INFLUENCE OF AQUATIC MICROBES ON MOSQUITO OVIPOSITION BEHAVIOR AND LIFE HISTORY

By

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ABSTRACT

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Mosquito-microbe interactions have major roles in mediating mosquito life histories and the capacity of mosquitoes to transmit pathogens to humans. While essential to better understanding mosquito life histories, our knowledge of how microbes influence oviposition behavior and development remains limited, especially in complex polymicrobial communities. This dissertation tested the relationship between aquatic microbial communities and mosquito oviposition behavior. Field studies were conducted in Michigan to investigate how different leaf-litter derived microbial communities (and modifications of the microbes present in those communities) effected mosquito oviposition and survivorship. Additionally, field (Ouidah, Benin) and laboratory assays were used to determine the effect of a bacterial toxin (mycolactone, produced by *Mycobacterium ulcerans*) on other aquatic microbes and the downstream impacts of those interactions on mosquito oviposition. *Ae. japonicus japonicus* (Theobald) mosquitoes preferred red oak (*Quercus rubra*) leaf-derived leachates over other leaf species tested (sycamore *Platanus occidentalis*, and honeysuckle *Lonicera maackii*). Leachates displayed distinct bacterial and fungal communities with sterilization of these communities causing a reduction in oviposition. Larval mosquito growth and survivorship were influenced by both leaf type and leachate concentration. The addition of mycolactone, hypothesized to inhibit microbial quorum sensing, to environmental water samples altered bacterial community composition and reduced *Aedes* egg laying. In addition to reducing oviposition of *Aedes aegypti* (L.) in a dose-dependent manner, mycolactone up- and down-regulated expression levels of multiple taxa (N = 13) and

functional groups (N = 13), suggesting the toxin plays an important role in interactions between *M. ulcerans*, other environmental microbes, and mosquito behavior. These studies highlight the essential role mosquito-microbe interactions play in oviposition behavior and life history and expand our knowledge of the microbial underpinnings of mosquito oviposition behavior. Additionally, experiments testing the relationship between the microbiota of aquatic macroinvertebrates and environmental conditions were conducted in Ostana, Italy using high-throughput amplicon sequencing. While both environmental conditions and macroinvertebrate species influenced the internal microbiome, species had a larger effect than environmental conditions, suggesting that physiological conditions in macroinvertebrate guts plays a role in what microbes are able to colonize and survive.

To all the excellent instructors I've had along the way

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PREFACE

Due to delays associated with the ongoing COVID-19 pandemic, there were three assays that were not completed as of the time this dissertation was submitted. Aims for chapter two (leaf litter microbial communities) included extraction and sequencing of mosquito gut bacterial and fungal communities (ten larvae from each leachate) to allow for comparison to the microbes present in leachates to determine what groups of microbes in the environment successfully colonize larval mosquitoes. Additionally, sequencing (bacterial and fungal) of antibiotic treated leachates is still in progress. In chapter three, additional samples were also collected to determine the effect of mycolactone dosages (0, 0.05, 0.5, 0.8 µg/ml) over time (four collection timepoints over seven days) on fungal communities. As of June 20, 2021, all samples for the above questions (N = 210) have been submitted to the MSU genomics core facility but sequencing has not been completed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ANOVA - Analysis of Variance

ASV - Amplicon Sequencing Variants

BTI - *Bacillus thuringiensis*

BU - Buruli Ulcer

CEV - Coincidental Evolution of Virulence

DNA - Deoxyribonucleic Acid

DO - Dissolved Oxygen

EtOH - Ethanol

FDR - False Discovery Rate

HSD - Honest Significant Difference

ITS - Internal Transcribed Spacer

KW - Kruskal-Wallis

MDA - Mean Decrease Accuracy

MI - Michigan

MS - Mean Squares

MU - *Mycobacterium ulcerans*

MW - Mann-Whitney

OOB - Out of Bag

ORP - Oxidation-Reduction Potential

PCoA - Principal Coordinates Analysis

PCR - Polymerase Chain Reaction

PERMANOVA - Permutational Multivariate Analysis of Variance

qPCR - Quantitative Polymerase Chain Reaction

RCF - Relative Centrifugal Field

RNA - Ribonucleic Acid

RNA-seq - Ribonucleic Acid Sequencing

RO - Reverse Osmosis

SD - Standard Deviation

SEM - Standard Error of the Mean

SS - Sum of Squares

UniFrac - Unique Fraction Metric

USA - United States of America

VOCs - Volatile Organic Compounds

WHO - World Health Organization

CHAPTER ONE:

INTRODUCTION

Our rapidly growing understanding of the impacts of microbial communities on disease systems and human health has led to numerous medical advances and potential solutions to major infectious diseases (Round and Mazmanian 2009, Honda and Littman 2012, The Human Microbiome Project 2012). Microbiome research has greatly expanded our knowledge of vector borne diseases and opened new avenues for their control and treatment (Bourtzis et al. 2014, Jupatanakul et al. 2014, Preidis and Hotez 2015). Arthropod vectors and the diseases they transmit, such as malaria, dengue and onchocerciasis rely on complex interactions between vectors biology, microbial communities, and hosts to spread; and controlling these diseases by traditional methods alone remains a challenge (Bourtzis et al. 2014, Coon et al. 2014). In the malaria transmission cycle, microbial communities have been found to impact every stage, from determining where mosquitoes will lay eggs (Trexler et al. 2003, Ponnusamy et al. 2015), to attracting mosquitoes to human hosts (Verhulst et al. 2011, Takken and Verhulst 2017) and mediating pathogenesis (Boissière et al. 2012, Dennison et al. 2014).

One of several emerging avenues of disease control is the manipulation of microbial symbionts. In mosquitoes, using microbial symbionts such as *Wolbachia* and *Asaia*, has the potential to control mosquito populations (Bourtzis et al. 2014, Dacey and Chain 2020) and limit or eliminate disease transmission (Xi et al. 2005, Hughes et al. 2011, Iturbe-Ormaetxe et al. 2011, Mousson et al. 2012). *Wolbachia* is widespread in insect species and can influence reproduction through a variety of mechanisms including parthenogenesis and cytoplasmic incompatibility (Bourtzis et al. 2003, Veneti et al. 2003, Bourtzis et al. 2014). In native mosquito hosts and when artificially introduced into mosquitoes that do not normally harbor it, *Wolbachia* can reduce

populations through cytoplasmic incompatibility and increase the ability of mosquitoes to resist infection (Dimopoulos 2003, Cirimotich et al. 2011, Bourtzis et al. 2014, Gomes et al. 2017). Symbiont mediated control may also be useful for the control of a variety of other neglected tropical diseases (Preidis and Hotez 2015, Adebayo et al. 2017). In the tsetse fly (Diptera: Glossinidae), naturally occurring endosymbionts mediate the fly's ability to become infected with trypanosomes (Weiss et al. 2013). Components of the gut microbiota of phlebotomine sandflies (Diptera: Psychodidae) and filarial worms (Nematoda: Onchocercidae) have been identified as a potential target for leishmaniasis control (McCarthy et al. 2011) and as novel targets for onchocerciasis treatment (Hoerauf et al. 2000).

In addition to improving our understanding of symbiont-mediated control efforts, a better understanding of mosquito-microbial community interactions may improve microbial and insecticidal methods of control as well (Dacey and Chain 2020). While *Bacillus thuringiensis israelensis* (Bti), the most commonly used microbe-based control agent for larval mosquitoes (Zhang et al. 2017), has limited effects on non-target organisms, it can alter the internal microbiome of mosquitoes (Receveur et al. 2018, Tetreau et al. 2018) and microbes in larval habitats (Duguma et al. 2015a). Similarly, other insecticides (e.g., malathion and permethrin) increase the gut bacterial richness of *Aedes albopictus* and *Culex pipiens* (Juma et al. 2020), but the downstream effects of these changes on mosquito life histories, and how microbes contribute to the breakdown of these control compounds remains largely unknown.

Interactions between mosquitoes and aquatic microbial communities

A substantial body of research has investigated the relationship between mosquito-microbe interactions, larval habitats, and oviposition behavior (Merritt et al. 1992, Walker et al. 1997, Kim et al. 2015, Day 2016). Larval mosquitoes use bacteria, fungi, and other microorganisms as major

components of their diet (Hinman 1930, Merritt et al. 1992, Wallace and Merritt 2004). Most mosquito species (with a few exceptions) are omnivorous as larvae, showing little discrimination in the type of food ingested (Merritt et al. 1992). With such behavior, it is unsurprising that recent investigations of the factors shaping *Ae. japonicus* gut microbial communities found strong influences of the aquatic habitat on internal bacterial communities (Kim et al. 2015, Muturi et al. 2018, Juma et al. 2021). Organic detritus (e.g., leaf litter) provides both a substrate and the nutrients needed for the development of complex microbial communities in larval mosquito habitats, with composition and abundance of microbes varying depending on the quality and quantity of organic inputs (Fish and Carpenter 1982, Walker et al. 1991, Kaufman et al. 2010, Muturi et al. 2013). Not all microbes present in larval habitats readily colonize mosquito guts. Multiple studies have found reduced bacterial richness in the gut compared to the aquatic environment suggesting gut physiology (e.g., pH, available space for colonization, etc.) plays a role in determining what microbes are able to successfully colonize larval mosquitoes (Dada et al. 2014, Kim et al. 2015, Coon et al. 2016).

Microbial impacts on growth and development

Egg hatching in *Aedes* mosquitoes can be stimulated by changes in water chemistry, namely a reduction in dissolved oxygen (DO)(Gjullin et al. 1941, Horsfall et al. 1958, Ponnusamy et al. 2011). Recent investigation has suggested that both a reduction in DO due to microbial activity and the presence of bacteria are involved in stimulating egg hatch behavior (Ponnusamy et al. 2011). After hatching, larval mosquitoes display distinct microbiomes by instar (Duguma et al. 2015b, Pennington et al. 2016, Receveur et al. 2018) with the presence of microbes and microbial induced hypoxia cues for larval development (Coon 2017). Without the presence of microbes, larval mosquitoes fail to molt but this can be rescued by the addition of bacteria (Coon

et al. 2014). The internal microbial communities present in mosquitoes differs substantially between developmental stage, sex, species, and environmental conditions (Minard et al. 2013). Generally, bacterial communities are more diverse in earlier instars compared to later instars and newly hatched adults, though some taxa from early instars can be found across developmental stages (Wang et al. 2011, Duguma et al. 2015b). Investigations of individual or small groups of microbes (e.g., yeasts, entomopathogenic bacteria/fungi) have shown strong positive and negative impacts on larval survival (Scholte et al. 2004, Bukhari et al. 2010, Steyn et al. 2016) but the effects of more complex communities on survivorship are poorly understood.

Microbial impacts on oviposition behavior

The selection of oviposition sites by gravid females is a critical component for the survival of their offspring (reviewed in Bentley and Day 1989, Day 2016). Mosquitoes use complex behaviors in conjunction with a wide range of receptors (e.g., visual, olfactory, and touch) to identify and evaluate the suitability of potential egg laying sites (Day 2016). Olfactory cues given off by these potential sites are largely in the form of Volatile Organic Compounds (VOCs). Mosquitoes use VOCs from a variety of sources including decaying plant material, aquatic microbes, biofilms, and conspecific larvae (Warburg et al. 2011, Ignell and Hill 2020). Most VOCs given off by microbes are products (or by-products) of metabolic pathways (e.g., production of hydrocarbons or breakdown of amino acids) with groups of microbes displaying distinct VOC profiles (Schulz and Dickschat 2007). The preference of mosquitoes for oviposition substrates and infusions with different microbes/volatile profiles is largely species specific (Day 2016). Some species have specific preferences for individual VOCs (e.g., aggregation pheromones produced by *Culex* species) while other species are dependent on a blend of volatile compounds being present and have only limited responses to individual compounds (Dawson et al. 1989, Du and Millar

1999a, Day 2016). Similarly, individual bacteria taxa can have positive influences on oviposition in one species while causing a reduction in oviposition in other species (reviewed in Girard et al. 2021). To add to the complexity of microbial influences, the attractiveness of single microbial isolates can differ depending on concentration and metabolic activity (Zhang et al. 2015, Girard et al. 2021). For example, the inhibition of quorum sensing in *Staphylococcus epidermidis* (induced through a gene mutant) leads to a reduction in attractiveness to *Ae. aegypti* compared to wild-type *S. epidermidis* (Zhang et al. 2015). With microbial communities in larval habitats changing in response to organic inputs, biomass, and fermentation time, there remains a need for additional studies of the influence of groups of microbes on mosquito oviposition (Ponnusamy et al. 2010c). From influencing pathogens to mediating host fitness and development, microbial communities influence a wide variety of vector-borne diseases, many of which represent unique challenges for human health. A better understanding of these critical interactions between microbes and mosquito species will improve our control strategies and progress towards the reduction or elimination of a variety of public health risks.

Research objectives

The primary goal of the following work was to investigate the relationship between aquatic microbes present in the larval environment and mosquito oviposition behavior and life history. The larval environment has long been recognized as a major determinant in the behavior and life history of mosquito species but there has been limited work combining mosquito assays using natural or near-natural bacterial and fungal communities with enumeration and identification of microbes present by high-throughput sequencing. Towards this goal, leaf-litter derived microbial communities were tested individually and manipulated in several ways (sterilization, antibiotic treatment, addition of a bacterial toxin) to examine the effects of changing microbial communities

on mosquito behavior. A secondary goal of this research was to further explore the relationship between aquatic macroinvertebrates, their microbiota, and their habitat and expand the ability of others to use sequencing tools to investigate these interactions and utilize publicly available sequencing data. The specific goals of this research were:

1. Test how microbial communities derived from several common types of leaf litter influences mosquito oviposition behavior and larval survivorship (Chapter 2).
2. Examine how mycolactone toxin (produced by *Mycobacterium ulcerans*) alters aquatic microbial communities (Chapter 3).
3. Investigate how the manipulation of natural aquatic communities with mycolactone alters mosquito oviposition behavior (Chapter 3)
4. Explore the relationship between aquatic macroinvertebrates microbiota and environmental conditions for several additional species of macroinvertebrates (Chapter 4)
5. Describe three case studies that provided hands-on training and workshop materials related to the analysis of high-throughput sequencing data (Chapter 5).

CHAPTER TWO:

LEAF LITTER DERIVED MICROBIAL COMMUNITIES IMPACT THE LIFE HISTORY AND BEHAVIOR OF *AEDES JAPONICUS JAPONICUS*

Abstract

Mosquito egg-laying preferences and larval growth have strong and long-lasting impacts on mosquito life histories. A major determinant of mosquito oviposition behavior and larval growth is the microbial community of the associated aquatic habitat; however, the specific mechanisms of this relationship remain unknown. This study evaluated the effects of leaf-litter (oak (*Querus rubra*), sycamore (*Platanus occidentalis*), and honeysuckle (*Lonicera maackii)*) derived leachate microbial communities on *Aedes japonicus japonicus* (Theobald) oviposition behavior and life history using a combination of field oviposition assays, laboratory growth experiments, and manipulations (autoclave sterilization and antibiotic treatment) of bacterial and fungal communities. Oviposition differed significantly among leaf-litter species with oak leaves attracting the highest oviposition by *Ae. j. japonicus*. The sterilization of leaf leachate led to a reduced egg-laying response compared to unmodified leachates, while treatment with antibiotics had a synergistic effect. Bacterial and fungal communities were distinct with multiple taxa differentially abundant among leachate types. For example, *Curvibasidum* and *Rhodosporidiobolus* were present in significantly greater abundance in oak leachates than all other leachates while *Sphingobium* and *Novospingobium* were significantly (greater and lower in abundance, respectively) in honeysuckle compared to all other leachates. A better understanding of the relationship between aquatic microbial communities and mosquito behavior and development will improve our ability to develop new control strategies for mosquito-borne diseases.

