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Influence of Nutrients and Integrated Mosquito Management Tactics on Mosquitoes and Their Habitat Microbiomes

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Influence of Nutrients and Integrated Mosquito Management Tactics on
Mosquitoes and Their Habitat Microbiomes

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

Dagne Duguma Demisse

December 2013

Dissertation Committee:
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Dedication

I would like to dedicate my dissertation to my late mother Asnakech Asefa. I also dedicate this work to my grandparents who raised and encouraged me to pursue my goals in education.

ABSTRACT OF THE DISSERTATION

Influence of Nutrients and Integrated Mosquito Management Tactics on
Mosquitoes and Their Habitat Microbiomes

by

Dagne Duguma Demisse

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, December 2013
Dr. William E. Walton, Chairperson

Mosquito management in wetlands is complex partly because some of the
wetland management operations are known to enhance mosquito production. I
aimed to describe the effects of nutrients and integrated mosquito management
strategies on mosquitoes produced in wetlands in the following three studies.
First, we evaluated the growth characteristics of alkali bulrush, an alternative
aquatic plant of potential bioremediation importance, and its associated mosquito
production across a gradient of nitrogen enrichment including the high
ammonium nitrogen regimens typically found in constructed wetlands. Mosquito
larvae abundance, dominated by the western encephalitis mosquito (*Culex
tarsalis* Coquillett), was significantly greater in enriched versus unenriched
mesocosms. Alkali bulrush survived high nitrogen loadings, but its biomass was
significantly suppressed at high (>50 mg/liter) ammonium nitrogen concentration
in the water. Second, we characterized microbiota associated with *C. tarsalis*

larvae, water column and epibionts of two (alkali and California) bulrushes using 16S rRNA gene sequences generated with Illumina sequencing platform. The diversity of microbiota in mosquitoes sampled from wetlands containing the two bulrushes did not differ appreciably, and was chiefly dominated by *Thorsellia* (*Gammaproteobacteria*). The epibionts of the bulrushes and bacterioplankton were dominated by members of Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria and Verrucomicrobia. Overall, nearly 49% of the *Bacteria* taxa found in the mosquito gut were also found in the habitat, suggesting a strong *Culex* larvae-*Bacteria* interaction. Finally, we evaluated the effects of a one-time application of two rates of a key mosquito biopesticide, *Bacillus thuringiensis* subsp. *israelensis* (a granular form of VectoBac G) on native microbiota and physicochemical variables in the feeding zone of *Culex* larvae in experimental mesocosms. Beta diversity of *Bacteria* communities revealed that samples from low *Bti* and untreated control mesocosms were significantly separated from high *Bti* mesocosms and were dominated by unidentified OTU of Cyanobacteria, *Cytophagales* and *Cyclobacteriaceae* (Bacteroidetes), and *Sphingomonas* (*Alphaproteobacteria*). Phytoplankton (chlorophyll *a*), sestonic particulates, nutrients, pH and other physicochemical variables in the water column were also significantly reduced in the high *Bti* mesocosms. Important implications of these studies on integrated mosquito management in treatment wetlands and other aquatic ecosystems are discussed.

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Chapter 1: Introduction

More than 2.5 billion people are at risk from mosquito-borne diseases such as malaria, dengue, yellow fever, West Nile fever, filariasis, encephalitis, etc. (Gubler 2002, Lemon et al. 2008). Malaria alone infects about 219 million humans worldwide with the majority of the cases and mortalities occurring in sub-Saharan Africa (WHO 2012). Mosquito-borne diseases continue to be a major burden in many developing countries in sub-Saharan Africa, Southeast Asia and Latin America despite intensified vector control efforts.

There is also growing public health concern in the United States and many other countries in temperate regions related to the increased incidence of mosquito-borne diseases such as arbovirus (arthropod-borne viruses) infections. The increased global connection via trade and travel (Kilpatrick 2011), resurgence and spatial expansion of already existing arboviruses, and lack of new and effective mosquito control tactics over the past four decades are among the most frequently mentioned risk factors of mosquito-borne diseases in temperate regions (Gubler 2002). There is an increased risk of movement of infectious mosquitoes or infectious vertebrate hosts to countries where mosquito-borne illnesses have been considered a low risk (Gubler 2002). A sporadic incidence of arboviruses such as West Nile virus in North America over the last decade is a notable example of such threats (Gubler 2002, Gould and Fikrig 2004, Kilpatrick 2011).

The biology and ecology of mosquitoes have been relatively well-studied primarily because mosquitoes vector pathogens that cause debilitating diseases

in humans and other animals (Clements 1992, 1999, 2012; Godfray 2013, Koenraadt and Takken 2013, Tolle 2013). However, the current knowledge of mosquito ecology comes from very few medically important mosquito species and some basic information about the ecology of these well-studied species is still far from complete (Ferguson et al. 2010, Godfray 2013, Koenraadt and Takken 2013). About 3,500 mosquito species occur worldwide and less than 5% of these mosquitoes are known to be potential vectors of pathogens that cause diseases in humans and other animals (Rueda 2008). Species in three genera *Anopheles*, *Culex* and *Aedes*, are considered the primary vectors of mosquito-borne pathogens.

Mosquitoes are also an important component of many aquatic and terrestrial food webs (Rueda 2008). The immature stages of mosquitoes are exclusively adapted to aquatic environments and can make up a significant portion of the aquatic food web either as prey, predators or competitors (Rueda 2008, Ferguson et al. 2010). Adult mosquitoes, on the other hand, are adapted to the terrestrial environment and interact with humans and other vertebrates. These life history traits of mosquitoes made them subjects of many studies focused to understand the ecological links between terrestrial and aquatic environments (Johnson et al. 2010).

Mosquitoes develop in habitats ranging from small microcosms such as tree holes or small containers (e.g. *Aedes*) to large natural or manmade wetlands (e.g. *Culex* and *Anopheles*) (Laird 1988, Rueda 2008). A myriad of environmental

variables is thought to influence immature mosquito development in their natural habitats, among which nutrients, temperature, aquatic plants and microorganisms play a significant role in affecting mosquito production (Rejmánková et al. 2013).

Influence of nutrients on mosquitoes

The dominant and most prevalent hypothesis is that elevated levels of nutrients in the aquatic habitats are linked to increased mosquito production (Mutero et al. 2004, Sanford et al. 2005, Chaves et al. 2009). Supporting the bottom-up regulation hypothesis, nutrients in aquatic habitats are thought to increase mosquito larval resources (Rejmánková et al. 2013). Increases of critical nutrients such as nitrogen and phosphorus are thought to increase the density and biomass of certain aquatic macrophytes and other phytoplankton that directly or indirectly influence mosquito production. It was unknown, however, if this phenomenon occurs in a dose-dependent manner (i. e., whether an increase in nutrient levels is directly related with increased mosquito production). Increase in nutrients in aquatic habitats has been linked to more disease occurrence (Johnson et al. 2010). Global increase of essential nutrients such as N and P in aquatic habitats as a result of agriculture runoff has been implicated influencing the mosquito and mosquito-borne disease ecology (Johnson et al. 2010, Kirkman et al. 2011). However, evidence directly linking nutrients to disease emergence is still scanty.

Interaction of aquatic plants and mosquitoes in constructed wetlands

Aquatic plants are an integral component of manmade treatment wetlands and

other aquatic restoration habitats. Aquatic macrophytes are thought to facilitate

the bioremediation processes in wetlands via (1) direct uptake and translocation

of nutrients from the wetlands (Brix 1997), (2) production of antimicrobial

compounds that enhance removal of pathogenic *Bacteria* from the aquatic

habitat (Vymazal 2011), and (3) production of exudates that enhance the

proliferation of particular *Bacteria* taxa (e.g. denitrifiers, sulfate- reducing

Bacteria) that are useful for toxic nutrient transformations (Vymazal 2011).

Overall, aquatic plants have potential to either promote or restrict the growth of

different microbial species in aquatic habitats (Ibekwe et al. 2007, Hempel et al.

2008, Sèrandour et al. 2008, Vymazal 2011).

While there is a growing demand for constructing new wetlands or restoring

existing wetlands to improve water quality to meet the needs of a growing human

population, these wetlands often pose a serious risk and challenges to humans

and animals residing in the vicinity of these wetlands due to an increased risk of

mosquito-borne diseases (Russell 1999, Walton 2002, Kirkman et al. 2011). The

risk of mosquito-borne diseases is primarily due to the following inherent

characteristics of these wetlands.

First, treatment wetlands are often constructed for multiple purposes such as

improving water quality, providing habitat for aquatic wildlife, providing other

ecosystem services (e.g., flood control), as well as educational and recreational

purposes (Walton 2003, 2012). The combination of the availability of suitable

egg-laying habitat and sources of blood meals from the wildlife inhabiting the

wetlands and humans residing in the vicinity of the wetlands can make

constructed wetlands suitable habitats for mosquito development. Second, most

of the emergent aquatic plants currently being utilized in many treatment

wetlands in California and elsewhere grow very large and dense [e.g., California

bulrush (*Schoenoplectus californicus*), cattails (*Typha* spp*.*), common reed

(*Phragmites australis*)]. These macrophytes have been associated with

enhanced mosquito production partly due to their physical interference to

mosquito biorational control agents and predators (Walton 2003). Finally, aquatic

plants harbor and influence microbial communities that may serve as larval

resources or provide habitat cues for egg-laying adult mosquitoes in these

wetlands. However, this role is not well explored. Emergent aquatic plants are

known to play an important role in shaping *Bacteria* community structure

associated with aquatic habitats (Hempel et al. 2008; Sèrandour et al. 2009,

Vymazal 2011). Selection and evaluation of alternative aquatic plants that fulfill

the important wetland functions (relatively to the currently used large

macrophytes) will likely enhance the value of the wetland and reduce the costs of

mosquito control and wetland operations.

Interaction of mosquitoes with microbial communities

Microbes are often implicated in influencing mosquitoes in variety of ways. Especially, *Bacteria* have been considered the most important component of mosquito larval diets (Merritt et al. 1992). The previous methods used to determine the interaction of mosquitoes with *Bacteria* and other microorganisms had technical limitations that often underestimated the extent of these interactions (Rani et al. 2009). With the advent of high throughput genomic technology, it has been possible to overcome several of these limitations and be able to fully describe the mosquito-microbiome interactions including rare and unculturable *Bacteria* species.

Bacteria associated with mosquitoes have several functions. *Bacteria* acquired during larval stages have been implicated to influence vector competence during the adult stages by enhancing basal immunity against parasites (Okech et al. 2007). The findings of Okech et al. (2007) highlight the importance of understanding the interaction of *Bacteria* associated not only with the adult stages but also with other developmental stages of mosquitoes, which has thus far been given little attention.

Bacteria found in adult mosquitoes have been shown to enhance immunity against parasites such as viruses and malaria pathogens, and are being considered for the development of paratransgenic control (Dong et al. 2009, Ricci et al. 2011, Minard et al. 2013). For instance, *Wolbachia*, a well-known endosymbiont of several arthropods, was originally discovered in *Culex pipiens*

and has currently been proposed to suppress dengue virus transmitted by *Aedes* mosquitoes (Minard et al. 2013). There has, therefore, been a growing interest in elucidating mosquito-microbiome interactions in natural habitats to understand the nature and extent of their interaction, and ultimately to exploit the knowledge to design novel approaches such as paratransgenic as well as "push-pull" mosquito control strategies (Dong et al. 2009, Ricci et al. 2011, Minard et al. 2013).

A considerable amount of effort has already been devoted to understanding the microbial associations of the major human disease-vectoring mosquitoes, mainly *Anopheles* and *Aedes*, species over the last decade (Minard et al. 2013). However, the *Culex*-microbiome interactions in natural conditions are less well understood. In North America (north of Mexico), about 174 species of mosquitoes are present, and about two dozen of these mosquitoes are *Culex* species (Darsie et al. 2005). *Culex* species are considered vectors of many enzootic arboviruses including West Nile virus in America.

Interaction of mosquito control operations with biotic and abiotic variables

Biopesticides such as *Bacillus thuringiensis* subsp. *israelensis* have been successfully used to control mosquitoes in much of North America, Europe and other developed countries over the past four decades. They are considered "environmentally safe" and no known detrimental impacts on eukaryotes including humans have been reported to date (Lacey and Merritt 2003, WHO

2009). However, there is a knowledge gap of the interaction of the most commonly applied mosquito biopesticides with microbiomes associated with mosquito habitats (Boisvert and Boisvert 2000). The impact of mosquito control operations specifically in wetlands of bioremediation importance is poorly understood.

In conclusion, understanding the ecology of mosquitoes is key to developing sustainable mosquito and novel control strategies (Ferguson et al. 2010, Godfray 2013). Ferguson and colleagues emphasized that the traditional approach of just aiming to control mosquitoes by focusing on a control tactic of one kind has fallen short of the target of reducing mosquito-borne diseases (Ferguson et al. 2010). Understanding larval ecology of mosquitoes primarily how they interact with environmental variables such as nutrients, aquatic plants and microorganisms will significantly enhance our ability to manage mosquitoes in wetlands. Moreover, it is crucial to understand the impacts and the underlying mechanisms of the current mosquito control operations on freshwater ecosystems specifically on the microorganisms associated with mosquito habitats that play an important role in core wetland processes and functions.

The aims of my dissertation studies were therefore to address aspects of larval ecology of *Culex* mosquitoes with the following main objectives: (1) evaluate the effects of nutrient enrichment on *Culex* mosquitoes and an alternative emergent macrophyte of bioremediation importance, (2) to characterize the microbiomes associated with *Culex* mosquitoes and aquatic

plants of bioremediation importance and (3) to describe the microbiomes and physicochemical properties of mosquito habitats following the application of a commonly used mosquito biopesticide.

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Chapter 2: Effects of nutrients on mosquitoes and an emergent

macrophyte, *Schoenoplectus maritimus,* **for use in treatment wetlands**

Abstract. *Schoenoplectus maritimus* (alkali bulrush) has desirable attributes, such as a short growth habit (height of mature stands < 1.5 m) and annual senescence, for a potential alternative to tall (height > 3 m) emergent macrophytes in shallow constructed treatment wetlands treating ammonium-dominated wastewater. The effects of different ammonium nitrogen (NH_4-N) levels on alkali bulrush growth and its ability to take up nutrients from the wastewater, as well as on mosquito production, across the range of NH_4-N found in constructed wetlands of southern California are unknown. We evaluated the effects of enrichment with NH_4-N on mosquito production, and on the nutrient uptake and growth of alkali bulrush in two studies. Overall, significantly greater numbers (> 50%) of immature mosquitoes (mainly *Culex tarsalis*) were found in mesocosms enriched with NH_4-N than in mesocosms receiving ambient (<0.3 mg/liter) NH_4-N. High NH_4-N enrichment (up to 60 mg/liter) did not adversely impact the height and stem density of *S. maritimus,* although a significant decrease in biomass was observed at the highest enrichment level. Nitrogen uptake by alkali bulrush increased directly with NH_4-N enrichment, whereas carbon was conserved in the above-ground biomass across the enrichment gradient. Alkali bulrush is recommended for use as part of integrated mosquito management programs for moderately enriched, multipurpose, constructed treatment wetlands that improve water quality as well as provide wetland habitat for waterfowl.

Keyword Index: *Culex*, ammonium nitrogen, alkali bulrush, nitrogen, carbon, mesocosms

INTRODUCTION

The management of emergent macrophytes in constructed wetlands is a significant concern for wetland managers. Macrophytes fulfill several important functions for water quality improvement, such as enhancing nitrification by increasing oxygen concentration in the sediments of treatment wetlands and enhancing removal of toxic compounds such as ammonia and pathogens from wastewater (Brix 1997, Stottmeister et al. 2003). The macrophytes used in treatment wetlands include California bulrush [*Schoenoplectus californicus* (C.A. Meyer) Palla], cattail (*Typha* spp.) or common reed (*Phragmites australis* (Cav.) Trin ex. Steud); with *Typha* being the most commonly planted species worldwide (Kadlec and Wallace 2009). Paradoxically, these perennial emergent macrophytes grow very large (> 3 m high) and dense (up to 800 stems/m^2; Thullen et al. 2002) in nutrient-rich habitats and consequently impede mosquito control agents such as aerial application of granular forms of mosquito biopesticides (Walton 2003). Furthermore, these macrophytes form mats of decaying matter that are known to increase food resources for larval mosquitoes (Berkelhamer and Bradley 1989, Walton and Jiannino 2005).

Nutrient-elevated sewage is often associated with enhanced mosquito production (Chaves et al. 2009, Walton 2012). Besides increasing the production of macrophytes that can produce detrital resources for mosquito larvae, nutrient enrichment increases epiphytic and planktonic algal populations and other

microbial assemblages that are food resources for mosquito larvae (Victor and

Reuben 2002, Johnson et al. 2010). Among the dominant nutrients in effluent

waters, nitrogenous compounds are known to increase mosquito abundance

(Sanford et al. 2005).

Nitrogenous nutrients are also known to influence the growth and survival of

emergent macrophytes. Ammonium (NH_4^+) is a predominant form of nitrogen in

flooded wetland soils and an initial form of nitrogen fertilizer readily taken up by

plants (Mitsch et al. 2001); however, ammonium can inhibit growth (Britto and

Kronzucker 2002). An increase in growth of *Schoenoplectus* (= *Scirpus*) *acutu*s

(Muhl. ex Bigelow) Á. Löve & D. Löve var. *acutus* was observed with the addition

of ammonium ranging between 30-50 mg/liter; whereas, a significant biomass

reduction was observed at high (> 60 mg/liter) ammonium nitrogen

concentrations (Hill et al. 1997). Excess total ammonia ($NH_3 + NH_4^+$) is thought to

interfere with core functions of plant physiology (e.g. inhibition of plant

respiration; Santamaria et al. 1994).

Schoenoplectus maritimus (L.) Lye (Cyperaceae), commonly known as alkali

(or cosmopolitan) bulrush, is a relatively short (< 1.5 m high) bulrush and has

been recommended for use in treatment wetlands (Tilley 2012). Synonyms

include *Scirpus maritimus* L. and *Bolboschoenus maritimus* (L.) Palla (Clevering

et al. 1995, Kantrud 1996). Its life cycle in northern California and the Pacific

Northwest includes dormancy in the winter, new shoot growth from corms in

March and April, flowering in May, peak growth rate in June and July, peak shoot

mass in August or September and senescence in October (Miller et al. 2009). Alkali bulrush produces larger achenes and more carbohydrate-rich corms than most bulrushes, and was ranked second to sea purslane (*Sesuvium portulacastrum* (L.) L.; Aizoaceae) as waterfowl food in northern California (Kantrud 1996, Miller et al. 2009).

The ability of *S. maritimus* to thrive in anaerobic aquatic substrate (Clevering et al. 1995) is a desirable attribute of this bulrush. The tuber of this species was reported to remain viable even after three months of anaerobic conditions (Clevering et al. 1995). Below-ground structures (roots, rhizomes and corms) of alkali bulrush occur within 0.2 m of the surface and produce a large surface area that harbors beneficial microbes involved in degradation of nutrients (Tilley 2012). In addition, *S. maritimus* is reported to enhance the removal of fecal pathogens (e. g. *Escherichia coli*) from sewage effluent and phenols from industrial wastes (Seidel 1971). Information on the effects of different nitrogen levels on alkali bulrush growth and its ability to take up nutrients from the wastewater is lacking. Moreover, the production of mosquitoes and other associated invertebrates from alkali bulrush within ammonium-dominated wastewater treatment wetlands is unknown.

The objectives of this study were to determine the effects of ammonium nitrogen enrichment on growth, biomass and nutrient uptake of *S. maritimus*, and on the abundances of mosquitoes and aquatic invertebrates in wetland mesocosms. We tested the null hypotheses that enrichment with ammonium

does not have an impact on alkali bulrush and on the abundance of invertebrates including mosquitoes.

MATERIALS AND METHODS

Study site and experimental design

Two independent studies (autumn 2009 and summer 2010) were conducted in fiberglass mesocosms (area= 1 m^2; volume = 0.5 m^3) at the Aquatic and Vector Control Research Facility of University of California, Riverside, USA (Figure 1). Soil mix (plaster sand mixed with peat moss; 0.17 m deep) and five uniform (average dry mass = 1.2 ± 0.15 g) seedlings of alkali bulrush were transplanted to each mesocosm. The seedlings were selected from a stock population established from seeds collected from natural stands in Riverside County, California in 2007. Transplantation was carried out during two periods of the annual cycle of alkali bulrush: late summer at peak shoot mass or early spring prior to peak growth rate. Seedlings were transplanted on 5 August 2009 and 22 March 2010 for the autumn (September-December 2009) and summer (June-September 2010) experiments, respectively. Replacement of seedlings from the nursery pond was conducted immediately for seedlings that failed to establish.

Water was supplied to the mesocosms from an irrigation reservoir. The water depth was kept constant during the studies at approximately 0.17 m except during the initial phase of plant establishment when the water depth was 0.1 m. A

PVC pipe [(½ inch (1.27 cm) diameter] inside each mesocosm was connected to an outside stand pipe for drainage and control of the water level in the tubs. The average inflow and outflow rates from the mesocosms were 2.2 ± 0.44 (SE, n = 16) and 1.4 ± 0.5 (SE, n = 16) mL/sec, respectively.

Three ammonium nitrogen (NH_4-N) treatments (low: 15 mg/liter, medium: 30 mg/liter and high: 50 mg/liter) were applied weekly to the mesocosms in the form of granular ammonium sulfate [$(NH_4)_2SO_4$; 21% nitrogen and 24% sulfur; Lilly Miller Brands, Walnut Creek, CA) for six consecutive weeks in autumn 2009 and for 11 consecutive weeks during summer 2010. These concentrations span the range of average NH_4-N concentration in the water column of treatment wetlands processing secondary- and tertiary-treated municipal effluent in southern California (Sartoris et al. 2000, Thullen et al. 2002, Sanford et al. 2005; Popko et al. 2009). The ambient NH_4-N concentration in the irrigation water entering the mesocosms was < 0.3 mg/liter (autumn 2009: 0.23 ± 0.07 mg/liter; summer 2010: 0.03 ± 0.002 mg/liter; SE, n = 4). The three enrichment treatments and an untreated control were assigned to the mesocosms in a completely randomized manner and replicated four times (n = 16 mesocosms).

Physicochemical variables

Water samples were taken in 500-mL dark plastic bottles 24 h and 7 d after enrichment with NH_4-N and transported to the laboratory on ice. Water also was collected from the control mesocosms. Ammonium-, nitrate- and nitrite-nitrogen

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concentrations were analyzed colorimetrically using a Hach DR™ 2800 spectrophotometer (TNT Plus tests, Hach Chemical Co., Loveland, CO). Nitrite and nitrate were not measured in autumn 2009. Water temperature was recorded using Taylor maximum-minimum thermometers in 2009, while in 2010 it was recorded every 0.5 h using a water temperature data logger (HOBO Temp Pro v2 (U22-001), Onset Computer Inc., Bourne, MA) in 2010.

Mosquitoes and invertebrates

A 350-ml plastic dipper was used to assess larval mosquito abundance in the mesocosms. Three dip samples were collected weekly from each mesocosm and filtered in a concentrator cup (screen mesh opening: 53 µm). However, during the first two weeks in the autumn experiment, only one dip sample per mesocosm was taken. The samples were then transferred into 20-mL plastic vials, preserved with 95% ethanol and transported to the laboratory for identification (Meyer and Durso 1998) and enumeration of the mosquitoes and other invertebrates (Merritt et al. 2008). Zooplankton was identified using Pennak (1989). Dip sampling was carried out for six weeks between 16 September and 11 November 2009 during the autumn experiment and for 10 weeks between 15 June and14 September 2010 during the summer experiment. Mosquito sampling ceased after the population declined both in autumn and summer experiments.

Plant parameter measurements

Plant growth parameters (stem density, height, number of inflorescences) were assessed monthly during both experiments. Plant density was estimated by counting the number of stems in three quadrats (size = 0.25 m^2) in the mesocosms. Height above the soil substrate for five flowered stems was measured from each mesocosm. Above-ground (leaves, stems and flowers) and below-ground (roots, rhizomes and corms) parts of five senesced stems per mesocosm were sampled in December for the autumn 2009 experiment and in September for the summer 2010 experiment. Samples were oven-dried at 50 °C for five days and weighed in the laboratory. The oven–dried plant samples were then ground into a fine powder and analyzed for elemental carbon and nitrogen using a Thermo-Finnigan Model EA1112 flash elemental analyzer (Thermo Finnigan LLC, San Jose, CA). The percentage concentration of elemental nitrogen and carbon in the plant tissues was determined in three replicate 10 mg subsamples of the ground plant tissues.

