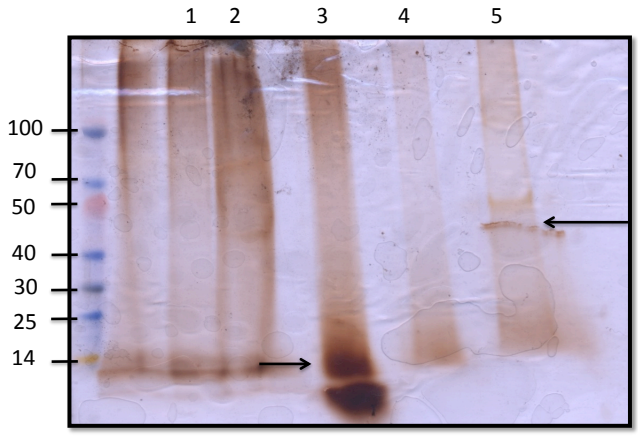

Figure 4.3. Protein gel with silver staining. Same gel used above but stained with silver nitrate to clearly show the protein bands

4.2.5 Sequence alignment and phylogeny

To visualize the relationship between the different adhesive proteins that are found throughout nature, a representative sample of adhesive protein sequences was used (Table 4.3). Accession numbers for the adhesive proteins of different glue laying animals was obtained from the excellent review by Elise Hennebert and collaborators. [12] Clustal Omega and the Simple Phylogeny tools from EBI were used to identify closely related sequences and how the group according to their similarity. [13] The ScanProsite tool from the SIB was used to scout for sequence motifs in the adhesive proteins. [14]

4.3 Results

4.3.1 Protein extraction

Extraction from highly cross-linked materials like oyster cement proved to be, and still is, a challenge. The concentration of the proteins extracted is not high enough to be seen on SDS-PAGE gels stained with Coomassie blue (Fig 4.2) but high enough to be detected by silver staining (Fig 4.3). From the extractions performed only the cement soluble matrix and the second extraction of the insoluble matrix of the shell had visible proteins. Most of the proteins in the cement sample were found at ∼14 kDa while the insoluble matrix of the shell had proteins with a molecular weight of ∼45 kDa.

4.3.2 Proteomic and bioinformatic analysis

Duke proteomic and metabolomics facility

Initially all samples that were extracted were sent to proteomic analysis regardless of protein concentration. Analysis was performed using the Scaffold proteome

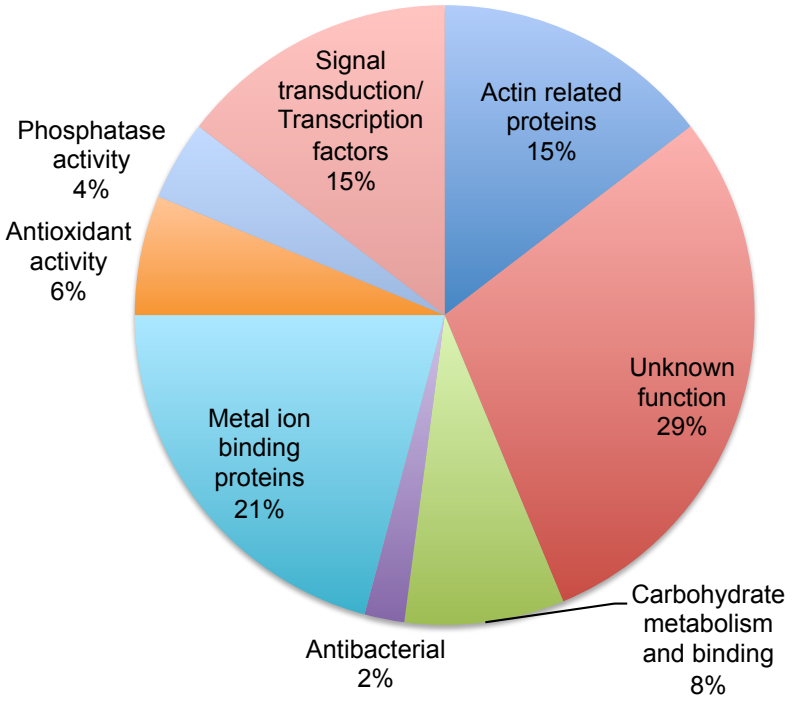

Figure 4.4. Protein identification Duke. Gene ontology (GO) terms were found for each of the 48 proteins identified at the Duke proteomics facility

software yielding a total of 428 protein hits. From the total identified matches, 230 belonged to unrelated organism, 198 matched to our species or related species of oysters and from those, 48 proteins had some function related to adhesion. Some functions identified by gene ontology include carbohydrate metabolism, metal ion binding, phosphatase activity, antioxidant and antibacterial activity (Fig 4.4).

Purdue Proteomics Facility

Samples sent to the Purdue Proteomics Facility yielded a total of 1064 protein hits all belonging to the oyster. From the total samples, 967 hits were exclusive to shell, 25 were identified in both shell and cement and 72 were exclusive to cement. Most of the proteins found on the cement had no annotated function (25 hits) followed by transcription factors (13 hits) and actin and microtubule activity (9 hits).

4.4 Discussion

4.4.1 Sample preparation

The most important part of a proteomics project is sample preparation and the complex matrix surrounding proteins in both shell and cement have proven to be a challenge in terms of protein extraction. In shell, for example, calcium carbonate has to be removed with a solution of 17% EDTA to make the proteins trapped within the matrix soluble. [15] Subsequent purification steps increase the probability of losing protein, especially when using EDTA since multiple dialysis had to be performed. However, the availability of many samples of shell make this problem easy to overcome. In contrast, cement samples are not abundant. Approximately 200 mg of cement could be extracted from a cluster of 10 to 15 oysters. In addition to the small sample size, this adhesive had to be treated with harsh extraction buffers containing high concentrations of Guanidine HCl and Urea [16] to obtain the highest amount of proteins possible.

