4-16-2014

Mosquito Larvicides from Cyanobacteria

Gerald A. Berry

Florida International University, jerryaberry@aol.com

DOI: 10.25148/etd.FI14071114

Follow this and additional works at: <https://digitalcommons.fiu.edu/etd>

Recommended Citation

Berry, Gerald A., "Mosquito Larvicides from Cyanobacteria" (2014). *FIU Electronic Theses and Dissertations*. 1449.
<https://digitalcommons.fiu.edu/etd/1449>

This work is brought to you for free and open access by the University Graduate School at FIU Digital Commons. It has been accepted for inclusion in FIU Electronic Theses and Dissertations by an authorized administrator of FIU Digital Commons. For more information, please contact dcc@fiu.edu.

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

MOSQUITO LARVICIDES FROM CYANOBACTERIA

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Gerald A. Berry

2014

To: Interim Dean Michael Heithaus
College of Arts and Sciences

This dissertation, written by Gerald A. Berry, and entitled Mosquito Larvicides from Cyanobacteria, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

Evelyn Gaiser

Miroslav Gantar

Kathleen Rein

John P. Berry, Co-Major Professor

Fernando G. Noriega, Co-Major Professor

Date of defense: April 16, 2014

The dissertation of Gerald A. Berry is approved.

Interim Dean Michael Heithaus
College of Arts and Sciences

Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2014

DEDICATION

This dissertation is dedicated to my family for supporting me in this long endeavor and to the Universe for being such a fascinating venue inspiring me to learn all that I can.

ACKNOWLEDGMENTS

I would like to thank Dr. Pat Gibbs for his efforts in the DNA analysis. I thank Mayra Exposito for instructions in regards to IR operations. I thank Dr. Wentian Wang for advice and all around help. I thank my committee Dr. Evelyn Gaiser, Dr. Miroslav Gantar, Dr. Kathleen Rein, and Dr. Fernando G. Noriega for their instrumental guidance in the design and overall direction of my dissertation. I would also like to thank members of the Noriega laboratory (Dr. Mario Perez and Dr. Marcela Nouzova) for guidance in regards to rearing mosquito larvae and overall assay setup. Lastly, I would like to thank Dr. John P. Berry and laboratory members (Dr. Asha Jaja, Katherine Walton, and Dalton Steel) for efforts far too numerous to mention here.

ABSTRACT OF THE DISSERTATION
MOSQUITO LARVICIDES FROM CYANOBACTERIA

by

Gerald A. Berry

Florida International University, 2014

Miami, Florida

Professor John P. Berry, Co-Major Professor

Professor Fernando G. Noriega, Co-Major Professor

Cyanobacteria (blue-green algae) produce a diverse array of toxic or otherwise bioactive metabolites. These allelochemicals may also play a role in defense against potential predators and grazers, particularly aquatic invertebrates and their larvae, including mosquitoes. Compounds derived from cyanobacteria collected from the Florida Everglades and other Florida waterways were investigated as insecticides against the mosquito *Aedes aegypti*, a vector of dengue and yellow fever. Screening of cyanobacterial biomass revealed several strains that exhibited mosquito larvicidal activity. Guided via bioassay guided fractionation, a non-polar compound from *Leptolyngbya* sp. 21-9-3 was found to be the most active component. Characterization revealed the prospective compound to be a monounsaturated fatty acid with the molecular formula $C_{16}H_{30}O_2$. This is the first evidence of mosquito larvicidal activity for this particular fatty acid. With larvicidal becoming more prevalent, fatty acids should be explored for future mosquito control strategies.

TABLE OF CONTENTS

CHAPTER	PAGE
Chapter 1: Background	1
1.1 Introduction.....	1
1.2 Mosquitoes as disease vectors	1
1.3 Targeting aquatic mosquito larvae instead of aerial adults.....	3
1.4 The need for novel insecticides.....	4
1.4.1 Inadequacies of pyrethroids	4
1.4.2 Human risk to pyrethroids	6
1.4.3 Insect resistance to pyrethroids.....	6
1.5 Cyanobacteria as a potential source for arthrocidal compounds	8
1.5.1 Introduction.....	8
1.5.2 Chemical defense against planktivores.....	8
1.5.2.1 Apparency theory.....	8
1.5.2.2 Quantitative defenses of <i>Microcystis aeruginosa</i> against <i>Daphnia</i>	9
1.6 Cyanobacteria as a potential source for mosquito larvicidal compounds.....	10
1.6.1 Cyanobacteria and mosquito sympatry.....	10
1.6.2 Grazer choice of food within periphyton	11
1.6.3 Mosquito larvae feeding on periphyton	12
1.6.4 Cyanobacteria: Novel insecticides and candidates for biological control agents	13
1.6.5 Effects of anatoxin-a and microcystin-LR on mosquito larvae	15
Chapter 2: Mosquito Larvicidal Assay	17
2.1 The larval developmental stage of choice.....	17
2.2 Development of the mosquito larvicidal assay	17
2.2.1 Treatments and resuspensions.....	17
2.2.2 Mosquito larvae: Egg production and handling of larvae.....	19
2.2.3 Evaluation of mosquito larvae	21
2.2.4 Evaluation of negative controls and resuspensions on mosquito larvae.....	21
2.2.5 Evaluation positive controls on mosquito larvae	23
Chapter 3: Bioassay Guided Fractionation	26
3.1 Background.....	26
3.2 Bioassays.....	26
3.3 Fractionation	27
3.4 Characterization	28
3.5 Proof of Principle.....	29
3.5.1 Introduction.....	29
3.5.2 Original purification of sulfated glycolipids.....	29
3.5.3 Re-purification and bioassay guided fractionation of sulfated glycolipids ..	31
3.5.4 Identification of re-purified of sulfated glycolipids.....	31
3.5.5 Discussion	32

Chapter 4: Isolation and Characterization of Mosquito Larvicidal Compounds from Cyanobacteria	34
4.1 Introduction.....	34
4.2 Methods.....	35
4.2.1 Screening of cyanobacterial isolates	35
4.2.2 Screening of cyanobacterial biomass.....	35
4.2.3 Bioassay guided fractionation of 3 prospective larvicidal cyanobacteria....	35
4.2.4 Purification of larvicidal compounds from <i>Leptolyngbya</i> sp. 21-9-3	37
4.2.5 Characterization of larvicidal compounds from <i>Leptolyngbya</i> sp. 21-9-3 ..	42
4.2.5.1 Liquid chromatography – mass spectrometry.....	42
4.2.5.2 Nuclear magnetic resonance	42
4.2.5.3 Infrared spectroscopy.....	43
4.2.5.4 Double Bond Localization	43
4.2.5.4.1 Derivatization.....	44
4.2.5.2 Gas Chromatography-Mass Spectrometry	45
4.3 Results.....	45
4.3.1 Screening of cyanobacterial isolates.....	45
4.3.2 Screening of cyanobacterial biomass.....	46
4.3.3 Bioassay guided fractionation of <i>Cocoid</i> sp. 53-2a, <i>Synechococcus</i> sp. 36-8, and <i>Leptolyngbya</i> sp. 21-9-3	46
4.3.4 Purification of larvicidal compounds from <i>Leptolyngbya</i> sp. 21-9-3	48
4.3.5 Characterization of larvicidal compounds from <i>Leptolyngbya</i> sp. 21-9-3 ..	50
4.3.5.1 Liquid chromatography – mass spectroscopy.....	50
4.3.5.2 Nuclear magnetic resonance	55
4.3.5.3 Infrared spectroscopy.....	68
4.3.5.4 Double Bond Localization	70
4.4 Structure determination larvicidal compounds from <i>Leptolyngbya</i> sp. 21-9-3	71
4.5 Sequencing of the 16s rRNA gene of <i>Leptolyngbya</i> sp. 21-9-3	77
4.5.1 Nomenclature Issues of Cyanobacteria.....	77
4.5.2 Methods.....	79
4.5.2.1 DNA Isolation and Purification	79
4.5.2.2 Gel Electrophoresis.....	80
4.5.2.3 Amplification of the 16S rRNA gene and Sequencing.....	80
4.5.2.4 Evaluation of sequences.....	81
4.5.3 Results.....	81
Chapter 5: Toxicological Analyses.....	84
5.1 Introduction.....	84
5.2 Methods.....	85
5.2.1 Mosquito larvicidal assay	85
5.2.1.1 Statistical analysis.....	85
5.2.1.2 Assay setup	85
5.2.2 Zebrafish assay.....	86
5.2.2.1 Rearing and breeding.....	86
5.2.2.2 Assay setup	87

5.2.3 Brine shrimp assay	88
5.2.3.1 Hatching.....	88
5.2.3.2 Assay setup	88
5.2.4 Bacterial assay	88
5.3 Results.....	89
5.3.1 Mosquito larvicidal assay	89
5.3.2 Zebrafish assay.....	95
5.3.3 Brine shrimp assay.....	95
5.3.4 Bacterial assay	97
5.4 Discussion.....	98
Chapter 6: Conclusions and Significance.....	100
6.1 Conclusions.....	100
6.1.1 Project Summary.....	100
6.1.2 Potential roles of unsaturated fatty acids in cyanobacteria.....	100
6.2 Significance.....	104
6.2.1 Novelty.....	104
6.2.2 Evaluation of the potential environmental effects of using unsaturated fatty acids to control mosquito populations	105
6.2.3 Palmitoleic acid as a mosquito control agent.....	108
Appendix 1: Cyanobacteria screened for mosquito larvicidal activity	110
Literature Cited	112
VITA.....	124

LIST OF FIGURES

FIGURE	PAGE
1. Distribution of dengue. Figure provided by WHO.....	2
2. Distribution of yellow fever. Figure provided by WHO.....	2
3. Well plate design.....	18
4. Non-polar extracts formed inedible precipitates when not re-suspended in solvent.. ..	22
5. Chemical structures of the control treatments.	24
6. (A) Bioassay results for polar controls anatoxin-a and microcystin-LR. (B) Bioassay results for the non-polar control DDT.	25
7. Sulfated glycolipids purified from <i>Synechococcus</i> sp. 21-10a	30
8. Mass spectra of prospective SGL-2 using negative ionization.	33
9. Mass spectra of prospective SGL-3 using negative ionization.	33
10. Chromatogram of the non-polar fractions derived from <i>Leptolyngbya</i> sp. 21-9-3 using a sloped MeOH gradient.	37
11. Chromatogram of the non-polar fractions derived from <i>Leptolyngbya</i> sp. 21-9-3 using a 65% isocratic acetonitrile gradient with H ₂ O in 0.1 % formic acid.	39
12. Chromatogram using the new biomass.	40
13. Chromatogram using a semi-preparative column.	41
14. Results of the cyanobacteria biomass screening. Red arrows.....	46
15. Simplified fractionation scheme for <i>Cocoid</i> sp. 53-2a.....	47
16. Chromatogram of the non-polar fractions derived from <i>Leptolyngbya</i> sp. 21-9-3 using a sloped MeOH gradient.	49
17. Chromatogram of the non-polar fractions derived from <i>Leptolyngbya</i> sp. 21-9-3 using a 65% isocratic acetonitrile gradient with H ₂ O in formic acid.....	49

18. Mass Spectra of (1) using positive ionization.....	50
19. Mass spectra of (2) using positive ionization.	51
20. Mass spectra of (2) derived from the new biomass using positive ionization	52
21. Mass spectra of (2) derived from the new biomass using a hot sleeve at 30 °C and positive ionization.....	54
22. High resolution mass spectra of (2) derived from the new biomass using a hot sleeve at 30 °C and negative ionization.....	55
23. Comparison of the isotope patterns from the simulation of C ₁₆ H ₂₉ O ₂ (top) and the mass spec data for ion 253.22 m/z (bottom).	56
24. ¹ H NMR spectra of (1) derived from the original biomass.....	57
25. ¹ H NMR spectra of (2) derived from the original biomass.....	58
26. ¹ H NMR spectra of (3) derived from the original biomass.....	58
27. ¹ H NMR spectra of (2) derived from the new biomass.	60
28. ¹ H NMR spectra of methyl elaidate. Figure provided by the AOCS lipid library.....	60
29. ¹ H NMR spectra with proton integration.	61
30. COSY spectra with coupling labeled.....	62
31. DEPT 135 spectra.	63
32. HMQC spectra	65
33. ¹³ C NMR derived from the HMQC that shows a closer view of the clustered area. ..	65
39. IR spectra of (2) analyzed as a solid.	70
40. DMDS addition reaction and potential observable ions with GC-MS. Scheme produced by Dunkelblum et al. (1984).....	71
41. GS-MS spectra of (2) after the derivatization procedure.....	72
42. Isotope simulation for C ₁₄ H ₂₇ ON ₃	73
43. Structure, mass, and isotopic values for palmitoleic acid.....	74

44. ^1H NMR spectra prediction (left) and ^{13}C NMR spectra prediction (right) of palmitoleic acid.....	75
45. Mass spectra of (3) using negative ionization.....	76
46. Results of the gel identifying the potential presence of genomic DNA.	82
47. Results of the gel identifying products from the PCR reaction.	82
48. DNA sequence amplified using primer CYA106F.....	83
49. DNA sequence amplified using primer CYA359F.....	83
50. Results of the probit analysis. Mortality was documented after 24 hours of initial exposure.	90
51. Comparison of dead larvae treated with 100 $\mu\text{g}/\text{ml}$ (top) and 5 $\mu\text{g}/\text{ml}$ (bottom). Pictures were taken 24 hours after initial exposure. Each large segment of the visible ruler is 1 mm (2.5 mm total are visible).....	92
52. Comparison of a control third instar (left) and a recently perished third instar from the 25 $\mu\text{g}/\text{ml}$ treatment after 7 days.....	94
53. Zebrafish assay after 24 hours. Two eggs failed to develop, one in the 20 $\mu\text{g}/\text{ml}$ treatment (left) and the other in the 35 $\mu\text{g}/\text{ml}$ treatments (right).	96
54. Zebrafish from the 35 $\mu\text{g}/\text{ml}$ treatments showing a slight curvature near the caudal peduncle (left) a normal zebrafish (right). The picture was taken on day four of the assay.....	96

Chapter 1: Background

1.1 Introduction

This chapter commences with the justification of controlling mosquito populations (section 1.2), targeting larvae instead of adults (section 1.3), and requiring novel insecticides (section 1.4). Section 1.5, a review of Berry et al. (2008), is a justification for the screening of cyanobacteria for arthrocidal compounds. Section 1.6 provides a justification for screening cyanobacteria for mosquito larvicidal compounds. Cyanobacteria are explored as a potential food source for mosquito larvae. Lastly, research conducted over the last two decades to identify mosquito larvicidal compounds from cyanobacteria is examined.

1.2 Mosquitoes as disease vectors

The adult female of the mosquito *Ae. aegypti* transmits dengue fever and yellow fever. Dengue fever is, after malaria, the second most prevalent mosquito-borne disease affecting human populations. Over a third of the world's population is at risk and every year 50-100 million people contract dengue fever (Figure 1). The World Health Organization (WHO) reports approximately 12,500 deaths annually most being children (WHO, 2012). The disease has been spreading and severe outbreaks have become more prevalent because vectors have been encroaching into new habitats expanding their ranges. There is currently no vaccine available for dengue fever. The virus exists as several serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) complicating the efforts for vaccine development (WHO, 2012).

Figure 1: Distribution of dengue. Figure provided by WHO.

A vaccine is available for yellow fever. However, the disease is still widespread in African and South American countries because many cannot afford the vaccine. Nearly 900 million people are at risk in tropical areas of Latin American and Africa (Figure 2). Cases have increased over the past two decades. Yellow fever is transmitted to about 200,000 people annually. Over 15% of cases result in mortality (WHO, 2013).

Figure 2: Distribution of yellow fever. Figure provided by WHO.

There is no cure for dengue fever or yellow fever. Once contracted, they can only be

treated with supportive therapy such as fluid replacement. The prevalence of these diseases demonstrates the enormous need worldwide for novel insecticides. With global warming, disease carrying strains could expand their ranges thus affecting more populations worldwide (Gage et al., 2008). Troubling evidence already exists of such a trend including the 2007 West Nile outbreak in Texas where 17 people died. The proceeding four years only had on average 4.75 annual deaths. However, in 2012, 89 deaths were attributed to West Nile with cases stretching across a majority of the state (Texas Department of State Health Services, 2013). Many cases of dengue have been reported in the Florida Keys. Most of these cases had been contracted in South America. However, that was not the case in the 2009-2010 outbreak. A recent study (Munoz Jordan et al., 2013), resulted in a particularly interesting find when examining 73 serum samples of suspected dengue patients from the outbreak. Specifically, these were the Monroe County samples of which 31 were confirmed as dengue virus infections via RT-PCR. None of these 31 patients had recently travelled outside the country. The authors found this strain of dengue to be genetically distinct enough from the Central American form of the virus that they classified it as a sublineage, the Key West DENV-1 strain.

1.3 Targeting aquatic mosquito larvae instead of aerial adults

Depending on the genus, mosquitoes can lay their eggs as rafts in stagnant bodies of water or they can be deposited directly onto moist substrates. Moist substrates may indicate opportunity for future inundation which triggers the eggs to hatch. Upon hatching, the resulting larvae spend much of their time near the surface because they must collect air through a breathing tube.

Mosquitoes, in their larval forms, are concentrated in pools of water increasing the effectiveness of insecticides compared to spraying for adult mosquitoes. Adult mosquitoes can have a flying range of 3 km in diameter from breeding sites (Hobbs et al., 1974; Lowe et al., 1975). Another benefit of controlling populations in the aquatic larval stage is that it reduces exposure to humans and other animals who can inhale aerially-applied insecticides (Harada et al., 2000).

Although aquatic-borne mosquito larvicides can potentially move into drinking water, they are mostly degraded by the time they reach human populations. The insect growth regulator (IGR) methoprene has a half-life of 2 days in water. The mammalian toxicity is relatively low, $LD_{50} = 5,000-10,000$ mg/kg in rats (Environmental Protection Agency [EPA], 2001). In contrast, some mosquito larvicides are a bit more hazardous such as organophosphates. Malathion is one of the safest organophosphates in regards to mammalian toxicity ($LD_{50} = 400-4,300$ mg/kg in mice) and also has a half-life of 2 days in water (Malathion Technical Fact Sheet, 2009). Despite the effectiveness of targeting the larval stage, mosquito control strategies still largely employ adulticiding. Sections 1.4.1–1.4.3 discusses the adulticiding of mosquitoes with pyrethroids.

1.4 The need for novel insecticides

1.4.1 Inadequacies of pyrethroids

Pyrethroids are synthetic compounds found in commercial insecticides that are used to spray for adult mosquitoes and other insects such as crop pests. Many believe that pyrethroids actually kill insects; however, this is a misconception. Pyrethroids have a quick knockdown effect, and it has been demonstrated that insects can completely

recover from high doses. The LD₅₀ values are misleading, stretching the definition of “lethal” as many such experiments consider paralysis 24-48 hours after exposure as being dead (Bloomquist and Miller, 1985). However, causing paralysis instead of mortality does not affect their usefulness in field applications because desiccation and predation are likely results before paralysis recovery (Khambay and Jewess, 2005). Desiccation is unlikely to affect mosquitoes as they proliferate during humid conditions and remain in the shade during the day. Also, considering how mosquitoes lack agility as flying insects, they are fairly vulnerable to predators without being paralyzed considering how easy it is to capture an adult mosquito versus nearly any other flying insect.

Unlike assertions of the insect specificity of pyrethroids, research conducted as early as the late 1980s and early 1990s demonstrates that pyrethroids are toxic to many non-target and beneficial organisms (Mueller-Beilschmidt, 1990; National Research Council of Canada, 1987; Smith and Stratton, 1986). Data from the analyses were quantified in the form of bioaccumulation factors. The bioaccumulation factor is the ratio of compound found within the organism when compared to the environment. The bioaccumulation factors for several important fish and mollusk species were measured to be as high as 1000 and 3000, respectively. Fish appear to be more susceptible to pyrethroids while mollusk species are fairly resilient (Maund et al., 2011). The resiliency in mollusks is particularly problematic because it could allow a considerable amount of the insecticide(s) to accumulate before being eaten by humans. Because of the lipophilic nature of these compounds, they accumulate in fatty tissues of higher trophic organisms particularly those with a steady molluscan diet rich in pyrethroids (Agency for Toxic Substances and Disease Registry, 2003). Bees are also highly susceptible to pyrethroids.

The LC₅₀ value for permethrin on honey bees was calculated to be 29 ng/bee (Toynton et al., 2009). Bees are extremely important economically. The exact annual dollar value estimated is highly variable but ranges from hundreds of millions (Rucker et al., 2005) to nearly 20 billion (Levin, 1983). The discrepancy in estimates is largely made on the basis of exclusion/inclusion, respectively, of indirect human impacts like ecosystem stability as these do not affect man made crops directly (Abrol, 2012).

1.4.2 Human risk to pyrethroids

Pyrethroids are known to be non-toxic to mammals because they are quickly metabolized via oxidation and hydrolysis pathways (Mikata et al., 2011). However, many studies involving mammalian toxicity are accomplished with acute toxicity experiments. Chronic toxicity studies performed by Narendra et al. (2008) demonstrate that pyrethroids can accumulate in different tissues such as adipose, nerve, and even in blood. The study showed chronic exposure to pyrethroids can alter human blood profiles which can lead to cardiovascular risk and damage to hepatic tissue. Such studies indicate a trade-off for human health by utilizing certain synthetic insecticides in the environment to control for disease vectors.

1.4.3 Insect resistance to pyrethroids

Resistance to pyrethroids is derived from three separate mechanisms, the primary of which is termed knockdown resistance (kdr) and is attributed to inherited point mutations in the target site; i.e., the *para*-type sodium channel. Target site mutations reduce the binding of such insecticides (Pauron et al., 1989) and has been developing in insects since the 1950s because of the use of dichlorodiphenyltrichloroethane (DDT)

which has the same target site as a mechanism of action (Busvine, 1951). The second resistance mechanism is metabolic degradation much of which is accomplished by the de-esterification reaction performed by cytochrome p450 enzymes. The exact isozyme responsible for the necessary de-esterification reaction is still not known (Khambay and Jewess, 2005). More research is required on this particular subject including the exact role of esterases. Metabolic resistance is linked to the overexpression of such enzymes (Funaki et al., 1994; Corbel et al., 2003). The third resistance mechanism comes in the form of reduced cuticular penetration. Reduction in cuticular penetration alone was found to have a marginal effect, but when in combination with the other forms of resistance can somehow decrease toxicity several-fold versus simply being an additive effect (Ahmad and McCaffery, 1999).

In 1977, synthetic pyrethroids were used in Australia for the first time. Six years later, resistance was already an issue (Forrester et al., 1993). In a 2003 study, 80 species of insects were found to be resistant to pyrethroids (Whalon et al., 2003). In Eastern and Southern Africa, many mosquito species have grown resistant to pyrethroids (Coetzee, 2006). Considering the prior use of DDT, the kdr mutation is present and being selected for once again.

Controlling for disease vectors is a complicated task as particular mosquito strains have grown resistant to certain insecticides. Thus, insecticides with different mechanisms of action are required to control different mosquito populations. Various mosquito vectors have ranges that overlap with each other. The rotation of insecticides with different mechanisms of action is a valuable technique to help prevent resistance

(Khambay and Jewess, 2005) and bioaccumulation in non-target organisms (Narendra et al., 2008).

A paradigm shift is emerging that advocates population control through larval instead of adult insecticides. With this new mindset and the overall need for insecticides with novel mechanisms of action, searches for mosquito control agents have led researchers to bioactive compounds from bacteria.

1.5 Cyanobacteria as a potential source for arthrocidal compounds

1.5.1 Introduction

Cyanobacteria produce many secondary metabolites that have been found to be toxic or otherwise bioactive. The purpose for the production of these compounds is merely speculative. One current hypothesis is the ecological roles of allelochemicals influence the outcome of competition among sympatric organisms such as macrophytes, algae, and microbes by inhibiting their growth (Kearns and Hunter, 2001). Others suggest these secondary metabolites serve as defensive strategies against grazers (Lüring, 2003). Grazers in aquatic communities include aquatic adult and larval invertebrates.

