New Methods for Imaging and Quantifying Dissolution of Pteropods to Monitor the Impacts of Ocean Acidification

A Thesis

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by

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ABSTRACT

Large-scale changes in climate and ocean ecosystems demand innovative and cost-effective ways to track changes in the marine environment and its living resources. During the past decade, ocean acidification has become recognized as a major threat to the biodiversity of marine ecosystems during the 21st century. However, an important constraint on modern ocean acidification research is the lack of accessibility to effective imaging techniques, as well as accurate analytical methods. Here, we compare several different microscopic techniques to evaluate the relative merits of each. Additionally, a new dissolution quantification method is developed that more completely assesses damage over an entire shell. These findings can help expand the toolbox for scientists engaged in studying the impacts of ocean acidification on marine invertebrates and enable more researchers to participate in this vital field.

BIOGRAPHICAL SKETCH

Sage was born and raised in Wharton, NJ and attended Morris Hills High School, where he found his interest in marine biology while taking several biology classes and working as a student teacher for Biology 1 during his senior year. He graduated in 2011 and started at Stockton University in Galloway, NJ that same year, majoring in Marine Science. During his undergraduate career, Sage interned at several institutions including the American Museum of Natural History in Manhattan, NY and the Rutgers University Marine Filed Station in Tuckerton, NJ. Sage was also a 2013 NOAA Ernest F. Hollings Scholar, where he worked at the Northwest Fisheries Science Center in Seattle, WA studying the contaminant toxicology of bottlenose dolphin mothers and calves. Sage graduated from Stockton University in 2015 with dual Bachelor of Science degrees in Marine Science and Biology and academic honors in each, and a minor in Computer Science. His baccalaureate thesis focused on the effect of external electrochemical charge on gene transcriptomes of deep-sea corals, with the goal of using genetic influence to help expedite reef recovery. In 2016, Sage attended Cornell University as a Master of Science student in the department of Earth & Atmospheric Sciences with a research focus in biological oceanography, while also serving as a graduate teaching assistant for Cornell's Introductory Oceanography course.

Sage currently lives in Ithaca, NY with his fiancée Diana and their cat, Waffles.

This thesis is dedicated to my mom Eileen, my dad Sage, and my fiancée Diana

for always encouraging my curiosity and my love for the ocean.

You have made me a better scientist and a better person. Thank you, I love you.

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LIST OF ABBREVIATIONS

OA ..……. Ocean Acidification

pH ……. Potential Hydrogen

CO_2 ……..…… Carbon Dioxide

CCE ……..…… California Current Ecosystem

$CaCO_3$ ……..……. Calcium Carbonate

EtOH …….……. Ethyl Alcohol

DI H_2O …….……..Deionized Water

CORALS ……... Cornell Ocean Research Apprenticeship for Lynch Scholars

SEM ……...……...…….. Scanning Electron Microscope

CT …..…… Computed Tomography

ANOVA ……..… Analysis of Variance

LIST OF SYMBOLS

Ω (Omega) …….…….. Aragonite saturation

INTRODUCTION

Ocean acidification (OA) is a potentially devastating global phenomenon that has locally variable biological, ecological, and chemical impacts. As anthropogenic emissions of carbon dioxide (CO_2) into the atmosphere increase, the ocean takes up some of the excess to achieve equilibrium, ultimately absorbing approximately one-third of all emissions (Doney et al. 2009, Bednaršek et al. 2014). These anthropogenic emissions have accounted for an increase of over 40% in atmospheric CO_2 concentrations since the Industrial Revolution (Bednaršek et al. 2014). CO_2 in the ocean undergoes hydrolysis to produce carbonic acid, which dissociates into bicarbonate, carbonate, and hydrogen ions (Orr et al. 2005). As the concentration of hydrogen ions increases (i.e., as pH decreases), the concentration of carbonate ions decreases, leading to dissolution of calcium carbonate ($CaCO_3$), a compound many organisms use to build their shells (Doney et al. 2009; Bednaršek et al. 2014). This change in seawater chemistry favoring the dissolution of $CaCO_3$ is depicted by the following series of equations:

$$CO_2 \text{ (atmospheric)} \rightleftharpoons CO_2 \text{ (aqueous)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$$

$$CaCO_3 \rightleftharpoons CO_3^{2-} + Ca^{2+}$$

While seawater is generally supersaturated with respect to $CaCO_3$ content, the saturation state is declining. This is highly pertinent to the shelf waters of the California Current Ecosystem (CCE), a highly productive coastal upwelling zone crucial for a number of ecologically and commercially important species (McClatchie 2014). Recent measurements of CO_2 in the CCE have found that CO_2 concentrations at 20 miles off the coast of California have reached approximately 405ppm (Ziese 2018, Sutton et al. 2011) (*Figure 1*). These levels, in addition

to the naturally higher CO_2 concentrations of deep-water upwelling to the surface, create unusually corrosive conditions for the local invertebrate fauna (Gruber et al. 2012).

