

**ECOLOGICAL IMPLICATIONS AND IDENTIFICATION OF A  
FRESHWATER RED ALGA CHEMICAL DEFENSE**

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**ECOLOGICAL IMPLICATIONS AND IDENTIFICATION OF A  
FRESHWATER RED ALGAE CHEMICAL DEFENSE**

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## SUMMARY

Aquatic generalist herbivores feed selectively when offered several species of macrophytes, preferring more nutritious macrophytes and those with minimal chemical or physical defense. This selective feeding plays an important role in structuring plant communities, sometimes completely altering communities of aquatic plants. While it is known that higher plants in freshwater systems produce chemical defenses against herbivores, the chemical deterrents themselves have rarely been identified, and none are known for freshwater red algae. *Batrachospermum helminthosum*, a freshwater red alga, exhibits evidence of chemical deterrents to feeding by crayfish. In this experiment, we evaluated whether crayfish unwillingness to consume *B. helminthosum* was due to morphological or structural feeding deterrents by drying, powdering, reconstituting the alga into sodium alginate gel pellets and offering these, versus pellets made with a different, palatable alga to crayfish. Crayfish consumed pellets made from the green alga *Cladophora glomerata* in preference to pellets made from *B. helminthosum*, suggesting that chemical rather than structural traits were responsible for crayfish selective feeding. Bioassay-guided fractionation and the application of proton NMR, carbon-13 NMR, liquid chromatography mass spectrometry (LCMS), and high resolution mass spectroscopy were used to attempt to isolate and identify the chemical compounds responsible for *B. helminthosum* herbivore defenses. We found evidence of several chemical deterrents ranging from polar to non-polar. Candidate molecular weights for one or two compounds in the most purified, active fraction were 339.5 and 268.2.

# CHAPTER 1

## BACKGROUND

Herbivory plays a significant role in determining macrophyte biomass, species composition, and relative abundance in aquatic ecosystems (Lodge et al. 1998, Hillebrand 2008), and rates of herbivory in freshwater ecosystems are at least as great as in more commonly studied marine and terrestrial ecosystems (Cyr and Pace 1993, Feminella and Hawkins 1995, Hillebrand 2009). Previous studies argue that short life spans and fast growth rates allow freshwater macrophytes to grow quickly enough to nullify the effects of selective pressures produced by aquatic herbivory (Steinman 1996). There is currently debate over the relative strength of selection pressures produced by the physical environment versus trophic selective pressures on the composition of algae in aquatic communities. However, aquatic generalist herbivores have been shown to feed selectively on several species of macrophytes preferring high nutrient sources and macrophytes with minimal chemical or physical defense (Hay 1994, Hay 1996, Cronin et al. 2002, Hay 2009). This selective feeding contributes substantially to aquatic food web structure and can alter aquatic ecosystem to dominance by unpalatable plants (Cronin et al. 2002, Parker et al. 2006, Parker et al. 2007).

Several generalist grazers consume freshwater red algae (Rhodophyta) (Sheath and Hambrook 1990, Sheath 2003). Freshwater red algae was found in the guts of 39 stream-dwelling animals including 26 aquatic insects, 9 amphipods, 2 snails, and 2 Asian carp (Sheath and Hambrook 1990, Sheath 2003). These data show evidence of freshwater red algae consumption yet provide limited information to the extent or importance of

these trophic interactions to the algae or the herbivores. This indication of consumption along with the evidence of selective feeding on nutrient rich, less-defended macrophytes signifies substantial importance for the study of herbivore-macrophyte relationships and their role in affecting the structure of aquatic community food webs.

While it is well known that freshwater macrophytes produce and utilize chemical defense strategies to deter herbivory (Bolser et al. 1998, Prusak et al. 2005, Parker et al. 2006, 2007, Morrison and Hay 2011, 2012, Goodman and Hay 2012), these chemical deterrents have been largely unexplored, especially in freshwater red algae (Goodman and Hay 2012). In fact, only twenty three freshwater vascular plants and one aquatic moss have shown evidence of producing a herbivore deterrent chemical defense (reviewed in Morrison and Hay 2011). Past studies on marine red algae suggest marine red algae may produce the highest variety and quantity of herbivore defense compounds (Blunt et al. 2009, Stout and Kubanek 2010). The evidence for the high abundance of chemical defenses in marine red algae suggests there may be a high abundance of defenses utilized by freshwater red algae as well. There has only been a single study on freshwater red algae chemical defenses. The study indicated the presence of chemical defenses in freshwater red algae, however, the defense was not isolated or identified (Goodman and Hay 2012).