1.5.2 Chemical defense against planktivores

1.5.2.1 Apparency theory

To establish the roles of cyanobacterial metabolites in ecosystems, it is important to consider the best studied plant/herbivore interactions because plants interact with their environment similarly to cyanobacteria as both are sessile organisms. Apparency theory describes how many plants have structures that are sessile, apparent, and thus simply

susceptible to herbivory unlike other plants mostly consisting of underground shoots and less obvious components (Feeney, 1976; Rhoades and Cates, 1976). These plants would produce quantitative and qualitative defenses, respectively. Quantitative defenses would be less specific but, as their name suggests, produced in larger quantities such as polyphenol compounds, e.g., tannins. Tannins bind many proteins, and thus make it difficult for herbivores to become tolerant given the lack of specificity of these compounds. Less specificity means multiple targets would need to be modified in order for tolerance to develop. In contrast, qualitative defenses are those produced in lower concentrations as their modes of action are highly specific giving them the true title of a “toxin” found in literature. A higher level of specificity means that co-evolution has a higher probability of occurring when compared to quantitative defenses as grazers would only require a single mutation in the target site to circumvent their effects.

1.5.2.2 Quantitative defenses of *Microcystis aeruginosa* against *Daphnia*

Microcystins are non-ribosomal peptides that inhibit serine/threonine phosphatases 1 and 2a making them hepatotoxic to humans (Dawson, 1998; Dittman and Wiegand, 2006). Although the mechanism of microcystins may seem specific for a quantitative defense, the targets are quite variable encompassing many proteins across most organisms (Brautigan, 1995). The high yield of these toxins also suggests a quantitative nature. Microcystins have shown feeding inhibition and the decline of population within *Daphnia* (Nizan et al., 1986; Rohrlack et al., 1999; Rohrlack et al., 2001; Lürling, 2003). Studies demonstrate that *Microcystis* may rely on other peptides that act as antifeedants for defense. These include cyanopeptolins, micropeptins, and

microviridins which have been linked to digestion inhibition. Their mechanisms have been linked to inhibition of trypsin like proteases (Dawson, 1998; Peñaloza et al., 1990).

Daphnia has shown evidence of co-evolution with *Microcystis* in that eggs obtained from microcystin infested waters have better survivorship when treated with microcystins compared to eggs reared without the presence of microcystins. Adults were given *Microcystis* as diet (Hairston et al., 1999; Hairston et al., 2001). Researchers believe this tolerance is caused by increased production of digestive enzymes and not resistance or the mutation in the target site (Pflugmacher et al., 1998; Adamovsky et al., 2007). The lack of specificity of the digestive enzymes for microcystins also portrays a quantitative nature. Also, researchers have found that glutathione production tends to increase (Chen, et al., 2005; Adamovsky et al., 2007) in microcystin resistant strains demonstrating once again that target site mutations have not been documented as a form of resistance.

These findings demonstrate that cyanobacteria can produce compounds that negatively affect the grazing activity of arthropods such as *Daphnia*. Section 1.6 will illustrate how the grazing of another arthropod, mosquito larvae, is leading to the discovery of novel mosquito control agents.

1.6 Cyanobacteria as a potential source for mosquito larvicidal compounds

1.6.1 Cyanobacteria and mosquito sympatry

Cyanobacteria can associate with eukaryotic algae, bacteria, and fungi forming benthic biofilms or mats called periphyton. Periphyton has been recognized as a significant source of primary productivity and habitat for invertebrates (McCormick *et*

al., 1998). In combination with detritus, periphyton acts as the base for many food webs (Browder *et al.*, 1994; Rader and Richardson, 1994). Cyanobacteria are present in many mosquito breeding areas (Laird, 1988) as mosquitoes will lay their eggs within cyanobacterial mats (Rejmankova *et al.*, 1996). Mats act as shelter, sources of food, and substrate for egg attachment during mosquito oviposition (Pope *et al.*, 2005). However, some debate the potential for cyanobacteria to be a potential source of food for aquatic invertebrates such as mosquito larvae.

1.6.2 Grazer choice of food within periphyton

Grazers within periphyton have been found to lack the discriminatory ability to reject certain sources of food (Resh and Houpp, 1986). These grazers were found to lack the sensory capability for food selection. For these particular grazers, food “selection” is likely determined by the morphology of their feeding parts (Karouna and Fuller, 1992) which influences the efficiency of feeding on particular algal species (Steinman, 1996). The appearance of selectivity is likely caused by random feeding leading to the discovery of compatible food items; i.e., food morphology versus feeding morphology (Steinman, 1996). However, most of the studies mentioned in this section do not encompass all the herbivores present within these communities. The most visible organisms, and thus the most abundant at the time of research, were normally chosen.

Cyanobacteria do not normally offer much nutritional values for grazers (Schmidt and Jonasdottir, 1997). Much research has shown that the production of secondary metabolites common to cyanobacteria would hinder their palatability (Porter, 1977; Gregory, 1983). However, some grazers do prefer cyanobacteria such as the chironomid

midge, *Cricotopus nostocicola*, which forms endosymbiotic associations with the cyanobacterium *Nostoc* in which the midge feeds on exclusively. *Nostoc* benefits from this association by somehow having a higher rate of photosynthesis when compared to midge-less strains.

1.6.3 Mosquito larvae feeding on periphyton

Mosquito larvae are often abundant in cyanobacterial mats (Lange and Lopez, 1996). Despite this knowledge, there is no conclusive information on whether mosquito larvae actually target specific food sources, like cyanobacteria, in these environments. Researchers have found cyanobacteria in the guts of mosquito larvae (Howland, 1930; Jones, 1960). Whether the mosquito larvae were targeting the cyanobacteria or ingesting it because of the association with other sources of food remains unclear. Other research involves rearing mosquito larvae with specific strains of cyanobacteria to observe ingestion. Results indicate that mosquito larvae can ingest cyanobacteria and that non-filamentous forms are preferred (Thiery et al., 1991). However, whether the cyanobacteria is filamentous or not may be of less importance within cyanobacterial mats. Large grazers can dislodge food particles allowing for previously inedible algal strains to be accessible to small grazers (Dudley, 1992). The determination of larval gut contents has been problematic in that partially digested food items are difficult to identify from larvae collected in the field. More recent techniques such as DNA barcoding and the use of stable isotopes offer tools that circumvent the problems in visual identification of partially digested food (Garros et al., 2008, Belicka et al., 2012). However, the technique is still plagued by the same shortcomings mentioned previously. The presence of

particular food items, confirmed with DNA or stable isotope analyses, does not confirm that mosquito larvae were targeting these food items specifically.

1.6.4 Cyanobacteria: Novel insecticides and candidates for biological control agents

Cyanobacteria produce a wide array of toxins which have been found to reduce the grazing rates of primary consumers. The true nature of these toxic compounds is unknown but has been linked to allelopathic associations (Berry et al., 2008). Toxins produced by cyanobacteria are much more likely to be detoxified by microorganisms than synthetic insecticides because they already occur naturally in the environment in which they are to be employed (Lange and Lopez, 1996). Given the variability of their secondary metabolites, cyanobacteria are recognized as an enormous source for future insecticides (Namikoshi and Rinehart, 1996). The use of cyanobacterial toxins as mosquito larvicides is an emerging field. Several advancements have taken place during the last two decades.

Nassar et al. (1999) discovered mosquito larvicidal activity against *Culex pipiens* using crude extracts in µg/ml concentrations derived from unspecified cyanobacteria. These extracts did not exhibit any mammalian toxicity at the same concentrations using mouse serum in an acetylcholinesterase assay. Rao et al. (1999) discovered serendipitously that cyanobacterial strains of the genus *Westiellopsis* inhibited mosquito larvae growth when investigating the biofertilizer properties of the cyanobacterium. Subsequent analysis revealed that the methanolic extracts contained the activity and showed to be larvicidal at µg/ml concentrations against various disease vectors including

strains of *Aedes aegypti* (dengue fever and yellow fever), *Anopheles stephensi* (malaria), and *Culex. quinquefasciatus* (encephalitis).

Kiviranta et al. (1993) screened 76 cyanobacterial isolates of which several were found to contain compounds with mosquito larvicidal activity against the larvae of *Ae. aegypti*. The most pronounced activity was associated with isolates containing the neurotoxic anatoxin-a and the hepatotoxic microcystins. These findings were not of much use for commercial application, considering the potential non-target toxicity, but demonstrate that purified compounds from cyanobacteria can act as mosquito larvicides. The following year, several compound were purified from *Oscillatoria agardhii* with mosquito larvicidal properties other than the neurotoxic and hepatotoxic compounds common to this species (Kiviranta and Abdel-Hameed, 1994).

One of the more notable discoveries of mosquito larvicidal compounds derived from cyanobacteria was the appearance of unsaturated fatty acids derived from *Oscillatoria agardhii* strain 27, which were found to be toxic to the larvae of *Aedes albopictus* (Harada et al., 2000). Compounds were characterized, and the common structure is relatively safe for mammals and the environment. Some of these fatty acids were found to have antimicrobial and enzyme inhibiting properties. To my knowledge, these compounds are the first to be characterized based on the bioassay guided fractionation of cyanobacteria using a mosquito larvicidal assay. Anatoxin-a and microcystin-LR will be discussed in section 1.6.5. Although they were not purified as a result of bioassays guided fractionation using a mosquito larvicidal assay, they still provide useful information. Only extracts have been mentioned so far and thus mosquito

larvicidal activity could have resulted from synergism of several components. It is important to demonstrate that purified compounds can produce the activity as well.

Beyond the use of cyanobacterial compounds for commercial mosquito larvicides, there is also considerable interest in allowing the cyanobacteria to propagate and produce desired compounds in mosquito breeding sites. Genes involved in the production of insecticidal proteins derived from *Bacillus thuringiensis* have been incorporated into four strains of the ubiquitous *Synechococcus* (Sangthongpitag et al., 1996). The authors chose *Synechococcus* because they collected several strains of it from mosquito breeding sites. Also, *Synechococcus* was found to be particularly appropriate as a toxin gene carrier because it is found worldwide and tolerant to various salinities, temperatures, and insecticides of biological and chemical origin. The unicellular organization of *Synechococcus* would facilitate its digestion by mosquito larvae.

Transgenics may be a premature approach in that many fear the release of such organisms into the environment could have unforeseen consequences. With more research on cyanobacteria derived mosquito larvicides, transgenics may not be necessary; i.e., inoculating mosquito breeding sites with cyanobacteria capable of producing mosquito larvicides.

1.6.5 Effects of anatoxin-a and microcystin-LR on mosquito larvae

No products derived from cyanobacteria are currently in use commercially as mosquito larvicides. However, several compounds have been explored, including the well documented cyanobacterial toxins anatoxin-a and the microcystin-LR. Although these are unlikely candidates for mosquito control considering their toxicities to non-target

organisms, it is important to illustrate that purified compounds (versus extracts discussed in section 1.6.4) from cyanobacteria can house mosquito larvicidal properties.

Anatoxin-a has a half-life of 14 days under normal photoperiods and its degradation is accelerated by direct sun exposure (WHO, 1999). The LC₅₀ range of purified anatoxin-a against *Ae. aegypti* was calculated to be 17–37 µg/ml (Kiviranta et al., 1993). Its mechanism of action is assumed to be the same as with other insects, a neurotoxin. Anatoxin-a is an acetylcholine analogue that is not degraded like acetylcholine causing muscle cells to be stimulated continuously.

The half-life of microcystin-LR is roughly 7 days (Codd and Bell, 1996). The LC₅₀ range of “purified” microcystin against *Ae. aegypti* was calculated to be 2.5-6.1 mg/ml (Kiviranta et al., 1993). These large values do seem to indicate a crude extract although the authors claim to have gone through several stages of purification. Microcystins are protein phosphatase inhibitors and are classified as hepatotoxins in regards to the location where they accumulate in vertebrates. However, classification as a hepatotoxin is not useful when describing its effects on organisms that do not have hepatic tissue. Saario et al. (1994), utilizing histopathology microscopy, discovered that microcystins lysed midgut epithelial cells in the mosquito larvae of *Ae. aegypti*.

The activity of both anatoxin-a and microcystin-LR on mosquito larvae demonstrate that purified compounds from cyanobacteria can house mosquito larvicidal activity. These results help to justify the rationale behind searching for mosquito larvicides within cyanobacteria.

Chapter 2: Mosquito Larvicidal Assay

2.1 The larval developmental stage of choice

Mosquito larvicidal assays commonly use third or fourth instars as the developmental stage of choice (Abdul Rahuman et al, 2008; Amer and Mehlhorn, 2006; Elango et al., 2011; Kiviranta and Abdel-Hameed 1993). The use of these late instars does not help describe natural settings because a mixture of different instars will be present at any given time within breeding sites (Rejmankova et al., 1996). For instance, if an insecticide is applied after a rain event, which can act as a trigger for mosquito eggs to hatch, there will be many first and second instars present within 48 hours. Thus, the exclusive use of third instars in laboratory assays would prevent measurement of toxin effects on first and second instar larvae, which may lead to the utilization of insecticides at miscalculated concentrations as susceptibility to insecticides can vary tremendously by life stage (Koziol and Witkowski, 1981).

2.2 Development of the mosquito larvicidal assay

2.2.1 Treatments and resuspensions

Bioassays were performed with 24 x 2.0 ml well plates made of polypropylene. Prior to adding mosquito larvae, toxin treatments were added to their respective wells. For crude extracts and their resulting fractions, small aliquots were placed into each well. Considering that toxicity has not been deduced at this stage, the volume of the aliquots is estimated. If too little is added one risks not seeing any activity and thus wasting time and materials. Small scale assays were performed with treatments consisting of serial

dilutions of the sample. From the resulting data, calculations were made to estimate the volume of aliquots used in subsequent bioassays including those from later fractionation steps. After the aliquots were placed into each well, solvents were allowed to evaporate in a fume hood. Polar extracts were resuspended directly into 500 μ l of water. Non-polar extracts were originally resuspended by first adding 10 μ l of methanol followed immediately by 500 μ l of water to prevent the solvents from evaporating. However, adding methanol first failed to resuspend a percentage of the sample. With colored extracts this was obvious by the treatments visibly adhering to the bottom of individual wells preventing them from interacting with the mosquito larvae. A new method was developed which involves first pipetting 500 μ l of water into each well and then adding the treatment with exactly 10 μ l of methanol. Treatments were performed in duplicate, using one row for each replicated serial dilution. An entire row was allocated for negative controls; i.e., just water, and another row for solvent controls (Figure 3).

Figure 3: Well plate design.

2.2.2 Mosquito larvae: Egg production and handling of larvae

Mosquitoes used in this study were *Aedes aegypti* of the Rockefeller strain. Eggs were acquired from 3 day old females following a blood meal. The blood meal was accomplished by circulating bovine blood through a heating apparatus. Blood was presented to the females through a layer of parafilm. Oviposition occurs on pieces of paper that line a container just above a water line. Before performing a bioassay, the number of required larvae must be calculated in order to estimate the amount of mosquito eggs to use. Egg density on the paper was estimated via microscopy and a 50% hatch rate assumed; i.e., if the assay requires 96 larvae, approximately 192 eggs were estimated (Dr. Mario Perez, personal communication, September 5, 2007). Once the size of the egg paper has been determined, it was placed in a capped bottle containing hypoxic water. The water was rendered hypoxic by boiling until the volume has been halved. The bottle was capped immediately upon removal from the heat source. Once the water cools down to the incubation temperature (28°C), the egg paper was added. The bottle was not capped during hatching. Mosquito eggs are buoyant and thus the egg paper required some assistance to submerge properly. Hatching took approximately 1.5-2 hours.

Immediately after hatching, four first instar larvae were transferred into each well with enough deionized water to make individual wells a total volume of 1 ml given consideration to treatment volumes (10-20 µl) and larval food. Each day, 120 µl of 0.1% liver powder solution was fed to each group of 4 larvae. Each larva requires 30 µl 0.1% liver powder solution daily as recommended by Dr. Mario Perez (personal communication, September 5, 2007). The liver powder solution was prepared by combining 10 g of liver powder with 100 ml of deionized water in a 250 ml autoclavable

bottle. The contents were mixed using a stirrer on medium velocity for 5 minutes. The resulting mixture was autoclaved as a liquid (121°C). The larvae are fed regardless of the presence of other potential sources of food such as cyanobacteria, because mosquito larvae have varying success feeding on cyanobacteria. As mentioned previously, the variability in feeding success is determined by the cellular organization (filamentous or non-filamentous) of the cyanobacteria. The amount of liver powder given was adjusted in order to compensate for dead larvae which are often removed as they can be eaten by other larvae. In cases where bioaccumulation is suspected, dead larvae were not removed and were subsequently eaten by other larvae. Bioaccumulation experiments can provide some useful information on the activity of treatments after being bound to the original target. Cannibalism among the mosquito larvae was not observed to be a potential source of mortality because mosquito larvae demonstrated an avoidance of feeding on anything that moves particularly if the movement is in response to physical contact. In extreme cases, a first and a fourth instar could co-occur in the same well which demonstrate the most likely scenario for a mosquito larvae to feed on another considering the small size of the first instar and the developed feeding parts of the fourth instar. Both first and fourth instars have been present within the same well several times and the first instar larvae were never eaten.

Bioassays were conducted with the use of a Precision low temperature illuminated incubator (model 818) with a 16:8 (day/night) photoperiod set at 28°C. The humidity was not controlled as in the case of many mosquito insectaries because the aquatic larvae have shown to develop at the same rate regardless of humidity (Dr. Mario Perez, personal communication, September 5, 2007). However, precautions were taken to limit

evaporation. Well plates used have covers that allow sufficient space for gas exchange but prevent the evaporation of water considerably.

2.2.3 Evaluation of mosquito larvae

Larvae were observed once daily for survivorship, current instar, and non-lethal effects such as deformities. Adult emergence occurs in approximately seven days post-hatching. Larvae were bleached to terminate the assay before adult emergence. The use of the term “activity” in reference to the mosquito larvicidal assay will be synonymous with mortality unless otherwise specified. Living first instars are easy enough to observe with the naked eye mainly because of their whipping like movements. Once movements have ceased, a dissecting microscope is required for their identification. Dead larvae were removed, except in bioaccumulation experiments, to prevent being eaten by other larvae.

2.2.4 Evaluation of negative controls and resuspensions on mosquito larvae

The negative controls using only water showed a 1% mortality rate by day 3. When mortality occurred early in the experiment, larvae were often not visible because of their small size. Solvents used as negative controls include methanol, ethanol, and chloroform. The threshold concentration for these solvents on larval mortality was found to be 5%. All larvae died at this level and at higher concentrations. Tests have shown that larvae reared in wells with previously evaporated solvent did not differ significantly in mortality when compared to controls containing only water. Solvent concentrations of $\leq 1\%$ were used for all bioassays requiring the re-suspension of a non-polar extract, fraction, or compound. Although some researchers did not require solvents other than water to resuspend non-polar extracts (Dr. Miroslav Gantar, personal communication,

September 10, 2008), resuspensions with water alone became an apparent issue. Non-polar extracts that were not re-suspended formed very rigid, plastic like, structures that remained on the bottom of each well. Even after being scraped off the bottom and subsequently cut into smaller pieces (Figure 4), the mosquito larvae seemed to not have the feeding apparatus that would allow them to consume any of these potential food items although they were observed trying to do so.

Figure 4: Non-polar extracts formed inedible precipitates when not re-suspended in solvent.

Another issue resulted from the use of chloroform for resuspensions. All larvae subjected to a 1% chloroform solution were anesthetized for 2-3 hours. Even though mosquitoes in the chloroform controls experienced zero mortality, nearly all treatments using chloroform had mortality, even those that normally did not demonstrate any mosquito larvicidal activity. Chloroform potentially stays bound to the treatments causing the seen mortality while in the controls it is evaporated more readily. Methanol became

the solvent of choice for resuspending non-polar compounds as it did not exhibit any anesthetic properties or suspiciously increase the mortality of any of the treatments.

The emulsification agent Tween 80 was also tested for dissolving non-polar compounds as it is used by many researchers in mosquito larvicidal bioassays (Silva et al., 2010; Kiviranta and Abdel-Hameed, 1993). However, on the basis of trials done during the current study, wells containing 0.5% Tween 80 had a mortality rate of 10%. The mortality rate of Tween 80 is approximately ten times that of the controls containing only water. Many of the wells containing Tween 80 developed white precipitates. Larval mortality was correlated with the presence of these precipitates. Dead larvae were found fused together on several occasions. Also, mortality increased considerably by day 3 (~50% mortality).

Lastly, controls examining density related mortality demonstrated that mortality tends to increase with more than 4 larvae per 1 ml of water. This increase in mortality may be caused by the lack of space for movement negatively affecting food acquisition and drinking.

2.2.5 Evaluation positive controls on mosquito larvae

Before commencing the experiments it was important to establish some control treatments to ensure the well plate design can produce data comparable to published research on cyanobacterial compounds tested with mosquito larvicidal assays. These control treatments simulate the properties of fractions derived from the polar and non-polar extracts.

Polar controls include anatoxin-a purchased from A.G. Scientific and microcystin-LR provided by Dr. Kathleen Rein's laboratory. The non-polar control, DDT, was purchased from Supelco. Solvents used to suspend the controls were allowed to evaporate, even for DDT, in order to minimize their effects. See Figure 5 for the structure of these compounds.

Figure 5: Chemical structures of the control treatments.

The results of the bioassays are shown in Figure 6. Kiviranta and Abdel-Hameed (1993) calculated LC_{50} values for purified anatoxin-a on *Aedes aegypti* (25-47 $\mu\text{g/ml}$) which are comparable to the results found in the current study (50% mortality at 50 $\mu\text{g/ml}$). Kiviranta and Abdel-Hameed (1993) also calculated LC_{50} values for “purified” microcystin (2.5-6.1 mg/ml). As mentioned previously, these large values are indicative of a crude extract being in the mg/ml range. Data collected in the current study showed 50% mortality at a concentration of 25 $\mu\text{g/ml}$ for purified microcystin-LR.

Figure 6: (A) Bioassay results for polar controls anatoxin-a and microcystin-LR. (B) Bioassay results for the non-polar control DDT.

Solubility was not an issue for DDT, as it was for the non-polar extracts. Without being resuspended, 100% mortality occurred in all treatments except the 1 µg/ml concentration which had 50% mortality.

Chapter 3: Bioassay Guided Fractionation

3.1 Background

Scientists have been purifying compounds from natural sources since ancient times. These efforts date back to 2600 B.C. and have been recorded on clay tablets found in Mesopotamia (Cragg and Newman, 2005). Oils were extracted from *Cupressus sempervirens*. These oils were used to treat coughs and inflammation and are still in use today. Many other examples exist from the ancient Egyptians and Chinese (Dias et al., 2012). The procedure has increased in efficiency over time with the advent of column chromatography, various characterization techniques, and an array of bioassays. Scientists attempt to pull everything they can out of the organism being studied and test individual components separately with several experiments, such as bioassays, in order to deduce the nature and utility of the derived compound(s). The use of bioassays in this manner is termed bioassay guided fractionation.

3.2 Bioassays

Well plates are often used in bioassay guided fractionation experiments. The well size must be considered on the basis of size of a single organism, including their potential for growth, as well as the number of organisms to be placed into each well (Robertson et al. 2007). The latter should be tested as adverse effects may be discovered as a result of group density issues. Mosquito larvae in particular are vulnerable to density related phenomena as delayed development is directly correlated to density (Clements, 1992).

