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## **Decline of Diporeia in the Great Lakes: Was Disease Associated with Aquatic Invasive Species the Primary Factor?**

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# **Honor's Thesis**

## **Decline of *Diporeia* in the Great Lakes: Was disease associated with aquatic invasive species the primary factor?**

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## I. ABSTRACT

Populations of the freshwater amphipod *Diporeia* spp. in the Great Lakes have steadily declined since the late 1980's. Prior studies have provided inconclusive data on possible reasons for their decline, but suggest that factors such as food competition, predation, toxic excretions, and potential diseases associated with aquatic invasive species (AIS), in particular zebra mussels (*Dreissena polymorpha*), may have caused the collapse of *Diporeia* throughout the Great Lakes. In this project, I examined the possibility of pathogens as the root cause of *Diporeia*'s collapse. Linear regression modeling showed a significant positive linear association between percent of *Diporeia* exhibiting a pathogenic infection and year ( $r=0.7202264$ ,  $p\text{-value}\leq 0.0124$ ). Chi-square testing for independence was also used to test if there was an association between year and percent infection. Values obtained were  $X^2 = 50$ ,  $df = 10$ ,  $p\text{-value}\leq 0.0001$ , implying significant association between year and infection. As such, the data indicates that zebra mussels and possibly other AIS (e.g. Quagga mussels; *Dreissena rostriformis*) may have acted as the vector for pathogen(s) that infected *Diporeia* and be the cause of their decline. Future research is needed to examine zebra and quagga mussel tissues for similar pathogens, including live studies of potential infection.

## II. INTRODUCTION

*Diporeia spp.* are freshwater amphipods that used to be the most dominant crustaceans in the benthic layer of the Laurentian Great Lakes. High in lipid content, *Diporeia* have previously been considered the primary food source for many bottom feeders in the Great Lakes including whitefish (*Coregonus clupeaformis*), bloater (*Coregonus hoyi*), and slimy sculpin (*Cottus cognatus*; (Nalepa et al. 1998) . Since the mid 1980's, however, populations of *Diporeia* began to disappear, declining over 95% in the last 15 years in some places. With the disappearance of *Diporeia*, fish populations consequently decreased as their sole source of food became scarce and perhaps exacerbated due to a shift to less nutritional food sources (Nalepa et al. 1998).

The disappearance of *Diporeia* has been postulated to be the result of the invasive Zebra (*Dreissena polymorpha*) and Quagga (*Dreissena rostriformis*) mussels deposited into the Great Lakes from ship ballast water and quickly becoming established as an aquatic invasive species (AIS) three years prior to the decline of *Diporeia*. Why *Dreissena* had this effect on *Diporeia* is still not completely understood. One hypothesis is that the mussels led to decreased food availability due to food competition (Nalepa, 1989). However, there are some inconsistencies with this hypothesis. *Diporeia* and *Dreissena* coexist in Lake Superior, Lake Cayuga and isolated areas of Lake Michigan. Such observations suggest that the relationship between *Diporeia* and *Dreissena* is more complex than simply competition for food.

A second possibility of *Dreissena* involvement is as a vector for pathogenic organisms infecting *Diporeia*; this hypothesis is still being explored (Fanslow, pers. comm.); anecdotal evidence indicate that during the early years of decline in *Diporeia*, crustaceans (shrimp) in many other locations were crashing, purportedly a consequence of disease. *Rickettsia*-like

infection, *Haplosporidia* spp. and *Microsporidia* spp. have all been observed in *Diporeia* tissues (Messick et al. 2004). The origin of these pathogens is not known. It is interesting to note that *Rickettsia* infections have, however, been found in *Dreissena* located in Greece (Molloy et al. 2001). Similarly, *Haplosporidium* pathogens have been identified as a primary disease causing significant death and decrease in populations of the bivalve *Crassostrea virginica* (Eastern oyster), on the east coast of North America and fresh water snails (*Physella parkeri*) in Douglas Lake in Michigan (Barrow, 1961). *Microsporidia* is also a common pathogen in freshwater shrimp (*Gammarus fasciatus*), an amphipod closely related to *Diporeia*. These associated pathologies suggest that one of these pathogens could infect and possibly be the cause of decline in populations of *Diporeia* in the Great Lakes.