Freshwater red algae occur over broad geographic regions and can constitute considerable aquatic plant biomass which implies they could be a valuable food source for aquatic grazers (Sheath and Hambrook 1990). Algae tend to be more nutritious (greater protein and lipid content as well as lower C:N ratios) than detritus and vascular plants (Frost et al. 2002). Consequently, when the algae bloom in the spring, invertebrate

grazers tend to switch feeding from detritus and vascular plants to the more nutrient rich algae (Frost et al. 2002). Grazing pressure from this event can completely eradicate filamentous green algae from these communities. These studies imply that freshwater red algae are potentially a valuable food source for a variety of aquatic organisms (Sheath and Hambrook 1990, Frost et al. 2002).

Several studies have shown that macrophytes use many strategies such as physical defense, poor nutrient value, chemical defenses, and mixtures of these strategies to reduce pressures from grazing (Bolser et al. 1998, Hay et al. 1994, Hay 1996, Cronin et al. 2002, Hay 2009). While it has not yet been investigated, it is believed that freshwater red algae most likely use many or all of these strategies to deter grazers and become less palatable (Lodge et al. 1998).

## CHAPTER 2

### INTRODUCTION

The functional role of red algal chemical defenses in aquatic ecosystems is poorly investigated or understood (Morrison and Hay 2010, Goodman and Hay 2012). Red algal chemical defenses are potentially important components contributing to the structure of freshwater aquatic foodwebs (Frost et al. 2002, Sheath and Hambrook 1990, Sheath 2003), yet, they have been completely unexplored (Goodman and Hay 2012). This study attempted to isolate and identify red algal chemical defenses and then draw ecological conclusions about their function and importance in freshwater aquatic ecosystems. Bioassay-guided fractionation and well-known chemical separation methodologies were used to identify the responsible chemicals. Because freshwater red algae are an ecologically important food source, essential to understanding aquatic food web structure, if we understand the properties of these chemical defenses, we may be able to extrapolate ecological consequences of being chemically defended. Once a chemical defense is identified, it is easier to explore similar species making related compounds. Thus, results from this investigation may not only elucidate properties of the focus species, but also aid in investigation of related freshwater algae.

In this experiment we, 1) assessed the palatability of *B. helminthosum* versus the palatable green alga *C. glomerata* following the elimination of structural and morphological traits to evaluate the likely effects of chemical traits alone, 2) conducted bioassay-guided fractionations to isolate the chemical feeding deterrent, and 3) used

proton NMR, carbon-13 NMR, high resolution mass spectroscopy, and 2 dimensional NMR to try to identify the compound that we isolated.

## CHAPTER 3

### METHODOLOGY

All physical defenses differences were first eliminated to ensure the feeding assays performed would test exclusively for chemical or nutritional defenses. Macrophyte chemistry was extracted after first freezing the plant at -80°C to assist in lysing algal cells. The macrophyte was then thoroughly crushed with a mortar and pestle while in deionized water. This process mimicked grazing and theoretically activated any grazer induced chemical defenses (Cetrulo and Hay 2000). The macrophyte was then soaked in methanol for 2+ hours. After soaking, the methanol/plant-extract mixture was filtered and collected. This process was repeated until the methanol being collected was clear signifying most of the macrophyte chemistry has been extracted. The methanol was then removed *in vacuo* using a rotary evaporator and Savant speed-vac leaving completely dry macrophyte extract. A palatable alga (*C. glomerata*) was then freeze dried and powdered. The macrophyte extract was distributed on the powdered palatable alga at the extract to dry-mass ratio of the macrophyte being tested by mixing both in enough methanol to bring everything into solution. The methanol was then removed *in vacuo* using a rotary evaporator and Savant speed-vac. This was then made into sodium alginate gel pellets using a well-established method (see Goodman and Hay 2012). Control pellets were made from *C. glomerata* powder with solvent, but without the extract. Crayfish, *Procambarus clarkii*, were trained to accept food pellets from small forceps. A crayfish was first fed a single control pellet ensuring the crayfish were currently willing to feed. Next, a treatment pellet containing the chemical extract of the algae being assayed was

administered to the same crayfish. It was then recorded if the crayfish accepted or rejected the treatment pellet. If the crayfish ingested the pellet without spitting, it was considered to be an accepting response. If the crayfish attempted ingesting the pellet but then either spit or vomited the pellet, it was considered to be a rejection. If the crayfish did not attempt to ingest the pellet, or there was an unobvious response, the event was discounted and the crayfish was not used until the following day. Lastly, another control pellet was administered to the same crayfish to ensure the rejection event was not due to the crayfish being full or for any other misleading reason. These steps were repeated until there were ten total responses of accept or reject. Data were evaluated using a Fisher's exact test.