The impacts of OA can lead to dynamical changes at the ecosystem level, affecting biogeochemical cycles, food web interactions, and ecosystem health, as well as fitness changes at the organismal level, affecting development, growth, and tolerance to other environmental stressors. During the past decade, ocean acidification has become recognized as a major threat to the biodiversity of marine ecosystems during the 21st century (Doney et al. 2009). Increased OA has caused a wide range of biological impacts, including the dissolution of calcareous structures in many marine invertebrates. The recent impacts of OA are unprecedented since the Late Pleistocene as calcifying organisms have not experienced the current level of undersaturation for at least the past 400,000 years (Pearson et al. 1999, Orr et al. 2005). Of particular concern are a group of marine planktonic snails collectively called pteropods (subgroup *pteropoda*, also known as "sea butterflies"); these organisms play a vital role in nutrient fluxes and trophic energy transfer of marine ecosystems in subpolar and polar regions including the California Current Ecosystem (CCE) (Bednaršek et al. 2014). Furthermore, they play a critical role in the carbon cycle; their calcified shells and carbon-rich fecal pellets sink into the deep ocean, thereby sequestering carbon at depths already susceptible to lower pH (Bergan et al. 2017).

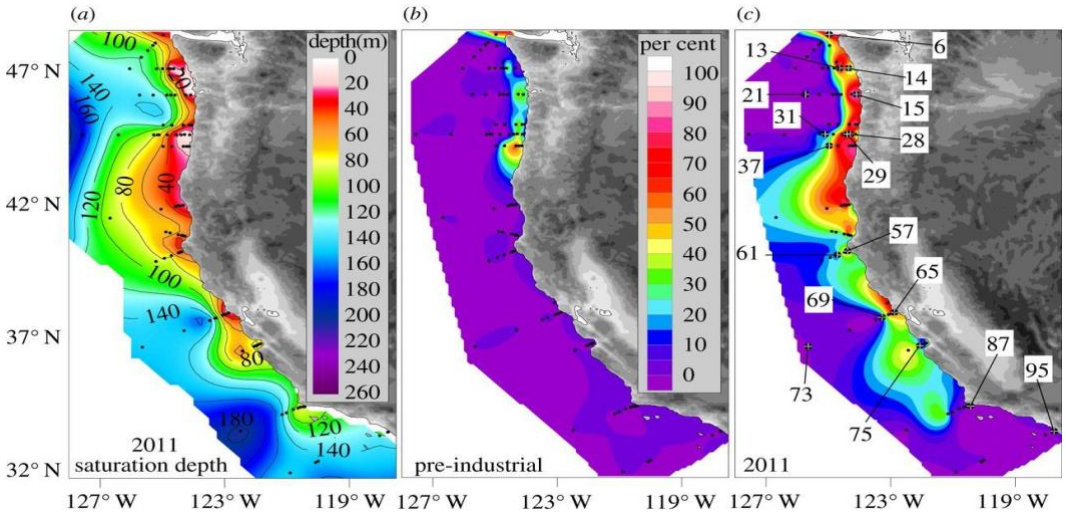

Figure 3: Planview maps. (a) Depth of the aragonite saturation horizon along the US West Coast. (b) Percent of upper 100 m of the water column in the CCE estimated to be undersaturated during the (b) pre-industrial time, and (c) the August–September 2011 time period. Pteropod station locations are indicated by numbers within the squares. From Bednaršek et al. (2014).

Pteropods are increasingly recognized as an important bioindicator of ocean acidification and are often referred to as the "poster child" of OA in temperate and subpolar climates. Their aragonite shells are more soluble than the calcite shells of many other calcifying organisms, making pteropods particularly sensitive to changes in ocean chemistry (Bednaršek et al. 2012a). Affected individuals show signs of dissolution and increasing opacity of their fragile shells. Generally, the first whorl of the pteropod shell is most affected by dissolution because it is composed of the oldest shell material. Bednaršek et al. (2012) have classified the extent of dissolution into four severity types that are useful for categorizing the effects of OA.

Scanning electron microscopic (SEM) techniques for observing and classifying levels of dissolution in pteropod shells have been successfully developed (Bednaršek et al. 2012). Using SEM, scientists are able to specify the locations of dissolution and record their observations in detail. However, a primary drawback of this method is cost. The cost of a

user-friendly JEOL SEM can reach as much as $90,000. Therefore, the accessibility of affordable SEM instruments is limited, and where common-use SEM facilities are available, training costs, supply costs, and user fees can be prohibitively expensive for many researchers, especially students (*Table 1*). These costs, combined with the relatively low number of institutions that own SEM machines, greatly restrict the possible number of researchers and potential amount of research that can be done in this vitally important field.

*Table 1: Sample comparison of SEM user fees of scanning electron microscopes across several American universities. Fees are taken from the respective university imaging laboratory websites. * = costs include operator/assistance fees.*

Institution	**SEM cost/hour ($)**
Cornell University	*63.50*
Oklahoma State University	55.00*
San Francisco State University	65.00
University of New Hampshire	88.00*
University of Virginia	110.00*

This study explores and compares the effectiveness of two alternative microscopic analytical methods: high-power light microscopy and computed tomography (CT) scanning for assessing the damage of OA on shells of the subpolar pteropod species *Limacina helicina*. By comparing cost and accessibility of each instrument with the final quality of the image data, we present alternative options for viewing OA damage. Light microscopy is less expensive and therefore more accessible than SEM; however, it is important to evaluate whether the resulting images retain sufficient resolution to classify the three dissolution severity types previously documented with SEM for pteropods. CT scanning is more expensive and

therefore less accessible than SEM; however, it is important to evaluate the potential benefits of the resulting three-dimensional imaging data.