Taxonomically, *Diporeia* spp. belongs to the Phylum Arthropod, Subphylum Crustacea, Class Malacostraca, Order Amphipoda, and Family Pontoporeiidea. In years past, all *Diporeia* were classified as *Pontoporeia hoyi* (anonomous with *P. affinis*), however, taxonomists today believe there may be as many as eight species in the Great Lakes (Cavaletto et al. 1996).

The objectives of this project were to:

- 1) Update the population density of the *Diporeia* in Lake Superior's Batchawana Bay.

This location has been identified in prior studies as a "safe haven" with high concentrations of *Diporeia* that coexist with a high abundance of *Dresseina* mussels. Studies of *Diporeia* at this location have not been done since 2008.

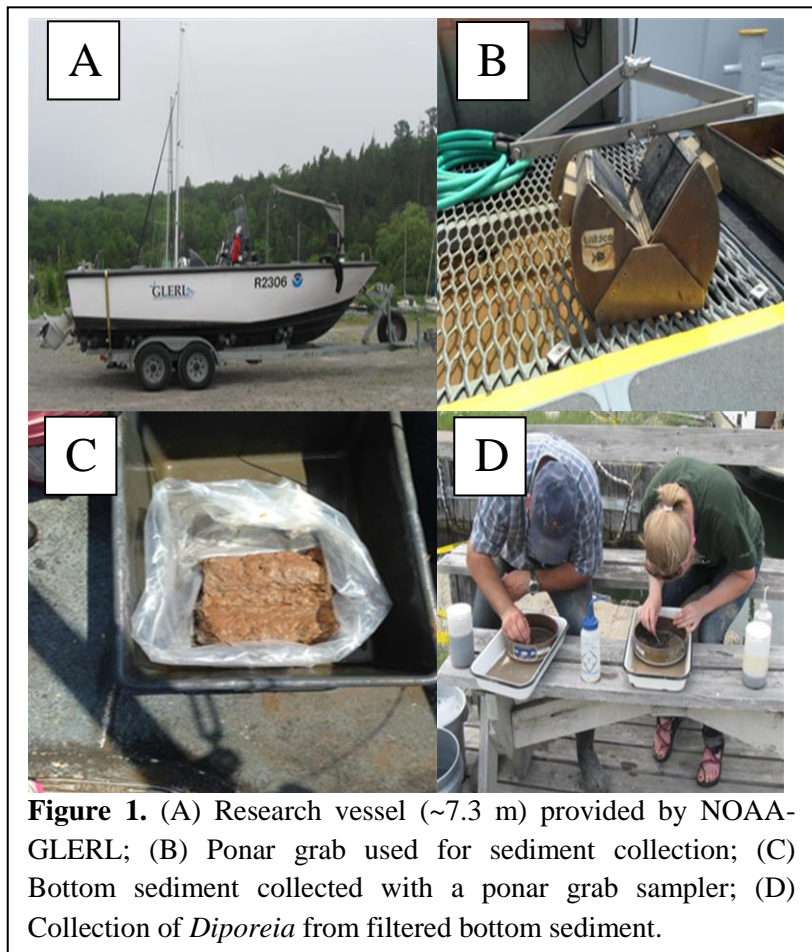
- 2) To examine archived and more recent samples of *Diporeia* tissue for pathogenic infection. Histological studies were done ~14 years ago (2000).

### III. METHODS

#### i. Field Work

In collaboration with the National Oceanic and Atmospheric Association (NOAA) and Great Lakes Environmental Research lab (GLERL) scientist Dave Fanslow, *Diporeia* samples were collected from Lake Superior's Batchawana Bay in Ontario, Canada. GLERL provided a ~7.3 meter research vessel (Fig. 1A) that was equipped with a ponar grab (Fig. 1B) which, when lowered, collects bottom sediment from the benthos (Fig. 1C). Using specific coordinates known by GLERL after many years of collecting *Diporeia*, the boat would be anchored and the ponar grab lowered to the bottom of the lake. The ponar opens and closes, collecting the bottom sediment which was then

retrieved. Through a series of water flushes, the sediment was filtered through a 500  $\mu\text{m}$  sieve until only a mixture of large material plus *Diporeia* remained. This was repeated ten times at each location to ensure an accurate abundance of *Diporeia* was collected. After filtering the sediment, *Diporeia* were manually removed with tweezers from the remaining material (Fig.