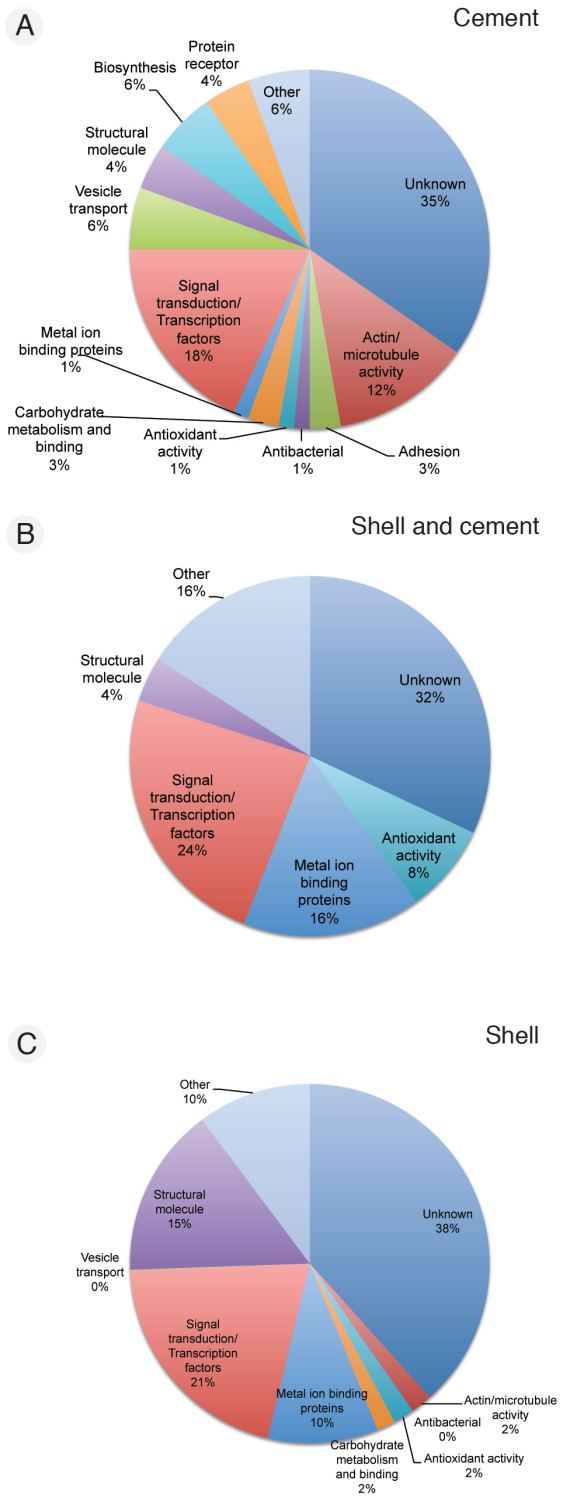

Figure 4.5. Protein identification Purdue. Gene ontology (GO) terms were found for the proteins identified exclusively in cement (A), shell & cement (B) and exclusively shell (C) at the Purdue Proteomics Facility

This approach yielded low molecular weight bands that were faintly visible using Coomassie stain for the raw cement extract (Fig 4.2). A streak at ∼14 kDa for the soluble extraction of the raw cement was observed using silver staining and a ∼50 kDa band from the insoluble fraction of shell (Fig 4.3). These differences might indicate the degradation of protein due to the reagents used in the extraction as well as the incubation times and changes in temperature during the procedure. While this method yielded some results, improvements can sill be done (See Future directions)

4.4.2 Proteomic data

Proteomic analysis was performed in two different proteomic centers. The Duke center for genomic and computational biology (proteomics and metabolomics core) and the Purdue Proteomics Facility in the Bindley Bioscience Center.

Initial results from the Duke proteomic core yielded a total of 428 proteins, 134 of them were uncharacterized and only 71 belong to oysters or related bivalve species. From those proteins belonging to marine bivalves 42 were potential adhesion protein candidates with carbohydrate, metal and phosphate binding functions (Table 4.1). Further analyses in these samples were stopped due to lack of information on how the samples were processed. Some results in the samples matched to unrelated organisms such as *Gorilla gorilla*, *Pan troglodites* (Chimpanzee) and *Pongo abelli* (Orangutan). While these results might appear amusing at first, there is a reasonable explanation. Most of the proteins identified for these organisms is keratin, a common contaminant in proteomic experiments. However, the fact that human keratin was matched to these related organisms means that: 1) the program used to analyze the data did not filter the contaminants from the final report. 2) It is possible that the whole nrNCBI database was used to search the proteins, which lowers the statistical significance of the hits, resulting in strange protein matches. For this reason the samples were submitted again, this time, to the Purdue Proteomics Facility.

Table 4.1.: List of proteins identified by proteomics. Data collected from the Duke proteomics facility

Protein ID	**Protein Name**	**Gene Ontology (GO)**
H9ZXX0	Major plasma protein 2	
A5LGG9	Calreticulin	endoplasmic reticulum calcium ion binding protein folding
O17320	Actin	cytoplasm cytoskeleton ATP binding
K1P9N7	14-3-3 protein zeta	
K1PBD3	Spore cortex-lytic enzyme	hydrolase activity
K1PCV0	Severin	
K1QSL2	Uncharacterized protein	
K1PG66	Coactosin-like protein (Fragment)	intracellular leukotriene biosynthetic process
K1PGX6	Uncharacterized protein	G-protein coupled receptor activity
K1PLG7	Interleukin-17 receptor D	
K1PLL1	Chitobiase	polysaccharide binding carbohydrate metabolic process
K1PNK7	Nephrocystin-3	
K1PPQ1	14-3-3 protein gamma	
K1PPU7	Hemicentin-1	

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Table 4.1 – *Continued from previous page*

Protein ID	**Protein Name**	**Gene Ontology (GO)**
K1PPV2	Uncharacterized protein	extracellular region chitin binding chitin metabolic process
K1PSZ8	G patch domain-containing protein 8	
K1PUJ1	Radixin	cytoplasm cytoskeleton extrinsic component of membrane
Q86FW9	Cavortin (Fragment)	metal ion binding oxidation-reduction process superoxide metabolic process
K1Q5Q2	Plastin-1	calcium ion binding
K1Q6D2	Putative tyrosinase-like protein tyr-1	metal ion binding oxidoreductase activity
K1QIY5	Dentin matrix protein 4	
K1QK19	Chorion peroxidase	heme binding peroxidase activity response to oxidative stress
K1QLH0	Peroxiredoxin-4	peroxiredoxin activity
K1QMV5	Annexin	calcium ion binding
K1QSY4	Tripartite motif-containing protein 56	intracellular zinc ion binding
K1QZJ9	Temptin	copper ion binding oxidoreductase activity
K1R2D6	Plastin-3	NAD biosynthetic process

Continued on next page

Table 4.1 – *Continued from previous page*

Protein ID	**Protein Name**	**Gene Ontology (GO)**
K1R3V2	Sushi von Willebrand factor EGF	extracellular region chitin binding chitin metabolic process
K1R5F2	14-3-3 protein epsilon	
K1R7L7	Kielin/chordin-like protein	
K1RAI3	Annexin	calcium ion binding negative regulation of coagulation
K1RFJ7	Alkaline phosphatase tissue-nonspecific isozyme	phosphatase activity
K1RFW1	Putative sulfite oxidase mitochondrial	heme binding molybdenum ion binding oxidoreductase activity nitrate assimilation
K1RH58	Alpha-actinin sarcomeric	calcium ion binding actin crosslink formation actin filament bundle assembly
K1RKR1	Uncharacterized protein	extracellular region chitin binding chitin metabolic process
K1RZH9	Purple acid phosphatase	acid phosphatase activity metal ion binding
Q1RQ16	Clp1 protein	chitinase activity carbohydrate metabolic process chitin catabolic process

For the new analysis a total of 8 samples from the soluble and insoluble matrices of both shell and cement were prepared for and analyzed by tandem mass spectrometry. A total of 1064 proteins were identified using the reference proteome of *Crassostrea gigas*. [11] By cross referencing the samples we found 72 proteins found exclusively on the cement, 25 localized in both shell and cement and 967 unique to the shell.

Compared to the previous round of proteomics all the proteins that have been identified on the cement samples are new entries. Only 47 proteins from the previous run were identified again in the current analysis, most of these belonging to shell samples. While in the initial proteomic search the proteins seemed to be scattered on both the cement and the shell the current analysis shows a clear separation between the proteins that are found in shell and cement (Table 4.2).