Additionally, Bednaršek et al. (2017) developed a monitoring protocol to assess the extent of ocean acidification by classifying pteropod dissolution into three types of varying severity. This current classification method for shell dissolution, while valuable for assessing populations, does not report on the entirety of shell dissolution coverage, nor has any previous research been done on the relative spatial extent or proportion of the different types of dissolution present on the shells of pteropods. Hence, based on the work of myself and the 2019 Cornell Ocean Research Apprenticeship for Lynch Scholars (CORALS) Program, this study proposes a new method for quantifying each type of dissolution based on average percent surface area coverage, but remaining consistent with standard categorization of shells based on severity types.

MATERIALS AND METHODS

Sample Cleaning and Preparation:

Limacina helicina samples were collected off the coast of Baja California and Mexico by Dr. Nina Bednaršek of the Southern California Coastal Water Research Project (SCCWRP). After collection, the samples were transported to The Harvell Laboratory at Cornell University for cleaning. Samples were cleaned by a multistep DI H_2O and bleach bath: samples were first removed from their 70% buffered ethyl alcohol (EtOH) storage and rinsed with two baths of DI H_2O, each three minutes long. Pteropods were then put in a 6% bleach solution for 90 minutes followed by another three baths in DI H_2O, each 30 seconds long. Samples were checked under a compound microscope after 90 minutes; if the shells appeared to retain some of their organic layer, they were placed in an additional 30-minute bleach bath. Finally, samples were exposed to two more rinses in DI H_2O. This cleaning process softened the body of the organism and flushed out any organic matter on the surface of the shell while preserving the integrity of the calcareous shell.

MICROSCOPY METHODS COMPARISON

Light Microscopy:

All samples were imaged using a Zeiss Stemi 2000 Stereo Microscope. Using a Moticam 3200 color camera at 5x zoom, images of each pteropod were captured at varying focal lengths. Images were taken in two separate ways, with lighting from behind and on a dark background with lighting on the front of the shell. All images were captured in a dark room

with very little ambient light. Once all images were captured, they were assessed for dissolution type and then imaged via CAT scanning and SEM.

Computed Tomography Scanning:

After examining shells under the light microscope, all samples were prepared for analysis via Bruker SkyScan 1173 micro computed tomography (CT) scanning. First, samples were packed inside a 1.5mL vial in layers organized by collection and separated by layers of foam; this ensured that shells would remain stationary during scanning without being crushed in the vial. After packing, vials were wrapped in cellophane for added protection and support and placed in the CT scanner. Scans were conducted at a resolution of 7µm for a duration of three hours per scan. After completion, scans were reconstructed and analyzed using CTVox software (*Figure 2*).

Scanning Electron Microscopy:

After analysis by light microscopy and CT scanning, all samples were examined using a JEOL Neoscope 4500 SEM. First, samples were mounted onto 12.5mm SEM specimen pin stubs, grouped by collection vial, and placed into a gold sputter coater. The sputter coater chamber was first pressure flushed at intervals between 0.4bar and atmospheric pressure to eliminate any remaining outside contamination prior to setting the desired pressure of 0.8bar. Once at this pressure, samples were coated in gold dust for two minutes to achieve even coating. Once placed in the SEM, images were taken of each shell following a protocol: (1) full-body image, (2) image of only the first whorl of the shell at 1000X magnification, (3) image at the shell's growing edge, and (4) additional images taken at all locations of dissolution and at the smallest possible magnification while retaining image sharpness.

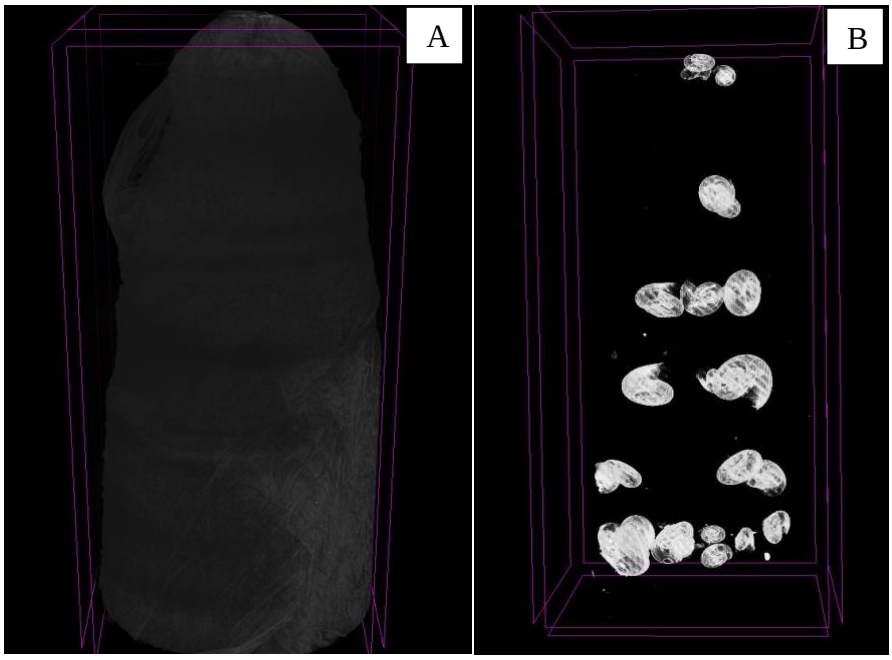

Figure 4: Reconstruction of CT scan data showing the 1.5mL vial wrapped in cellophane (A), and after altering density contrast to expose shells within the vial (B).