Rejection is highly distinctive and easy to identify. If crayfish do not accept both controls, the data were not used. The crayfish were kept on a feeding schedule that promotes willingness to feed during bioassays. However, they were fed enough to prevent significant hunger, as this may lead them to consume even chemically defended food items. The crayfish were kept on an almost purely herbivorous diet to eliminate a preference for high protein or highly nutritious food items.

Deterrent extracts were then fractionated using a variety of well-known chemical separation techniques including column chromatography with HP20SS or C18 silica gels, thick plate liquid chromatography, size exclusion column, liquid-liquid separation, or high performance liquid chromatography. The method of separation was determined by the content of the extract, the extracts' chemical properties, and its purity. After separation, the chemical fractions were each individually bioassayed using the feeding process described above to determine which fraction contained the chemical deterrent. Once the deterrent fraction was identified, it was further separated using the chemical

approaches listed above. Fractions from the parent fraction were again bioassayed at natural plant concentrations. Each fractionation process eliminated non-deterrent chemicals and further isolated the chemicals of interest. By repeating this process of separating the deterrent fractions and eliminating chemicals not responsible for a defensive property, eventually a relatively pure chemical deterrent was isolated. Once the chemical defenses were isolated, proton NMR, carbon-13 NMR, liquid chromatography mass spectrometry (LCMS), and high resolution mass spectroscopy were used to attempt identify the structure of the compound in question.