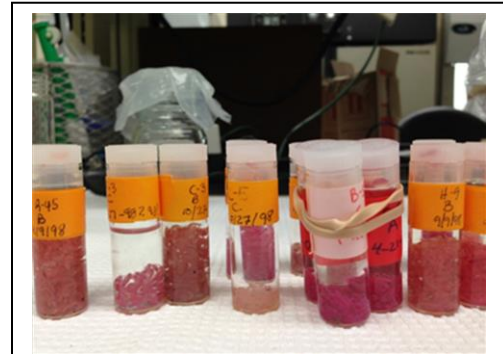
**Figure 1.** (A) Research vessel (~7.3 m) provided by NOAA-GLERL; (B) Ponar grab used for sediment collection; (C) Bottom sediment collected with a ponar grab sampler; (D) Collection of *Diporeia* from filtered bottom sediment.

1D.). The collected *Diporeia* were subdivided into two aliquots, one sample was store in liquid

nitrogen and later transferred to a -80°C freezer for future analyses while the second sample was used for analyses for this project.

ii. Labwork: Histology

*Diporeia* tissue collected from this study plus samples provided by NOAA were prepared for histological studies (Fig. 2). Samples of *Diporeia* provided by NOAA were collected from Lake Michigan since the late 1980's. The organisms examined in this project were archived Lake Michigan *Diporeia* from 2005 and 2010. All tissues used in this study were collected, prepared, and analyzed using similar methodologies.



**Figure 2.** Archived samples of *Diporeia* stained in Rose Bengal dye and stored in Formalin solution

To begin the fixing processes to preserve the tissue, the tissue samples were stained in Rose Bengal dye (Sigma-Aldrich, USA) and then were placed in 10% formalin (Sigma-Aldrich, USA) which maintains and preserves the tissue.

The following steps in preparing *Diporeia* tissue samples for microscopy involves processing. The purpose of processing tissue samples is to remove water from the tissue and replace it with a solid medium that will allow for thin sectioning. Individual *Diporeia* were removed from the formalin solution and placed into a histology cassette. Each cassette held ten *Diporeia*. The *Diporeia* in these cassettes were then processed using a series of increasing graded ethanol (Sigma-Aldrich, USA) solutions to help dehydrate the tissue. Once complete, tissue was then placed in xylene (Sigma-Aldrich, USA), which is a clearing agent that removes the alcohol from the prior step. Each cassette was placed, in order, in an 80%, 90%, 95%, and

two changes of 100% ethanol solutions, followed by two washes of 100% xylene. Each cassette was incubated for 20 min. in each respective solution (Bergman, per comm.).

Following this process, the cassettes were placed in liquid paraffin (wax) baths where *Diporeia* was incubated for ~30 minutes. This allowed the tissue to become infiltrated with wax. Once the tissue had been infiltrated, the tissue was embedded (Fig. 3). Each individual sample of *Diporeia* was placed into a metal embedding tray using tweezers. Before placement of tissue, each tray was sprayed with HistoPrep Mold Releasing Agent (Fisher Scientific, USA) to help with the removal of the solid wax block after it cools; blocks not prepared in this manner chipped and fell apart and were too difficult to remove. Samples were then placed flat on their side in the embedding tray. This allowed for a sagittal cut when tissue was sectioned. The paraffin wax was melted in a vacuum infiltrator and paraffin dispenser (Lipshaw Inc.) at 55°C. Once the tissue was placed appropriately, liquid wax was dispensed into the embedding tray until the tissue was completely immersed. After ~ 30 sec. when the first wax layer became firm, additional wax was dispensed to completely fill the embedding tray. The wax blocks were left to cool for at least 3 hrs. at room temperature.



**Figure 3.** *Diporeia* tissue embedded in wax block held in microtome.

Following incubation for 3 hrs. at room temperature, wax blocks were removed from the embedding tray. Due to the small size of *Diporeia* the wax outside a 1 cm radius of the organism was removed. This assisted in the later processes of sectioning the wax block with a sliding microtome (Bausch & Lomb, Rochester, USA). Each block was secured in the microtome and cut into 5- 8  $\mu$ m sections. Each section was then transferred to a warm water bath at 36°C bath to

ensure the wax section was free of wrinkles. The sections were then placed on poly-prep-lysine coated glass slides (Sigma-Aldrich, USA) followed by placement of the slides on a slide warmer (Sigma-Aldrich, USA) at 46°C for 24 hours. This procedure ensured that the thin sections adhered to the slides.

