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Validation of Pilot Protocol: Damage Scoring of Puget Sound Mollusk Shells

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Abstract:	The Marine Sediment Monitoring Team at the Washington State Department of Ecology observed damage to mollusk shells while studying the benthic communities of Puget Sound. A pilot protocol was written to allow researchers to quantify the damage. Two researchers independently followed the protocol, including reference photos, for a set of samples collected in 2019 from an urban bay, East Possession Sound. Two scores were given to each specimen: highest-level damage and extent of highest-level damage. An additional score was given to bivalve species: rust/stain. The sets of scores were compared to determine if the protocol yielded similar values between the two researchers. The mean for highest-level damage (0.750, P-Value <0.001) and extent of highest-level damage (0.637, P-Value <0.001) showed significant correlation. Stain scoring was not found to be repeatable (0.544, P-Value = 0.036). The protocol was validated and reproducible at the station level for highest-level damage and extent of highest-level damage. This confirmation will allow the Marine Sediment Monitoring Team to use this protocol to quantify the observed damage on mollusk species, providing the ability to track changes both spatially and over time. This will be important in the future as environmental stressors related to climate change, including increasing atmospheric CO_2 in marine waters, potentially interfere with the ability of mollusk species to build and repair their shells.
Key Words:	ocean acidification, shell damage scoring, protocol, Mollusk, bivalve, pH
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This project was a part of an undergraduate research internship with the Marine Sediment Monitoring Team at the Washington State Department of Ecology. I completed volunteer work during the summer of 2021 and was interested in continuing to work at the Ecology lab. At the time, the team had been working on the pilot protocol for scoring shell damage and they needed someone to validate the procedure. I would like to express my gratitude to Julie Masura for having the idea to turn this work at the Ecology lab into an internship and eventually a capstone project. In addition, I am grateful for all her support in the development and editing of this manuscript. I would also like to Sandy Weakland and Dany Burgess at the Ecology lab for overseeing my internship and supporting me throughout the data collection and analysis process.

Introduction

Puget Sound is an estuary, formed by geological deformation and glacial activity. As the glaciers receded, they left behind a variety of inlets, fjords, and bays (Kennish 1998). This variability in the landscape created a wide array of ecosystems ranging from shallow bays to deep marine waters. The Puget Sound estuary plays an integral role in the overall health of the region. Communities rely on it for food, water, and power. Flora and fauna rely on it for the stability of their environment and food webs.

The quality of these waters is integral to the health of marine ecosystems, and, with increasing anthropogenic pressures, monitoring is necessary to detect change over time. The Washington State Department of Ecology's Marine Sediment Monitoring Team (MSMT) is at the forefront of this effort. They use measures of sediment quality to determine whether environmental pressures, such as carbon and nutrient loading, climate change, and chemical contaminant inputs, are having an effect on sediment-dwelling (benthic) invertebrate communities (Dutch et al. 2021).

Over the last decade, the MSMT noticed increased shell damage during sorting and identification of benthic mollusks, although the cause of this damage has not been determined. Benthic organisms have a limited capacity for movement, so they are vulnerable to both natural and anthropogenic environmental pressures. One such pressure is increased anthropogenic CO_2 concentrations. This increase in CO_2 absorption into marine waters reduces the available carbonate ions and calcium carbonate ($CaCO_3$) minerals, making the creation of biogenic $CaCO_3$ difficult for marine organism that produce skeletons, shells, or tests (Orr et al. 2005). In the subarctic Northwest Pacific, between the years of 1999-2018, the pH was found to have decreased by 0.0011 ± 0.0004/yr (Ishizu et al. 2021; figure 1). This was caused by the absorption and circulation of atmospheric CO_2 into the surface and depths of the oceanic waters (Goethel et al. 2017). Other factors that cause mollusk shells to experience increase susceptibility to damage include erosion from anoxic sediments or natural shell deterioration from age.