Statistical analyses

The data for the autumn and summer experiments were analyzed separately using JMP version 10 (SAS Institute, Cary, NC). Repeated-measures ANOVA was carried out to discern the effects of ammonium nitrogen enrichment on plant parameters (culm density and height), mosquito production and physicochemical parameters across time. Wilks' Lambda was used to test the significance of

treatment effects and time interactions. Normality was assessed using a Sphericity test and, if the test was significant, adjusted F-values (Univar Geisser-Greenhouse Epsilon) were used instead of Wilks' Lambda to determine the significance of the treatment effects and time interactions. Numbers of mosquitoes were $\log_{10} (x+1)$ transformed before analysis. The effects of nitrogen enrichment on plant biomass, percent nitrogen and carbon content of the plant tissue were analyzed using one-way ANOVA. Mosquito counts from the first two weeks of the autumn 2009 experiment were analyzed separately because the sampling regimen for these two weeks was different. If the treatment effect was significant, Tukey's HSD test was conducted to assess the significance of differences among the means.

RESULTS

Water physiochemical characteristics

In 2009, the average NH_4-N concentrations in the water column at 24 h after enrichment were between 86 to 93% of the nominal enrichment treatments of 15, 30 and 50 mg/liter and differed significantly among the treatments (Tables 2.1 and 2.2). In summer 2010, the mean NH_4-N concentration at 24 h after enrichment for the two lower enrichment treatments was about 85% of the target levels; whereas, the NH_4-N concentration in the High treatment exceeded the target of 50 mg/liter by about 18% (Table 2.1). The water column NH_4-N concentration differed significantly among the treatments (Table 2.2). Seven days

after enrichment, NH_4-N was considerably reduced but still in the high treated mesocosms the concentration was significantly greater than the concentration in the medium, low and control mesocosms (Table 2.1; 2009: $F_{3,12}$= 3.96, P<0.05; 2010: $F_{3,11}$ = 3.2, P=0.05).

The nitrite-nitrogen (NO_2-N) concentration differed significantly among the enrichment treatments (Table 2.3; 24h: $F_{3, 9}$ = 6.7, 7d: F3,9=4.4;P<0.05).The High and Medium enrichment levels resulted in significantly greater NO_2-N levels than were recorded in the Low enrichment and control mesocosms (Tables 2.2 and 2.3). Similarly, there was also a significant difference in nitrate-nitrogen (NO_3-N) levels among the four treatments (24h: $F_{3,12}$ = 4.8, P<0.05; 7d: $F_{3,12}$ = 8.2, P<0.05; Tables 2.2 and 2.3). There was a peak in NO_3-N level in the middle of August that coincided with the peak nitrite level, suggesting the presence of more nitrification during that period.

Water temperature in the experimental mesocosms varied among months during the study periods. The maxmium temperatures were recorded in August (28.6 ± 0.78 °C (SE); n = 12) and September (29.0 ± 0.88 °C; n = 10) during the 2009 experiment, and in July (25.3 ± 0.38 °C; n = 31) and August (24.8.± 0.28 °C; n = 31) during the 2010 experiment.

Mosquitoes and other invertebrates

The immature (larvae and pupae) mosquitoes found in the experimental mesocosms were *Culex tarsalis* Coquillett and *Anopheles hermsi* Barr and

Guptavanij, with the former being the dominant mosquito species found during both experiments. The mean of the total number of immature mosquitoes per dip sample varied significantly among the four enrichment treatments and by sampling date during both studies ($P < 0.01$; Control < Low, Medium, High NH_4-N treatments). However, repeated measures ANOVA showed that the treatment effect on mosquito counts during the first two weeks in 2009 experiment was not significantly different among treatments (Date x treatment effect, $F_{3,12} = 1.4$, $P > 0.05$). Less than 16% of the total immature mosquitoes were produced in control treatments while about 27%, 32%, 25% of the total mosquitoes were produced in mesocosms that received low, medium and high NH_4-N enrichments, respectively, in autumn 2009. Immature mosquito abundance in the enriched mesocosms was greater (> 50%) than in the control mesocosms at three weeks after the initial application of ammonium nitrogen in the autumn experiment ($P < 0.001$; Figure 2.2). However, NH_4-N ranging from 15 mg/liter to 50 mg/liter did not influence the total number of immature mosquitoes in the mesocosms during autumn 2009.

In summer 2010, the mean numbers of immature mosquitoes produced from the four treatments were also significantly different (Table 2.4). Mosquitoes from control mesocosms accounted for nearly 17% of the total, which was comparable to mesocosms that received low NH_4-N (16%) enrichment. Mesocosms that received medium and high NH_4-N enrichments produced about 28% and 39%, respectively, of the total immature mosquitoes sampled during summer 2010.

Sampling date also significantly influenced the number of immature

mosquitoes collected per dip sample, with more mosquitoes produced during

early weeks of enrichments (Figure 2). The abundance of the mosquito

population plummeted towards the end of the 2009 experiment in response to

cool temperatures at the end of the mosquito production season. The abundance

of *An. hermsi* averaged less than one individual per dipper sample for the 2009

study and was not separately analyzed. During summer 2010, the treatment

effects on immature mosquito abundance were also significantly different from

three days after the initial enrichment continuously for five weeks, after which the

treatment effect was no longer significant (Figure 2). *Culex* mosquitoes were

more abundant than *An. hermsi* in the study mesocosms. *Anopheles* dominated

late in the summer during the 2010 experiment (Figure 3).

The abundance of the immature stages of the predator community (the

majority of which were in the order Odonata) was not significantly different

among the four NH_4-N treatments in both experiments. In the 2009 experiment,

the average number of naiads collected was less than one individual (mean ±

SE: 0.05 ± 0.02; *n* = 96) per dip sample; whereas, in the summer 2010

experiment, the mean number of naiads per dip sample was considerably greater

(2.1 ± 0.14; *n* = 192); but was not significantly different ($P > 0.05$, $F_{3, 44} = 0.59$)

among the four treatments. Naiads first appeared in the dip samples three weeks

following the initial enrichment of mesocosms (data not shown). The predators

tended to colonize the mesocosms following the primary colonizers such as

mosquitoes and crustaceans (dominantly cladocerans). The decline of mosquito population coincided with an increase in the predator abundance.

The total number of crustaceans (copepods, cladocerans and ostracods) per dip sample did not differ significantly among the enrichment treatments ($P > 0.05$; data not shown). Cladocerans predominated (~88% of the total abundance) in the mesocosms. Both Cladocera and Ostracoda increased significantly 3 weeks after the first enrichment; whereas, Copepoda abundance stayed constant throughout autumn 2009. In summer 2010, Cladocera was present in a proportion (> 90%) higher than the two other crustacean groups and was negatively affected by the highest ammonium nitrogen enrichment.

The abundance of chironomid larvae ranged between 0.5-1 individuals per dip sample and did not differ significantly among the four enrichment treatments (data not shown). Chironomids are adapted to benthic substrates and were not expected to occur in large numbers on the plant surfaces and in the water column. Benthic samples were not taken in this experiment.

Growth characteristics of *S. maritimus*

Culm height, density and inflorescences

Culm height was greatest in the mesocosms enriched at low levels of NH_4-N, and differed significantly among the four treatments in autumn 2009 ($P < 0.05$) (Figure 4A; Table 2.5). The mean culm heights in all the enriched mesocosms were significantly higher than the control treatments in summer 2010, with the

greatest mean culm heights in the mesocosms enriched with low and medium levels of NH_4-N (Figure 4B).

The mean culm density did not differ significantly among the four enrichment levels ($P > 0.05$) during the autumn 2009 (Figure 4C). However, the mean culm densities in enriched mesocosms were significantly greater than the control mesocosms during 2010 with the maximum density attained in July (Figure 4D).

The mean number of flowers did not differ among treatments in both experiments; although, low and medium enrichment treatments tended to produce slightly higher number of flowers in July and August 2010 ($P > 0.05$).

Biomass

The above-ground (Figure 6A) mean dry mass of five senesced *S. maritimus* plants (stems and inflorescence) was not significantly different among treatments in autumn 2009 ($P > 0.05$). However, the mean dry weight of below-ground plant structures (Figure 5A) was significantly different among the treatments in autumn 2009 ($F_{3, 12} = 4.41$; $P = 0.026$). The above-ground mean dry weight of *S. maritimus* was not significantly different among treatments ($P > 0.05$) in July 2010 (data not shown) but was significantly different among enrichment treatments in September 2010 ($F_{3,12} = 7.58$; $P = 0.024$; Figure 5B). No statistically significant difference was found in the weights of below-ground structures among the treatments in September 2010. The mean total dry weight (above- and below-ground masses combined) of the plants grown in the four

treatments was significantly different in both year studies. Mesocosms enriched

at the high treatment level yielded a significantly lower dry biomass compared to

the low and medium enrichment levels (Figure 5).

Elemental nitrogen and carbon

The mean percentage of nitrogen in above-ground biomass differed

significantly among treatments both in autumn 2009 ($F_{3, 44}$ = 41.8, P < 0.001) and

summer 2010 ($F_{3,92}$ = 11.5, P < 0.001)(Figures 2.6A and 2.6C, respectively).

Similarly, the mean percentage of nitrogen in below-ground biomass also differed

significantly among treatments in autumn 2009 ($F_{3, 44}$ = 28.1, P < 0.001) and

summer 2010 ($F_{3, 44}$ = 4.5, P < 0.001) (Figures 2.6B and 2.6D). The percentage

of N found in above-ground plant biomass increased directly with ammonium

enrichment in both studies. Overall, plants that were raised in mesocosms

enriched with NH_4-N incorporated a greater percentage of nitrogen than did alkali

bulrush plants in the control mesocosms (Figure 2.6).

Elemental carbon in the above-ground biomass did not vary significantly

among the treatments in each of the two studies (Figure 6). However, mean

percentage of carbon in below-ground alkali bulrush tissues differed significantly

among treatments (autumn 2009: $F_{3, 44}$ = 3.0, P = 0.040; summer 2010: $F_{3,44}$ =

23.9, P < 0.001) (Figures 2.6B and 2.6D, respectively). The percentage of carbon

in below-ground biomass of nitrogen-enriched plants was significantly greater

than in the control plants (Figure 2.7). A greater percentage of carbon

accumulated in the above-ground structures (stem, leaf and flowers) than in the

below-ground (rhizomes and corms) structures (Figure 2.7). Although there was

variability among treatments, overall, the ratio of carbon to nitrogen (C:N; mass)

was significantly greater in flowers (35.7 ± 1.38, n = 48) and roots (34.9 ± 1.40)

than in stems and leaves (22.1 ± 1.49) of the plants. Nitrogen was more

concentrated in the above-ground photosynthetic structures particularly in the

stems and leaves than in other parts of the plants (Figure 2.8).

DISCUSSION

Effect of nutrient enrichment on mosquitoes and other invertebrates

Mosquito production, primarily *Culex tarsalis*, increased by nearly two-fold in

the enriched mesocosms and this finding is in agreement with the findings

(Beattie 1932, Victor and Reuben 2000, Sunish et al. 2003, Mutero et al. 2004)

that enrichment with ammonium nitrogen is associated with enhanced oviposition

by mosquitoes. Ammonia also has been shown to attract host-seeking adult

Anopheles mosquitoes (Meijerink et al. 2001). Sanford et al. (2005) observed a

similar effect of nutrient enrichment on immature mosquito abundance even at a

very low (<1 mg/liter) ammonium nitrogen concentration in wetlands treating river

water derived primarily from tertiary-treated municipal effluent.

Nitrogenous effluents are known to change trophic cascades in wetlands by

increasing detritus and microbial communities that are primary food resources of

mosquito larvae. Johnson et al. (2010) linked an increase of critical nutrients

such as nitrogen and phosphorus into the aquatic environment to an increased

incidence of debilitating diseases vectored by mosquitoes. Nutrients leaching

from agriculture and released from other industrial activities can significantly

influence the composition and abundance of aquatic macrophytes, which can in

turn reduce mosquito predator activities while increasing microbial communities

that are food resources for mosquito larvae (Johnson et al. 2010). In addition,

high levels of nitrogen compounds such as ammonia can directly inhibit survival

and reproduction of mosquito predators such as fish (Walton 2003).

The abundance of immature mosquitoes in our study could have been

influenced by a combination of reduced predation rates under enriched

conditions as well as enhancement of mosquito production from bottom-up

processes. Mosquito abundance was greater in enriched mesocosms that also

had higher plant biomass and bulrush culm densities compared to the control

mesocosms. High culm (stem) densities are known to reduce mosquito predator

efficiency (Orr 1991, Thullen et al. 2002). The abundance of invertebrate

predators (primarily zygopterans) did not differ significantly among the

enrichment treatments in our study. Because predator abundance did not change

across the enrichment gradient, predation efficiency in the enriched mesocosms

could have been reduced by the enhanced physical structure provided by the

alkali bulrush as compared to the control mesocosms. Greater habitat complexity

reduces predator efficiency and reduces prey vulnerability (Saha et al. 2009).

Increased macrophyte production has been known to support large mosquito

populations (de Szalay and Resh 2000). Low dissolved oxygen levels in the water column are characteristic of dense stands of emergent macrophytes in eutrophic treatment wetlands (Sartoris et al. 2000, Thullen et al. 2008, Walton et al. 2012). Mosquito predator abundance declines at low dissolved oxygen concentrations (Walton 2012) and top-down effects on mosquito populations appear to decrease with enrichment as predator abundance is decoupled from enrichment gradients in hypereutrophic constructed treatment wetlands (Peck and Walton 2008). Immature *Culex* abundance increases directly with bottom-up enrichment (Chaves et al. 2009, Walton 2012).

We did not quantify water column resources available to mosquito larvae across the enrichment gradient and ammonium nitrogen concentration in enriched mesocosms. However, mosquito abundance declined to ambient levels in the control treatment by one week after enrichment. A combination of factors could have contributed to the enhancement of mosquito populations with ammonium nitrogen enrichment. Our sampling technique —standard dip sampling mainly used for immature mosquito and zooplankton sampling— might have underestimated the abundance of odonates, especially the dragonfly naiads which tend to inhabit the benthic substrates below the water column. Substrate sampling was not suitable in this mesocosm study because it could interfere with plant growth.

Abundances of other invertebrates, such as cladoceran populations did not respond differently to nitrogen enrichment in autumn 2009; however, a significant

(20%) reduction of cladoceran abundance occurred in mesocosms that received the highest enrichment level during the summer 2010. Prolonged enrichment at high levels of ammonium nitrogen was known to have a detrimental effect on *Daphnia magna* developmental stages (Yang et al. 2012). Cladocera were not identified to species in our study.

Effects of nitrogen on *S. maritimus*

An understanding of the growth response and nutrient uptake of an alternative macrophyte species under different nutrient conditions is required before utilizing the plant in constructed treatment wetlands for wastewater water quality remediation. Lack of this knowledge has been the most frequently reported problem for the failure and poor survival of plants in treatment wetlands (Kadlec and Wallace 2009). In this study, low and medium levels (~15-30 mg/liter) of NH_4-N enrichment maximized culm density, stem height and the overall plant biomass. At greater than 50 mg NH_4-N/liter, alkali bulrush biomass was significantly reduced. This is consistent with the typical relationship reported for plant growth response and nutrient enrichments reported elsewhere (Hill et al. 1997, Kadlec and Wallace 2009). For example, the growth of *S. acutus* was reduced in wetlands when ammonium nitrogen concentration exceeded 60 mg/liter. The highest NH_4-N concentration (~50 mg/liter) in our experiment suppressed *S. maritimus* biomass (Figure 5). Ammonium nitrogen is known to settle or bind to the substrate (clay) and is likely to increase toxic ammonia

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production in the rhizosphere of plants, which in turn affects the root physiology of the plant (Wang 1991). We observed significantly higher culm mortality and below-ground biomass reduction in the mesocosms enriched at the highest ammonium nitrogen treatment.

Transplantation of *S. maritimus* in late summer (at peak shoot mass) or early spring (prior to peak growth rate) was successful. The rate of growth of alkali bulrush decreased over time, with the maximum growth attained two and three months after planting in autumn and summer experiments, respectively, after which alkali bulrush reached maturity. Both enriched and control treatments exhibited a similar growth pattern except that plants in the control treatments were significantly shorter and less dense than enriched treatments (Figures 5B and 5D).

However, the percent nitrogen content in the alkali bulrush tissues increased directly with nitrogen enrichment (Figures 2.6 and 2.7). The concentration measured in alkali bulrush was within the range of nitrogen composition found in most emergent macrophytes used in treatment wetlands (Reddy and Delaune 2008). Emergent macrophytes generally incorporate less than 5% of nitrogen into their tissues (Reddy and Delaune 2008). The majority of nutrient removal takes place within the microbial communities that inhabit these emergent macrophytes (Stottmeister et al. 2003). The nitrogen concentration in vegetative portions of alkali bulrush ranged between 8-24 g/kg of dry weight and compares reasonably well with the amount reported for other *Scirpus* species (8-27g/kg: Reddy and

Delaune, 2008). In our study, alkali bulrush in the enriched mesocosms

contained about 77g N/m^2, which was three times greater than the amount (24 g

N/m^2) found in plants in the control mesocosms. This suggests that the mass-

specific uptake rates of nutrients by alkali bulrush is comparable with the large-

stature emergent macrophytes (e.g. California bulrush, cattail, etc.) used in

wastewater treatment wetlands. The carbon content of alkali burush was also

comparable to the carbon content in other congeners (Reddy and Delaune 2008)

and even to oak leaves (Walker et al. 1997).

In this study, we observed a greater percentage of carbon and nitrogen (by

mass) in flowers and roots than in the vegetative portion (stems and leaves) of

the plants. However, carbon did not change across the enrichment gradient

(Figure 2.7). Similar to our observations, Santamaria et al. (1994) reported that

the net photosynthesis of other submerged macrophytes did not change under

different levels of enrichment. This supports our observation that carbon was

conserved across different enrichment levels in the above-ground biomass

(Figures 2.6 and 2.7).

Because the above-ground biomass of *S. maritimus* dies off each winter and

its below-ground biomass (rhizomes and tubers) can persist in the soil for several

years for future regeneration, this bulrush does not likely require the costly

routine management (harvesting and removal) of biomass similar to the other

emergent macrophytes currently being utilized in many treatment wetlands. The

annual senescence and die back of *S. maritimus* can also be considered an

important trait of a species to be utilized in constructed wetlands because it can provide detritus and carbon to the denitrifying bacteria and other microbial communities when mosquito activity is limited by cool weather conditions. Moreover, *S. maritimus* is considered a preferred diet for waterfowl (Kantrud 1996, Miller et al. 2009).

In conclusion, mosquito production was increased by ammonium nitrogen enrichment, and the enrichment effect varied across time and between seasons. Mosquito production in the enriched mesocosms was enhanced soon after inundation when insect predators were comparatively rare. The differences in mosquito abundance between the enriched vs. control mesocosms lessened across time as the wetland plots aged, especially during the summer. Although the abundance of immature mosquitoes was not directly related to ammonium nitrogen treatment during the autumn, immature mosquito abundance in mesocosms of all the enrichment treatments was comparatively greater than in the control mesocosms. Overall, mosquito abundance increased by nearly two-fold in the enrichment treatments (15-50 mg NH_4-N/liter). Our findings also indicated that ammonium concentration up to 60 mg/liter has no detrimental effect on survival of *S. maritimus* although plants enriched at the high (>50 mg/liter) ammonium nitrogen regimen had lower biomass than did plants exposed to lower NH_4-N levels. We found ammonium nitrogen levels ranging between (15-30 mg/liter) to be more favorable for alkali bulrush growth than were the ambient (< 0.3 mg NH_4-N/liter) and highest NH_4-N concentrations;

consequently, we can recommend that alkali bulrush be planted in constructed wetlands exhibiting a wide range of reduced nitrogen concentrations, but especially for moderately enriched treatment wetlands. However, our experiments were carried out in shallow (depth < 0.2 m) experimental mesocosms and further research on the impact of water depth on alkali bulrush survival and growth is warranted. Moreover, *S. maritimus* has a cosmopolitan distribution and investigations of populations from different habitat types across the geographic range of this species might be helpful.

ACKNOWLEDGMENTS

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Table 2.1. Mean (± SE) ammonium nitrogen concentration in mesocosms 24 h and 7 d after weekly ammonium sulfate applications during autumn 2009 and summer 2010.

Year	Treatment	Mean (± SE) concentration (mg/liter)			
		N	24 h	N	7 d
2009	Control	19	0.19 ± 0.08^a	21	0.27 ± 0.12^a
	Low	20	14.0 ± 2.18^b	20	0.89 ± 0.39^a
	Medium	22	26.6 ± 3.58^c	20	2.34 ± 1.04^a
	High	24	42.8 ± 4.33^d	20	5.35 ± 1.78^b
2010	Control	44	0.03 ± 0.002^a	44	0.03 ± 0.002^a
	Low	44	12.6 ± 1.09^b	43	0.50 ± 0.13^a
	Medium	44	25.8 ± 2.70^c	44	1.90 ± 0.82^a
	High	42	59.0 ± 4.72^d	42	10.9 ± 3.23^b

[a-d]For each column, different letters within each year differ by $P < 0.05$ (Tukey test).

Table 2.2. Repeated-measures ANOVA of enrichment effect on nitrogen species 24 h after enrichments.

Year	Sources	Ammonium-N			Nitrite-N			Nitrate-N		
		F	df	*P*	F	df	*P*	F	df	*P*
2009	Between groups									
	Treatment	15.8	3,12	0.0002						
	Within groups									
	Date	41.8	3,40	0.0001						
	Date x treatments	8.8	10,40	0.0001						
2010	Between groups									
	Treatment	12.8	3,9	0.0014	6.73	3,12	0.006	4.75	3,12	0.021
	Within groups									
	Date	19.5	3,24	0.0001	5.80	6,75	0.0001	4.63	5.56	0.002
	Date x treatments	4.8	8,24	0.0013	1.29	19,75	0.218	1.12	14.56	0.357

Table 2.3. Mean (± SE) nitrate-nitrogen and nitrite-nitrogen concentration (mg/liter) in mesocosms 24 h and 7 d

after weekly applications of ammonium sulfate during summer 2010.

Treatments	Nitrate			Nitrite			
	N	24 h	7 d	N	24 h	N	7 d
Control	44	0.94 ± 0.11^a	0.66 ± 0.08^a	43	0.03 ± 0.004^a	44	0.03 ± 0.005^a
Low	44	1.28 ± 0.10^b	1.21 ± 0.08^a	43	0.05 ± 0.01^b	43	0.04 ± 0.006^a
Medium	44	1.70 ± 0.11^c	1.37 ± 0.09^a	43	0.13 ± 0.03^{bc}	42	0.07 ± 0.01^a
High	44	1.66 ± 0.12^{bc}	1.36 ± 0.10^b	44	0.31 ± 0.06^c	43	0.29 ± 0.06^b

[a-d]For each column, different letters differ by $P < 0.05$ (Tukey test).

Table 2.4. Repeated-measures ANOVA of the effect of four ammonium nitrogen levels on the abundance of immature mosquitoes (larvae and pupae) in mesocosms planted with *Schoenoplectus maritimus*.

Year	Sources	F	df	*P*
2009	Between groups			
	Treatment	7.0	3, 44	0.0006
	Within groups			
	Date	77.6	3,42	0.0001
	Date x treatments	2.9	9, 102	0.0046
2010	Between groups			
	Treatment	7.6	3, 38	0.0004
	Within groups			
	Date	23.1	9, 30	0.0001
	Date x treatments	2.5	27, 88	0.0008

Table 2.5. Repeated-measures ANOVA of the effect of four ammonium nitrogen levels on culm height and density of *Schoenoplectus maritimus*.

Year	Sources	Height			Culm density		
		F	df	*P*	F	df	*P*
2009	Between groups						
	Treatment	5.53	3, 74	0.002	0.64	3,12	0.603
	Within groups						
	Date	28.8	2, 136	0.100	39.5	2,22	0.001
	Date x treatments	0.64	5, 136	0.954	1.35	5,22	0.194
2010	Between groups						
	Treatment	71.5	3, 76	0.001	12.7	3,12	0.005
	Within groups						
	Date	332	4, 73	0.001	390	7,84	0.001
	Date x treatments	11.6	12, 193	0.001	14.4	21,84	0.001

Figure 2.1. Schematic diagram of the experimental set up of the two studies (autumn 2009 and summer 2010). Treatments were assigned in a completely randomized design.