3.3 Fractionation

The protocol commences with the identification of the organism to be analyzed for potential natural products. In the case of deriving mosquito larvicidal compounds from cyanobacteria, a small aliquot of cyanobacterial biomass is placed with the larvae. This exposure repeated with as many strains of cyanobacteria as possible to help ensure the most useful (in housing desired compounds) will be discovered in the screening process. Once the most effective cyanobacteria have been identified, in regards to mosquito larvicidal activity, active components are purified. The biomass is subjected to polar and non-polar solvent extractions which are tested individually for activity. Active extracts are separated into numerous fractions via column chromatography which in turn are tested individually for activity. The solvent system is determined by those giving the best resolution with thin layer chromatography (TLC). If the activity is lost at this step or seems to not be in any particular location, the protocol should be revised. Fractionation can be done in small scale at first with the use of solid phase extraction cartridges and scaled up for a large glass column once the active fractions have been identified. Active fractions are separated by another column and then tested once again, hence the term "bioassay guided fractionation". Column chromatography is performed until samples are pure enough for high pressure liquid chromatography (HPLC). To consider fractions to be HPLC ready, they are required to have baseline separation between peaks which will allow for their individual collection. Standard acetonitrile/water or methanol/water sloped gradients are first used and are then fine-tuned to achieve satisfactory baseline separation. Finally, individual peaks are collected and tested for activity. During the HPLC phase, all wavelengths should be considered when dealing with unknown compounds. It is

possible for a compound to have several chromophores; i.e., visible at more than one wavelength. If multiple chromophores are suggested, one must be sure the peaks at the multiple wavelengths have exactly the same retention time and are similarly symmetrical otherwise several compounds may still be present at that specific retention time. Since elution via HPLC likely means the sample contains water, a rotary evaporator should be used to remove the more volatile solvents such as methanol and acetonitrile. Once the sample can freeze, the remainder of the water can be removed with a freeze dryer.

3.4 Characterization

If compound purity is still in question, the verification that a single mass is present via mass spectrometry will help confirm that it is indeed pure. High resolution mass spectrometry can give masses up to four decimal places. With this information molecular formulas can be generated. However, the amount of formulas generated can be quite numerous if the atomic composition is not known. Discovering the functional groups with infrared spectroscopy (IR) adds another layer to characterization. In combination with proton and carbon nuclear magnetic resonance (NMR) techniques the remainder of the structure can be deduced. This section is an overly simplistic view of characterization as NMR spectra do not look as clean as shown in text books and many complications can arise when working with unknowns as the properties of prospective compounds are obtained through trial and error. Important discoveries can result from mistakes particularly if meticulous observations are constantly being made.

3.5 Proof of Principle

3.5.1 Introduction

The re-purification of sulfated glycolipids (SGL) derived from a strain of *Synechococcus* sp. 21-10a was performed (Figure 7). The original purification of these compounds was guided using a hemolysis assay and was performed by Dr. Tianying An while a postdoc in Dr. Kathleen Rein's laboratory at Florida International University (FIU). Subsequent to their original purification, the glycolipids were tested by our laboratory for mosquito larvicidal activity using the assay described in Chapter 2. Glycolipids SGL 1 and SGL 3 had the most activity, 75% and 100%, respectively. Both were tested at a concentration of 100 µg/ml. However, the mosquito larvicidal activity was deduced in small scale. No official LC₅₀ values were produced as small quantities were obtained and subsequent yields were low. Being the case, it was decided to re-purify these glycolipids following the fractionation given by Tianying and also by bioassay guided fractionation to demonstrate a "proof of principle" that bioassay guided fractionation can accomplish this specific goal in regards to discovering compounds with certain properties of interest.

3.5.2 Original purification of sulfated glycolipids

Living culture of *Synechococcus* sp. 21-10a was obtained from Dr. Miroslav Gantar's laboratory located at FIU. Biomass was drained of medium and subsequently freeze dried. An EtOH (ethanol) extraction was performed and solvent evaporated in vacuo. The resulting residue was resuspended in 90% MeOH (methanol) and fractioned using a Biotage unit (flash chromatography) using a C18 cartridge. Two fractions were

Figure 7: Sulfated glycolipids purified from *Synechococcus* sp. 21-10a

collected using 90% and 95% MeOH. A precipitate was collected from the 90% fraction and reconstituted into MeOH:H₂O, 70% and 30% respectively, which was applied to a second C18 column. Four fractions were collected using a step gradient of 72%, 74%, 76%, and 78% MeOH. The 78% fraction contained SGL-1. The supernatant from the 90% and 95% MeOH fractions, derived from the first fractionation, were combined and reconstituted in 50% MeOH and was also applied to a second C18 column. Five fractions were collected using 60%, 70%, 80%, 90%, and 100% MeOH. The 90% and 100% fractions were combined, dried, and reconstituted in 50% MeOH and applied to a third

C18 column. Four fractions were collected using 65%, 66%, 67%, and 68% MeOH. SGL-4 was found in the 66% fraction, SGL-3 in the 67% MeOH fraction, and SGL-2 in the 68% MeOH fraction.

3.5.3 Re-purification and bioassay guided fractionation of sulfated glycolipids

Unused freeze dried biomass from the original purification was obtained. All steps from the previous section were followed for the re-purification except those involving the precipitate as none was observed. The lack of a precipitate was unfortunate as the second most active of the sulfated glycolipids (SGL-1) would not be able to be re-purified. Bioassays were performed on the biomass, the EtOH extract, and the two subsequent C18 columns confirming the mosquito larvicidal activity was present and located approximately where the sulfated glycolipids were described to be found by Dr. An's fractionation scheme. As for the second C18 column, SGL-1, SGL-2, and SGL-3 should have been found in the 90% and 100% MeOH fractions but the bioassay suggested that they may be found in the 80% and 100% MeOH fractions. Although the solvent volumes and flow rates used were identical to the original purification, some discrepancies were expected considering different Biotage units were used.

3.5.4 Identification of re-purified of sulfated glycolipids

Although the original protocol called for yet another C18 column it was decided to run the samples through an LC-MS (Liquid Chromatography-Mass Spectrometry). The Kinetex C18 column used for the LC-MS can act as the final C18 column of the original protocol as masses can be analyzed at various retention times. Mass spectra does support the presence of SGL-2 and SGL-3 (Figure 8 and Figure 9, respectively). Both analyses

were performed in negative ionization mode using heated electrospray ionization (HESI). HESI is a fairly gentle ionization technique which allows for most molecular ions to remain intact for subsequent mass analysis.

3.5.5 Discussion

Given the structure of these sulfated glycolipids, it was deduced that in negative ionization mode Na^+ would be removed causing a loss of 23 mass units in both SGL-3 and SGL-3. The isotope pattern as well as their abundance in the chromatogram does support their re-purification. Lastly, the surfactant nature of these compounds was apparent when drying them. Detergent like bubbles were produced making it difficult to evaporate the solvent in which the glycolipids were suspended in. In combination with the mass spec analyses and the mosquito larvicidal activity, these observations help to confirm the re-purification of sulfated glycolipids from *Synechococcus* sp. 21-10a.

Glycolipids are integral components in the thylakoid membranes of chloroplast and cyanobacteria. They can replace phospholipids in order to conserve phosphate and may play a direct role in photosynthesis (Dörmann and Benning, 2012). Interestingly, through the results of bioassay guided fractionation, the most mosquito larvicidal activity resides within membrane bound constituents (Harada et al., 2000; Rahuman, 2008; Komansilan, 2012, Ahmed et al., 2014)

Chapter 4: Isolation and Characterization of Mosquito

Larvicidal Compounds from Cyanobacteria

4.1 Introduction

Over a million deaths are attributed to mosquito-borne diseases every year (American Mosquito Control Association (AMCA), 2013). States along the Gulf Coast are potentially susceptible to encroachment of pathogen carrying strains of mosquitoes considering the recent West Nile outbreaks in Texas and locally acquired dengue fever in the Florida Keys (Texas Department of State Health Services, 2013; Munoz Jordan et al., 2013).

Inadequacies of adulticiding include the use of pyrethroids which affect all insects including beneficial insects such as bees (Toynton et al., 2009). A paradigm shift is underway targeting the aquatic larvae instead of the aerial adults. Mosquitoes are concentrated in pools of water while in their larval form thus increasing the effectiveness of applied insecticides. Insecticides are placed in remote bodies of water during larviciding which diminishes human exposure when compared to adulticiding which affects human population immediately through inhalation.

Cyanobacteria produce a wide array of secondary metabolites. These compounds potentially help outcompete other photosynthetic organisms and/or act as deterrents to grazing invertebrates (Kearns and Hunter, 2001; Lüring, 2003). Considering the sympatry of cyanobacteria with mosquito larvae (Pope et al., 2005), cyanobacteria are a potential source for compounds with mosquito larvicidal activity (Harada et al., 2000). With this sympatric association and the benefits of larviciding, cyanobacteria were

evaluated for the potential of containing mosquito larvicidal compounds. Compounds were characterized and their biological activities were described.

4.2 Methods

4.2.1 Screening of cyanobacterial isolates

The original screening involved polar and non-polar extracts, provided by Dr. Miroslav Gantar. These were previously extracted and screened for activity against developing zebrafish embryos in Dr. John Berry's laboratory. Since the remainder of these extracts were no longer in use, it was decided to test them for mosquito larvicidal activity. Small aliquots were tested using the mosquito larvicidal assay.

4.2.2 Screening of cyanobacterial biomass

Cyanobacterial biomass was provided by Dr. Miroslav Gantar. Strains were collected from the Florida Everglades and other Florida waterways. Cyanobacteria are identified through microscopy, isolated, and then grown as unialgal cultures. Biomass was obtained from 120 different cultures. Samples were freeze dried subsequent to being drained of medium. A list of the cyanobacterial strains used in the current study can be found in Appendix 1. Small aliquots of the freeze dried biomass were tested in quadruplicate using the mosquito larvicidal assay.

4.2.3 Bioassay guided fractionation of 3 prospective larvicidal cyanobacteria

Three strains of cyanobacteria were selected on the basis of the highest mosquito larvicidal activity. Extractions were performed using 70% MeOH and 100% CHCl₃

giving rise to a polar and non-polar extract for each prospective strain. These were tested for mosquito larvicidal activity.

Polar extracts exhibiting mosquito larvicidal activity were fractionated by solid phase extraction (SPE) using C18 2 ml prepsep cartridges. Solvent systems were developed by utilizing normal phase thin layer chromatography (TLC) plates in order to obtain resolution of components within each extract. Fractions were produced using water and MeOH (20%, 40%, 60%, 80%, and 100% MeOH). Each fraction obtained from SPE was bioassayed for larvicidal activity. Fractions with activity derived from *Synechococcus* sp. 36-8 were recreated in large quantities by increasing the scale of the SPE separation which was accomplished with a Horizon biotage unit with 48 ml C18 cartridges. It should be noted that this volume is given by the manufacturer and apparently the pore volume has been subtracted (e.g. 2.5 cm x 15 cm [$\pi r^2 h$] = 74 ml).

Non-polar extracts exhibiting mosquito larvicidal activity were fractionated as the polar extracts, by SPE, but using silica 2 ml hypersep cartridges instead. Fractions were produced using hexane and ethyl acetate as solvents (100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, and 0% hexane) and were subsequently bioassayed. The 90% hexane fraction derived from *Leptolyngbya* sp. 21-9-3 was dried using a rotary evaporator and reconstituted in hexane:ethyl acetate (9:1). The resulting sample was separated using a 500 ml glass column packed with 150 ml of silica gel. The same stepwise hexane/ethyl acetate gradient was used as in the SPE separation. Solvent volumes were determined by the movement of specific colored bands. An orange band was first produced followed by a dark green band. These were allowed to elute separately. Several other colored bands were collected in this manner but did not show

any activity. The 75% hexane fraction (black band) was chosen for analysis based on activity. The fraction was dried and reconstituted in 100% MeOH. The sample was now ready to be purified via high pressure liquid chromatography (HPLC).

4.2.4 Purification of larvicidal compounds from *Leptolyngbya* sp. 21-9-3

An HPLC method was developed to separate the remaining components of the *Leptolyngbya* derived fractions now reconstituted in 100% MeOH as mentioned in the previous section. Many methods were explored including sloped gradients and isocratic concentrations of both methanol and acetonitrile with water. The purification protocol was developed using an analytical column (Phenomenex Luna 5u C18 100A 250x4.60mm 5micron). The sloped methanol gradient was abandoned because it lacked resolution (Figure 10). Although an isocratic methanol gradient could have been explored to resolve the peaks, the chromophore overlap of methanol and the prospective compounds was not an ideal situation (λ max ~ 200 nm).

Figure 10: Chromatogram of the non-polar fractions derived from *Leptolyngbya* sp. 21-9-3 using a sloped MeOH gradient.

Acetonitrile was adopted as the solvent of choice because it offered improved resolution and did not overlap with the prospective compounds within the chromatogram (190 nm). In addition, a Kinetex column was found to aid in the separation. The unique particle size (2.6 microns) of the Kinetex column C18 stationary phase offered improved resolution when compared to that of a standard analytical column (particle size of 5 microns). It was also discovered that resuspending this sample in acetonitrile eliminated a significant amount of minor peaks from the chromatogram of which could have contained the mosquito larvicidal activity. It was later determined that these minor peaks did not contain any activity. The resuspension acted like a solvent extraction cleaning up the sample considerably for purification. Most of the contents of the injected sample eluted at 60% - 80% acetonitrile while running a sloped acetonitrile gradient. These lacked resolution. Isocratic concentrations were experimented with, following the 60% - 80% acetonitrile range, in 5 % increments; i.e., 60%, 65%, 70% et cetera. An isocratic concentration of 65% acetonitrile produced 3 distinct peaks (Figure 11) which will be referred to in this dissertation as **(1)**, **(2)**, and **(3)**. The optimal conditions for baseline resolution consisted of an oven temperature of 30 °C, 0.1% formic acid in H₂O, flow rate of 0.5 ml/min, and as mentioned previously resuspending the sample in acetonitrile prior to injection. Prospective compounds **(1)**, **(2)**, and **(3)** were subsequently tested individually for mosquito larvicidal activity. Unfortunately the Kinetex column is small in volume (4.6 mm x 100 mm [$\pi r^2 h$] = 1661 mm³ or 166.1 ml) and no more than 25 µl of sample could be injected per run. Analytical columns, although have the same width of the Kinetex column, are more than twice as long, and thus contain more than double the stationary phase (4.6 mm x 250 mm [$\pi r^2 h$] = 4152.65 mm³ or 415.265 ml). An ideal

Figure 11: Chromatogram of the non-polar fractions derived from *Leptolyngbya* sp. 21-9-3 using a 65% isocratic acetonitrile gradient with H₂O in 0.1 % formic acid.

scenario would call for a semi-preparative column which has a much larger stationary phase (10 mm x 250 mm [$\pi r^2 h$] = 19625 mm³ or 1962.5 ml) and thus allow for the injection of considerably more sample. However, while using the semi-preparative column contaminants were observed overlapping with (1) and (2). Potential contaminants were observed at 240 nm and 400 nm. These overlaps did not occur while using the Kinetex column. Although purity was increased significantly, the slowest protocol was thus adopted.

After a hundred runs on the HPLC, the sample was finally exhausted giving total masses of 516 µg for (1), 753 µg for (2), and 225 µg for (3). These purified samples were utilized for subsequent characterization analyses. These samples did not provide clear mass spec and NMR data. Thus, more biomass was obtained and the purification was commenced anew. All steps were followed as with the original biomass except the SPE step was not performed. The discovery of utilizing acetonitrile for resuspending the

sample prior to purification with the HPLC (cleaned up the sample) led to the idea of performing the resuspension at the crude extract stage prior to the first separation; i.e., the 500 ml glass column. The early resuspension with acetonitrile shortened the purification by an entire step with nearly the exact same HPLC chromatogram as a result (Figure 12). Unfortunately cyanobacteria do not always produce the same amount of their associated compounds. The first peak, **(1)**, was not found at the same concentrations seen previously. Because of this new turn of events it was decided that **(2)** would be the focus for the remainder of the project as sufficient sample could be acquired and the prospective compound exhibited mosquito larvicidal activity (see results section).

Figure 12: Chromatogram using the new biomass.

Because the NMR data were not conclusive using the original biomass, the HPLC protocol was slightly improved. All wavelengths at the retention time for **(2)** were examined. Potential contaminants were observed at 240 nm and 400 nm. The contaminants were identified as such because their peaks were not centered with **(2)**. Although they seemed minor, <5% of **(2)**, their exclusion should ameliorate the

characterization data. The contaminants were avoided by narrowing the collection time preventing as much of their elution as possible. After performing this slightly revised HPLC protocol 110 additional times, exclusively collecting (2), a total of 1.273 mg was collected and subsequently used for NMR and IR analyses.

A semi-preparative method was developed to collect (2) in bulk to be utilized in a large scale bioassay to calculate its LC_{50} value on mosquito larvae. The semi-preparative method is not advised to be used for characterization because the contaminant overlap is slightly greater with the semi-preparative column which would likely give subpar results similar to the first round of NMR data. The stationary phase for the semi-preparative column has nearly double the individual particle size of that in the Kinetex column (5 microns versus 2.6 microns) which is the same particle size as the analytical column and in both cases produce a tailing effect seen in the chromatogram (Figure 13).

Figure 13: Chromatogram using a semi-preparative column.

Although many alterations to this method were employed (solvent concentrations, flow rate, and oven temperature), none significantly ameliorated peak resolution. The

final conditions offering the best resolution were found to be an isocratic acetonitrile gradient of 80%, an oven temperature of 30 °C, the use of 0.1% formic acid in H₂O, and a flow rate of 4.5 ml/min. To avoid contamination from (3), (2) was only collected from 19-21 minutes. Mass spec data confirmed its purity. After 40 runs, over 3 mg was obtained for the LC₅₀ analysis.

4.2.5 Characterization of larvicidal compounds from *Leptolyngbya* sp. 21-9-3

4.2.5.1 Liquid chromatography – mass spectrometry

The first step in characterization involved liquid chromatography mass spectrometry (LC-MS) in an attempt to discover the molecular ion and associated fragments of the compounds in question. Individual peaks were evaluated using a TSQ Quantum Access LC-MS system and an Orbitrap LC-MS system. Both positive and negative ionization modes were performed using a heated electrospray ionization (HESI) source. Since much of the original purification was done with the Kinetex column, which is the standard column used for the LC portion of the LC-MS, the exact method used for the HPLC purification was also used here (65% isocratic acetonitrile gradient, 0.1% formic acid in water, and a flow rate of 0.5 ml/min). The column was heated using a hot sleeve to simulate the oven temperature of the HPLC method (30 °C). Thermo Xcalibur software was used for molecular formula generation and to create isotope simulations.

4.2.5.2 Nuclear magnetic resonance

Samples were concentrated into an HPLC vial using a drying apparatus consisting of air blown through a cotton filter, Drierite, another cotton filter, and finally the sample.

To remove any remaining solvent, samples were dried overnight using a Drierite desiccator attached to a vacuum. Deuterated methanol (CD_3OD) and acetone ($((\text{CD}_3)_2\text{CO})$) were used as solvents. Deuterated methanol was mainly used as it dissolves the prospective compounds more readily. Acetone was used to view potential hydroxyl groups that would overlap with the signal from methanol. Nuclear magnetic resonance (NMR) analyses were accomplished using a Bruker 400 MHz NMR. Experiments performed include ^1H NMR, ^{13}C NMR, Distortionless Enhancement by Polarization Transfer (DEPT 135), Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC), and Heteronuclear Multiple Bond Correlation (HMBC). For comparative purposes, chemical shift predictions were performed using ChemDraw software.

4.2.5.3 Infrared spectroscopy

Samples used in the NMR experiments were analyzed with infrared (IR) spectroscopy using a Nicolet iS5 FT-IR Spectrometer. Samples were analyzed as solids but as a precaution were suspended separately in methanol and acetone prior to analysis. This would prevent false positives for hydroxyl and carbonyl functional groups.

4.2.5.4 Double Bond Localization

A derivatization of (**2**) paired with gas chromatography-mass spectrometry was necessary to locate a potential site of unsaturation. The following method was modified from Dunkelblum et al. (1984).

4.2.5.4.1 Derivatization

Esterification. Reactions were performed in a 5 ml v-vial with a solid-top cap. All steps requiring the sample to be concentrated were performed with streamed nitrogen. Reagents were purchased from Sigma-Aldrich. One milligram of sample was concentrated in hexane to a volume of 30 μ l which was subjected to methyl esterification using 450 μ l of methanolic HCl. The reaction was kept at 65 °C for 2 hours and was subsequently cooled down to room temperature. Methanol was evaporated. The sample was diluted into 750 μ l of hexane and was washed with 750 μ l of distilled water. The organic layer was separated and dried with Na₂SO₄. The sample was concentrated to 30 μ l and 90 μ l of acetyl chloride was added. The reaction was kept at room temperature for 20 minutes. Excess reagent and solvent were evaporated and the sample was reconstituted into 75 μ l of hexane.

Dimethyl disulfide adducts. The sample derived from the esterification reaction was combined with 300 μ l of dimethyl disulfide and 90 μ l of iodine solution. The iodine solution was prepared using 60 mg of iodine in 1 ml of diethyl ether. The reaction mixture was kept in an incubation chamber set at 40 °C for 22 hours. The sample was cooled down to room temperature and diluted with 600 μ l of hexane. Removal of the iodine was accomplished by shaking the mixture with 1.5 ml of 5% aqueous Na₂S₂O₃. The organic layer was separated and a hexane extraction was performed on the aqueous layer using 300 μ l. The organic layer and the hexane extract were combined and dried with Na₂SO₄. The sample was then concentrated to a volume of 120 μ l and was stored at 4 °C until GC-MS analysis.

4.2.2.5.2 Gas Chromatography-Mass Spectrometry

The analysis was performed using a Thermo Scientific DSQ™ II Series single quadrupole GC/MS with a flame ionization detector and an Rxi-5Sil MS column. The column was set at 50 °C for 2 minutes and was heated to 250 °C using a 30 °C/min gradient. Two microliters of sample was injected. Mass acquisition commenced after 4.5 minutes

4.3 Results

4.3.1 Screening of cyanobacterial isolates

The isolates derived from polar and non-polar extracts provided by Dr. Gantar's laboratory lacked any significant mosquito larvicidal activity. The screening did reveal that extracts known to be cytotoxic did not induce mortality as *Ae. aegypti* may have grown resistant to particular cyanobacteria toxins. A hypothesis being considered is that these secondary metabolites may be produced specifically to suppress the feeding of mosquito larvae and other invertebrate grazers (Berry et al., 2008). Mosquito larvae exposed to these secondary metabolites may subsequently develop resistance by the overexpression of genes involved in detoxification, cuticular mutations limiting toxin uptake, and target site mutations decreasing toxin efficiency. Other extracts have shown to hasten the development of mosquito larvae such as those derived from *Chlorococcum*. All extracts in the isolate screening were deemed ineffective for the current study and it was decided to commence the search for mosquito larvicidal compounds from living cultures.

4.3.2 Screening of cyanobacterial biomass

Out of 120 tested strains, 31 exhibited 25% mortality or greater (Figure 14). *Coccoid* sp. 53-2a, *Leptolyngbya* sp. 21-9-3, and *Synechococcus* sp. 36-8 had the most activity and were chosen for further analysis. Although *Lyngbya* sp. 15-2 had more activity than *Coccoid* sp. 53-2a, it was not used because it is known to produce pahayokolide A which was previously linked to vertebrate toxicity when tested in a zebrafish teratogenicity assay (Berry et al., 2004).