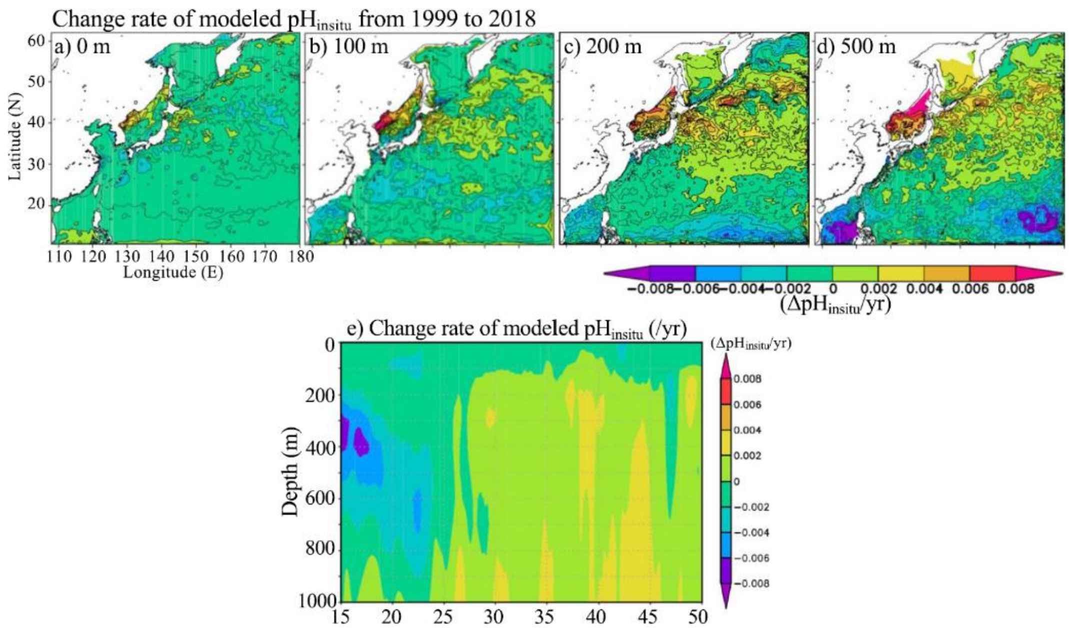

Figure 1. Fluctuations in pH in marine waters of the subarctic Northwest Pacific between 1999-2018 (Ishizu et al. 2021).

Statement of the Problem

No protocol previously existed for quantifying the level of damage to mollusk shells from benthic samples collected in Puget Sound, and therefore, no data existed with which to make correlations with potential contributing factors. A pilot protocol was designed to determine if it was possible to reliably collect data on shell damage, with methods that could be reproduced over time, by different parties. These data could then be compared to changing chemical and physical marine water and sediment properties to gain a greater understanding of what might be contributing to mollusk shell dissolution in Puget Sound.

Purpose of the Study

The purpose of this study was to determine the validity of a pilot shell scoring protocol developed by the MSMT. This protocol was developed in collaboration with paleobiologists from the University of Chicago (Kidwell and Kokesh, pers. communication, 2021). The pilot protocol was initially applied to a set of benthic samples by a single Ecology staff member. If the protocol was found to be repeatable in an independent re-scoring of the same samples by a second person, it could be finalized and used as an additional monitoring tool for the Washington State Department of Ecology.

Review of Literature

Field studies of shell damage can be difficult to do because of the variety of confounding factors. Many studies have been done in laboratory settings (Hettinger et al. 2013; Goethel et al. 2017; Yang et al. 2021), but few have been done with environmental samples. One field study conducted in 2019 on the Southern Brazilian Shelf categorized the damage characteristics of several different groups including whether the margin of the shell was damaged or if evidence of bioerosion or predation were present (Ritter et al. 2019).

Though shell damage has not been very well researched in the field, the effects of ocean acidification are well documented both in regard to sea water chemistry (Bates et al. 2014) and physical effects on shelled organisms and their life cycles (Hettinger et al. 2013; Martins et al. 2021; Yang et al. 2021). Reduced pH was found to have a significant effect on the growth of mollusks (Martins et al. 2021). An archipelago called the Azores have volcanic vents that pumps

CO_2 into the surrounding ocean creating naturally-occurring environments where there are areas of high and low pCO_2. In areas where the carbon dioxide levels were high, the pH was found to be between 6.81-6.92. Comparing these values to the reference sites reveal a significant different pH range of 8.16-8.2 (Martins et al. 2021). Ash weight of organisms were found to be significantly lower in sites containing high carbon dioxide concentrations (Martins et al. 2021). This decrease in weight, in comparison to the reference sites, could be attributed to the decrease availability of $CaCO_3$ minerals that occurs when the pH of a system is low (Bates et al. 2014). Low survival rates have been found when certain species were exposed to low pH systems. A study observing the effects of acidic conditions on the bivalve, *Patinopecten yessoensis*, revealed that after 28 days, 80% of the individuals in the low pH environment had died (Yang et al. 2021). The remaining individuals were classified as pH tolerant. The DNA of these two groups were compared to determine if there was any variation in genetic expression. Genetic variation was found between the two groups revealing that the species had the capacity to adapt to changes in seawater chemistry (Yang et al. 2021).

Studies in areas where there is sea ice melt have shown that increased pCO_2 can also reduce the $CaCO_3$ levels in the waters (Bates et al. 2014). Increases in pCO_2 have also been shown to affect the life cycles of shell organisms. A study observing the effects of variable pCO_2 and food availability revealed that the growth of Olympic oyster larvae was found to be slower in environments with high pCO_2 and low food supply. The percentage of larvae that underwent metamorphosis into adult oysters was also affected by pCO_2 levels. The experiments with higher pCO_2 had significantly lower percent metamorphosis, even when food supply was increased (Hettinger et al. 2013).

If the shell scoring protocol being tested in this research is determined to be viable, some of the previously mentioned studies could be modified to include damage scores. Instead of observing the larvae of oysters, an experiment could be done looking at a species of bivalve to find out if their shell become more susceptible to damage when pCO_2 levels are high and food supply varies (Hettinger et al. 2013). Instead of grouping the organisms into pH-sensitive and -resistant based on survival in a high pCO_2 environment, genetic testing could be done on groupings of individuals within a species that have low damage scores and high damage scores (Yang et al. 2021). It is possible that the species have adapted to the reduction of $CaCO_3$ mineral saturation as a result of high pCO_2 and are able to maintain shell integrity. This protocol could be used as an additional tool in understanding how mollusk communities are being affected by ocean acidification.