DISSOLUTION QUANTIFICATION

Pteropod Imaging:

Once pteropods were properly prepared, 15 individuals were subsampled and analyzed over the course of three days by myself and six different CORALS researchers using the SEM at Friday Harbor Laboratories. All researchers followed the imaging identical to that used for comparison of microscopy methods, described above. If samples were broken or unviable, only a single full body image was captured.

Classification:

Samples were first classified by the currently accepted typing method in order to establish a basis for comparison with the new method; all researchers agreed to the definition of each dissolution type as followed in Bednaršek et al. (2017). Type 0 indicates a shell with an

inferred exposure to an aragonite saturation greater than 1.3 ($\Omega > 1.3$) with no visible dissolution signs and high calcification. Type 1 dissolution represents inferred exposure to an aragonite saturation of $1.1 < \Omega < 1.3$ with visible signs of dissolution exposing the prismatic layer of the shell. Type 2 dissolution represents inferred exposure to an aragonite saturation of $1.1 < \Omega < 0.9$ with visible dissolution exposing the inner crossed-lamellar shell layer with minimal calcification. Finally, Type 3 dissolution represents inferred exposure to an aragonite saturation of $\Omega < 0.9$ and extreme dissolution signs through crossed-lamellar layer with notably thicker and visibly deformed aragonite crystals (*Figure 3*). The current method may be described as a "whole-shell" method, i.e. each shell is classified by the most severe type of dissolution detected and is only given one singular type for the entire shell.

After classifying each shell via the currently accepted method, a new method, dubbed the "surface area" method, for assessing percent cover of each dissolution type was devised. As there is currently no imaging software available to assess percent cover of pteropod shell dissolution, an average estimate of percent area of each dissolution type as recorded by each student researcher was used. Each researcher examined the images taken of each pteropod individual per mount and then estimated the percent cover of each dissolution type in order from Type 3, Type 2, Type 1, and Type 0. Percentages were estimated to the nearest whole integer from 0-5%. If estimates exceeded 5% cover, they were rounded to the nearest 5% (5%, 10%, 15%, 20%...). Once Type 3, Type 2 and Type 1 cover was assessed, Type 0 percent cover was assumed to be the remaining shell cover and thus every individual estimate totaled to 100% cover. Then to account for variation in human bias, the average estimate of

each type for each individual pteropod was calculated independently by the seven student researchers.

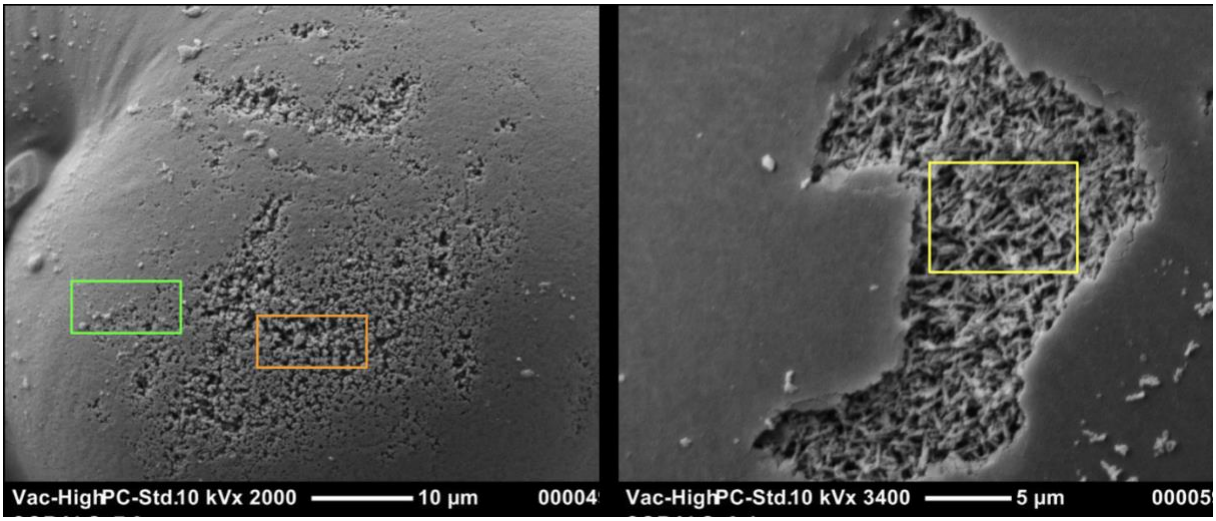

Figure 3: Comparison of dissolution types 1 (green), 2 (orange), and 3 (yellow), across the same pteropod shell. Detecting different dissolution types within the same individual allows for clearer differentiation of the types and the physical damage each causes.

Statistical Methods:

All graphs and statistical tests were done using Microsoft Excel. Standard error for average percent surface area coverage was calculated, and an Analysis of Variance (ANOVA) test was run on the percent surface areas for each dissolution type (method 2).

RESULTS

MICROSCOPY METHODS COMPARISON:

Light Microscopy versus SEM:

Analysis of images taken via light microscopy and SEM showed that while dissolution was detectable in light microscopic images, determination of the specific type of dissolution was impossible at the resolution used. In nearly all samples, dissolution was detectable as a collection of blotchy, discolored patches, which strongly correlated to detected dissolution under SEM (*Figure 4*). This indicates that a binary detection of dissolution is possible via light microscopy alone, though higher resolution microscopy would be needed to determine type of dissolution.