Figure 14: Results of the cyanobacteria biomass screening. Red arrows indicate the cyanobacteria used for further analysis. *Oscillatoria* was the original genus assignment for strain 21-9-3

4.3.3 Bioassay guided fractionation of *Coccoid* sp. 53-2a, *Synechococcus* sp. 36-8, and *Leptolyngbya* sp. 21-9-3

All 3 prospective strains had activity in their polar and non-polar extracts. The bioassay performed on the first fractionation step of the polar extracts using the biotage unit showed some moderate activity occurred in the 60% methanol fraction from *Coccoid*

sp. 53-2a and the 80% methanol fraction from *Synechococcus* sp. 36-8. See

Figure 15 for a simplified representation of this fractionation step. In both cases, as much as 50% mortality occurred but only did so when using older eggs. Larvae derived from older eggs (≥ 2 months) are more susceptible to toxins, when compared to larvae derived from new eggs (≤ 2 weeks), considering they have less nutritional reserves hindering their overall fitness (Perez and Noriega, 2012). However, using older eggs can be seen as a valuable tool in bioassay guided fractionation with mosquito larvae because less sample can be used and thus less is wasted. Caution should be taken in that one must ensure that higher concentrations will indeed affect larvae derived from newer eggs (≤ 2 weeks).

Figure 15: Simplified fractionation scheme for *Coccoid* sp. 53-2a.

The bioassays performed on the first fractionation step of the non-polar extracts using SPE showed the 90% hexane fraction from all 3 prospective cyanobacteria

exhibited 100% mortality regardless of the age of the eggs. *Leptolyngbya* sp. 21-9-3 also had activity in the 70% hexane fraction, but it was found to be difficult to resolve its components using TLC plates. The aforementioned bioassays on the polar and non-polar fractions were repeated numerous times particularly in regards to the polar extracts as mortality varied tremendously. Because of this variability in toxicity and the potential for dereplication confirming the identity of an already known compound, as many polar compound have already been purified from the cyanobacterial taxon in question (anatoxin-a, microcystin-LR, and even the glycolipids re-purified in this study), it was decided that the non-polar extracts were to become the focus of the dissertation.

The 90% hexane fractions from the non-polar extracts caused 100% mortality in every subsequent bioassay. After running these through the glass column, no activity was observed in any of the resulting fractions. Samples were concentrated and the assay was performed again leading to the discovery that *Leptolyngbya* sp. 21-9-3 had some activity in the 75% hexane fraction which was then analyzed with HPLC.

4.3.4 Purification of larvicidal compounds from *Leptolyngbya* sp. 21-9-3

Using a MeOH gradient, fractions were collected at two minute intervals and the subsequent bioassays confirmed that activity resided in the only pronounced portion seen in the chromatogram, 36-40 min (Figure 16). Active fractions (36-40 min) were resolved into (1), (2), and (3) using a 65% isocratic acetonitrile gradient (Figure 17). The most activity was produced by (1) causing 100% mortality in approximately 5 hours. Larvae were noted to lose mobility instantaneously while subjected to the treatment. Compound (2) showed the second most activity causing 100% mortality in 24 hours. There was no

observed activity associated to **(3)** even when tested at annihilation concentrations used for **(2)**, see section 5.2. However, **(3)** was still purified as it could help elucidate the structures of **(1)** and **(2)** given their potential for being chemically similar as they elute in similar conditions.

Figure 16: Chromatogram of the non-polar fractions derived from *Leptolyngbya* sp. 21-9-3 using a sloped MeOH gradient.

Figure 17: Chromatogram of the non-polar fractions derived from *Leptolyngbya* sp. 21-9-3 using a 65% isocratic acetonitrile gradient with H₂O in formic acid.

4.3.5 Characterization of larvicidal compounds from *Leptolyngbya* sp. 21-9-3

4.3.5.1 Liquid chromatography – mass spectroscopy

The first set of LC-MS data encompasses purified products derived from the first batch of cyanobacterial biomass used in the current study. Analysis of **(1)** in positive ionization mode revealed a molecular ion $[M+H]^+$ found at 584.72 m/z (Figure 18). Compound **(2)** in positive ionization mode revealed a molecular ion $[M+H]^+$ at 700.71 m/z (Figure 19).

Figure 18: Mass Spectra of **(1)** using positive ionization.

No peak was discernable from the chromatogram associated with the mass spectra of **(3)**. Compound **(3)** may be difficult to ionize under normal conditions. Even when its specific retention time was examined, no isotope pattern was observed with any similarity to those seen for **(1)** and **(2)**. The isotope pattern, specifically the +1 mass from the molecular ion, is a good indication of the number of carbons in a molecule as it is caused

Figure 19: Mass spectra of **(2)** using positive ionization.

primarily by the frequency of the ^{13}C isotope (1.1% abundance versus 98.9% for ^{12}C). Thus, a 20% abundance of that +1 mass is suggestive of a molecule consisting of 17-18 carbon atoms. Since **(1)** was found in low yield with the new biomass and **(3)** was not active (and proven to be difficult to analyze), their characterization were no longer pursued.

After analyzing **(2)** derived from the new biomass, it was apparent that the previously identified molecular ion, 700.71 m/z, was no longer observed (Figure 20). Although, from the HPLC chromatograms, clearly all 3 peaks have identical retention times when compared to those derived from the original biomass. Regardless if the second peak derived from the new biomass was different from the original biomass, it was of little consequence considering the only characterization done was obtaining its mass and the newly purified **(2)** demonstrated identical mosquito larvicidal activity to the originally collected **(2)**.

Figure 20: Mass spectra of (2) derived from the new biomass using positive ionization.

From the mass spectra of the new biomass we see 3 ions; potential fragments 292.72 m/z, 333.70 m/z, and potential molecular ion $[M+H]^+$ at 351.57 m/z. The loss of 18 (351-333) is likely the loss of water. The loss of 41 (333-292) is a little more problematic considering the limited candidates and how unusual they are. One of the solutions provided by the formula generator of the Thermo Xcalibur software is the loss of a propenyl group (C_3H_5). The 838.87 m/z ion seems to be related to (2) as it has increased in abundance while using the semi-preparative method for purification.

Several issues were apparent from these data. Firstly, the discrepancies of the peak shapes from the HPLC and the mass spec chromatograms are suggestive of different compounds. Even when duplicating the exact conditions of the HPLC method using a hot sleeve to simulate the oven temperature (30°C), the chromatogram from the mass spec

remains disfigured (Figure 21) Secondly, the mass spec instruments are highly sensitive and yet a large amount of sample was required for any apparent ionization (>50 ug). When ionization of the prospective compound is inefficient, its abundance in the mass spec derived chromatogram may be the same as impurities that ionize well. Thirdly, heavy ions (668–838 m/z) seem to change abundances dramatically from one analysis to the next while the HPLC and NMR suggest a much more stable and purified compound. Lastly, the isotope pattern of the suspected molecular ion $[M+H]^+$ of 351 does not support the NMR analyses suggesting a 16 carbon molecule and thus a 16% abundance of the $[M+H+1]^+$ ion. The abundance of the $[M+H+1]^+$ was actually quite variable ranging from 20–35%. Another aspect of the isotope pattern that seems to be contradicting is the $[M+H+2]^+$ ion which has 15% abundance relative to the molecular ion. A 15% abundance could suggest the presence of sulfur. Given the negative results using Ellman's reagent, sulfur does not appear to be present at least in the form of an aliphatically attached sulfhydryl group that the reagent specifically identifies. All of these discrepancies led to a more robust evaluation of **(2)** using negative ionization. Negative ionization was previously abandoned for analyzing samples derived from the original biomass because the results consisted of too many ions. With positive ionization, all though variable, results consisted of a single molecular ion. However, samples from the original biomass did not offer any clear NMR data. Samples derived from the new biomass appeared much cleaner using negative ionization.

Negative ionization of **(2)** using high resolution mass spectrometry yielded a molecular ion $[M-H]^-$ of 253 m/z (Figure 22). The isotope pattern is consistent with a

Figure 21: Mass spectra of (2) derived from the new biomass using a hot sleeve at 30 °C and positive ionization.

molecule of 20 carbons. There is no apparent $[M-H+2]^-$ area which would eliminate the possibility of sulfur being in the compound. The 299 m/z ion is likely a formic acid adduct (253+46) considering it is used in the mobile phase for purification and the mass spec analyses (Ginter et al., 2007). The 507 m/z ion could be a dimer of (2). The isotope pattern does support this showing less than 40 carbons in regards to the abundance of the $[M-H+1]^-$ ion. Another important feature is that the shape of the peak in the chromatogram is not deformed as with positive ionization and more closely matches the shape found in the HPLC protocol. These factors are much more suggestive of acquiring the correct molecular ion when compared to the findings using positive ionization.

With mass obtained in high resolution (4 decimal places), a molecular formula can be generated with the Thermo Xcalibur software. The formula can be fairly accurate especially if the atoms included in generating the formula are correct. All significantly

Figure 22: High resolution mass spectra of **(2)** derived from the new biomass using a hot sleeve at 30 °C and negative ionization.

abundant isotopes of carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur were included as candidate atoms. The formula generated for the mass of 253.2159 was $C_{14}H_{27}ON_3$ which has a delta mmu of 0.009. Even though the delta mmu is very low, the isotope pattern does not match that seen in the 1H NMR data. The next formula generated was $C_{16}H_{29}O_2$ with a delta of -1.333 milli mass units (mmu). Given this is the $[M-H]^-$, the actual formula would be $C_{16}H_{30}O_2$ with 2 degrees of unsaturation. The isotope pattern of $C_{16}H_{30}O_2$ is nearly an identical match to that of **(2)**. The isotope patterns are compared in Figure 23. On the basis of this information, $C_{16}H_{30}O_2$ was closely scrutinized for the potential of being the prospective compound housing mosquito larvicidal properties.

4.3.5.2 Nuclear magnetic resonance

1H NMR. From the 1H NMR spectra of **(1)** using the original biomass, there are no peaks that are resolved enough for subsequent characterization steps (Figure 24). Peaks at 3.34

Figure 23: Comparison of the isotope patterns from the simulation of $C_{16}H_{29}O_2$ (top) and the mass spec data for ion 253.22 m/z (bottom).

ppm and 4.87 ppm are attributed to methanol and water, respectively. Although methods were employed to limit the concentrations of these solvents, they prove to be difficult to eliminate given their solubility in deuterated methanol and potential interactions with the prospective compounds. Their chemical shifts do not seem to interfere with those from (1) of which all seem to have some degree of overlap. Even the cleanest peak observed at 2.16 ppm has irregular broadening upon closer inspection. Low sample yield (516 μ g) could potentially have been the issue. The NMR engineer of FIU recommended that at least 1 mg be used especially when the actual size of the molecule is still unknown (Yali Hsu, personal communication, June 20, 2012). At 500 μ g, a larger molecules will have a lower molarity compared to a smaller molecule. Another potential cause for this lack of resolution could be sample degradation and/or contamination. It was determined that sample degradation was not the issue because when the sample was analyzed with the HPLC method used in the purification process, one sole peak is observed in the resulting

chromatogram. However, this would not determine whether the sample was contaminated because the contaminant may not elute with a 65% isocratic acetonitrile gradient. Subsequent mass spec analysis did suggest contamination.

Figure 24: ¹H NMR spectra of **(1)** derived from the original biomass

Much of the same discrepancies were seen in the ¹H NMR spectra of **(2)** (Figure 25). Compounds **(1)** and **(2)** were hypothesized to be structurally similar given their mosquito larvicidal activity and their elution proximities in the HPLC protocol. For comparative purposes only, the ¹H NMR spectra for **(3)** is also quite similar to those of **(1)** and **(2)** suggesting that all three compounds are structurally related (Figure 26).

As mentioned previously, because of lower yield of **(1)**, the lack of ionization of **(3)**, and time constraints, **(2)** will now be the focus for the remainder of the characterization data.

With new biomass, and a slightly more stringent HPLC protocol, new ¹H NMR

Figure 25: ¹H NMR spectra of **(2)** derived from the original biomass.

Figure 26: ¹H NMR spectra of **(3)** derived from the original biomass.

spectra was obtained and can be seen in Figure 27. Although the structure has not been elucidated in this text so far, the proposed structure has been included to facilitate the viewing of the data. The new spectra is much cleaner than that obtained from the original biomass even though some areas still have some broadness associated to them. The peaks from the spectra are labelled alphabetically from A–F starting with the lowest chemical shift. Abbreviations in parenthesis are indicators of the splitting pattern (s = singlet, t = triplet, q = quartet, quin = quintet). It is clear that the chemical shift for peak A is likely a

terminal methyl group coupled to its sole neighbor, a methylene, making it a triplet. Upon looking up chemical shifts for peak F, several possibilities were discovered including carbon bound hydrogens in close proximity to a phenyl ester. However, hydrogen signals from within the speculated phenyl group were not visible in the spectra. Another possibility is that the hydrogen atoms are bound to carbon atoms involved in a double bond which is supported by the triplet splitting pattern. Each hydrogen from the methines in the double bond are bordered by methylenes splitting the signal. With these findings and having presented them at a committee meeting, Dr. Kathleen Rein brought to my attention the similarities between this spectrum and that of a fatty acid (Figure 28). Assuming **(2)** is a fatty acid, when comparing Figure 27 and Figure 28 it is apparent that the designation of the terminal methyl and the alkene groups seem to be correct. The methylene chain would be group (B) in Figure 27 as it has the same chemical shift as (d) in Figure 28. These signals have some broadness associated to them because the methylene groups are not perfectly equivalent considering minute differences in their environments. The alkene portion likely contributes to this slight unevenness in deshielding. Every methylene in the chain is adjacent to 4 hydrogens, from neighboring carbons, except for the methylene adjacent to the terminal methyl group. Because of these conditions no discernable splitting pattern is present. Carbons adjacent to the double bond have a chemical shift around 2 ppm seen by (D) in Figure 27 and supported by (e) Figure 28. The quartet splitting pattern does suggest each being coupled by 3 hydrogens. Peaks C and E correspond to those closest to the heteroatom portion of the molecule as the deshielding increases within proximity to this group. These assignments

Figure 27: ^1H NMR spectra of (2) derived from the new biomass.

Figure 28: ^1H NMR spectra of methyl elaidate. Figure provided by the AOCS lipid library.

are supported by (b) and (c) in Figure 28 and their splitting patterns coincide as well. The proton integration demonstrates that all assignments made thus far are feasible (Figure 29). However three questions remain:

- 1) Is the methylene chain actually multiple chains as in Figure 27?
- 2) What is the heteroatom containing functional group?
- 3) What is the location of the double bond?

Figure 29: ¹H NMR spectra with proton integration.

Correlation Spectroscopy. Figure 30 contains the COSY spectra. These data confirm everything mentioned previously as all groups (A-F) solely couple with every adjacent group in the proposed structure. Since the methylene chain (B) couples with three different groups (A, C, and D), it must be composed of two separate chains.

Distortionless Enhancement by Polarization Transfer. All of the spectra derived from the various ¹³C NMR experiments will use the same letter assignments for each CH_x group as in the ¹H NMR. From the DEPT 135 analysis (Figure 31) the most obvious

Figure 30: COSY spectra with coupling labeled.

features are the carbons with odd numbers of hydrogens (methine and methyl) which would appear above the baseline. These would consist of the terminal methyl and the alkene groups seen on the extremities of the spectra as they are in the ^1H NMR. Two peaks appear for (F) supporting the double bond hypothesis encompassing 2 carbons with a chemical shift around 130 ppm. Only one peak appears for (A) supporting the identity of a single terminal methyl group. Considering the proposed structure, all other carbons would have to be methylenes which is exactly what the DEPT 135 illustrates in that these signals are all below the baseline. There are 10 individual peaks in this particular region giving a total of 13 carbons for the structure. Subtracting the 4 methylene groups that are not found in group (B) suggests the methylene chains consist of 6 total carbons assuming that all carbons are visible on the spectra. Carbons that are equivalent can potentially be seen as one peak. Particularly, carbons in methylene chains would have potential for being equivalents. Methanol appears around 49 ppm and appears on both sides of the

baseline because of deuterium exchange causing the number of hydrogens to fluctuate between odd and even.

Figure 31: DEPT 135 spectra.

Heteronuclear Multiple Quantum Coherence. The HMQC spectra (Figure 32) justifies all of the assignments made thus far in that all of the proposed hydrogen assignments are coupled to their respected carbon assignments. Solely using the DEPT 135, it was not possible to make specific assignments for many of the methylene groups centered at 30 ppm. The assignments can be made using the HMQC data as the exact ^{13}C NMR chemical shift can be deduced on the basis of the group assignments made in the ^1H NMR. Interestingly, 2 small and broad peaks that were not included as potential carbon signals using the DEPT 135 spectra are shown to be groups (C) and (E). See Figure 33 for a closer view of these assignments. Identifying the correct shifts for (C) and (E) increases the total number of suggested carbons from 13 to 15 meaning the methylene

chains have increased to 8 total carbons. Another interesting feature apparent on the HMQC spectra is that groups (C) and (D) have lower chemical shifts than (B) in the ^{13}C NMR and not in the ^1H NMR. The same results are obtained when comparing the ^{13}C NMR and the ^1H NMR chemical shift predictions (Figure 34). Comparing the spectra to the predictions, the most telling feature is how much the chemical shifts from the ^{13}C NMR seem to fluctuate within group (B). Specifically carbons 14 and 15 (23.1 and 32.6 ppm, respectively) are the source of the fluctuation. The variability in chemical shift could be the result of differential shielding on the carbon nuclei versus their attached hydrogens.

^{13}C NMR. The ^{13}C NMR could provide information in regards to quaternary carbons as only primary, secondary, and tertiary are visible in the DEPT 135. No such carbons are apparent in Figure 35. Although, quaternary carbons are not always visible using ^{13}C NMR.

Heteronuclear Multiple Bond Correlation. Seen in Figure 36, the HMBC spectra does not provide much more information than did the COSY analysis. It does demonstrate that no mistakes are apparent from the assignments made thus far. Groups E and C had very weak carbon signals and do not appear here at all. The HMBC should show 2 sequential bonds for each group but this only occurs for group A. The 2 adjacent carbons to group A have the largest difference in chemical shifts than any other methylene groups (Figure 34). The other groups are all paired and likely did not show bonds 2 atoms away because none of these bonds were exactly the same thus hindering those signals.

Figure 32: HMQC spectra

Figure 33: ^{13}C NMR derived from the HMQC that shows a closer view of the clustered area.

Figure 34: ^1H NMR and ^{13}C NMR chemical shift predictions for palmitoleic acid.

Figure 35: ^{13}C NMR spectra.

Summary of NMR Analyses. All assignments match the proton integration (Figure 29) except for the methylene chains which shows 16-17 hydrogens. Since the HMBC revealed that eight carbons produce this signal and the DEPT 135 shows that they must all be methylene groups, 16 is likely the correct number of hydrogens. There could be another hydrogen on a heteroatom somewhere overlapping with this signal which would explain why it is visible in the proton and not the carbon NMR, however, the only other

Figure 36: HMBC spectra.

possibility is a sulfhydryl group. A sulfhydryl group would have a chemical shift that would support this ($\delta = 1.6\text{--}1.8$ ppm) while a hydroxyl or an amine would have a much higher chemical shift.

From all the NMR analyses we can generate the following formula for the hydrocarbon portion of the molecule:

And the proposed structure:

Additional ^1H NMR data were obtained in order to reveal the presence of potential hydroxyl groups that would overlap with the MeOH signal in the original experiments. These were performed in deuterated acetone. From the resulting ^1H NMR

spectra (Figure 37), which does appear to contain such a peak (3.3 ppm) which integrates to 2 hydrogens. Also, if these are hydroxyls they should not couple with anything given that they are likely being exchanged rapidly with deuterium. The lack of coupling is supported by the COSY seen in Figure 38. However, the chemical shift of 3.3 is identical to that of methanol in deuterated acetone. Even though measures were taken to rid any traces of methanol, it is feasible that enough was still present to produce such a signal. If the heteroatom portion of the molecule is in fact a carboxylic acid, hydrogen bonding can occur between the hydroxyl proton found on methanol and the carbonyl oxygen found on the carboxylic acid. Considering the formula generated from the mass spec data ($C_{16}H_{30}O_2$) with an RDB of 2.0, a carboxylic acid is quite feasible.

4.3.5.3 Infrared spectroscopy

From the IR spectra (Figure 39) it is apparent that the previously suggested sulfhydryl group, potential adding an extra hydrogen to the signal designated to the 2 methylene chains, is not present. A sulfhydryl group would create a peak around 2600 cm^{-1} . The lack of a sulfhydryl group was also confirmed with negative results using Ellmans's reagent. The broad peak from $3100\text{--}3800\text{ cm}^{-1}$ is indicative of an O–H stretch specifically for a carboxylic acid. The O–H stretch of an alcohol would appear less broad and as a sharper signal. Also, (**2**) cannot be an alcohol because the ^{13}C NMR chemical shift for a carbon adjacent to a hydroxyl group is around 60 ppm which does not occur in any of the spectra. Lastly, an alcohol would require the hydroxyl to be located away from the carbonyl creating too many signals that were not observed from the NMR data. The

Figure 37: ^1H NMR spectra using $(\text{CD}_3)_2\text{CO}$.

Figure 38: COSY spectra using $(\text{CD}_3)_2\text{CO}$.

C–H stretch can be seen around 2900 cm^{-1} . The small protrusion just left of this signal, around 3000 cm^{-1} , shows a site of unsaturated ($=\text{C}-\text{H}$ stretch). The signal at 1709 cm^{-1} ($\text{C}=\text{O}$ stretch) seemed small for a carbonyl but when considering the size of the C–H stretch from a fairly long aliphatic chain, proportionally it seemed appropriate. The only other signal that could occur around 1700 cm^{-1} would be that of imine ($\text{C}=\text{N}$) which could encompass an N–H stretch likely masked by the O–H stretch. With all visible

signals considered, the IR supports the identity of a hydrocarbon molecule with a site of unsaturation and a carboxylic acid functional group.

Figure 39: IR spectra of **(2)** analyzed as a solid.

4.3.5.4 Double Bond Localization

Figure 40 shows the derivatization using DMDS. Methyl sulfide is incorporated at the alkene portion of the molecule giving rise to a predictable molecular ion (M^+) and 3 distinct fragments (A^+ , B^+ , and C^+) when subjected to GC-MS. Assuming **(2)** was palmitoleic acid, the M^+ , A^+ , B^+ , and C^+ were calculated to be 362 m/z, 145 m/z, 217 m/z, and 185 m/z, respectively. The GC-MS spectra is in Figure 41 and strongly supports the identity of **(2)** to be palmitoleic acid. Fragments A^+ , B^+ , and C^+ appear in large abundance. The M^+ is present but with low relative abundance. From the results of Dunkelblum et al. (1984), it was found that the M^+ would only appear at a 10-30% relative abundance and would not be present in some cases. Dunkelblum et al. (1984) tested several 16 carbon fatty acids one of which had a double bond on carbon 9 as does palmitoleic acid. The ions they documented for this particular fatty acid were identical to those obtained here. The authors also mention that the E and Z isomers were readily

distinguishable because of slightly different elution times. Unfortunately that level of resolution was not obtained here. However, it was suspected for some time that (2) contained isomers because of strange tailing effects seen in the HPLC chromatograms which is also slightly visible in Figure 41. The tailing effect is more pronounced when conditions are altered such as lowering the flow rate. Efforts were made to resolve these potential isomers earlier in the current study but these attempts were not very fruitful. All things considered, (2) potentially contains both the E and Z isomers of palmitoleic acid.

Figure 40: DMDS addition reaction and potential observable ions with GC-MS. Scheme produced by Dunkelblum et al. (1984).

4.4 Structure determination larvicidal compounds from *Leptolyngbya* sp. 21-9-3

From the high resolution mass spec data, a mass of 254.2159 was obtained giving a chemical formula of $\text{C}_{16}\text{H}_{30}\text{O}_2$. The structure has 2 degrees of unsaturation which would be explained by the proposed alkene and carbonyl groups. The IR spectra indicates that the carbonyl is part of a carboxylic acid. The IR also supports the presence of an alkene.

Figure 41: GS-MS spectra of (2) after the derivatization procedure.