Methods

Sampling

Since 1989, the Washington State Department of Ecology's Marine Sediment Monitoring Team (MSMT) has conducted long-term sediment and benthos monitoring as part of the legislatively mandated Puget Sound Ecosystem Monitoring Program (PSEMP). The current sampling design includes 50 stations throughout Puget Sound, sampled annually between early April and early May (Dutch et al. 2018). The MSMT also takes samples from stations in six major urban bays within Puget Sound. Each urban bay is sampled once every six years, rotating each year. The bays include Bellingham Bay, Bainbridge Basin, Commencement Bay, Elliot Bay, East Possession Sound, and Budd Inlet (Dutch et al. 2018).

For this study, field sampling occurred in 2019 in the East Possession Sound urban bay sampling area (figure 2). A double van Veen grab was used to collect 0.1 m^2 of surface sediment and benthos. The sample was rinsed through a 1.0 mm screen to collect benthic organisms. All organisms were place in jars and preserved in a 10% borax-buffered formalin-seawater solution (Dutch et al. 2018).

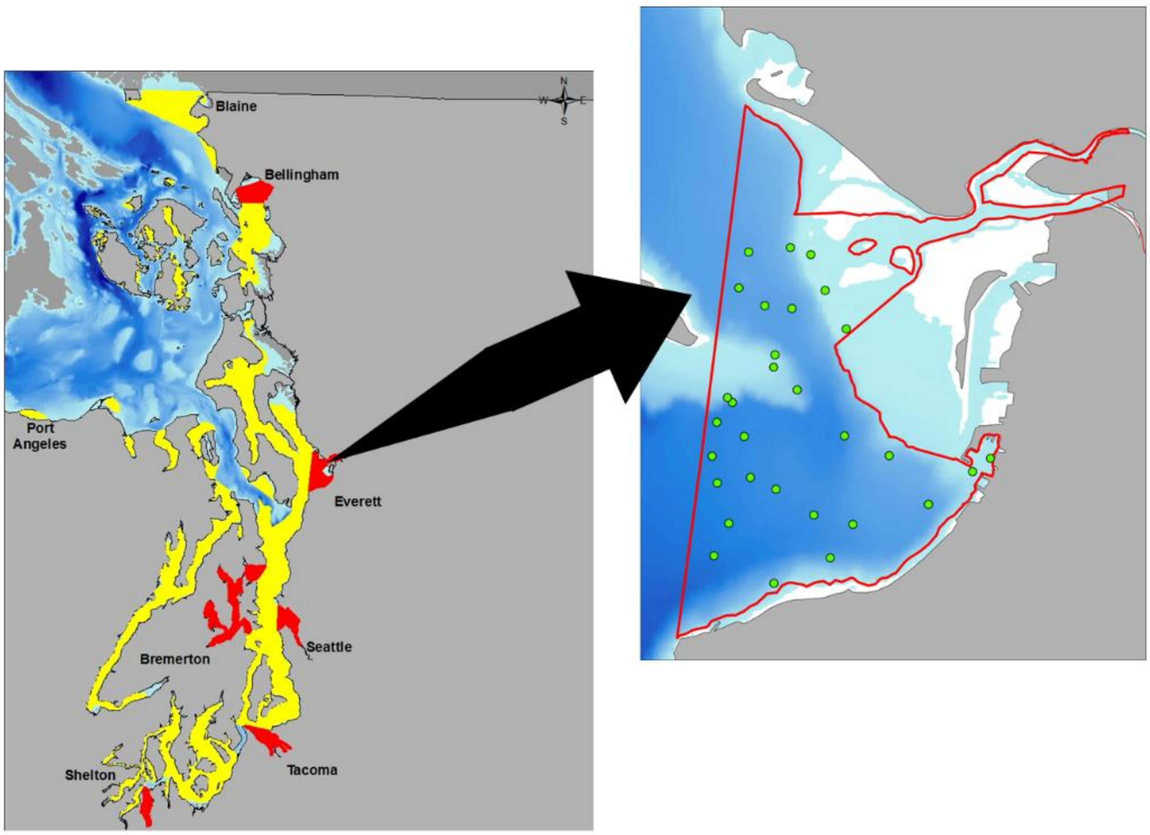

Figure 2. Sampling area by the Marine Sediment Monitoring Team. Left: Yellow and red indicates entire sampling area; red = Urban Bays only. Left: Pop-out shows locations of sampling stations within the East Possession Sound urban bay (Dutch et al. 2018**).** The location of the stations are denoted by green dots.

Sample Preparation

Organisms were transferred to 70% ethanol in the laboratory. The samples were then sorted into five phyla groups: Arthropoda, Annelida, Mollusca, Echinodermata, and miscellaneous phyla. After the samples were collected and sorted, Ecology's taxonomist sorted them further into species and size classes, keeping the organisms separated by station.

Individual vials only contained one species each. All organisms were stored in 70% ethanol through the sorting process. For this study, we focused on only the vials containing the phylum Mollusca, specifically the classes Bivalvia and Gastropoda.