Figure 2.2. Immature (larvae and pupae) *Culex* mosquito abundance (mean ± SE, n = 4) in 0.17-m^3 mesocosms containing *Schoenoplectus maritimus* and four levels of ammonium nitrogen during autumn 2009 (A) and summer 2010 (B).

Figure 2.3. Abundance of immature *Anopheles hermsi* (mean ± SE, n = 4) in 0.17-m^3 mesocosms containing *Schoenoplectus maritimus* and low and medium (C) or high and control (D) levels of ammonium nitrogen during summer 2010.

Figure 2.4. Mean (± SE, n = 4) height (cm) and density (number m^{-2}) of *S. maritimus* in mesocosms containing four levels of ammonium nitrogen during autumn 2009 (A and C) and summer 2010 (B and D), respectively.

Figure 2.5. Dry weight (mean ± SE, n = 4) of five above-ground (light gray) and below-ground (dark gray) parts of *Schoenoplectus maritimus* culms in autumn 2009 (A) and summer 2010 (B) among four enrichment treatments.

Figure 2.6. Percentages of carbon (mean ± SE, n = 12) and nitrogen in above- (A and C) and below-ground (B and D) plant tissues of *S. maritimus* across a gradient of ammonium nitrogen enrichment.

Figure 2.7. Carbon and nitrogen content (mean ± SE, n = 12) in three (roots, stem and leaves, and flowers) plant tissues of *S. maritimus* raised under four enrichment treatments in September 2010. Roots are composed of rhizomes and corms.

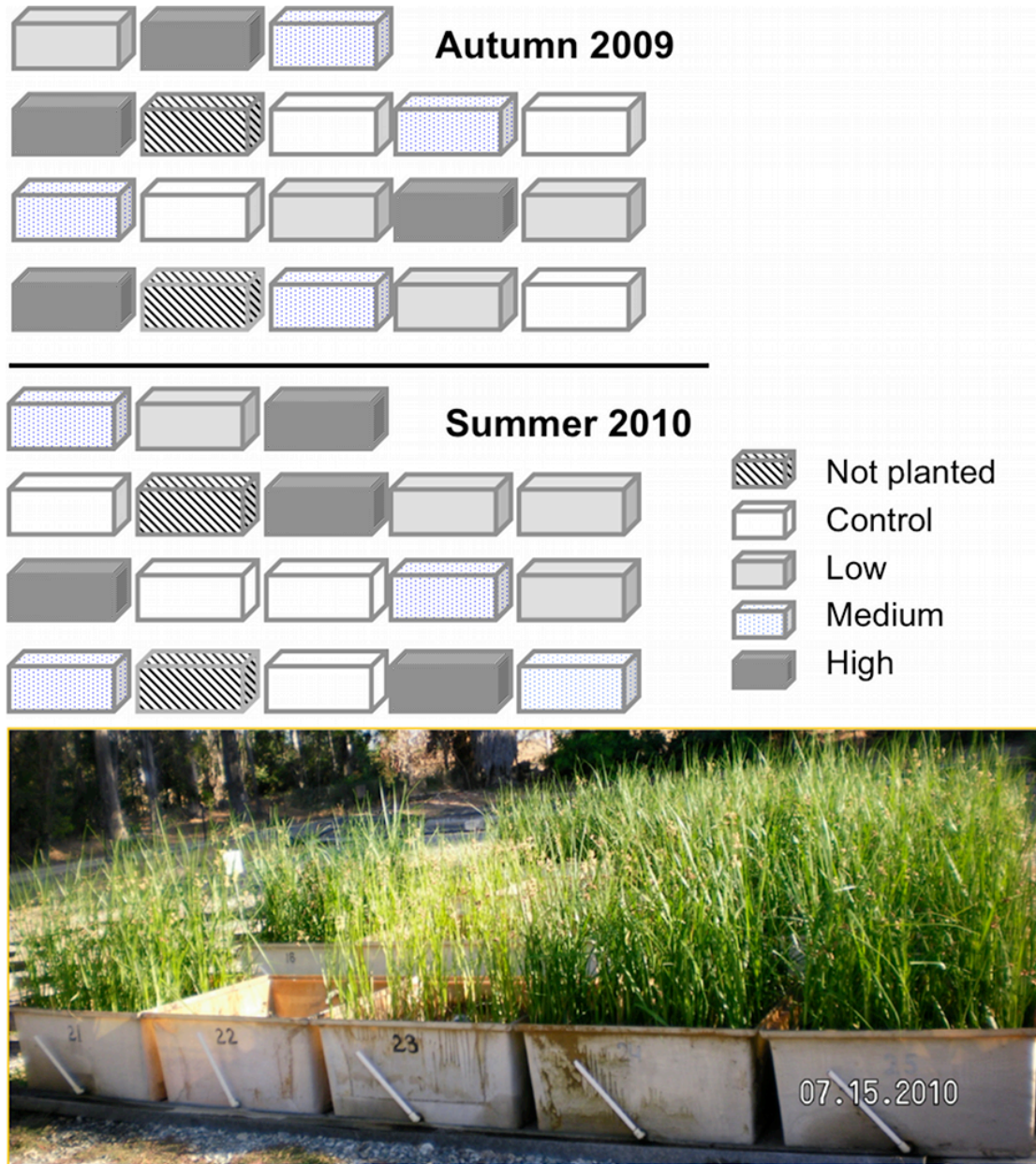Figure 2.1

Autumn 2009

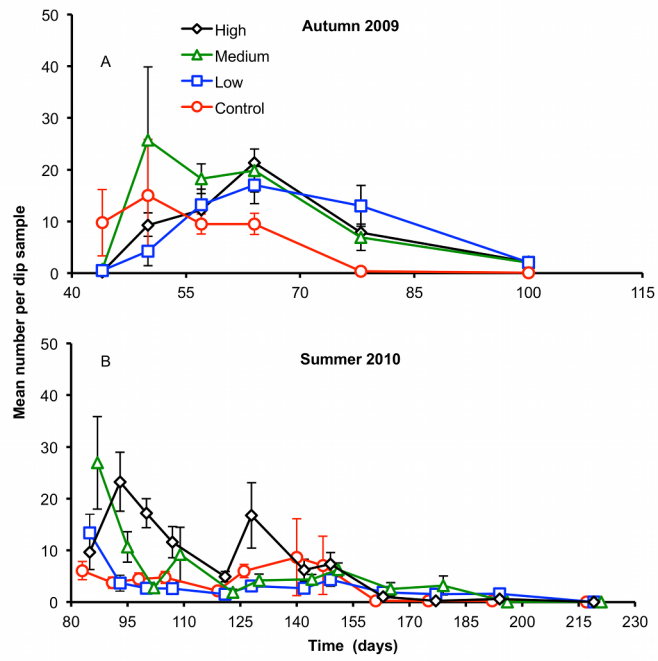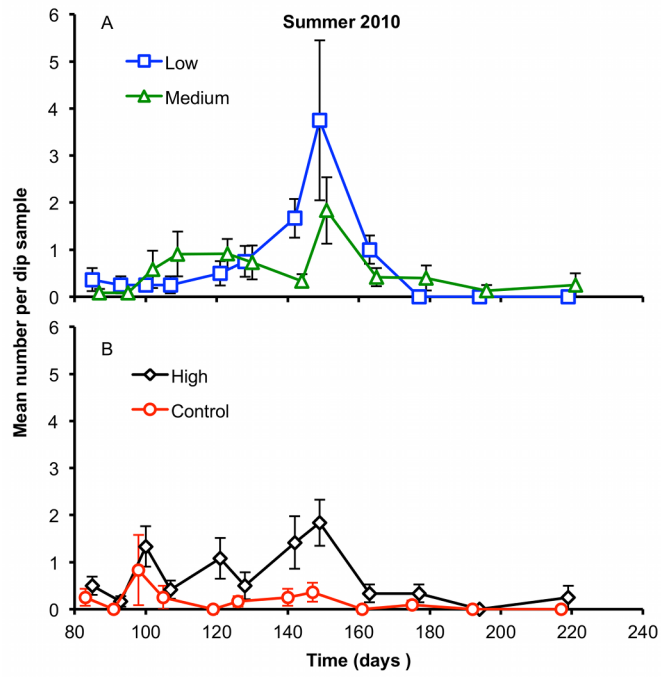Summer 2010

Not planted
Control
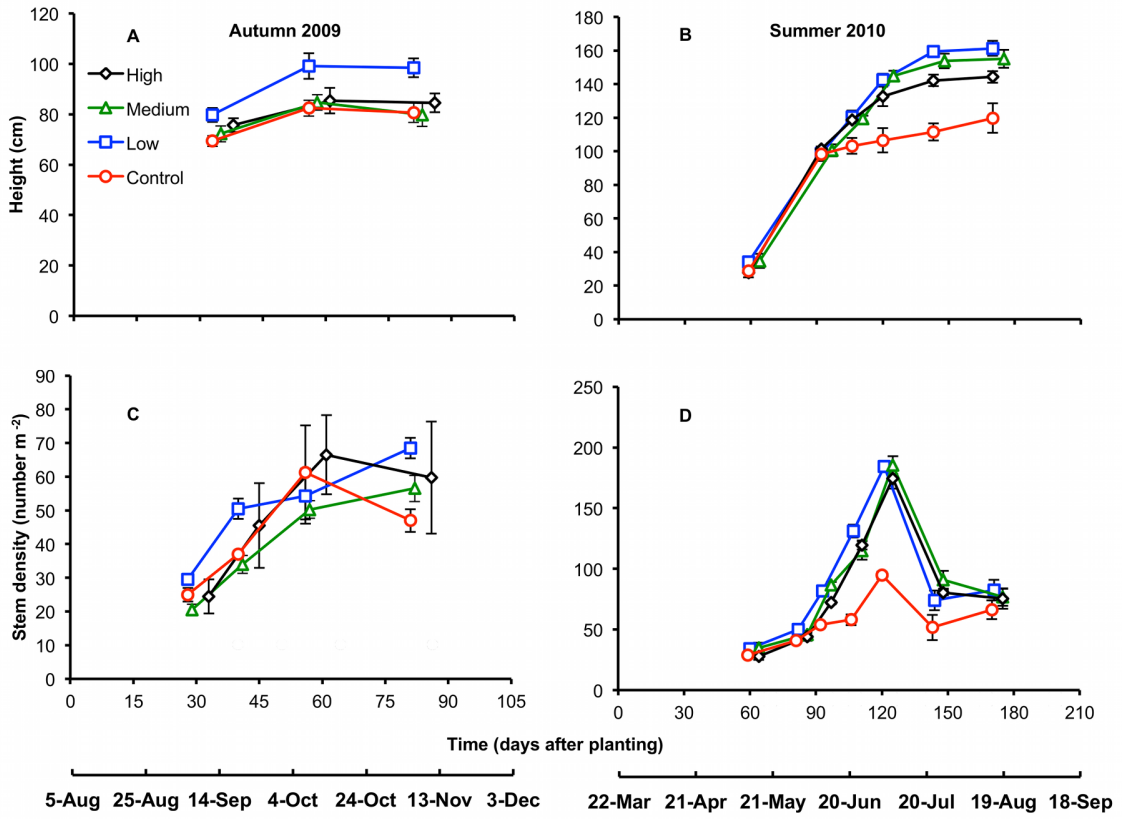Low
Medium
High

07.15.2010

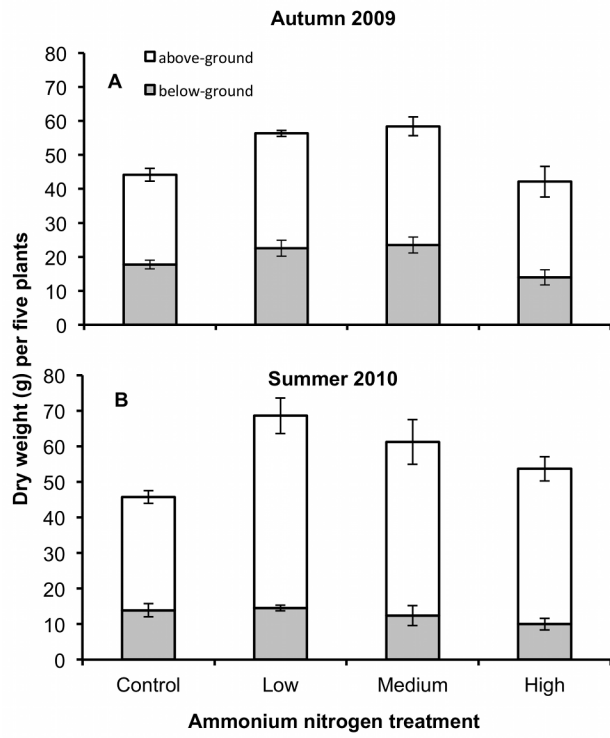54

Figure 2.2

Figure 2.3

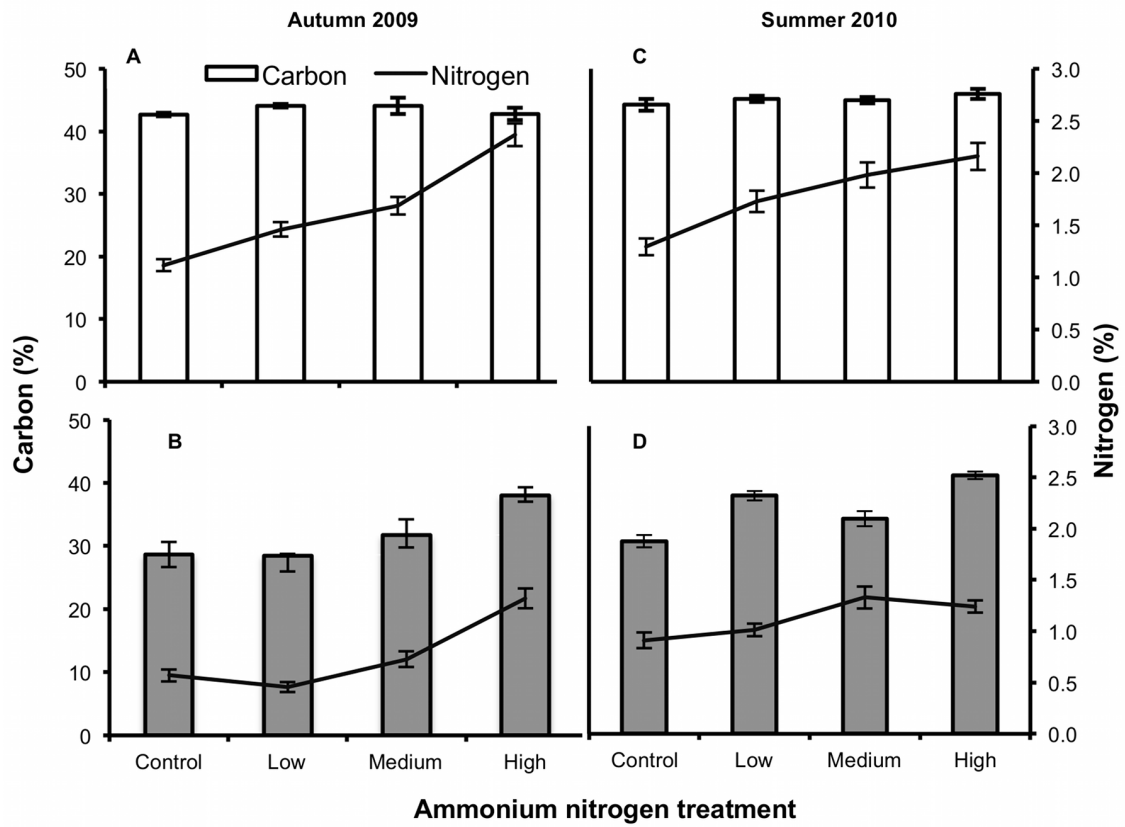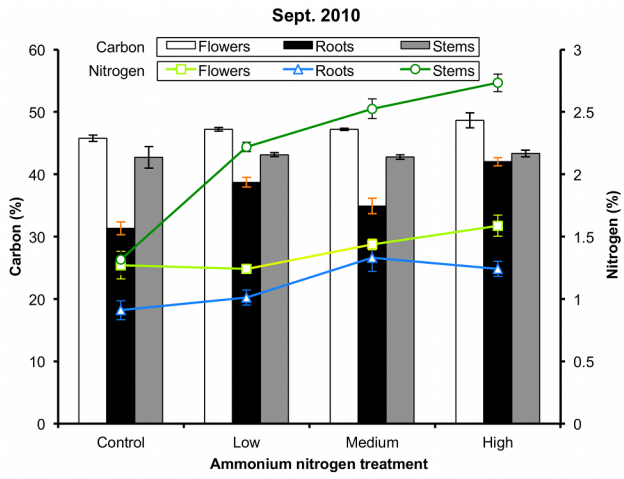Figure 2.4

Figure 2.5

Figure 2.6

Figure 2.7

Chapter 3: Bacterial Communities Associated with *Culex* Mosquito Larvae

and Two Emergent Aquatic Plants of Bioremediation Importance

Abstract. Microbes are important for mosquito nutrition, growth, reproduction and control. In this study, we examined bacterial communities associated with larval mosquitoes and their habitats. Specifically, we characterized bacterial communities associated with late larval instars of the western encephalitis mosquito (*Culex tarsalis*), the submerged portions of two emergent macrophytes (California bulrush, *Schoenoplectus californicus* and alkali bulrush, *Schoenoplectus maritimus*), and the associated water columns to investigate potential differential use of resources by mosquitoes in different wetland habitats. Using next-generation sequence data from 16S rRNA gene hypervariable regions, the alpha diversity of mosquito gut microbial communities did not differ between pond mesocosms containing distinct monotypic plants. Proteobacteria, dominated by the genus *Thorsellia* (*Enterobacteriaceae*), was the most abundant phylum recovered from *C. tarsalis* larvae. Approximately 49% of bacterial OTUs found in larval mosquitoes were identical to OTUs recovered from the water column and submerged portions of the two bulrushes. Plant and water samples were similar to one another, both being dominated by Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria and Verrucomicrobia phyla. Overall, the bacterial communities within *C. tarsalis* larvae were conserved and did not change across sampling dates and between two distinct plant habitats. Although *Thorsellia* spp. dominated mosquito gut communities, overlap of mosquito gut, plant and water-column OTUs likely reveal the effects of larval

feeding. Future research will investigate the role of the key indicator groups of *Bacteria* across the different developmental stages of this mosquito species.

INTRODUCTION

Recent studies have focused on understanding the role of microorganisms in mosquito biology and ecology, with the prospect of designing effective control strategies for species that vector debilitating agents (Ponnusamy et al. 2008, Ricci et al. 2011, Wang et al. 2011). Among these microorganisms, *Bacteria* play an important role, not only as major components of larval diet (Laird 1988, Merritt et al. 1992), but also in generating volatiles that attract mosquitoes for oviposition (Ponnusmay et al. 2008, 2010]. *Bacteria* are thought to provide valuable nutrition for the growth of mosquito larvae, but this is likely dependent on the particular species of *Bacteria* present in the larval habitat and ingested by larvae throughout their development (Laird 1988, Kaufman et al. 2000). A complex microbial consortium is considered fundamental for normal survival and complete development of mosquito larvae to adults (Merritt et al. 1992, Kaufman et al. 2000, Ponnusmay et al. 2008).

Larval *Culex* mosquitoes are generally considered filter-feeders and consume *Bacteria* and many other microorganisms in the water column (Merritt et al. 1992). Studies have shown that the microbial communities isolated from the mid-gut of laboratory-reared fourth instar *Culex tarsalis* Coquillett (a vector of western encephalitis and West Nile viruses) using conventional culturing techniques

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included several species including *Lactobacillus*, *Micrococcus* sp., *Micrococcus candidus*, *Saccharomyces*, *Proteus rettgeri*, *Geotrichum*, *Pseudomonas*, and other unidentified Gram-negative bacteria (Chao and Wistreich 1959, Wistreich and Chao 1960). Among these, *Micrococcus* sp. (Actinobacteria), *Lactobacillus* (Firmicutes: *Bacilli*) and *Pseudomonas* (*Gammaproteobacteria*) associated most frequently with *C. tarsalis* guts (Chao and Wistreich 1959, Wistreich and Chao 1960). Most genera of *Bacteria* found in the gut of larval *C. tarsalis* were also found in the adults, with the exception of *Aerobacter*, *Escherichia*, and *Flavobacterium* (Chao and Wistreich 1959, Wistreich and Chao 1960). However, these studies were based on laboratory-reared mosquitoes, and therefore it is difficult to extrapolate the bacterial composition of the gut of this mosquito species in the natural habitat. *Bacteria* found in the water column can be free-living, single cells, but also occur in clumps, and attached to sediment particles or submerged parts of aquatic plants (Velji and Albright 1993). In our own work, we have often observed the aggregation of *Culex* larvae at the surfaces of aquatic plants and on the sides of mesocosms during active mosquito production seasons. Others have shown biofilms to be important food resources for the larvae of other mosquito genera e.g., *Aedes* (Kaufman et al. 1999, 2001). However, the extent to which *Culex* species feed on the biofilm attached to these substrates is unknown.

Emergent macrophytes provide attachment sites, carbon and oxygen for microorganisms, and are purposely planted in treatment wetlands to facilitate the

remediation of wastewater (Brix 1997, Kadlec and Wallace 2009). In addition,

macrophytes produce high amounts of organic matter (Kröger et al. 2007, Kadlec

and Wallace 2009), thereby increasing wetland food resources for mosquito

larvae (Walton 2003, Walton and Jiannino 2005). Large macrophytes (height > 3

m) such as bulrushes, cattail and common reed, are used extensively in

constructed treatment wetlands in California and elsewhere in North America.

However, dense stands of large emergent macrophytes such as California

bulrush (*Schoenoplectus californicus* [C.A. Mey.] Palla) also enhance mosquito

oviposition (Orr 1991) and reduce the effectiveness of mosquito control

strategies by forming a physical barrier to "mosquitocides" and providing shelter

from predators such as fish (Walton 2003).

Therefore, macrophytes that interfere less with conventional mosquito control

tactics may be preferable to the large macrophytes planted in constructed

treatment wetlands. *Schoenoplectus maritimus* L. ("alkali bulrush" or

"cosmopolitan bulrush") has a short growth habit (height: 0.5 -1.5 m) and, in most

habitats, the above-ground biomass dies off annually in winter (Kantrud 1996).

Schoenoplectus maritimus reproduces primarily by a matrix of rhizomes that

provides surface area for beneficial *Bacteria* used in wastewater treatment, while

removing fecal pathogens such as *Escherichia coli* from the water column

(Kantrud 1996). In addition to supporting larger populations of predaceous

insects, the annual phenology, morphology and growth patterns of *S. maritimus*

are predicted to interfere less with integrated mosquito management strategies than do large emergent macrophytes.

Macrophytes interact with aquatic microorganisms and zooplankton in various ways. Some macrophytes release secondary plant compounds, such as phenols and alkaloids, which affect bacterial communities (Kröger et al. 2007, Borchardt et al. 2008, Hempel et al. 2008, Sérandour et al. 2008, Berg and Smalla 2009, Hansen et al. 2011) These properties are likely to influence the pollutant removal efficiency of treatment wetlands and, more importantly, the growth of mosquitoes and beneficial invertebrates. Some macrophytes are known to have antimicrobial and zooplankton-repellent properties (Borchardt et al. 2008). For instance, root exudates from *Schoenoplectus lactularis*, *Phragmites communis* and *Juncus maritimus* have been reported to alter bacterial composition in wetlands (Borchardt et al. 2008). Furthermore, macrophytes are thought to provide different structural complexes that affect macroinvertebrate composition and abundance (Orr 1991, Hansen et al. 2011). Larval *Anopheles* abundance differed among three structurally different emergent macrophytes in northern California (Orr 1991). However, many of these studies failed to address the underlying cause of these variations in invertebrate production.

In this study, we addressed the following objectives: 1) characterize bacterial communities within the larvae of the western encephalitis mosquito sampled from semi-natural habitats; 2) characterize the bacterial communities found in the water column from which the mosquitoes were sampled, and, 3) detect evidence

for larval grazing by comparing the epiphytic bacterial composition of two aquatic plants of phytoremediation importance to larval mosquito gut communities.

MATERIALS AND METHODS

Experimental mesocosms and sampling

Five young bulrush seedlings were transplanted into each of eight simulated wetlands [fiberglass mesocosms; area = 1 m^2] containing 17 cm (depth) of soil mix (plaster sand mixed with peat moss) at the University of California Riverside Aquatic Research Facility on 21 April 2011 (i.e., four replicate alkali bulrush mesocosms and four replicate California bulrush mesocosms). Water was supplied from an irrigation reservoir and water depth was maintained at 17 cm using float valves. The plants received an ambient ammonium nitrogen level of approximately 0.2 mg/L. Microbiota associated with plants, water, and mosquitoes were sampled monthly from September through November. Mosquito and predator abundance was estimated using three 350 mL "standard dip" samples, taken diagonally across each mesocosm every two weeks in September and October, and once in November, after which the mosquito population declined.