Table 4.2.: List of proteins extracted from oyster adhesive. Data collected from the Purdue Proteomics Facility

Protein ID	**Protein Name**	**Gene Ontology (GO)**
K1PGM3	Serine-protein kinase ATM	ATP binding protein serine/threonine kinase activity
K1PBV0	UDP-glucose 6-dehydrogenase	NAD binding UDP-glucose 6-dehydrogenase activity
K1Q233	Kinesin-like protein KIF16B	ATP binding microtubule motor activity phosphatidylinositol binding
K1S2X4	Serine/threonine-protein kinase DCLK3	ATP binding protein serine/threonine kinase activity

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Table 4.2 – *Continued from previous page*

Protein ID	**Protein Name**	**Gene Ontology (GO)**
K1QCB5	Poly [ADP-ribose] polymerase (PARP)	NAD+ ADP-ribosyltransferase activity
K1QJA1	Cell division control protein 42-like protein	GTP binding
K1R915	Glycine receptor subunit alpha-2	extracellular ligand-gated ion channel activity
K1RH81	Uncharacterized protein	GTP binding
K1PEP8	Uncharacterized protein	G-protein coupled receptor activity
K1PBC0	Non-neuronal cytoplasmic intermediate filament protein	structural molecule activity
K1RHM2	Low-density lipoprotein receptor-related protein 6	
K1RRQ1	Eukaryotic translation initiation factor 5B	GTPase activity GTP binding translation initiation factor activity
K1Q5S6	Protocadherin Fat 4	calcium ion binding
K1QRE7	TBC1 domain family member 4	metallopeptidase activity
K1R7U9	Multiple epidermal growth factor-like domains 10	
K1PR10	Uncharacterized protein C12orf65-like protein	hydrolase activity translation release factor activity

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Table 4.2 – *Continued from previous page*

Protein ID	**Protein Name**	**Gene Ontology (GO)**
K1R0S1	Growth arrest-specific protein 8	
K1PA49	Putative ferric-chelate reductase 1	
K1RHY2	Uncharacterized protein	oxidoreductase activity
K1R752	E3 ubiquitin-protein ligase MYLIP	ligase activity zinc ion binding
K1PPY9	Putative 39S ribosomal protein L24; mitochondrial	structural constituent of ribosome
K1QPP2	Elongation factor Tu; mitochondrial	GTPase activity GTP binding translation elongation factor activity
K1Q680	Clathrin interactor 1	
K1QA61	Histone-lysine N-methyltransferase PRDM9	methyltransferase activity
K1QTN1	Nuclear valosin-containing-like protein	ATP binding
K1R9W6	Uncharacterized protein	nucleic acid binding zinc ion binding
K1S3Z7	Gametogenetin-binding protein 2	
K1R217	Tripartite motif-containing protein 45	zinc ion binding
K1R2G7	Ran-binding protein 3	

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Table 4.2 – *Continued from previous page*

Protein ID	**Protein Name**	**Gene Ontology (GO)**
K1QGX3	Fucolectin-1	
K1QYM1	Thymidylate synthase	thymidylate synthase activity
K1PLK6	BTB/POZ domain-containing protein 9	
K1RZ99	Filamin-A	
K1QPZ9	Amyloid protein-binding protein 2	
K1S1N8	Coiled-coil and C2 domain-containing protein 1-like protein	
K1R7N1	Tripartite motif-containing protein 3	
K1QUX6	4-hydroxybutyrate coenzyme A transferase	transferase activity
K1QEN3	NFX1-type zinc finger-containing protein 1	
K1QCJ2	UPF0638 protein B	
K1PRM3	IMPACT-like protein	
K1QRN4	Zonadhesin	
K1RBJ3	DnaJ-like protein subfamily C member 13	
K1QSA9	Uncharacterized protein	GTPase activator activity
K1PF44	Receptor-type tyrosine-protein phosphatase R	protein tyrosine phosphatase activity
K1Q7Y2	Nuclear receptor coactivator 7	
K1QQ20	Kyphoscoliosis peptidase	

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Table 4.2 – *Continued from previous page*

Protein ID	**Protein Name**	**Gene Ontology (GO)**
K1Q5H2	Protein dopey-2	
K1Q3I9	Spore cortex-lytic enzyme	hydrolase activity
K1Q3E1	Hyaluronan mediated motility receptor	hyaluronic acid binding
K1RQ92	Coiled-coil domain-containing protein 60	
K1QWJ0	Nuclear mitotic apparatus protein 1	
K1R2P3	VAC14-like protein	

4.4.3 Bioinformatic analysis

Identification and functional annotation for the oyster samples was collected from the Uniprot database. [17] The initial mass spectrometry run from the Duke Proteomic center identified only 71 proteins belonging to *Crassostrea gigas*, none of those belonging exclusively to cement. For this reason the identification of potential adhesive proteins was based on functional Gene Ontology terms (GO Terms). [18] From the total proteins found for oyster 42 were identified as potential adhesive candidate proteins (Fig. 4.4). 7 metal ion binding proteins, 7 glyco-proteins, 7 proteins involved in antibacterial responses, 5 related to protein binding, 5 antioxidant proteins, 6 proteins related to carbohydrate and phosphate binding and 7 with other functions (oxidoreductases, transcription factors, etc.). From these matches, glycoproteins, metal ion binding proteins and oxidoreductases have also been found in other adhesive-laying organisms. [19–21]

Analyses from the samples sent to the Purdue Proteomics Facility showed us that from the 72 proteins identified in the cement 23 are uncharacterized proteins that need further BLAST analysis to assign a function. The remaining 49 proteins still need to be annotated with GO terms (Fig 4.5). It is worth mentioning that between the samples sent to Duke and the samples sent to Purdue there was no overlap in the identification of proteins in the cement. Most of the proteins sent to Duke were also found in shell samples analyzed at Purdue, but none of the proteins identified at Duke were present in the cement samples analyzed at Purdue. To further confirm that the proteins identified in the cement are different from those found in the internal organs of the oyster a comparison was made between our dataset and a recent paper that analyzed the mucosal secretions within the eastern oyster. [6] From the list of proteins found in the paper only 5 matched with our dataset indicating that the majority of the proteins we found in the second round belong exclusively in the cement of the animal. Since the time of the analysis of the samples, new research has delved into the characterization of the cement of a closely related species of oyster *Crassostrea gigas*. [22]

Table 4.3.: Adhesive proteins across the tree of life. Protein names and accession numbers of a variety of glue laying organisms

Species	**Protein**	**Protein Accession Number**
Asterias rubens	sfp-1	AHN92641.1
Gasterosteus aculeatus	spiggin	NP_001254619.1
Euperipatoides rowelli	Er_P1	ADI48487.1
	Er_P2a	ADI48488.1
	Er_P2b	ADI48489.1
	Er_P3	ADI48490.1
Megabalanus rosa	Mrcp-19k	BAE94409.1

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Table 4.3 – *Continued from previous page*

Species	**Protein**	**Protein Accession Number**
	Mrcp-20k	BAB18762.1
	Mrcp-52k	BAL22342.1
	Mrcp-100k	BAB12269.1
Fistulobalanus albicostatus	Balcp-19k	BAE94410.1
	Balcp-20k	BAF96022.1
Balanus improvisus	Bicp-19k	BAE94411.1
Nephila clavipes	ASG1	ACI41238.1
	ASG2	ACI41239.1
	PySp2	ADK92884.1
Latrodectus hesperus	AgSF1	AFP57565.1
	AgSF2	AFP57562.1
	PySp1	ACV41934.1
Rhipicephalus appendiculatus	RIM36	AAK98794.1
	64P	AAM09648.1
Rhipicephalus haemaphysaloides	RH50	AAS66656.1
Dreissena Polymorpha	Dpfp1	AAF75279.1
	Dpfp2	EST sequence no protein data
Mytilus californianus	Mfp-3S	AAZ94729.1
	Mcfp-5	ABE01084.1
	Mcfp-6	ABC84186.1
Mytilus edulis	Mefp-1	AAX23968.1
	Mefp-2	AAX23970.1
	Mefp-3	AAF89278.1
	Mefp-4	N/A
	Mefp-5	AAL35297.1
	Mefp-6	N/A