Introduction

In lentic aquatic habitats, such as vernal ponds and tree holes, leaf detritus commonly comprises a large portion of the resident organic matter (Baldy et al. 2002) and contributes significantly to productivity of these habitats (Yee and Juliano 2006). Aquatic insects are highly dependent on both the quantity and quality (Carpenter 1983, Walker et al. 1997, Yee and Juliano 2006) of organic matter and use a variety of cues to select suitable habitats for oviposition (Day 2016). Mosquitoes for example, use both short (contact to cm) and long (meters) distance cues to identify and evaluate oviposition sites (Day 2016). These cues include a variety of visual (Beehler et al. 1992, Gibson 1995), tactile (Thavara et al. 2004) and olfactory sources (Huang et al. 2006, Choo et al. 2015). Although individual chemical attractants, such as octenol, are strong enough to elicit a response and commonly used in trapping efforts, previous research has suggested that female attraction in natural settings may be more a response to blends of compounds rather than a single powerful attractant (Du and Millar 1999b, Day 2016). Many of the chemical cues used by mosquitoes are derived from volatile organic compounds (VOCs) given off during the decomposition of leaf litter or the microbes associated with those processes (Ponnusamy et al. 2008a, Ponnusamy et al. 2010a). These complex decomposer communities can change considerably as decomposition progresses (Benbow et al. 2018), and can alter the attractiveness of oviposition sites in a species dependent manner (Lampman and Novak 1996, Trexler et al. 1998a).

As the potential for co-occurrence of invasive mosquitoes and invasive plant species rises with climatic change (Dukes and Mooney 1999, Going and Dudley 2008), there is a timely need for additional research into how mosquitoes respond to leaf litter (and associated microbes) from both native and invasive plants (Reiskind et al. 2010, Muturi et al. 2015). For example, the invasive honeysuckle (*Lonicera maackii*) has been shown to increase the survivorship and body size of

Culex pipiens mosquitoes (Shewhart et al. 2014) and alter the life history traits of other vector species (Gardner et al. 2015, Muturi et al. 2015). All mosquito life stages, from egg to adult, are influenced by environmental microbes, though there is a need for more information regarding what taxa drive these interactions (Merritt et al. 1992, Kaufman et al. 1999, Kaufman et al. 2008, Ponnusamy et al. 2008b, Coon et al. 2014, Receveur et al. 2018).

Leaf leachates from oak and other native plant species have commonly been used in oviposition traps targeting *Aedes* mosquitoes (Trexler et al. 1998b) and research has repeatedly identified oviposition attractants and stimulants in leaf infusions with different mosquito species showing preference for different leaf species, infusion ages, and concentrations (Allan and Kline 1995, Lampman and Novak 1996, Trexler et al. 1998b, Ponnusamy et al. 2010c). In laboratory assays, mosquito oviposition behavior was significantly affected by total bacterial concentration as well as bacterial species composition (Ponnusamy et al. 2010b). Fungal communities also play major roles in aquatic leaf litter decomposition (Pascoal et al. 2005); however, there has been limited investigation of how these communities influence mosquito oviposition behavior or life history traits (Kaufman et al. 2008, Malassigné et al. 2020). Much of the recent investigation into fungal impacts on oviposition has been related to single fungal isolates or secondary metabolites towards the goal of identifying attractants (Sivgnaname et al. 2001, Eneh et al. 2016).

In addition to influencing the oviposition behavior of gravid females, aquatic microbes have strong effects on the growth and development of larval mosquitoes (Merritt et al. 1992, Coon et al. 2017, Strand 2017, Valzania et al. 2018) and subsequent adult traits (Dickson et al. 2017). Mosquito larvae largely acquire their gut microbiome through feeding (Strand 2017, Juma et al. 2021). It is well established that microbes are important for mosquito larval growth and development, where reducing concentrations of bacteria or the sterilization of rearing water has

deleterious effects on growth and mortality (Hinman 1930, Strand 2017). For instance, larvae provided nutritionally complete but sterile food were unable to reach pupation (Coon et al. 2014). However, many of these reported effects have yet to be investigated under natural settings, which can differ substantially from assays conducted under laboratory conditions (Day 2016), as the microbial communities between natural and laboratory settings are presumably different. The food and habitat available to larval mosquitoes has long lasting impacts on mosquito life histories including impacts on adult size (Lyimo et al. 1992), longevity (Joy et al. 2010), and the ability to transmit or become infected with arboviruses or malarial parasites (Alto and Lounibos 2013, Vantaux et al. 2016).

A better understanding of how leaf species and associated microbial communities mediate mosquito behavior and life histories has the potential to lead to new integrated control strategies for mosquito borne diseases, such as baited attract and kill traps (Muema et al. 2017). The objectives of this study were: i) To test mosquito oviposition responses to the microbial communities derived from various leaf litter species types; ii) Investigate how different communities and concentration of microbes influenced larval mosquito growth and survivorship and iii) Test how different leaf litter types affected aquatic microbial communities.

Materials and Methods

Leaf collection

Leaves from northern red oak (*Quercus rubra*), sycamore (*Platanus occidentalis*), and honeysuckle (*Lonicera maackii*) were collected with a gloved hand from the campus of Michigan State University (East Lansing, MI, USA). Leaves were collected from several trees and combined to form a single composite for each species. The leaves were visibly inspected for the absence of insect activity or discoloration. After collection, leaves were dried at 70 ◦C until constant mass then stored in paper bags at room temperature until use (within 2-3 weeks of collection).

Leachate preparation

For Assay 1, each preparation of leachate leaf material (8.4 g/L RO purified water) of each species was placed in open containers, crushed with a gloved hand into small pieces, and incubated for seven days. A seven-day incubation period was chosen based on previous studies examining the effect of incubation length on leachate attractiveness (Trexler et al. 1998b, Ponnusamy et al. 2010c). The leaves were incubated with constant aeration for seven days at room temperature as described previously (Shewhart et al. 2014). To account for evaporation during the incubation, the water level of each container was checked each day and sterile water was used to return each container to the starting volume. For Assay 2, mixed leachate was incubated similarly but with 2.8 g/L of each oak, sycamore, and honeysuckle leaves (8.4 g/L leaf material total).

To remove any influence of large plant material on results, before use, all leachates were filtered through sterile 125 µm mesh. Four separate preparations of leachate were made as describe above, one used for each assay listed below. Immediately before use, 50 ml water samples (N = 3) were

taken from each leachate to provide baseline microbial community profiles for each leachate type (see below).

Impacts of leachates on mosquito oviposition

Field Assay 1: Replicates (N= 15) of each leachate species (red oak, sycamore, honeysuckle, and a control (reverse osmosis purified water) were arranged up in a complete block design in Baker Woodlot on the campus of Michigan State University, a wooded old growth forest in East Lansing, MI, USA (August 24-27, 2017). Each block was at minimum 50 m away from any other block. Within each block, 250 ml of each treatment were placed in a 1 L black plastic container and randomly placed in a cardinal direction 1 m from a central point. Seed germination paper was used to line the inside of each container to collect eggs. Containers were open to mosquito oviposition for 72 hours, during which no measurable precipitation was observed, before collection. Each germination paper was stored separately and transported to the lab to count eggs and for use in laboratory assays. A subset of mosquito eggs (> 100 individuals) collected from each oviposition assay were reared to 4^{th} instars or adults for identification. Additionally, larvae used in survivorship and growth assays (see below) were also identified when possible (i.e., those that survived or grew beyond first instars). All mosquitoes were identified using dichotomous keys from Darsie and Ward (2005).

Influence of leachate microbial manipulation on oviposition

Field Assay 2: In August 2018 to test for microbial effects on mosquito oviposition, oak and mixed species (2.8 g/L of each oak, sycamore, and honeysuckle leaves, 8.4 g/L total) leachates were prepared as described above. Two different manipulations of the leachates were performed, sterilization and an antibiotic treatment. For the sterilization manipulation, aliquots of oak and

mixed leachates were sterilized by autoclaving (121◦C at 15 psi for 45 minutes). The two sterilized leachates were set out in a blocked design (11 blocks) with unsterilized aliquots of the same leachate and egg-laying was measured similarly as above. The antibiotic manipulation used two broad spectrum antibiotics Ampicillin (antibacterial 200 µg/ml, inhibition of gram - and + bacteria, Sigma Aldrich) and Amphotericin B (antifungal, 5 µg/ml, ThermoFisher) added individually and in combination to oak leachate (chosen due to oak having the highest oviposition response in previous assays) following the seven-day incubation. Each block for this experiment (N = 8) contained one oviposition cup containing unmodified oak leachate, one containing ampicillin only, one with amphotericin B, and one with ampicillin and amphotericin B. Concentrations of ampicillin and amphotericin B were double the recommended dosages for cell culture work to ensure the antibiotic maintained efficacy in the leachates. To limit the microbial breakdown of the antibiotics before the trial while still allowing the antibiotics to alter the microbial communities, leachates were incubated for 24 hours prior to being placed in Baker woodlot, similar to previous assays.

Larval survivorship

To determine the survivorship of larvae exposed to each leachate, single second instar larvae (collected during assay one and hatched in RO water with fish food (TetraMin) were placed in 20 ml of a leachate treatment (N = 20 per leachate and leachates were prepared as described above) and placed in a growth chamber, similar to previously described methods (Shewhart et al. 2014). Larval survivorship was measured every 12 hours for 72 hours. Two separate assays were conducted, one using only undiluted leachates and a separate assay conducted with undiluted leachate compared to two other dilutions of leachate (1/2X and 1/4X) and a sterilized leachate (undiluted). Dilutions were made using sterile water. All experiments conducted in a growth

chamber occurred at 29◦C with a 16:8 light dark cycle. Using the same preparations of leachate as the survivorship assays, water chemistry parameters (pH, DO, conductivity, ORP) were also recorded. Parameters were recorded from a 250 ml aliquot of each leachate/concentration every 12 hours for 72 hours using a YSI multiparameter probe (Yellow Springs, Ohio).

Larval growth

Assay 4: To measure the effect of each leachate and concentrations on larval growth, five first instar larvae were placed in 20 ml of leachate (N = 10 replicate containers per leachate) and placed in a growth chamber. After seven days, mosquito larvae were removed from the leachate and preserved. Only larval mosquitoes alive at the time of collection were used for growth measurements with collected mosquitoes preserved in 90% EtOH. Head capsule width was measured using a stage micrometer calibrated to mm. Larval mass was measured after removing ethanol and allowing an additional six hours for any residual ethanol to evaporate. Additionally, larval length was measured from the anterior-most point of the thorax to the posterior margin of the 10^{th} abdominal segment using a dissecting scope and ImageJ software (Schindelin et al. 2015). Water chemistry (Temperature, Dissolved Oxygen, pH, conductivity) during larval survivorship assays was measured every twelve hours over the course of the experiment using a YSI multiparameter probe.

DNA extraction and sequencing

Water samples (50 ml, taken after removal of large leaf material) were centrifuged at 7,500 RCF for 15 minutes. The resulting pellet was then extracted using the PowerSoil DNA Isolation kit (Qiagen). Lysozyme (15 mg ml^{-1}, Invitrogen) was added during the lysis step, with the rest of the extraction process following the manufacturer's protocol. Extracted DNA was quantified using

a Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen) and a Qubit 2.0 (ThermoFisher). All DNA preparations were stored at -20 ◦C until sequencing. Region-specific primers 515F/806R (5′-GTGCCAGCMGCCGCGG-3′, 5′-TACNVGGGTATCTAATCC-3′) were used to amplify variable region 4 of the 16S rRNA gene according to previously described methods (Claesson et al. 2010, Caporaso et al. 2011b). Fungal sequencing was performed using the fITS7/ITS4 primer set (5′- GTGARTCATCGAATCTTTG-3', 5′-TCCTCCGCTTATTGATATGC-3′) (Bokulich and Mills 2013), Invitrogen Platinum Hot Start master mix (Thermofisher), and QIAquick (Qiagen) PCR cleanup kit according to manufacturers instructions. Library construction and sequencing (2 x 250 bp paired-end reads, Illumina MiSeq) was performed by the Michigan State University Genomics Core Facility using a modified version of the protocol adapted for the Illumina MiSeq, described by Caporaso et al. (2011a).

Data analysis

Raw reads were demultiplexed, assembled, and quality-filtered in QIIME 2 (v 2020.11), using default settings (Bolyen et al. 2019). DADA2 was used to filter chimeric reads and artifacts (Callahan et al. 2016b). To classify filtered reads to taxanomic groups, a Naive Bayes classifier was trained using against the 16S rRNA region (V4, SILVA database v13.8) and the fungal Inter-Transcribed Spacer (ITS, UNITE database v8.99) region (Abarenkov et al. 2010, Quast et al. 2012). These trained classifiers were then used on their respective sets of reads and used to assign taxonomy. Bacterial reads (V4) assigned to mitochondria or chloroplast were removed from each sample before analysis. Sequencing reads were rarified in QIIME 2 based on alpha rarefaction plots.

Differences in taxanomic composition and alpha diversity (species richness, Simpson's diversity, Shannon diversity) among groups were tested with Kruskal-Wallis and Mann-Whitney

tests (FDR corrections) in R (v 2.4.4)(R Development Core Team 2017). Diversity estimates were calculated using phloseq (McMurdie and Holmes 2013). Egg counts met the assumptions of normality and differences among groups/blocks were analyzed with ANOVA tests and Tukey's Honest significant difference tests. Larval suvivorship among leachates was evaluated using Kaplin-Meyer tests calculated with the Survminer package (v0.4.9)(Kassambara et al. 2017). Differences in variability in bacterial and fungal beta diversity were tested using the weighted UniFrac distance (Lozupone et al. 2011) and PERMANOVA/ Betadispr tests using the Vegan R package(v 2.5-7) (Oksanen et al. 2015). Random forest modeling, to classify samples to treatment based on their microbial communities, was conducted using Out of Bag (OOB) error, 1000 trees, with top indicators chosen by Mean Decrease Accuracy (MDA) in the randomForest R package (v 4.6-14) (Liaw and Wiener 2002). Plots were created using a combination of base R, ggplot2 (v3.3.3), ggpubr (v 0.4.0), and phylseq (v 1.32.0)(Wickham 2011, McMurdie and Holmes 2013, Kassambara 2017). R code used in this analysis are avialable at https://github.com/JPReceveur/AedesLeafCommunities.

Results

Leaf leachate species effects on mosquito oviposition

Both leaf species leachate (Figure 2.1A) and block location had significant effects on mosquito oviposition (ANOVA, P > 0.05, Table 2.1), with leachate species ($F_{3,36}$ = 21.5) having a much stronger effect than block location ($F_{14,36}$ = 3.4). Oak leachate had the greatest mean oviposition (466, SEM ± 77), followed by sycamore (218 ± 44.1) and honeysuckle (202 ± 29.6), that had similar numbers of eggs (Tukey HSD, P-adj = 0.99, Figure 2.1B). Significantly fewer eggs were laid in RO water (68.9 ± 18.6 eggs) than oak and sycamore leachates (Tukey HSD, P-adj < 0.05), but the control was not different from honeysuckle (202 ± 29.6) (P-adj = 0.06).

Table 2.1. Impact of leachate type and block on mosquito egg laying. Test = ANOVA, SS= Sum of Squares, MS = Mean Squares.