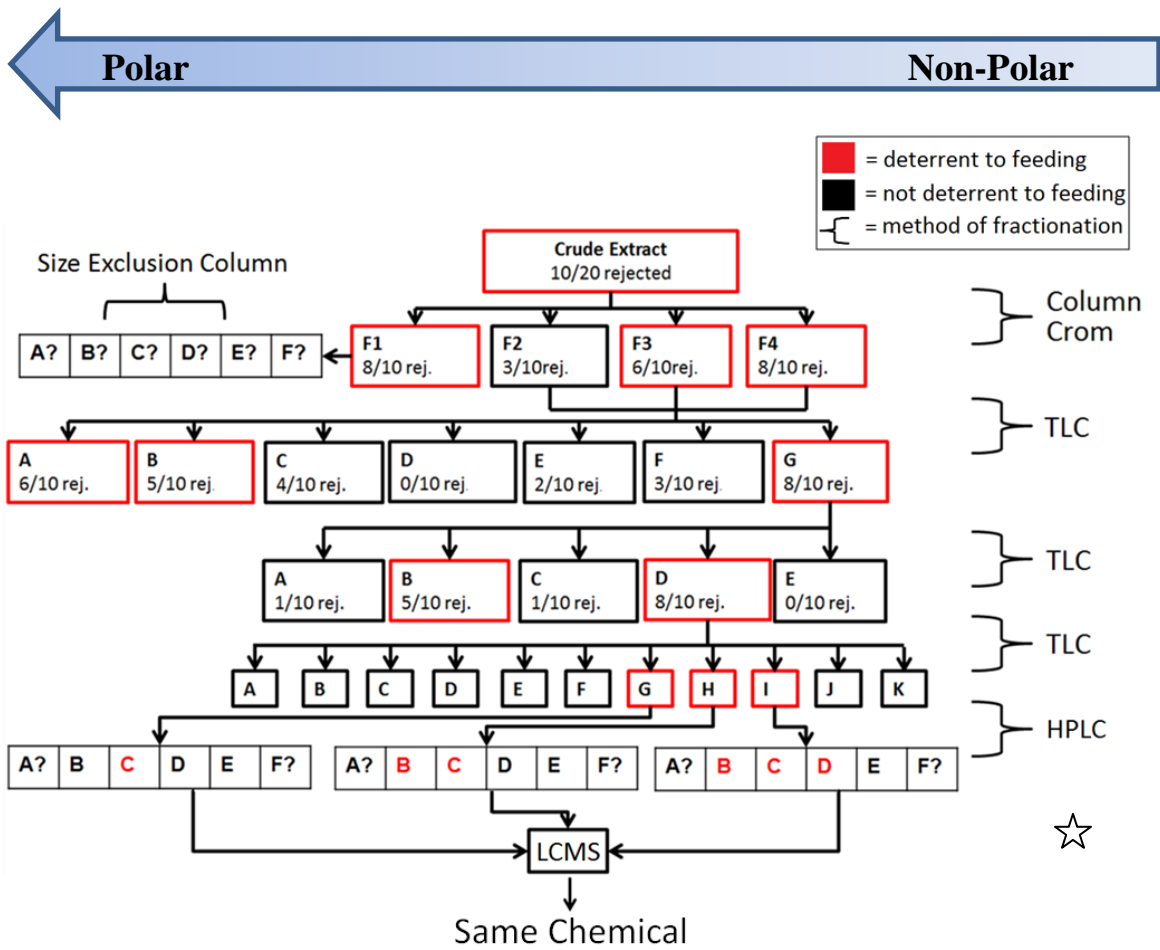
## CHAPTER 4

### RESULTS

We found evidence of several different chemical deterrents ranging from extremely polar to extremely non-polar. Data were gathered on one chemical deterrent that we were able to separate and purify to an acceptable degree. This compound of interest showed candidate molecular weights of 339.5 and 268.2. The presence of two mass peaks may mean that the active fraction has two different compounds in it, and is therefore not pure. These two peaks may also indicate that one of those masses, likely the greater mass of 339.5, represents the actual compound, and the smaller mass represents a fragment ion of that larger ion. If these two peaks represent a single compound, the fraction is acceptably pure. If this fraction is a mixture of two compounds, our mass spectrometry data do not indicate relative abundance of the compounds. Peak sizes are unreliable because different compounds ionize to different extents.

The proton NMR spectrum is weak due to a low abundance of the compound but the spectrum shows probable aromatic signals. Spectral analysis indicated the compound absorbing at  $\lambda_{\text{max}} = 244\text{nm}$  also suggesting the presence of aromatic groups. Figure 1, below, displays the scheme of bioassay-guided fractionation, results from feeding assays for each fraction, and relative polarities of deterrent fractions.

**Figure 1. Fractionation Scheme** - Each box represents a feeding event for a specific fraction of *B. helminthosum* crude extract in the bioassay-guided fractionation process. Numbers in the boxes indicate rejections of treatment pellets. If the treatment was rejected at least 5/10 times ( $P \leq 0.016$ , Fisher's exact test), the fraction was highlighted in red above, considered to contain a deterrent chemical, and then further fractionated.



*C. glomerata* pellets containing *B. helminthosum* extract at natural concentration were assayed with control pellets of *C. glomerata* containing no extract. As depicted in Figure 1, 10/20 crayfish rejected pellets containing the *B. helminthosum* extract while 0/20

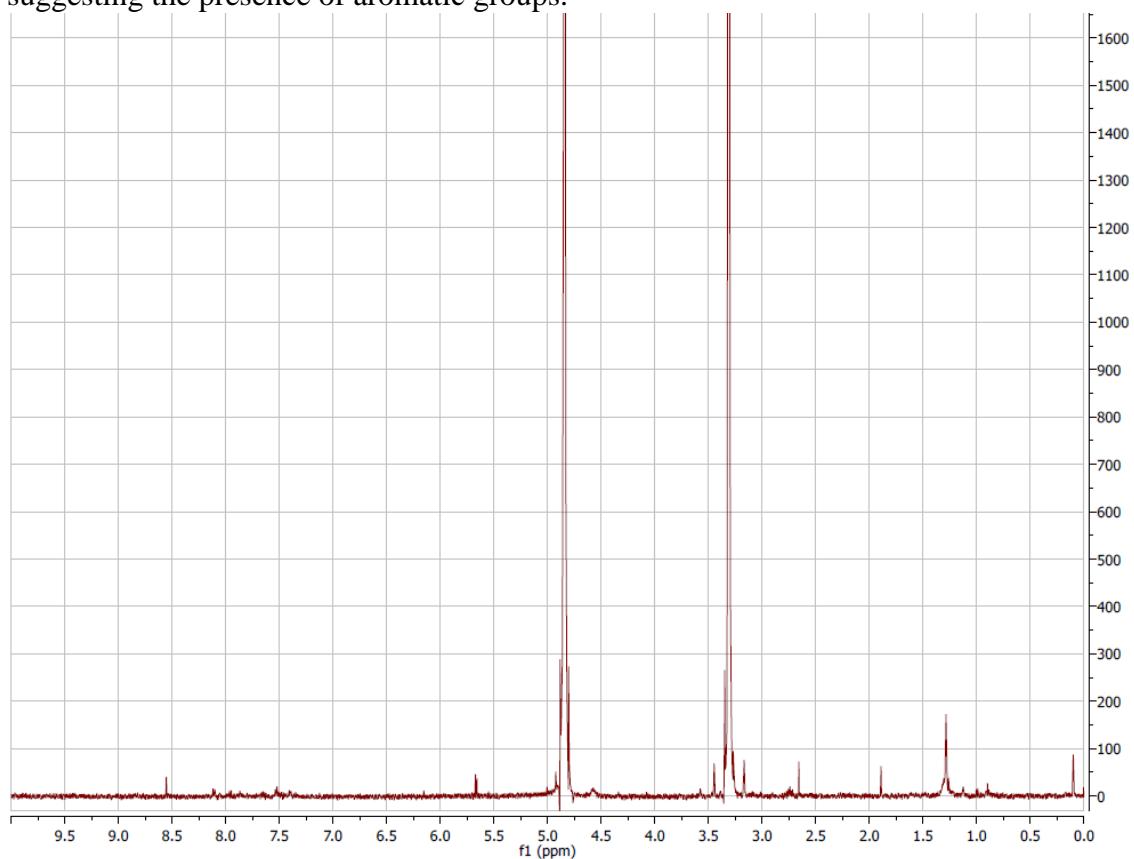
crayfish rejected the control pellets indicating the presence of chemical deterrents in *B. helminthosum* ( $P \leq 0.001$  Fisher's exact test). We then proceeded with fractionation.