Although light microscopy did not resolve the type of dissolution that SEM revealed, time to image samples was drastically lower. Time taken to image all samples by light microscope averaged approximately 30 minutes per six pteropods, whereas the same samples took approximately two hours via SEM, resulting in a 300% increase in time. Additionally, light microscopy using the Zeiss Stemi 2000 stereo microscope was significantly less expensive than the LEO 1550 FE SEM, both in per-hour costs and base expense of each machine.

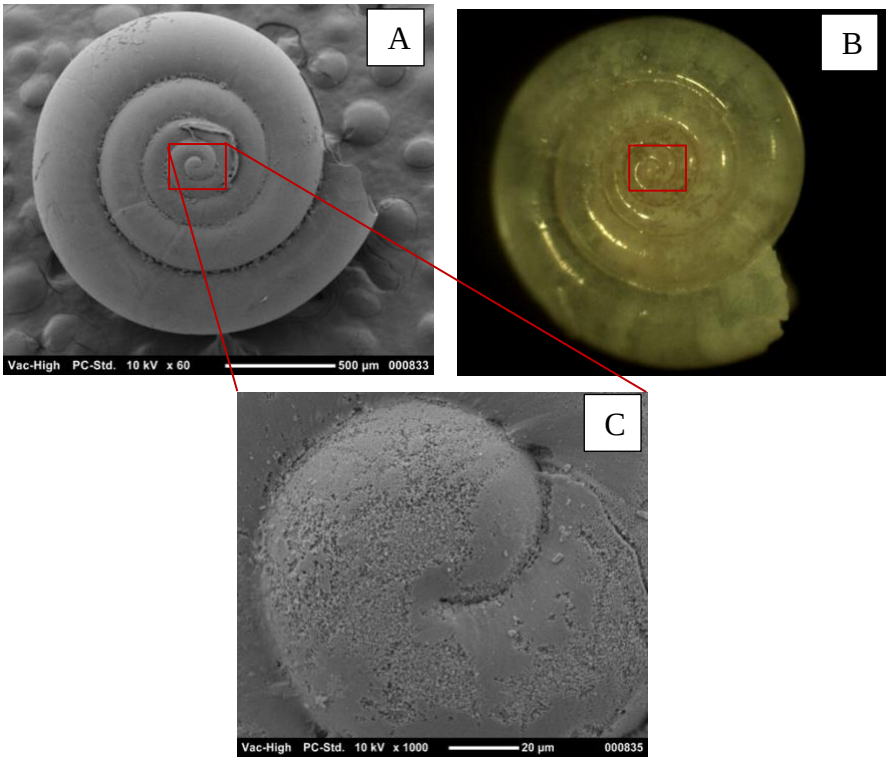

Figure 4: Comparison of SEM and light microscopy images of individual 7,2 (A and B). Although dissolution is detectable in both SEM and light microscope images, specific dissolution type at the first whorl (C) is indistinguishable with only light microscopy.

Light Microscopy versus CT Scanning:

After reconstruction, CT scan images created a three-dimensional model of the vial containing the shells, which were exposed through the cellophane layer after altering the density contrast via CTVox (*Figure 5*). Creating a 3-D image of the shells allowed us to examine an additional metric of depth penetration into each shell, which is vital in determining overall damage to each shell.

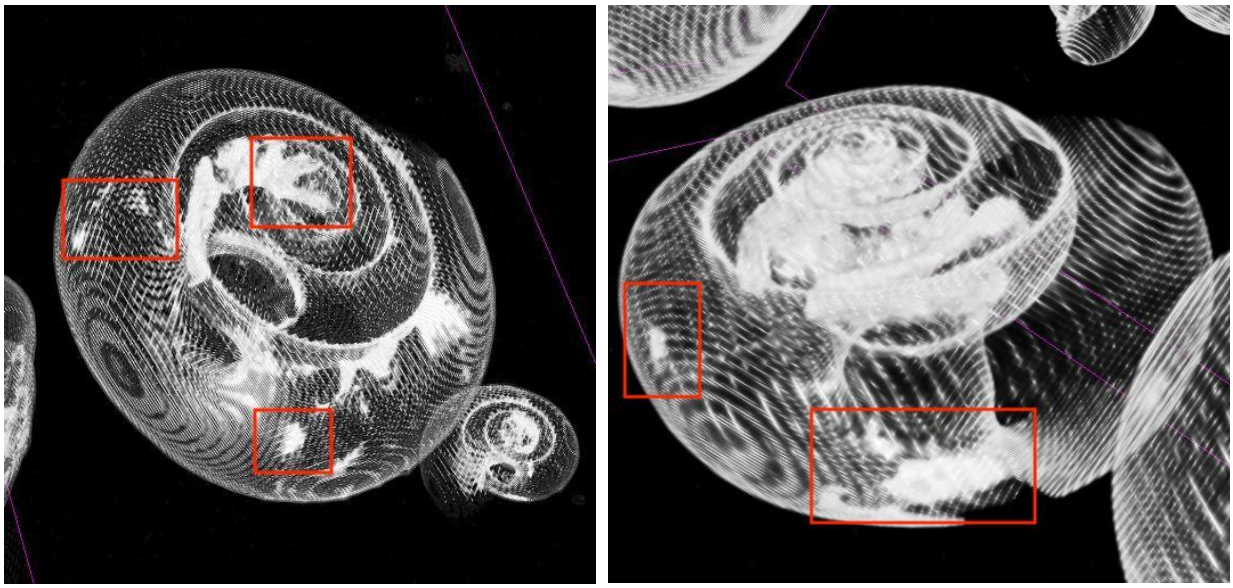

Figure 5: CT scan results showed visible patches of dissolution on 3-D rendered models of shells. These white patches correlated to areas of dissolution detected by other microscopic methods.