Observation

Each vial was poured into a petri dish and observed under a dissection microscope using 10x-40x magnification. For bivalves, only the side of the shell facing up was scored. The entire shell of gastropods was scored. Two scores were given to each individual: highest-level damage and extent of highest-level damage; a third score, rust/stain extent was given only for bivalves. Reference photos (figures 3, 4) were used to maintain consistency in scoring. The scoring levels for damage were based on the level of etching, delamination, and shell loss. Level one was classified as surface-level damage from etching or scratching, but the shell maintained shape when touched by tweezers (figure 3a). Level two was classified as subsurface level damage from deep pitting or decalcification (figure 3b). Often layers of shell were missing, but the shell remained rigid when touched by tweezers. Level 3 was classified by the breakdown of the shell's overall structure from cracking or crushing (figure 3c) and level four was classified as shell loss, whether partial or complete (figure 3d).

Extent of shell damage was scored over three levels. Level one was localized to less than half the shell, level two was approximately half, and three was widespread across the entire shell. For bivalves, the rust level was also scored. The extent of the staining of the shell was scored over three levels as seen in figure 4. In addition to scoring each individual, the count of each sample was verified, and any missing individuals were noted.

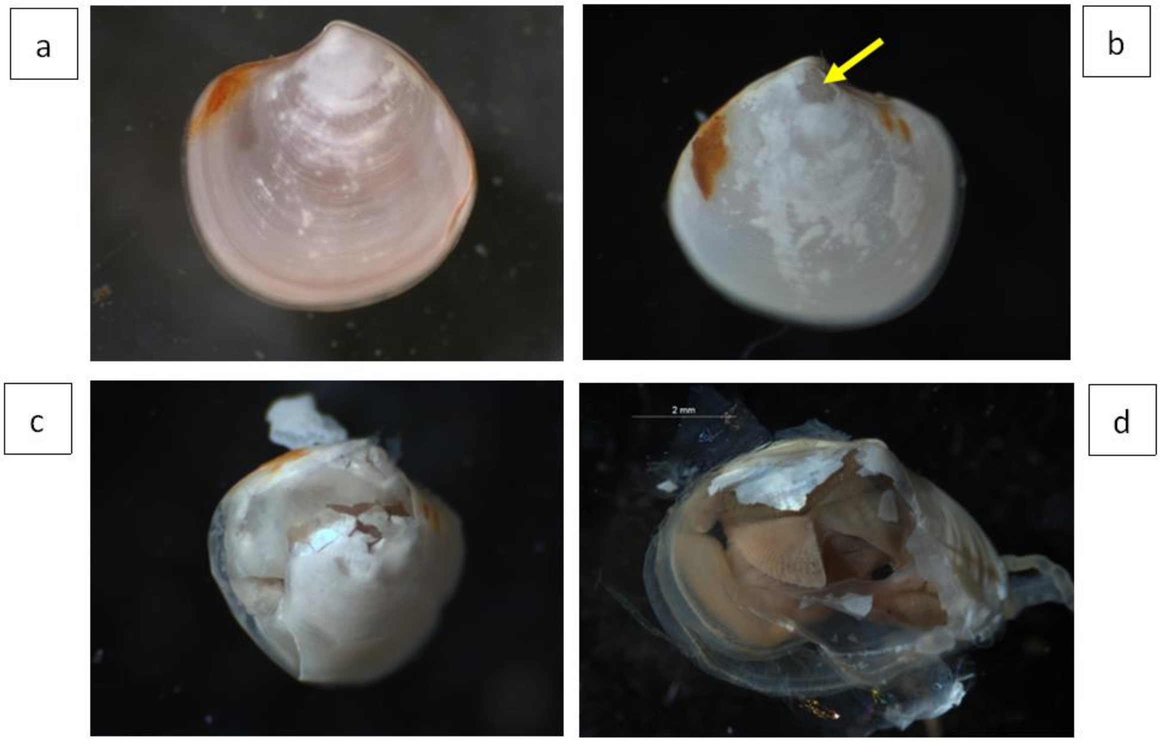

Figure 3. Shell damage exemplars taken from Shell Scoring Protocol. a. Score 1, surface level damage, b. Score 2, decalcification, deep pitting, c. Score 3, crushing damage, cracking, d. Score 4, shell missing.

This procedure was conducted by two researchers at the Washington State Department of Ecology Marine Monitoring lab. Each used the same protocol and reference photos to reduce the subjectivity of the score. Researcher 1 (R1) collected scores first, followed by Researcher 2 (R2). The data was documented on separate spreadsheets to ensure that the scores were taken independently.