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Table 4.3 – *Continued from previous page*

Species	**Protein**	**Protein Accession Number**
Mytilus galloprovincialis	Mgfp1	BAA09851.1
	Mgfp5	AAS00463.1
Perna viridis	Pvfp-1	AAY46226.1
	Pvfp-2	AGZ84282.1
	Pvfp-3	AGZ84285.1
	Pvfp-5	AGZ84279.1
	Pvfp-6	AGZ84283.1
Lehmannia valentiana	Sm40	ABR68007.1
	Sm85	ABR68008.1
Phragmatopoma californica	Pc-1	AAY29115.1
	Pc-2	AAY29116.1
	Pc-3A	AAY29119.1
	Pc-3B	AAY29122.1
	Pc-4	EST sequence no protein data
	Pc-5	EST sequence no protein data
Sabellaria alveolata	Sa-1	CCD57439.1
	Sa-2	CCD57460.1
	Sa-3A	CCD57480.1
	Sa-3B	CCD57502.1

4.4.4 Potential adhesive proteins

One of the risks of working with non-model species is the lack of genomic, transcriptomic and proteomic information that can be acquired. [7] In our case this information is found for *Crassostrea virginica* and *Crassostrea gigas*. [11] However, the

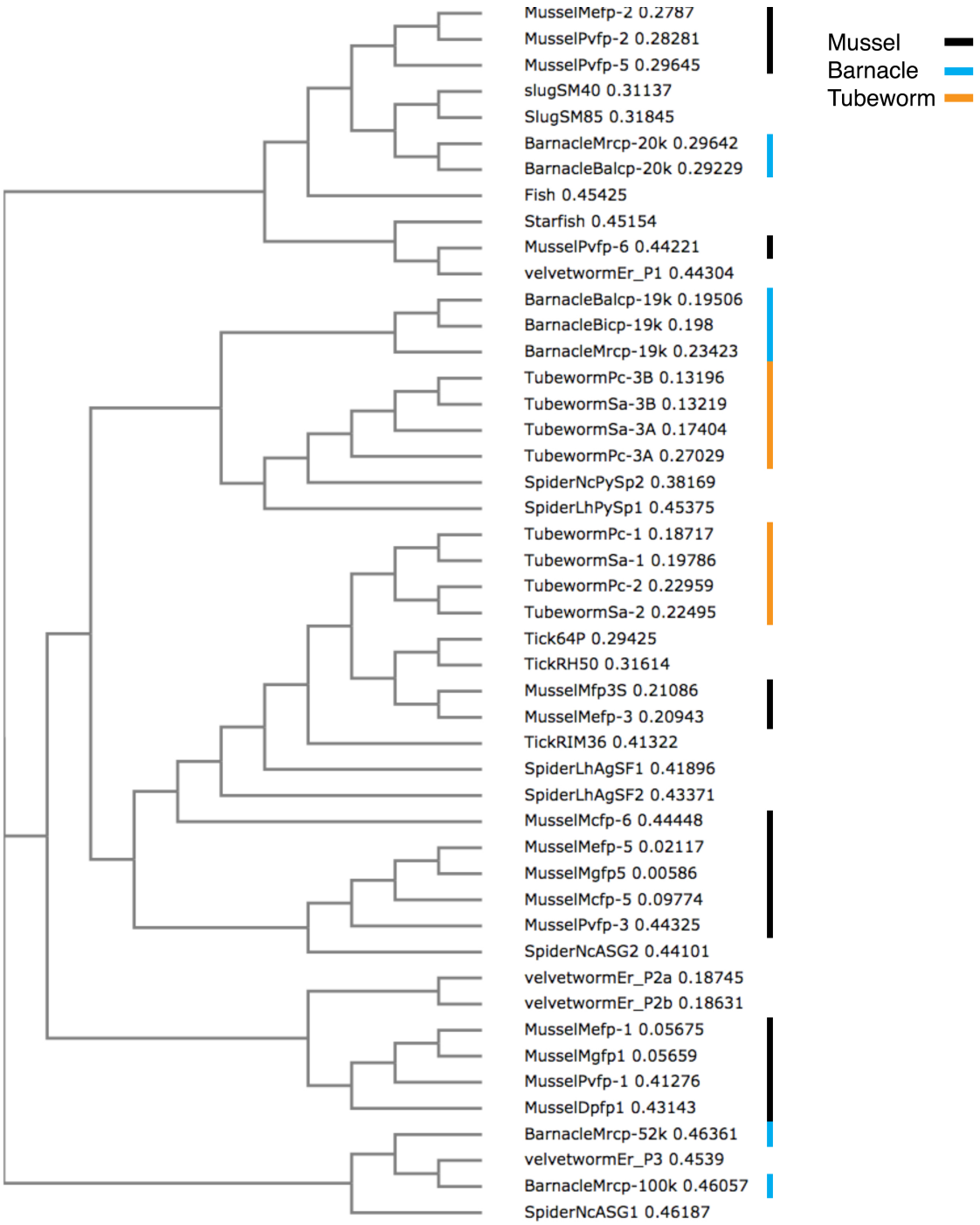

Figure 4.6. Phylogenetic tree of adhesive proteins found on glue laying organisms. A simple phylogenetic tree was generated with known sequences of adhesive proteins from multiple organisms.

challenge lies in the correct identification of adhesive proteins, our main target. Biological adhesives can be found throughout the tree of life (Fig 4.6) in different organisms but their function and complexity vary greatly. [12] To be able to identify common motifs it is necessary to look for closely related species whose adhesive proteins have been studied. For this purpose, sequences of adhesive properties from different animals in the Metazoan phylogeny were used (Table 4.3). The first step is to align all the sequences looking for conserved regions and then use them to organize them in a phylogeny using the Clustal Omega and simple Phylogeny tools from EBI. Results from this showed that there were almost no conserved regions between the proteins. An example of such difference is the adhesive protein from the sea star *Asterias rubens* that has 3853 residues compared to the 100 to 200 residues from the rest of the species analyzed. [23] Another challenge that was observed is the fact that none of the adhesive proteins identified so far in glue laying organisms cluster in one particular node in the phylogeny. Even if the approach of using closely related species was useful for the identification of adhesive proteins in the tubeworm *Sabellaria alveolata* [24],it seems that for the eastern oyster other strategies will be needed.