CT scans allowed for an additional metric of assessing how deep into a shell OA had penetrated. Opaque, white spots on CT images correlated to areas of dissolution seen via light microscopy and confirmed via SEM (*Figure 5*). Additionally, these spots of dissolution were visible in the shells' inner layers, penetrating the outer layer (*Figure 6).* As with light microscopy, determination of the type of dissolution detected was impossible via CT scanning, although confirmation of dissolution presence was clear. Time to scan samples was significantly higher than light microscopy, taking approximately three hours to scan each vial. However, this process is passive, allowing researchers to examine other samples by light microscopy or SEM while the CT scanner is running. In this sense, CT scans proved more efficient, creating a "conveyer belt" of analyzing completed scans while the other procedures were in progress.

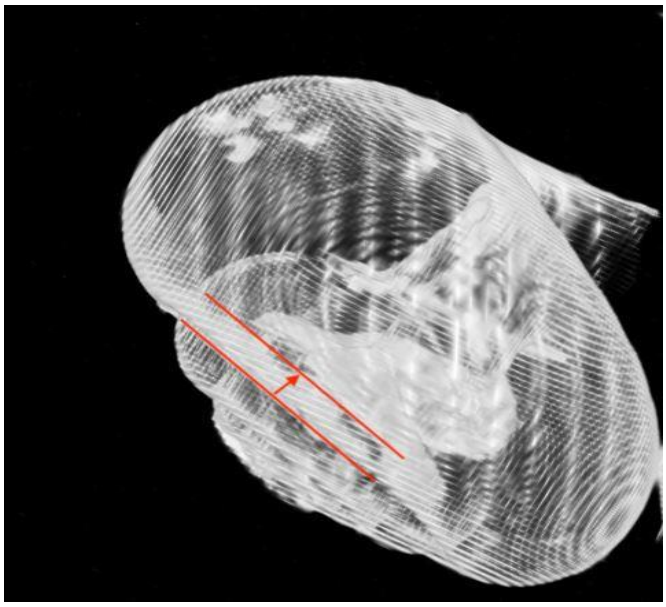

Figure 6: By creating 3-D renderings of shell samples, depth of erosion into the shell could be seen. Dissolution penetrated shells' outer layers to a maximum average depth of several microns.

DISSOLUTION QUANTIFICATION

Method 1- the "whole shell" method:

Results using the current "whole-shell" typing method are based on the consensus of the seven student researchers who agreed on the maximum extent of dissolution based on SEM images. More than half of the shells (53%) are classified as Type 3, three (20%) of the shells are classified under Type 2, and only two shells are classified under Type 1 (13%) and Type 0 (13%), respectively (*Figure 7)*. Based on this qualitative analysis of pteropod shells, more than half of the shells analyzed were experiencing severe levels of dissolution (type 2 or 3), and nearly all (86.6%) of them were experiencing some level of dissolution.

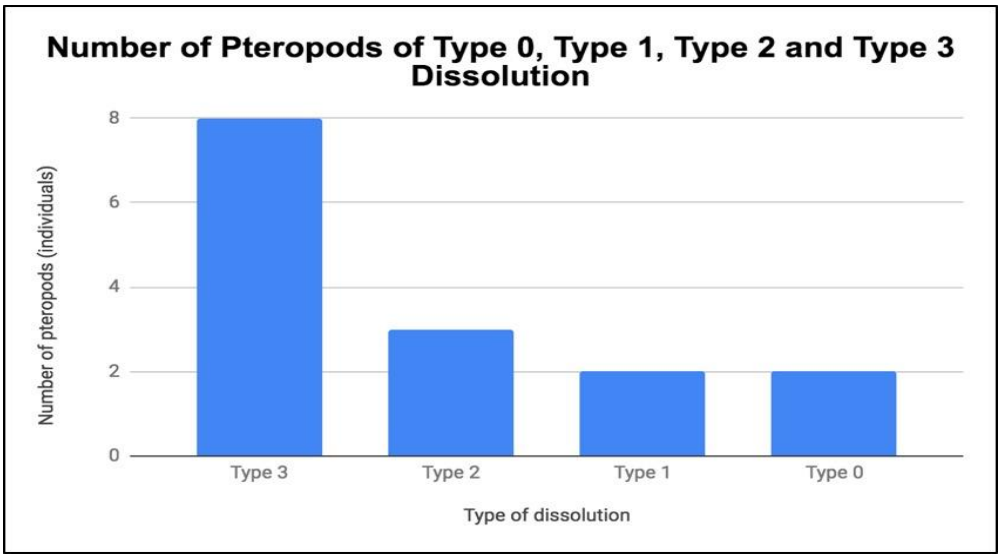

Figure 7: Distribution of subsampled pteropod shell types via method 1 ("whole shell"). Based on whole-shell classification, shells appear to exhibit severe dissolution.