Water temperature was recorded at 0.5 h intervals throughout the study using a water temperature data logger (HOBO Water Temp Pro V1, Onset Computer Co.). The average monthly temperatures of the water were 24.2°C, 19.4°C and 12.8°C, in September, October, and November, respectively.

Bulrush Leaves

Five leaf disks, each 15 mm diameter (oven-dry weight was ~ 0.05 g), were collected monthly from submerged leaves of alkali bulrush and California bulrush in each of the experimental mesocosms, using a cork borer on sterile Petri plates. The disks were placed in sterile 15-mL centrifuge tubes with sterile water. The "rinsates" (detached biofilm) from the Petri plates were also added to the tubes. The cork borer was rinsed with ethanol and flamed between samples. Samples were transported to the laboratory in a cooler on ice. The tubes immediately were sonicated in ice water using an ultrasonic cleaner (Branson 1510) for 10 min to detach bacterial biofilm from the plant surfaces as described previously (Pelz-Stelinski et al. 2011). After sonication, plant material was removed from the tubes and oven-dried for weight measurement. The suspension left in the tube was centrifuged at 2900 x g in an Allegra 25 centrifuge (Beckman Coulter) at 4°C for 30 min. The majority of the supernatant was then discarded, and the pellet was resuspended in approximately 3 mL of water.

Water Column

Two water samples from each mesocosm were collected in sterile 50-mL centrifuge tubes on each of the three sampling days and transported to the laboratory on ice. The samples were then centrifuged at 2900 x g for 30 min at

4°C using an Allegra 25 centrifuge. The majority of the supernatant was again

discarded and the pellet resuspended in approximately 3 mL of water.

Mosquito Larvae

Late (third and fourth) instars of *C. tarsalis* were sampled from each

mesocosm using 350-mL standard dip samples. Five larvae were selected and

euthanized immediately in 95% ethanol in sterile 15 mL centrifuge tubes.

Samples were transported on ice to the laboratory. The larvae in the tubes were

sonicated in an ultrasonic cleaner bath for 2 min at room temperature to detach

any *Bacteria* and biofilm from the exoskeleton of the mosquito larvae. The larvae

were then rinsed with phosphate buffered saline (PBS) and allowed to air-dry

before DNA extraction.

DNA Extraction from Leaves and Water Samples

Two replicate 0.75-mL volumes from each mesocosm were transferred to

individual 2-mL microcentrifuge tubes. Biological material was then pelleted by

centrifugation at 9300 x g for 10 min, followed by removal of the supernatant and

resuspension in 0.5 mL of nuclease-free water. DNA extraction was carried out

with the Ultraclean Soil DNA kit (MoBio Laboratories, Inc. Carlsbad, CA, USA)

with some modification to the standard protocol (Pelz-Stelinski et al. 2011).

Specifically, after suspension in nuclease-free water, samples were poured into

the 2-mL bead solution provided by the manufacturer. Samples were

homogenized using a vortex with a vortex adapter (Va12G20-24; MoBio

Laboratories, Inc. Carlsbad, CA, USA) for 10 min at maximum speed, after which

425-µL of the homogenate was transferred to a new 2-mL tube.

DNA Extraction from Mosquitoes

The DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) was used to extract

DNA from a pool of three mosquito larvae per sample following the

manufacturer's protocol with a single final elution in 200 µL of Buffer AE.

Polymerase Chain Reaction (PCR)

Following DNA extraction, the hypervariable region 3 (V3) of the 16S rRNA

gene was amplified using bacterial primers 341F (5'-CCTACGGGAGGCAGCAG-

3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993). For each

sample, two replicate 25 µL reactions were conducted, each containing 1X HF

Buffer, 200 µM of each dNTP, 1 µM of each primer, 0.5 U of Phusion DNA

polymerase (New England Biolabs, Ipswich, MA, USA) and 2 µL of DNA template

(concentration 1.4-14.2 ng/µL). Reactions were run on a MasterCycler Gradient

5331 thermocycler (Eppendorf, Hamburg, Germany) with amplification cycle

conditions as described previously (Bartram et al. 2011). Products from the two

replicate amplifications were pooled and 20 µL of the combined PCR product

was electrophoresed on 1.5% (wt/vol) agarose gel. PCR products of the

expected size (170-190 bp) were excised from the gel and cleaned using the

High Pure PCR Product Purification Kit (Roche Applied Sciences). The cleaned

PCR product was then quantified using an ND-1000 spectrophotometer

(NanoDrop Technologies, USA) and kept at -20°C for library preparation.

Illumina Library Preparation and Sequencing

Illumina libraries were generated for each sample using NEXTflex DNA

sequencing kits (and protocols) and an identifying NEXTflex DNA barcode with 6-

base indices (Bioo Scientific, Inc., Austin, TX). Library quality was checked using

the 2100 Bioanalyzer (Agilent Technologies, Palo, Alto, CA, USA) and the molar

concentration of each library (n=60) was normalized to 7 nM with 10 mM Tris-HCl

(pH 8.5). The resulting libraries were pooled, in equimolar quantities, to create

three multiplexed libraries and subjected to 101-base paired-end sequencing on

a HiSeq 2000 (Illumina, Inc. San Diego, CA) at the Institute for Integrative

Genome Biology, Core Instrument Facility, University of California Riverside.

Sequence Analysis, Alignment and Taxonomy Assignment

Analysis of the sequence reads was carried out using (Quantitative Insights

Into Microbial Ecology) QIIME (Caporaso et al. 2010) version 1.4.0 and AXIOME

(Lynch et al. 2013) version 1.6.0 pipelines. Non-small subunit and 18S rRNA

gene sequences were excluded by aligning all OTUs against a small subunit

model using ssu-align (Nawrocki 2009). Chloroplast OTUs were excluded with

Metaxa (Bengtsson et al. 2011). Clustering of identical (0.97 similarity)

sequences to operational taxonomic units (OTUs) was carried out using CD-hit-est (multi-threaded version) (Li and Godzik 2006). Taxonomy assignment was conducted using the Ribosomal Database Project (RDP-II) (Cole et al. 2007) via QIIME parallel assign_taxonomy_rdp_py script with a confidence level of 0.8. All sequences and associated sample metadata were submitted to MG-RAST (Metagenome Rapid Annotation Using Subsystems Technology) (Meyer et al. 2008) with accession number 4984.

Statistical analysis

Alpha diversity indices were estimated using QIIME v1.4.0 and the Phylogenetic Diversity (PD) Whole Tree method for bacterial communities sampled from mosquitoes and each of the two aquatic plants. For analyses which compare between samples, all samples were rarefied down to the smallest library size (9,104 sequences). Differences between OTUs of bacterial communities from three bacterial DNA sources (mosquito larvae, water and plants) were assessed by principal coordinate analysis (PCoA) using an R script (Lynch et al. 2013, Oksanen et al. 2012, Paradis et al. 2004). Beta diversity measures based on both Bray-Curtis dissimilarities of sample OTU profiles and UniFrac distances assessed differences between samples from each of the DNA sources (mosquito, plant and water) and sampling dates. Indicator species analysis was carried out using the R package (Roberts 2010) to determine OTUs that were significantly associated with each of the three sampled habitats. Venn

diagrams were also generated to demonstrate OTUs common to both mosquitoes and plant/water habitats. Repeated-measures ANOVA on mosquito and predator abundance, data summary and tables of the bacteria sequences were generated using JMP version 10 (SAS 2010]. Numbers of mosquito larvae and odonate naiads were transformed by $\log_{10}(x+1)$ prior to analysis.

RESULTS

Mosquito and Predator abundance

Significantly greater (>2-fold) numbers of mosquitoes were observed in mesocosms planted with California bulrush than alkali bulrush ($F_{1, 17}$= 8.24, $p <$ 0.01; Figure 3.1A). In contrast, mesocosms planted with alkali bulrush produced significantly greater numbers of invertebrate predators (predominantly damselflies, Zygoptera) than did mesocosms containing California bulrush ($F_{1, 22}$= 36.8, $p <$ 0.001; Figure 3.1B).

Data Summary of HiSeq2000 Illumina Sequences

We generated a total of 135,838,727 sequences from 60 samples. A large proportion of OTUs identical to eukaryotic 18S rRNA genes (7.0%) and plant chloroplast 16S rRNA genes (1.7%), and all OTUs that did not align to any small subunit model (84.2%) were discarded. A total of 12,177,876 sequences aligned to the bacterial small subunit models and were used in the analysis, which

resulted in a total of 123,814 bacterial OTUs. The number of contributed

sequences ranged between 9,104-1,336,522 reads per sample.

Bacteria Diversity and Associations

Alpha diversity based on the PD_Whole tree method revealed that bacterial

communities from mosquito samples were significantly less diverse than

communities derived from leaf and water samples (Figure 3.2). There was no

significant difference between bacterial communities derived from water column

or plant leaves (Figure 3.2).

Beta-diversity measures using principal coordinate analysis (PCoA) plots of

Bray-Curtis OTU profile distances showed that bacterial communities from the

two bulrush species did not differ significantly (Figure 3.3). Axis 1 of the Bray-

Curtis PCoA represented 24.2% of the total variation of the bacterial communities

derived from mosquitoes and the two (water and leaf) habitat components.

Clustering of samples based on bacterial communities of water and on leaves

were primarily influenced by Cyanobacteria (OTU #243), Actinobacteria

(*Actinobacteriadae*: *Microbacteriaceae* OTU#969), Bacteroidetes (OTU #1002)

and Proteobacteria (*Beta-* and *Alphaproteobacteria*; Figure 3.4). Among the

Betaproteobacteria subdivision, a member of *Comamondaceae* (OTU #23) and

an *Incertae sedis* 5 (*Burkholderiales*; OTU #30) dominated the water and leaf

samples. From the *Alphaproteobacteria*, *Bosea* (OTU #10) dominated the water

and leaf samples. Similar to the Bray-Curtis based plot (Figure 3.3), bacterial

communities of mosquito larvae were clustered distinctly from those derived from water and leaves based on weighted UniFrac distances (Figure 3.4).

The sequences of 16S rRNA genes of *Bacteria* from both the mosquito gut and shared plant and water habitats showed that a relatively high proportion (49%) of bacterial OTUs in the habitat was found in the guts (Figure 3.5). Mosquito larvae shared 42% (816 bacterial OTUs) of the bacterial communities with the water column and bulrush leaves (Figure 3.5) and these shared OTUs accounted for 99% of all sequences recovered from the larvae.

Bacterial Communities Associated with the Gut of *C. tarsalis* Larvae

The thirteen larval mosquito samples yielded a total of 1,498,438 bacterial 16S rRNA gene sequences that were assigned to 12,640 OTUs and analyzed to describe the bacterial community of *C. tarsalis* larval guts. Of these, 9,514 bacterial OTUs were unique to mosquitoes and were not recovered from other plant and water samples (Figure 3.5). Among these mosquito-specific OTUs, 22% (2,669 OTUs) were unclassified singletons. Overall, the bacterial communities recovered from the larval guts were classified into 20 phyla and these accounted for12% of the total sequences (Table 3.1).

Proteobacteria dominated the guts of mosquito larvae (Table 3.1) with three abundant subdivisions: *Gammaproteobacteria* (57%), *Betaproteobacteria* (24%) and *Alphaproteobacteria* (13%). Other members of the Proteobacteria accounted for the remaining 6%. Of the *Gammaproteobacteria,* the family

Enterobacteriaceae accounted for about 51% of all the larval sequences. The next most common family, *Burkholderiales incertae sedis* 5 (*Betaproteobacteria*), accounted for 9% of all the sequences identified to the family level (Figure 3.6). Overall, 108 families of *Bacteria* were recovered from the guts of *C. tarsalis* larvae. Firmicutes, the second most abundant phylum, was dominated by *Clostridiales* (79%).

A total of 738 OTUs of *Thorsellia* (*Gammaproteobacteria*: *Enterobacteriaceae*) were recovered from larval guts, with just three OTUs accounting for 99.5% of *Thorsellia* sequences. Moreover, among the most abundant OTUs (>100 sequences) that were classified to the genus level, 64% were identified to the genus *Thorsellia* (Table 3.2). Overall, ~250 genera were affiliated with the gut profiles of *C. tarsalis*. Amongst our samples, 7 genera were unique to the mosquito gut and the remaining 244 genera were also recovered from the environment (water and leaves). Overall, the larval mosquito gut maintained a fairly stable microbial community regardless of the differences in sampling date and habitats (Figure 3.4).

Dominant Bacterial Communities Associated with Habitats

A total of 22 bacterial phyla were found in water samples whereas 23 phyla were recovered from the bulrushes. A small number of Planctomycetes was detected in the plant samples, but not found in the water samples. Proteobacteria, Bacteroidetes, Actinobacteria and Cyanobacteria were the most

abundant phyla found in the water column and on the submerged leaves of alkali

and California bulrushes (Table 3.5). These four phyla accounted for 84% of all

sequences from water column and unclassified bacterial OTUs accounted nearly

15% of water column sequences (Table 3.5). Plant and water samples were

specifically clustered with *Comamonadaceae*, *Microbacteriaceae*, *Bosea*,

Burkholderiales and *Cyanobacteria* species (Figure 3.4).

Indicator Species

Analysis of indicator species (Dufrêne and Legendre 1997) of bacterial OTUs

from mosquito (Table 3.3) and plant/water (Table 3.4) samples revealed OTUs

that strongly associated with one group over the others. The indicator species

concept has widely been applied in vegetation ecology studies over the last two

decades (Dufrêne and Legendre 1997) to typify habitats or groups by taking into

account the abundance and frequency of a species that occur in these habitats

or groups. A maximum indicator species value of 1.0 represents the species

occurrences in all samples of a treatment group (fidelity) and only in samples

from that treatment group (specificity); lower indicator values indicate that OTUs

are not good predictors of treatment groups or habitats (Dufrêne and Legendre

1997). There were 99 bacterial OTUs identified as indicator species from

mosquito larvae ($p < 0.05$), of which 81 were classified to taxa below phyla level.

The remaining 18 indicator OTUs were unclassified. Of the identified indicator

OTUs, the genus *Thorsellia* (19 OTUs) was the dominant predictor of the

Bacteria community from *Culex* larvae. Members of Proteobacteria (71%, by sequence abundance), Firmicutes (20%), Bacteroidetes (3%) were also among the dominant predictors of bacterial communities from the gut of *Culex* larvae. In addition, among the OTUs that had indicator values greater than 0.8 (31 OTUs), 63% of these bacterial OTUs (by sequence abundance) were originated from mosquito larvae whereas water and the bulrushes had only 25% and 12%, respectively, suggesting that there was consistency within bacterial communities associated with mosquito larvae.

Members of the Actinobacteria and Proteobacteria phyla sequenced from water samples had the highest indicator values, whereas several members of Proteobacteria and Bacteroidetes were good predictors of the bacterial communities associated with bulrush leaves (Table 3.4). *Alphaproteobacteria* specifically dominated the bulrushes while *Betaproteobacteria* dominated the water column.

DISCUSSION

This study represents the first use of next-generation sequencing to explore the bacterial communities associated with the guts of mosquito larvae and associated habitats (water column and emergent macrophytes). Although it has been suggested that immature mosquitoes feed on microbial assemblages (Merritt et al. 1992), existing evidence supporting this hypothesis is mostly limited to characterization of microorganisms based on traditional cultivation,

morphology and Sanger sequencing techniques (Wistreich and Chao 1960, Veiji and Albright 1993, Kaufman et al. 1999, 2001). These methodologies undoubtedly under-sampled the diversity of microflora found in mosquito guts and the environment.

Although the majority of ingested microbes are likely to be quickly digested and/or passed through the gut, at the time of sampling, almost half (49%) of the bacterial OTUs (representing 99% of sequences) associated with plant and aquatic samples were found in the mosquito gut (Figure 3.5). *Culex* species are not known to be inherently selective feeders, but they could be, and further their gut might be selective and only provide suitable medium for the proliferation of a few *Bacteria*. Three OTUs of the most abundant genus, *Thorsellia,* were among those recovered both from larval guts, water and plant samples. All the phyla of *Bacteria* originated from mosquitoes were represented in the water column and on leaves.

Kaufman and colleagues compared the bacterial communities found in treeholes inhabited by mosquitoes, to treeholes that lacked mosquitoes and found differences in the bacterial communities of the two habitats (Kaufman et al 2001). However, no effort was made to eliminate mosquitoes from pond mesocosms and make a comparison of bacterial communities in the presence and absence of mosquitoes in our study. To circumvent this limitation, we created simulated wetlands planted with two bulrush species that influenced the abundance of mosquitoes and their predators (Figure 3.1). It is well known that

aquatic plants of different structural complexities have a differential effect on macroinvertebrate community assemblages (e.g., Hansen et al. 2011). Alkali and California bulrushes have different structural complexities that likely influence the presence and abundance of mosquitoes and their predators (odonate naiads). Alkali bulrush has a more structurally complex growth habit with significantly greater number (5.8 ± 0.23 SE) of leaves than California bulrush (2.2 ± 0.12 SE). Interestingly, the bacterial communities in the guts of larval mosquitoes from mesocosms planted with the two bulrushes species did not differ significantly between monotypic plots of the two bulrushes (Figure 3.3). Our results indicated that differences in the abundance of mosquitoes and mosquito-predators did not affect the diversity of the microbial community in the two bulrushes, contrary to the top-down predation model. However, we did not examine other bacterivores (e.g., protists), the presence or absence of which might have also affected bacterial community composition.

Similar to our findings, a previous study did not observe changes in bacterial communities in two species of syrphid flies that fed on different larval resources and concluded that the insect midgut might be selective for certain species of microbes (Martínez-Falcón et al. 2012). That study also found *Enterobacteriaceae* (different genera from what were found in our study) were the predominant colonizers of larval guts, suggesting that the gut of insects might be conducive for the proliferation of this group of *Bacteria*. Additional studies of dipteran larvae showed that the bacterial communities in *Drosophila* spp. and

Musca domestica were dominated by several members of *Enterobacteriaceae* (Chandler et al. 2011, Gupta et al. 2012).

This study identified for the first time that *Thorsellia* (Proteobacteria, *Enterobacteriaceae*) OTUs dominated the gut of *C. tarsalis* larvae collected from natural habitats (Figure 3.4). *Thorsellia anophelis* was first isolated from the midgut of adult *Anopheles arabiensis* mosquitoes (Lindh et al. 2005). It has been suggested that this dominant bacterium may be acquired from rice paddies via ingestion by the larvae, and transferred transstadially from larvae to adults (Rani et al. 2009). *Thorsellia* are Gram-negative, rod-shaped bacteria (Kämpfer et al. 2006) and have recently been suggested for manipulation of mosquitoes that transmit malaria parasites (Briones et al. 2008). Although the current taxonomical category using RDP and NBCI databases places *Thorsellia* in the *Enterobacteriaceae* family, the correct taxonomical assignment of this genus within *Gammaproteobacteria* is still unresolved (Kämpfer et al. 2006, Briones et al. 2008).

The *Thorsellia* genus has never been reported from mosquitoes outside *Anopheles* and this was the first report of their presence in *Culex* mosquitoes. Of all the *Thorsellia* sequences obtained in this study, 99.6% were from *C. tarsalis* larvae; leaf samples and water column contained only ~0.2% and ~0.3% of *Thorsellia* sequences, respectively. This suggests that the genus is a well-established mosquito gut inhabitant, although the habitat may still serve as a reservoir (Briones et al. 2008).

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It is currently unknown whether members of *Thorsellia* are obligate symbionts of mosquitoes but their close association with the gut of the mosquito might indicate that this genus is at least a commensal that can be acquired from the habitat reservoir and proliferates in the insect midgut. It is evident from our study and that of Briones et al. (2008) that this genus is likely free living because it was recovered from the environment. The fact that this genus is found in both *Culex* and *Anopheles* supports it as a potential candidate for manipulating disease vectors across genera. Midgut *Bacteria* have been known to prime the native immunity of *Anopheles* (Dong et al. 2009, Boissière et al. 2012) and whether the genus *Thorsellia* has the capacity to make mosquitoes refractory to parasites and could be used for the symbiotic control strategy in *Culex* genera has yet to be explored.

The bacterial communities dominating the water column included Actinobacteria, Bacteroidetes, Cyanobacteria, *Betaproteobacteria*, and other phyla typically found in freshwater habitats (Tanaka et al. 2009, Okafor 2011). The submerged plant leaves were dominantly colonized by *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, Cyanobacteria and *Sphingobacteria* also typically found on plant leaves (Sérandour et al. 2008, Tanaka et al. 2009). Similar to our findings, Tanaka and coworkers also found the family *Comamonadaceae* (*Betaproteobacteria*) to be the most abundant family predominating in pond water (Tanaka et al. 2009). However, the roots of common reeds, an aquatic plant commonly planted for bioremediation

importance, contained a separate group of *Betaproteobacteria* from the pond water indicative of the plant rhizosphere having an influence on the surrounding microbial communities (Berg and Smalla 2009, Tanaka et al. 2009). In our study, we did not find significant separation of the bacterial communities associated with submerged portion of plants from those found in the water column (Figure 3). We also did not characterize the microbial communities associated with the roots of the two bulrushes, which might be an important area of future study and significant in understanding their role in treatment wetlands (Tanaka et al. 2009). In this study, we also characterized for the first time the bacterial communities associated with the submerged portions of two bulrushes that are used in wastewater treatment processes.

Next-generation technologies, such as Illumina, provide unprecedented access to environmental bacterial communities, including those from the guts of disease vectoring insects (Bartram et al. 2011, Wang et al. 2011, Su et al. 2012). Future research will investigate the role of these dominant groups of *Bacteria* identified in this study across the developmental stages of *C. tarsalis.* Future studies will also investigate whether congeneric *Culex* species that share common niches also share similar gut microbiota*.*

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Table 2.1. Phylum-level classification of bacterial communities from *C. tarsalis* larvae.

Phylum	No. of OTUs	Relative abundance
Proteobacteria	6185	0.6568
Firmicutes	1908	0.2055
Unclassified bacteria	3614	0.0722
Bacteroidetes	479	0.0464
Cyanobacteria	196	0.0159
Actinobacteria	196	0.0028
Chloroflexi	6	0.0002
Others*	56	0.0002

* Other bacterial phyla include (Chlamydiae, Acidobacteria, TM7, Gemmatimonadetes, Spirochaetes, Fusobacteria, Nitrospira, Chlorobi, Verrucomicrobia, Deinococcus-Thermus, SR1, WS3, and OP10)

Table 3.2. Most abundant (> 100 sequences) genera of *Bacteria* found in *C. tarsalis larvae*.

Phylum	Class	Family	Genus	a	No. of OTUs
Bacteroidetes	*Bacteroidetes*	*Porphyromonadaceae*	*Dysgonomonas*	6.0	1
	Flavobacteria	*Cryomorphaceae*	*Fluviicola*	0.2	1
		Flavobacteriaceae	*Flavobacterium*	1.1	1
	Sphingobacteria	*Flexibacteraceae*	*Flectobacillus*	0.3	1
Firmicutes	*Clostridia*	*Lachnospiraceae*	*Lachnospiraceae Incertae sedis*	0.4	1
Proteobacteria	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Bosea*	1.1	2
		Rhizobiaceae	*Rhizobium*	0.2	1
		Rhodobacteraceae	*Pseudorhodobacter*	0.2	1
			Rhodobacter	9.1	2
			Rubrimonas	1.0	1
		Sphingomonadaceae	*Erythrobacter*	0.3	1
	Betaproteobacteria	*Comamonadaceae*	*Hydrogenophaga*	0.9	3
		Incertae sedis 5	*Leptothrix*	0.2	1
			Rubrivivax	0.5	1
		Oxalobacteraceae	*Duganella*	0.1	1
		Rhodocyclaceae	*Azonexus*	1.9	1
			Azovibrio	8.9	2
	Gammaproteobacteria	*Aeromonadaceae*	*Aeromonas*	2.6	1
		Halothiobacillaceae	*Thiovirga*	1.2	1
		Enterobacteriaceae	*Thorsellia*	63.6	6
		Pseudomonadaceae	*Pseudomonas*	0.2	4

[a] relative percentages (only percentages ≥ 0.1% are shown).

Table 3.3. Indicator species of bacterial OTUs from the guts of *Culex* mosquito cluster ($p < 0.05$).