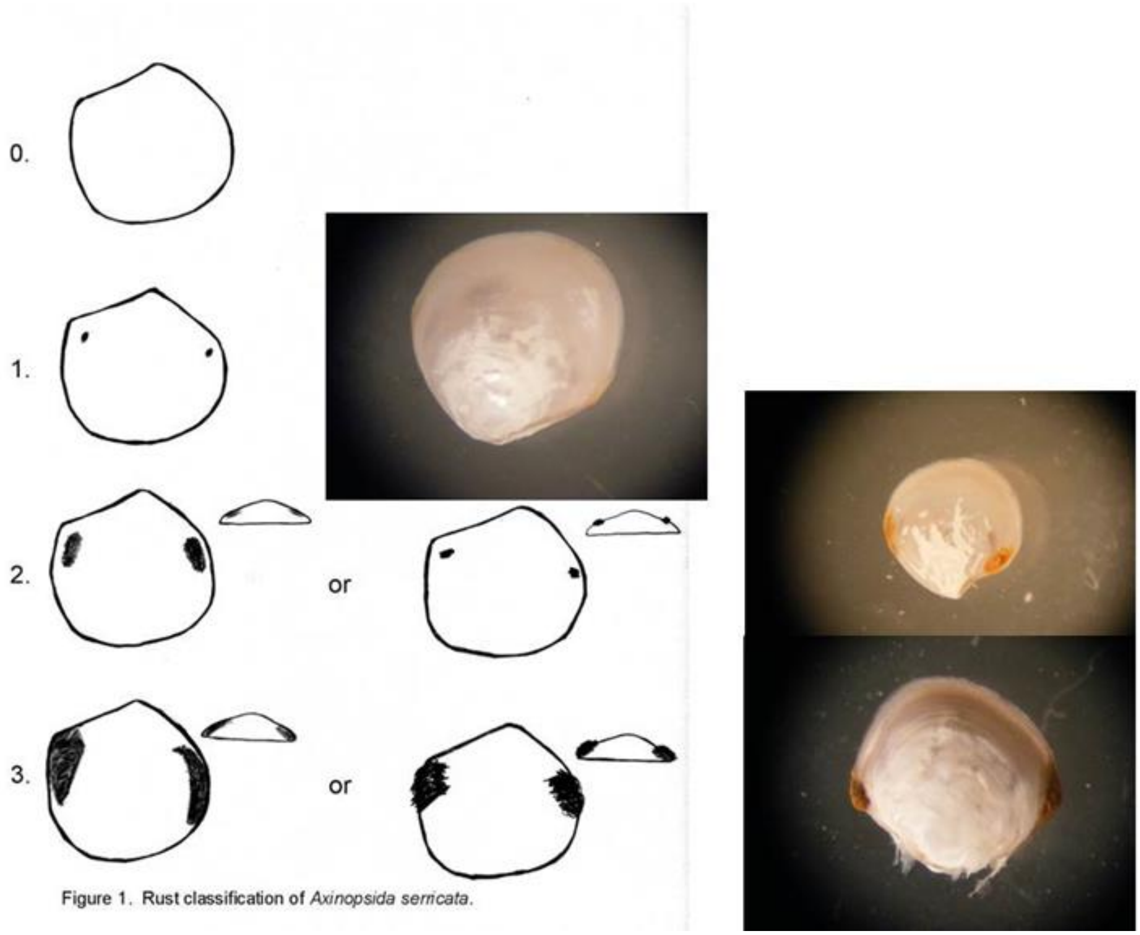

Figure 4. Rust classification exemplars taken from Shell Scoring Protocol.

Data Analysis

Once data collection was complete, data for both R1 and R2 were combined into one set. The average values of the three individual scores were calculated at the station level, regardless of class (table 1). These averages were plotted using box-and-whisker plots and the means were recorded (figures 5 and 6). The stain scores were analyzed at an overall level and at species level, specifically the species *Axinopsida serricata*, using both box-and-whisker and scatterplots (figure 7). Pearson correlations were used to determine statistical significance. Average values for individual species were correlated using Excel and Minitab. Species level correlations were plotted with scatterplots and the R-value was recorded (Figure 8).

Results and Discussion

Results

The mean damage found by R1 was 2.28, and for R2, was 2.39 (table 1, figure 5). This was a difference of 0.11. Upon statistical analysis, the Pearson correlation was found to be 0.750 (P-Value <0.001). For the extent of highest-level damage, the mean found by R1 was 1.83 and R2 was 1.86 (table 1, figure 6). This is a difference 0.03 with a Pearson correlation of 0.637 (P-Value <0.001). For the stain value, R1 found a mean of 1.68 and R2 found a mean of 1.95 (table 1, figure 7). The difference in means was 0.27 with a Pearson correlation of 0.544 (P-Value = 0.036).

Table 1. Summary data for station level

Person	Variable	N	N*	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum	Range
R1	Damage	181	3	2.28	0.08	1.03	0	1.53	2	3	4	4
	Extent of Highest level damage	181	3	1.83	0.06	0.75	0	1	1.9	2.36	3	3
	Stain	3160	0	1.68	0.01	0.8	0	1	2	2	4	4
R2	Damage	181	3	2.39	0.07	0.98	0	1.88	2	3.15	4	4
	Extent of Highest level damage	181	3	1.86	0.05	0.66	0	1.31	2	2.21	3	3
	Stain	3987	0	1.95	0.01	0.8	0	1	2	3	3	3

Four species were selected to represent the correlation in highest damage data (figure 8). *Parvilucina tenuisculpta* (figure 8a) and *Axinopsida serricata* (figure 8b) were the two most abundant bivalve species found in the sampling area. For both species, the correlations were found to be significant with R-values of 0.991 (P-Value <0.001) and 0.932 (P-Value <0.001),