New insights on oyster adhesion have been published which show similar results to the ones described here. [22] Multiple transcription factors have been found in the proteomic analysis of the adhesive of larvae from *Crassostrea gigas*. These proteins are identified easily due to the abundance of them in relation to the life stage of the oyster, where protein production is at maximum capacity to aid the transition from larvae to young oyster. [25] However, as was discussed in the first chapter of this thesis, oysters produce two very distinct adhesives. [26] The first adhesive resembles a fibrous material that cures very rapidly, similar to what has been observed in other marine animals. [27, 28] The second adhesive is more like a secretion that mineralizes over time. [29] More experiments are needed, but, it would seem logical to assume that the actin and other structural proteins might be playing a role in this first stage

adhesive (K1RH58, Table 4.1). While the abundance of transcription factors in the second stage adhesive might be attributed to the oyster constant shell growth. [30]

4.5 Future Directions

There is still a lot of work to be done in this particular system. A protein solubilization protocol for these highly cross-linked proteins was developed and will require further optimization. Other bioinformatic analysis such as BLAST of uncharacterized proteins, domain and motif analysis and amino acid analysis, among others, need to be performed. The final goal of this project is to accomplish the same level of characterization of the adhesive material that has been done in other well studied organisms like Mytilus edulis, the blue mussel. [31] Here I mention a few things to consider.

Extraction methods

EDTA and Guanidine HCl extractions had to be changed. The extraction with EDTA proved to be tedious since it required multiple rounds of dialysis while extraction with acetic acid only needed one round. Multiple chaotropic agentes were used (Hydroxilamine, Urea, Guanidine HCl). However, the only one that is suitable for proteomics is Urea since hydroxilamine cleaves proteins and Guanidine HCl is not compatible with the 2-mercapto ethanol from the loading buffer in SDS-PAGE.

Other alternatives are needed but it seems that Urea 8M with a low percentage of detergent (Tween, Triton X, SDS) has been used with relative success and is somewhat compatible with the instruments in the proteomics facility. You would have to look for what the limits allowed for this detergents are in your own instrument or facility.

Bioinformatic analysis

You might have realized by now that the section on bioinformatics was missing in the results section of this chapter. Well, there is a reason for that.

Sequence analysis was performed for the adhesive proteins found on Table 4.3. However, no significant match was found. When multiple sequence alignment was performed across multiple organisms no similarity was found, even when comparing the adhesive proteins of mussels. Looking at the protein sequences it is clear that there are certain motifs repeating throughout the proteins, similar to the patterns found in spider silk. [32]

It would be very interesting to continue with and in depth bioinformatic analysis of the sequences that are available and compare those to the sequences that are unknown proteins in the proteomics results. I have performed a domain analysis using the ScanProsite tool from SIB but there are only a few domains that appear, like von willebrand factor domain and EGF domains but they don't tell a complete story yet. This is going to require collaboration with someone that actually does work with bioinformatics.

Samples from different life stages (and organs too)

This chapter focused on the extraction of proteins from adult oysters. This poses a challenge since the samples that we are collecting are from their natural habitat. In the first chapter we could observe some algae growing in the cement layer between oysters. Even if the library that we are using for proteomics is based on the genome of the oyster only, the inclusion of proteins from other organisms lowers the probability of finding the proteins that we actually want. Which is why I think it would be interesting to analyze the samples from the footprints of oyster spat (as they were collected after the experiments performed in the third chapter of this thesis). This is still a challenge due to the quantity of material that can be recovered, but it will give a cleaner sample of the actual stage 1 adhesive, narrowing down the search of the proteins involved.

Another sample that would be interesting to analyze is the "nacre" of the shell. Takahashi and collaborators [33] report a protein involved in shell repair. If this

protein is indeed located there, then we should be able to identify it using proteomics. So far none of our trials have shown this protein. However, one of our collaborators was able to identify it using solid state NMR. [34]

Something to try as well during the proteomic analysis is to look for post-tranlational modifications. In particular phosphorylation and glycosylation. There are a number of phosphoproteins involved in mineralization and we found some proteins here that have some functions involving carbohydrates. Since the oyster seem to combine mineralization and adhesion this relationship might be worth to look for.

4.6 Acknowledgements

Being a newcomer to the realm of proteomics, I would like to thank Vicky Hedrick, Tiago Sobreira and Uma Aryal from the Purdue Proteomics Facility for their help. I am also grateful to professor Mark Hall for helping me troubleshoot many of the challenges associated with protein extraction.

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VITA

Andrés Mauricio Tibabuzo Perdomo, observer of nature, wanderer of forests, climber of trees, helper of apicomplexans, collector of venoms, extruder of liposomes, father of oysters, breaker of shells, son of Hugo Mauricio Tibabuzo and Diana Esther Perdomo, was born on August 2nd of 1989 in Bogotá, Colombia.

Given his interest in nature, he studied biology at Universidad de los Andes where he explored the different ecosystems of his home country. At this moment of his life, he wandered cloud forests, drank water from the hearts of mountains, captured crocodiles (*Caiman crocodilus*), was bitten by a bat, shared a room with giant spiders and loved every second of it.

Later in his career, he had the opportunity to learn biochemistry and molecular biology in the laboratory of Dr. Barbara H. Zimmermann. His passion was to study venoms, so to his parents and advisor's surprise, he embarked on an adventure to the coast of Colombia to collect snake venom. He later joined the laboratory of Dr. Chad Leidy and started a collaboration by studying the effect of snake venom phospholipases on membranes, combining the expertise of both laboratories. This led him to present his work at the World Congress of the International Society on Toxinology in 2012 and to graduate as a biologist in 2013.

Curious and wanting to gain a deeper knowledge of the natural world, he started his graduate studies in chemistry at Purdue University. He joined Dr. Jonathan J. Wilker's laboratory because he saw the opportunity to translate basic research into useful applications. Here, he applied all the knowledge gathered in his toolbox throughout the years to tackle the question, "How do oysters stick"?

After living many experiences and lessons during his time at Purdue University and eager to use his skills to help the world, Andrés received his Doctor of Philosophy degree in Chemistry in August, 2019.

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Changes in Cementation of Reef Building Oysters Transitioning from Larvae to Adults

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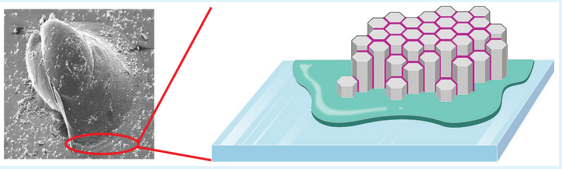
Supporting Information

ABSTRACT: Oysters construct extensive reef communities, providing food, protection from storms, and healthy coastlines. We still do not have a clear picture of how these animals attach to surfaces. Efforts described herein provide the first examination of adhesion at the transition from free swimming larvae to initial substrate attachment, through metamorphosis, and on to adulthood. Two different bonding systems were found to coexist. Larvae use an organic, hydrated glue that persists while the animal progresses into the juvenile phase, at which point a very different adhesive emerges. Juveniles bond with an organic−inorganic composite system, positioning the organic component for maximum adhesion by residing between the animal and substrate. Beyond understanding our marine environment, these insights may aid efforts in aquaculture, reef restoration, and adhesive design.



KEYWORDS: *adhesion, biomineralization, cement, oyster, reef*

Oysters have been influencing human culture and our livelihood for centuries. Native Americans and European settlers in North America relied upon these shellfish to provide a major source of food.[1,2] Oyster reef structures protect coasts by absorbing the energy of storm surges, creating an environment for other species to live within, and filtering large volumes of water.[3,4] Once they settle onto a surface, these bivalves remain in place for their entire lives and, even after death, their shells provide a substrate for future generations. Consequently, we are particularly interested to watch how the animals transition from free swimming larvae to attached juveniles and then become macroscopic reef builders. Our current view of oysters bonding to surfaces is sparse and does not provide a consistent story. Efforts described herein examine the interface between shell and substrate while the animal develops from larvae through to adults. Oysters are shown to create two, strikingly different adhesive systems, depending upon the animal's stage of life. Contrary to prior proposals, juvenile attachment is neither simply shell nor the periostracum shell coating. An all organic material starts and then gives way to an organic−inorganic composite system, all the while differentiating shell on top from adhesive on the bottom.