LCMS of fractions F2, F3, and F4 indicated the fractions were very similar chemically. Since F3 and F4 exhibited a defense and F2, F3, and F4 were very similar, it was assumed that they all may contain a similar defense chemical. Therefore, F2, F3, and F4 were recombined before further fractionation to prevent the risk of diluting the chemical of interest.

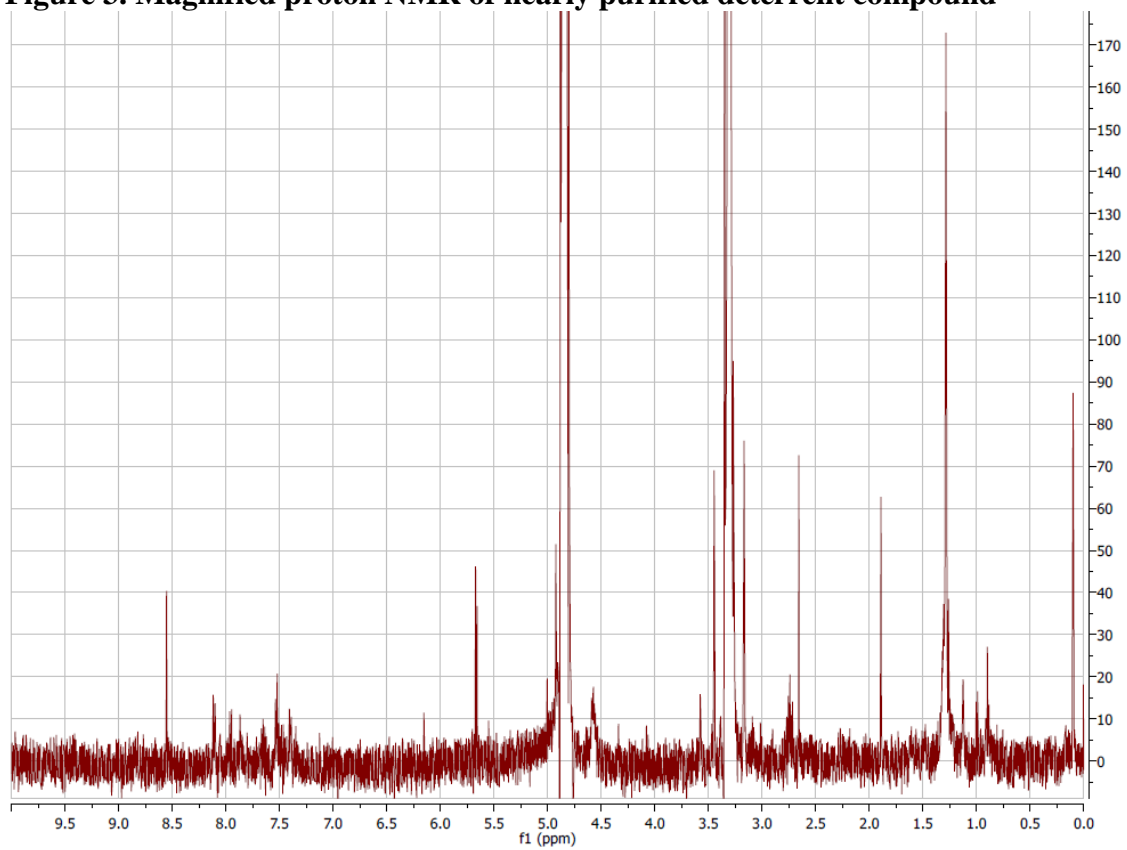
High performance liquid chromatography (HPLC) analysis of the bottom-most fractions in Fig. 1 (all fractions in the shaded row of Fig. 1) indicated the deterrent fractions contained extremely similar and relatively pure chemistry. These fractions were combined to preserve and combine as much of the chemical of interest as possible. HPLC was performed again and each large peak was collected separately and then bioassayed. One of these fractions produced 8 rejections for the 10 crayfish assayed; none of the 10 crayfish rejected control pellets ( $P < 0.001$ , Fisher's exact test). HPLC analysis showed the vast majority of the fraction eluting at a single peak ( $\lambda_{\text{max}} = 244\text{nm}$ ). We further purified the rejected fraction via HPLC by collecting only the peak of interest. After purifying the chemical, proton NMR (Fig. 2) and high resolution mass spectroscopy (Fig. 3) were used to obtain information on the structure of the active compound.