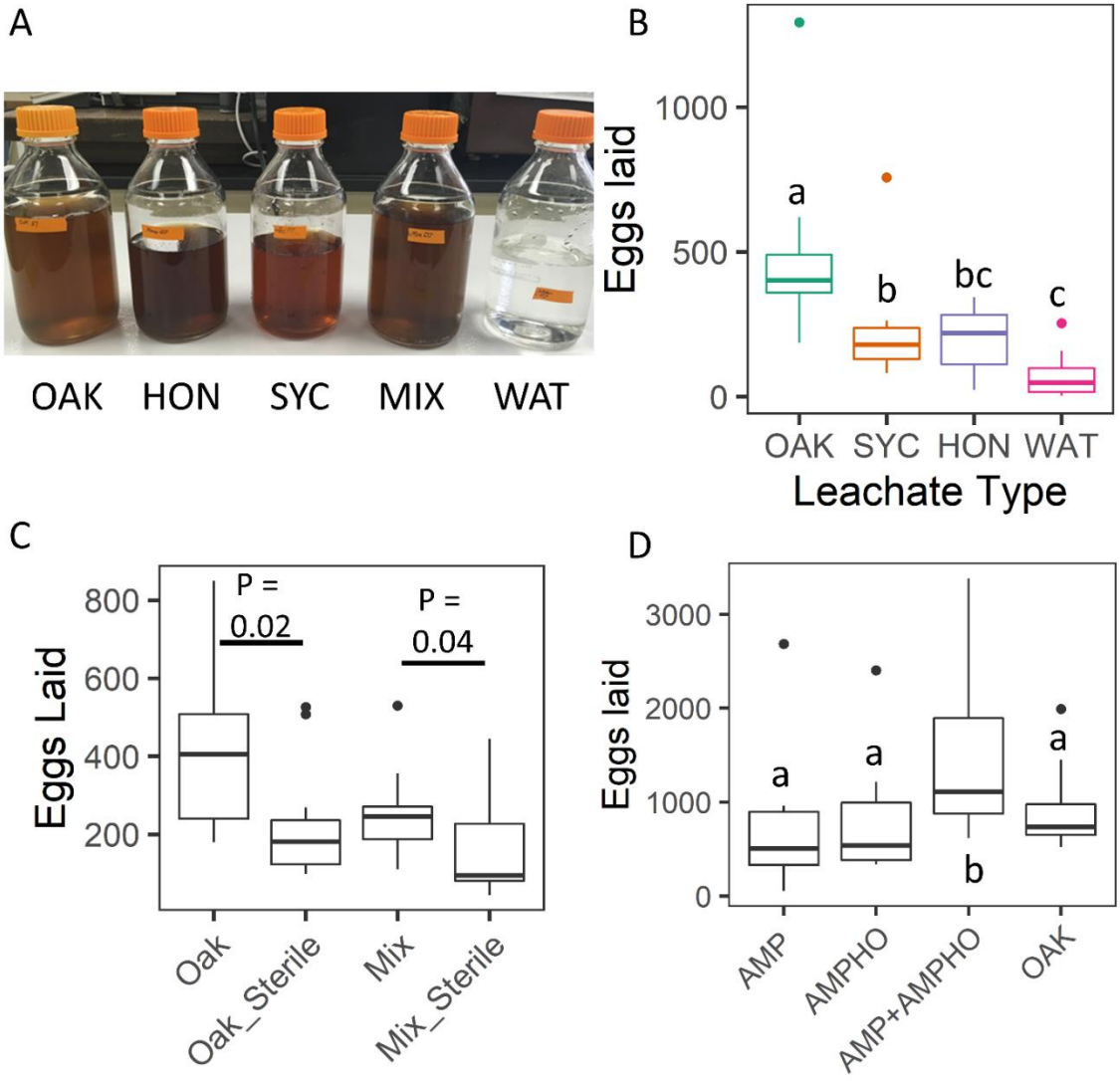
	Df	**SS**	**MS**	**F**	**P**
Leachate Type	3	1123039	374346	21.45	< 0.001
Block	14	838457	59890	3.43	0.001
Residuals	36	628078	17447		

Leaf leachate manipulation effects on mosquito oviposition

Manipulating leaf leachate microbial communities using both autoclave sterilization and antibiotic treatment changed oviposition compared to untreated leachates (Figure 2.1C, D). Sterilizing oak leachate significantly reduced mean oviposition by 192 eggs (Sterile = 226.2 SEM ± 45.8, Unsterilized = 418.0 ± 67.5, ANOVA, $F_{1,8}$ = 7.9, P = 0.02). Similarly, sterilizing the mixed leachate (equal combinations of oak, honeysuckle, and sycamore leaves) resulted in lower oviposition (158.9 ±37.9 eggs) than unsterilized leachate (255.8 ± 42.1, ANOVA, $F_{1,8}$ = 5.8, P = 0.04). Block did not have an effect on either oak or mixed trials (ANOVA, F < 2, P > 0.25).

The antibiotic treatments used to modify the leachate microbial communities also affected oviposition in oak leachates. Sample block (ANOVA, $F_{7,21} = 18.8$, $P < 0.001$) had a stronger effect than treatment ($F_{3,21} = 7.7$, $P < 0.01$). Neither the individual addition of ampicillin (anti-bacterial) or amphotericin B (anti-fungal) to oak leachates significantly altered the number of eggs laid compared to the control oak leachate (Tukey HSD, $P > 0.5$, Figure 2.1D). However, the combination of the two antibiotics resulted in increased oviposition (1476 ± 328 eggs) than in the oak leachate (944 ± 179.9 eggs, Tukey HSD, P-adj =0.02) or individual treatments (P-adj > 0.05).

Figure 2.1. Impact of leaf leachate species on mosquito oviposition, expressed as the number of eggs laid in a leachate or control. A) Representative photo of different leachate types after filtering. HON =honeysuckle, SYC = sycamore, MIX = mixed leachate containing 2.8 g/L of oak, sycamore and honeysuckle leaves (8.4g/L total), WAT = control water. B) Eggs laid by leachate type during Assay 1. Lowercase letters indicate significant differences in pairwise comparisons among groups (Tukey HSD, P-adj < 0.05). C) Impact of sterilization on mosquito egg laying. P values are from ANOVA tests testing number of eggs laid ~ Treatment+ Block. Block did not have a strong impact on either comparison (P> 0.25). D) Influence of antibiotic treatment on oviposition. AMP = Ampicillin (250 µg/ml, antibacterial) in oak leachate, AMPHO = Amphotericin B (5 µg/ml, antifungal) in oak leachate, AMP + AMPHO = combined ampicillin and amphotericin B in oak leachate, OAK = oak leachate only.



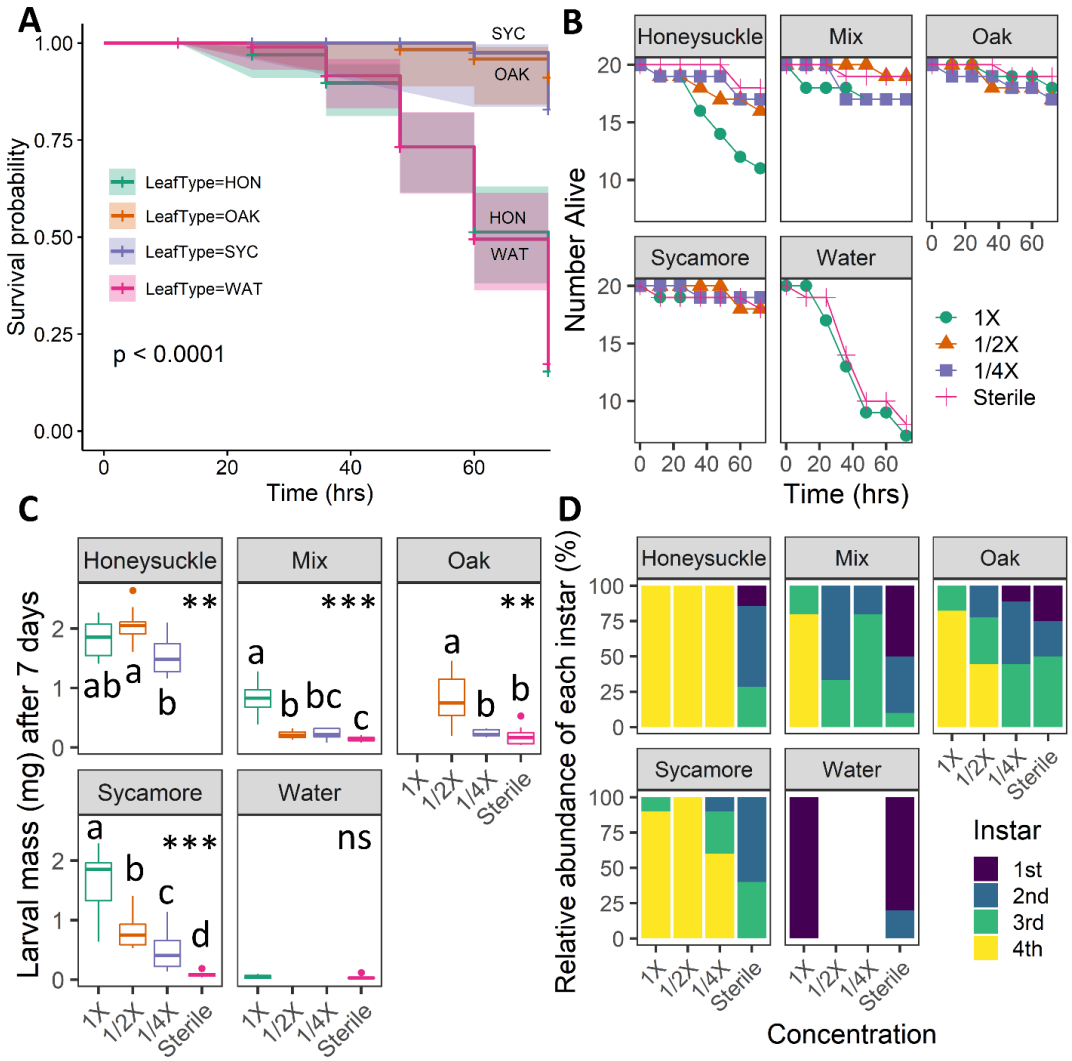
Leaf leachate effects on survivorship and growth

Of the more than 600 larvae that were hatched and reared for identification, greater than 95% were *Aedes japonicus japonicus* (Theobald) with the remainder having ambiguous characters due to damage and were not identified. Leaf leachate species and concentration both influenced larval survivorship and growth. Honeysuckle leachate and control water had significantly lower survivorship compared to oak and sycamore leachates (Kaplin-Meyer, $P < 0.01$, Figure 2.2A), with less than 25% of mosquito larvae surviving to 72 hours. When different concentrations of leachate were tested (1X, 1/2X, 1/4X, and 1X sterile), the 1X honeysuckle leachate and control water both resulted in fewer larvae surviving to 72 hours compared to other treatments (Figure 2.2B). Interestingly, larvae in lower leachate concentrations and sterilized honeysuckle leachate had similar survivorship to other leachates, with > 75% larvae surviving to 72 hours.

Larval mass at seven days differed by leachate and concentration (Kruskal-Wallis, $P < 0.05$). In general, reduced concentrations of leachates were associated with significantly lower larval mass (Figure 2.2C). Exceptions to this trend were the 1X and 1/2X honeysuckle as well as the 1/2X and 1/4 X mixed leachates (Mann-Whitney, $P > 0.05$). Larvae in sterile oak (0.20 mg ± 0.06) and mixed (0.14 mg ± 0.02) leachate had significantly higher mass than larvae reared in sterile control water (0.05 mg ± 0.02, Mann-Whitney, $P < 0.05$). While not quantified, multiple instances of cannibalism were observed during the growth experiments, predominantly in the sterile leachates. Larval instar at the end of the growth assay (seven days) differed by concentration with 1X concentration (8 g/L leaf material) of all leachate species resulting in predominantly 4^{th} instar larvae at seven days (with the exception of the water only treatment, Figure 2.2D). At lower concentrations, the predominant instar depended on leachate type. While 1/2X and 1/4X concentrations for honeysuckle and sycamore leachates resulted in predominantly 4^{th} instars,

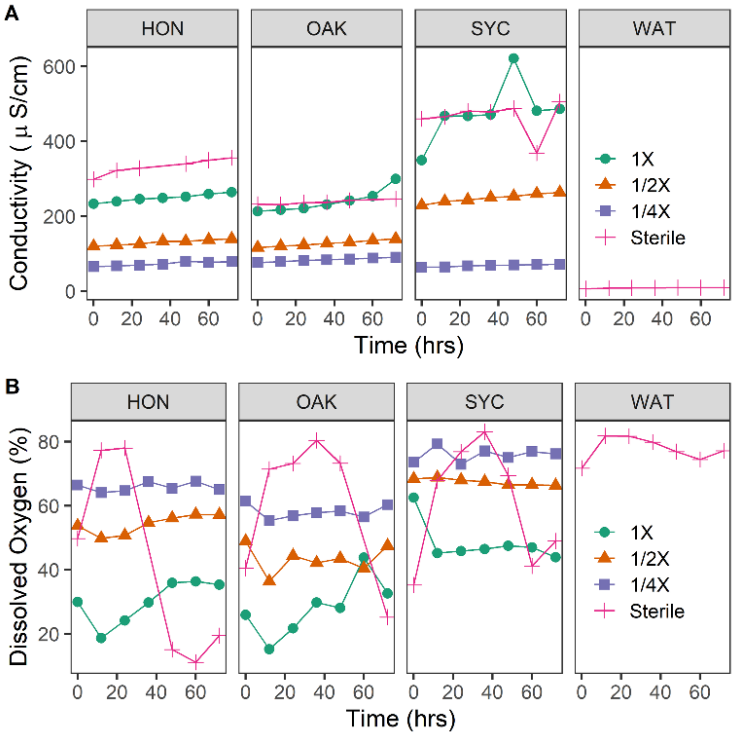
mixed and oak leachates had lower proportions of 4th instars. Notably, no larvae reached the 4th instar in the mixed leachate except for at 1X concentration.

Figure 2.2. *Aedes j. japonicus* larval survivorship and mass. A) Survival probability of larvae reared in 1X leachates. HON = honeysuckle, OAK= oak, SYC = sycamore, WAT = water. Shaded areas indicate 95% confidence intervals (Kaplan-Meier estimator). B) Number of larvae surviving by time and concentration. Sterile = sterilized 1X leachate. C) Larval mass at the end of seven-days growth. Overall tests were Kruskal-Wallis tests with a FDR correction. ** = P-adj < 0.01, *** = P-adj < 0.001, ns = not significant (P-adj > 0.05). Lowercase letters indicate significance in pairwise comparisons between groups (Mann-Whitney U test, FDR correction). D) Relative abundance of each instar by leachate and concentration at the end of growth trials. Oak 1X and honeysuckle sterile samples became desiccated during storage and mass was not measured



Leaf leachate species and concentration both influenced water chemistry. Conductivity was stable over 72 hours assay with honeysuckle, oak, and sycamore leachates showing similar trends, while having different ranges of conductivity (e.g., sycamore conductivity range: 63.8-621.1 µS/cm, Oak range: 76.3-299.9). Across all leachates, lower concentrations had lower conductivity than higher concentrations (Figure 2.3A). Dissolved oxygen showed the opposite trend of conductivity, with higher leachate concentrations having lower dissolved oxygen (Figure 2.3B). Sterile (autoclaved) leachates had higher variability in DO with concentrations decreasing during later timepoints in the sterilized leachates but not in the sterilized water treatment. pH was relatively stable over time and concentration with sycamore (7.73 ± 0.04) and water (7.78 ± 0.14) treatments having higher average pH than honeysuckle (7.40 ± 0.02) and oak (6.97 ± 0.03) leachates.

Figure 2.3. Water chemistry of leaf leachates by species and concentration. A) Conductivity by leachate species and concentration. B) Dissolved oxygen (%) by leachate type and concentration. Sterile indicates autoclaved leachate or water.



Leaf leachate bacterial communities

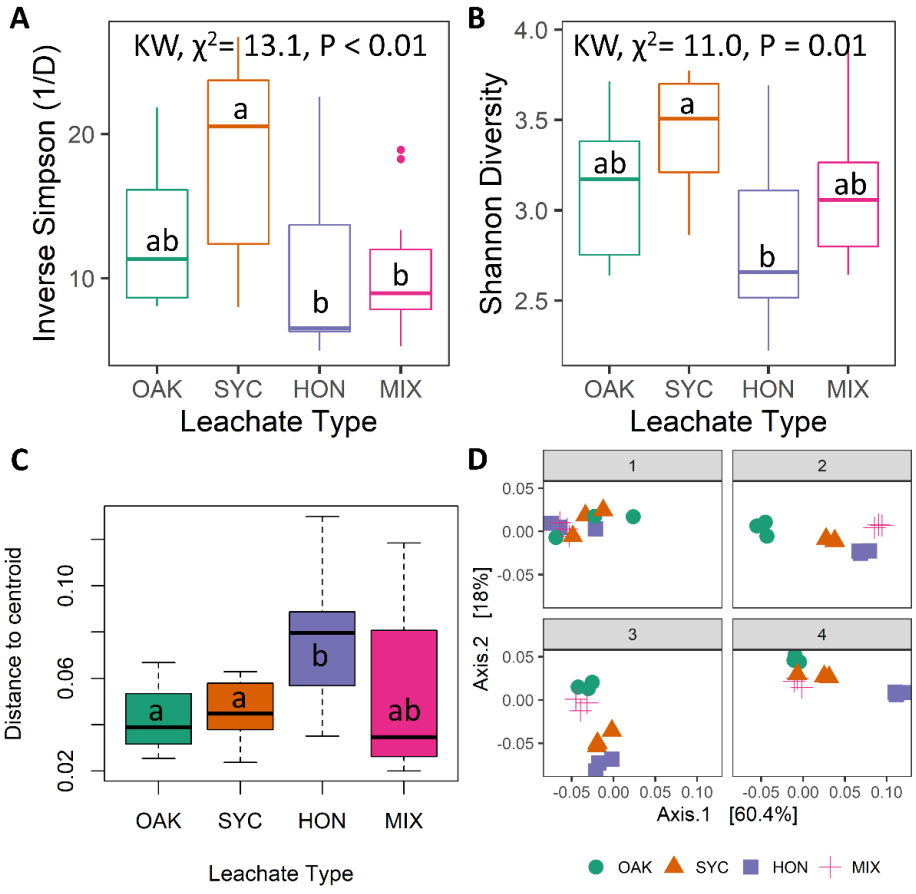
A total of 3,633,874 sequencing reads were obtained from 61 water and leachate samples with an average of 59,571 reads per sample. After rarefaction to 10,000 reads and removal of control samples (No sequencing or extraction control had greater than 1,400 reads, Figure S2.1), 48 samples and 640 Amplicon Sequencing Variants (ASVs) remained. While the number of sequencing variants did not significantly differ (Kruskal-Wallis, $\chi^2 = 5.9$, $P = 0.11$), both Shannon (KW, $\chi^2 = 10.99$, $P = 0.01$) and Simpson's diversity ($\chi^2 = 13.1$, $P < 0.01$) were different among leachate species (Figure 2.4A, B). Sycamore leachate community diversity was significantly greater than honeysuckle (Simpson's and Shannon) and mixed leachate (Simpson's only) (Mann-Whitney, P-adj < 0.05).