Reef communities are built by oysters producing an adhesive, often called a "cement," for sticking to one another (Figure 1A).[5] As a result of fishing, pollution, and disease, a mere 2% of indigenous reef habitats remain in the US.[3] Consequently, after each major storm such as hurricanes Katrina and Sandy, there is often a flurry of media attention calling for oyster restoration to protect our coasts. Restoring marine ecosystems relies upon our ability to coax these shellfish into settling and producing glue. Conversely, aquaculturists wishing to sell single oysters instead of clusters for food and ship owners seeking minimal drag on their hulls are looking for ways to prevent cementation. Given the importance of oysters in our lives, we are compelled to understand how these shellfish stick together.

Decades ago, we learned that initial larval settlement relies upon a fibrous adhesive containing proteins and mucopolysaccharides.[6−10] Separate studies with the adhesives of juveniles or adults for different oyster species are somewhat contradictory, with some reports of predominantly inorganic, crystalline materials being responsible for bonding.[11−14] Alternatively, the periostracum, an organic outer coating for protecting shells,[15] may contribute to surface attachment.[16,17] Our most recent insights on adult cement found the adhesive to be derived from organic components within an unstructured inorganic matrix.[18−20] In addition to proteins and polysaccharides, the material contained phospholipids,[19] possibly creating an analogy to larval barnacle adhesive.[21] Both the structure and composition of adult oyster cement differed dramatically from the surrounding shell.[18−20] Results discovered here show that

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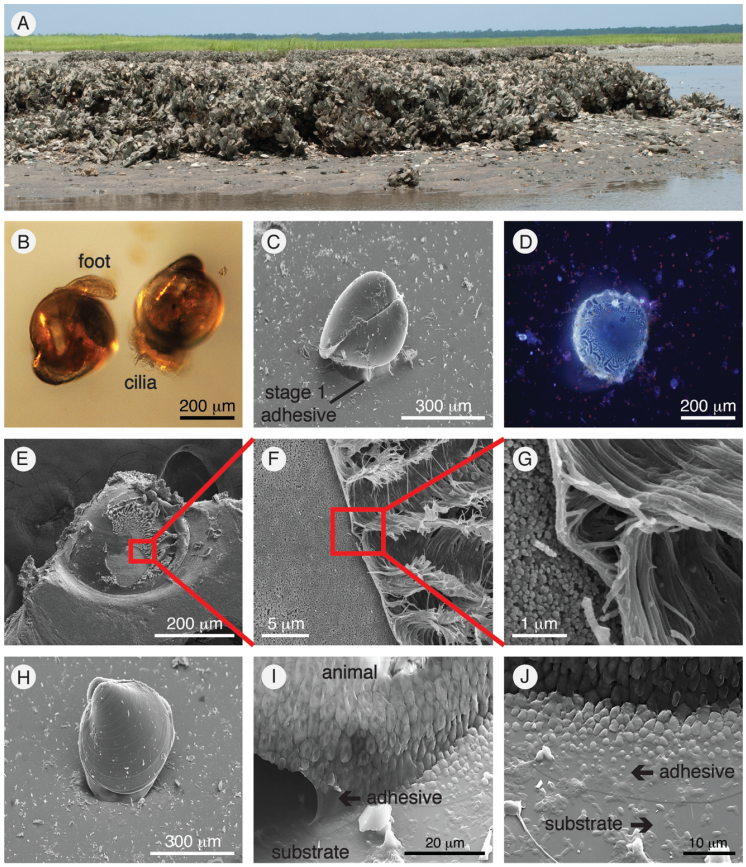

Figure 1. Adhering oysters. (A) Reef in South Carolina, US. The top animals are living and bonded to the remaining shells and cement of prior generations. (B) Two larval oysters exploring a surface. (C) Scanning electron micrograph (SEM) of an oyster after initial attachment. Note the adhesive residing between shell and surface. (D) Fluorescence microscopy of initial larval adhesive, looking up through the glass substrate at the bottom of the animal ($\lambda_{excitation}$ = 310−390 nm, $\lambda_{emission}$ = 420+ nm). (E−G) SEM images from the bottom of a ∼6 month old oyster, after detachment from a plastic substrate. The adhesive has taken on a highly fibrous structure. (H−J) At 1−2 days after initial settlement, a "stage 2" adhesive, different from the larval glue, begins to emerge from in between the animal's shells. This adhesive appears to be pliable and making efficient contact with the surface for bonding.

oysters do not use shell or periostracum for contacting the substrate. An all organic material is responsible for larval adhesion. After metamorphosis into juveniles, a structured composite of organic and inorganic components is then generated for surface attachment.

Studies began with free swimming larvae of the Eastern oyster (*Crassostrea virginica*), the dominant species of the US east coast and Gulf of Mexico. At less than 14 days old, larvae were grown in aquaria with glass microscope slides lining the tank bottoms. Animals were observed before, during, and post settlement. Figure 1B and Video S1 show larvae moving freely prior to settlement, consistent with a prior description.[22] Within hours of contacting the substrate, an adhesive material was visible. A scanning electron micrograph (SEM) in Figure 1C showed what happened immediately after attachment, with material placed between shell and substrate. Prior studies with a different oyster species, the European flat oyster (*Ostrea edulis*), found initial settlement to use an unstructured organic glue.[6−9] Results here for the Eastern oyster appeared to be generally similar.

Looking up at the bottom of the animal through the glass substrate showed that the outer adhesive edge fluoresced (Figure 1D, Figure S1). This light emission indicated the presence of a system with reactive chemistry as opposed to, for example, the generally sticky polysaccharides used by bacteria for attachment.[23] In mussels organic radicals have been shown to be involved in the curing of their protein-based adhesive.[24] Radicals tend to be quite reactive and, among other things, generate radical−radical couplings.[25] The resulting products are often conjugated aromatic compounds. Once these species are formed, the systems will often fluoresce.[26−28] The images shown here and below are all without any added dyes. Such observations of autofluorescence indicate that these oysters form conjugated organics by way of curing chemistry that may involve radical species or other means of, for example, coupling aromatic amino acids.