As a result of combining all the different NMR analyses, the molecular formula ($C_{15}H_{29}$) and a structural formula were generated for the aliphatic portion of the molecule. The identity of the heteroatom containing functional group was elusive for some time considering no quaternary carbons were visible from the NMR. Many functional groups were explored containing sulfur and phosphorous. When the molecular ion was finally discovered using negative ionization, it was clear that sulfur could not be in the molecule considering the lack of an $[M-H+2]^-$ ion caused by the frequency of ^{34}S isotope (4.21%). In fact, with the high resolution mass it was possible to eliminate many possibilities simply based on the isotope pattern. From the formula generator, only one solution had a lower delta mmu, $C_{14}H_{27}ON_3$. Because it has 14 carbons the abundance of the $[M-H+1]^-$ ion should be around 15% (Figure 42) which does not coincide with the 18% abundance found from the molecular ion (253.2159 m/z) and the isotope simulation for $C_{16}H_{29}O_2$ (Figure 23). Even though the NMR analyses only revealed 15 carbons, the IR supports

the presence of a quaternary carbon. The isotope pattern also supports the presence of an additional carbon. Quaternary carbons can be difficult to visualize because the intensity of ^{13}C NMR signals are weakened without attached hydrogens (Joseph and Wong, 1979). This property is known as the Nuclear Overhauser Effect.

Figure 42: Isotope simulation for $\text{C}_{14}\text{H}_{27}\text{ON}_3$.

All things considered, it appeared that the prospective compound was likely a monounsaturated fatty acid with the molecular formula $\text{C}_{16}\text{H}_{30}\text{O}_2$. One such fatty acid does exist, palmitoleic acid. Its structure is provided in Figure 43. Making things even more like that palmitoleic acid is indeed the prospective compound is the fact that it has already been purified from cyanobacteria (Matsunaga, 1995). The NMR spectra for **(2)** are nearly identical to those predicted for palmitoleic acid (Figure 44). The missing signals from the OH proton and the quaternary carbon, seen in the NMR spectra for **(2)**, are the only differences. The signal from the OH proton cannot normally be seen because of deuterium exchange. A quaternary carbon does produce a weak signal. It is suspicious that only carbons 2 and 3, those adjacent to the carboxylic acid functional group, have

extremely weak and broad signals. If these two methylenes can produce such weak signals, the adjacent quaternary carbon will likely not produce any kind of measurable signal. However, as mentioned previously, the IR spectra strongly supports that (2) encompasses a carboxylic acid and thus a quaternary carbon must be present.

Figure 43: Structure, mass, and isotopic values for palmitoleic acid.

Given the good results using negative ionization for identifying the molecular ion of (2), (1) and (3) were also observed using the same method. Unfortunately, even when concentrating (1) to visible residue, no mass was obtained. A molecular ion of 278.52 m/z was discovered for (3). The experiment was not done with the high resolution mass spectrometer which was higher in mass by nearly 4/5 of a mass unit when analyzing (2). Thus, the molecular ion of (3) is closer to ~279.34 m/z and the uncharged mass of the molecule is roughly 280.34 amu. Structurally, (3) is likely similar to (2) considering their proximities in the purification protocol. Also, the original NMR data for (1) and (3) does

Figure 44: ^1H NMR spectra prediction (left) and ^{13}C NMR spectra prediction (right) of palmitoleic acid.

show the same chemical shifts as those found from (2) (Figure 25 and Figure 26). The only shift missing is that of the alkene portion. Although these data did not allow for structural characterization, it does demonstrate the potential for (1) and (3) being fatty acids of different lengths and potentially with different degrees of unsaturation. The 26 mass unit difference between (2) and (3) does support this identity. Two additional carbons and an extra site of unsaturation would explain the difference. The isotope pattern does appear slightly lower than expected (Figure 45). Considering the same results were found for (2) when using the low resolution mass spectrometer, it would appear that everything matches up to (3) being a longer chain fatty acid with an additional site of unsaturation such as linoleic acid. These findings are also consistent with the fact that (3) elutes after (2). The longer chain would have a higher level of interactivity with the C18 stationary phase of the HPLC columns used. Palmitoleic acid has been found in fairly large quantities within the cyanobacteria *Phormidium* sp. NKBG 041105 and *Oscillatoria* sp. KKBG 091600 (Matsunaga, 1995). In fact, the total fatty acid composition of these two organisms was mostly made up of palmitoleic acid, over 54% in both cases. In a study conducted by Sharathchandra and Rajashekhar (2011),

palmitoleic acid and linoleic acid were present in many isolates spanning several freshwater cyanobacteria including species of the genera *Oscillatoria* and *Lyngbya*. It is thus in the realm of possibility that **(2)** and **(3)** are indeed these two fatty acids.

Figure 45: Mass spectra of **(3)** using negative ionization.

With the results of the double bond localization procedure, it can be said with much more confidence that **(2)** is in fact palmitoleic acid. It is also likely that both E and Z isomers are present given the co-occurrence of these in biological samples (Dunkelblum et al., 1984) and the apparent co-elution seen in the chromatograms as tailing effects. No further characterization was performed on **(3)** but its identity as linoleic acid is also strengthened by the characterization of **(2)** considering the likelihood that these 2 fatty acids can be found within the same cyanobacterium (Sharathchandra and Rajashekhar, 2011), the characterization done on **(3)** (MS, NMR, IR), and their proximities in the purification protocol.

4.5 Sequencing of the 16s rRNA gene of *Leptolyngbya* sp. 21-9-3

4.5.1 Nomenclature Issues of Cyanobacteria

At low magnification, cyanobacteria resemble eukaryotic algae as they share similarities in cellular organization; i.e., both can exist as linear filaments, branched filaments, and unicellular conformations. In combination with the similarities observed by scientists between green algae (recently placed into the plant kingdom) and higher plants, it is not surprising that the classification of cyanobacteria has been in the form of botanical methods. Botanical classification is based on ecological and morphological characteristics (Geitler, 1932; Desikachary, 1959). Since the late 1970s, some have argued that cyanobacteria should be classified, as other bacteria, on type cultures deposited in culture collections (Stanier et al., 1978; Rippka et al., 1979). The justification of such an argument is that differing environmental conditions of cultured strains induce phenotypic plasticity observable in cellular morphology. Phenotypic plasticity has made classification by botanical methods quite variable and has shown to result in taxonomic errors (Nelissen et al., 1995; Neilan et al., 1997). Other non-molecular techniques that have acted as taxonomic deterrents include the use of cyanophage susceptibility and sheath properties, both of which also vary in culture (Rippka et al., 1979; Whitton, 1992; Rippka, 1988).

In the late 1980s, Anagnostidis and Komarek (1988) made many taxonomic changes in the order Oscillatoriales based on thylakoid arrangement and cell division classifications which lead to a debate on the endemism of many polar oscillatorian strains. Although these techniques are still regarded as some of the more useful

characterization protocols based on morphological characteristics, the human error component can be mostly eliminated with a genetic analysis.

The 16S rRNA gene has been the target of choice to study the phylogeny of cyanobacteria on a genetic level. It is useful in distinguishing organisms at the genus level and has more broad taxonomic applications as well (Casamatta et al., 2005). The problem with molecular tools is that the cyanobacteria need to be identified first by morphology which is then followed by the ribosomal RNA analysis; i.e., if the morphological identification is incorrect, the phylogenetic assignment based on molecular tools is misplaced. Casamatta et al. (2005) used the International Code of Botanical Nomenclature (Greuter et al., 2000) for species identification which incorporates information on the immobilized state of the sample as opposed to the bacterial code with type cultures. They also used Wiley and Mayden's (2000) evolutionary concept for new species recognition. The concept maintains that a new species must keep its identity disregard to changes in the environment or over time and that this identity is sufficiently different from like populations to be considered "new".

Cassamatta et al. (2005) found good evidence that many *Leptolyngbya* spp. are actually polyphyletic. Conversely, *Phormidium* sp. IAM M-99 was found to be a strain of *Leptolyngbya*. Also, the authors found that the type of cell division combined with thylakoid arrangement concurs with their ribosomal RNA analysis which supports the classification methods of Anagnostidis and Komarek (1988). These new techniques finally offer means of establishing correct taxonomic classification of cyanobacteria combining visual cues that lack plasticity and gene sequence assignments. The prospective cyanobacteria of the current study was assigned a genus based on its non-

branched filaments and the lack of heterocyst. Any genera from the order Oscillatoriales could potentially be its identity based on morphological characteristics. *Leptolyngbya* sp. 21-9-3 was thus originally given the title of *Oscillatoria* sp. 21-9-3. A closer examination of the identifiable morphological features that resist phenotypic plasticity derived from differing culture conditions, mentioned previously, could provide a more accurate designation. However with the advent of 16S rRNA sequencing and the ever growing Gene Bank database, this was the tool of choice for genus identification.

4.5.2 Methods

4.5.2.1 DNA Isolation and Purification

Four replicates of frozen biomass, with medium drained, were subjected to a digestion solution (dH₂O, Tris pH8, NaCl, EDTA pH8, SDS, DTT, Proteinase K) equal to 4 times their original volumes of around 100 µl. Samples being digested were placed in a water bath at 55 °C overnight and inverted periodically. Contents were shaken and enough NaCl was added to obtain a concentration of 0.7 M. An extraction with phenol:chloroform:isoamyl alcohol was performed followed by a chloroform:isoamyl alcohol extraction. Extracts were combined, mildly shaken, and then spun for ~10 minutes in a microfuge until a sharp interface resulted. Supernatants were collected, while completely avoiding the interface, and placed into clean tubes. To precipitate the DNA, sample volumes were doubled by the addition of isopropyl alcohol. Contents were mildly shaken. Glycogen was added to each sample, 1 µl of a 20 mg/ml solution. Samples were then placed at -70 °C for 15 minutes (-20 °C for 1 hour would also work). Samples were spun on a microfuge for 15 minutes. The resulting DNA pellets were

washed with 70% ethanol. Ethanol was then removed by pipette and by the samples being spun down. The ethanol should be removed as much as possible while avoiding drying out the DNA pellets as they will be difficult to solubilize otherwise. Pellets were resuspended in TE buffer (Tris-HCl pH8 + EDTA).

4.5.2.2 Gel Electrophoresis

For confirmation of DNA isolation the resuspended pellets were analyzed using gel electrophoresis. Gels were prepared using 1% agarose and TAE. Ethidium bromide was added as a dye. Each of the 4 replicates were placed in individual wells. Confirmation of PCR products were performed in similar fashion. Bands were visualized using long wave UV. DNA was separated from the gel using glass wool and alcohol.

4.5.2.3 Amplification of the 16S rRNA gene and Sequencing

Primers were purchased from operon.com and are specific for the cyanobacterial 16S rRNA gene (CYA106F and CYA359F). Their sequences can be seen in Figure 48 and Figure 49, respectively. Magnesium chloride, Go Taq buffer, dNTP, PCR H₂O and 1 of the 2 primers were combined in thin wall tubes. Purified DNA and mineral oil were then added. Tubes were placed into the PCR thermal cycler which was programmed for 94 °C/30 seconds, 55 °C/30 seconds, and 72 °C/30 seconds. Once the temperature reached 80 °C, Taq polymerase was added (hot to 80). Thirty cycles were allowed to be performed. PCR products were confirmed with gel electrophoresis and sent to GENEWIZ, Inc. for sequencing.

4.5.2.4 Evaluation of sequences

Data received from GENEWIZ, Inc. was analyzed using DNA Baser computer software. Contiguous DNA sequences were produced using the results from both primers. BLAST searches were accomplished by GenBank and incorporated all available nucleotide sequences with no organismal specificity in the search filter.

4.5.3 Results

As mentioned previously, 4 replicates were performed and correspond to the 4 bands circled in Figure 46. All other lanes in the gel correspond to purified DNA from other cyanobacteria that were tested in the same experiment. The DNA ladder on the far right has a mass range of 15 kb – 100 bp from top to bottom. Although these bands are smeared, the primers are specific and should still function as long as the smearing is not caused by a contaminant that could potential hinder the PCR reaction.

Both primers used, CYA106F and CYA359F, produced PCR products (Figure 47). Their amplified products correspond to the red circles from left to right, respectively. All other lanes in the gel correspond to PCR products amplified from the DNA of other cyanobacteria that were tested in the same experiment. The DNA sequences derived from the PCR reaction using primers CYA106F and CYA359F are shown in Figure 48 and Figure 49, respectively. The subsequent GenBank blast for both sequences and contiguous sequences support that the identity of *Oscillatoria* 21-9-3 was actually *Leptolyngbya boryana* with a 99% identity match. This find was not a shocking revelation considering the cellular morphology is quite similar in both genera having non-branched filaments lacking heterocysts.

Figure 46: Results of the gel identifying the potential presence of genomic DNA.

Figure 47: Results of the gel identifying products from the PCR reaction.

Figure 48: DNA sequence amplified using primer CYA106F.

Figure 49: DNA sequence amplified using primer CYA359F.

Chapter 5: Toxicological Analyses

5.1 Introduction

A comparative analysis was conducted on the toxicity/bioactivity of (2). Toxicological analyses were performed using mosquito larvae, zebrafish embryos, brine shrimp, and bacteria. The analysis of the mosquito larvae was the most comprehensive as it has higher relevance to the current study and thus deserving of a large portion of the purified sample.

Zebrafish (*Danio rerio*) are a widely used vertebrate model encompassing such subjects as embryonic development, genetics, natural products, and toxicology (Hill et al., 2005 and Crawford et al., 2008). Many cyanobacteria derived metabolites have been evaluated using zebrafish assays (Berry et al., 2008 and 2009; Wright et al., 2006). The assay has proven to be a useful tool in the purification of bioactive metabolites via bioassay guided fractionation (Berry et al., 2008). The zebrafish model can potentially detect the bioactivity of many types of molecules because embryos will not develop properly if any of the many processes involved in embryogenesis are affected. Other benefits of the model include the small embryo size which allows for their evaluation in 24-96 well plates, transparency of the embryos allowing for microscopic analyses, and quick embryogenesis (3-5 days) allowing for more evaluations hastening the bioassay guided fractionation process.

Prior research suggests a link between the toxicity of fatty acids to mosquito larvae and brine shrimp. Studies have shown the ability of unsaturated fatty acids to reduce $\text{Na}^+/\text{K}^+ \text{-ATPase}$ activity in *Artemia salina* (Morohashi et al. 1991). Harada et al.

(2000) correlated this activity with that observed on mosquito larvae. Unsaturated fatty acids were found to have toxicity comparable to microcystin on *Daphnia magna* (Reinikainen et al., 2001). Cumulatively, these studies suggest the potential for arthrocidal effects of unsaturated fatty acids. Considering linoleic acid was among these fatty acids to be tested for arthrocidal activity, **(3)** was also tested in the brine shrimp assay.

The antibacterial activity of unsaturated fatty acids has been well documented (McGaw et al., 2002; Mundt et al., 2003; Zheng et al., 2005). Considering this, it would be useful to test **(2)** and **(3)** for antibacterial activity and compare the results with previously published data (Zheng et al., 2005). Confirmation of antibacterial activity would strengthen the proposed structures as unsaturated fatty acids.

5.2 Methods

5.2.1 Mosquito larvicidal assay

5.2.1.1 Statistical analysis

Resulting data were analyzed with a probit regression model using IBM SPSS Statistics 20 software.

5.2.1.2 Assay setup

A large scale bioassays was performed on **(2)** using the same mosquito larvicidal assay discussed in Chapter 2. All treatments were conducted for a minimum of 7 days. The vast majority of mortality occurred within 24 hours for all treatments. Given the mortality and the fact that control larvae can potentially die later in the experiment,

mortality will be documented here as 24 hour exposures unless otherwise specified. A small scale experiment was performed prior to determine the concentrations to be used for each treatment. Three concentrations were tested: 25 µg/ml, 50 µg/ml, and 100 µg/ml. Each of the 3 treatments were tested with 1 replicate consisting of 4 mosquito larvae. Larvae were derived from 2 week old eggs. The 50 µg/ml and 100 µg/ml treatments caused 100% mortality while the 25 µg/ml treatment caused 75% mortality. This find was surprising considering preliminary data using 2 month old eggs also caused 75% mortality at a concentration 25 µg/ml. Larvae derived from newer eggs are known to be more resilient to toxins considering their larger lipid reserves increasing their capability to produce enzymes used in detoxification. Interestingly, the data collected from these preliminary assays does suggest that toxicity of (2) is the same regardless of the age of the eggs. Considering the results of the small scale experiment, treatments were selected ranging from 5-45 µg/ml in 5 µg increments; i.e., 5, 10, 15, 20, 25, 30, 35, 40, and 45 µg/ml. One replicate was performed for each treatment consisting of 5 subsets of 4 larvae each (total of 20 larvae per treatment).

5.2.2 Zebrafish assay

5.2.2.1 Rearing and breeding

Zebrafish were maintained and bred in the Marine Science Building at FIU's Biscayne Bay Campus (BBC). The facility houses several tanks attached to a water filtration system (Benchtop System - Aquatic Habitat AHBTRACK-4). Zebrafish were reared in 10 L tanks with a water temperature of 28 °C and a 14:10 light/dark cycle. Breeding commences within 15 minutes prior to the next light cycle. A mixture of male

and females are placed into two 10 L tanks. Mesh separates the adults from their falling eggs to prevent them from being eaten. After being collected from the bottom of the tanks, eggs are washed with E3 medium and deposited into petri dishes containing E3 medium. Subsequently, the eggs are analyzed for mortality and developmental issues using an Olympus SZX7 dissecting microscope. Viable eggs at this stage of development (just after breeding) should be in the 32-64 cell stage. These are sorted away from unfertilized eggs and any other eggs that appear deformed. After screening, eggs are now ready to be used for rearing another generation or to be incorporated into an assay.

5.2.2.2 Assay setup

The well plates used are identical to those used for the mosquito larvicidal assay (discussed in section 2.2). Since the non-polar compounds tested are buoyant and the zebrafish embryos remain near the bottom (unlike most mosquito larvae), treatments were placed on the bottom of each well. This method was devised to maximize the interaction of non-polar compounds with zebrafish embryos. Each treatment was transferred into its corresponding well with 10 μ l of methanol which was allowed to evaporate in a fume hood. Two treatments (20 μ g/ml and 35 μ g/ml) were tested as single replicates made up of 2 subsets consisting of 5 zebrafish eggs each (10 zebrafish per treatment). These concentrations are based on the effects of **(2)** on mosquito larvae (LC_{50} and annihilation concentrations, respectively). Zebrafish eggs were pipetted with enough medium to make each well exactly 1 ml. Solvent controls were performed for each treatment. The assay was conducted for 5 days.

5.2.3 Brine shrimp assay

5.2.3.1 Hatching

Dried *Artemia* eggs were purchased from Brine Shrimp Direct. Eggs were placed in a 1 liter flask containing 500 ml of artificial seawater (2% NaCl). A 60 watt lamp was used to reach the desired incubation temperature of 25-28 °C. Eggs were allowed to hatch overnight. Larvae were separated from egg fragments using a perforated lid as a filter. The larvae at this stage were ready to be incorporated into the assay.

5.2.3.2 Assay setup

Compounds **(2)** and **(3)** were tested as 2 treatments (20 µg/ml and 35 µg/ml) tested as single replicates made up of 4 subsets consisting of 5 *Artemia* larvae each (20 brine shrimp per treatment). These concentrations were based on the effects of **(2)** on mosquito larvae (LC₅₀ and annihilation concentrations, respectively). The experiment was conducted in 96-well plates. Treatments were placed into the wells as described with the mosquito larvicidal assay discussed in section 2.2. Larvae were transferred with enough seawater to make each well 300 µl. The assay was conducted for 5 days.

5.2.4 Bacterial assay

Liquid bacterial cultures of *Bacillus megaterium*, *Escherichia coli*, *Micrococcus luteus* and *Staphylococcus* sp. were acquired from the laboratory of Dr. Miroslav Gantar. Each bacterium was inoculated onto a petri dish containing nutrient agar to ensure the cultures were capable of growth and axenic. Growth occurred on all plates and which were used to create new liquid cultures by inoculating 4 ml of nutrient broth. The new

liquid cultures were incubated at 30°C overnight. Each liquid culture was used to inoculate respective petri dishes containing nutrient agar. Treatments were placed on 2 mm absorbent disks. Neomycin was used as a positive control and was tested at 100 µg/disk and 500 µg/disk. Based on the minimum inhibitory concentrations for antibacterial activity, provided by Zheng et al. (2005), (2) and (3) were added at 100 µg/disk. All treatments were added with MeOH and thus a disk was used for the solvent control. Plates were incubated at 30°C overnight.

5.3 Results

5.3.1 Mosquito larvicidal assay

Probit analysis. After 24 hours, treatments at 35 µg/ml and higher caused 100% mortality and the 5 µg/ml treatments had 0% mortality. These values were not used in the LC₅₀ calculations as they do not correspond to probit values. Only percentages other than 0% and 100% can offer information for this type of analysis. The data can be found in Table 1 and the results of the probit can be seen in Figure 50. From the regression line, the estimated LC₅₀ for (2) is calculated using the probit value of 0 (50% mortality). The inverse log of 1.318 ($10^{1.318}$) is equal to 20.794 µg/ml with 95% confidence limits 18.529 - 23.374 µg/ml. Therefore, the estimated LC₅₀ value for (2) the mosquito larvae of *Aedes aegypti* is 20.8 µg/ml after 24 hours of exposure using 2 week old eggs. This LC₅₀ value is reminiscent of those calculated for anatoxin-a (25-47 µg/ml) by Kiviranta and Abdel-Hameed (1993). This comparison is not entirely justifiable considering first instar larvae were used for (2) while third and fourth instars were used in studies of anatoxin-a. A more appropriate comparison can be made based on data collected for anatoxin-a in the

current study; i.e., 50% mortality using 50 µg/ml on first instar larvae. Another similarity was apparent when observing the effects on larval mobility. All higher concentrations (≥ 50 µg) of anatoxin-a and (2) inhibited movement nearly instantaneously upon exposure. Movements were reduced to very slight lateral contractions when compared to the controls. Given these comparisons, it does appear that (2) is as potent a toxin as anatoxin-a in regards to mosquito larvicidal activity.

	No.	Conc (µg/ml)	Log (Conc)	Number of Subjects	Observed Responses	Expected Responses	Residual
PROBIT	1	10	1.000	20	1	.500	.500
	2	15	1.176	20	2	3.819	-1.819
	3	20	1.301	20	10	9.170	.830
	4	25	1.398	20	15	13.782	1.218
	5	30	1.477	20	16	16.736	-.736

Table 1: Data from the probit analysis using IBM SPSS Statistics 20 software.

Figure 50: Results of the probit analysis. Mortality was documented after 24 hours of initial exposure.

Cuticular effects. It was also observed that larvae treated with 100 µg/ml seemed elongated when compared to those treated with 50 µg/ml (Figure 51). This was an obvious observation apparent with the naked eye which ultimately led to its documentation. The variability in length could not be explained by differences in instar, as mentioned in the previous paragraph, these high concentrations impaired larval mobility immediately upon exposure and mortality occurred within 2-3 hours. This short amount of time would not allow the larvae to develop into second instars and no remnants from ecdysis were visible under magnification. Length measurements were taken 8 hours after initial exposure. Curvature was incorporated into the length calculations by taking measurements along the sagittal plane and breaking down curved areas into several straight segments. The mean larval length from the 100 µg/ml treatment was 12.35% higher than larvae derived from the 50 µg/ml treatment (Table 2). Also, the variability in length was higher in the 100 µg treatment. Although these results were obtained with few replicates, as it was part of the small scale experiment discussed in section 5.2.1.2, the paired t-test revealed a p-value of 0.028 in favor of larvae treated with 100 µg/ml to be significantly longer than those treated with 50 µg/ml. Figure 51 shows a comparison of larvae acquired from both treatments. Both larvae are slightly curved and thus actually longer than the comparison demonstrates. These 2 larvae were chosen for the figure because they were the most straight and did not require any manipulation for the comparison. Seeing that their tissues could be affected by the treatments, manipulating them could alter their lengths. Small and large particles visible in the figure are pieces of liver powder.

Figure 51: Comparison of dead larvae treated with 100 µg/ml (top) and 50 µg/ml (bottom). Pictures were taken 24 hours after initial exposure. Each large segment of the visible ruler is 1 mm (2.5 mm total are visible).