Both leachate species and trial (each trial being a separate preparation of leachate) had strong effects on beta diversity (weighted UniFrac). Combining all trials, bacterial communities from honeysuckle leachates had significantly greater variability than communities from oak (Vegan: betadisper, $F_{1,22} = 14.3$, $P = 0.002$, Bonferroni-corrected $\alpha = 0.0083$) and sycamore ($F_{1,22} = 10.67$, $P = 0.002$) (Figure 2.4C). Individual trials had similar variability in beta diversity ($F_{3,44} = 0.63$, $P = 0.58$). Species, trial, and their interaction all influenced bacterial beta diversity (Table 2.2); however, because of a lack of true replication within treatment in a trial (i.e., all samples within a trial come from the same preparation of leachate) pairwise comparisons within individual trials were not tested (Figure 2.4D). Across all trials, bacterial community structure of oak leachate was significantly different than honeysuckle (PERMANOVA, $F_{1,22} = 7.02$, $P = 0.003$, Bonferroni-corrected $\alpha = 0.0083$) and sycamore ($F_{1,22} = 5.05$, $P = 0.003$) with no differences among other leachate species or interactions.

Table 2.2. Impact of leachate species and trial on bacterial beta diversity. PERMANOVA test (weighted UniFrac distance) was conducted with 999 permutations. SS = Sum of Squares, MS = Mean Squares.

Factor	DF	SS	MS	F.Model	R^2	Pr(>F)
Species	3	0.11291	0.037638	27.083	0.21277	0.001
Trial	3	0.1877	0.062567	45.02	0.3537	0.001
Species:Trial	9	0.18559	0.020621	14.838	0.34972	0.001
Residuals	32	0.04447	0.00139	0.0838		
Total	47	0.53068	1			

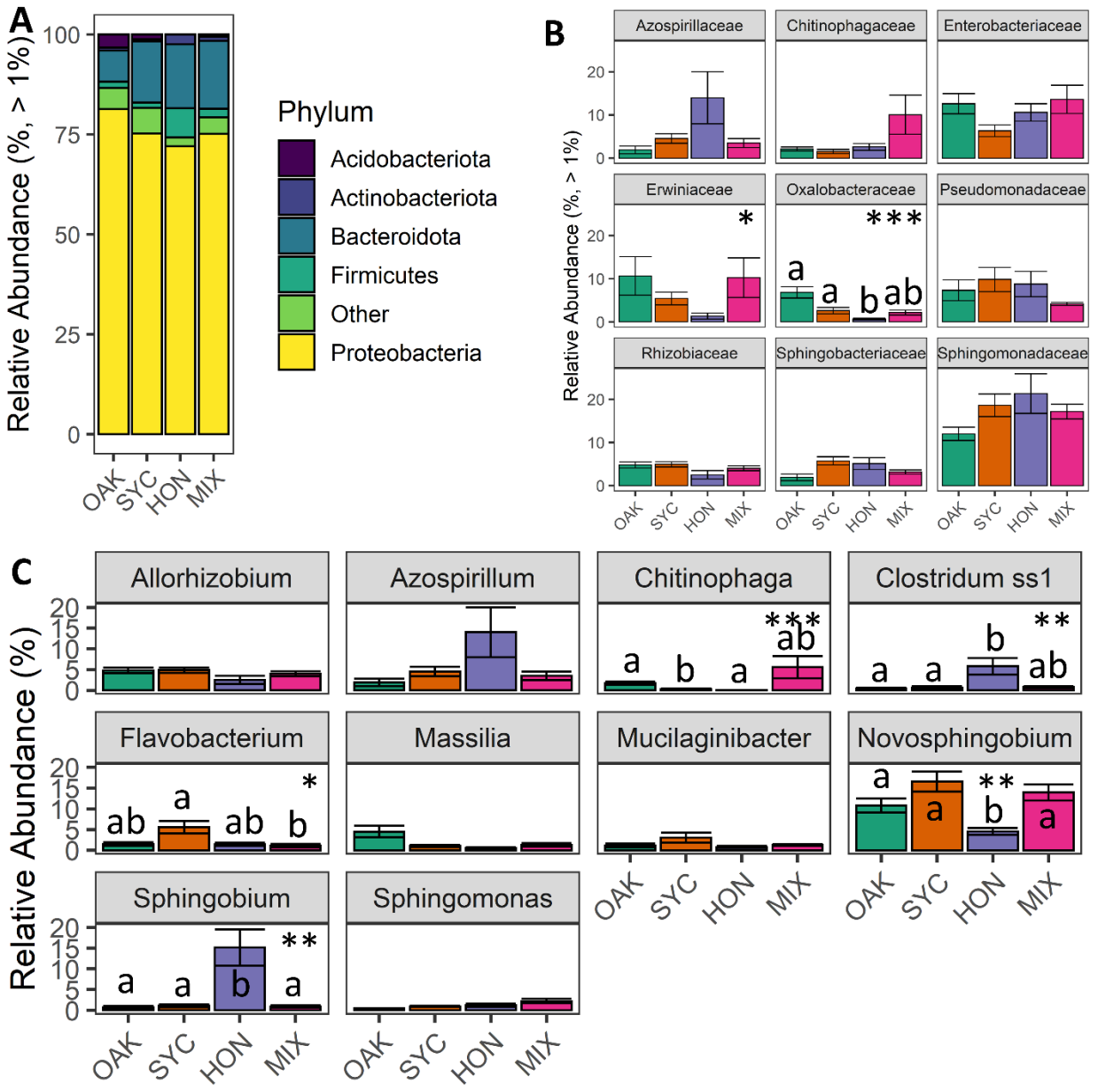
Figure 2.4. Leaf leachate bacterial community diversity. A) Inverse Simpson's diversity by leachate type. HON = honeysuckle, OAK = oak, SYC = sycamore, MIX= mixed. KW = Kruskal-Wallis. Lowercase letters indicate pairwise comparisons between groups (Mann-Whitney U test, FDR adjusted P values < 0.05). B) Shannon diversity by leachate species. C) Variability in beta diversity (weighted UniFrac distance). Distance to centroid indicates the distance between an individual sample and the mean of that group (i.e., higher numbers indicate greater variability in microbial communities). D) Principal Coordinates Analysis (PCoA) plot of bacterial beta diversity. Numbers indicate leachate preparations (1-4).



Among all samples, Proteobacteria was the most abundant phylum (75.9% of relative abundance), followed by Bacteroidota (formerly Bacteroidetes, 14.1%) and Firmicutes (3.1%). Two out of the five phyla which comprised greater than 1% of the total relative abundance across all samples, were significantly different among leachate species: Firmicutes and Acidobacteria, KW, P-adj < 0.05, Figure 2.5A). Firmicutes comprised 7.3% (SEM ± 2.3) of relative abundance in honeysuckle leachate, while only 1.3% (± 0.56) in sycamore (MW, P-adj < 0.05). Acidobacteria were nearly significantly different among leachate species and the mixed leachate (P-adj < 0.06), being largely absent from honeysuckle (0.03% relative abundance, ± 0.01). At the family level, two (Erwiniaceae and Oxalobacteraceae) out of the nine families which comprised greater than 1% of the total relative abundance differed significantly by leachate (KW, P-adj < 0.05, Figure 2.5B). The relative abundance of Oxalobacteraceae was higher in oak (6.84% ± 1.3) and sycamore (2.62% ± 0.73) leachate compared to honeysuckle (0.64% ± 0.19, Mann-Whitney, P-adj < 0.05), while none of the pairwise comparisons for Erwiniaceae reached statistical significance (P-adj > 0.05).

Random Forest modeling of bacterial communities revealed distinct differences among trials and leachate species with multiple taxa as strong indicators for individual leachate species. Using genus level bacterial taxonomy, models predicted leachate species (4% Out of Bag error rate, two samples misclassified) and trial (All samples classified successfully to trial) with a high degree of accuracy. Of the top ten bacterial indicators for leachate, five (*Sphingobium*, *Novosphingobium*, *Chitinophaga*, *Clostridum sensu stricto*, *Flavobacterium*) genera significantly varied by leachate species (KW, P-adj < 0.05, Figure 2.5C). Notably, *Sphingobium* and *Novosphingobium* abundances were significantly higher and lower in honeysuckle, respectively, compared to other leachate types (Mann-Whitney, P-adj < 0.05).

Figure 2.5. Bacterial community composition by leaf leachate species. A) Phylum level bacterial abundance. Only phyla which comprise greater than one percent of the total relative abundance across all samples are shown. B) Family level relative abundance by leachate species. Error bars indicate Standard Error of the Mean (SEM). Overall group differences were compared with Kruskal-Wallis tests (FDR correction). * = P-adj < 0.05, *** = P-adj < 0.001. Pairwise comparisons are denoted by lowercase letters (MW, P-adj < 0.05). No pairwise comparisons for Erwiniaceae were significant. C) Top genus level indicator taxa for classifying samples to leachate type. Indicators were chosen by mean decrease accuracy in the random forest model. *Clostridium* ss1 = *Clostridium sensu stricto* 1.



Leaf leachate fungal communities

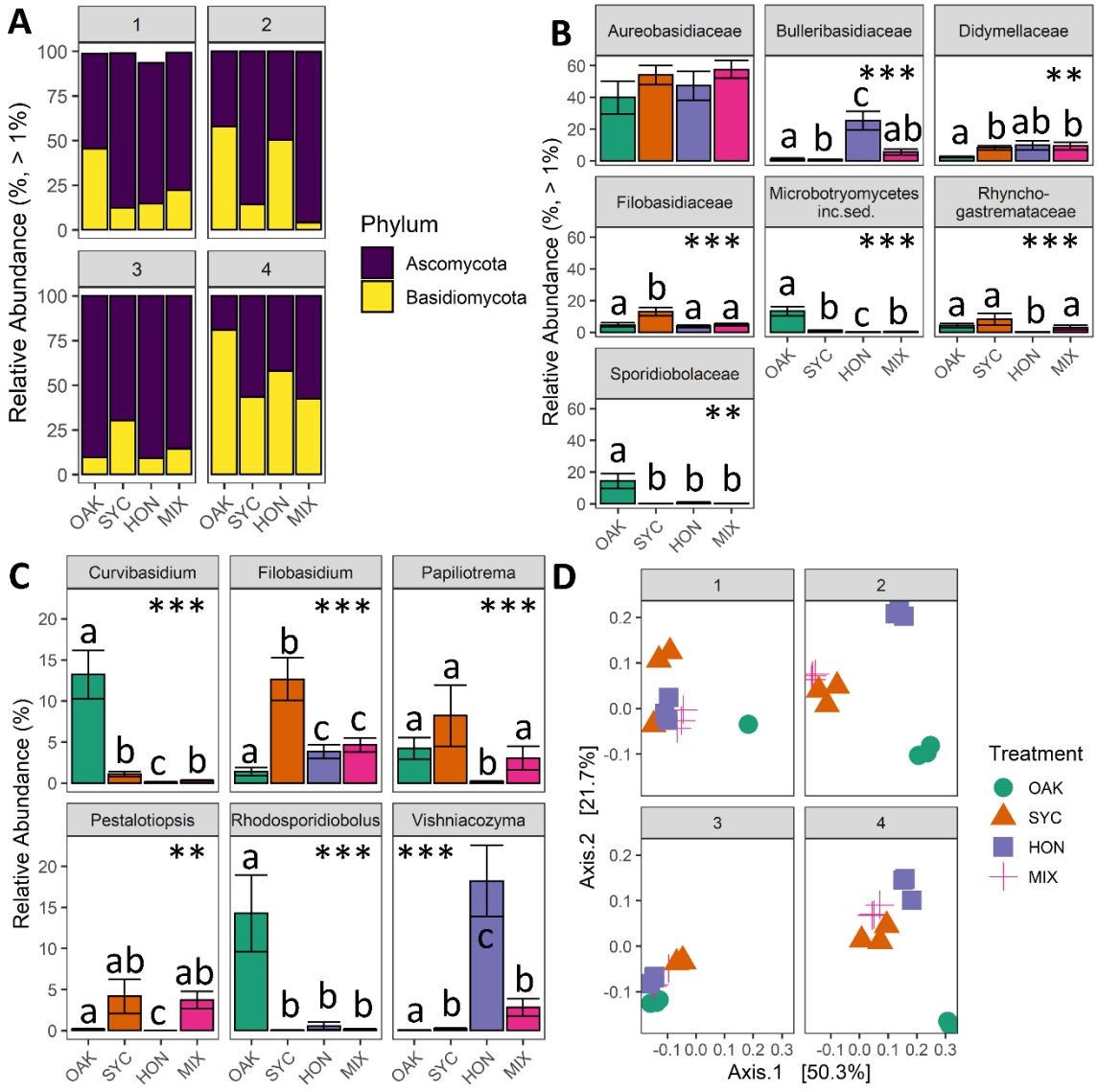
A total of 2,519,215 sequencing reads were obtained from 58 samples with an average of 43,434 reads/sample. After filtering, rarefaction to 4,000 reads, and removal of controls (sequencing controls and water samples, Figure S2.2), 46 samples and 306 Amplicon Sequencing Variants (ASVs) remained. There was no difference in fungal species richness, Shannon, or Simpson's diversity (1/D) among the communities of leaf leachate species (KW, $P > 0.05$). The combined abundance of the top two phyla, Ascomycota and Basidiomycota, represented greater than 99% of the total relative abundance across all samples. Across all trials, neither of these phyla differed significantly among leachate species (KW, $P > 0.5$, Figure 2.6A). Of the seven fungal families which each comprised greater than 1% of the total relative abundance, six differed in abundance among leachate species (Figure 2.6B). Microbotryomycetes *Incertae sedis* (*Inc. sed.* indicates relationship to broader taxonomic groups unclear) and *Sporidiobolaceae* were both higher in oak leachate, while *Bulleribasidiaceae* was higher in honeysuckle than all other leachate species (MW, P-adj < 0.05).

Using genus level taxonomy, random forest modeling was able to predict leachate species, with a 13% Out Of Bag (OOB) error rate (six samples misclassified). Five of the six misclassifications were associated with the mixed treatment: two sycamore leachates and one honeysuckle were misclassified as mixed with two true mixed samples incorrectly classified (one as each sycamore and honeysuckle). Of the top ten genus level indicator taxa, eight significantly varied in abundance among leachate species (KW, P-adj < 0.05, Figure 2.6C). *Curvibasidium* and *Rhodosporidiobolus* were strong indicators of oak leachate, both comprising greater than 10% average relative abundance in oak, while less than 2.5% in all other leachate species (MW, P-adj

< 0.05). *Filobasidium* and *Vishniacozyma* were present at higher abundances in sycamore and honeysuckle leachate, respectively, than all others.