**Figure 2. Proton NMR of nearly purified detergent compound** - The spectrum is weak due to a low abundance of the compound but the spectrum shows probable aromatic signals. Spectral analysis indicated the compound absorbing at  $\lambda_{\text{max}} = 244\text{nm}$  also suggesting the presence of aromatic groups.

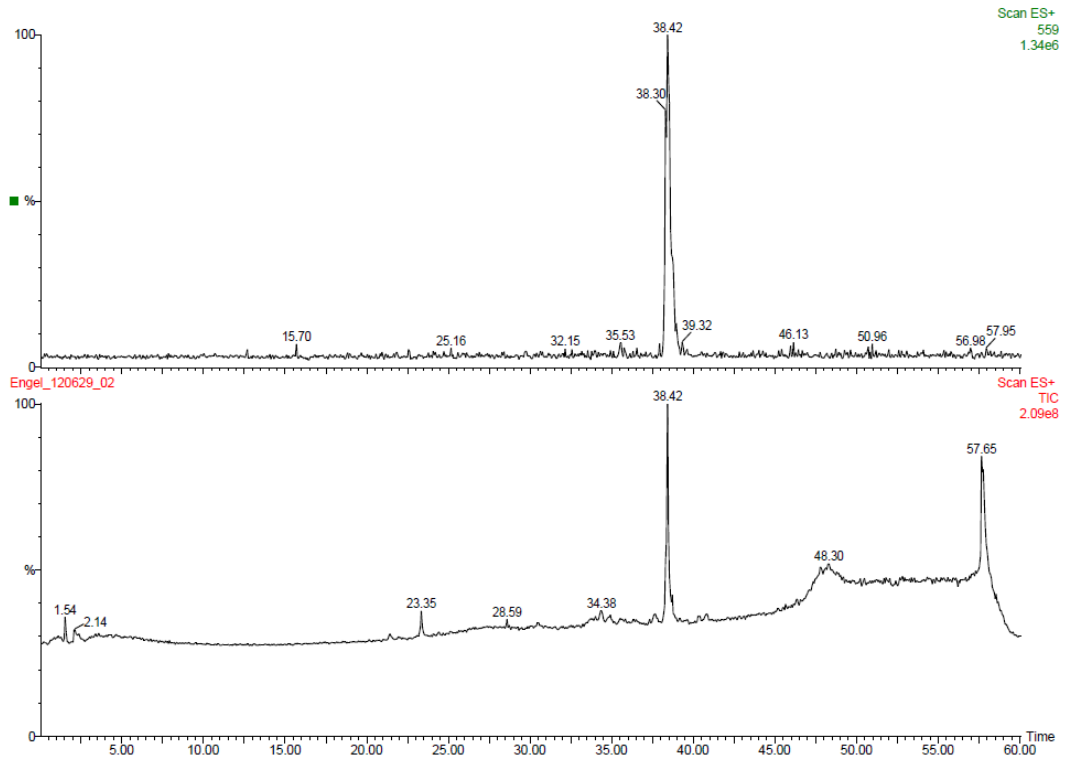


**Figure 3. Magnified proton NMR of nearly purified detergent compound**



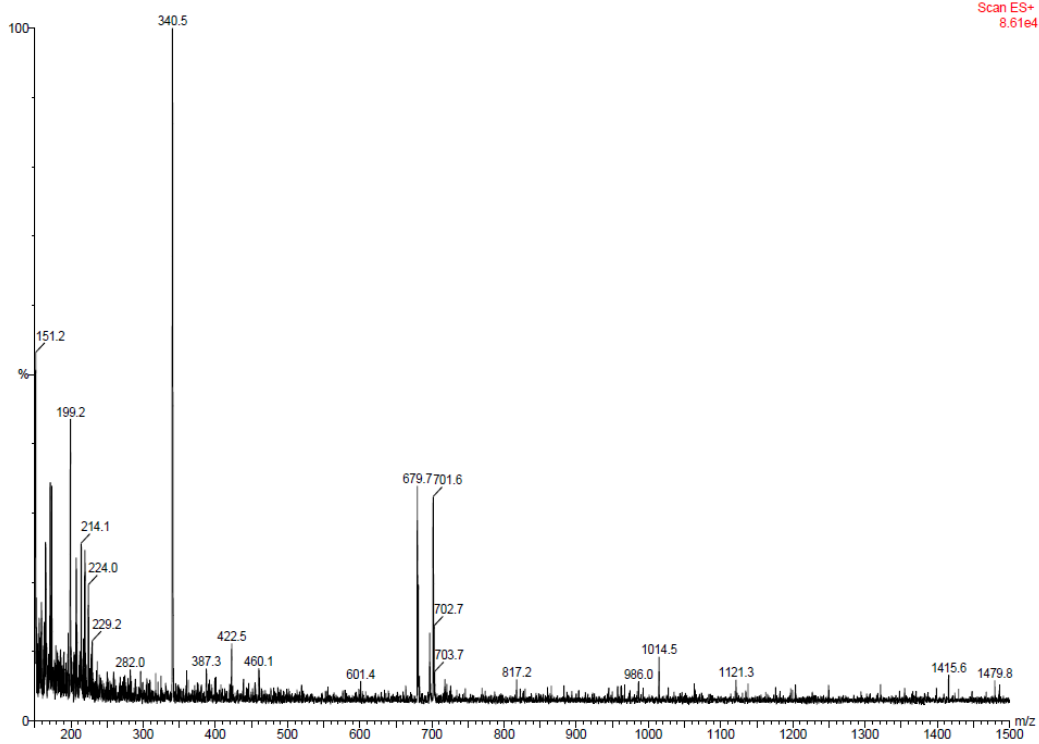
