

Assessing the Health of Regional Marinas by Measuring the Lysosomal Stability of Deployed Atlantic Ribbed Mussels, *Geukensia Demissa*

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Abstract

Using the neutral red dye assay and a microplate reader the comparative health for mussels deployed in regional marinas, Bradley Creek Marina, Carolina Beach Municipal Marina, and Sea path Yacht Club, were assessed via lysosomal stability. In the month of October mussels located in marinas showed a significant decrease in percent health when compared to the reference site. For the months November through April there were no statistical decreases in percent health for the three marinas when compared to the reference site.

Introduction

The marine organisms found in the inland waters of Eastern North Carolina have increasingly been exposed to stressful situations over the past decade. This is due to a constant advance in population. Over the past 14 years the population in New Hanover County has increased by more than 40%.⁵ Many of the New Hanover residents are also boaters; in 2002 12,285 boats were registered in New Hanover County alone.⁵ With this rise in boating activity areas such as the Carolina Beach Municipal Marina, Bradley Creek Marina, and Seapath Yacht Club are likely to have high levels of contaminants in their water.

The research conducted during the 2004-2005 academic year was designed to assess qualitatively the stress on these three ecosystems. An efficient, accurate and low cost method for measuring the effects of toxins on marine organisms was developed by using the neutral red dye retention method and a microplate reader.

The Atlantic Ribbed Mussel, *Geukensia demissa*, was used to analyze the health of the marine environment in the three marinas. The Atlantic Ribbed Mussel was chosen because it is a common marine bivalve found in waters along the east coast. It is a filter feeding organism that does not possess the ability to excrete toxic build up, therefore making it sensitive in highly polluted areas.² This capability of bioconcentration for mussels allows them to indicate presence of toxins in the water without the cost involved with traditional chemical analyses.

The neutral red dye method involves the destabilization of lysosomes in cells of the Atlantic Ribbed Mussels. Neutral red dye a weak base amine is able to diffuse across the cell membrane into the lysosome. In the lysosome the dye becomes protonated and can no longer diffuse across the membrane.⁴ Over time the build up of protonated red dye increases the pH in the lysosome thus losing the acidic nature.⁴ This results in a change in membrane function, and leads to destruction of the cell which can be monitored using a microplate reader. The destruction is exasperated by the collection of pollutants by the lysosomes, and thus the retention time becomes an indirect measurement of pollutants in the organisms.

Methodology

Mussels were collected from the inland side of Masonboro Island, Wrightsville Beach North Carolina. Mussels used for in lab exposure experiments were allowed to acclimate in the laboratory for 24 hours. Mussels used for the assessment of marina health were deployed in minnow traps and allowed to acclimate for 30 days before analysis were performed.

Atlantic Ribbed Mussels were deployed in the four locations; Carolina Beach Municipal Marina, Bradley Creek Marina, Seapath Yacht Club and in a reference site on the inland side of Masonboro

Island. Six mussels were removed from each location for monthly analyses throughout the months of October through April.

Neutral Red Dye Method

Using a microplate reader set at 540nm, the following method was used to determine the amount of red dye retained in cells from mussels deployed in the marinas and in cells from mussels taken from the reference site behind Masonboro Island. The absorbance measured correlates with the comparative health of the respective mussels.

- From each mussel 500µL of hemolymph was extracted from the adductor muscle with a syringe containing 500µL of physiological saline solution.
- 80µL of the hemolymph solution was placed into individual wells of a 96-well plate, five wells per mussel. After 45 minutes excess cells were removed by decanting the 96-well plate.
- 200µL of neutral red dye was added to each well and the plate was incubated in a lightproof chamber for three hours. After three hours the excess neutral red solution was decanted.
- 100µL of physiological saline solution was used to wash away lysed cells. This was performed three times.
- 200µL of 50/50/1 ethanol/water/acetic acid was added to the individual cells to destroy the remaining cell membranes to release the red dye contained in the lysosomes.
- The absorbance was measured by the plate reader at 540nm with higher absorbances indicating healthier cells.

Reagent Preparation

Marine bivalve Physiological Saline Solution (PSS)

1. Hepes	4.77g
2. Sodium Chloride	25.48g
3. Magnesium sulfate	13.06g
4. Potassium chloride	0.75g
5. Calcium chloride	1.47g

The above compounds were combined and brought to 1 liter with de-ionized water. The pH was adjusted to 7.36 by adding 1.0M NaOH or 1.0M HCl as needed. Physiological Saline Solution was kept at 4 ° C and made up monthly. Physiological Saline Solution was used at room temperature to minimize the shock to the mussels.

Neutral Red Dye Preparation

Three methods were tested in order to maximize the absorption of red dye by the plate reader while lowering the standard deviations for the average absorption of each mussel. Method three proved to be the best technique to accomplish this.

Method (1) 0.5000g Neutral Red Dye was added to 50 mL of (PSS) and allowed to dissolve for 30 min.

Method (2) 0.5000g Neutral Red Dye was added to 50mL of (PSS) and sonicated until all NRD was dissolved and used immediately..