All leachates had similar variability in their fungal beta diversity (Jaccard distance, Vegan: Betadispr, $F_{3,42} = 2.19$, $P = 0.11$). Treatment (PERMANOVA, $F_{3,30} = 22.7$, $P < 0.001$) trial ($F_{3,30} = 26.0$, $P < 0.001$), and their interaction ($F_{9,30} = 10.4$, $P < 0.001$) had strong effects on beta diversity with treatment accounting for 25.1% (R^2) of the variability in beta diversity (Figure 2.6D).

Figure 2.6. Fungal communities by leachate type. A) Phylum level fungal abundance by leachate preparation (1-4). Only taxa which comprised greater than one percent of the total microbial communities are shown. B) Family level fungal abundance. Overall test for differences between groups was a Kruskal-Wallis test with an FDR correction. ** = P-adj < 0.01, *** = P-adj < 0.001. Pairwise comparisons (MW, P-adj < 0.05) are denoted by lowercase letters. C) Abundance of top fungal indicators (determined by Mean Decrease Accuracy) in a random forest model classifying samples to leachate type. Overall test for differences was a Kruskal-Wallis test with Mann-Whitney test used for pairwise comparisons. D) Principal Coordinates Analysis (PCoA) plots of fungal beta diversity (Jaccard distance). Numbers (1-4) indicate individual preparations of leachate.



Discussion

In this study, the effect of different species of leaf leachate was found to influence mosquito oviposition (in outdoor settings) and the growth/survivorship of subsequent larvae. Leachates derived from oak, sycamore, and honeysuckle leaves all affected *Ae. j. japonicus* oviposition. While the importance of understanding mosquito oviposition and larval growth to vector control efforts has led to a substantial body of research regarding the attractiveness of various leaf leachates to mosquitoes (reviewed in Day 2016), there remains a need to couple field-based oviposition assays with assessments of the microbial communities from complex leachates under environmental conditions (Ponnusamy et al. 2010b, Ponnusamy et al. 2010c, Muturi et al. 2013, Mwingira et al. 2020). Laboratory oviposition assays provide ample opportunities to test relationships between mosquitoes and specific oviposition stimulants (e.g., VOCs produced by microbes), but findings may not be directly applicable to natural oviposition behavior (Day 2016). By necessity, most laboratory assays focus on short-range and contact cues (i.e., oviposition stimulants and deterrents), which represent only the final component of site-seeking behavior for gravid females (Day 2016). Before encountering stimulants or deterrents in natural settings, females must first detect and orient towards longer range cues (i.e., attractants). This influence of both short- and long-range cues on oviposition behavior makes studies of oviposition in natural settings an important step in investigating the influence of individual volatile compounds or microbial communities (Benzon and Apperson 1988). The observation of increased egg-laying numbers due to a compound in a laboratory assay may suggest utility as bait for an attract/kill trap, but without longer range attractants causing gravid females to orient and close the distance to a source, the influence of close-range stimulants is limited. To that end, we combined field mosquito assays with sequencing of the bacterial and fungal communities associated with leaf-litter derived

leachates as a step towards better understanding the role microbes play in mosquito life histories and behavior.

Our observation that greater than 99% of individual larvae reared for identification were from a single species *Ae. j. japonicus* while other species are commonly observed in the same area (e.g., *Culex* spp, *Ae. triseriatus*) (Kaufman et al. 2014) was surprising. This was likely due to a combination of timing (late summer), habitat/methodology (artificial containers in an old growth woodlot), and a greater preference of *Ae. j. japonicus* for artificial containers (relative to other species in the area). While Kaufman et al. (2014) observed relative abundances of hatched *Ae. j. japonicus* ranging from 43- 95% during a three-year period in lower Michigan (MI), Lorenz (2012) observed similar abundances as our oviposition assays with greater than 99% of hatched larvae being identified as *Ae. j. japonicus*. Additionally, while the storage conditions after collection (7 days at 23 ˚C were adequate for ending diapause for both *Ae. j. japonicus* and *Ae. triseriatus* (Lorenz 2012, Kaufman et al. 2014) it may not be adequate for hatching all species found in lower MI.

Ae. j. japonicus preferred to oviposit in oak leachates compared to other types. Oviposition in sycamore and honeysuckle leachate was similar with control water having the lowest average number of eggs. These results are largely in line with previous investigations of leaf litter impacts, where different leaf species have variable impacts on oviposition rates (Reiskind et al. 2009, Afify and Galizia 2015, Muturi et al. 2015). While not an exhaustive comparison of leaf types found in southern Michigan, the observed preference of *Ae. j. japonicus* for oak leaves over other species suggest that leachates from northern red oak (*Querus rubra*) leaves are a readily available attractant for oviposition traps targeting *Ae. j. japonicus* in Michigan.

While filtering out large particulates (above 150 µm) removed the effect of larger pieces of leaf material, our investigations of microbial effects on oviposition and larval growth cannot be completely uncoupled from effects of the leaves themselves. Microbial signals represent a major component of the cues gravid females use when seeking an oviposition site but other plant derived cues (i.e., tannins, phenolic compounds etc.) can also contribute to site selection and suitability (Day 2016). Similar to Reiskind et al. (2009), fresh, rather than senesced, leaves were used in the current study. Investigations of fresh vs senescent leaves have been previously shown to have variable effects on larval aquatic macroinvertebrate growth (Walker et al. 1997, Kochi and Kagaya 2005, Pelz-Stelinski et al. 2010). With most soluble leaf components being released early in decomposition (within 24 hours) (Carpenter 1982, Webster and Benfield 1986), the soluble secondary compounds (e.g., tannins from oak leaves or the variety of allelopathic compounds present in honeysuckle leaves) (Cipollini et al. 2008) were likely completely leached by the end of the seven day incubation. Honeysuckle leachate led to high levels of growth and development with a higher mass and proportion of larvae reaching 4th instar by the end of the experiment than any other leachate (regardless of concentration), similar to the positive impacts on larval growth found by Shewhart et al. (2014) for *Culex pipiens*. While Shewhart et al. (2014) found that honeysuckle concentrations more than double that used in the current study (20 g/L senesced leaf material) had high levels of survivorship (*Culex,* > 80% over 72 hours), we observed lower survivorship at an 8.4 g/L concentration (1X). This discrepancy and observed dose dependent effect (by honeysuckle leachate only) on survivorship may be due in part to an increase in secondary products and metabolites present in fresh honeysuckle leaves, which have been shown to influence insect feeding behavior (Cipollini et al. 2008).

The finding that individual treatment with an antibacterial or antifungal agent did not reduce oviposition and even increased oviposition when the two agents were combined was surprising and not in line with previous oviposition experiments which show marked decreases in oviposition when leachates are treated with antibiotics (Benzon and Apperson 1988, Navarro et al. 2003). Anecdotally, the combined ampicillin and amphotericin B treatment had a much stronger smell after the 24-hour incubation than the other leachates. Our observation that sterilization reduced oviposition behavior for both the oak and mixed leachates is consistent with previous investigations (Benzon and Apperson 1988, Navarro et al. 2003) and supports the hypothesis that microbial attractants/ stimulants are only a single part of the varied set of cues female mosquitoes use when identifying suitable oviposition sites (Afify and Galizia 2015, Day 2016, Mwingira et al. 2020).

Leachate microbial communities

There were some differences in diversity that were consistent across multiple preparations (i.e., higher Simpson and Shannon bacterial diversity in sycamore vs honeysuckle leachates) while diversity also varied between different preparations of leachates. These differences were likely at least partially due to differences in the microbes colonizing starting leaf material and chemical composition (%N, C:N ratios, etc.,), shown previously to impact the microbial communities of decomposing leaf litter and soil (Bray et al. 2012, Stanek and Stefanowicz 2019). There were numerous taxonomic groups that differed between leachates across all trials. For example, *Sphingobium* and *Novosphingobium,* two genera of Alphaproteobacteria, differed in abundance between honeysuckle and all other leachates. Though our sequencing method did not allow for determination of exact species, many of the described species within these two genera have the ability to degrade volatile aromatic and phenolic hydrocarbons (Liang and Lloyd-Jones 2010,

Wang et al. 2018), components found in high abundance in honeysuckle leaves (Cipollini et al. 2008). Similar to bacteria, the abundances of number of fungal families and genera differed between leachates. *Rhodosporidiobolus* (Sporidiobolales), members of which have been isolated from a variety of leaf species (Valério et al. 2002, Lorenzini and Zapparoli 2019), was present in oak leachates, but largely absent from other leachate types. Both bacterial and fungal taxa differed between leachate type and our investigation has identified several taxa differentially abundant between leachates which may warrant future investigation to better explain the response of mosquitoes to particular leachates and aquatic microbes.

Microbial species richness and diversity did not have a clear association with oviposition behavior, suggesting that the composition (beta diversity) of microbial communities may be more important than simply the number of taxa. Oak leachates, while having similar bacterial and fungal diversity as sycamore and honeysuckle leachates, had significantly greater oviposition, though our comparisons were likely influenced by other differences between leachates that can influence mosquito egg laying (e.g., tannins, phenolic compounds in leaves, differences in bacterial concentrations). Previous investigations using a single species of starting material (bamboo) have found mosquito oviposition response to be strongly impacted by bacterial diversity (Ponnusamy et al. 2010b). With the internal microbial communities of *Ae. j. japonicus* largely dependent on their environment (Juma et al. 2021), a better understanding of the microbes present in larval environments and how they influence growth and development is essential for increasing our understanding of vector competence and mosquito life histories.

Conclusion

In this study, we observed that leachates derived from different leaf species had variable and dose-dependent impacts on *Ae. j. japonicus* growth and survivorship. Similarly, oviposition behavior differed between leachates with modification of leachates (i.e., sterilization or antibiotic treatment) altering egg-laying response. Leaf type had strong impacts on the bacterial and fungal communities with multiple taxa such as *Sphingobium, Novosphingobium, and Rhodosporidiobolus* differentially abundant between leachate types representing potential targets for future study. A better understanding of the interactions between microbial communities and mosquitoes will increase our knowledge of mosquito oviposition and development, critical components of developing integrated control programs for vector-borne diseases.

CHAPTER THREE:

MYCOLACTONE TOXIN ALTERS AQUATIC MICROBIAL COMMUNITIES AND ASSOCIATED MOSQUITO OVIPOSITION BEHAVIOR

Abstract

Interactions between environmental microbes and toxins produced by opportunistic pathogens remain an understudied area of community ecology especially in the context of neglected tropical diseases. Mycolactone, the major toxin and determinant of virulence for the opportunistic pathogen *Mycobacterium ulcerans* (causative agent for Buruli Ulcer, a neglected tropical skin disease), impacts the gene expression of other individual bacteria species and mosquito oviposition behavior, though the effects of this toxin on complex communities of microbes remain largely unknown. This study investigated the impact of mycolactone toxin on complex microbial communities and mosquito oviposition behavior using laboratory and field (Ouidah, Benin) behavior assays, amplicon sequencing of aquatic bacterial and fungal communities, and transcriptomics. The addition of mycolactone to aquatic microbial communities led to a dose-dependent reduction in mosquito (*Ae. aegypti*) oviposition. Bacterial and fungal communities were significantly affected by the addition of mycolactone with richness, diversity, and the abundance of multiple taxa differing significantly among treatments. The expression levels of several organisms (including *Mycobacterium spp.*) and thirteen functional groups were differentially abundant between ecologically relevant low (0 + 0.05 µg/ml mycolactone) and high (0.5 + 0.8 µg/ml) concentrations. Investigations into the interactions between mycolactone, other aquatic microbes, and a hypothesized vector (mosquitoes) will improve our understanding of the ecological role of mycolactone toxin in *M. ulcerans* fitness individually and opportunistic pathogens more generally. The *M. ulcerans*-Buruli ulcer system provides an ideal system to investigate microbe-toxin interactions in the context of a coincidental evolution of virulence

hypothesis, where the presumed primary role of toxins is to improve fitness of environmental microbe in natural habitats and only opportunistically cause human disease.

Introduction

Buruli ulcer (BU) is a necrotizing skin disease caused by the pathogen *Mycobacterium ulcerans* (MU). MU is naturally found in aquatic habitats and its DNA (via qPCR) has been reported from a range of invertebrate and vertebrate hosts and environmental reservoirs (Merritt et al. 2010, Röltgen and Pluschke 2015, Combe et al. 2017). The disease is endemic to approximately 30 countries, predominantly in Africa and the South Pacific (Benbow et al. 2017, WHO 2017) and symptoms are caused by mycolactone, a polyketide toxin encoded by a large plasmid (George et al. 1999, Yip et al. 2007, Walsh et al. 2008). BU is one of only two diseases (closely related leprosy is the other) on the WHO's list of neglected tropical diseases where the predominant mode of transmission remains unknown (Benbow et al. 2017, WHO 2017). Epidemiological data have shown that proximity to disturbed water bodies and skin punctures are risk factors for contracting BU (Williamson et al. 2014, Wallace et al. 2017), while wearing long sleeves, using bed netting, and cleaning injuries with alcohol reduce this risk (Merritt et al. 2010, Röltgen and Pluschke 2015). These risk factors, combined with case and pathogen prevalence, as well as the association of disease with both human communities and aquatic environments, have led to a significant body of research into the potential of mosquitoes acting as vectors of MU, though it is only one competing hypothesis among several (Receveur et al. IN PRESS).

While there is no support for the biological transmission of MU, or any other bacteria by mosquitoes, previous research has shown that a puncture can lead to MU transmission in animal models (Williamson et al. 2014), and that mosquitoes can mechanically infect mice through skin punctures when the animal skin is coated with MU (Wallace et al. 2017). In aquatic environments, mosquito larvae readily take up MU through feeding, though it does not remain detectable in adult mosquitoes after pupation (Wallace et al. 2010). Once they emerge as adults, it has been shown in

laboratory assays that mosquitoes can reacquire MU on their external surfaces, including their proboscis, but this has not been tested using environmental water sources containing MU, only using monocultures of MU (Johnson et al. 2007, Wallace et al. 2010). If this hypothesis holds true under field conditions, while MU does not survive pupation, when the adults emerge from the water it is plausible that they may be coated with MU and other microbes from the larval habitat. However, the ability of adult mosquitoes to acquire enough viable MU to be infective or successfully disperse MU to a different habitat remains unknown. Due to the slow growing nature of MU, and difficulties to isolate as a pure culture, most research into the MU-BU disease system has focused on using amplicon-based methods such as PCR (Williamson et al. 2012, Benbow et al. 2015). While highly sensitive, PCR confirmation of the presence of MU on external surfaces of mosquitoes does not by itself indicate whether the pathogen is viable or that mosquitoes are involved in its transmission. In addition, previous work suggests that mycolactone is a cue for mosquito oviposition and has impacts on growth and development (Sanders et al. 2017, Mashlawi et al. 2020); however to date all published research on the role of MU in mosquito behavior has focused on MU or mycolactone in isolation and not as part of a dynamic, naturally occurring microbial community. Therefore, there is a need for experiments to elucidate how the interactions between MU, and the more complex biotic environment influence the behavior of mosquitoes.

Mycolactone toxin is immunosuppressive with well-known effects on human cells and disease pathology (Hall et al. 2014) but its role in the environment remains largely unknown (Sanhueza et al. 2019). Mycolactone is involved in the down-regulation or disruption of a number of cell processes including cell adhesion and membrane properties (López et al. 2018) as well as blocking the production of proinflammatory mediators post-transcriptionally (Simmonds et al. 2009, Hall et al. 2014). The strong effect of mycolactone on human virulence, environmental

lifestyle of *M. ulcerans*, lack of human-to-human transmission, and expression of mycolactone in the environment makes the MU-BU system an ideal system to study a variety of theories about pathogen evolution (Dhungel et al. 2021).

One of these hypotheses, the Coincidental Evolution of Virulence (CEV) challenges the idea that toxins which cause virulence in humans are present due to a positive selection pressure towards involvement in human disease. Generalist microbes in the environment are challenged by a varied array of conditions and interactions for which novel or unique methods of suppressing other microbes may provide a reproductive advantage (Brown et al. 2012). In the case of MU, the negligible role of human-to-human interactions on transmission (O'Brien et al. 2017), and broad distribution of mycolactone production in other mycobacteria not commonly associated with human disease (Yip et al. 2007), suggest an environmental role is more likely as the main utility of the mycolactone toxin to MU. Comparable environmental associations can be seen in other taxa which product toxins with pathological effects in humans. *Clostridium botulinum* (producer of Botulinum, the most potent known neurotoxin) commonly occurs in aquatic sediments and interacts with both benthic invertebrates and microbes (Shukla and Sharma 2005, Rocke and Bollinger 2007, Pérez-Fuentetaja et al. 2011). Similarly, a number of *Vibro cholerae* virulence factors are produced and active in aquatic environments independent of disease, contributing to the persistence and survival of *V. cholerae* (Sakib et al. 2018).