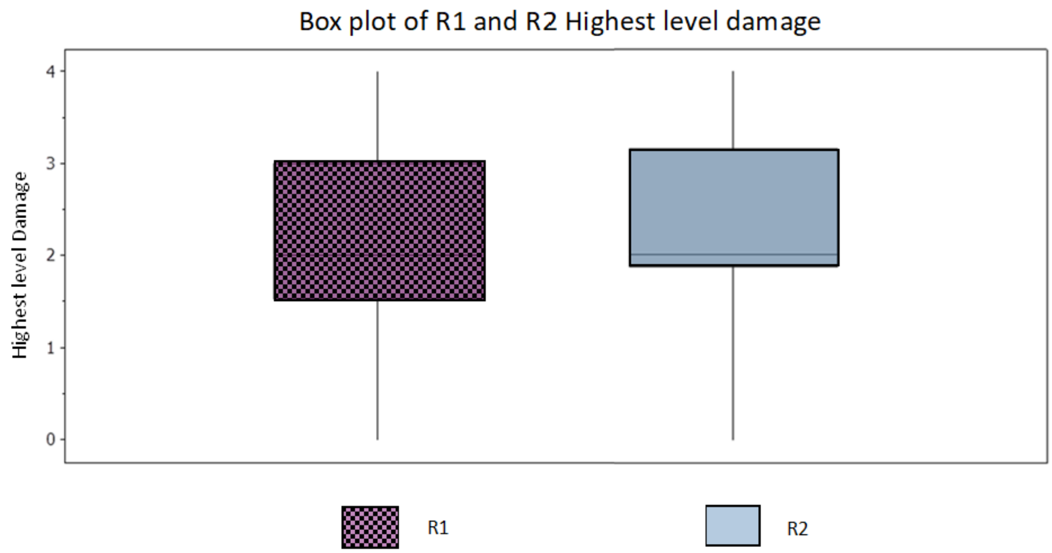

Figure 5. Boxplot comparing average highest damage values for both researchers at station level.

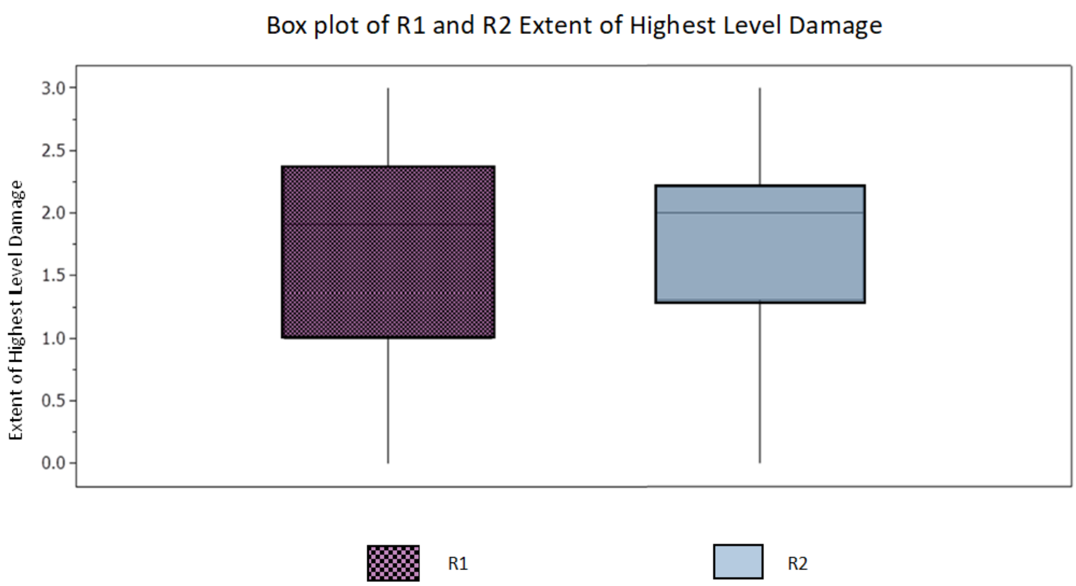

Figure 6. Boxplot comparing average extent of highest-level damage values for both researchers at station level.

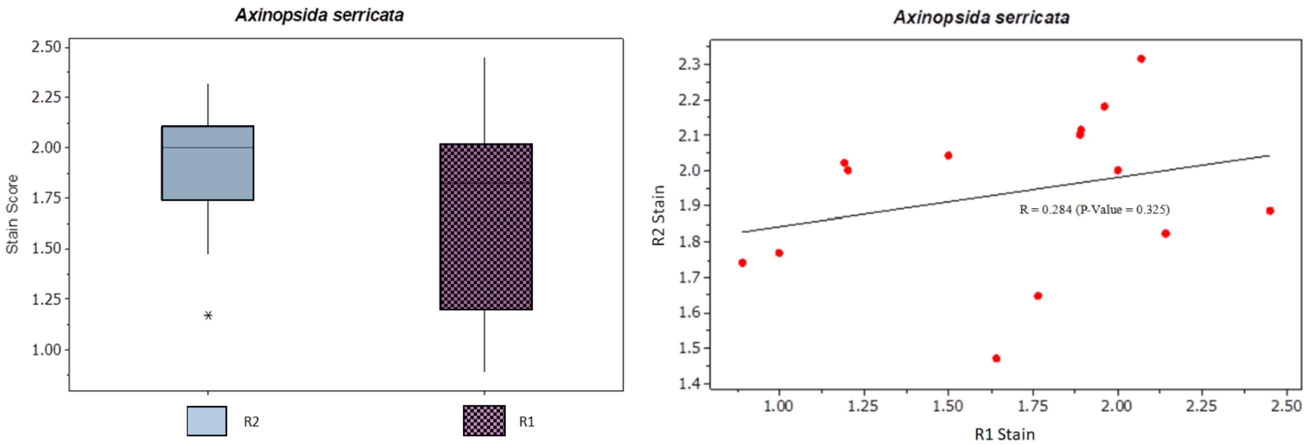

Figure 7: Boxplot (left) and scatterplot (right) comparing average stain scores for *Axinopsida serricata* for both researchers (R1 and R2).