After heating the prepared sections for 24 hrs., the slides were then exposed to a graded series of ethanol and xylene solutions in reverse order as described above. This removes the wax from the slide, leaving only the tissue to be stained. The slides were then incubated in a solution consisting of 65% ethanol and 5% hydrochloric acid for 5 min. The purpose of the aforementioned step was performed to remove the Rose Bengal dye that the tissue had originally been fixed in.

Mayer's Hematoxylin and Eosin Y stains (Sigma-Aldrich, USA) were then used to stain tissues adhering to the glass slides, followed by light and fluorescent microscopy to identify and characterize infected *Diporeia* tissue. The protocol used was a modified version from Lillie (1965). The protocol I developed is as follows:

1. Immerse tissue with Mayer's Hematoxylin;
2. Incubate for 10 min;
3. Rinse and run room temperature tap water over sections for 10 min;
4. Immerse with working Eosin Y Stain;
5. Allow to incubate for 30 sec;
6. Rinse with tap water until water runs clear off of slide;
7. Clear, and mount tissue

After completion of these seven steps, the tissue mounted on the slide is ready for histological examination.

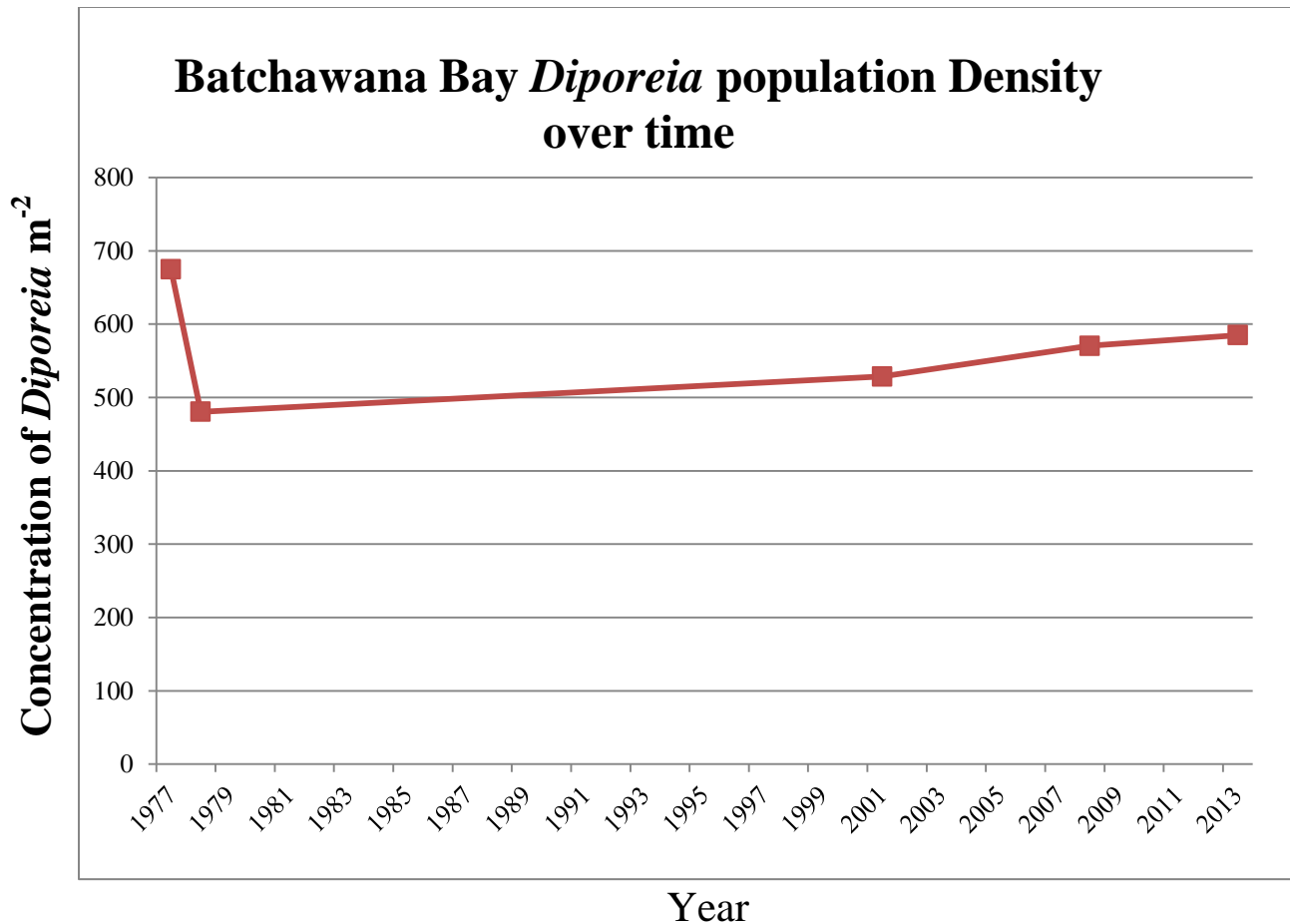
### iii. Statistical Analysis

Data was analyzed by Grand Valley's Statistical Consulting Center via Dr. Sango Otieno. Two types of statistical analyses were performed: (1) linear regression using year as the predictor and percent of pathogens present, and (2) Chi-square test for Independence to determine if there is association between year and presence of pathogens. All analyses were completed using the R-Statistic (The software is R for Statistical Computing). In the Analysis, we compared our finding of *Diporeia* from the years 2005 and 2010, to data collected in earlier years in order to look for a trend over time. The data for *Diporeia* tissue prior to 2005 was collected by Messick et al. 2004.

## IV. RESULTS

### i. Field Work

The purpose of collecting *Diporeia* from Batchwana Bay was to update the status of the *Diporeia* population that had been monitored since 1977 and to provide researchers at AWRI and GLERL with relatively "healthy" *Diporeia* for future research. Based on the average number of *Diporeia* that were collected in the ponar sample, the observed population was found to be 285 *Diporeia* m<sup>-2</sup>. Figure 5 shows that this concentration appears consistent with prior sampling years.