Though the effects of mycolactone on diverse communities of microbes has not been previously investigated, several studies have sought to understand the interactions of MU or mycolactone and pure culture isolates of fungi and bacteria taxa. Hammoudi et al. (2019) observed that mycolactone enhanced spore germination of several fungal species and was attractive to *Mucor circinelloides* colonies. Dhungel et al. (2021) found that mycolactone reduced *S. aureus*

hemolytic activity and greatly influenced *S. aureus* metabolism. The previously observed effects on mosquito oviposition and microbe-microbe interactions led to the hypothesis that adding mycolactone to environmental water samples would reduce mosquito oviposition by altering the community composition and gene expression of aquatic microbial communities in a dose dependent manner. To test this hypothesis, the effects of mycolactone on mosquitoes and aquatic microbes were examined using two undefined communities of aquatic microbes (one created from the leaching of leaf material, and a second from environmental water sources in an endemic region). The objectives of this study were to: i) Determine how mycolactone would alter the oviposition behavior when introduced to polymicrobial communities; and ii) Characterize the effects of mycolactone toxin on microbial composition and gene expression in complex aquatic communities.

Materials and Methods

Mosquito assays: laboratory

Northern red oak (*Quercus rubra*) leaves without visible insect activity or discoloration were collected from the campus of Michigan State University, (MI, USA) with a gloved hand as described previously (see Chapter 2) for creating a leachate for mosquito oviposition (Shewhart et al. 2014). Leaves were dried to constant mass at 80 °C and stored in paper bags out of direct sunlight at room temperature until use. To create the leaf leachate, 8.4 g of dried leaves were added to 1 L of distilled water and crushed with a gloved hand. Leaves were incubated with constant aeration for seven days at room temperature with water (purified by reverse osmosis) added throughout incubation to account for evaporation. Four preparations of leachate were prepared, each corresponding to a separate generation of mosquitoes used for the laboratory assays. Immediately prior to the addition of mycolactone, leachates were filtered through sterile 125 µm mesh to remove any large plant material. Purified mycolactone, prepared as described previously (Adusumilli et al. 2005, Sanders et al. 2017), was suspended in EtOH and added at three concentrations (final concentrations = 0.8 µg/ml, 0.5 µg/ml, and 0.05 µg/ml) while EtOH was added to the control at the same concentration as the highest treatment (12.8 µl/L solvent). These concentrations, and the use of EtOH as a solvent, were chosen based on previous work to represent a high, intermediate, and low environmental dose of mycolactone (Williamson et al. 2012, Sanders et al. 2017). After the addition of mycolactone, leachates were incubated for 24 hours at room temperature, out of direct sunlight. Immediately before oviposition assays, 50 ml aliquots were collected and preserved at -20 °C until DNA/RNA extraction.

Laboratory colonies of *Aedes aegypti* (Rockefeller strain) were maintained at 37 °C and 70% relative humidity with a 16:8 light-dark cycle. Aliquots (40 ml) of each treatment (0.8 µg/ml,

0.5 µg/ml, 0.05 µg/ml and control) were added to cups lined with filter paper (Whatman No 1) to collect eggs. Treatments were randomly assigned a corner of each cage (30 x 30 x 30 cm). For each trial (N = 4), 20 female mosquitoes (four days post blood meal) were added to five cages with each cage given a random location in the incubator. Mosquitoes were given 24 hours to oviposit before eggs were counted. In addition to the trials using oak leachate, a separate trial was conducted to investigate the impact of mycolactone in isolation (without the influence of any other microbes) to distilled water similar to the experiments conducted above but with distilled water (including 12.8 µl/L EtOH as a solvent control) and distilled water with 0.8 µg/ml mycolactone as the two treatments.

Field trial

Water was collected from several stagnant pools in Ouidah, Benin, a region endemic for BU, and mixed to create a composite sample. This water was used in a blocked design consisting of 10 replicate blocks placed randomly located on campus of the Université d'Abomey-Calavi (Ouidah, Benin). Each block contained two containers (each filled with 200 ml of the composite water sample) with the containers lined with seed germination paper to collect mosquito eggs. For the mycolactone treatment, purified mycolactone was added at a final concentration of 0.8 µg/ml while EtOH was added to the control, to mimic any effects of suspending mycolactone in EtOH (12.8 µl/L). After three days, the number of eggs on the seed germination paper was counted. At the end of the trial, 50 ml water samples were collected and stored at 20 °C until DNA extraction. RNA samples were collected, immediately pelleted, and stored in RNAlater (Qiagen) at -20 °C until extraction.

Bacterial and fungal sequencing and quantification

Water samples were centrifuged at 7,500 RCF for 15 mins with the pellet used for subsequent DNA extraction. DNA extractions were performed using the PowerSoil (Qiagen) DNA extraction kit with the addition of lysozyme (15mg ml^{-1}, Invitrogen) during the lysis step. For the bacterial communities, library preparation and indexing were performed by the Michigan State Research Technology Support Facility according to previous described methods using primers targeting the V4 region of the 16S rRNA gene (5′-GTGCCAGCMGCCGCGG-3′, 5′-TACNVGGGTATCTAATCC-3′) (Claesson et al. 2010, Caporaso et al. 2011a). Fungal sequencing was performed using the fITS7/ITS4 primer set (5′- GTGARTCATCGAATCTTTG-3', 5′-TCCTCCGCTTATTGATATGC-3′) (Bokulich and Mills 2013), Invitrogen Platinum Hot Start master mix (Thermofisher), and QIAquick (Qiagen) PCR cleanup kit according to manufacturers instructions. Library construction and sequencing of both bacterial and fungal communities (2 x 250 bp paired-end reads, Illumina MiSeq) was performed by the Michigan State University Genomics Core Facility using a modified version of the protocol adapted for the Illumina MiSeq, described by Caporaso et al. (2011a). Total bacterial and fungal DNA were quantified using the Femto Bacterial and Femto Fungal Quantification kits (Zymo Research). Assays were conducted using manufacturer's instructions and the QuantStudio 7 Flex qPCR Platform (Applied Biosystems).

RNA sequencing

RNA from twelve samples (trials two, three, and four, 1 sample per treatment from each trial) were extracted using the TRIzol method (ThermoFisher) according to manufacturer instructions. Pelleted samples were mixed with TRIzol reagent and homogenized with 0.2 mm beads. The aqueous phase was precipitated with isopropanol before washing with 75% ethanol.

Resulting pellet was dried and suspended in nuclease-free water before being cleaned using the RNeasy PowerClean Pro kit (Qiagen). RNA was treated with Turbo DNase (Invitrogen) before library preparation. The NEBNext Ultra RNA Library prep kit and NEBNext Multiplex Oligos were used according to manufacturer's protocols for library preparation. Sequencing was performed on an Illumina HiSeq2000 platform (2 x 150 bp reads) by St. Jude Children's Research Hospital and initially trimmed using TrimGalore (v0.4.2)(Andrews et al. 2015).

Data processing

Demultiplexed amplicon (16S, ITS) sequences were filtered and chimeric sequences were removed using DADA2 (Callahan et al. 2016a) implemented in QIIME 2 (v 2020.11)(Bolyen et al. 2018). After singletons were removed, bacterial taxonomy was assigned using a naïve Bayesian classifier trained against the SILVA database) at a 99% similarity (Quast et al. 2012). For the 16S sequences, reads assigned to chloroplasts or mitochondria were removed before rarefaction (see below). Fungal samples were processed similarly as above using DADA2 with the UNITE database (v8.99) used for classification (Abarenkov et al. 2010).

RNA sequences were filtered and trimmed using SAMSA2 (Westreich et al. 2018). After paired end reads were merged with PEAR (Zhang et al. 2014), they were trimmed and filtered using Trimmomatic and SortMeRNA (Kopylova et al. 2012, Bolger et al. 2014). DIAMOND and SMASA2 scripts were used to annotate sequences against the NCBI RefSeq database (Buchfink et al. 2014, Tatusova et al. 2014). Functional characterization was performed similarly against the SEED database (Overbeek et al. 2014). Clustering of samples and heatmap visualization were performed using pheatmap (v 1.0.12)(Kolde and Kolde 2015).

Statistical analysis

Differences in mosquito oviposition (as number of eggs per container) were compared using Kruskal-Wallis and ANOVA tests implemented in R (v 2.4.4, R core development team, 2014). ANOVA tests were also used to test for differences in bacterial and fungal variant richness and diversity. Diversity metrics were calculated using the phyloseq R package (v 1.32.0) (McMurdie and Holmes 2013). Tests of pairwise significant differences among groups were conducted with Kruskal-Wallis and Mann-Whitney U tests (False Discovery Rate (FDR) correction) or with Tukey's Honest Significant Difference (Tukey HSD, Bonferroni adjusted P values) tests. Differences in bacterial and fungal relative abundance were tested using Kruskal-Wallis (FDR-adj) and Mann-Whitney U tests (FDR-adj) implemented in the ggpubr package (v 0.4.0) (Kassambara 2017). Random forest models built using the randomForest package (v 4.6-14)(Liaw and Wiener 2002) with 1000 trees and out of bag (OOB) error rates were used to identify genus level indicator taxa for different treatments (chosen by mean decrease accuracy). To explore differences in beta diversity among treatments and controls, Permutational multivariate analysis of variance (PERMANOVA) and beta dispersion tests (betadispr) were conducted using the vegan package (v2.5-7) (Oksanen et al. 2015). DESeq2 (v 1.28.1) and SAMSA2 scripts (Love et al. 2014, Westreich et al. 2018) were used to identify differentially expressed microbes and functional pathways between sample groups. All tests were considered significant at an adjusted P value < 0.05, with the exception of testing for differential gene expression between groups (RNA-seq data, DEseq2, significant at $P < 0.01$).

Results

Laboratory oviposition assays

There were no differences in the number of eggs laid in distilled water with mycolactone (0.8µg/ml) added compared to the ethanol control (Kruskal-Wallis, $\chi^2 = 0.27$, P = 0.60, Figure 3.1). Trial did not significantly affect oviposition (KW, $\chi^2 = 0.90$, 0.82, Table 3.1) so all trials were combined to increase statistical power. Treatment had a strong negative dose response effect on oviposition (KW, $\chi^2 = 17.2$, $P < 0.001$, Figure 3.2). While there was no difference between control (oak leachate + EtOH) and 0.05 µg/ml treatments (Mann-Whitney U test, P-adj = 0.4), the higher doses (0.5 and 0.8 µg/ml) received significantly fewer eggs than the control (MW, P-adj < 0.05). Control samples had on average 142.7 eggs (± 19.1 SEM), approximately double the average number of eggs laid in the 0.5 µg/ml (74.9 ± 13.5) and 0.8 µg/ml (62.9 ± 8.4) treatments.

Figure 3.1. Differences in egg laying between distilled water with EtOH added (Ethanol control) and water with a 0.8µg/ml dose of mycolactone.

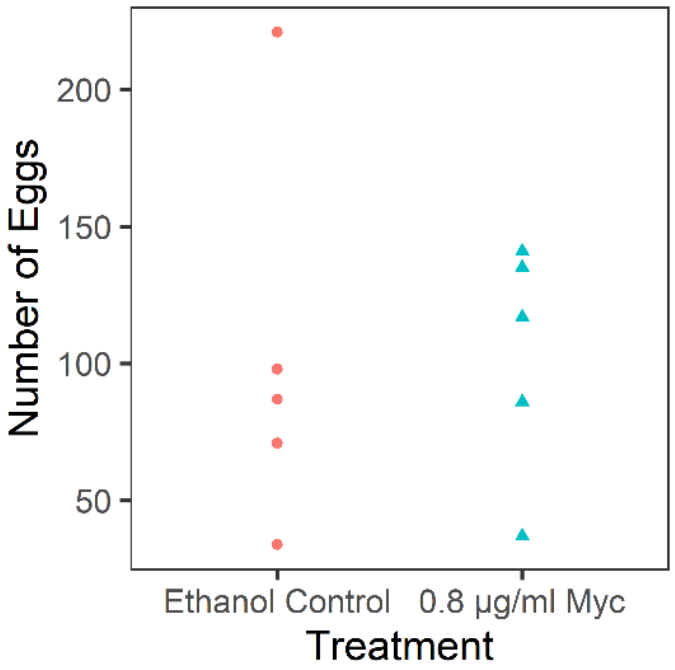
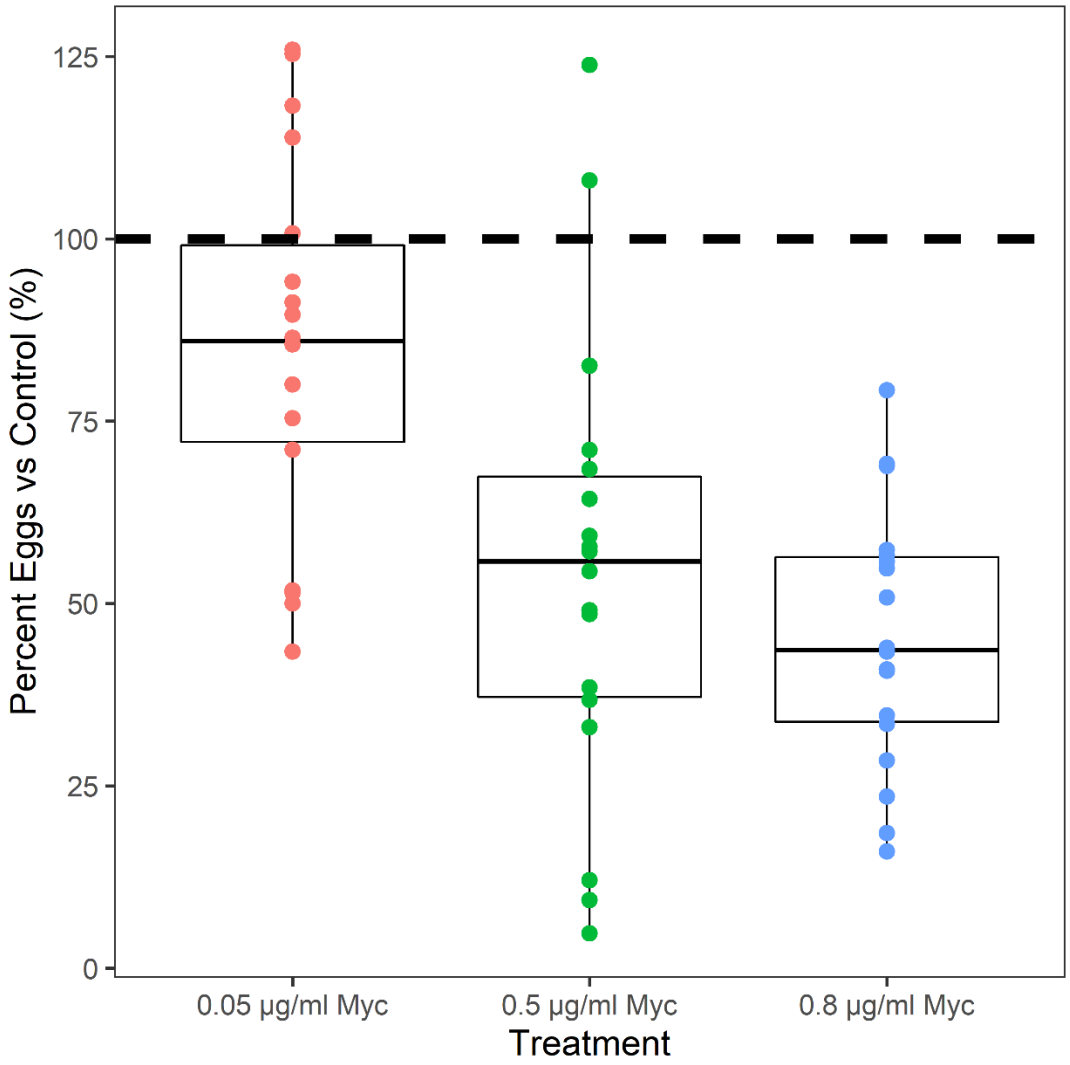


Table 3.1. Oviposition by treatment and trial. Each trial represents a separate preparation of leachate. Treatment indicates dose of mycolactone, controls included ethanol to account for solvent. N =Number of cages within the trial, SD = Standard Deviation, SEM = Standard Error of the Mean. All eggs collected were from the genus *Aedes*.