OTU ID	No. of sequences	Consensus Lineage	Ind Val.[a]
3	303441	Proteobacteria; *Gammaproteobacteria*; *Enterobacteriales*; *Enterobacteriaceae; Thorsellia*	0.998
25	216058	Proteobacteria; *Gammaproteobacteria*; *Enterobacteriales*; *Enterobacteriaceae; Thorsellia*	0.998
272	52381	Proteobacteria; *Betaproteobacteria*; *Rhodocyclales*; *Rhodocyclaceae*; *Azovibrio*	0.99
4	80284	Proteobacteria; *Alphaproteobacteria*; *Rhodobacterales*; *Rhodobacteraceae; Rhodobacter*	0.98
528	4212	Proteobacteria	0.92
82	56986	Firmicutes; "*Clostridia*"; *Clostridiales*	0.92
5	49398	Bacteroidetes; *Bacteroidetes*; *Bacteroidales*; *Porphyromonadaceae*; *Dysgonomonas*	0.92
27	44364	Firmicutes	0.92
384	20555	Firmicutes; "*Clostridia*"; *Clostridiales*	0.92
30	127850	Proteobacteria; *Betaproteobacteria*; *Burkholderiales*; Incertae sedis 5	0.88
1284	431	Proteobacteria; *Gammaproteobacteria*; *Enterobacteriales*; *Enterobacteriaceae; Thorsellia*	0.85
31	44685	Firmicutes; "*Clostridia*"; *Clostridiales*; "*Ruminococcaceae*"	0.84
12	75489	Firmicutes; "*Clostridia*"; *Clostridiales*	0.84

[a] Only OTUs with indicator values > 0.8 and identified to phylum level and below are shown.

The entire indicator value table for all bacterial OTUs from this study included in the supplementary material.

Table 3.4. Top indicator species values for bacterial OTUs from water column and bulrush leaves ($p < 0.05$).

OTU ID	No. of sequences	Consensus Lineage	Cluster	Ind Val.[a]
10551	27680	Proteobacteria; *Alphaproteobacteria*; *Rhodospirillales*	plant	0.90
8931	4490	Proteobacteria; *Betaproteobacteria*; *Burkholderiales*	plant	0.89
3058	14532	Proteobacteria; *Alphaproteobacteria*; *Rhizobiales*; *Hyphomicrobiaceae; Devosia*	plant	0.89
10374	10421	Proteobacteria; *Betaproteobacteria*; *Burkholderiales*	plant	0.88
3595	30290	Proteobacteria; *Alphaproteobacteria*; *Rhizobiales*	plant	0.88
16356	4868	Proteobacteria; *Alphaproteobacteria*	plant	0.87
8991	39373	Bacteroidetes; *Flavobacteria*; *Flavobacteriales*; *Flavobacteriaceae; Mariniflexile*	plant	0.85
8986	4399	Bacteroidetes; *Sphingobacteria*; *Sphingobacteriales*	plant	0.84
1252	7869	Bacteroidetes	plant	0.84
10964	16015	Proteobacteria; *Betaproteobacteria*; *Rhodocyclales*; *Rhodocyclaceae; Zoogloea*	plant	0.83
9696	10452	Bacteroidetes; *Flavobacteria*; *Flavobacteriales*; *Flavobacteriaceae*	plant	0.83
22257	4776	Bacteroidetes; *Sphingobacteria*; *Sphingobacteriales*; *Flexibacteraceae; Runella*	plant	0.83
4395	3113	Bacteroidetes; *Sphingobacteria*; *Sphingobacteriales*; *Flexibacteraceae; Dyadobacter*	plant	0.82
2649	43461	Proteobacteria; *Alphaproteobacteria*; *Sphingomonadales*; *Sphingomonadaceae; Sphingobium*	plant	0.80
969	151929	Actinobacteria; *Actinobacteria*; *Actinobacteridae*; *Actinomycetales*; *Micrococcineae; Microbacteriaceae*	water	0.87
3802	67333	Proteobacteria; *Betaproteobacteria*; *Burkholderiales*	water	0.86
1002	229663	Bacteroidetes	water	0.82

[a] Only OTUs with indicator values > 0.8 and identified to phylum level and below are shown.

Table 3.5. Major phyla of bacterial OTUs associated with the habitat of *Culex*

mosquito larvae.

Phylum	Number of OTUs		Relative proportion	
	water	bulrush	water	bulrush
Acidobacteria	144	238	0.0030	0.0064
Actinobacteria	1910	993	0.0505	0.0095
Aquificae	1	1	<0.0001	<0.0001
BRC1	16	13	<0.0001	<0.0001
Bacteroidetes	4557	4750	0.1796	0.1426
Chlamydiae	32	31	0.0001	0.0002
Chlorobi	2	2	0.0001	<0.0001
Chloroflexi	58	76	0.0005	0.0007
Cyanobacteria	1793	2726	0.0624	0.0902
Deinococcus-Thermus	3	6	<0.0001	<0.0001
Firmicutes	926	912	0.0078	0.0043
Fusobacteria	8	8	0.0001	<0.0001
Gemmatimonadetes	35	38	0.0014	0.0033
Nitrospira	11	8	0.0001	<0.0001
Unclassified	18963	22643	0.1504	0.1540
OP10	8	16	0.0001	0.0004
Planctomycetes	-	1	-	<0.0001
Proteobacteria	30879	38346	0.5425	0.5862
SR1	7	5	0.0002	0.0002
Spirochaetes	43	49	0.0009	0.0010
TM7	16	31	0.0002	0.0009
Tenericutes	2	1	<0.0001	<0.0001
Verrucomicrobia	15	15	<0.0001	<0.0001
WS3	7	5	0.0002	0.0001

Figure Legends

Figure 3.1. Mosquito and invertebrate predator abundance. Repeated-measures analysis of variance showed that mean numbers of mosquitoes, Panel A and invertebrate predators (zygopteran predators), Panel B varied significantly ($P<$ 0.05) between mesocosms planted with alkali bulrush and California bulrush. Alkali bulrush significantly harbored more predators and fewer mosquitoes as compared to the California bulrush. The x-axis represented time after the onset of the experiment. Error bars represent the standard error of the mean based on four replicate mesocosms per plant species.

Figure 3.2. Alpha diversity measures. Alpha diversity measures based on PD_Whole tree of the bacterial communities from mosquito larvae, water column and leaves. Sequences from mosquito samples are significantly less diverse than sequences from water and plant samples. The x-axis for the phylogenetic diversity of *Bacteria* communities from leaf samples is offset by 100 sequences for better illustration.

Figure 3.3. Community similarity of OTU profiles representation. PCoA plots based on Bray-Curtis distances of OTU profiles mosquitoes, water and leaf samples from mesocosms containing the two bulrushes from the different sampling dates. Panel A shows points colored by DNA source. Panel B shows

points colored by the plant present in the mesocosm. Panel C shows points colored by sampling date.

Figure 3.4.Taxonomic profiling of mosquito-water-plant microbiome profiles. PCoA plots of weighted UniFrac distances of bacterial communities in mosquitoes, water and leaf samples from mesocosms containing the two bulrushes (alkali and California bulrushes) from the three sampling dates. Panel A shows the OTUs associated with that region on the plot, scaled based on sequence abundance. Panel B shows a PCoA plot based on three DNA sources (mosquitoes, water and plant leaves), Panel C recolors samples of Panel B to highlight two plant species, Panel D recolors the same samples based on the three sample dates.

Figure 3.5. Overlap of bacterial communities across habitats. Venn diagram illustrating overlapping of *Bacteria* OTUs and sequences between mosquito larvae and habitat (*C. tarsalis* larvae; habitat = leaves of alkali and California bulrushes; water = water column samples). The first number represents the number of OTUs, while the number in parentheses represents the number of sequences.

Figure 3.6. Family-level classification of bacterial communities in mosquitoes. Family-level classification of bacterial communities in *C. tarsalis* larvae and their relative proportions.
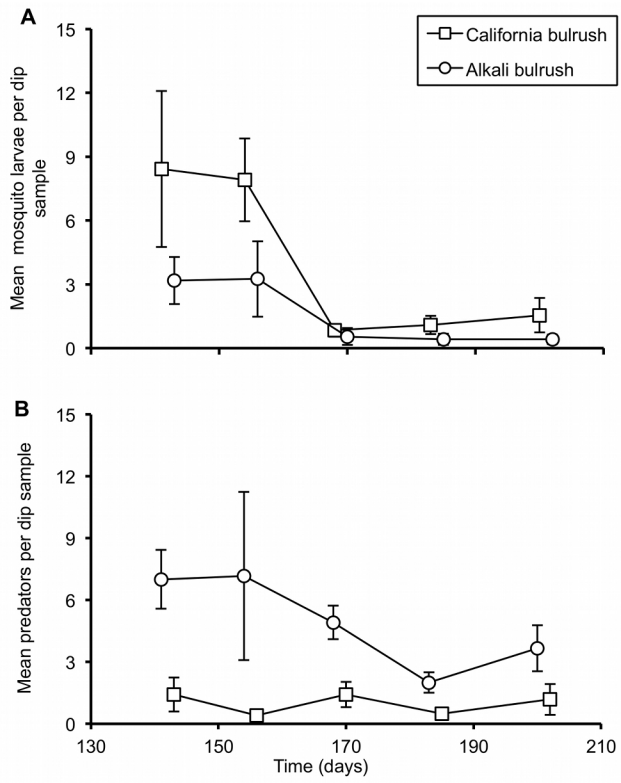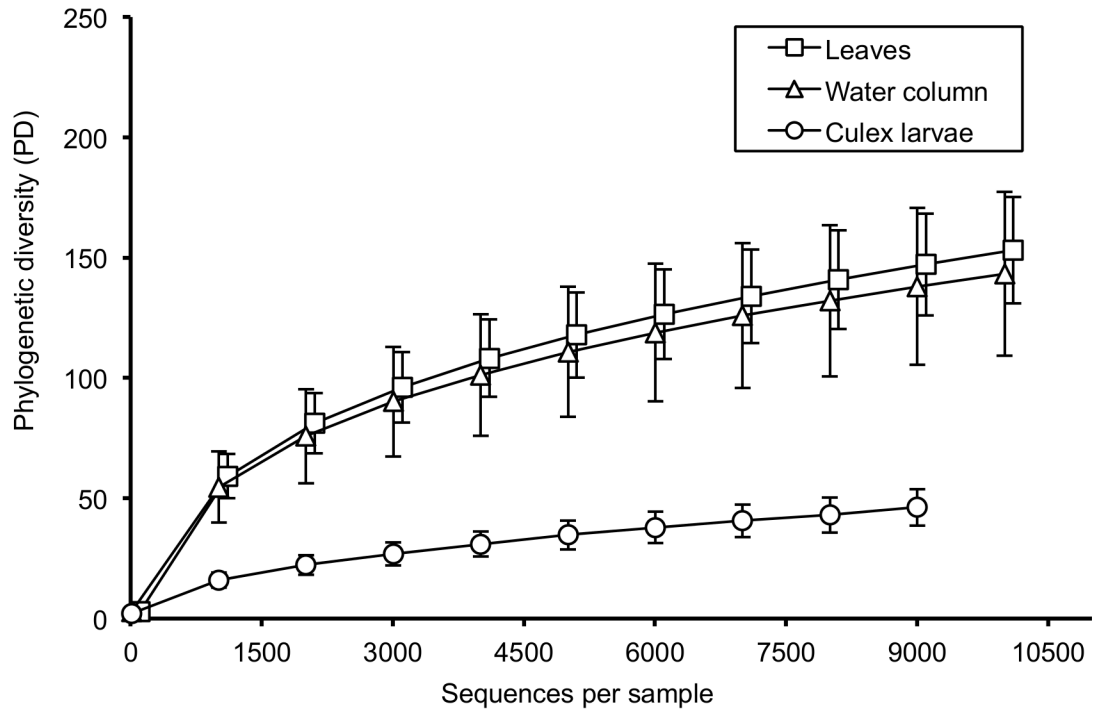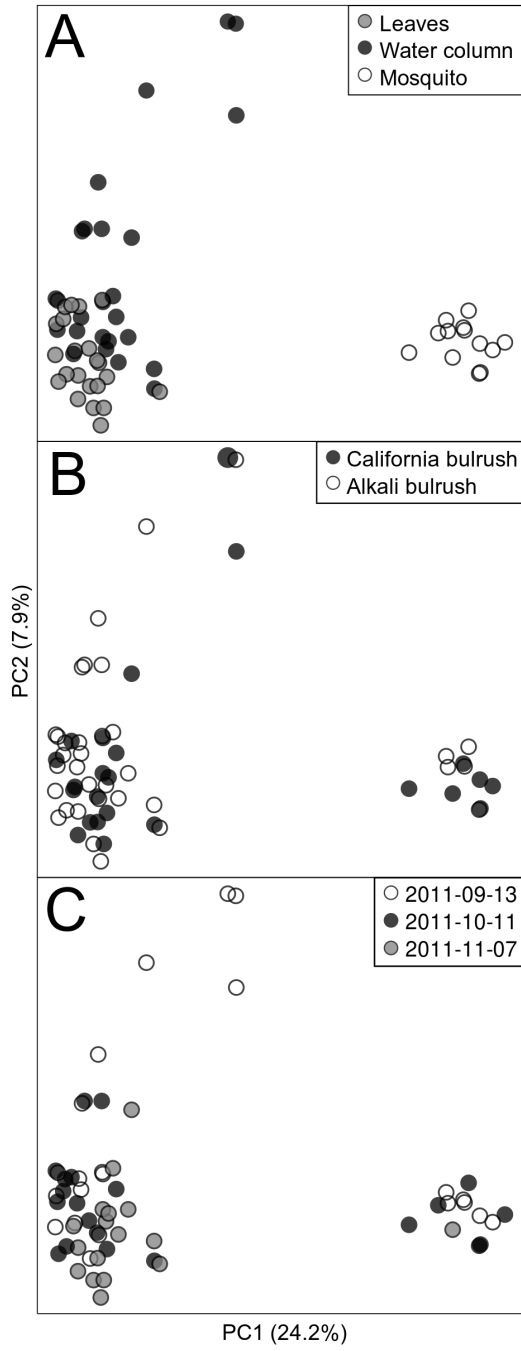
Figure 3.1
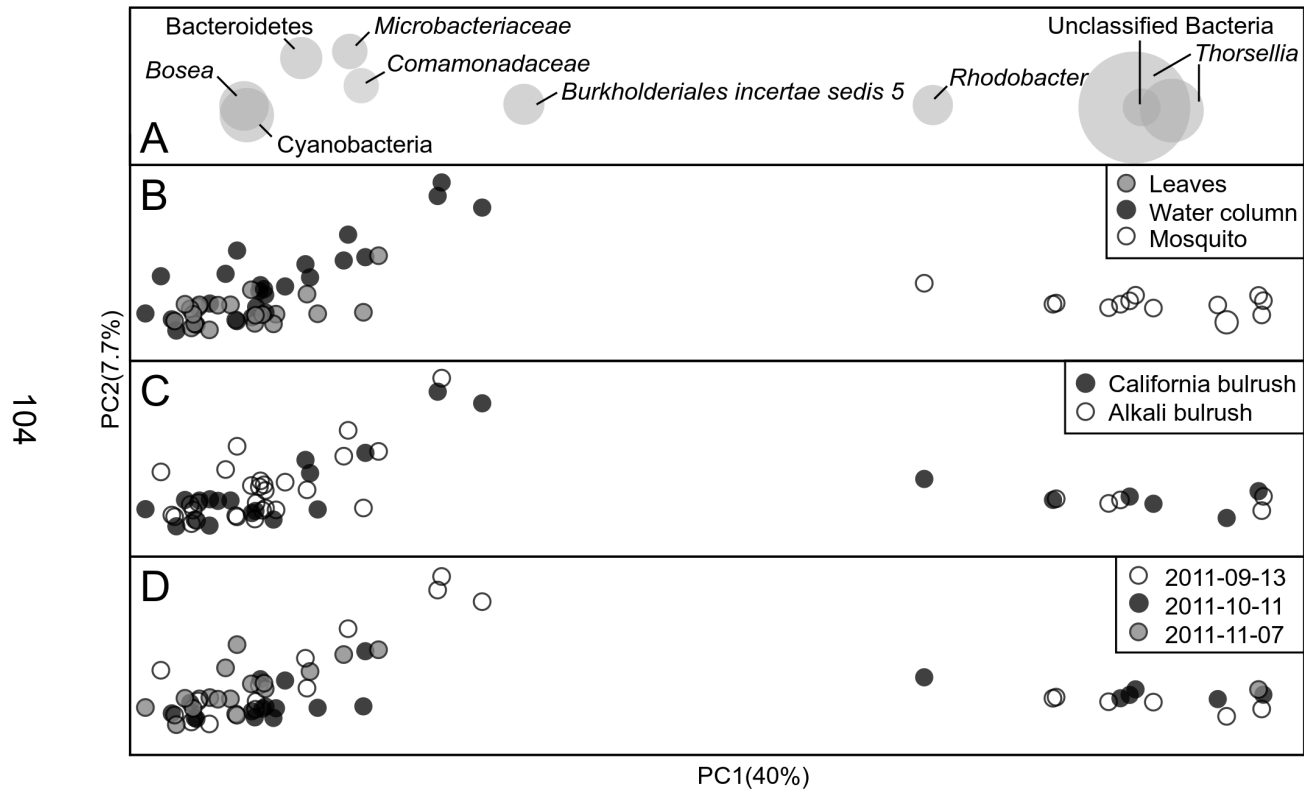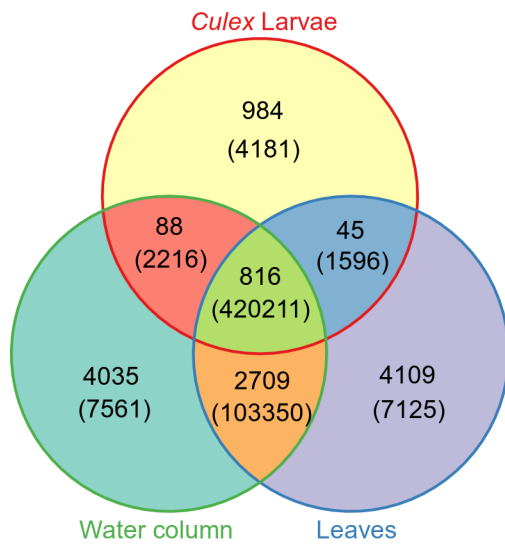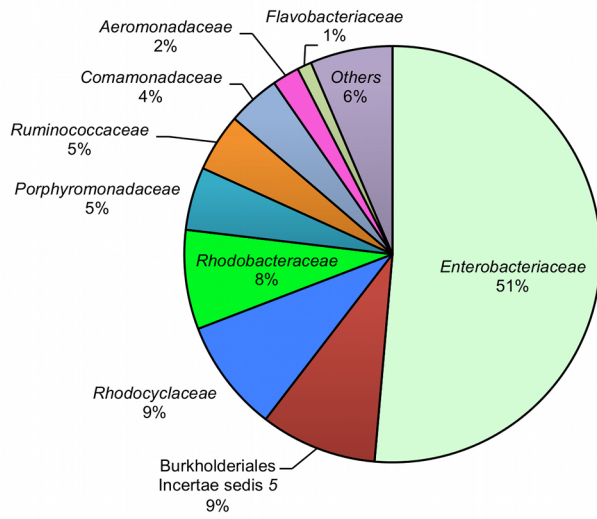
Figure 3.2

Figure 3.3

Figure 3.4

Figure 3.5

Figure 3.6

Chapter 4 Use of *Bacillus thuringiensis* subsp. *israelensis* for mosquito control alters the aquatic microbial community and nutrient dynamics

Abstract. *Bacillus thuringiensis* var. *israelensis* (*Bti*) is the most widely used

biopesticide against mosquitoes and blackflies with a history of high specificity

and efficacy. Interactions of *Bti* with native microfauna in the environment are

poorly known. We tested whether *Bacteria*, algae and physicochemical variables

in the mosquito larval habitats are unaffected by different rates of *Bti* application.

To test the null hypothesis, an application of either a high or a low rate of a *Bti*

(VectoBac G) treatments and an untreated control were assigned to 1 m^2

mesocosms in a completely randomized design under field conditions. The V3

region of the 16S rRNA genes of *Bacteria* samples taken from water column

were targeted and analyzed using next-generation sequencing technology to

compare *Bacteria* communities among treatments and across different sampling

dates. *Culex* mosquito abundance, phytoplankton, sestonic particulates, nutrients

and other water quality parameters in the water were assessed during a 78-day

field study in autumn 2012. Beta diversity analysis revealed that *Bacteria*

communities in the water column were significantly influenced by the high *Bti*

treatment and sampling date. *Bacteria* in the water column of the low *Bti*

treatment and untreated control mesocosms were dominated by Cyanobacteria,

Cytophagales and *Cyclobacteriaceae* (Bacteroidetes), *Sphingomonas*

(*Alphaproteobacteria*)*,* and *Polaromanas* (*Betaproteobacteria*). These taxa were

all suppressed in mesocosms subjected to the high *Bti* application rate. The high

Bti application rate significantly reduced mosquito abundance, phytoplankton

biomass (chlorophyll *a*), sestonic particulates, nutrients, pH and other

physicochemical variables in the water column. Important implications of this study for aquatic ecosystems could be significant.

Keywords: *Bti*, *Bacteria*, phytoplankton, *Culex*, water physicochemistry

INTRODUCTION

Bacillus thuringiensis subsp. *israelensis* (*Bti*) is the most commercially successful microbial control agent for disease-vectoring Diptera (mainly mosquitoes and black flies) (Lacey and Merritt 2003). The widespread use of *Bti* is due largely to its high specificity and effectiveness in targeting and controlling pestiferous mosquitoes and closely related nematocerans (Lacey and Merritt 2003).

The effects of *Bacillus thuringiensis* subsp. *israelensis* on nontarget invertebrates and vertebrates (fish, mammals and birds) have been intensively studied and no known detrimental effects have been found to date (Lacey and Merritt 2003). However, possible interactions of *Bti* with microbial communities, and on ecosystem-level processes associated with aquatic mosquito habitat, have largely been unexplored (Boisvert and Boisvert 2000; EFSA 2013).

A few studies indicated an indirect positive effect of *Bti* application on certain members of aquatic microbial communities. A 4.5-fold increase in abundance of protozoans (size>10 μm: Amoeba, Ciliophora, Zoomastigophora) was observed in *Bti* -treated wetlands as compared to untreated control wetlands (Östman et al.

2008). Similarly, a significant increase in some *Bacteria* (e.g. *Flavobacteriaceae*) taxa in treehole mosquito larval habitats resulted from the exclusion of *Aedes triseriatus* (*Ochlerotatus triseiatus*) larvae by *Bti* (Kaufman et al. 2008, Xu et al. 2008). The changes in microbial communities were attributed directly to the removal of mosquitoes (Kaufman et al. 2008, Östman et al. 2008, Xu et al. 2008) and support the hypothesis of "top-down" regulation of resources by larval mosquitoes. Mosquito larvae are known predators of microorganisms (Protozoa, *Bacteria*, algae) and feed on other organic matter as well (Merritt et al. 1992).

Contrary to the expected outcome of "top-down" hypothesis, Su and Mulla (1999) reported a significant reduction in the abundance of two microalgae species (*Closterium* sp. and *Chlorella* sp.) in mesocosms treated with *Bti* to control *Culex* mosquito larvae. Algal biomass was expected to increase as a result of *Bti* application, but the opposite occurred. The authors suggested that the *Bti* treatment applications improved water quality as a result of phytoplankton suppression. However, Su and Mulla (1999) did not characterize *Bacteria* communities and measure other key water quality indicators to support their hypothesis.

Understanding the effects of *Bti* applications on microbial communities and water physicochemistry in mosquito habitats (treatment wetlands or other aquatic ecosystems) has immense importance for several reasons: 1) the reduction in primary producers such as algae that comprise the base of many food chains might have significant direct or indirect effects on other aquatic ecosystem

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inhabitants such as fish (Jackson et al. 2013). Reductions of primary production are known to reduce the abundance and production of the insect groups that are significant components of wetland food webs (e.g., Poulin et al. 2010). 2) Microbial communities including *Bacteria*, Archaea and some algae play important roles in recycling nutrients in treatment wetlands. The algicidal effect and potential antibiotic effect of the *Bti* could have a dramatic effect on the efficacy of the treatment wetland. 3) Finally, the current regulatory climate of mosquito control operations on water quality is under ever-increasing scrutiny. Environmental regulatory agencies (e.g. in California) have recently required that vector control agencies assess the impacts of mosquito control agents on aquatic ecosystems. The current assessment protocols do not consider the biotic consequences of application of mosquito control agents. By focusing on only a few physicochemical variables and "visual inspection", the assessment protocols will likely fail to recognize potentially important changes in the communities and processes in aquatic ecosystems caused by some mosquito control agents.