Method 2 – the "Surface Area" method:

Using a percent surface area analysis of each shell showed that, contrary to results reported by the "whole shell" method, the vast majority of pteropod individuals have an intact, Type 0 shell. Hence, the highest average percent surface area of dissolution observed was Type 0 for all individuals, with a percent area of Type 0 ranging from 88% to 100%. There was a significantly lower difference in average percent area of the remaining dissolution types (*Figure 8A*), with Type 1 ranging from 0 to 5.29%, Type 2 ranging from 0 to 5%, and Type 3 ranging from 0 to 6.86% (outlier in individual 1,6 - removing this outlier would result in a 0 to 3% area of dissolution). The ANOVA in Table 2 (*Appendix*) confirms that the differences in percent area of dissolution between each of the dissolution categories are statistically significant, and the standard error is minimal across all individuals

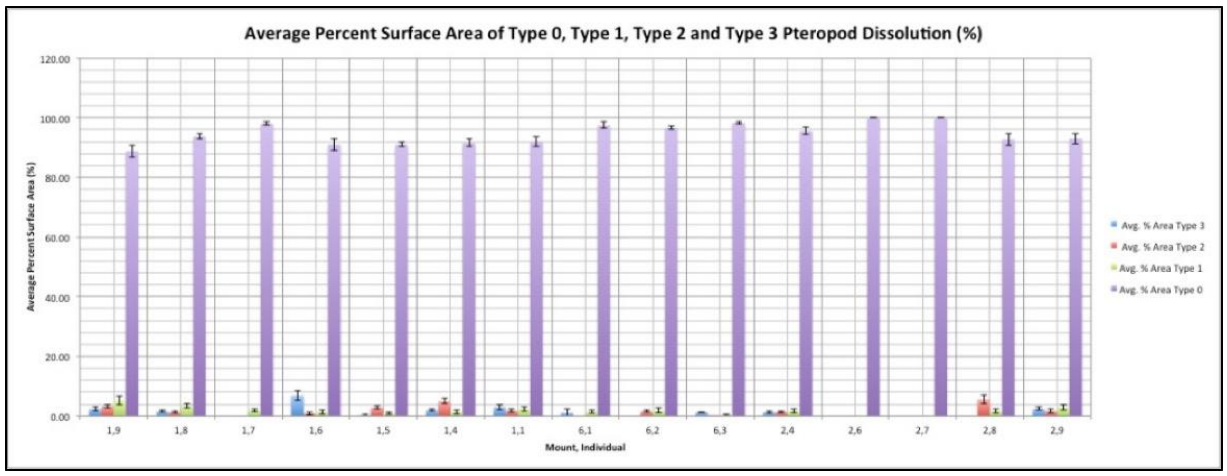

Figure 8A: Average percent surface area of Type 0, Type 1, Type 2 and Type 3 pteropod dissolution for each individual, based on classification by percent surface area coverage.

Removing Type 0 from the surface area averages allowed for a more effective visualization of the variance in actual dissolution across all individuals (*Figure 8B*). After removing Type 0, individuals 2,6 and 2,7 are anomalies as they are 100% Type 0 (no dissolution). Removing Type 0 from the percent analysis also confirmed a low proportion of individuals with Type 3 dissolution relative to the proportion of Type 1 and Type 2 dissolution, with the exception of individuals 1,6 and 6,3, which experienced a relatively higher proportion of Type 3 dissolution.

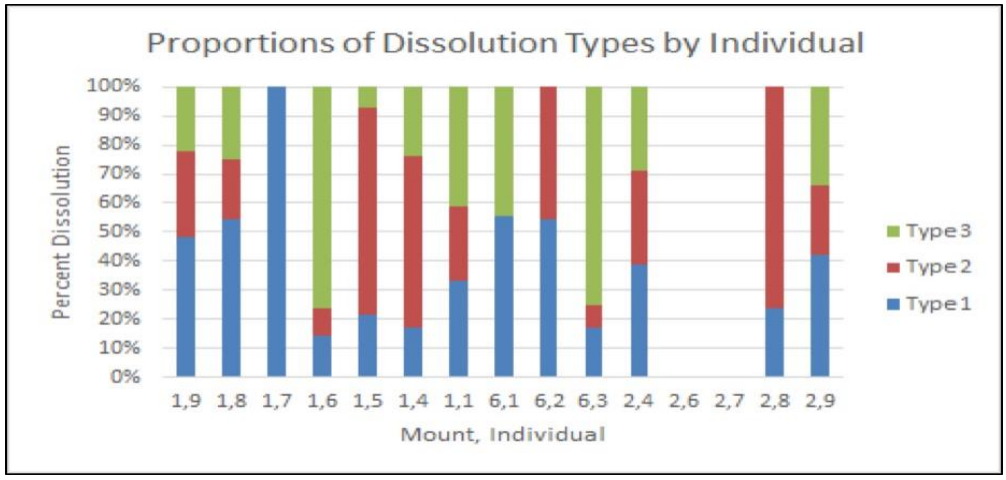

Figure 8B: Average percent surface area of Type 1, Type 2 and Type 3 (removing Type 0) dissolution for each individual as a proportion of the total dissolution area. Removing Type 0 showed that a majority of dissolution detected fell into lower severity types.

DISCUSSION

Analysis of Microscopic Methods

Evaluation of the different microscopic methods revealed a stark contrast between sharpness of image resolution and amount of time and funds needed to produce images. While light microscopy proved to take approximately 25% of the time to image the same number of pteropods, image sharpness was much lower and could not achieve the same resolution as SEM. This resulted in an inability to classify the specific type of dissolution for each shell with light microscopy in comparison to SEM. However, based on the lower cost-per-hour use of the light microscope and the strong correlation of dissolution presence in light versus SEM images, this method may prove to be sufficient for sample analysis if the objective is a binary determination of whether or not dissolution is present. Using light microscopy to simply determine whether or not dissolution is present on a sample may save researchers time and expense in comparison to the investment required in use of SEM. However, if the objective is to quantify the frequency and incidence of different types of dissolution, than the use of SEM will yield more accurate and reliable results.