Trial	Treatment (µg/ml)	N	Mean Eggs	SD	SE
One	0.05	5	105.2	51.0	22.8
	0.5	5	41.0	29.6	13.2
	0.8	5	55.8	30.0	13.4
	Control	5	157.4	102.9	46.0
Two	0.05	5	110.4	49.6	22.2
	0.5	5	92.2	61.3	27.4
	0.8	5	64.6	26.3	11.8
	Control	5	119.6	42.7	19.1
Three	0.05	4	102.8	33.5	16.8
	0.5	4	77.5	55.2	27.6
	0.8	4	55.0	21.9	11.0
	Control	4	136.3	81.1	40.6
Four	0.05	4	148.0	101.2	50.6
	0.5	4	93.0	80.0	40.0
	0.8	4	77.5	64.4	32.2
	Control	4	160.0	111.9	56.0

Figure 3.2. Percent eggs laid in mycolactone treatments compared to control. Dashed line indicates 100% of control treatments. Egg laying response for each treatment is shown relative to the control (oak leachate only) within each cage.



Laboratory assay bacterial communities

Total bacterial abundance in water samples did not differ between treatments (one day incubation, KW, $P > 0.05$, qPCR). Bacterial sequencing resulted in 4,424,364 reads from 49 samples (average reads per sample = 90,293). After filtering and rarefaction to 5,000 reads (based on alpha diversity rarefaction curves, Figure S3.1), 48 samples and 321 Amplicon Sequencing Variants (ASVs) remained (the only sample lost during rarefaction was the extraction control,

1,546 reads before filtering). Treatment and trial both influenced the number of ASVs present in water samples (ANOVA, P< 0.05, Table 3.2, Figure 3.3a). Trial two (85.1 ± 1.8 ASVs) had fewer ASVs than trials one (104.5 ± 1.33), three (103.5 ± 2.6), and four (109.6 ± 2.07, Tukey's HSD, P-adj < 0.05). Control and 0.05 µg/ml treatments had significantly higher bacterial ASV richness than the 0.5 µg/ml treatment (Tukey HSD, P-adj < 0.05, Differences and 95% CIs shown in Table S3.3). Similar to richness, treatment and trial had strong effects on Shannon diversity (Table 3.2). In addition to the significant main effects, the interaction between trial and treatment influenced Shannon diversity so the effects were examined within each trial individually. Trials two (ANOVA, $F_{3,8}$ = 12.3, P = 0.002) and three ($F_{3,8}$= 4.6, P = 0.037) had significant responses to treatment while trial one ($F_{3,8}$= 1.27, P = 0.35) and four ($F_{3,8}$= 3.82, P = 0.057) did not. Within trial two, 0.05 µg/ml and 0.5 µg/ml treatments had lower diversity than control samples while 0.5 µg/ml had lower diversity than 0.05 µg/ml samples in trial four (Tukey HSD, P-adj < 0.05, Figure 3.3b).

Table 3.2. ANOVA of bacterial alpha diversity during lab trial. Model = Response ~Trial x Treatment. SS = Sum of Squares, MS =Mean Squares.

Response		**Df**	**SS**	**MS**	**F**	**P**
ASV Richness	Trial	3	4141	1380.3	35.65	< 0.0001
	Treatment	3	443	147.7	3.82	0.019
	Trial:Treatment	9	434	48.2	1.25	0.303
	Residuals	32	1239	38.7		
ASV Shannon diversity	Trial	3	0.64	0.21	71.15	< 0.0001
	Treatment	3	0.1	0.03	10.6	< 0.0001
	Trial:Treatment	9	0.07	0.01	2.68	0.019
	Residuals	32	0.1	0.001		

Table 3.3. Tukey HSD test comparing differences in ASV richness between treatment groups. Model: ASV richness ~ Trial x Treatment. Diff = difference between groups, lwr = lower bound for 95% confidence intervals. Upr = upper bound for 95% confidence intervals.

Comparison	Diff	Lwr	Upr	P-adj
0.05-Con	0.417	-6.465	7.298	0.998
0.5-Con	-6.917	-13.798	-0.035	0.048
0.8-Con	-4.167	-11.048	2.715	0.371
0.5-0.05	-7.333	-14.215	-0.452	0.033
0.8-0.05	-4.583	-11.465	2.298	0.290
0.8-0.5	2.750	-4.132	9.632	0.702

The two major phyla across all samples were Proteobacteria and Bacteroidota (formerly Bacteroidetes) which collectively represented 96.8% of the total relative abundance across all samples (Figure 3.3c). At the family level, only Spirosomaceae (out of nine families comprising greater than 3% of the total relative abundance) was differentially abundant between treatments when all trials were combined (KW, P-adj < 0.05). Due to the strong effects of trial, taxonomic composition was also examined separately within individual trials, but no taxa were significantly different among treatments within trial (KW, P-adj > 0.05). At the genus level, while some taxa, (e.g., *Sphingobium* in trial one or *Herbaspirillum* in trial two) displayed a visually dose dependent response, no groups were significantly different after correction for multiple comparisons (Figure S3.2).

Variability in bacterial beta diversity (Jaccard distance) was not different between treatments (Vegan: Betadispr, $F_{3,44} = 0.02$, $P = 0.99$) but differed by trial ($F_{3,44} = 6.06$, $P = 0.002$). Trial four had greater variability than trial two ($F_{1,22} = 16.3$, $P < 0.001$, Bonferroni-adj α value = 0.008). Bacterial community composition was significantly impacted by treatment, trial, and their interaction (Table 3.4). Treatment had significant impacts on beta diversity in all trials (PERMANOVA, $P < 0.02$) with treatment accounting for a substantial proportion of variation within each trial (R^2, Range: 56%-77%, Figure 3.3d).

Figure 3.3. Differences in bacterial community composition between mycolactone treatments. a) Amplicon sequencing variant richness by treatment and trial (1-4). b) Shannon diversity between treatment and trial. * = P-adj < 0.05, ** = P-adj < 0.01 in overall ANOVA test for differences between treatment. Pairwise significance (Tukey HSD) is denoted by lowercase letters. c) Phylum level relative abundance. Ony phyla which comprised greater than 3% of total abundance are shown. d) PCoA plot of differences in bacterial beta diversity (Jaccard distance).

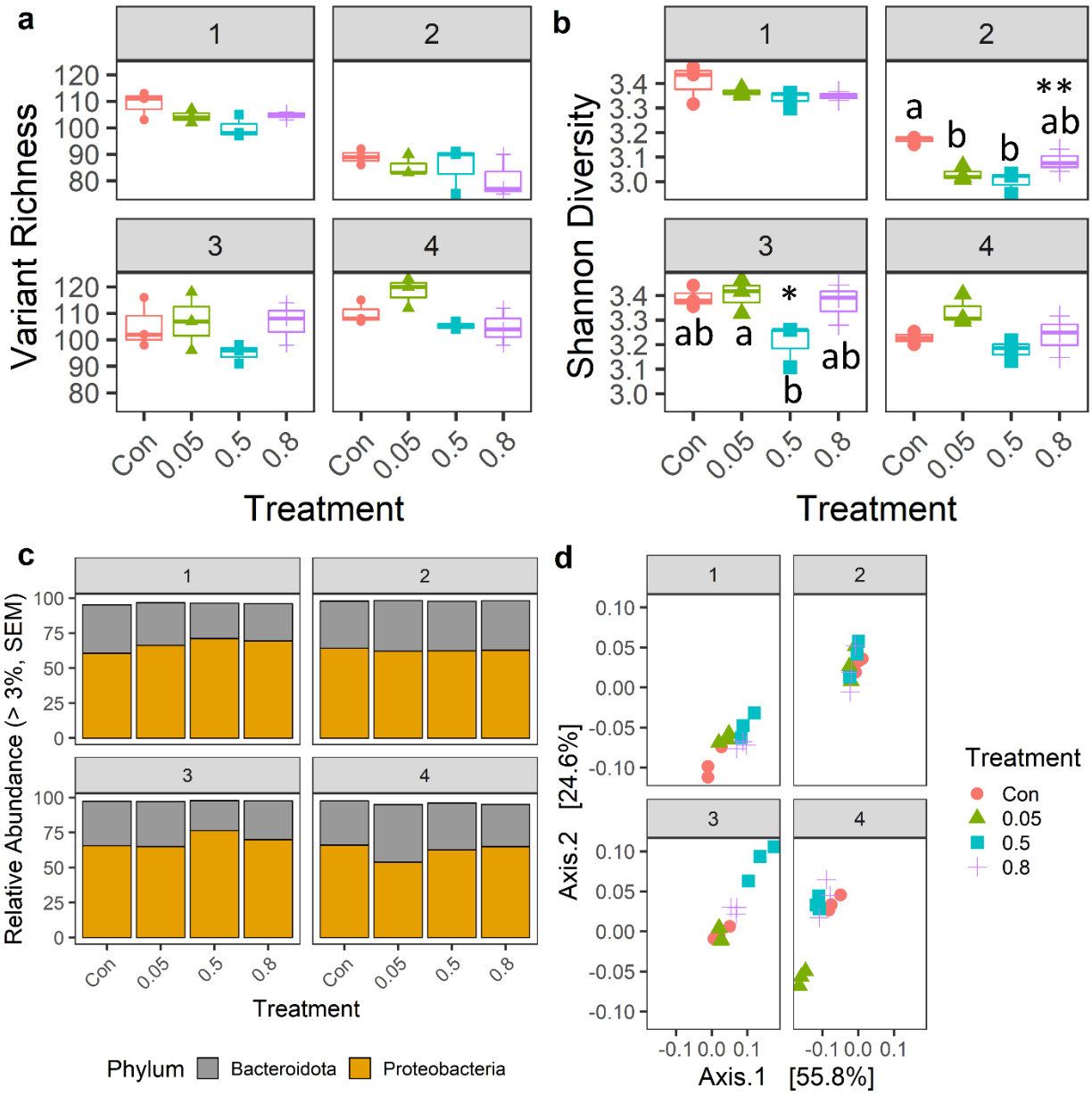


Table 3.4. PERMANOVA of bacterial beta diversity. PERMANOVA test was conducted using Jaccard distance and 999 permutations. SS = Sum of Squares, MS = Mean Squares.

	Df	SS	MS	F.Model	R2	Pr(>F)
Treatment	3	0.24	0.08	5.66	0.02	0.001
Trial	3	8.51	2.84	199.75	0.87	0.001
Treatment:Trial	9	0.62	0.07	4.81	0.06	0.001
Residuals	32	0.45	0.01	0.05		
Total	47	9.82	1.00			

Laboratory assay fungal communities

There were 2,618,252 fungal reads obtained from 48 samples (average reads = 54,546). After filtering and rarefaction to 10,000 reads (Figure S3.3), 247 ASVs and all samples remained. Fungal ASV richness was influenced by both treatment and trial (Table 3.5**,** Figure 3.4a). Trial four had fewer fugal ASVs than all other trials (Tukey HSD, P-adj < 0.05) with trial one having fewer than trail two and three (All pairwise differences between groups are shown in (Table S3.1). Among treatments, 0.8 µg/ml had lower richness than 0.05 µg/ml samples (Tukey HSD, P-adj = 0.04). While other comparisons were nearly significant (Con-0.05, 0.05- 0.5, 0.05- 0.8, P-adj < 0.065), none of them reached statistical significance (Table S3.1). Similarly, both treatment and trial influenced Shannon diversity (ANOVA, P > 0.05) with trial having a much stronger effect than treatment (F = 31.6 vs 4.09, Figure 3.4b). Trial two had higher fungal diversity than all other trials (Tukey HSD, P-adj < 0.05). The highest concentration of mycolactone (0.8 µg/ml) had lower diversity than 0.05 and 0.5 µg/ml concentrations (Tukey HSD, P-adj < 0.05). Trial accounted for a substantial proportion ($R^2 = 0.77$) of the total variation in beta diversity (PERMANOVA, Jaccard distance, P < 0.001), with treatment only accounting for a small amount of total variation ($R^2 = 0.03$, P = 0.047, Figure 3.4c, Table 3.6).

Table 3.5. ANOVA tests of impacts on fungal richness. SS = Sum of Squares, MS = Mean Squares.

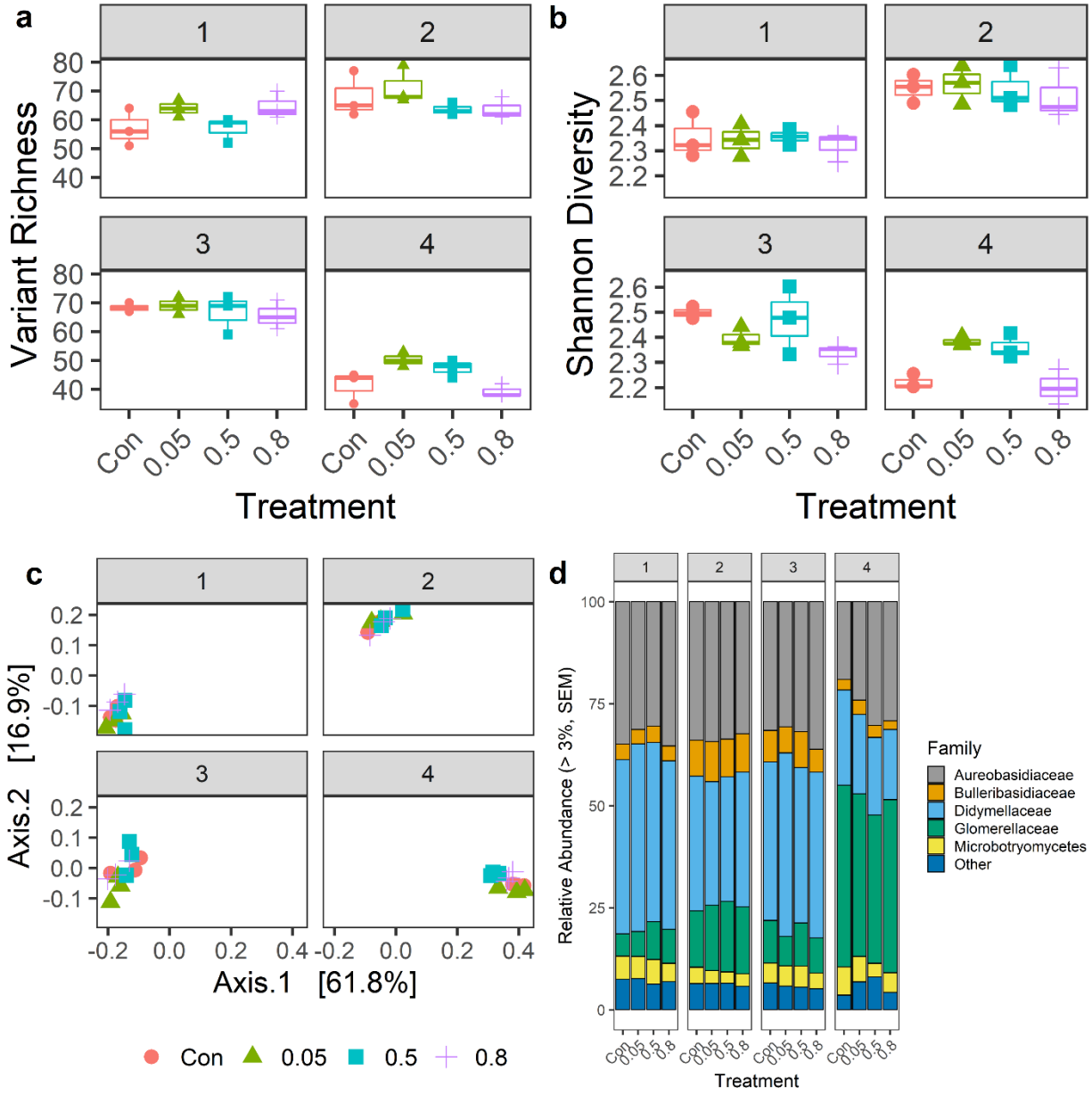
	Factor	Df	SS	MS	F	P
Observed Variants	Trial	3	4050	1349.8	61.067	< 0.001
	Treatment	3	236	78.7	3.559	0.025
	Trial:Treatment	9	308	34.2	1.547	0.174
	Residuals	32	707	22.1		

Table 3.6. Impact of treatment and trial on fungal beta diversity. PERMANOVA tests used Jaccard distance and 999 permutations. SS = Sum of Squares, MS = Mean Squares.