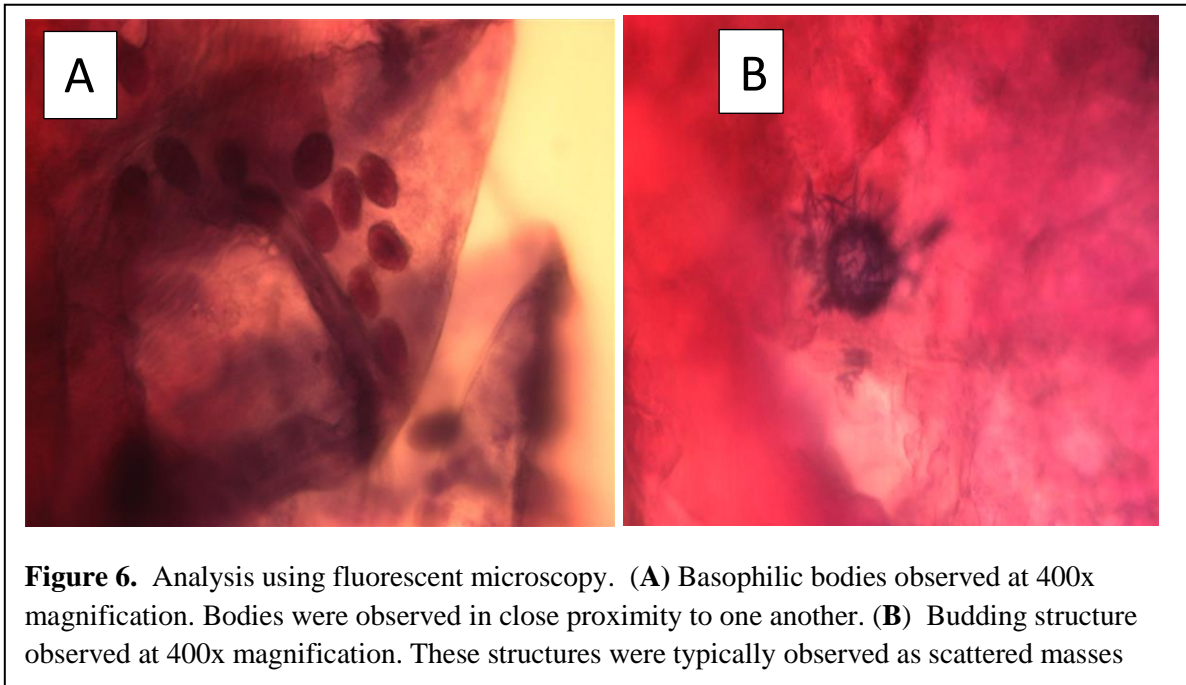


**Figure 5.** The concentration of Bactchawana Bay *Diporeia* population over time

ii. Labwork: Histology

The immune response of *Diporeia* was also assessed using both light and fluorescent microscopy. Tissue was determined to have an immune response if they met one of the following conditions: (1) A basophilic body or mass was present and/or (2) A cellular body with projections or a budding structure was present. A basophilic body (or mass) is defined as a structure within the tissue that is abnormal and is darker in color because it absorbed more dye (Fig. 6A). These masses involve in the innate immune system of an amphipod's response to pathogens (Martinez, 2007). Basophilic bodies were often found in the legs, and along the spine

of *Diporeia*. The second prevalent structure found in *Diporeia* tissues were budding structures (Fig. 6B). These structures had projections off of the cellular body suggesting possible fungal infection.