Treatment	Mean	SD
50 µg	2.025	0.043
100 µg	2.275	0.148

Table 2: Mean larval length (mm) and standard deviation of larvae derived from the 2 highest concentrations tested.

Effects after 24 hours. After 48 hours from initial exposure, an additional mortality occurred in the 15 µg/ml treatments and 2 additional mortalities occurred in the 20 µg/ml treatments. Larvae that survived the 30 µg/ml treatments took nearly twice as much time to develop into third instars when compared to the controls, 6 days versus 3 days, respectively. Of the larvae that survived the 25 µg/ml and the 30 µg/ml treatments, 50% died as third instars after 7 days. This observation is interesting in that many larvae that survive a treatment and can develop into third instars normally do not suddenly die. In fact, from previous bioassays with other toxic compounds (anatoxin-a, microcystin-LR, sulfated glycolipids, pahayokolide etc.), larvae exposed to these tend to develop and

pupate faster. When larvae die early in the experiment it is difficult to say how much of their designated food was not eaten. Recall that each larva receives 30 μ l of a 0.1% liver powder solution. Extra food is not visible enough to be removed and thus sole survivors of a treatment simply have more food which may explain why these larvae matures faster. Also, several wells with 100% mortality were given 4 additional larvae, 24 hours after the original exposure, to see if (2) would still be active but was found to not be the case. This finding suggests that after 24 hours of exposure, (2) is likely denatured or rendered inaccessible by being bound to the dead larvae. Dead larvae were not removed in the last experiment because bioaccumulation was suspected. Dead larvae were eventually eaten and still no subsequent activity occurred.

Effects on tissue. Figure 52 shows a comparison of a control third instar and one of the dead third instars from the 25 μ g/ml treatments. The curvature seen in the dead larva is not necessarily linked to the activity of (2) as many dead larvae are found in this manner. One observation that does seem to be linked to (2) is the visible distortion of the hemolymph and digestive system. All control larvae have very predictable and homogenous coloration of these systems while treated larvae are patchy and disorganized. This pattern was found to be the case with every larvae from the 25 μ g/ml and 30 μ g/ml treatments that survived the first week. All of these treated larvae died as third instars by the 9th day of the assay.

Old eggs versus new eggs. In addition to the experiment performed with 2 week old eggs, it was decided to perform the same experiment with 2 month old eggs. These experiments

Figure 52: Comparison of a control third instar (left) and a recently perished third instar from the 25 µg/ml treatment after 7 days.

would be a good indication of larvae derived from the wet season and dry season, respectively, as the age of the eggs at the time of hatching is largely dependent on being triggered by rain events. The probit analysis on 2 month old eggs revealed an estimated LC_{50} of 18.951 µg/ml with 95% confidence limits 17.470 - 20.411 µg/ml. This LC_{50} is slightly lower than that calculated using 2 week old eggs (20.794 µg/ml). These results support data collected from the small scale experiments suggesting similar toxicity to both new and old eggs. Larvae derived from older eggs have used up much of their lipid reserves (Perez and Noriega, 2012). Considering this, these findings do suggest that lipid reserves do not play a significant role in the detoxification of fatty acids such as (2). Lipid mobilization in insects has been linked to immune responses (Arrese and Soulages, 2010). Although the exact role of lipids in this response is not well understood, it is thought to potentially fuel the reactions involved in enzyme synthesis. The activity of (2) was nearly identical regardless of lipid reserves suggesting that detoxification pathways

that require lipid mobilization have only a small beneficial affect when compared to lipid depleted larvae.

5.3.2 Zebrafish assay

After 24 hours, both concentrations tested had 1 embryo fail to develop. While under a microscope, dead embryos appear as dark blue-green masses within the chorion (Figure 53). No further mortalities occurred in any of the treatments for the remainder of the experiment. No mortalities occurred in any of the controls. By day 4, all but 1 control and all the zebrafish in the 20 µg/ml treatments hatched successfully. Only 50% of the zebrafish in the 35 µg/ml treatments were hatched at that same time but were all completely hatched by the end of day 4. Considering this, it does appear that the 35 µg/ml treatments delayed development for some of the zebrafish by 8-10 hours. This is also supported by these zebrafish lacking the same pigmentation found in the controls. A 10% mortality in each treatment is not very conclusive that **(2)** is toxic to zebrafish at the tested concentrations. Even with the selection process for viable eggs, it is still possible that these two failed embryos (Figure 53) were caused by defective eggs. One last observation includes a slight curvature of one zebrafish found in the 35 µg/ml treatments (Figure 54). This feature can be characteristic of a toxins effect but the zebrafish in question had straightened by the end of the assay.

5.3.3 Brine shrimp assay

No significant mortalities occurred in any of the treatments when compared to the controls. This would appear to contradict the potential arthrocidal effects of unsaturated

Figure 53: Zebrafish assay after 24 hours. Two eggs failed to develop, one in the 20 $\mu\text{g/ml}$ treatment (left) and the other in the 35 $\mu\text{g/ml}$ treatments (right).

Figure 54: Zebrafish from the 35 $\mu\text{g/ml}$ treatments showing a slight curvature near the caudal peduncle (left) a normal zebrafish (right). The picture was taken on day four of the assay.

fatty acids mentioned previously. However, Morohashi et al. (1991) did not discuss mortality but Na^+/K^+ -ATPase inhibition. The *Artemia* assays documenting mortality were conducted with crude extracts and likely contained a pool of fatty acids (Kiviranta et al., 1991; Kiviranta and Abdel-Hameed, 1994). Determining the quantity of

palmitoleic acid and linoleic acid in these “pools” would be merely speculative. It seems likely that fatty acids other than palmitoleic acids and linoleic acid are responsible for the previously documented activity against *Artemia*.

5.3.4 Bacterial assay

The results of the bacterial assay can be seen in Table 3. Both **(2)** and **(3)** had strong inhibitory effects against the only gram-negative bacterium in the assay, *E. coli*. These values are remarkably higher than the antibiotic control and are in far contrast to those acquired by Zheng et al., (2005). These authors found that unsaturated fatty acids purchased from Sigma, including palmitoleic and linoleic acid, to be effective only on gram-positive bacteria. Conversely, McGaw et al. (2002), found linoleic acid purified from a plant (*Schotia brachypetalato*) to be effective against gram-positive bacteria and gram-negative strains such as *E. Coli*. The source of the fatty acid does seem to affect its activity. Perhaps the small amount of impurities retained during the purification process has more utility than mere additive effects such as increasing membrane permeability of gram-negative bacteria. It was noted that in combination with ethanol, the antibacterial activity of palmitoleic acid was synergistically increased (Wille and Kydonieus, 2003). Also, different bacterial strains of the same species can exhibit various levels of membrane hydrophobicity increasing or decreasing membrane permeability to fatty acids (Desbois and Smith, 2010). Although **(2)** houses the mosquito larvicidal activity, **(3)** has a wider range of antibacterial activity encompassing inhibition of gram-positive bacteria as well. These results are supported by Zheng et al., (2005) which demonstrated greater

inhibitory properties of linoleic acids towards gram-positive bacteria than palmitoleic acid.

	Neomycin (100 µg)	Neomycin (500 µg)	(2)	(3)	MeOH
<i>E. coli</i>	1.5	2	8	5	0
<i>Staphylococcus</i>	0.9	1.5	0.2	2	0.1
<i>B. megaterium</i>	2	3	0.1	1.2	0
<i>M. luteus</i>	0.1	0.1	0.1	0.1	0.1

Table 3: Results of the bacterial assay. Inhibition zones are given in mm.

5.4 Discussion

The LC₅₀ for (2) on mosquito larvae was found to have little influence from the age of the eggs (2 week old: 20.8 µg/ml, 2 month old: 19.0 µg/ml). This demonstrates that any benefits mosquitoes receive from their lipid reserves, in regards to toxin defense, play little part in managing unsaturated fatty acids when used as a control agent. The toxicity of (2) was comparable to that of other cyanobacteria derived compounds such as anatoxin-a. Both (2) and anatoxin-a can render larvae motionless after 1-2 hours. There could be a neurotoxic component considering this. However, the only evidence of a mechanism exists with studies involving the Na⁺ /K⁺ -ATPase inhibition (Ahmed and Thomas, 1971; Morohashi et al., 1991). Unfortunately the role of these enzymes is poorly understood in insects mainly because they normally do not appear to have any activity associated with them (Blanco, 1997).

The zebrafish assay and the brine shrimp assay did not reveal any significant activity. This is an important find in regards to the applicability of unsaturated fatty acids

in the environment considering the annihilation concentration for mosquito larvae was used as treatments.

The bacterial assay was fairly surprising considering the high levels of activity against *E-coli*. Long chain fatty acids rarely show activity against gram-negative bacteria. Membrane permeability is the issue (Desbois and Smith, 2010). Some variable in the sample (isomers, impurities etc.) may be influencing the activity at the membrane level. Researchers have found comparable inhibition of gram-positive bacteria using palmitoleic acid and linoleic acid (Zheng et al., 2005). Results from this dissertation does suggest that **(2)** is a more potent inhibitor of gram-negative bacteria and linoleic acid a more potent inhibitor of gram-positive bacteria.

Chapter 6: Conclusions and Significance

6.1 Conclusions

6.1.1 Project Summary

Freshwater cyanobacteria were explored for the production of mosquito larvicidal compounds. Of 120 tested strains, 31 showed 25% mortality or greater when freeze-dried biomass was used as treatments. Bioassay guided fractionation using the mosquito larvicidal assay was performed on strains with the highest activity (*Cocoid* sp. 53-2a, *Leptolyngbya* sp. 21-9-3, and *Synechococcus* sp. 36-8). *Cocoid* sp. 53-2a and *Synechococcus* sp. 36-8 did not yield satisfactory results and were thus abandoned in favor of *Leptolyngbya* sp. 21-9-3. Bioassay guided fractionation revealed 3 compounds derived from the non-polar extract that potentially housed the activity. Compound (1) had the most activity but was not produced in large quantities in the new biomass and its characterization data were inconclusive. The second Compound (2) was found to be the second most active and characterization supports its identify as palmitoleic acid. Compound (3) showed no activity and characterization supports its identity as linoleic acid. These results demonstrate that cyanobacteria do produce compounds with mosquito larvicidal properties. Through bioassay guided fraction it appears that the most toxic components of *Leptolyngbya* sp. 21-9-3 are monounsaturated fatty acid such as (2). The toxicity of (2) to mosquito larvae was found to be comparable to anatoxin-a.

6.1.2 Potential roles of unsaturated fatty acids in cyanobacteria

Unsaturated fatty acids are implicated in the maintenance of membrane fluidity.

In a study conducted by Wada and Murata (1990), the membrane fatty acid composition of *Synechocystis* PCC6803 was altered in response to temperature. Lower temperatures caused desaturation as a result of desaturase enzymes (Nishida and Murata, 1996). Although unsaturated fatty acids have a structural function, they are also linked to many types of competitive and defense roles.

Heterotrophic bacteria can outcompete cyanobacteria for nutrients (Drakare, 2002). The production of certain fatty acids may be defenses for certain types of bacteria. Considerable research has been conducted investigating the antibacterial properties of fatty acids (Mundt et al., 2003; Dahmsa et al., 2006; Desbois and Smith, 2010). Desbois and Smith (2010) suggests that fatty acids disrupt oxidative phosphorylation, electron transport, enzyme functionality, nutrient uptake, and cause cell lysis thus largely targeting cell membranes in general. Direct competition for nutrients also occurs between cyanobacteria and eukaryotic algae. Ikawa et al. (1996) found that unsaturated fatty acids purified from the cyanobacterium *Aphanizomenon flos-aquae* inhibited the growth of *Chlorella*. These findings do suggest that unsaturated fatty acids could be used by cyanobacteria to outcompete sympatric organisms.

Lastly, from the results of this and several other studies, fatty acids appear to be the most toxic component to mosquito larvae given a variety of sources including cyanobacteria (Harada et al., 2000), plant leaves (Rahuman, 2008), and plants seeds (Komansilan, 2012). Toxicity to unsaturated fatty acids may be arthropod specific considering research done on crustaceans *Artemia salina* (Morohashi et al., 1991; Kiviranta and Abdel-Hameed, 1994) and *Daphnia magna* (Reinikainen et al., 2001). Even though no activity occurred with *Artemia* using (2) and (3), other fatty acids could

have been the culprit in the *Artemia* assays done by Kiviranta and Abdel-Hameed (1994) because they used extracts containing pools of fatty acids. These pools may work synergistically with each other and potentially to other compounds. Linoleic acid was found to work synergistically with microcystin-LR when used against *Daphnia* (Reinikainen et al., 2001). These assays also demonstrated that linoleic acid was acutely toxic in concentrations of 9 $\mu\text{l/ml}$. This is in far contrast to the 35 $\mu\text{l/ml}$ used on *Artemia* in this dissertation which produced no significant mortality. Recall that 35 $\mu\text{l/ml}$ was the annihilation concentration for (2) on mosquito larvae while (3) had no mosquito larvicidal activity at any concentration. These conflicting results do not necessary discredit the potential for arthrocidal specificity but perhaps demonstrate that different unsaturated fatty acids affect different arthropods. Potentially the functions of individual unsaturated fatty acids have little overlap with each other; i.e., economic in regards to fitness cost.

It has been noted for quite some time that fatty acids can affect Na^+/K^+ -ATPase activity (Ahmed and Thomas, 1971). Morohashi et al. (1991) found a dose dependent correlation to fatty acid concentration and Na^+/K^+ -ATPase inhibition using *Artemia salina*. In fact palmitoleic acid induced the fourth most inhibition of 10 tested fatty acids. Linoleic acid provided the third most inhibition, nearly 3 times that of palmitoleic acid. This is an interesting find considering only (2) showed any mosquito larvicidal activity. Thus, there is an inverse correlation with Na^+/K^+ -ATPase inhibition and mosquito larvicidal activity. Harada et al. (2000) noted the similarities between the inhibition of *Artemia salina* and *Aedes albopictus* by unsaturated fatty acids and had linked the activity with Na^+/K^+ -ATPase inhibition referencing Morohashi et al. (1991). However,

the *Artemia* assays they discuss involved lyophilized cells and crude extracts as treatments (Kiviranta et al., 1991; Kiviranta and Abdel-Hameed, 1994). These treatments likely contained several fatty acids. From the results of this dissertation it would appear that mosquito larvicidal activity and Na^+/K^+ -ATPase inhibition would be caused by different fatty acids, palmitoleic and linoleic acid, respectively. Although, palmitoleic acid does contain some Na^+/K^+ -ATPase inhibitory properties. This activity would seem to not be the sole culprit for mosquito larvicidal activity because linoleic acid was found to be a more potent Na^+/K^+ -ATPase inhibitor. It is possible that the functionality of each fatty acid would not overlap much with others. This specificity could be based on the ability of the fatty acid to enter the target organism. Joint roles in defense would help explain why so many fatty acids are synthesized. Palmitoleic acid may inhibit Na^+/K^+ -ATPase on certain grazers, like mosquito larvae, while linoleic may inhibit Na^+/K^+ -ATPase on another grazer. Perhaps linoleic acid is used more as an antibacterial agent as (3) inhibited more gram-positive bacteria than did (2). Unfortunately, the exact mechanism of Na^+/K^+ -ATPase in insects is not well documented as many of these enzymes are found to be present but with little to no activity (Blanco, 1997). Research done by Gatto et al. (2001) does suggest that Na^+/K^+ -ATPases in insects may play roles in transport for intracellular organelles because fully functional enzymes were discovered in such locations. More research is necessary to answer many of these questions, particularly Na^+/K^+ -ATPase activity should be measured in mosquito larvae in response to treatments with unsaturated fatty acids. The correlation between Na^+/K^+ -ATPase inhibition and mortality should be clarified for both *Artemia* and mosquito larvae

and differences in activity may revolve around the ability of individual fatty acids to enter the organism whether the route of entry is cuticular or ingestive.

6.2 Significance

6.2.1 Novelty

The current study encompassed a search for mosquito larvicides derived from cyanobacteria. The idea emerged as an enticing prospect particularly with the occurrences of cyanobacteria in mosquito breeding sites. Photosynthetic organisms such as cyanobacteria produce chemical defenses as inhibitors to competitors and potentially as deterrents to grazing pressure. An experiment emerged from this concept with prospect in discovering novel control agents for disease vectors that could be used in rotation with contemporary strategies.

Considering the vast amount of compounds produced by cyanobacteria, the exact nature of the discovered mosquito larvicide was highly speculated. Would purification yield another peptide like molecule such as a microcystin, a small neurotoxic alkaloid such as anatoxin-a, or something never before seen? As in the case of Harada et al. (2000), when all cyanobacterial components are screened based on bioassay guided fractionation using a mosquito larvicidal assay, the results indicate that the most toxic components are in fact unsaturated fatty acids. Harada et al. (2000) also demonstrated that unsaturated fatty acids (oleic acid and linoleic acid) were toxic to the larvae of *Aedes albopictus* while saturated fatty acids (palmitic acid and stearic acid) were none toxic. This trend in unsaturation causing higher levels of activity seems to hold for other types

of lipids including the sulfated glycolipids (SGL-3) tested in the current study. As a result of Harada et al. (2000), the first cyanobacteria derived compounds were discovered using a mosquito larvicidal assay and bioassay guided fractionation. The characterization of these fatty acids is not entirely conclusive. A mass spectrum is provided and some ^1H -NMR spectra comparisons are made between a 90% methanol fraction and that of γ -linolenic acid. The NMR spectrum of the fraction does show appropriate chemical shifts but fails to integrate as the spectra of γ -linolenic acid. As a result, it does appear that the current study resulted in the first thorough characterization of a cyanobacteria derived compound using a mosquito larvicidal assay and bioassay guided fractionation. Although linoleic acid has been documented to be mosquito larvicidal when used in combination with oleic acid and/or as part of a crude extract (Harada et al., 2000; Rahuman, 2008), the results of assays performed with **(3)** suggest that linoleic acid alone is unlikely capable of mosquito larvicidal activity. The results of the current study also encompass the first evidence of the mosquito larvicidal activity of palmitoleic acid. Lastly, the results of the toxicology analysis indicate that **(2)** acid has the lowest LC_{50} range on mosquito larvae than any other cyanobacteria derived compound tested so far.

6.2.2 Evaluation of the potential environmental effects of using unsaturated fatty acids to control mosquito populations

As mentioned in the introduction to Chapter 6, inferences made in this section are all based on assays done in well plates. Although the results of these assays have limited applicability to mosquito breeding sites, the information nonetheless provides useful insight into potential environmental effects.

After 24 hours of exposure, four additional larvae were placed into wells with 100% mortality (annihilation concentrations of $>30\text{ }\mu\text{g/ml}$) and no subsequent mortalities occurred. The lack of activity could indicate that (2) has a half-life of less than 24 hours. However, a short half-life is unlikely the case considering during bioassay guided fractionation these fatty acids were left in water several times. During purification the last solvent to evaporate would be water which meant that the fatty acids were solely in water for hours every time they were dried down and no difference in mosquito larvicidal activity was observed. Also, if the fatty acids were somehow denaturing in water it would have been readily apparent in the chromatograms during purification. The stability of these fatty acids in water was later confirmed by a treatment consisting of (2) in which the larvae were added after 24 hours. The activity was identical to treatments that added larvae immediately.

Considering observations of (2) affecting the organization of tissues within mosquito larvae, it is likely bound to dead larvae. Dead larvae were not removed in these experiments because bioaccumulation of the unsaturated fatty acids was suspected. Being bound meant being ingested once dead larvae are eaten by other larvae but no additional mortalities occurred. It is possible that upon being bound to the larvae, the structure and thus chemical nature of (2) is altered enough to render it ineffective. From these observations, it would appear that this naturally occurring fatty acid would not permeate in the environment once incorporated into its target organism.

Considering the results of the zebrafish assay, there is no concrete evidence that concentrations for field application would affect even the most vulnerable state of a non-target organism such as fish embryos. Compound (2) was specifically placed on the

bottom of the well plates to make contact with the zebrafish embryos and no significant mortalities were documented. In natural conditions the fatty acids would float and thus not interact with any organism located below the microfilm layer such as fish embryos.

There have been no documented cases of mammalian toxicity with these types of fatty acids. In fact, they are linked to decreased blood glucose levels in diabetes research (Heydarizadeh et al., 2013). These results include the fatty acid most closely related to (2), palmitoleic acid (Yang et al., 2011). Other beneficial properties of unsaturated fatty acids to mammals include but are not limited to: cardio-protection (Lee et al. 2010), anti-obesity (Micallef et al., 2009), and anti-cholesterol (Valdivielso et al., 2009). Matsunaga et al. (1995) showed prospect for cyanobacteria being a great source of palmitoleic acid for pharmaceutical use. With all these studies considered, a prospective mosquito larvicide with medicinal uses to mammals would seem like a strong candidate to combat disease vectors with little impact on the environment. Research exists as far back as 1970 in regards to the application of fatty acids as mosquito repellents (Skinner et al., 1970) and ovipositional repellents (Hwang et al. 1984). It is somewhat surprising that these efforts in mosquito control using fatty acids have not been more fruitful. However, because of the advent of synthetic antibiotics and insecticides, many of the plant/algae derived compounds were not fully explored (Cowan, 1999). With increased demand for novel antibiotics and insecticides to prevent future bacterial and disease vector resistance, these venues should be considered more carefully in large part because the prospective compounds have beneficial properties to mammals and already exist in the environments that they are to be used.

6.2.3 Palmitoleic acid as a mosquito control agent

Before making conclusive remarks in regards to the use of unsaturated fatty acids as mosquito larvicides, it is important to compare them to mosquito larvicides used in the field today. The highly insect specific Cry toxins from *Bacillus thurengiensis* are some of the most prospective mosquito larvicides to date. LC₅₀ values were calculated to be 3.8-7.54 ng/ml (Sun et al., 1996). They have no known toxicity to vertebrates or plants and they are naturally degraded (Bravo et al., 2005). Cry toxins consist of multidomain proteins. The different domains contribute to a stepwise mechanism responsible for its activity. However, any one of these components that are required for its performance can be a source of tolerance and even potential future resistance (Bravo et al. 2005). The first of these components is the requirement for proteolytic activation by proteases derived from the insect's midgut. Resistance of this kind has been documented as early as the late 90s and corresponded to reduced production of particular proteinases (Oppert et al., 1997). The second component involves the interaction with receptors in the midgut. Mutations in these receptors have corresponded to the lack of toxin binding (Ferre and Van Rie, 2002). The third component involves carbohydrate like binding domains found on the Cry toxins. These are integral in the binding of the toxin to target receptors and can be impaired by products derived from oligosaccharide synthesis (Griffits et al., 2001). These were, however, mostly speculated means of resistance using organisms in optimal conditions to promote selection and no there are no examples of resistance in the field thus far. The only shortcoming of these proteins that exists today is that they are susceptible to sedimentation and have to be reapplied periodically. Sedimentation is likely negligible in smaller bodies of water considering the extremely low LC₅₀ values

and the fact that commercial formulations (mosquito dunks) are composed of bacterial spores and not just the toxic proteins. The juvenile hormone analogue methoprene is used in larger bodies of water where the Cry proteins would sediment out. However, methoprene is more toxic to non-target organisms as it inhibits emergence from the pupa stage in all insects (EPA, 2003).

In conclusion, current mosquito control strategies targeting the larval stage are not without shortcomings. With the advent of range expansion of disease vectors into areas surrounding the gulf coast of the United States, it is hardly the time and place to be content with Cry proteins and methoprene. These mosquito larvicides need to be rotated with other insecticides immediately to help prevent resistance and negative impacts on the environment. Unsaturated fatty acids could be a good candidate considering they lack the ability to sediment, do not bioaccumulate in active form, lack toxicity towards non-target organisms, already exist in the environment that they are to be used, and could be produced continuously by allowing particular strains of cyanobacteria to propagate in mosquito breeding sites. Resistance is inevitable and is merely delayed by control strategies. The delay may only be long enough to discover the next generation of insecticides. Considering the encroachment of disease vectors aided by changes in global climate, the issue should be addressed with great urgency.