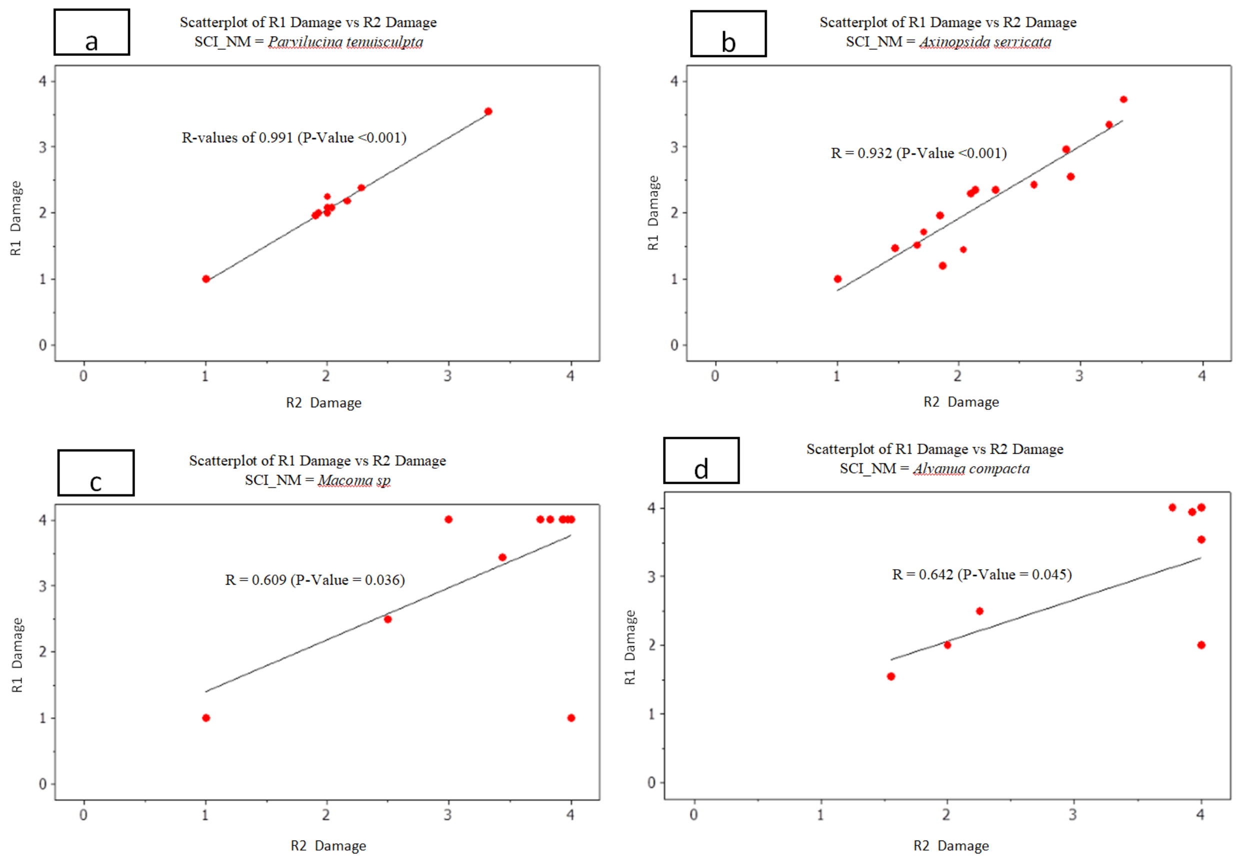

Figure 8. Correlated highest damage data for four species. Plots a and b are for the two most abundant species found in the sampling area. Plot c, *Macoma* sp., have naturally soft shells and plot d is for the most abundant gastropod.

respectively. The scores for bivalve *Macoma* sp. did not yield a significant correlation, with an R-value of 0.609 (P-Value = 0.036, figure 8c). *Alvania compacta* was the most abundant gastropod. The scores for this species did not yield a significant correlation, with an R-value of 0.642 (P-Value = 0.045; figure 8d).

Discussion

The goal of this study was to determine if the shell scoring protocol developed by the MSMT at the Washington State Department of Ecology was repeatable and, therefore, viable as a tool for measuring observed damage to mollusk shells in Puget Sound. After comparing the results of the three variables (highest-level damage, extent of highest-level damage, and stain) from R1 and R2, the protocol was found to be reproducible at the station level for damage and extent, but not for stain.

When looking at the species level, the values revealed some variability. Occasionally, the damage on one side of a bivalve's shell was significantly higher than on the other. This could skew the data if the sample size was too small for a particular species.

The scores for *Macoma* sp. were less consistent between the set of scores recorded by R1 and R2. This could have been caused by the increased number of handlings of this shelled organism. *Macoma* sp. tend to have thinner shells than some of the other bivalve species investigated, and it is possible that further damage was done between the two scoring observations.