**Figure 4. Mass Spectrometry data of sample** – The compound of interest is most likely responsible for the peaks eluting at either 38.42 min. or 23.35min. The compound therefore has candidate molecular weights of or 268.2 or 339.5 respectively.

GT Mass Spectrometry Laboratory



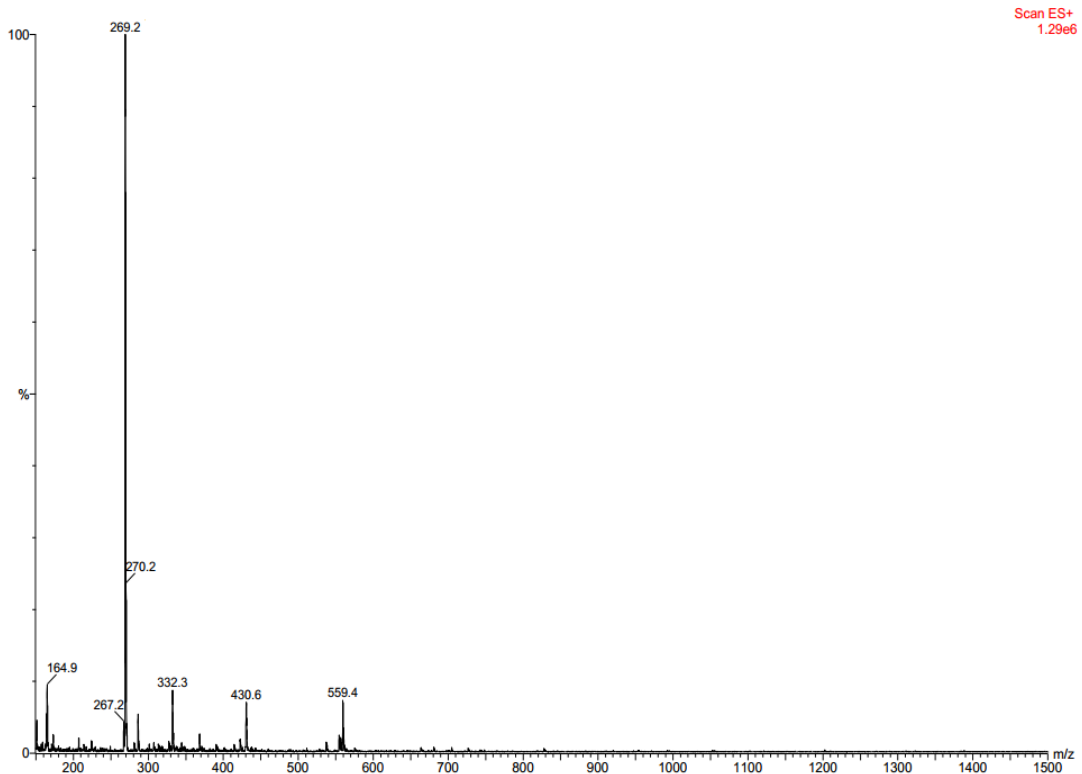
**Figure 5. Ion masses responsible for the peak eluting at 23.35min.**