Appendix 1: Cyanobacteria screened for mosquito larvicidal activity

Genus	Strain number	Genus	Strain number
<i>Aphanotheca</i>	0-1-10 (Ev-1-10)	<i>Microcystis smithii</i>	36-1
<i>Anabaena</i>	55-1	<i>Microcystis</i>	36-5-1
<i>Anabaena</i>	53-1	<i>Microcystis</i>	22-6
<i>Anabaena</i>	40-3	<i>Microcystis</i>	46-2
<i>Anabaena</i>	45-5	<i>Microcystis</i>	UCFF 299
<i>Anabaena</i>	48-2	<i>Microchaeta</i>	22-7
<i>Anabaena</i>	43-2-1	<i>Nostoc</i>	23-2
<i>Anabaena</i>	66-2	<i>Nostoc</i>	23-2a-1
<i>Ankistrodesmus</i>	45-2	<i>Nostoc</i>	23-2a
<i>Arthronema</i>	21-10-20	<i>Nostoc</i>	23-2b
<i>Arthronema</i>	37-8-1	<i>Nostoc</i>	23-2c
<i>Arthronema</i>	64-16	<i>Nostoc</i>	47-2-1
<i>Arthrospira</i>	0-25 (Ev-25)	<i>Nostoc</i>	47-3
<i>Calothrix</i>	3-6	<i>Nostoc</i>	58-2
<i>Calothrix</i>	13-3	<i>Nostoc</i>	53-2
<i>Calothrix</i>	23-1	<i>Nostoc</i>	41-1
<i>Calothrix</i>	22-7	<i>Nostoc</i>	37-7
<i>Calothrix</i>	28-2-2	<i>Nostoc</i>	37-2
<i>Calothrix</i>	30-1-13	<i>Nostoc</i>	2S9
<i>Calothrix</i>	33-2	<i>Nostoc</i>	34-5
<i>Calothrix</i>	38-3	<i>Nostoc</i>	Ev-7
<i>Calothrix</i>	3-27	<i>Nostoc</i>	64-15
<i>Calothrix axenic</i>	67-1	<i>Oscillatoria</i>	21-9-3
<i>Calothrix bacteria</i>	67-2	<i>Oscillatoria</i>	37-2-1
<i>Calothrix</i>	69-4	<i>Oscillatoria fragilis</i>	46-3
<i>Chlorella</i>	2-4	<i>Oscillatoria</i>	48-3
<i>Chlorococcum</i>	5-1	<i>Oocystis prochloro</i>	37-10
<i>Chlorococcum</i>	55-5	<i>Phormidium</i>	64-13
<i>Chlorococcum</i>	45-3	<i>Phormidium</i>	69-9
<i>Chlamidomonas</i>	0-29 (Ev-29)	<i>Phormidium</i>	49-1
<i>Coccoid cyano</i>	21-11	<i>Pseudoanabaena</i>	69-7
<i>Coccoid cyano</i>	53-2a	<i>Pseudoanabaena</i>	37-8-1

<i>Cocoid green</i>	64-12	<i>Pseudocapsa</i>	30-1-8
<i>Cocoid green</i>	56-4	<i>Pseudocapsa</i>	37-8-1
<i>Coelastrum</i>	46-4	<i>Plectonema</i>	33-7
<i>Cyanosarcina</i>	33-3	<i>Plectonema</i>	21-9-3
<i>Cylindrospermopsis</i>	42-1	<i>Prochloro</i>	37-9
<i>Cylindrospermopsis</i>	42-1	<i>Prochloro</i>	0-7 (Ev-7)
<i>Fremyella diplosihpon</i>	23-1	<i>Prochloro</i>	37-10
<i>Gleocapsa</i>	43-3	<i>Rhizoclonium</i>	0-17 (Ev-17)
<i>Gleocapsa</i>	30-1-15	<i>Scytonema hophmanii</i>	26-1
<i>Gleocapsa</i>	37-8	<i>Scytonema</i>	26-1
<i>Gleotheca</i>	47-1	<i>Spirulina</i>	21-0
<i>Hapalosiphon</i>	52-1	<i>Scenedesmus</i>	3--4
<i>Hapalosiphon</i>	52-1	<i>Scenedesmus</i>	66-1
<i>Limnothrix</i>	39-1	<i>Synechococcus</i>	36-2
<i>Limnothrix</i>	48-1	<i>Synechococcus</i>	36-9
<i>Limnothrix</i>	16-1	<i>Synechococcus</i>	36-9
<i>Limnothrix</i>	60-1	<i>Synechococcus</i>	36-2
<i>Lyngbya major</i>	15-2	<i>Synechococcus</i>	31-2
<i>Lyngbya</i>	23-3	<i>Synechococcus</i>	47-1
<i>Lyngbya</i>	29-3	<i>Synechococcus</i>	22-1
<i>Lyngbya</i>	30-1-2	<i>Synechococcus</i>	21-10a
<i>Lyngbya brachynema</i>	33-1	<i>Synechococcus</i>	22-11
<i>Lyngbya</i>	64-2	<i>Synechococcus</i>	55-3
<i>Leptolyngbya</i>	71-2	<i>Synechococcus</i>	40-4
<i>Leptolyngbya</i>	69-2	<i>Tolypothrix</i>	30-1-4
<i>Leptolyngbya</i>	69-5	<i>Tolypothrix</i>	30-1-4
<i>Leptolyngbya</i>	33-7	<i>Tolypothrix</i>	38-6
<i>Merismopedia</i>	46-1	<i>Tolypothrix</i>	64-11

Literature Cited

- Abdul Rahuman, A., Venkatesan, P., Kannappan Geetha, Geetha Gopalakrishnan, Bagavan, A. and Kamaraj, C. (2008) Mosquito larvicidal activity of gluanol acetate, a tetracyclitriterpenes derived from *Ficus racemosa* Linn. Parasitology Research. Vol.103, 333-339.
- Abrol, D.P. (2012) Pollination Biology: Biodiversity Conservation and Agricultural Production. Chapter 7.
- Adamovsky, O., Kopp, R., Hilscherova, K., Babica, P., Palikova, M., Paskova, V., Navratil, S., Marsalek, B., and Blaha, L. (2007) Microcystin Kinetics (Bioaccumulation and Elimination) and Biochemical Response in Common Carp (*Cyprinus carpio*) and Silver Carp (*Hypophthalmichthys molitrix*) Exposed to Toxic Cyanobacterial Blooms. Environmental Toxicology and Chemistry. Vol.26, 2687-2693
- Agency for Toxic Substances and Disease Registry [ATSDR]. (2003) Toxicological profile for Pyrethrins and Pyrethroids. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.
- Ahmad, M. and McCaffery, A.R. (1999) Penetration and metabolism of trans-cypermethrin in a susceptible and pyrethroid-resistant strain of *Helicoverpa armigera*. Pesticide Biochemistry and Physiology. Vol. 65, 6-14
- Ahmed, A.M., Abdel-Megeed, A.A.M. and Al-Qahtaney, H.M. (2014) A Novel Mosquitocidal Bacterium as a Biocontrol Agent in Saudi Arabia: I - A Promising Larvicide Against *Aedes caspius* Mosquito. Pakistan Journal of Zoology. Vol.46, No.1, 191-201
- Ahmed, K. and Thomas, B.S. (1971) The effect of long chain fatty acids on sodium plus potassium ion-stimulated adenosine triphosphatase of rat brain. Journal of Biological Chemistry. Vol.246, 103-109
- AMCA (2013) Mosquito Borne Diseases
<http://www.mosquito.org/mosquito-borne-diseases>
(Accessed May 1, 2014)
- Amer, A. and Mehlhorn, H. (2006) Larvicidal effects of various essential oils against *Aedes*, *Anopheles*, and *Culex* larvae (Diptera, Culicidae). Parasitology Research. Vol.99, 466-472.
- Ames, B., Gold, L.S. and Willett, W.C.(1995) The cause and prevention of cancer. Proceedings of the National Academy of Sciences. Vol.92, 5258–5265

- An, T., Suresh Kumar, T.K., Wang, M., Liu, L., Lay Jr, J.O., Liyanage, R., Berry, J., Gantar, M., Marks, V., Gawley, R.E. and Rein, K. (2007) Structures of Pahayokolide A and B, cyclic peptide from *Lyngbya* sp. *Journal of Natural Products*. Vol.70, 730-735.
- Anagnostidis, K. and Komárek, J. (1988). Modern approach to the classification system of cyanophytes. 3. Oscillatoriales. *Archiv für Hydrobiologie*. Supplement 80. 327-472
- AOCS Lipid Library (2006)
<http://lipidlibrary.aocs.org/nmr/1NMRdb/index.htm>
 (Accessed June 13, 2013)
- Arrese, E.L. and Soulages, J.L. (2010) Insect Fat Body: Energy, metabolisms, and regulation. *Annual Review of Entomology*. Vol. 55: 207–225.
- Aupali, T., Ismid I.S. and Wibowo H. (2006) Estimation of the prevalence of lymphatic filariasis by a pool screen PCR assay using blood spots collected on filter paper. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. Vol.100, No.8 753–759.
- Belicka, L.L., Sokol, E.R. Hoch, J.M., Jaffé, R. and Trexler, J.C. (2012) A Molecular and Stable Isotopic Approach to Investigate Algal and Detrital Energy Pathways in a Freshwater Marsh. *Wetlands*. Vol.32, No.3, 531-542
- Berry, J.P., Gantar, M., Gawley, R.E., Wang, M. and Rein, K.S. (2004) Pharmacology and toxicology of pahayokolide A, a bioactive metabolite from a freshwater species of *Lyngbya* isolated from the Florida Everglades. *Comparative Biochemistry and Physiology part C: Toxicology & Pharmacology*. Vol.139, No.4, 231-238
- Berry, J.P., Gantar, M., Perez, M.H., Berry, G. and Noriega, F.G. (2008) Cyanobacteria Toxins as Allelochemicals with Potential Applications as Algacides, Herbicides and Insecticides. *Marine Drugs*. Vol.6, 117-146
- Berry J., Gibbs P., Schmale M., Saker M. (2009) Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. *Toxicon*. Vol. 53, 289–299
- Blanco, G., Hatfield, W.R., Minor, N.T., Sánchez, G., Koster, J.C., DeTomaso, A.W. and Mercer, R.W. (1997) Studies of Na,K-ATPase structure and function using baculovirus. *Annals of the New York Academy of Science*. Vol.834, 88-96

- Bloomquist, J.R. and Miller, T.A. (1985) Carbofuran triggers flight motor output in pyrethroid-blocked reflex pathways of the house-fly. *Pesticide Biochemistry and Physiology*. Vol. 23, 247-255
- Bowerman, W.W., Best, D.A., Grubb, T.G., Zimmerman, G.M. and Giesy, J.P. (2004) Trends of contaminants and effect in bald eagles of the Great Lakes basin. *Environmental Monitoring and Assessment*. Vol.53, No.1, 197-212
- Brautigan, D.L. (1995) Flicking the Switches: Phosphorylation of Serine/Threonine Protein Phosphatases. *Seminars in Cancer Biology*. Vol.6, 211-217
- Bravo, A., Soberon, M. and Gill, S.S. (2005) *Bacillus thuringiensis*, mechanism and use. *Comprehensive Molecular Insect Science*. Vol.6, 175-197
- Browder, J.A., Gleason, P.J. and Swift, D.R. (1994) Periphyton in the Everglades: spatial variation, environmental correlates, and ecological implications. *Everglades: The Ecosystem and its Restorations*. 379
- Burke, D.S. and Monath, T.P. (2001) Flaviviridae. *Fields virology*. 4th ed. 1043–1125
- Busvine, J.R. (1951) Mechanism of resistance to insecticide in houseflies. *Nature*. Vol. 168, 193-195
- Casamatta, D.A., Johansen, J.R., Vis, M.L. and Broadwater, S.T. (2005) Molecular and morphological characterization of ten polar and near-polar strains within the Oscillatoriales (cyanobacteria). *Journal of Phycology*. Vol.41, No.2, 421-438
- Cebrián, M.E. (1988) Efectos de los plaguicidas sobre la función reproductiva humana: una asignatura pendiente. *Avance y Perspectiva* Vol.17, 205-213
- Chaufaux, J., Marchal, M., Gilois N., Jehanno I., and Buisson, C. (1997) Investigation of natural strains of *Bacillus thuringiensis* in different biotypes throughout the world. *Canadian Journal of Microbiology*. Vol.43, 337–343
- Chiu, T.C., Yen, J.H., Liu, T.L. and Wang W.S. (2004) Anaerobic degradation of the organochlorine pesticide DDT and heptachlor in river sediments in Taiwan. *Bulletin of Environmental Contamination Toxicology*. Vol.72, 821-828
- Clements, A.N. (1992) The biology of mosquitoes. Vol.1 Development, nutrition and reproduction. Chapman & Hall, New York, NY.
- Coats, J.R. (1994) *Annual Review of Entomology*. Vol.39, 499-515

- Codd GA, Bell SG (1996) The occurrence and fate of blue-green algal toxins in freshwaters. London, Her Majesty's Stationery Office (National Rivers Authority R and D Report 29).
- Coetzee, M. (2006) Malaria and dengue vector biology and control in Southern and Eastern Africa. Bridging laboratory and field research for genetic control of disease vectors. 101-109
- Cohen, Y. and Gurevitz, M. (2006) The Cyanobacteria—Ecology, Physiology and Molecular. The Prokaryotes. 1074-1098
- Coppings, L.G. (1996) Crop protection agents from nature: Natural products and analogues. Royal Society of Chemistry. Cambridge, UK.
- Corbel, V., Chandre, F., Darriet, F., Lardeux, F. and Hougard, J.M. (2003) Synergism between permethrin and propoxur against *Culex quinquefasciatus* mosquito larvae. Medical and Veterinary Entomology. Vol. 17, 158-164
- Cowan, M.M. (1999) Plant Products as Antimicrobial Agents. Clinical Microbiology Reviews. Vol.12, No.4, 564-582
- Cragg, G.M.; Newman, D.J. (2005) Biodiversity: A continuing source of novel drug leads. Pure and Applied Chemistry. Vol 77, 7–24.
- Crawford, A.D., Esguerra, C.V. and de Witte, P.A. (2008) Fishing for drugs from nature: zebrafish as a technology platform for natural product discovery. Planta Medica. Vol.74, 624–632
- Curtis, C. F. (2002) Should the use of DDT be revived for malaria vector control? Biomedica Vol.22, 455-61
- Dahmsa, H-U., Yinga, X. and Pfeifferb, C. (2006) Biofouling. The Journal of Bioadhesion and Biofilm Research. Vol.22, No.5, 317-327
- Dawson, R.M. (1998) The Toxicology of Microcystins. Toxicon. Vol.36, 953-962
- Desbois , A.P. and Smith, V.J. (2010) Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Applied Microbiology and Biotechnology. Vol.85, No.6, 1629-642
- Desikachary, T.V. (1959) *Cyanophyta*. Indian Council of Agricultural Research, New Dehli, 686.
- Devlin, J.P., Edwards, O.E., Gorham, P.R., Hunter, N.R., Pike, R.K. and Stavric, B. (1977) Anatoxin-a, a toxic alkaloid from *Anabaena flos-aquae* NRC-44h. Canadian Journal of Chemistry. Vol.55, No.8, 1367-1371.

- Dhadialla, T.S., Retnakaran, A. and Smagghe, G. (2005) Pyrethroids. *Comprehensive Molecular Insect Science*. Vol.6, 55-100
- Dias, D.A., Urban, S. and Roessner, U. (2012) A Historical Overview of Natural Products in Drug Discovery. *Metabolites*. Vol. 2, 303-336.
- Dittman, E. and Wiegand, C. (2006) Cyanobacterial Toxins – Occurrence, Biosynthesis and Impact on Human Affairs. *Molecular Nutrition and Food Research*. Vol.50, 7-17
- Dörmann, P. and Benning, C. (2002). Galactolipids rule in seed plants. *Trends in Plant Science*. Vol.7, No.3, 112–118
- Drakare, S. (2002) Competition between picoplanktonic cyanobacteria and heterotrophic bacteria along crossed gradients of glucose and phosphate. *Microbial Ecology*. Vol.44, No.4, 327-35.
- Dudley, T.L. (1992) Beneficial effects of herbivores on stream macroalgae via epiphyte removal. *Oikos*. Vol.65, 121-127.
- Dunkelblum, E., Tan, S.H. Silk, P.J. (1984) Double-bond location in monounsaturated fatty acids by dimethyl disulfide derivatization and mass spectrometry: Application to analysis of fatty acids in pheromone glands of four lepidoptera. *Journal of Chemical Ecology*. Vol.11, No.3, 265-277.
- Elango, G., Abdul Rahuman, A., Kamaraj, C., Bagavan, A. and Abduz Zahir, A. (2011) Screening for feeding deterrent activity of herbal extracts against the larvae of malaria vector *Anopheles subpictus* Grassi. *Parasitology Research*. Vol.109, 715-726.
- Environmental Protection Agency. (2012) DDT - A Brief History and Status
- Eritja, R., Escosa, R., Lucientes, J., Marquès, E., Roiz, D. and Ruiz, S. (2005) Worldwide invasion of vector mosquitoes: present European distribution and challenges for Spain. *Biological Invasions*. Vol.7, No.1, 87-97
- Environmental Protection Agency: Insecticide Fact Sheet. (2001) Methoprene R.E.D. Fact Sheet. <http://www.epa.gov/pesticides/factsheets/chemicals/ddt-brief-history-status.htm> (accessed October 30, 2013)
- Feeney, P. (1976) Plant Apparency and Chemical Defense. *Recent Advances in Phytochemistry*. New York, USA, Plenum Press.
- Ferre, J. and Van Rie, J. (2002) Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology*. Vol. 47, 501-533

- Forrester, N.W., Cahill, M., Bird, L.J. and Layland, J.K. (1993) Management of pyrethroid and endosulfan resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. Bulletin of Entomological Research. Supplement 1.
- Funaki, E., Dauterman, W.C. and Motoyama, N. (1994) In-vitro and in-vivo metabolism of fenvalerate in pyrethroid-resistant houseflies. Journal of Pesticide Science. Vol. 19, 43-52
- Finney, D. J., Ed. (1952). Probit Analysis. Cambridge, England, Cambridge University Press.
- Gage K.L., Burkot, T.R., Eisen, R.J. and Hayes, E.B. (2008) Climate and Vectorborne Diseases. American Journal of Preventive Medicine. Vol.35, No.5, 436-450
- Gambel, J.M., DeFraites, R., Hoke C., Jr Brown, A., Sanchez. J., Karabatsos, N., Tsai. T. and Meschievitz, C. (1995) Japanese encephalitis vaccine: persistence of antibody up to 3 years after a three-dose primary series (letter). Journal of Infectious Disease 1071-1074
- Garros, C., Ngugi, N., Githeko, A.E., Tuno, N. and YAN, G. (2008) Gut content identification of larvae of the *Anopheles gambiae* complex in western Kenya using a barcoding approach. Molecular Ecology Resources. Vol.8, 512–518
- Gatto, C., McCloud, S.M. and Kaplan, J.H. (2001) Heterologous expression of Na⁺-K⁺-ATPase in insect cells: intracellular distribution of pump subunits. American Journal of Physiology Cell Physiology. Vol. 281, 982-992
- Geitler, L. (1932) Cyanophyceae. In Rabenhorst, L. [Ed.] Kryptogamen-flora von Deutschland, Österreich und der Schweiz. Akademische Verlagsgesellschaft, Leipzig. Vol.14, 1196.
- Gerwick, W. H., Tan, L., and Sitachitta, N. (2001). Nitrogen-containing metabolites from marine cyanobacteria. In The Alkaloids, G.A.Cordell, ed. San Diego, Academic Press. 75-184
- Ginter, J.M., Shackman, H.M., Fox, J.P., Nishimura, M., Taniguchi, M., Usami, Y. and Lee, K-H. (2007) Natural Product Analysis using the LC-MS-IT-TOF: Technical Report vol.4. Shimadzu Scientific Instruments. Chapel Hill, NC
- Gregory, S.V. (1983) Plant-herbivore interactions in stream systems. Stream Ecology. 157-189
- Greuter, W., McNeill, J., Barrie, F.R., Burdet, H.M., Demoulin, V., Filgueiras, T.S., Nicolson, D.H., Silva, P.C., Skog, J.E., Treharne, P., Turland, N.J. and Hawksworth. D.L., Ed. (2000) International Code of Botanical Nomenclature (Saint Louis Code)., Konigstein, Germany, Keoltz Scientific Books. 474

- Grier, J.W. (1982) Ban of DDT and subsequent recovery of reproduction in bald eagles. *Science*. Vol.212, 1232-1235
- Griffits, J.S., Whitacre, J.L., Stevens, D.E. and Aroian, R.V. (2001) *Bacillus thuringiensis* toxin resistance from loss of a putative carbohydrate-modifying enzyme. *Science*. Vol.293, 860-864
- Hairston, N. G., Jr., Lampert, W., Caceres, C., Holtmeier, C., Weider, L., Gaedke, U., Fischer, J., Fox, J. A. and Post, D. M. (1999) Rapid Evolution Revealed by Dormant Eggs. *Nature*. Vol.401, 446
- Hairston, N. G., Holtmeier, C. L., Lampert, W., Weider, L. J., Post, D. M., Fischer, J. M., Caceres, C. E., Fox, J. A. and Gaedke, U. (2001) Natural Selection for Grazer Resistance to Toxic Cyanobacteria: Evolution of Phenotypic Plasticity? *Evolution*. Vol.55, 2203-2214
- Harada, K., Suomalainen, M., Uchida, H., Masui, H., Ohmura, K., Kiviranta, J., Niku-Paavola, M-L. and Ikemoto, T. (2000) Insecticidal compound against mosquito larvae from *Oscillatoria agardhii* strain 27. *Environmental Toxicology*. Vol.15, No.2, 114-119
- Heydarizadeh, P., Poirier, I., Loizeau, D., Ulmann, L., Mimouni, V., Schoefs, B. and Bertrand, M. (2013) Plastids of Marine Phytoplankton Produce Bioactive Pigments and Lipids. *Marine Drugs*. Vol.11, 3425-3471
- Hill, A. J., Teraoka, H., Heideman, W. and Peterson, R. E. (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences*. Vol.86, No.1, 6-19
- Hobbs, J. H., Lowe, R.E. and Schreck, C.E. (1974) Studies on flight range and survival of *Anopheles albimanus* Wiedemann in El Salvador: I. Dispersal and survival during the dry season. *Mosquito News*. Vol.34, 389-393.
- Howland, L. J. (1930). The nutrition of mosquito larvae, with special reference to their algal food. *Bulletin of Entomology Research* Vol.21, 431-439
- Hwang, Y-S., Schultz, G.S. and Mulla, M.S. (1984) Structure-activity relationship of unsaturated fatty acids as mosquito ovipositional repellent. *Journal of Chemical Ecology*. Vol.10, No.1, 145-151
- Ikawa, M., Haney, J.F. and Sasner, J.J. (1996) Inhibition of *Chlorella* growth by lipids of the cyanobacterium *Microcystis aeruginosa*. *Hydrobiologia*. Vol.331, 167-170
- Jones, J. C. (1960) The anatomy and rythmical activities of the alimentary canal of *Anopheles* larvae. *Ann. Entomological Society of America*. Vol.53, 459-474

- Joseph, J.T. and Wong, J.L. (1979) Determination of protonated and quaternary carbons in H-coal liquids from quaternary carbon suppressed and nonsuppressed CMR spectra. 177th National Meeting of the American Chemical Society, Honolulu, HI, USA.
- Karouna, N. K. and Fuller, R.L. (1992) Influence of four grazers on the periphyton community associated with clay tiles and leaves. *Hydrobiologia*. Vol.245, 53-62
- Kearns, K.D. and Hunter, M.D. (2001) Toxin-Producing *Anabaena flos-aquae* Induces Settling of *Chlamydomonas reinhardtii*, a Competing Motile Alga. *Microbial Ecology*. Vol.42, 80-86.
- Khambay, B.P.S. and Jewess, P.J. (2005) Pyrethroids. *Comprehensive Molecular Insect Science*. Vol.6, 1-21
- Kiviranta, J., Sivonen, K., Niemelä, S.I., Huovinen, K. (1991) Detection of toxicity of cyanobacteria by *Artemia salina* bioassay. *Environmental Toxicology and Water Quality*. Vol.6, No.4, 423–436
- Kiviranta, J. and Abdel-Hameed, A. (1993) Toxicity of cyanobacteria to mosquito larvae – Screening of active compounds. *Environmental Toxicology and Water Quality: An International Journal*. Vol.8, 63-71
- Kiviranta, J. and Abdel-Hameed, A. (1994) Toxicity of the blue green algae *Oscillatoria agardhii* to the mosquito *Aedes aegypti* and the shrimp *Artemia salina*. *World Journal of Microbiology and Biotechnology*. Vol.10, 517-520
- Komansilan, A., Abadi, A.L., Yanuwadi, B. and Kaligis, D.A. (2012) Isolation and Identification of Biolarvicide from Soursop (*Annona muricata* Linn) Seeds to Mosquito (*Aedes aegypti*) Larvae. *International Journal of Engineering & Technology*. Vol.12, No.03, 28-32
- Koziol, F.S. and Witkowski, J.F. (1981) Dosage-mortality response of the first three instars of European corn borer (Lepidoptera: Pyralidae) larvae to seven insecticides. *Journal of the Kansas Entomological Society*. Vol.54, No.2, 327 – 330
- Kurane, I., Rothman, A.L., Livingston, P.G., Green, S., Gagnon, S.J., Janus, J., Innis, B.L., Nimmannitya, S., Nisalak, A. and Ennis, F.A. (1994) Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome. *Archives of Virology Supplementum*. Vol 9, 59-64
- Laird, M. 1988. The natural history of larval mosquito habitats. Academic Press, Inc. (London), Ltd., London.