Method (3) 0.5000g Neutral Red Dye was added to 50mL of (PSS) and sonicated until all NRD was dissolved, filtered with a Whatman # 1. This solution was diluted to 50% and transmittance was measured with a Spectronic 20 at 540nm ensuring a transmittance of less than 5%.

Acetic Acid/Ethanol Solution

1mL of acetic acid and 50mL of ethanol were added to 50 mL of de-ionized water.

Bradford Assay

The Bradford assay was used to quantitatively and qualitatively determine the presence of hemolymph in each sample. A calibration curve ranging from 0.2mg/mL to 1mg/mL of bovine serum albumin was constructed using Bio Rad protein standard (2.0mg/ml) and Bio Rad protein assay (Lot # 72729A). For quantitative assessment the average absorbance values for each mussel were normalized by the amount of protein present. For qualitative assessment the average absorbance values were not used for further analyses if less than 0.95mg/mL of protein was present for that mussel.

The protein concentrations were determined with 10 uL of the samples and standards were pipetted into individual wells in a 96-well plate. Five samples per mussel and three standards for each concentration. Using a multichannel pipetor 200uL of filtered BioRad solution were added to each well containing a standard or sample. The solution was allowed to incubate at room temperature for 15 minutes. To determine the protein concentration the absorbance for each cell was measured at 620 nm with the Multiakan MCC/340. A calibration curve was determined for the protein concentration from The BSA standards, and the concentration of the protein in the hemolymph samples was calculated.

Results and Discussion

The comparative health for Atlantic Ribbed mussels were determined monthly for Bradley Creek Marina, Carolina Beach Municipal Marina, Seapath Yacht Club, and a reference site. The average

absorbances for each group of mussels obtained from the neutral red dye assay were normalized with the amount of protein withdrawn from each sample. Protein concentration was determined by the Bradford assay, and the microplate reader at 620nm. The control was assumed to represent 100% health and the three marinas were adjusted to this scale as shown in Figure (1).

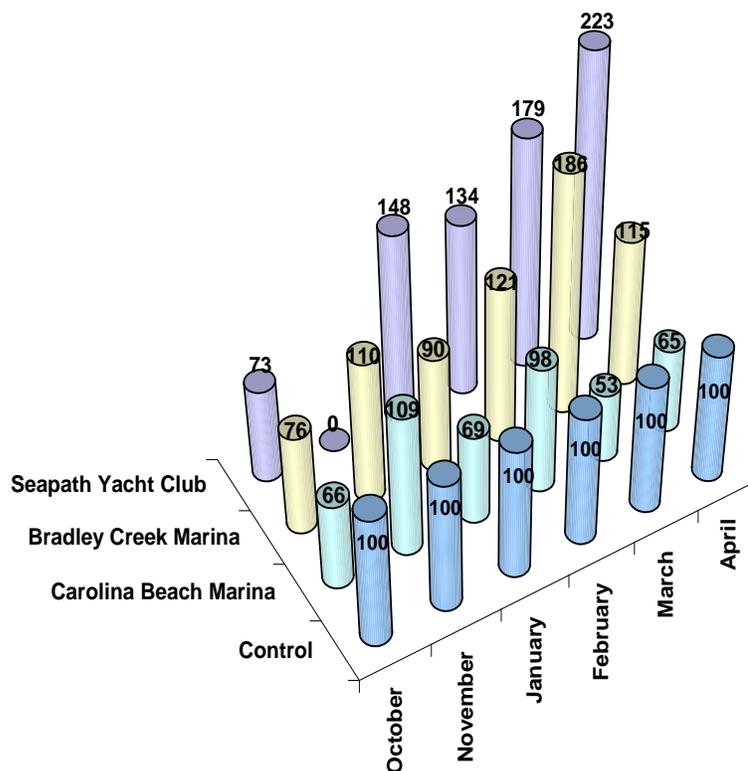


Figure 1: Monthly comparison of % Health of Marinas from October – April. All data collected was normalized with protein concentrations.

Using a 95% confidence level a significant difference in percent health for October was observed for mussels located in each marina when compared to the reference site. For the months of November, January and February there were no statistical difference in values. In March, Seapath Yacht Club showed a significantly higher percent health when compared to the reference site; this was also seen for April data. For the other marinas in March and April there were no significant differences in percent health.

Conclusion

The data obtained showed that mussels deployed in marinas did exhibit a significant decrease in comparative health in October when compared to the reference site. For the months of November through April there were no significant decreases in percent health. In fact, Seapath Yacht Club began to show a higher comparative health value when compared to the reference site in March and April. For the other

marinas there were no other statistical changes in percent health. In order to determine if Atlantic Ribbed mussels are good biomarkers a continuation of data collection throughout the summer months should be conducted. If a decrease in health is noted during month of high boating activity this would prove the transient biomarker nature of the Atlantic Ribbed Mussel.

Other methods for treating data such as using protein concentrations as a qualitative assay instead of quantitative were examined. When using protein in a qualitative manner the deviations in the absorbance values decreased greatly however there were still no statistical differences between marinas and the reference site during the winter months, and data followed the same trends in comparative health changes as when absorbance values were normalized with protein concentrations.

References

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