In this study, we addressed the unexplored effects of the most commonly used biorational (*Bti*) mosquito control agent on freshwater ecosystems primarily on the microbial communities (*Bacteria* and algae) responsible for nutrient transformations and recycling in wetlands. We tested the null hypothesis that *Bti* has no effect on microbial community structure and water physicochemistry in the water column. The objectives of this study were 1) to characterize changes in the mosquito-associated microbial (*Bacteria* and algae) communities over time

after a one-time application of *Bti*, 2) to determine effects of a one time *Bti*

application on water quality variables (nutrients, sestonic particulates and

physicochemistry), and 3) to evaluate the effects of different *Bti* application rates

on the biotic and abiotic factors of aquatic ecosystems.

MATERIAL AND METHODS

Experimental design and treatments

This study was conducted in fiberglass mesocosms at the Aquatic and Vector

Control Research Facility of the University of California Riverside Agricultural

Experiment Station. Twelve mesocosms (area = 1 m^2) were flooded to 0.3 m

depth on September 28, 2012. Ammonium sulfate [$(NH_4)_2SO_4$; 21% nitrogen and

24% sulfur; Lilly Miller Brands, Walnut Creek, CA)] and rabbit pellets (17% crude

protein) (Brookhurst Mill, Riverside, California) were added to the mesocosms at

the rate of 400 and 500 kg/ha, respectively to promote mosquito colonization

(Mutero et al. 2004, Nguyen et al. 1999). Two *Bti* treatments (High= 48.1 kg/ha,

Low= 0.6 kg/ha) and an untreated control treatment were assigned (4 replications

each) using a completely randomized experimental design to the mesocosms on

October 2, 2012. A corncob granule formulation of *Bti* (VectoBac® G) with a

toxicity of 200 ITU per mg was used. The application rates were based on a

previous study (Su and Mulla 1999) of prolonged mosquito control using this

formulation. The high *Bti* treatment used in this study was approximately twice

the currently recommended concentration (20 lb/acre) of *Bti* for mosquito control

in enriched habitats whereas the low is below the recommended rate for mosquito control.

Bacteria DNA extraction and polymerase chain reaction

Two 40-mL water samples were taken in 50-mL sterile, centrifuge tubes on four sampling dates (October 1, 11, 18 and on Nov 15, 2012) from each of the mesocosms and taken to the laboratory for genomic DNA extraction. The procedure of DNA extraction, amplification of the target gene using Polymerization Chain Reaction (PCR) followed similar procedure described in chapter 3. Briefly, after centrifugation of the water samples at 4°C for 30 min, DNA was extracted using MOBIO Soil DNA Extraction Kit from the pellets (pooled from the two tubes) after discarding the supernatant. Following DNA extraction, the V3 region of the 16S rRNA gene was amplified using universal primers 341F (5'-CCTACGGGAGGCAGCAG-3') and reverse primer 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993). The PCR conditions were described in chapter 3. Products from the two replicate amplifications were pooled and 20 µL of the combined PCR product was electrophoresed on 1.5% (wt/vol) agarose gel. PCR products of the expected size (170-190 bp) were then excised from the gel and cleaned using the High Pure PCR Product Purification Kit (Roche Applied Sciences) following manufacturer's protocol. The cleaned PCR product was then quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, USA) and kept at-20°C for Illumina preparation.

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Library preparation and sequencing

Illumina libraries were generated for each sample using Nextflex DNA sequencing kits and an identifying NEXTflex DNA barcode with 6-bp indices (Bioo Scientific, Inc., Austin, TX). Library quality was checked using a 2100 Bioanalyzer (Agilent Technologies, Palo, Alto, CA, USA) and the molar concentration of each sample was determined. All samples were normalized to 10 nM using Tris-HCl (10 mM, pH 8.5) and combined to create two multiplexed samples (lane 1 = 22 samples, lane 2 = 20 samples). The multiplexed samples were then subjected to a 2 X150 bp pair-end sequencing on MiSeq Illumina sequencing platform at GENOSEQ (Sequencing and Genotyping Core) of the University of California Los Angeles, Los Angeles.

Sequence analysis, alignment and taxonomy assignment.

Analysis of the sequence reads was carried out using (Quantitative Insights Into Microbial Ecology) QIIME version 1.7.0 using PyNast version 1.2.0 (Caporaso et al. 2010). The sequences were assembled using PANDSeq version 2.5.0 (Masella et al. 2012). Non-small subunit and 18S sequences were filtered out by aligning all OTUs against a small subunit model using ssu-align (Quast et al. 2013). Clustering of identical (≥0.97 similarity) sequences to operational taxonomic units (OTUs) was carried out using CD-hit-est (version 4.5.4) (Li and Godzik 2006). Taxonomy assignment was conducted using Blastn against the

SILVA 111 reference database with (Quast et al. 2013) via QIIME parallel

assign_taxonomy_rdp_py script with maximum e-value of 0.001.

Algae and Sestonic particulates

Phytoplankton biomass was measured as chlorophyll *a* in duplicate 25- to

100-mL samples. Whole water samples were filtered through 45-mm membrane

filters (0.8-µm pore size) under darkened conditions in the laboratory and then

frozen at -20 ºC. Filters were ground using a Teflon mortar and pestle and then

pigments were extracted in 90% alkaline acetone (Wetzel and Likens 1991).

After centrifugation at 262 × *g* for 5 min, pigment concentration was determined

using a Biospec-1601 UV-Visible spectrophotometer (Shimadzu Scientific,

Columbia, MD) following method 10200H of APHA (1995).

Sestonic particle distributions (equivalent spherical diameter (ESD): 0.6 to

224 µm) were enumerated using an electronic particle counter (Multisizer™4

Beckman Coulter, Inc. Brea, CA). The particles in bulk water samples were

quantified in 3-8 replicate samples using 100- µm and 280-µm apertures.

Approximately 10 mL of bulk water was filtered through 10-µm aperture mesh

and the particle distribution (0.6 to 10 µm ESD) in the filtered water was

determined in three replicates using a 20-µm aperture. Blanks consisted of either

unfiltered (for 100- and 280- µm apertures) or filter-sterilized (0.2-µm pore size;

for 20-µm aperture) electrolyte (ISOTON® II).

Physicochemical parameters

Water samples were collected just below the water surface in 500-mL dark

plastic bottles placed on ice in a cooler and transported to the laboratory to

determine the concentration of nutrients (ammonium-nitrogen, nitrate-nitrogen,

nitrite-nitrogen, total nitrogen, phosphate as soluble reactive phosphorus (SRP),

total phosphorus, chemical oxygen demand (COD) and sulfate). These

parameters were measured colorimetrically using a Hach DR™ 2800

spectrophotometer (TNT Plus tests, Hach Chemical Co., Loveland, CO). Water

temperature was recorded continuously every 0.5 h using a water temperature

data logger (HOBO, Onset Computer Inc., Bourne, MA). Average (± SE) hourly

temperature on the sampling days was 25.6 (0.43) °C, 21.2 (0.32) °C and 12.4

(0.21) °C on day 4, 9, 16 and 44 after the onset of the experiment, respectively.

Other water physicochemical parameters (pH, temperature, total dissolved

solids, dissolved oxygen concentration) were measured in the morning (~08:30)

and afternoon (~15:30) of each sampling date using an electronic sensor array

(ICM AquaCheck™, Perstop Analytical, Wilsonville, OR). The experiment was

terminated on 20 December 2012.

Mosquitoes and other macroinvertebrates

Five dip samples per mesocosm were taken to enumerate and identify

immature mosquitoes and other macroinvertebrates using 350-mL standard

dipper 24 h after water sample was analyzed for nutrient concentration. Samples

were taken from each corner and one from the center from each of the
mesocosms.

Statistical analysis

Beta diversity analyses: Principal Coordinate Analysis using Bray-Curtis
similarity measures and Non-Metric Multidimensional Scaling analysis) based on
the standardized (smallest library size=6139 reads per sample) sequences were
carried out to determine differences in *Bacteria* communities between samples
using AXIOME version 1.6.0 pipeline (Lynch et al. 2013) which used R "vegan"
library (Paradis et al. 2004; Roberts 2010). Principal coordinate analysis using
weighted UniFrac distances was carried out using QIIME. Repeated-measures
ANOVA on counts of mosquito abundance in the first three dip samples, algal
biomass and physiochemical variables was carried out using JMP version 10
(SAS Inc. 2011). Counts of mosquito larvae were transformed by $\log_{10}(x+1)$ prior
to analysis. Logarithmic transformation was carried out when necessary for
physicochemical data prior to analysis to assume normality.

RESULTS

MiSeq sequence data summary

We generated 4,095,139 *Bacteria* sequences using MiSeq Illumina platform
that resulted in 42,413 OTUs from 41 water samples taken from the experimental
mesocosms on four (once before treatment and three times post treatment

application) sampling dates. About 62% and 13% of the *Bacteria* sequences recovered from the water column were singletons and doubletons, respectively. Overall, a total of 45 *Bacteria* phyla were recovered from samples taken from the water column, with over 98% of *Bacteria* sequences classified in to only 7 phyla: Bacteroidetes (35.4%), Proteobacteria (30.1%), Cyanobacteria (14.5%), Firmicutes (7.5%), Actinobacteria (7.2%), Verrucomicrobia (1.5%) and Planctomycetes (1.4%) in descending order of abundance (Figure 1).

Pre-treatment bacterial communities

Water samples (before *Bti* application (3 d after initial flooding) included 26,801 *Bacteria* OTUs containing 1,892,941 sequences. Based on rarified sequences, about 99.9% of the sequences recovered on this date (denoted by sample identification numbers 0 to 10, Figure 4.1) were classified in to just eight phyla: Proteobacteria (53.6%), Bacteroidetes (23.5%), Firmicutes (19.7%), Cyanobacteria (1.3%), Verrucomicrobia (0.8%), BD1-5 (0.7%), Fusobacteria (0.2%) and Actinobacteria (0.1%) in order of sequence abundance (Figure 4.1). Proteobacteria accounted for 39-61% while Bacteroidetes accounted for 16-31% of the sequences per sample on this sampling date. Firmicutes was the third most abundant phylum and accounted for 9-23% of the sequences per sample on this sampling date.

Proteobacteria was primarily dominated by *Gamma-* and *Betaproteobacteria* accounted for 26% and 23% of sequences per sample, respectively, whereas

each of *Epsilon-* and *Alphaproteobacteria* accounted for only 2% of sequences per sample.

At the genus level, an uncultured OTU of *Aeromonas* bacterium (*Gammaproteobacteria*) followed by an uncultured OTU of *Comamonadaceae* (*Betaproteobacteria*) dominated the *Bacteria* community in water column on this sampling date. *Flavobacteria, Cytophagia* and *Sphingobacteria* (Bacteroidetes) accounted 19.9%, 2.5% and 1.5%, respectively of the sequences per sample. *Bacilli* and *Clostridia* (Firmicutes) accounted for 9.7% and 9.6% sequences per sample.

Bacterial communities post-treatment application

To discern the effects of *Bti* treatments on *Bacteria* communities (both free living and *Bacteria* communities likely adhered to the particles and phytoplankton), sequences from pre-treatment applications were removed from all analyses unless otherwise stated. Overall 30 sequences from 30 samples taken on three sampling dates were analyzed after sequences from pretreatment level were removed. *Bacteria* sequences from the 30 post-treatment application resulted in 17, 049 *Bacteria* OTUs, which were classified into 43 phyla: Bacteroidetes (42.5 %), Proteobacteria (21.8%), Cyanobacteria (19.0%), Actinobacteria (7.2%), Firmicutes (3.0%), Chlorobi (2.2%), Verrucomicorbia (1.98%), and Planctomycetes (1.0%) in descending order of abundance. These eight phyla accounted for 98.7% of all sequences from the samples taken on the

three sampling dates. The remaining 34 phyla accounted only for 1.3% of the sequences.

Beta diversity of *Bacteria* communities

Principal coordinate analysis using Bray-Curtis similarity distance measures revealed a significant separation of samples by application treatment and sampling date (Figure 4.2). The greatest separation of samples, as shown by the first principal-component axis, was by sampling date, while the separation of samples by treatments was explained by the second axis (Figure 4.2). Samples taken 44 d after *Bti* application were significantly (PC 1=34%) differentiated from samples taken on the 9 and 16 d after treatment application. Similarly, samples from the high *Bti*-treated mesocosm separated significantly (PC 2 =17%) from samples taken from low and untreated control treatments (Figure 4.2). The separation of the samples by sampling date and treatments was even more evident in non-metric multidimensional scaling plot of *Bacteria* profiles from the three treatment applications (Figure 4.3). *Bacteria* communities from the high *Bti* treatment were significantly differentiated from the untreated control and low *Bti* treatments (P=0.002)

Principal coordinate analysis of a weighted UniFrac distances revealed that samples from low *Bti* and untreated control treatments were clustered together on a given sampling date and were dominated by *Proteomonas sulcata* (Cyanobacteria), *Cyclobacteriaceae* and *Polaromonas* (*Betaproteobacteria*),

Sphingomonas sp. (*Alphaproteobacteria*) and two Bacteroidetes species:

Leadbetterella (*Cytophagia*) and *Solitalea* (*Sphingobacteria).* Samples from high

Bti treated mesocosms were separated from the two other treatments and were

dominated by *Muciiaginbacter*, *Sedminibacterium* (*Sphingobacteria*) and

Polaromonas (*Betaproteobacteria*) (Figure 4.4).

A Cyanobacteria species (OTU #10) was drastically reduced in the high *Bti*

treated mesocosms (Table 4.1). It occurred in significantly greater abundance

(proportion per sample) in samples from untreated control [average: 24%

(±4.6%SE), n=11] and low [20.6% (±4.2%), n=9] *Bti* treatments than in samples

from high [5% (±1.3%, n=10] *Bti-* treated mesocosms (Table 4.1).

Cyclobacteriaceae sp. (*Sphingobacteria*) was another dominant species in the

low *Bti* and untreated control mesocosms that was significantly suppressed in

high *Bti* treated mesocosms (Table 4.1). The abundance of *Bacillus funiculus*

(Firmicutes) and other congeneric *Bacilli* members were also significantly

suppressed in the high *Bti*-treated mesocosms. On average, the *B. funiculus*

sequences accounted for 1.8 (± 0.4% SE) and 1.1 (± 0.2%) per sample in

untreated control and low *Bti* treated mesocosms, respectively, while it only

accounted 0.4 (± 0.1%) sequences per sample in the high *Bti* treated

mesocosms (Table 4.1).

Overall, members of Cyanobacteria, Bacteroidetes, and Firmicutes

proliferated in the low *Bti* treated and untreated control mesocosms while these

taxa were severely suppressed in the high *Bti* treated mesocosms. Specifically,

members Cyanobacteria, *Sphingobactertiales*, *Cytophagales*, *Flavobacteria* (Bacteroidetes), *Bacillales* (Firmicutes), *Sphingomonadales* (*Alphaproteobacteria*) and *Burkaholderiales* (*Betaproteobacteria*) dominated the low *Bti* and untreated control mesocosms while these taxa were suppressed in the high *Bti* treated mesocosms. We observed a slight increase in abundance in some members of *Beta-*, *Gamma-* and *Deltaproteobacteria*, *Verrucomicrobiae* and Actinobacteria in the high *Bti* treated mesocosms (Table 4.1). *Frankiales* were also among the dominant *Bacteria* found in the high *Bti* treated mesocosms.

Nearly 13% of the OTUs were shared among samples from the three treatments and sequences of these OTUs accounted for 93% of all the sequences recovered from this study (Figure 4.5). About 34% of the *Bacteria* OTUs were unique to samples from the water column of the high *Bti* treated mesocosms, while 15% and 24% of the OTUs were unique to low *Bti* and untreated control treatments, respectively (Figure 4.5).

Effect of *Bti* on phytoplankton and suspended sestonic particulates

Water in mesocosms that received high *Bti* treatments appeared crystal clear compared with the dark-green color observed in mesocosms that received low *Bti* and control treatments 16 d after treatment application (Figure 6). The water in all three treatments appeared transparent and similar in color 74 d after treatment applications.

Repeated measures ANOVA revealed significant differences in mean chlorophyll concentration in the water column between treatments ($F_{2, 9}$=12.0; P<0.05) and this varied across time (Table 4.2). The significant difference among the treatments in the mean chlorophyll concentration occurred 16 d after the *Bti* application (Figure 4.7). At day 16 after treatment applications, algal biomass in the mesocosms treated with the high concentration of *Bti* was significantly lower than the control and low *Bti*-treated mesocosms. Algal growth in mesocosms that received the high *Bti* treatment ceased growing at 9 d after application whereas algae biomass continued to increase exponentially (Exp (log Chl *a* (μg L^{-1})) = 1.47 ($\pm$ 0.45) + 0.74 ($\pm$ 0.02) *d; R^2=0.96) for the first 34 days in the low *Bti*-treated and untreated control mesocosms. The chlorophyll biomass abruptly declined after 34 days. A maximum difference of 50-fold greater total chlorophyll concentration was found in untreated control mesocosms than in the high *Bti*-treated mesocosms at 30 d after *Bti* treatment was applied (Figure 4.7). Chlorophyll concentrations in the mesocosms treated with low *Bti* and control treatments were not significantly different throughout the study period and attained maximum algal biomass at 34 d after initial flooding (Figure 4.7).

Similarly, counts of sestonic particulates suspended in the water column were also significantly different among the different treatments (Table 4.2). The number of particulate size range: 1.01-10 μm, referred to as "medium" size class in the text hereafter, was significantly reduced in the high *Bti* treated mesocosms and showed a similar pattern as the chlorophyll concentration (Figure 4.8). The

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counts of medium size range particles and chlorophyll were positively correlated (r=0.8). Similarly, the differences in mean number of particulates between size ranges of 0.6-1.0 μm was also significant among treatments (P=0.029), while particle counts of the size range 10.1-240 μm were not significantly different among the three different treatments (P=0.619) (Table 4.2).

High *Bti* application rate reduced inorganic nutrients in the water column

A statistically significant reduction of inorganic nutrients (nitrogen and phosphorus) was observed in mesocosms that received high *Bti* treatments (Figures 9-11). Total nitrogen and nitrate in the mesocosms differed significantly among treatments 18 d after *Bti* application, whereas differences in ammonium nitrogen and nitrite nitrogen were not statistically significant (Figures 4.9 and 4.10). Total nitrogen deceased at a rate of 46%, 43% and 39% per week in mesocosms treated with low, high *Bti* treatment, and untreated control, respectively for the first 18 days. At 34 d after *Bti* application, the reduction in total nitrogen drastically changed in mesocosms in the high *Bti* treatment (Figure 4.9). Both the bioavailable form of nitrogen and total nitrogen in the high *Bti*-treated mesocosms declined by 44% while nitrogen in the low and control mesocosms remained unchanged for the remaining experimental period (Figure 4.9 and 4.10).

Similarly, total and soluble reactive phosphorus (SRP) were significantly reduced in high *Bti* treated mesocosms (Figure 4.11). Soluble reactive

phosphorus is biologically available in the water column whereas total

phosphorus both inorganic forms and phosphorus bound to organic matter.

Sulfate concentration in the water column was measured twice (38 and 44 d

after treatment application) during the study, and was also significantly reduced

in mesocosms that received high *Bti* treatments (Table 4.3).

Effects of *Bti* on other physicochemical variables

High *Bti* application rate reduced pH, dissolved oxygen and chemical oxygen

demand in the water column. At 14 d after *Bti* application, significantly greater pH

was measured at 15:30 PM in mesocosms of the low *Bti* and control treatments

than in mesocosms in the high *Bti* treatment (Figure 4.12). There was no

statistically significant difference in pH levels measured at 8:30 AM between

treatments (Figure 4.12).

Similarly the mean concentration of dissolved oxygen (DO) was significantly

higher in mesocosms with low *Bti* and untreated control mesocosms 14 d after

the treatments (Figure 4.13). The mean DO concentration during morning

measurements did not differ significantly among the three different treatments.

The chemical oxygen demand, which is often considered a useful indicator of

the amount of organic matter in the water column, was significantly reduced

approximately 75% and 50% in the high *Bti* application rate 44 and 74 d,

respectively, after *Bti* application (Table 4.3).

Effects of *Bti* on mosquitoes

Three *Culex* mosquito species (*Culex tarsalis* Coquillett, *C. stigmatosoma* Dyar and *C. quinquefasciatus* Say) were found in the mesocosms during the study. Two other dipteran species in the families Ephidridae and Chironomidae were also among the primary colonizers found in this study. Nearly 90% and 97% of the total numbers of Chirinomids and Ephydrids, respectively, were sampled during the first week after flooding and enrichment of the mesocosms. The abundance of Chirinomids and Ephydrids was not significantly different among the three treatments (*P*<0.05).

The early instar mosquito abundance among the three treatments was not significantly different (F $_{(2, 33)}$ = 2.2; *P*=0.126) (Figure 4.14A). However, the mean number of the late (3rd and 4th) instar *Culex* larvae in the high *Bti* treatment was significantly lower than in the control and low *Bti* mesocosms (F $_{(2, 33)}$ = 4.0; *P*=0.030). The late instar larvae abundance was reduced by nearly 54% in the high *Bti* mesocosms 3 d after the treatments were applied (Figure 4.14B). The suppression of the late instar larvae lasted nearly for a month in the high *Bti* mesocosms (Figure 4.14B). *Culex* pupae abundance was also significantly lower than in the control and low *Bti* application (F $_{(2, 33)}$ = 19.2; *P*=0.007) in the high *Bti* mesocosms. Pupae were first seen 14 d after initial flooding of the mesocosms (Figure 4.14C).

DISCUSSION

To our knowledge, this is the first in depth characterization of microbial communities and physicochemical characteristics in aquatic habitats treated with the most commonly used mosquito biopesticide (*Bti*) in the environment. We employed a deep sequencing technology to characterize microbial communities to overcome technical difficulties and biases of the commonly used culture-dependent techniques. The method enabled us to discern the potential effects of *Bti* on *Bacteria* community structure in lentic aquatic mosquito habitats.

Suppression of some *Bacteria* taxa in the high *Bti* application rate

A significant suppression of *Bacteria* taxa that include Cyanobacteria, *Cytophagales* and *Cyclobacteriaceae* (Bacteroidetes), and *Sphingomonas* (*Alphaproteobacteria*) was observed in mesocosms that received a high dose of a mosquito larvicide, VectoBac G granules, in our study (Table 4.1). As depicted in PCoA and MDS plots, samples from untreated control and low *Bti* treated mesocosms were clearly separated from samples taken from high *Bti* treated mesocosm, showing a significant effect of high *Bti* treatment (Figures 4.2-4.4).

Previous laboratory studies showed contradictory results of the effects of *Bti* on microorganisms (Yudina et al. 2003; Koskella and Stotzky 2002, Revina et al. 2005). Yudina et al. (2003) demonstrated the antibacterial activities of the endotoxins of *Bti* on six gram-positive species of Actinobacteria (three *Micrococcus* spp., *Nocardia calcaea* and two *Steptomyces* spp.) in laboratory

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studies. Contrary to Yudina et al. (2003), Koskella and Stotzky (2002) reported

that toxins from *Bti* have no negative effect on some selected species of

Bacteria, algae and fungi. The results of these studies were based on laboratory-

controlled bioassays and on very few cultivable microbial species. Their results

do not likely reveal the *Bti* interaction with the more diverse microbial

communities found in aquatic habitats.

Influence of high *Bti* application on phytoplankton

Our study revealed that algal biomass (estimated by total chlorophyll

concentration) was significantly suppressed in the high *Bti* treatments

approximately two weeks after *Bti* application (Figure 4.7) and agrees with the

findings of Su and Mulla (1999). Algal biomass ceased growing 9 d after high *Bti*

application and the biomass 74 d after application was comparable to the

pretreatment level (Figure 4.7). The high *Bti* treatment used in this as well as in

Su and Mulla's study was twice the concentration of *Bti* currently recommended

for mosquito control in wastewater treatment wetlands. It is unknown whether the

suppression of algal biomass in the water column observed in this study, or in Su

and Mulla (1999), was directly related to the active substances of VectoBac G,

other proprietary components of the formulation, or indirectly via application of *Bti*

favoring the proliferation of *Bacteria* taxa that may have algicidal effects.