Examination of Lake Michigan *Diporeia* has been done previously on samples that were collected in 2000 and earlier (Messick, 2004). The samples examined in my work were from 2005 and 2010. The intent was to provide more recent insight into the current state of *Diporeia* tissue and disease. For the *Diporeia* collected in 2005, it was found that ~18.90% (Table 1) of *Diporeia* was found to be exhibiting pathogenic infection. For the 2010 samples, ~29.20% (Table 1) of *Diporeia* was exhibiting pathogenic infection.

**Table 1.** Percent *Diporeia* Exhibiting an Immune Response

Year	Number Examined	Number Exhibiting Immune Response	Percent (%) exhibiting Immune Response
2005	58	11	18.90%
2010	24	7	29.20%

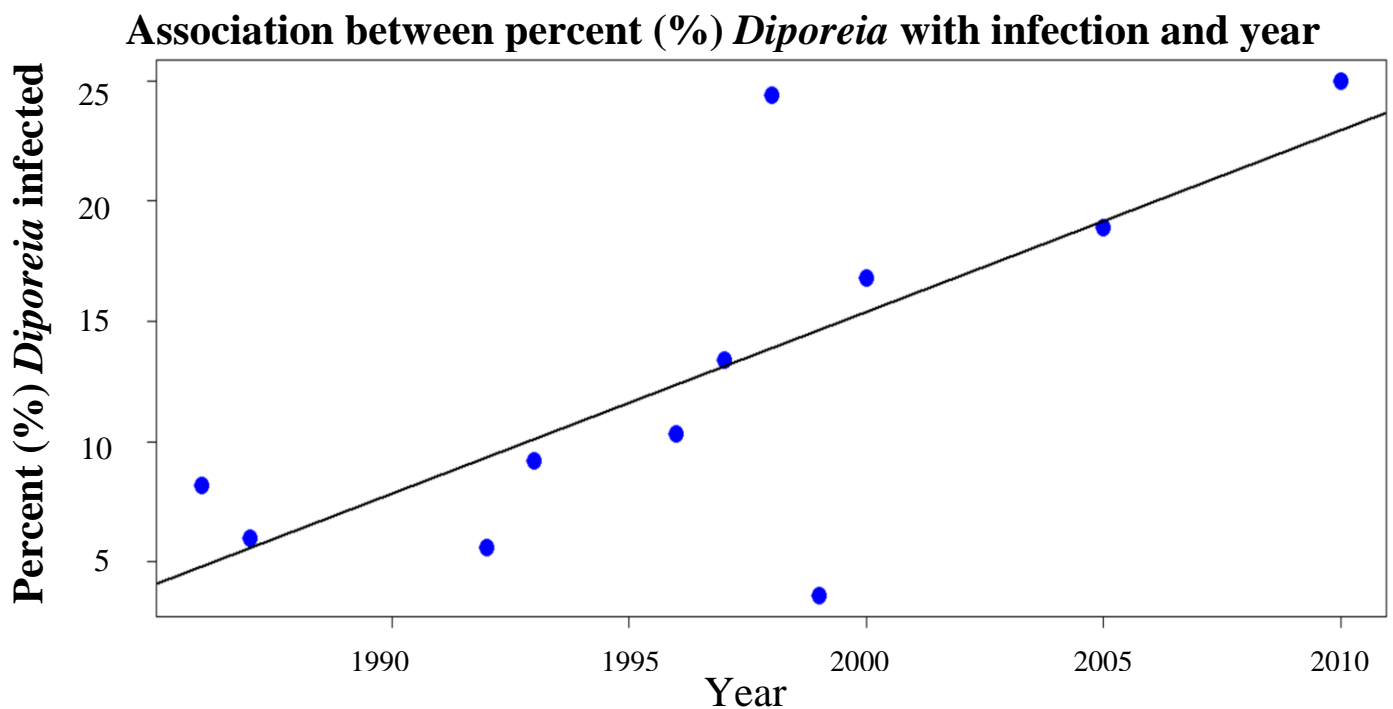
*Diporeia* that had pathogenic infections were further categorized into those that had (1) basophilic masses or bodies, *verse* those that had (2) budding structures. In 2005, ~63.60% (Table 2) of *Diporeia* exhibiting an immune response had basophilic bodies or masses, with the remaining being associated with budding structures. In 2010, ~71.40% (Table 2) exhibited basophilic bodies or masses.