- Lange, L. and Lopez, C.S. (1996) Protection agents from nature: Natural products and analogues. Cambridge, UK, Royal Society of Chemistry. Chapter 1
- Lee, S.H., Kang, H.J., Lee, H.J., Kang, M.H. and Park, Y.K. (2010) Six-week supplementation with *Chlorella* has favorable impact on antioxidant status in Korean male smokers. *Nutrition*. Vol.26, 175–183
- Levin, M. (1983) Value of bee pollination to US agriculture. *Bulletin of the Entomological Society of America* Vol.29, 50–51
- Lipkind, G.M. and Fozzard, H.A. (2000) KcsA crystal structure as framework for a molecular model of the Na⁺ channel pore. *Biochemistry*. Vol.39, 8162-9170
- Lowe, R. R., Schreck, C.E., Hobbs, J.H., Dame, D.A. and Lofgren, C.S. (1975) Studies on flight range and survival of *Anopheles albimanus* Wiedemann in El Salvador: II. Comparisons of release methods with sterile and normal adults in wet and dry seasons. *Mosquito News*. Vol.35, 160-168
- Ludwicki, J.K. and Goralczyk, K. (1994) Organochlorine pesticides and PCB in human adipose tissue in Poland. *Bulletin of Environmental Contamination Toxicology*. Vol. 52, 400-403
- Lüring, M. (2003) Effects of Microcystin-Free and Microcystin-Containing Strains of the Cyanobacterium *Microcystis aeruginosa* on Growth of the Grazer *Daphnia magna*. *Environmental Toxicology*. Vol.18, 202-210
- Malathion Technical Fact Sheet (2009) National Pesticide Information Center.
- Margalith, Y. and Ben-Dov, E. (2000) Biological control by *Bacillus thuringiensis* subsp. *israeliensis*. *Insect Pest Management: Techniques for Environmental Protection*. 243 – 301
- Matsunaga, T., Takeyama, H., Miura, Y., Yamazaki, T., Furuya, H. and Sode, K. (1995) Screening of marine cyanobacteria for high palmitoleic acid production. *FEMS Microbiology Letters*. Vol.133, No.1-2, 137-141
- Maud, S.J., Campbell, P.J., Giddings, J.M., Hamer, M.J., Henry, K., Pilling, E.D., Warinton, J.S., and Wheeler, J.R. (2011) Ecotoxicology of Synthetic Pyrethroids. *Topics in Current Chemistry*. Vol. 314, 137-166.
- McCormick, P.V. Shuford III, R.B.E., Backus, J.G. and Kennedy, W.C. (1998) Spatial and seasonal patterns of periphyton biomass and productivity in the northern Everglade, Florida, USA. *Hydrobiologia* 362, 185.
- McGaw, L.J., Jager, A.K. and van Staden, J. (2002) Isolation of antibacterial fatty acids from *Schotia brachypetala*. *Fitoterapia*. Vol.73, 431–433

- Micallef, M., Munro, I., Phang, M. and Garg, M. (2009) Plasma n-3 polyunsaturated fatty acids are negatively associated with obesity. *British Journal of Nutrition*. Vol.102, 1370-1374
- Mikata, K., Isobe, N. and Kaneko, H. (2011) Biotransformation and Enzymatic Reactions of Synthetic Pyrethroids in Mammals. *Topics in Current Chemistry*. Vol. 314, 113-136.
- Miller, J.R., Huang, J., Vulule, J. and Walker, E.D. (2007) Life on the edge: African malaria mosquito (*Anopheles gambiae s. l.*) larvae are amphibious. *Naturwissenschaften*. Vol.94, No.3, 195-199
- Mitsui, A., Rosner, D., Goodman, A., Reyes-Vasquez, G., Kusumi, T., Kodama, T. and Nomoto, K. (1987) Hemolytic toxins in marine cyanobacterium *Synechococcus* sp. Red tide: biology, environmental science and toxicology. Elsevier, New York, 367–370
- Morohashi, M., Tsuchiya, K., Mita, T. and Kawamura, M. (1991) Identification of (Na,K)ATPase inhibitor in brine shrimp, *Artemia salina*, as long-chain fatty acids. *Journal of Comparative Physiology B*. Vol.161, 69-72
- Mueller-Beilschmidt, D. (1990) Toxicology and environmental fate of synthetic pyrethroids. *Journal of Pesticide Reform*. Vol.10, No.3, 32-37
- Mundt, S., Kreitlow, S. and Jansen, R. (2003) Fatty acids with antibacterial activity from the cyanobacterium *Oscillatoria redekei* HUB 051. *Journal of Applied Phycology*. Vol.15, 263–267
- Munoz Jordan, J.L., Santiago, G.A. and Margolis, H. (2013) Genetic Relatedness of Dengue Viruses in Key West, Florida, USA, 2009–2010. *Emerging Infectious Diseases*. Vol.19, No.4
- Namikoshi, M. and Rinehart, K.L. (1996) *Journal of Industrial Microbiology*. Vol.17, 373-384
- National Research Council of Canada [NRCC] (1987) Pyrethroids: their effects on aquatic and terrestrial ecosystems. No. 24376. Ottawa (Canada): National Research Council of Canada, Associate Committee on Scientific Criteria for Environmental Quality. 248
- Narendra, M., Kavitha, G., Helah Kiranmai, A., Raghava Rao, N. and Varadacharyulu, N.C. (2008). Chronic exposure to pyrethroids-based allethrin and prallethrin mosquito repellents alters plasma biochemical profile. *Chemosphere*. Vol.73, No.3, 360-364
- Nassar, M.M., Hafez, S.T., Nagaty, I.M. and Khalaf, S.A. (1999) The insecticidal activity of Cyanobacteria against four insects, two of medical importance and two agricultural

- pests with reference to the action on albino mice. Journal of Egyptian Society of Parasitology. Vol.29, No.3, 939-49
- Neilan, B.A., Jacobs, J., Del Dot, T., Blackall, L.L., Hawkins, P.R., Coz, P.T. and Goodman A.E. (1997) rDNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. International Journal of Systematic Bacteriology. Vol. 47, 693-697.
- Nelissen, B., Van der Peer, Y., Wilmotte, A. and De Wachter, R. (1995) An early origin of plastids within the cyanobacterial divergence is suggested by evolutionary trees based on complete 16S rDNA sequences. Molecular Biology and Evolution. Vol. 12, 1166-1173
- Nishida, I. and Murata, N. (1996) Chilling Sensitivity in Plants and Cyanobacteria: The Crucial Contribution of Membrane Lipids. Annual Review of Plant Physiology and Plant Molecular Biology. Vol.47, 541-568
- Nizan, S., Dimentman, C., and Shilo, M. (1986) Acute Toxic Effects of Cyanobacterium *Microcystis eruginosa* on *Daphnia magna*. Limnology and Oceanography. Vol. 31, 497-502
- Ooi, T., Kusumi, T., Kakisawa, H. and Watanabe, M.M. (1989) Structure of cyanoviridin RR, a toxin form the blue-green alga, *Microcystis viridis*. Journal of Applied Phycology. Vol.1, No.1, 31-38.
- Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D. and McGaughey, W.H. (1997) Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. Journal of Biological Chemistry. Vol.272, 23473-23476
- Pauron, D., Barhanin, J., Amichot, M., Pralavorio, M., Berge, J.B., et al. (1989) Pyrethroid receptor in the insect Na⁺ channel – alteration of its properties in pyrethroid-resistant flies. Biochemistry. Vol. 28, 1673-1677
- Peñaloza, R., Rojas, M., Vila, I. and Zambrano, F. (1990) Toxicity of a Soluble Peptide from *Microcystis* sp. to Zooplankton and Fish. Freshwater Biology. Vol.24, 233-240
- Perez, M.H. and Noriega, F.G. (2012) *Aedes aegypti* pharate 1st instar quiescence affects larval fitness and metal tolerance. Journal of Insect Physiology. Vol.58. No.6, 824-829
- Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K. A., Krause, E., Codd, G. A. and Steinberg, C. E. W. (1998) Identification of an Enzymatically-Formed Glutathione Conjugate of the Cyanobacterial Hepatotoxin Microcystin-LR. The First Step of Detoxication. Biochimica et Biophysica Acta. Vol.1425, 527-533.

- Pope, K., Masuoka, P., Rejmankova, E., Grieco, J., Johnson, S. and Roberts, D. (2005) Mosquito habitats, land use, and malaria risk in Belize from satellite imagery. Vol.15, No.5, 1223 – 1232.
- Porter, K.G. (1977) The plant-animal interface in freshwater ecosystems. American Journal of Science. Vol.65, 159-170
- Rader, R.B. and Richardson, C.J. (1994) Response of macroinvertebrates and small fish to nutrient enrichment in the northern Everglades. Wetlands. Vol.14, 134
- Rahuman, A.A., Venkatesan, P. and Gopalakrishnan, G. (2008) Mosquito larvicidal activity of oleic and linoleic acids isolated from *Citrullus colocynthis* (Linn.) Schrad. Parasitologyresearch. Vol.103, 1383–1390
- Rao, D.R., Mani, T.R., Rajendran, R., Joseph, A.S. and Gajanana, A. (1995) Development of high level resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. Journal of the American Mosquito Control Association. Vol.11, 1-5
- Rao, D.R., Thangavel, C., Kabilan, L., Suguna, S., Mani, T.R. and Shanmugasundaram, S. (1999) Larvicidal properties of the cyanobacterium *Westiellopsis* sp. (blue-green algae) against mosquito vectors. Transactions of the Royal Society of Tropical Medicine and Hygiene. Vol.93, No.3, 232
- Reich, A.R., Perkins, J.L. and Cutter, G. (1986) DDT contamination of north Alabama aquatic ecosystems. Environment Toxicological chemistry. Vol.5, 725-736.
- Reinikainen, M., Meriluoto, J.A., Spoof, L. and Harada, K. (2001) The toxicities of a polyunsaturated fatty acid and a microcystin to *Daphnia magna*. Environmental Toxicology. Vol.16, No.5, 444-448
- Rejmankova, E., Roberts, D.R., Manguin, S., Pope, K.O., Komarek, J. and Post, R.A. (1996) *Anopheles albimanus* (Diptera: Culicidae) and cyanobacteria: an example of larval habitat selection. Environmental Entomology. Vol.25, No.5, 1058-1067
- Resh, V. H. and Houpp, R.E. (1986) Life history of the caddisfly *Dibusa angata* and its association with the red alga *Lemanea australis*. Journal of the North American Benthological Society Vol.5, 28-40
- Rhoades, D.F. and Cates, R.G. (1976) Toward a General Theory of Plant Antiherbivore Chemistry. Recent Advances in Phytochemistry. Vol.10, 168-213

- Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979) Genetic assignments, strain histories, and properties of pure cultures of cyanobacteria. *Journal of General Microbiology*. Vol.111, 1-61.
- Rippka, R.J. (1988) Recognition and identification of cyanobacteria. *Methods in Enzymology*. Vol. 167, 34-72
- Robertson, J.L., Russell, R.M., Preisler, H.K. and Savin, N.E. (2007) *Bioassays with Arthropods: Second edition*. CRC Press. Boca Raton, FL.
- Rohrlack, T., Dittman, E., Henning, M., Börner, T., Kohl, J. (1999) Role of Microcystins in Poisoning and Food Ingestion Inhibition of *Daphnia galeata* Caused by the Cyanobacterium *Microcystis aeruginosa*. *Applied Environmental Microbiology*. Vol.65, 737-739
- Rohrlack, T., Dittman, E., Borner, T. and Christofferson, K. (2001) Effects of Cell-Bound Microcystins on Survival and Feeding of *Daphnia* spp. *Applied. Environmental. Microbiology*. Vol.67, 3523-3529
- Rucker, R.R., Thurman, W.N. and Burgett, M. (2005) Internalizing reciprocal benefits: the economics of honeybee pollination markets. North Carolina State University, Department of Agricultural and Resource Economics, Raleigh, Mar 6, 2005. Unpublished manuscript.
- Saario E, Abdel-Hameed A, Kiviranta J. (1994). Larvicidal microcystin toxins of cyanobacteria affect midgut epithelial cells of *Aedes aegypti* mosquitoes. *Medical Veterinary Entomology*. Vol.8, 398–400.
- Sato. N. and Murata, N. (1980) Temperature shift-induced responses in lipids in the blue-green alga, *Anabaena variabilis*. *Biochimica et Biophysica Acta*. Vol.619, 353-366
- Sato. N. and Murata, N. (1981) Studies on the temperature shift-induced desaturation of fatty acids in monogalactosyl diacylglycerol in the blue-green alga (cyanobacterium) *Anabaena variabilis*. *Plant and Cell Physiology*. Vol.22, 1043-1050
- Sangthongpitag, K. Delaney, S.F. and Rogers, P.L. (1996) Evaluation of four fresh-water unicellular cyanobacteria as potential hosts for mosquitocidal toxins. *Biotechnology Letters*. Vol.18, No.2, 175-180
- Schlegel, I., Doan, N.T., de Chazal, N. and Smith, G.D. (1999) Antibiotic activity of new cyanobacterial isolates from Australia and Asia against green algae and cyanobacteria. *Journal of Applied Phycology*. Vol.10, 471-479.
- Scott, J.G. (1989) Cross resistance to the biological insecticide abamectin in pyrethroid resistant strains of house flies. *Pesticide Biochemistry Physiology*. Vol.34, 27-31

- Sharathchandra, K. and Rajashekhar, M. (2011) Total lipid and fatty acid composition in some freshwater cyanobacteria. *Journal of Algal Biomass Utilization*. Vol.2, No.2, 83– 97
- Silva, A.G., Almeida, D.L., Ronchi, S.N., Bento, A.C., Scherer, R., Ramos, A.C. and Cruz, Z.M. (2010). The essential oil of Brazilian pepper, *Schinus terebinthifolia* Raddi in larval control of *Stegomyia aegypti* (Linnaeus, 1762). *Parasites & Vectors*. Vol.3, Retrieved from <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2936394/>
- Skinner, W.A., Tong, H.C., Maibach, H.I. and Skidmore, D. (1970) Human skin-surface lipid fatty acids — Mosquito repellents. *Experientia*. Vol.26, No.7, 728-730
- Schmidt, K. and Jonasdottir, S.H. (1997) Nutritional quality of two cyanobacteria: How rich is 'poor' food? *Marine Ecology Progress Series*. Vol.151, 1-10
- Smith, T.M., and Stratton, G.W. 1986. Effects of synthetic pyrethroid insecticides on nontarget organisms. *Residue Reviews*. Vol.97, 93-120
- Stanier, R.Y., Sistrom, W.R., Hansen, T.A., Whitton, B.A., Castenholz, R.W., Pfennig, N., Gorlenko, V.N., Kondratieva, E.N., Eimhjellen, K.E., Whittenbury, R., Gherna, R.L. and Truper, H.G. (1978). Proposal to place the nomenclature of the cyanobacteria (blue-green algae) under the rules of the International Code of Nomenclature of Bacteria. *International Journal of Systematic Bacteriology*. Vol. 28, 335-336
- Steinman, A.D. (1996) Effects of grazers on freshwater benthic algae. *Algal ecology*, 1st edition. Academic Press, San Diego. 341–373
- Subcommittee on Pesticides and Industrial Organic Pesticides, Associate Committee on Scientific Criteria for Environmental Quality, National Research Council of Canada. 1986. *Pyrethroids: Their effect on aquatic and terrestrial ecosystems*. NRCC No.24376. Ottawa, Canada: Environmental Secretariat, National Research Council Canada.
- Sun, M., Luo, X., Dai, J., Qu, K., Liu, Z., Yu, L., Chen, Y. and Yu, Z. (1996) Evaluation of *Bacillus thuringiensis* and *Bacillus sphaericus* Strains from Chinese Soils Toxic to Mosquito Larvae. *Journal of Invertebrate Pathology*. Vol.68, No.1, 74-77
- Tan, L.T. (2007) Bioactive natural products from marine cyanobacteria for drug discovery. *Phytochemistry*. Vol.68, No.7, 954–979
- Texas Department of Health Services (2013) West Nile Virus in Texas <http://www.dshs.state.tx.us/idcu/disease/arboviral/westnile/> (accessed on August 8th 2013)

- Thiery, I., Nicolas, L., Rippka, R. and Tandeau de Marsac, N. (1991) Selection of cyanobacteria isolated from mosquito breeding sites as a potential food source for mosquito larvae. *Applied and Environmental Microbiology*. Vol.57, No.5, 1354-1359
- Toynnton, K., Luukinen, B., Buhl, K. and Stone, D. (2009) Permethrin Technical Fact Sheet; National Pesticide Information Center, Oregon State University Extension Services. <http://npic.orst.edu/factsheets/Permttech.pdf>. (accessed October 30, 2013)
- Valdivielso, P., Rioja, J., Garcia-Aras, C., Sanchez-Chaparro, M.A. and Gonzalez-Santos, P. (2009) Omega 3 fatty acids induce a marked reduction of apolipoprotein B48 when added to fluvastatin in patients with type 2 diabetes and mixed hyperlipidemia: A preliminary report. *Cardiovascular Diabetology*. Vol.8, 1-7
- Vázquez-Martínez, M.G., Rodríguez, M.H., Arredondo-Jiménez, J.I., Méndez-Sánchez, J.D., Bond-Compeán, J.G. and Gold-Morgan, M. (2002) Cyanobacteria associated with *Anopheles albimanus* (Diptera: Culicidae) larval habitats in southern Mexico. *Journal of Medical Entomology*. Vol.39, 825–832.
- Wada, H. and Murata, N. (1990) Temperature-induced Changes in the Fatty Acid Composition of the Cyanobacterium, *Synechocystis* PCC68031. *Plant Physiology*. Vol.92, 1062-1069
- Whalon, M., Mota-Sanchez, D., Hollingworth, R.M., Bills, P. and Duynslager, L. (2003) The Database of Arthropods Resistance to Pesticides. Michigan State University.
- Whitton, B.A. (1992) Diversity, ecology, and taxonomy of the cyanobacteria. *Photosynthetic Prokaryotes*. Plenum Press, New York. 1-51
- WHO (1999) Toxic Cyanobacteria in Water. A guide to their public health consequences, monitoring and management. 416
- WHO (2000) World Health Organization Expert Committee on Malaria. Twentieth Report. Geneva, Switzerland
- WHO (2001) Malaria in Africa.
http://www.rbm.who.int/cmc_upload/0/000/015/370/RBMInfosheet_3.htm
(accessed October 27, 2006)
- WHO (2012) Dengue and severe dengue. Fact sheet N° 117
<http://www.who.int/mediacentre/factsheets/fs117/en/index.html>
(accessed June 27, 2013)

- WHO (2013) Yellow fever. Fact sheet N° 100
<http://www.who.int/mediacentre/factsheets/fs100/en/index.html>
 (accessed June 27, 2013)
- Wiley, E.O. and Mayden, R.L. (2000) The evolutionary species concept. In: Wheeler, Q.D., Meier, R. (Eds.), *Species Concepts and Phylogenetic Theory: A Debate*. Columbia University Press, New York, 70–89
- Wille, J.J. and Kydonieus, A. (2003) Palmitoleic acid isomer (C16:1 Δ 6) in human skin sebum is effective against gram-positive bacteria. *Skin Pharmacology and Applied Skin Physiology*. Vol.16, No.3, 176-87
- Wirth (2010) Mosquito Resistance to Bacterial Larvicidal Toxins. *The Open Toxinology Journal*. Vol.3, 126-140.
- Wright, A.D., Papendorf, O., König, G.M. and Oberemm, A. (2006) Effects of cyanobacterium *Fischerella ambigua* isolates and cell free culture media on zebra fish (*Danio rerio*) embryo development. *Chemosphere*. Vol.65, 604-608
- Yang, Z-H., Miyahara, H. and Hatanaka, A. (2011) Chronic administration of palmitoleic acid reduces insulin resistance and hepatic lipid accumulation in KK-Ay Mice with genetic type 2 diabetes. *Lipids in Health and Disease*. Vol.10, 120
- Zheng, C.J., Yoo, J-S., Lee, T-G., Cho, H-Y., Kim, Y-H. and Kim, W-G. (2005) Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *FEBS Letters*. Vol. 579, 5157–5162

VITA

GERALD ARTHUR BERRY

Born, Carol Stream, Illinois

2000-2005

B.A., Biology
Florida Gulf Coast University
Estero, Florida

2007-2014

Doctoral Candidate
Florida International University
Miami, Florida

Teaching Assistant
Florida International University
Miami, Florida

PUBLICATIONS AND PRESENTATIONS

Gerald A. Berry, John P. Berry, and Fernando G. Noriega. Mosquito Larvicides from Cyanobacteria. (Poster). 79th Annual Meeting, American Mosquito Control Association. Atlantic City, N.J., February 24-28, 2013.

Gerald A. Berry, John P. Berry, and Fernando G. Noriega. Mosquito Larvicides from Cyanobacteria. (Oral Presentation) 14th Annual Biology Research Symposium. Miami, FL, January 28, 2012.

Gerald A. Berry, John P. Berry, and Fernando G. Noriega. Mosquito Larvicides from Cyanobacteria. (Oral Presentation) 33rd Annual Southeastern Phycological Colloquy. Miami, FL, October 15, 2011.

Gerald A. Berry, John P. Berry, and Fernando G. Noriega. Cyanobacteria Toxins: Novel Insecticides for Disease Vector control. (Poster). Joint Annual Meeting of the American Society of Pharmacognosy & the Phytochemical Society of North America. St. Petersburg, FL, July 10-14, 2010.

John P. Berry, Miroslav Gantar, Mario H. Perez, Gerald Berry, and Fernando G. Noriega. (2008) Cyanobacterial Toxins as Allelochemicals with Potential Applications as Algaecides, Herbicides, and Insecticides. Marine Drugs. Vol.6, 117-146.