Some *Bacillus* species have been known to produce algicidal toxins. For

example, Reim and coworkers (1974) reported that the antibiotics produced by

Bacillus bevis (Firmicutes: *Bacillaceae*) directly inhibited the growth of blue-green algae, *Plectonema boryanum* (Cyanobacteria) and other congeneric species. Other *Bacteria* species such as *Bacillus cerus* (Firmicutes: *Bacillaceae*), *Planomicrobium* sp. (Firmicutes: *Planococcaceae*) and several species in the genera *Pseudoalteromonas (Gammaproteobacteria*: *Pseudoalteromonaceae*) and *Cellulophaga* (*Falvobacteria*: *Flavobacteriaceae*) were also reported to produce algicidal toxins (Skerratt et al. 2002; and references cited therein). In general, there is a lack of knowledge on basic biology of spores of many *Bacillus* species including *Bti*, and their interaction with the microorganisms in the environment (Nicholson 2002).

Bacteria and algae are also known to interact with one another depending on the availability of resources (carbon: nutrient ratios) and presence or absence of bacterial predators (Cole 1982; Hulot et al. 2001; Doucette 2006; Amin et al. 2012). Carbon rich resources are generally considered favorable for *Bacteria* proliferation whereas nutrient-rich (e.g. nitrogen, phosphorus) media are thought to encourage algal growth (Cole 1982; Danger et al. 2007). The suppression of some *Bacteria* taxa occurred in the high *Bti* treatments might also be directly linked with the suppression of algae. The surfaces and surroundings of algae, also known as phycosphere, are known to influence *Bacteria* communities as well (Eigemann et al. 2013).

Impacts of high *Bti* application rate on physicochemical variables

This study is the first to characterize physicochemical characteristics of the water following a *Bti* application to control mosquitoes in aquatic habitat. Su and Mulla (1999) indicated that *Bti* application improved water quality. However, their study did not measure key water quality parameters. Inorganic nutrients (phosphorus and nitrogen) which are essential for growth of phytoplankton and thereby zooplankton were significantly reduced in high *Bti* treatment applications (Figures 4.9-4.11). Our study (and that of Su and Mulla [1999]) was conducted in experimental mesocosms with very small amounts of substrate [0.02% (W/vol) rabbit pellet and 0.01% Ammonium sulfate]. The nitrogen lost from the high *Bti* treated mesocosoms might have been due to denitirification but the loss of phosphorus from water column in high *Bti* treated mesocosms is unknown.

The reduction in the nutrients (nitrogen and phosphorus) as result of high *Bti* application that we observed in our study (Figures 4.9-4.11) might have been responsible for the reduced phytoplankton biomass produced seen in the high *Bti* treatments. Phosphorus for example has often been reported to limit the growth of algal communities in fresh water lakes (Mazumder 1994). In our study, total phosphorus was positively correlated (r=0.58) with algal biomass [log (Chlorophyll *a*) = 1.26 (± 0.21) − log (TP) 1.38 (±0.6), R^2=0.34] and compares reasonably well with the algal growth model in response to total phosphorus concentration described by Stow and Cha (2013).

Our study also revealed a suppression of the abundance of the low and medium size sestonic particulates in the high *Bti* treatment applications. The number of the medium size particles correlated very well (r=0.8) with the algal biomass and showed a similar pattern as algal concentration dynamics during the study.

Chemical Oxygen Demand (COD), which is another useful parameter of oxygen availability and indicator of the amount organic matter, was significantly reduced in high *Bti*-treated mesocosms. Similarly the Dissolved oxygen level in the high *Bti* treated mesocosms also decreased in the high *Bti* treated mesocosms. This happened in the afternoon when respiration from the phytoplankton was at its peak. Algal biomass, which is a source of organic matter and oxygen as result of photosynthesis, was also significantly reduced in the high *Bti* treated mesocosms.

Fate of *Bti* formulations in the water column

Bti formulations are known to rapidly disappear rapidly from the water column and settle and bind to substrates soon after application (Ohana et al. 1987). In our study, we also did not attempt to scrape the bottom of the mesocosms to determine the presence of *Bti* spores. The primary cause of the loss of the toxicity of *Bti* was due the immediate settling and binding of the spores with the substrate (soil or particulates) (Ohana et al. 1987). However, a significant portion of the *Bti* toxicity (up to 90% larvae mortality) was restored after three weeks by

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subsequent stirring and filtering of the substrates (Ohana et al. 1987), suggesting

that some recycling of *Bti* spores might occur in the environment. Ohana and

colleagues carried out their study only for two months. More recent and long-term

studies showed that *Bti* spores persist in the environment bound with soil

particles (Guidi et al. 2011, 2013) and leaf litter (Tilquin et al. 2008;Tetreau et al.

2012a, 2012b) longer than the short time span previously reported in Ohana et

al. (1987) and Walton and Mulla (1992).

Guidi and colleagues monitored spores of *Bti* over long (> two decades)

period and found that the spores were frequently recovered from the environment

(soil substrate) after several months (up to 275 days) after the last treatment

application (Guidi et al. 2011, 2013). Although the number of spores recovered

from the aquatic habitats in their study sites was influenced by elevation and

frequency of *Bti* treatments per year, the numbers of spores remained

unchanged overtime and were comparable to the spore concentration in the

substrate two days after the *Bti* application (Guidi et al. 2013). In this study, we

did not recover *Bti* sequences from the water column using *Bacteria* specific 16S

rRNA gene primers 9 d after application, and we did not attempt to recover *Bti*

from the substrate (rabbit pellets).

Effects of *Bti* on mosquitoes and the "top-down" hypothesis

In our study, the high *Bti* application nearly eliminated *Culex* mosquitoes (late

instars and pupae) for nearly a month. However, when the number of these

stages are broken down to species, the proportion of the three *Culex* mosquitoes varied between treatments. *Culex tarsalis* tended to increase over time in mesocosms treated with high *Bti* treatments, whereas the number of *C. stigmatosoma* were significantly reduced in high *Bti*-treated mesocosms.

Previous studies that used *Bti* for mosquito exclusion experiments attributed changes in *Bacteria* taxa in the aquatic habitats to the removal mosquitoes, agreeing with the predominant top-down population regulation hypothesis (e.g., Nugyen et al. 1999, Kaufman et al. 2008, Xu et al. 2008). Although the feeding habits of mosquitoes (e.g. *Culex* vs *Aedes*) varied from one mosquito genus to another, it is unlikely that the "top- down" hypothesis (removal of mosquitoes) resulted in significant changes as significant as would be expected from changes in bottom up resources such as bacterioplankton and phytoplankton.

Xu et al. (2008) reported a proliferation of *Flavobacteriaceae* in treehole habitats as a result of *Aedes* mosquito removal by *Bti* application. Contrary to their findings, the abundance of *Flavobacteriaceae* found in control mesocosms was 3X greater than the abundance found in high *Bti* mesocosms suggesting that this group of *Bacteria* were in fact suppressed as a result of high *Bti* application. Overall, the top down regulation by mosquitoes as hypothesized by several studies (Kaufman et al. 2008, Xu et al. 2008, Östman et al. 2008) was not evident in our study. The potential influence of protozoan grazing on *Bacteria* communities was not explored in this study. However, the abundance of the large

(> 10- 240 um) sestonic particulates, which likely includes the protozoan abundance, did not differ significantly among the treatments (Figure 4.8).

Our study suggests some effect of a high application rate of *Bti* (granular form of VectoBac G) on microbial communities in aquatic habitat. However, it is currently unknown what aspect of *Bti* caused the suppression of some bacterial taxa and reduction of nutrients in the water column. The consequences of the suppression of phytoplankton, certain *Bacteria* taxa and reduction of physicochemical variables on natural or agricultural (e.g. rice field) aquatic ecosystems are unknown.

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Table 4.1. Mean (±SE) abundance (proportion per sample) of 11 *Bacteria* taxa found in all samples.

Phylum	Class	Species	Treatments		
			Control n=11	Low n=9	High n=10
Bacteroidetes	*Cytophagia*	*Cyclobacteriaceae*	5.2% (0.98%)a	6.4% (2.1%)a	0.2% (0.1%)b
	Sphingobacteria	*Sediminibacterium*	12.2% (3.9%)a	10.6% (4.7%)a	9.8% (3.5%)a
		Sphingobacterium	1.9% (0.7%) a	1.2% (0.6%)a	0.7% (0.3%)b
Cyanobacteria	*Chloroplast*	*Proteomonas_sulcata*	24.5% (5%)a	20.4% (4.2%)a	5.7% (1.3%)b
		Eutreptiella_sp._LIS_2000	0.3% (0.1%)a	0.2% (0.1%)a	0.6% (0.3%)a
Firmicutes	*Bacilli*	*Bacillus*	1.7% (0.4%)a	1.0% (0.3%)a	0.4% (0.1%)b
	Clostridia	*Clostridium*	0.6% (0.1%)a	0.4% (0.1%)a	0.6% (0.1%)a
Actinobacteria	*Micrococcales*	*Candidatus_Aquiluna*	3.4% (0.5%)b	4.5% (1.6%)a	6.4% (1.5%)a
		Agrococcus jejuensis	0.95% (0.4%)b	0.8% (0.3%)b	0.7% (0.2%)b
Proteobacteria	*Alphaproteobacteria*	*Sphingomonas Sp.*	5.2% (2.6)%a	0.97% (0.5%)b	2.0% (0.8%)b
	Betaproteobacteria	*Polaromonas*	4.6% (0.6%)b	5.2% (0.5%)b	7.0% (1.0%)b

Means followed by same letters within a row are not significantly different (*P*>0.05).

Table 4.2. Repeated measures analysis of variance of water quality parameters.

Sources		Between	*Within subjects	
		Treatments	Time	Treatment x Time
Total N	F	42.5 (2,9)	65.8 (9,81)	4.4(18,81)
	P	0.0001	0.0001	<0.0001
NO_3-N	F	17.5 (2,9)	163.6 (6,54)	8.5(12,54)
	P	0.0008	<0.0001	<0.0001
NO_2-N	F	13.9 (2,9)	18.5 (6,63)	1.4(14,63)
	P	0.0018	<0.0001	0.159
NH4-N	F	2.0 (2,7)	60.6 (6,42)	2.2(12,42)
	P	0.212	<0.0001	0.0268
Soluble reactive	F	18.7(2,8)	51.0 (7,56)	18.0(14,56)
phosphorus (SRP)	P	0.001	<0.0001	<0.0001
TP	F	30.2 (2,9)	32.9(7,63)	9.2(14,63)
	P	0.0004	<0.0001	<0.0001
pH (time,8:30 AM)	F	6.1 (2,9)	53.3(6,54)	1.9 (12,54)
	P	0.0212	<0.0001	<0.0001
pH (time, 15:30 PM)	F	50.1(2,9)	95.6 (6,54)	6.8 (12,54)
	P	0.0212	<0.0001	<0.0001
DO (time, 8:30 AM)	F	5.3 (2,9)	42.8 (6,54)	1.2 (12,54)
	P	0.0311	<0.0001	<0.0001
DO (time, 15:30 PM)	F	14.1(2,9)	11.9 (5,45)	5.4 (10,45)
	P	0.0017	<0.0001	<0.0001
Particles (0.6-1.0 µm)	F	12.0 (2,9)	112.2 (6,54)	16.5 (12,54)
	P	0.0029	0.0003	0.0128
Particles (1.001-10µm)	F	37.8 (2,9)	58.1 (6,54)	21.7 (12,54)
	P	0.0001	0.0001	0.0001
Particles (10-241 µm)	F	0.5 (2,9)	5.1 (6,54)	2.4 (12,54)
	P	0.6193	0.0003	0.0128
Total chlorophyll	F	12.0 (2,9)	112.2 (6,54)	16.5 (12,54)
	P	0.0029	0.0003	0.0128

*F values from within subjects are Univariate unadjusted Epsilon values unless

otherwise stated.

Table 4.3. Mean (±SE, n=4) sulfate and chemical oxygen demand concentration

(mg L^{-1}).

Parameters	Treatment	Time (days after *Bti* application)		
		38	44	74
Sulfate	Control	445.8 (13.24)a	425.3 (6.69a)	N/A
	Low	400.3 (14.10)a	400.0 (14.02)a	N/A
	High	342.0 (18.83)b	363.0 (13.74)b	N/A
COD	Control	N/A	*549.3 (18.0)a	269.8 (32.83)a
	Low	N/A	482.3 (10.66)a	252.3 (5.31)a
	High	N/A	137.5 (8.0)b	135.3 (2.10)b

* n= 3

Means followed by same letters within a column are not significantly different

(*P*>0.05).

Figure Legends

Figure 4.1. Percentage (≥1%) of *Bacteria* Phyla found in each of the 41 samples taken from water column. Samples 0-10 are from pre-treatment applications whereas samples 11-41 are post-treatment applications.

Figure 4.2 Bray-Curtis principal coordinate analysis (PCoA) plot comparing *Bacteria* communities from different *Bti* treatments (Panel A) and three sampling dates (Panel B).

Figure 4.3 Non-meteric multidimensional scaling (NMDS) of Bray-Curtis distance matrix illustrating divergence of *Bacteria* communities by treatments (Panel A) and sampling date (Panel B).

Figure 4.4 A weighted UniFrac distance PCoA plot comparing *Bacteria* communities in water column samples from three different treatments. Panel A) 10 most dominant taxa clustering the samples B) samples separated by treatments C) samples separated by sampling dates.

Figure 4.5. Venn diagram illustrating the overlapping of *Bacteria* species from untreated control, Low and High *Bti* treatments. Numbers in parenthesis are the abundance of *Bacteria* sequences.

Figure 4.6. Water samples from untreated controls (A) and mesocosms treated with high *Bti* (B) at 44 days after *Bti* application in autumn 2012.

Figure 4.7. Phytoplankton biomass (total chlorophyll: mean ± SE; *n* = 4) in the water column of mesocosms assigned to two application rates of larvicide treatments and untreated control. The y-axis is on logarithmic scale.

Figure 4.8. Sestonic particle (range; 0.6-224 μm equivalent spherical diameter) concentration (mean ± SE) in mesocosms assigned to two application rates of mosquito larvicide treatments and untreated control.

Figure 4.9. Total and ammonium nitrogen concentration (mg L^{-1}: mean ± SE). A significant difference in total nitrogen was observed nearly three weeks (18 days) after *Bti* application.

Figure 4.10. Nitrite and nitrate nitrogen concentration (mg L^{-1}: mean ± SE) in the water column of mesocosms assigned to two application rates of mosquito larvicide treatments and untreated control.

Figure 4.11. Soluble reactive phosphorus and total phosphorus (mg PO_4 L^{-1}: mean ± SE) in the water column of mesocosms assigned to two application rates of mosquito larvicide treatments and untreated control.

Figure 4.12. pH levels in the water column of mesocosms assigned to two application rates of mosquito larvicide treatments and untreated control at two time intervals (AM: 08:30; PM: 15:30).

Figure 4.13. Dissolved oxygen levels (mg L^{-1}: mean ± SE) in the water column of mesocosms assigned to two application rates of mosquito larvicide treatments and untreated control at two time periods (AM: 08:30; PM: 15:30).