**Table 2.** Type of Response found in infected *Diporeia* samples.

Year	Number with immune response	Percent with basophilic masses or bodies	Percent with budding structures	Percent with both structures
2005	11	63.60%	27.30%	0.00%
2010	7	71.40%	0.00%	14.28%

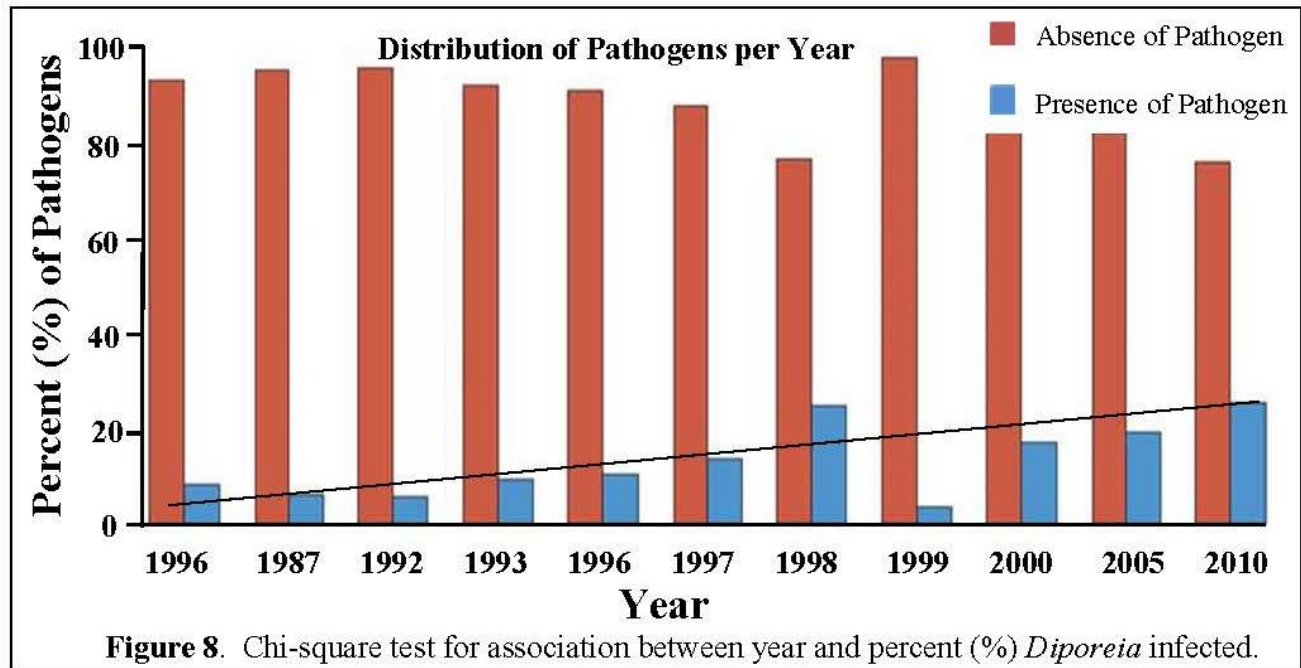
Figure 7 displays a linear regression model using year as predictor for percent pathogens found in *Diporeia* tissue. There is a significant positive linear association between percent of *Diporeia* exhibiting a pathogenic infection or immune response and year ( $r = .0.7202264$ ,  $p\text{-value} \leq 0.0124$ ).