Energy-dispersive X-ray (EDX) spectroscopy of this early stage glue showed pronounced differences compared to the animal's shell (Figure S2). The elements C, Cl, and S were all elevated in the adhesive whereas Ca and O were lower. Oyster shells are comprised of ∼98% inorganic $CaCO_3$,[29] but larval

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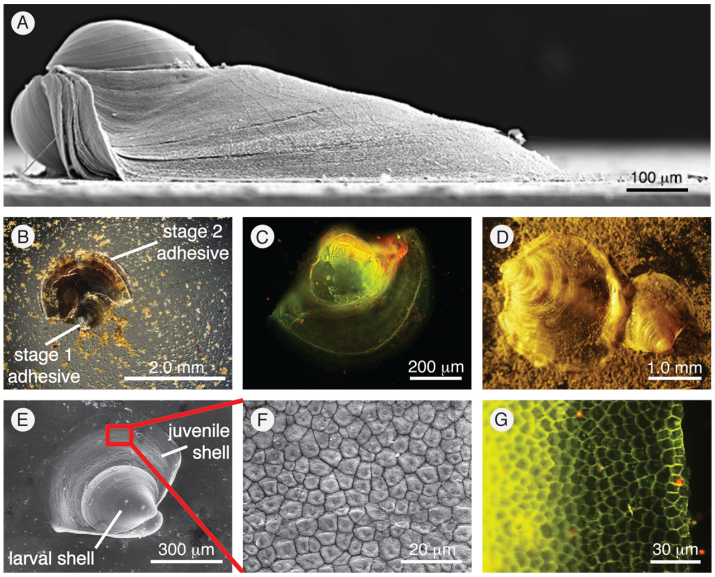

Figure 2. Adhesion changes after metamorphosis. (A) Side-on view of an oyster growing after ∼2 months. (B) Optical microscopy of the bottom of a juvenile oyster, 5 days after settlement, looking through the glass substrate. Both stage 1 and stage 2 adhesives can be seen. (C) Different animal examined by fluorescence microscopy ($\lambda_{excitation}$ = 450−490 nm, $\lambda_{emission}$ = 520+ nm), also through the glass substrate. This oyster is 4 days post settlement. (D) Two oyster spat, ∼2 months old, aggregating together in the first stages of reef construction. (E) SEM image of the whole animal and (F) a close-up of the top shell growth. Inorganic columns with gaps in between can be seen. (**G**) Fluorescence microscopy indicating that organics provide a binder, holding together the inorganic columns. The animals in E, F, and G are shown 7 days after settlement ($\lambda_{excitation}$ = 450−490 nm, $\lambda_{emission}$ = 520+ nm).

adhesive appeared to be more organic. The nature of this material was generally sticky, with diatoms becoming attached occasionally (Figure S3). Although larval adhesive appeared unstructured in the first few hours after deposition (Figure 1C, Figures S1−S3), a highly fibrous structure could be seen in this same material ∼6 months later. Growth on a plastic slide allowed the substrate to be bent for releasing the animal with minimal damage to the adhesive. Figure 1E−G shows a view of the bottom, formerly in contact with the surface. The fibrous structure was produced by several animals of this age (Figure S4).

At 1−2 days after settlement, the animals underwent metamorphosis from a prodissoconch to a dissoconch.[16] Coincident with this transition to juvenile (i.e., spat) was a dramatic change in the oyster's adhesive system. The "stage 1" larval adhesive seen in Figure 1C−G and Figures S1−S3 did not grow any bigger. Rather, a totally new "stage 2" system appeared. Figure 1H−J shows the very first evidence of a new adhesive system emerging from in between the two shells. A pliable, organic material originated from the animal, reached the substrates, and conformed to the surfaces with an adhesive layer.

Beyond the first 1−2 days, animals became larger and deposited more of this stage 2 material (Figure 2A). All subsequent growth occurred at this second adhesive, with the animal no longer adding to the stage 1 glue. Figure 2B−C shows optical and fluorescence microscopy images of juveniles less than a week after settling, viewed from the bottom of glass substrates. Both the stage 1 and stage 2 systems were seen here by SEM (Figure S5). At this point in the growth cycle, the animals aggregated by cementing to each other (Figure 2D, Figure S6). Here, we were able to witness the earliest steps of reef formation.

This stage 2 adhesive differed from stage 1 in both structure and composition. Looking down onto a growing oyster, columns with narrow gaps in between were visible (Figure 2E, F). Fluorescence microscopy showed the presence of an organic binder holding together columns of inorganic material (Figure 2G). Energy-dispersive X-ray scans across this assembly attested to the organic−inorganic composite character (Figure S7). When moving from the inorganic columns to the organic binder, Ca and C decreased while Cl and Na increased. These results indicated that the columns were predominantly $CaCO_3$, whereas the binder was organic and hydrated with seawater. Bradford staining provided evidence that protein was present in the binder (data not shown). Overall, this assembly looked to be simply an oyster shell.[30−33] However, almost every known biological or synthetic adhesive is predominantly organic. A material such as shell comprised of ∼98% $CaCO_3$[29] is not likely able to generate sufficiently strong adhesive contacts. Closer inspection was required to understand how these juvenile oysters can attach in the face of intertidal forces.

With oysters a week or less after settlement, examination of the newest leading edge material (Figure 3A, B) provided distinction from shell (Figure 2E−G). This new growth appeared to have the columns of shell on top. However, an amorphous layer making direct adhesive contact with the substrate was clearly visible. Fluorescence microscopy (Figure 2C) attested to the organic nature of this adhesive material and that curing chemistry was likely present. Much like the stage 1 adhesive seen in Figure 1D, autofluorescence seen here indicates that reactive chemistry, perhaps from organic radicals, is generating conjugated organic compounds. Furthermore, this

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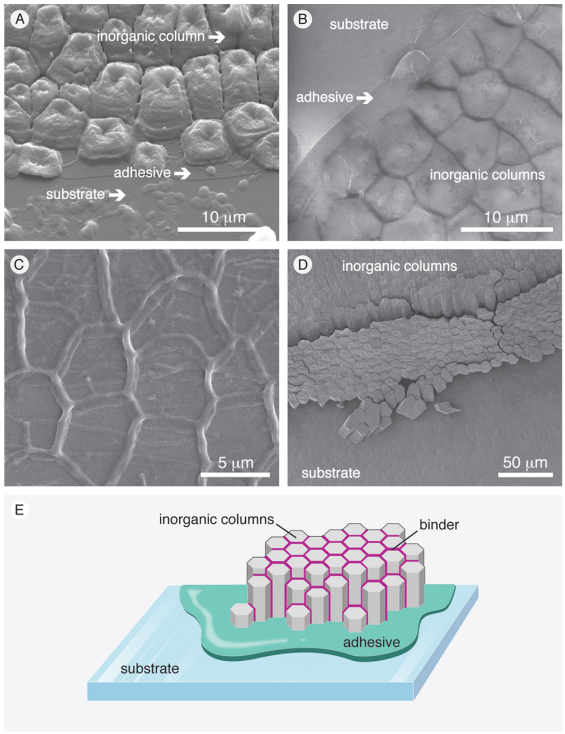

Figure 3. Juvenile oysters creating adhesive contacts. (A, B) Leading edge of spat, depositing new material as they grow. Inorganic columns, looking like shell, are on the top. Immediately below the inorganics are layers of amorphous material making adhesive contact with the surface. These images are from different animals, 2 days post settlement. (C) EDTA etch of a juvenile oyster for leaving behind organics after selective inorganic $CaCO_3$ removal. Organic adhesive at the surface and the binder between $CaCO_3$ columns persisted. (D) Partial bleach etching for selective removal of organics. This treatment often destroyed samples because of the binder and adhesive no longer being present. Here, the inorganic columns can be seen held together only loosely. See image A for a contrast of when the organics were present. (E) Model of how oysters adhere immediately after metamorphosis.

chemical activity did not appear to be distributed evenly across all of the new material. Rather, the fluorescence was strongest where each animal contacted the surface most recently. At ~6 months, the leading edge of growth took on a less structured, but still familiar, appearance (Figure S8). The top, right valve (i.e., shell) was visible and distinct from the lower, left valve. At the substrate, organics were visible as well as inorganics of the lower valve. Oysters always settle with their left valve onto a substrate.[16] Here we were able to observe differentiation of left versus right valves.