Analyzing pteropods using CT scanning provides an additional metric for assessing shell dissolution damage by creating a three-dimensional image, allowing researchers to see how far deep into a particular shell OA has eroded away. Using a 3-D depth metric for erosions analysis can provide a valuable supplement to the established method of assessing surface area coverage of a shell while simultaneously determining the risk of OA dissolution penetrating into the actual body of the organism. Additionally, CT scanning allows for multiple shells to be scanned simultaneously, as samples can be packed into CT tubes that can

hold potentially several dozen shells. Using CT scanning to analyze erosion depth shows that the threat of OA is not only detrimental to a major portion of the shell surface area, but also does significant damage through the entire structure of the shell. However, drawbacks of CT scanning are similar to SEM; both machines require much higher financial input and specialized training, including use of the software required to reconstruct and manipulate CT scans. Nevertheless, CT scanning and SEM both offer greater benefits with their superior image resolution and ability to view samples in three dimensions. Consequently, the decision regarding which microscopic method to use depends on the needs of the researcher and available funding and equipment. The benefit of this comparison lies in the new, wider range of options and potential results available from each method.

Comparison of Dissolution Classification Methods

Results from the implementation of a percent surface-area coverage method suggest that using only the current "whole -shell" method may result in overestimation of the sample's overall dissolution severity. Additionally, these results indicate that classifying entire samples based only on the most severe dissolution found on each shell may result in inaccurate quantification, especially of lower-severity types. The newly proposed method is more quantitative and recognizes that significant percentages of each shell actually remain healthy; for example, in observed samples, only 14% of total surface area showed any dissolution beyond Type 0. However, despite its drawbacks, the "whole -shell" method does retain certain merits. This method is much more time efficient for analyzing large quantities of pteropods, thus making it more efficient for rapidly assessing samples with large numbers of pteropods. For example, a single tow may yield several hundred pteropods, which, without

automated image analysis, could potentially take several weeks to analyze via the surface-area method.

Classifying each shell by percent coverage of each type of dissolution is a more accurate approach in comparison to classifying each solely on the basis of the most intense dissolution type present. The “surface -area” method suggests an overall, relatively low exposure of sampled pteropods to dissolution by ocean acidification in the CCE. The use of the “surface-area” method in relatively more aragonite saturated waters and therefore a lower overall severity of dissolution may be more useful than the “whole -shell” method, which may overestimate dissolution severity in a population. However, this new method may prove less efficient when sampling large numbers of shells in regions where much higher OA levels and more severe dissolution are expected, such as the subpolar and polar seas. Therefore, as both methods offer solutions to the other’s drawbacks, we conclude that using a combination of both methods yields the most complete and accurate dataset while still preserving time and financial resources.

FUTURE WORK

The strengths of the methods presented in this study lie not only in their usefulness and accessibility, but also in their potential for ongoing improvement and expansion into research in other locations and with other target species.

Potential future studies could also examine not only the physical damage of the shell, but also the biological health of the organism within. Although the results showed that a small percentage of each individual shell surface is affected, the biological effects on the organism remain undetected and may be more severe than the outer shell layer indicates. Understanding how this mechanical damage translates into functional impacts on things such as reproduction, mobility, and feeding, is critical to our understanding of the greater ecological impacts of OA on the physiology of calcifying marine invertebrates. For example, although research on the potential relationship between mortality in juvenile pteropods due to dissolution and aragonite unsaturation is lacking, we would still expect veligers and larvae to be most susceptible (Bednaršek et al. 2014). This is because signs of dissolution can appear on larval shells within two weeks of exposure to acidic waters (Bednaršek et al. 2014), thereby affecting a weak and developing invertebrate immune system. Moreover, shell dissolution at the reproductive stage may hinder the exchange and storage of gametes and hence compromise reproductive capacity of the individual (Bednaršek et al. 2014).

The development of new imaging and monitoring strategies is valuable for pteropod research; however, a greater benefit will be realized as these methods are applied to other key

invertebrate species in other regions around the world. As many marine invertebrates experience a calcifying stage either for their entire life cycle or during some point in their development, these same methodologies can be applied to other species that hold significant ecological or socioeconomic impact in their respective local ecosystems. Applying these monitoring strategies to other vulnerable locations and other vital invertebrate species can lead to the development of an OA monitoring network. It is hoped that further development and wider implementation of these methods, combined with the creation of future invertebrate-based monitoring networks, will contribute greatly to our understanding and mitigation of OA and its impacts on marine ecosystems.

APPENDIX

Table 2: ANOVA table for method 2 (surface area method)

ANOVA: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Avg. % Area Type 3	15	22.3	1.486666667	3.278109524		
Avg. % Area Type 2	15	25.58	1.705333333	3.194498095		
Avg. % Area Type 1	15	26.73	1.782	1.921888571		
Avg. % Area Type 0	15	1419.56	94.63733333	12.84164952		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	97258.71438	3	32419.57146	6106.488794	0	2.769430949
Within Groups	297.30604	56	5.309036429			
Total	97556.02042	59				

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