However, due to small sample size for some years, one needs to be cautious in analysis.



**Figure 7.** Linear regression with year as predictor for percent (%) *Diporeia* infected

Alternatively a Chi-square test for independence, (Fig. 8) was also used to test if there was an association between year and percent pathogens. Values obtained were  $X^2 = 50$ ,  $df = 10$ ,  $p\text{-value} \leq 0.0001$ , implying significant association between year and pathogen incidence.



## V. DISCUSSION

The population in Batchawana Bay remained unaffected by the introduction of Zebra mussel and has had a slight increase in abundance over the same period of time. A population density of 285 *Diporeia* m<sup>-2</sup> in Batchawana Bay follows a basic trend/average where the natural population for that locality of *Diporeia* continues to exhibit stabilized growth since the 1970's. This is an interesting observation as the *Diporeia* populations elsewhere have declined.

The microscopy data from 2005 and 2010 compared with prior tissue analysis show an overall increase in the prevalence of pathogens found in *Diporeia* since 1986. This is apparent in both the linear regression model (Fig. 7) and the chi-square test analysis (Fig. 8). This trend is

consistent with the hypothesis that the invasion of zebra mussels in the Great Lakes has caused *Diporeia*'s population crash. Zebra and quagga mussels invaded the Great lakes in the late 1980's (Nalepa et al. 1998); *Diporeia* populations have crashed in most areas since that time. The data also shows an overall increase in pathogens found in *Diporeia* tissue since the introduction of the AIS zebra mussel, suggesting that competition for food may have been a secondary effect caused by the primary effect, namely disease. The correlation between population decline and increase in pathogenic infection and immune response over time supports this hypothesis.

Although most areas in the Great Lakes have experienced a decline in *Diporeia* populations since the introduction of zebra mussels, there are some locations that have not been affected by declining populations. Isolated areas of Lake Michigan and Lake Huron still support minimal *Diporeia* populations (Nalepa et al. 1998). Lake Superior's population of *Diporeia* has remained largely unchanged, as supported by our sampling of *Diporeia* at Batchawana Bay. This suggests that the population crashes observed elsewhere may be attributed to multiple factors including pathogenic infection. However, it is also possible that the greater depths of Lake Superior have provided a "safe-haven" compared to other shallower areas that *Diporeia* typically inhabit. Another possibility is that the *Diporeia* populations in Lake Superior may have naturally adapted over time or through mutation from breeding with other healthier populations and have developed some type of immunity (or tolerance) to the specific diseases that are causing population crashes elsewhere; tolerance may be a better description than immunity as it is well known that "shrimp" lack an adaptive immune system.

It should also be noted that the data for the years 2005 and 2010 were obtained from *Diporeia* collected from three specific locations in Lake Michigan. The data prior to 2005 was

collected from Lake Michigan and Lake Huron *Diporeia*. This could possibly be a source of error, however, the populations in both of these regions experienced similar trends in decline and both had similar exposures to zebra mussels.

Lastly, it is possible that the budding structure found in the tissue may not be pathogenic and/or may inhabit *Diporeia* tissue as a commensal (Messick et al. 2004). As a consequence, more studies are needed to confirm speculation. Because the identity of these budding structures is unknown, it should not be assumed that they are necessarily harmful to *Diporeia*. Future research needs to specifically identify what these budding structures are and whether they are “infecting” *Diporeia* tissue and having a negative consequence.

## VI. CONCLUSION

Analyses in this study have shown a significant increase in pathogenic infection and immune-type response since the invasion of zebra mussels in 1986. This suggests that zebra and possibly quagga mussels may have acted as a vector for pathogen(s) that infected *Diporeia*. Some inconsistencies exist with this hypothesis, however. Healthy *Diporeia* populations have remained steady since the invasion of zebra and quagga mussels in certain areas. It is apparent that an increase in pathogenic infection in *Diporeia* tissue, however, does have a significant role in the population abundance and overall health. Future research should involve specifically identifying these pathogens and how they are affecting *Diporeia* physiology. In addition, zebra and quagga mussel tissues should be analyzed for similar pathogens that have been found in *Diporeia* tissue.

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