GT Mass Spectrometry Laboratory



**Figure 6. Ion masses responsible for the peak eluting at 38.42min.**

GT Mass Spectrometry Laboratory



## CHAPTER 5

### DISCUSSION

During the fractionation process, some of the sample was lost to LCMS, mass spectrometry, and feeding assays. Based on our yields from our extraction, the compound naturally exists at low concentrations in the macrophyte. For these reasons, after purification of the one of the compounds, we were left with less than a microgram of the major active compound. This was not enough for a carbon-13 NMR which would be necessary to elucidate a chemical structure. To completely confirm this chemical is responsible for a chemical defense, the remaining sample was bioassayed. Because we were left with less than a microgram, the bioassay was performed using far less of the chemical than would naturally exist in *B. helminthosum*. The bioassay yielded 5/10 rejections of treatment pellets with none of the 10 crayfish rejecting control pellets suggesting the presence of a chemical defense (  $P = 0.016$ ). Since we were left with less than a microgram of the compound, and because we had to make enough treatment pellets to perform a feeding assay, this bioassay was performed at far below the natural concentration of the chemical. If the chemical were tested at natural concentration, the rejection likely would be more pronounced.

Using the data gathered, we hope to eventually isolate more of the compound and elucidate its structure. Future studies should attempt to isolate chemicals responsible for deterrent activity in the other fractions.

Once a chemical defense is isolated and the structure of the chemical is elucidated, we can look for the presence of the compound or similar compounds in a variety of freshwater red algae. Many red algae may use a similar chemical defense or a completely

different defense. With elucidation of the chemical structure, we may be able to draw inferences about the specific function of the molecule and determine if it simply tastes bad to herbivores, or if the molecule is a defense by some other interesting or ecologically important mechanism.

Bioassays confirmed crayfish feeding preference for the green alga *C. glomerata* over the red alga *B. helminthosum* with all structural and morphological traits eliminated. A previous study showed *B. helminthosum* to be nutrient deficient when compared to *C. glomerata* (Goodman and Hay 2012). However, here we assayed *B. helminthosum* extract on *C. glomerata* plant material eliminating nutrient content as a variable. With nutrient content between treatment and control pellets being nearly identical, our bioassays still confirmed preference for *C. glomerata* pellets without *B. helminthosum* extract present. Therefore, our data indicate preferential feeding on the green alga *C. glomerata* over the red alga *B. helminthosum* due to, at least in part, chemical defenses in *B. helminthosum*. Low palatability due to nutritional inadequacy and chemical deterrents are possibly used synergistically by *B. helminthosum* to deter herbivory similar to what has been shown for many marine macrophytes (Hay 1996, 2009, Cruz Riveras and Hay 2003).

Bioassay-guided fractionation revealed the likelihood of at least four deterrent compounds ranging broadly in polarity. Similar to what we found in this study, previous studies have indicated that freshwater macrophytes contain complex mixtures of both lipophilic and water-soluble chemical defenses (Bolser et al. 1998, Kubanek et al. 2001). These complex mixtures of compounds suggest a complex mediation of plant-herbivore interactions.

The presence of both lipophilic and more water soluble compounds imply a diverse array of chemical defenses, possibly for diverse functions. Goodman and Hay (2012) demonstrated the likely presence of activated defenses in *B. helminthosum*. Perhaps this complex mixture of defenses plays a role in defense activation. It is well known that many plants store water soluble secondary metabolites that often function as allelochemicals or herbivore defense compounds inside their vacuoles (Wink 1993). Many of these chemicals are toxic to the plants themselves, and only released upon cell lysis (Wink 1993). Furthermore, it may be beneficial to produce both lipophilic and water soluble defense. In an aquatic system, lipophilic compounds may act as better defenses present on the exterior of the macrophyte as they would be far less likely to elute into the water column. More water-soluble chemicals would most likely stored inside the macrophyte and perhaps inside cell vacuoles (Wink 1993). In this way, a macrophyte could contain a water-soluble chemical defense without constantly losing it to the water column. Future studies of freshwater chemical defenses could examine the location of these chemical deterrents in and on the surface of freshwater macrophytes to lend understanding to the functional role of this diverse suite of chemical deterrents.

The high prevalence and diversity of freshwater macrophyte chemical defenses (Bolser et al. 1998, Kubanek et al. 2000, 2001, Cronin 2002, Prusak et al. 2005, Goodman and Hay 2012) suggest grazing pressure in freshwater systems is significant enough to select for chemically defended macrophytes. This implies grazing pressures in freshwater systems have been ecologically important over evolutionary time scales for freshwater macrophytes.

Freshwater red algae are an ecologically important food source and refuge for many aquatic animals and essential to understanding aquatic food web structure. If we understand the properties of these chemical defenses, we may be able to extrapolate ecological consequences of being chemically defended. Once a chemical defense is identified, it is much easier to find in other algae. We hope to find similar chemical defenses in other plants. This way we can begin to use our results to contribute to the understanding of entire freshwater aquatic systems.

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