Treating these young oysters (e.g., 1 month) with the chelating agent ethylenediaminetetraacetic acid (EDTA) removed inorganic $CaCO_3$ selectively, leaving behind the binder between columns as well as organic adhesive atop the substrate (Figure 3C). With the exception of adhesive remaining at the surface, this image looks much like what happens with analogous chelation of shell.[30,34,35] A different story emerged with bleach etching for digestion of the organics (Figure 3D, Figure S9). Most often, bleach destroyed the samples completely by removing the binder between columns and adhesive at the substrate. The persisting inorganic columns remained occasionally, but these structures did not look to be bound together well, and attachment to the substrate appeared tenuous (Figure 3D). By contrast, the untreated animals of Figure 3A, B seemed to be anchored more robustly. These data provide a model for juvenile oyster attachment in which a layer of organic adhesive provides an interface between the substrate and left valve (Figure 3E). The common view of shell with $CaCO_3$ columns tied together by organics persists.[30,34,35] This shell is then attached to the surface via a flowing, pliable, organic material present for maximizing interfacial contacts.

The animals continued to grow larger and deposit more material. When ~6 months old, they became big enough to allow cutting of complete cross sections (Figure 4A). Such

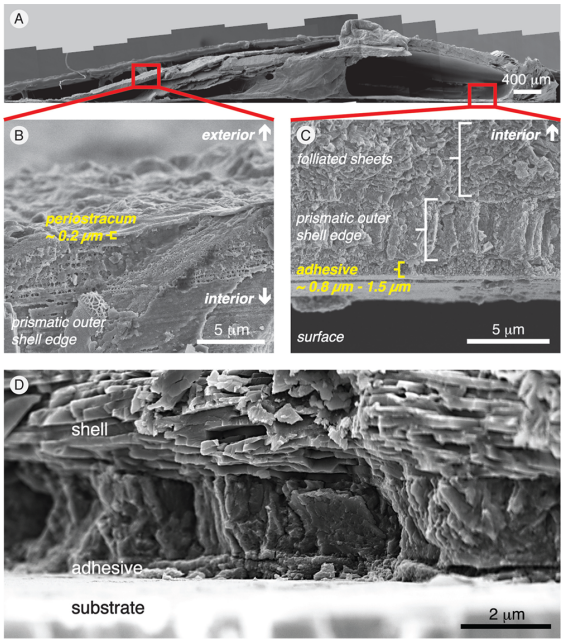

Figure 4. Adhesion of ~6 month old oysters. (A) Animal cut into a cross-section. This picture is a composite of several SEM images. (B) Close-up of the top, thick shell with a thin periostracum coating. (C) Bottom of the animal at the surface, showing that the adhesive layer is significantly thicker than the periostracum of the top shell in frame B. (D) Interface between shell and substrate of another animal. This sample was prepared by fracturing the animal and substrate.

samples permitted examination of the top, right valve in direct contrast to the surface contacting left, bottom valve. In general, the upper shells were found to be thicker, whereas the lower shells closest to the surface were thinner. The top shell was covered with a very thin periostracum coating, typically only ~0.2 μm (Figure 4B). This organic barrier is particularly narrow for oysters relative to other shellfish.[15] Another layer was observed beneath the animal, often of ~1 μm or greater thickness. This underlayer was always more substantial than the right valve periostracum (Figure 4C). Fluorescence microscopy of spat cross sections showed strong emission in the space between shell and surface, further indicating the presence of organics providing interfacial binding (Figure S10). Looking underneath the animal here also yielded contrasts in shell, adhesive, and the substrate (Figure 4D). The crystalline, foliated sheet structure of shell[33] was observed. Intermediate

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between this shell and the surface was an adhesive layer, devoid of any conspicuous structure. With the animal being older, the adhesive was more mineralized. Further growth into adulthood was consistent with prior findings of an unstructured adhesive layer bonding shell to substrate.[18−20]

Results reported here indicate that the adhesive is a distinct part of the animal and not simply periostracum or shell. Regular shell plus thin periostracum of the animal's thicker top valve differed significantly from the lower valve, which comprised a thick organic layer residing between substrate and thin shell. We can now see how oysters differentiate one side from another with regards to shells and binding atop surfaces.

These observations provide our first comprehensive view of how oysters mature throughout their lives with regard to adhesion. The nature of their glue changes from initial settlement, on to juvenile adhesion, and then into adulthood. An amorphous, organic, hydrated material sticks larvae to surfaces. After metamorphosis, the animals switch over to an organic−inorganic composite adhesive system. The structure is defined by inorganic columns atop a thick, organic, underlying layer of glue. This adhesive contrasts with shell in being more organic and lacking microstructure. Growth of full-sized adults and their extensive reef structures then builds upon the materials seen here. By describing oyster adhesion through all major life stages, we hope to provide insights to those working in adhesive design, aquaculture, and reef restoration.

MATERIALS AND METHODS

Culturing of Oysters. About 150 000−200 000 pediveliger larvae of Eastern oysters (*Crassostrea virginica*) were obtained several times, over multiple spawning seasons and years from the Virginia Institute of Marine Sciences and Louisiana State University. Upon arrival in the laboratory, larvae were acclimated in aged seawater (18 parts per thousand salinity) for 15−30 min prior to transferring into an aerated 10 gallon glass aquarium at the same salinity, maintained at room temperature. Beds of plastic (vinyl, Rinzl) and glass (Thermo Scientific Gold Seal) slides were placed at the bottom of the aquaria for larval settlement. Oysters where fed daily with an algae mixture (Shellfish Diet 1800) and water changes performed every 3 days to ensure growth into spat. After 48 h, most larvae were in the crawling-settling stage (see Video S1).

Instrumentation. Optical and fluorescence microscopies were carried out on an Olympus BX51 with USH-102DH and USHIO lamps as well as an Olympus DP71 CCD camera. Wavelengths for the filters used to change the excitation and emission wavelengths are provided in the figure captions.

An FEI Quanta 3D FEG dual-beam scanning electron microscope (SEM) as well as an FEI Nova NanoSEM, both with Everhart-Thornley and through-the-lens detectors (TLD), were used. Typical parameters included 5−20 kV accelerating voltages and 4.5−10 μm working distances. Oysters were covered with platinum via a sputter coater prior to imaging.

Energy-dispersive X-ray (EDX) spectroscopy was accomplished with an Oxford INCA Xstream-2 on a Quanta 3D FEG microscope. For EDX parameters, 20 kV, 50 μm objective aperture, and 100 s of collection time were used most often. Oxford AZtecEnergy EDS software was employed for data analyses.

Etching. In order to differentiate between organic and inorganic materials, EDTA and bleach etching was performed. For removal of the inorganic material, a solution of 10% EDTA at pH 7.4 was used. These oysters were treated for 1 week. To remove the organics, we made diluted solutions of commercial bleach. Overnight treatment resulted in complete removal of samples from the slides. Thus, shorter incubation periods of 1 h or less were used for allowing material to persist.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b01305.

Electron microscopy images and energy-dispersive X-ray data (PDF)

Video S1, larval oysters swimming, probing the surface, and depositing glue (AVI)

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Notes

The authors declare no competing financial interest.

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