Alvania compacta was the most abundant species of gastropod so it was used as a reference for all gastropod scores. The damage scores collected were not as closely correlated as the bivalve species. The main issue with the gastropod species was the tips of their shells. They were often broken off, but the rest of the shell had very little damage. Since a part of the shell was missing, the individual had to be scored as a level 4 for damage. The survival rate of gastropods with missing tips is unknown. The variability found in the *Alvania compacta* data could also be contributed to overhandling. If the tips of the shells were broken off between the first and second scoring observations, the scores would be skewed. Even with the variability between species, the scores collected using the protocol was found to be overall, reproducible. The only species that was consistently scored between the two researchers was *Axinopsida serricata*, but the scores were not found to be significantly correlated.

Suggested Future Work

Initial analysis shows that the shell scoring protocol is reproducible, and this means that it could be applied to the remainder of the benthic samples. Some variability was found from species to species, which could be explored further. It may be more practical to look only at the most abundant species in an area instead of all species. Shell damage scoring data could be examined spatially to see what parts of the Sound are the most severely affected (figure 9). The Marine Sediment Monitoring Team at the Washington State Department of Ecology also hopes to use the shell scoring data to compare to water quality data at the same location to form a more detailed picture of how the benthos is being affected by natural and anthropogenic pressures.

The map shows that the average scores were consistent at nine out of the 15 stations for which both researchers collected scores. For four stations, the average damage score increased between observations from R1 and R2. This type of mapping could be used to compare damage and extent to chemical composition or other data that the monitoring team has collected to draw further conclusions about the health of the ecosystem around each station.

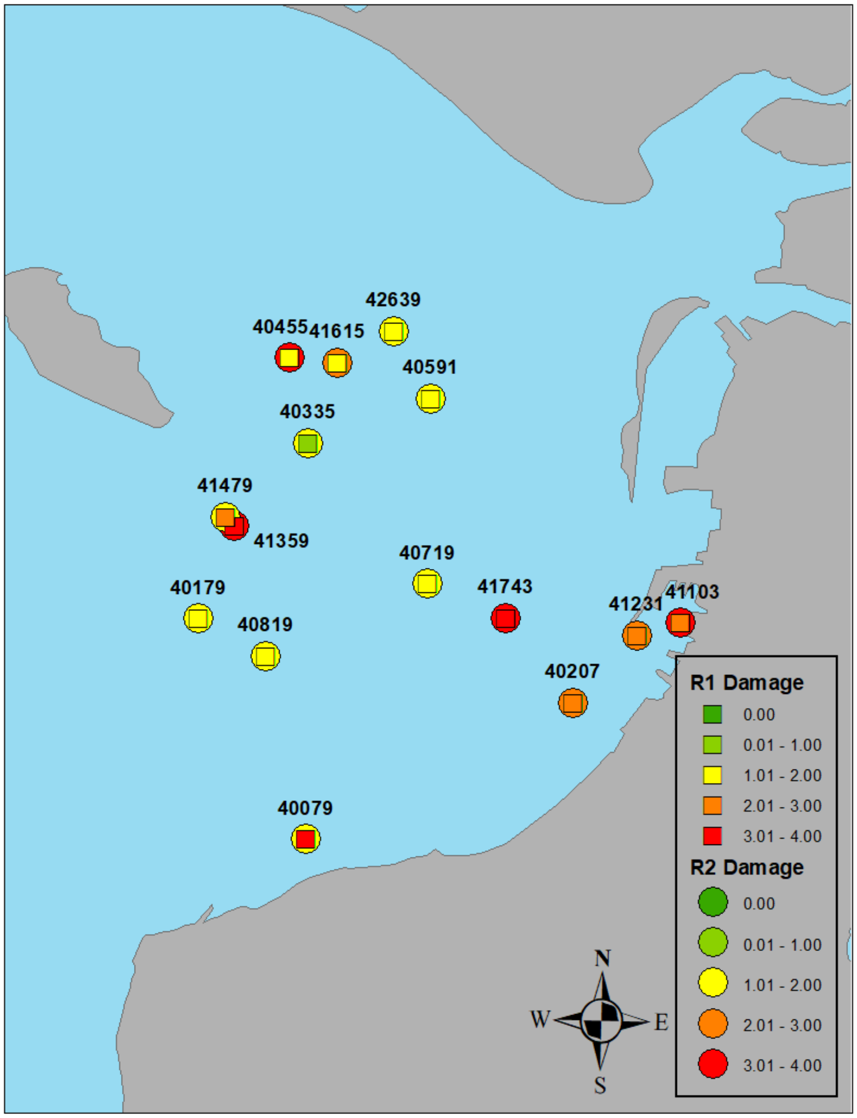

Figure 9. Average damage scores of each researcher at each station location within the East Possession Sound urban bay.

Conclusion

This study revealed that the pilot shell scoring protocol developed by the Marine Sediment Monitoring Team is viable and could be used to quantify observed shell damage both spatially and over time. It is well known that the ocean is warming and becoming more acidic, so it is important to continue to develop new data collection tools to track potential changes. The monitoring of Puget Sound's marine waters can help us gain a broader understanding of what is happening farther inland, and inform policy decisions surrounding restoration and management of our natural resources.

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