Figure 4.14. *Culex* abundance (numbers per dip sample) of early instar (Panel A) and late instar (Panel B) and pupae (Panel C) in mesocosms assigned to two application rates of mosquito larvicide treatments and untreated control.
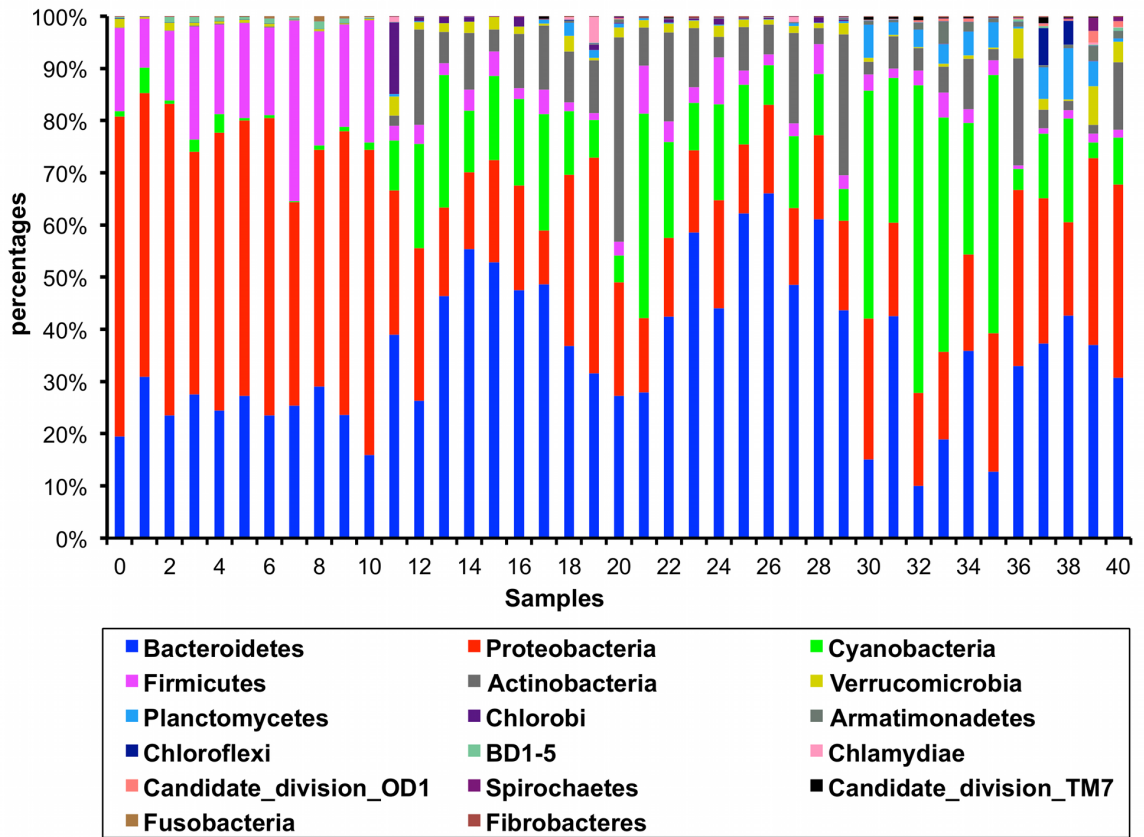
Figure 4.1
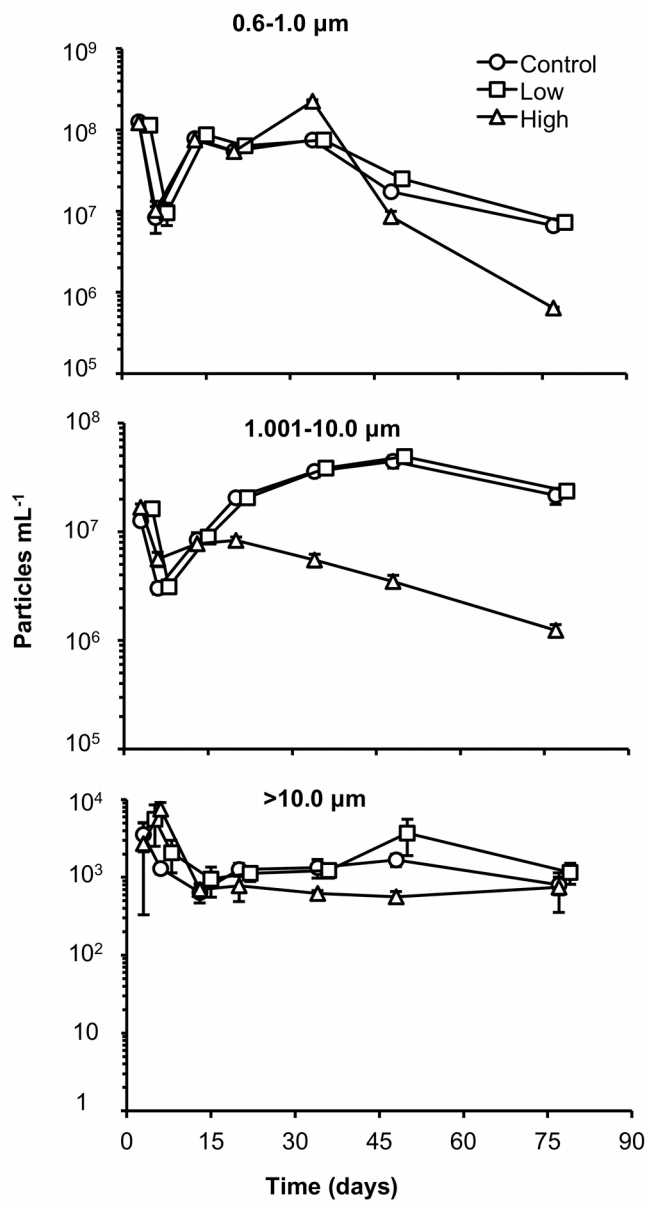
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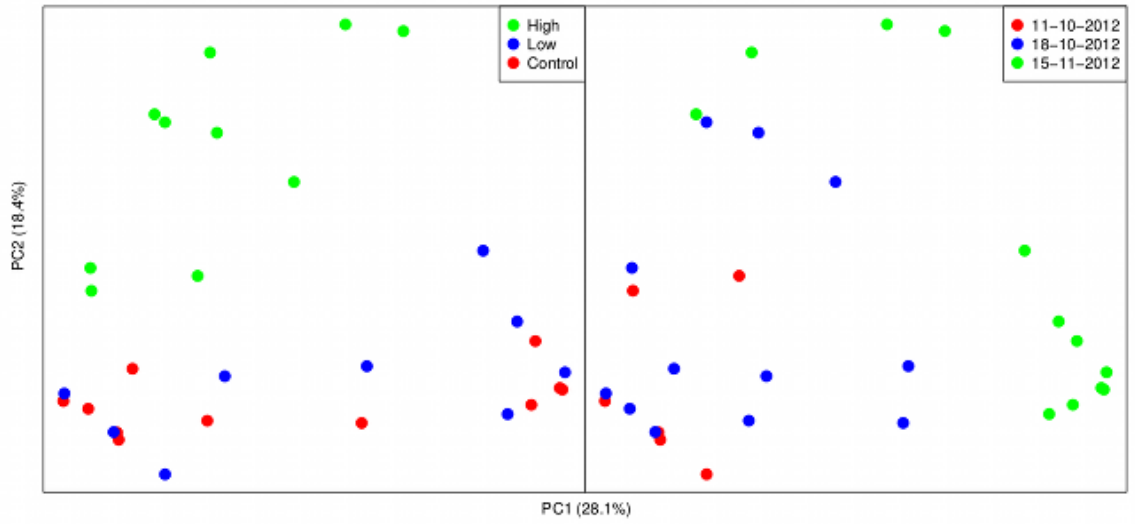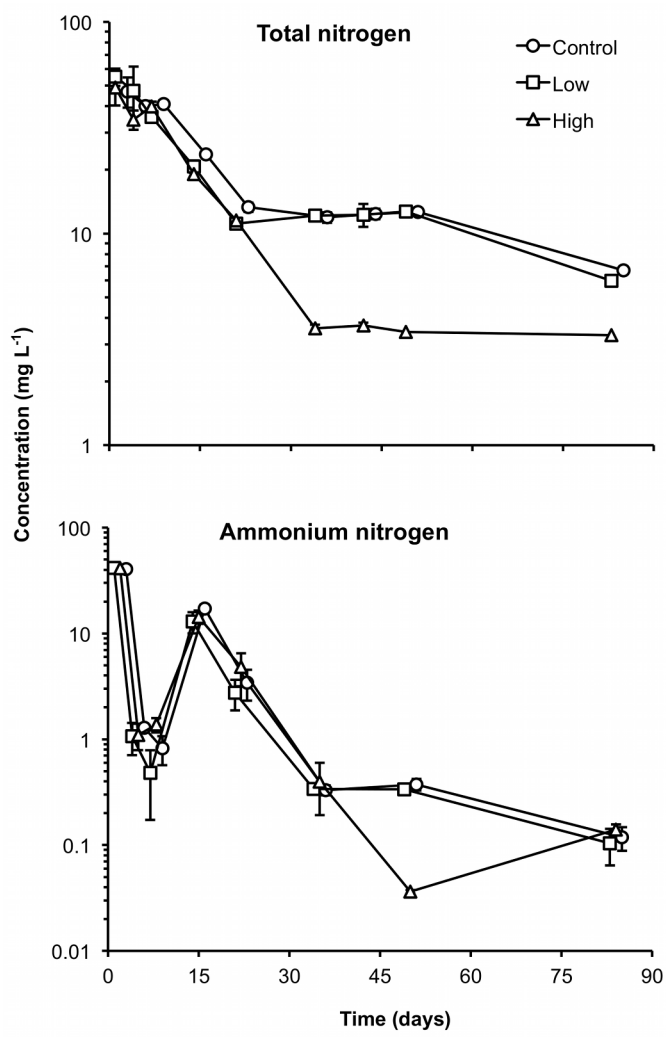
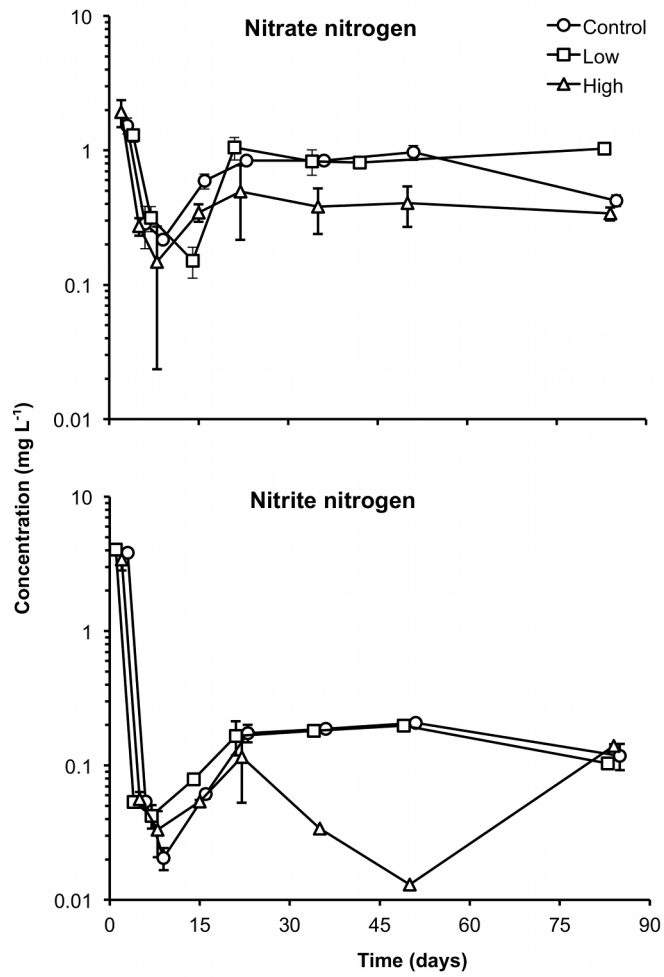Figure 4.3
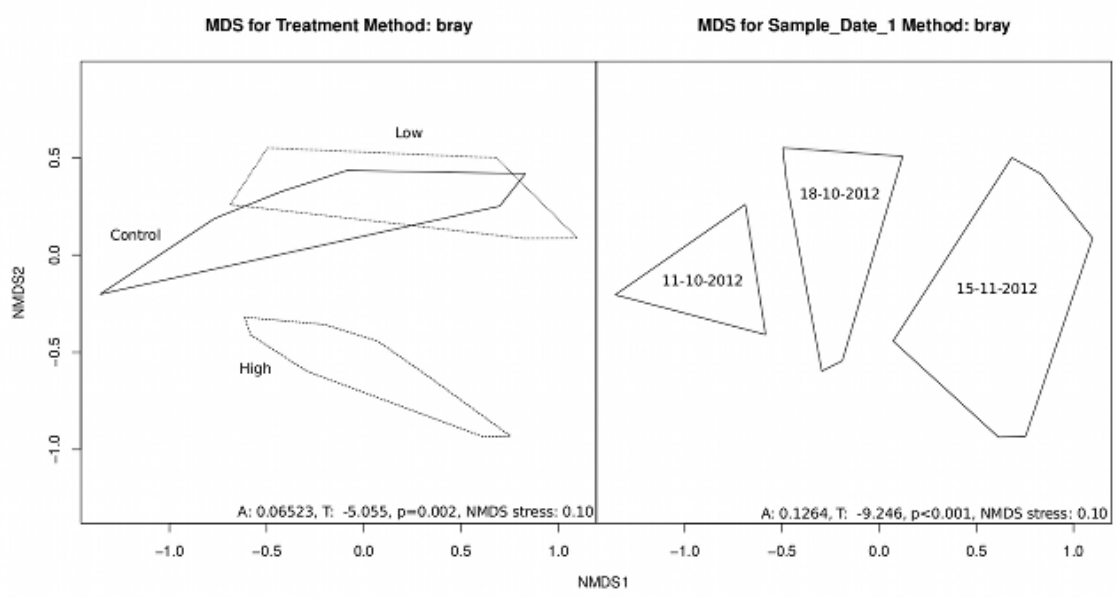
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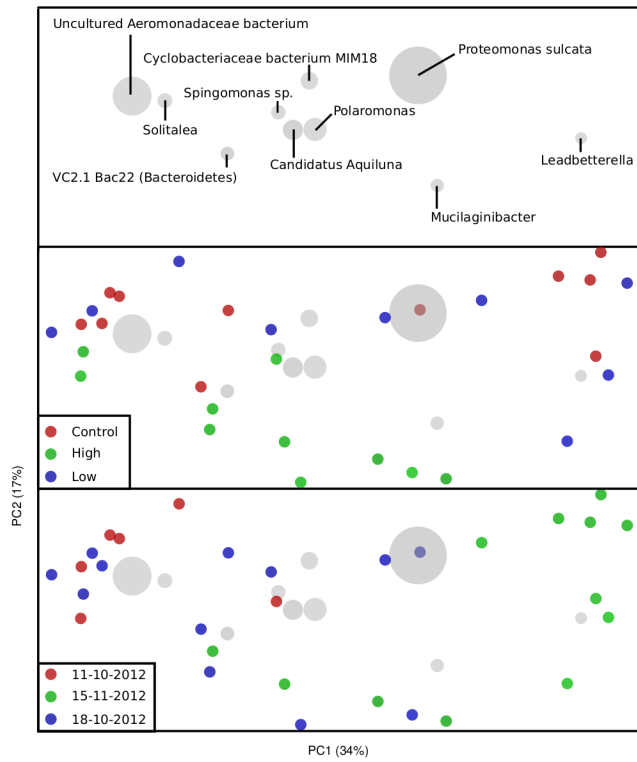
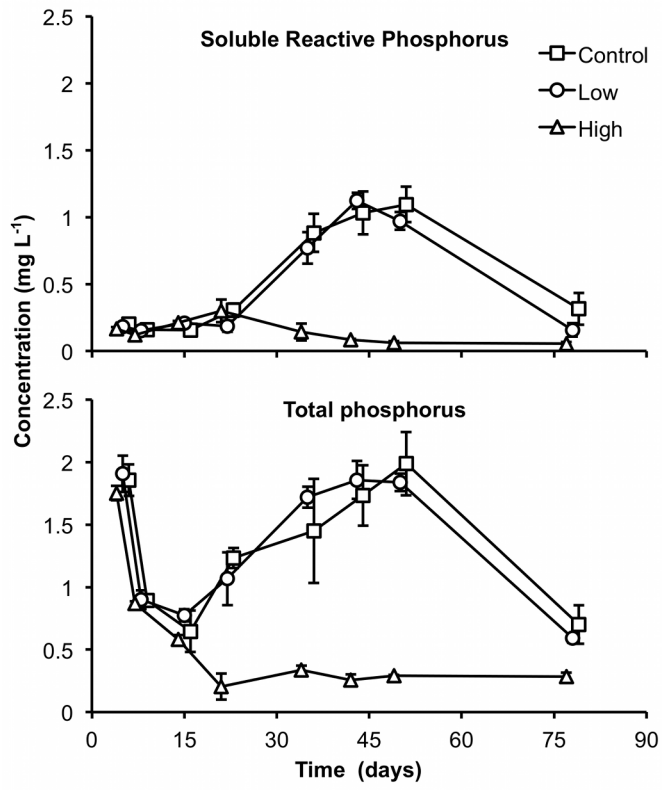Figure 4.5
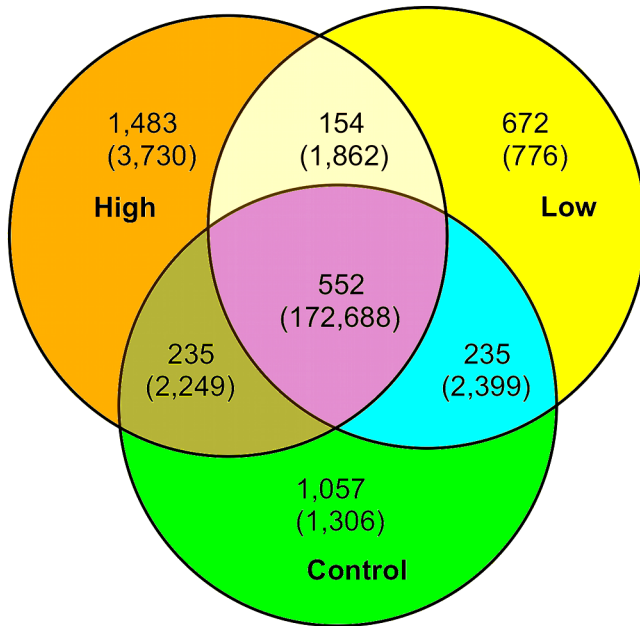
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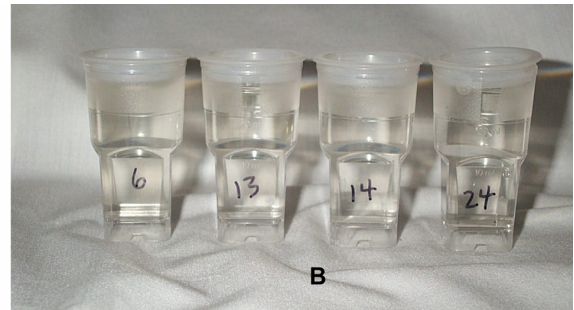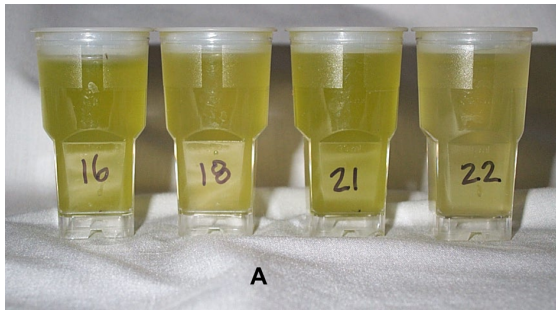
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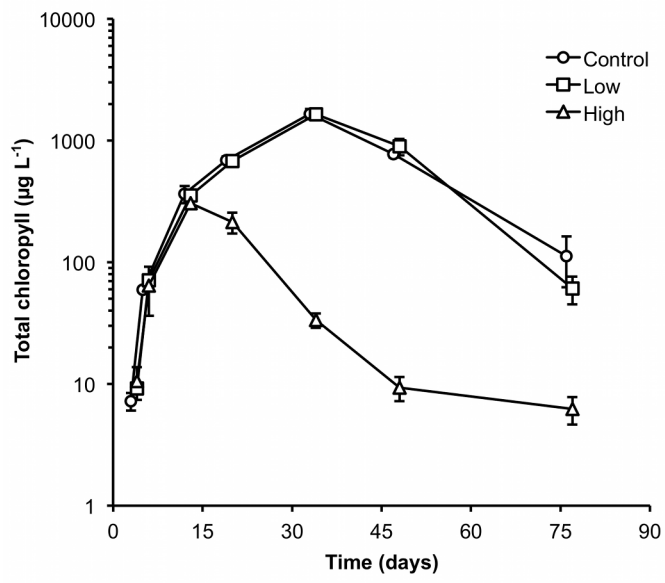
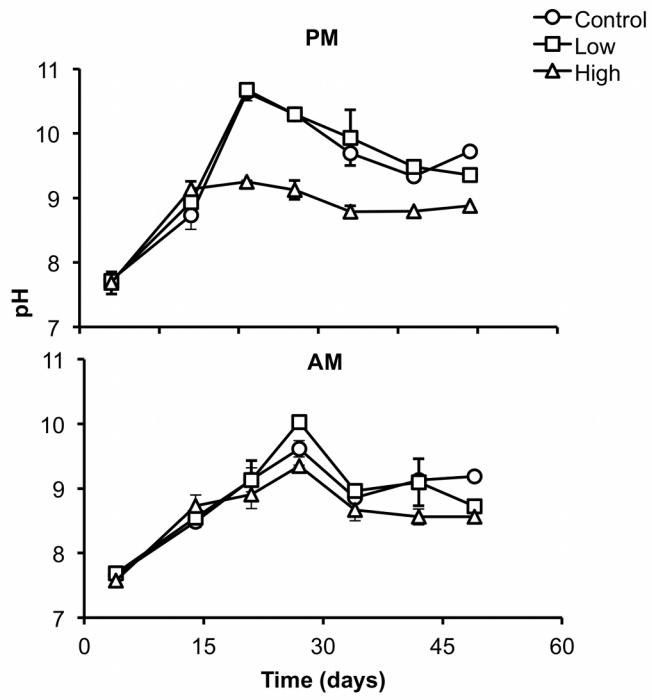Figure 4.8

Figure 4.9

Figure 4.10

Figure 4.11

Figure 4.12

Figure 4.13
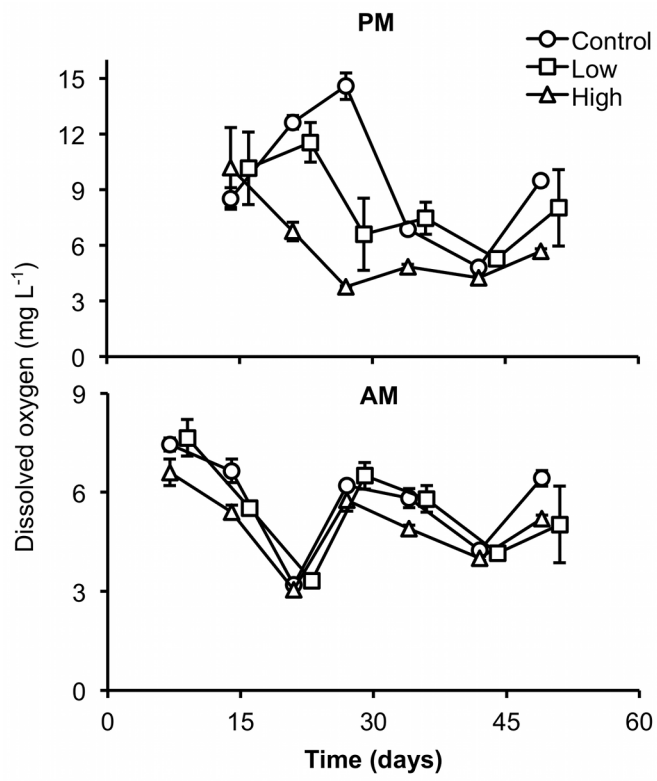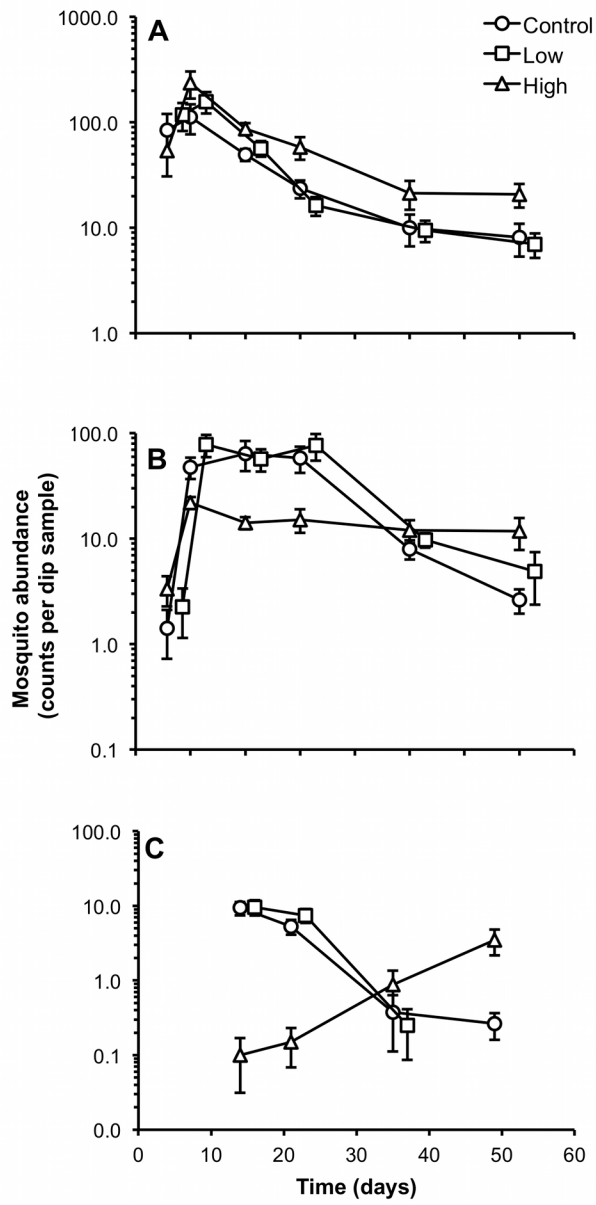
Figure 4.14

Chapter 5: Conclusions

Managing mosquitoes in wetlands is very complex because some of the wetland management operations that are intended to improve the core functions of wetlands (e.g. bioremediation) often enhance mosquito production (Walton 2003). Mosquito production in wetlands in general, and in constructed wetlands in particular, is a function of several environmental variables but is dominantly influenced by nutrients, aquatic plant species and microbial communities associated with these habitats. Knowledge of the influence of these variables on mosquito development in their habitat will not only enhance our understanding of the basic biology of mosquitoes but also help in designing a more sustainable and integrated mosquito control programs that reduce mosquito production while maximizing the functions of these wetlands.

Moreover, the existing knowledge on the impacts of mosquito control operations (e. g. biopesticides) has largely been limited to their effects on vertebrates and macroinvertebrates. Information on potential impacts (positive or negative) of these control tactics on the dominant (native microbiota) components of wetland ecosystems is scarce (Boisvert and Boisvert 2000, Lacey and Merritt 2003). Microorganisms in aquatic habitats are considered indicators of the health of freshwater ecosystems.

We evaluated the effects of nutrients on mosquito production and growth characteristics of alkali bulrush across a gradient of nitrogen enrichment typically found in treatment wetlands. Alkali bulrush is an alternative emergent macrophyte to the large stature species routinely planted in constructed

treatment wetlands (Kanturd 1996). Nitrogen enrichment (in the form of ammonium nitrogen) significantly influenced mosquitoes and the growth parameters of alkali bulrush in our study. Mosquito abundance, dominated by the western equine encephalitis mosquito (*Culex tarsalis* Coquillett), in the enriched mesocosms was nearly twice the immature mosquito abundance found in unenriched mesocosms. Although the effect varied over time, nutrient enrichment over the gradient used in the study was not directly related to mosquito abundance. This suggests that mosquitoes production enriched wetlands is not necessarily a function of increase in nutrient levels but also reflects the type of aquatic plants and other associated variables (e. g. microbial communities) found in the larval habitats. A concentration range between 15 and 30 mg of ammonium nitrogen per liter was found the most favorable for growth of alkali bulrush, while high nutrient levels (> 50 mg of ammonium nitrogen per liter) suppressed the biomass of alkali bulrush. Nitrogen uptake by the plants increased directly with ammonium nitrogen levels, suggesting that this bulrush can be used as an alternative macrophyte in moderately enriched treatment wetlands.

We also characterized microbiomes associated with the western encephalitis mosquito and its larval habitats in simulated wetland mesocosms planted with two structurally different bulrush (California bulrush and alkali bulrush) species of bioremediation importance to understand mosquito-microbiome interaction in natural habitats. *Culex tarsalis* is naturally associated with vegetated wetlands

low in organic nutrients. Most previous studies on mosquito-*Bacteria* interactions were based on laboratory-reared mosquitoes using culture-dependent techniques (e.g. Chao and Wistreich 1959, Wistreich and Chao 1960), which have underestimated the extent of these interactions.

California bulrush is one of the dominantly planted aquatic macrophytes in many treatment wetlands in California and elsewhere and can be associated with high mosquito production (Walton 2003, Jiannino and Walton 2004). While California bulrush is almost three times the height of alkali bulrush, alkali bulrush is more structurally complex and harbors more invertebrate mosquito predators.

Although *Culex* species are generally considered filter feeders in the water column (Merritt et al. 1992), it was unknown whether the larvae of this mosquito feed on sestonic microorganisms or on epibionts of the two bulrush species. *Bacteria* diversity of mosquitoes sampled from the two plant habitats did not differ significantly, but nearly 49% of the *Bacteria* species found in the gut of *C. tarsalis* were similar to the *Bacteria* found in the water column and on the plants, revealing a strong mosquito-*Bacteria* interaction in their habitats. Mosquito larvae are known to ingest *Bacteria* (Merritt et al. 1992).

A Gram-negative *Bacteria* genus known as *Thorsellia* dominated (~64%) *Bacteria* communities found in the larvae of *C. tarsalis* collected from these habitats. This was the first report of *Thorsellia* from *Culex* genus and mosquitoes from North America. This genus was originally described from adults of malaria mosquito vectors (*Anopheles*) in Africa (Lindh et al. 2005, Kämpfer et al. 2006). It

was found to be the dominant *Bacteria* taxa found in the wild adult mosquitoes of *Anopheles gambiae* (Briones et al. 2009) in Kenya and was also present in the *Anopheles stephensi* larvae in India (Rani et al. 2009). The *Bacteria* is also known to be associated with rice field habitats (Briones et al. 2009). In our study it was also found associated at very low (<1%) abundance with the water and submerged surfaces of bulrushes from which *C. tarsalis* larvae were sampled.

There is a growing interest in using native microbiota associated with mosquitoes to combat vector-borne diseases (Ricci et al. 2011, Wang and Jacobs-Lorena 2013). However, one of the major hurdles of the current symbiotic control efforts has been the lack of efficient and stable symbionts that will carry anti-parasite effector molecules because mosquito-originated obligate symbionts are apparently rare (Wang and Jacobs-Lorena 2013). *Wolbachia*, a ubiquitous endosymbiont of many arthropods, is lacking in most wild mosquito species including in *C. tarsalis*. Our finding of *Thorsellia* from *Culex* species establishes the candidacy of this bacterium for further mosquito paratransgenesis studies.

A one-time application of high (twice the recommended label rate for polluted habitats) application of a commonly applied granular form of *Bti* formulation (VectoBac G) significantly suppressed phytoplankton (chlorophyll *a*), sestonic particulate ranging between 0.6- 1.0 um and 1.0 - 10.0 μm, inorganic nutrients (phosphorus and nitrogen) as well as the abundance of some *Bacteria* taxa. The taxa that were primarily affected by the high *Bti* application were one OTU of Cyanobacteria, *Cytophagales* and *Cyclobacteriaceae* (Bacteroidetes),

Sphingomonas (*Alphaproteobacteria*)*,* and *Polaromanas* (*Betaproteobacteria*)*.*

Mosquitoes were effectively controlled for nearly a month in mesocosms treated

with the high *Bti* application. It is currently unknown what component of the *Bti*

formulations is responsible for the changes in biotic and abiotic variables in the

water column observed above. The findings of our study highlight the need and

importance of assessing the impacts of mosquito control operations using the

currently available novel approaches. Traditional mosquito control assessment

techniques may have underestimated the potential impact of these control

tactics.

Deep sequencing of the hypervariable regions of the 16S rRNA genes using

next-generation technologies (e.g. Illumina platforms) has become the most

popular technique used to explore and characterize *Bacteria* communities from

the environment including the guts of disease vectors. Despite some limitations

(e.g. biases due to various methods of DNA extraction), this method overcomes

the major hurdles (e.g. detecting rarely occurring *Bacteria* species) previously

faced with the traditional PCR and other culture dependent techniques

(Caporaso et al. 2012). Moreover it is believed to be cost effective for the amount

of data generated per sample (Caporaso et al. 2012).

Mosquito management in their habitats has historically been the most

effective strategy to reduce mosquito-borne diseases. Integrated control

measures that targeted mosquito larvae and their habitats were responsible for

the eradication of mosquito-borne diseases (e.g. malaria) from the United States

and many other countries in the past. There is a renewal of interest in mosquito

larval ecology and control measures because control tactics that have dominantly

targeted adult stages over the past several decades have thus far fallen short of

established goals of eradicating mosquito-borne diseases worldwide (Ferguson

et al. 2010, Bukhari et al. 2013).

The studies described in this dissertation have important implications not only

for integrated management of mosquitoes in constructed wetlands but also for

other wetland mosquito habitats such as in irrigated agriculture (e.g. rice field

ecosystems) where interactions of aquatic plants, fertilizer and mosquito control

applications are likely to occur.

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