	Df	SS	MS	F.Model	R2	Pr(>F)
Treatment	3	0.096	0.032	2.089	0.026	0.047
Trial	3	2.882	0.961	62.812	0.771	0.001
Treatment: Trial	9	0.269	0.030	1.953	0.072	0.015
Residuals	32	0.489	0.015	0.131		
Total	47	3.736	1			

At the phylum and family level, there were no any taxonomic groups affected by treatment when all trials were combined (Kruskal-Wallis, P-adj > 0.05, Figure 3.4d). Similarly, there were no significantly different families among treatments within individual trials (KW, P-adj < 0.05). Genus level communities were distinct between trial with random forest models able to classify samples to trial with an 8.33% error rate (Out of Bag error, 4 samples misclassified). Within individual trials, random forest models did a poor job of classifying samples to treatment with errors rate ranging from 41.6% -91.6%.

Figure 3.4. Impact of mycolactone on fungal communities. a) ASV richness by trial and treatment. Each box (1-4) indicates a separate trial b) Shannon diversity by trial and treatment. c) Treatment impacts on fungal beta diversity (Jaccard). d) Relative abundance of family level fungal communities. Only taxa which comprise greater than 3% of the total communities are shown.



Differences in gene expression

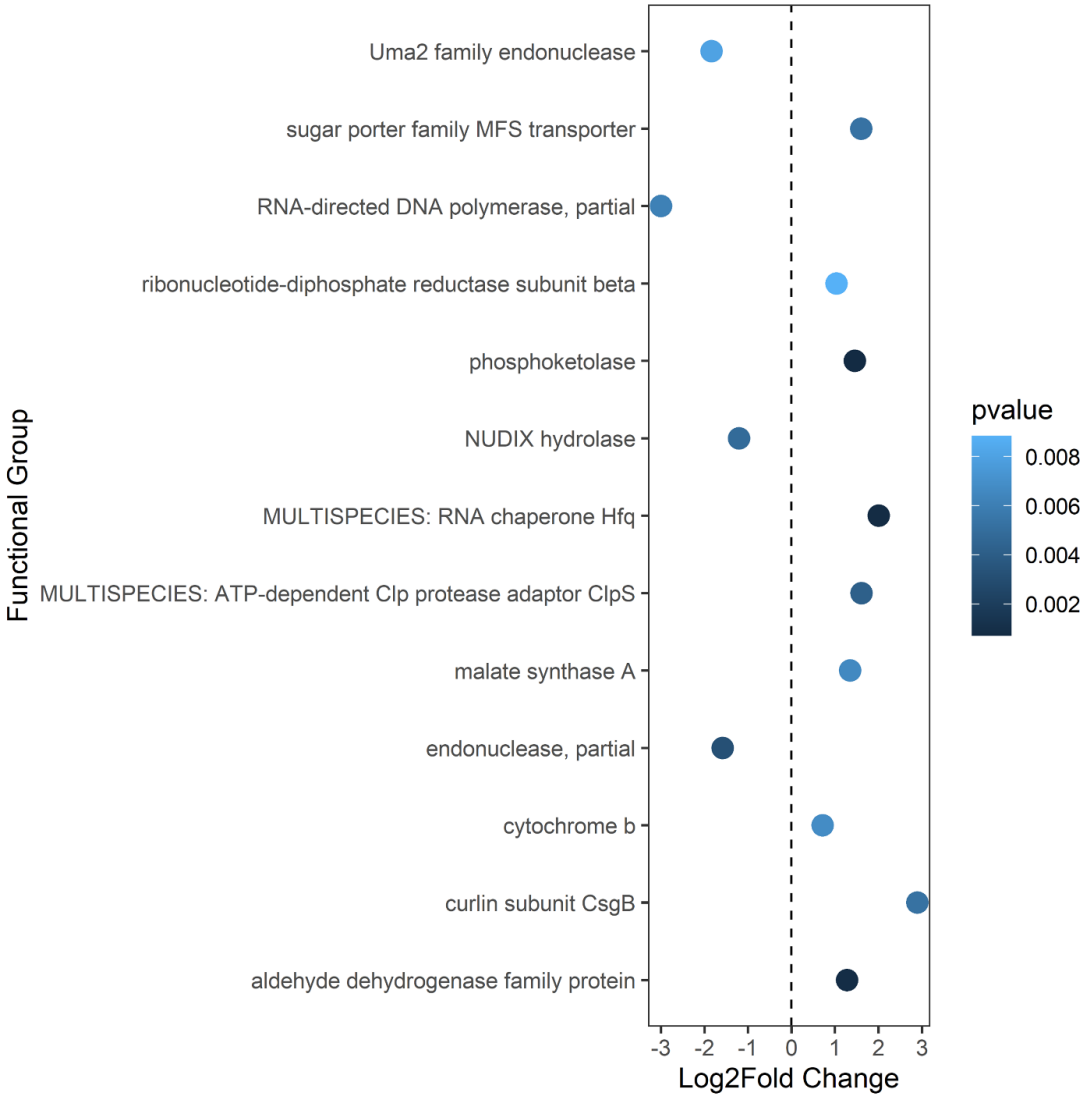
Due to the limited number of samples (N = 12) and differences between trials, differences in expression between treatments were compared between low (0 and 0.05 µg/ml) and high doses

(0.5 and 0.8 µg/ml) of mycolactone rather than among all treatments. Thirteen functional groups were differentially expressed between high and low doses (DeSeq2, $P < 0.01$, Table 3.7) with four functional groups (Uma2 family endonucleases, RNA-directed DNA polymerase, NUDIX hydrolase, and endonuclease (partial)) higher in lower concentrations while the remaining gene families were expressed at higher levels in higher mycolactone concentrations (Figure 3.5).

Table 3.7. Differences in functional groups between low and high concentrations of mycolactone. Low dose = 0 and 0.05 treatments, high dose = 0.5 and 0.8 treatments. DESeq model = Matrix ~ Trial+ Treatment. Base Mean = mean value across all samples. lfcSE = Standard Error of log-fold chance. stat = statistic from DESeq model. Groups with positive log_2Fold change values are higher in higher concentrations of mycolactone.

Functional Group	Base Mean	Low Dose Mean	High Dose Mean	log_2Fold Change	lfcSE	stat	P
phosphoketolase	14.87	7.88	21.86	1.46	0.43	3.38	0.001
MULTISPECIES: RNA chaperone Hfq	7.10	3.33	10.87	2.01	0.60	3.35	0.001
aldehyde dehydrogenase family protein	35.11	20.26	49.95	1.28	0.39	3.32	0.001
endonuclease, partial	7.73	11.70	3.76	-1.58	0.54	-2.95	0.003
MULTISPECIES: ATP-dependent Clp protease adaptor ClpS	4.96	2.43	7.50	1.61	0.56	2.87	0.004
NUDIX hydrolase	8.06	11.14	4.99	-1.21	0.43	-2.82	0.005
curlin subunit CsgB	1.67	0.16	3.18	2.89	1.04	2.79	0.005
sugar porter family MFS transporter	8.29	4.04	12.54	1.60	0.57	2.79	0.005
RNA-directed DNA polymerase, partial	1.34	2.47	0.21	-3.00	1.09	-2.74	0.006
malate synthase A	11.37	5.82	16.91	1.35	0.50	2.72	0.007
cytochrome b	41.94	30.84	53.03	0.72	0.27	2.71	0.007
Uma2 family endonuclease	2.55	4.02	1.08	-1.84	0.69	-2.65	0.008
ribonucleotide-diphosphate reductase subunit beta	10.92	7.05	14.80	1.04	0.40	2.62	0.009

Figure 3.5. Differences in functional group expression between low (0 + 0.05 µg/ml) and high (0.5 + 0.8 µg/ml) mycolactone doses. Positive log2fold change values are higher in high dose mycolactone treatments while negative values have higher expression at lower doses. Only groups with a P-value less than 0.01 are shown. DESeq model: Community ~ Trial + Treatment.



When samples were clustered by organism activity, gene expression between high and low mycolactone treatments clustered separately (Figure 3.6). The top three taxa, *Legionella pneumophila* (15.5 ± 1.9), *Klebsiella pneumoniae* (11.3 ± 1.6), and *Virgibacillus senegalensis* (5.7 ± 0.73) comprised 32.5% of the total relative activity across all samples (Figure 3.7a). There were thirteen organisms which displayed differential activity between low and high concentrations of

mycolactone (DESeq, P value < 0.01). Seven had higher activity in low doses of mycolactone while six had higher activity in higher concentrations of mycolactone (Figure 3.7b, Table 3.8). Interestingly *Mycobacterium sp.* was one of the groups which had higher activity at lower doses (P value = 0.0001).

Figure 3.6. Log_2Fold Change heatmap of expression levels for the 50 organisms with the greatest variance across all samples (DESeq model: Matrix ~ Treatment). Tree building and clustering of samples was conducted using Ward's clustering implemented in SAMSA and DESeq. Higher log2fold change values indicates groups that were higher in higher doses.

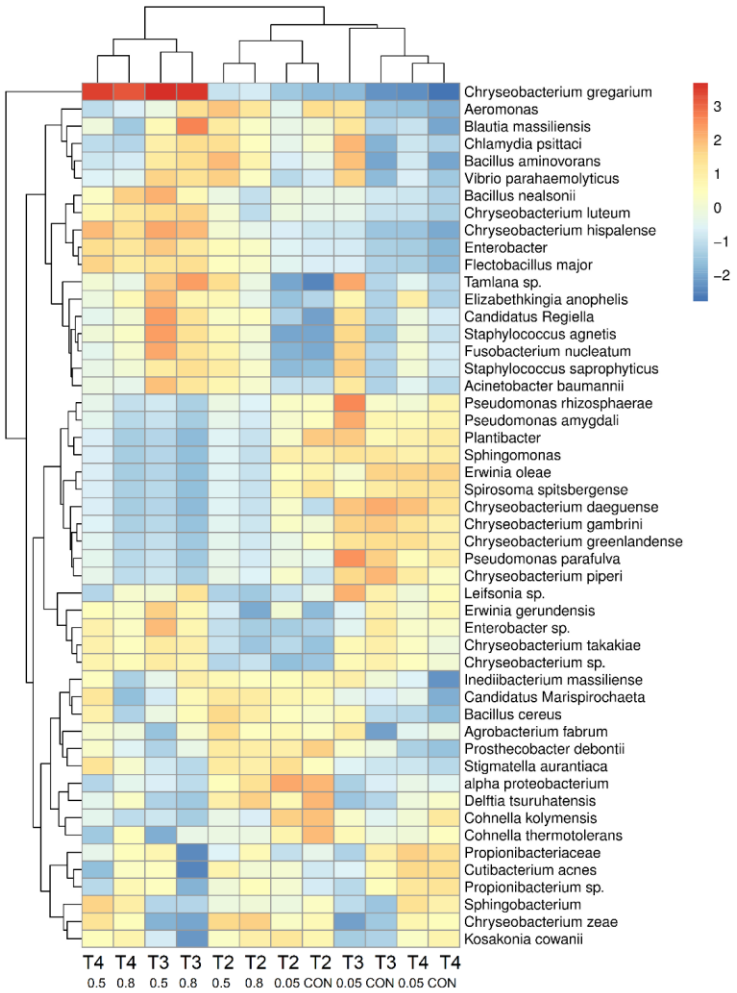


Figure 3.7. Difference in relative gene expression between treatment. a) Relative expression activity. Numbers (2-4) indicate individual trials. b) Log2Fold change between organisms.

Log2Fold change values above one indicate higher in higher doses while negative values had greater activity at lower doses.

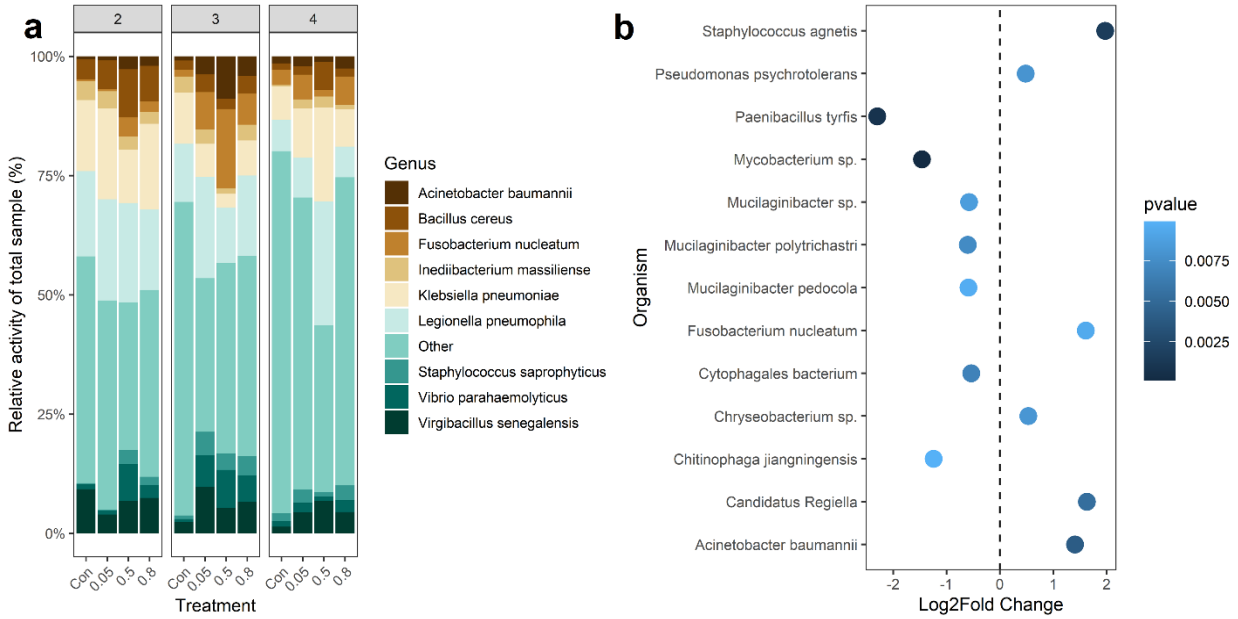


Table 3.8. Differences in organism activity between low and high concentrations of mycolactone. Low dose = 0 and 0.05 treatments, high dose = 0.5 and 0.8 treatments. DESeq model = Matrix ~ Trial+ Treatment. Base Mean = mean value across all samples. lfcSE = Standard Error of log-fold chance. stat = statistic from DESeq model. Groups with positive log2Fold change values have higher activity in higher concentrations of mycolactone.

Organism Name	Base Mean	Low Dose Mean	High Dose Mean	log2Fold Change	lfcSE	stat	pvalue
Mycobacterium sp.	15.97	23.66	8.28	-1.46	0.38	-3.87	0.0001
Paenibacillus tyrfis	6.99	11.52	2.46	-2.30	0.69	-3.35	0.0008
Staphylococcus agnetis	1457.52	750.72	2164.32	1.97	0.62	3.163	0.0016
Acinetobacter baumannii	3428.99	1850.60	5007.37	1.40	0.49	2.88	0.0040
Candidatus Regiella	342.62	176.88	508.36	1.63	0.58	2.788	0.0053
Cytophagales bacterium	207.52	248.72	166.32	-0.54	0.20	-2.71	0.0067
Mucilaginibacter polytrichastri	885.69	1082.67	688.71	-0.60	0.22	-2.68	0.0073
Pseudomonas psychrotolerans	192.23	163.07	221.39	0.48	0.18	2.654	0.0079
Chryseobacterium sp.	938.64	809.46	1067.82	0.53	0.20	2.647	0.0081
Mucilaginibacter sp.	1178.81	1436.43	921.20	-0.58	0.22	-2.63	0.0086
Fusobacterium nucleatum	5875.15	3457.21	8293.09	1.61	0.62	2.596	0.0094

Table 3.8 (cont'd)

Mucilaginibacter pedocola	292.93	362.78	223.07	-0.59	0.23	-2.59	0.0097
Chitinophaga jiangningensis	9.65	14.15	5.15	-1.24	0.48	-2.58	0.0099

Field oviposition assay

The field oviposition assay, conducted in Ouidah, Benin, showed a similar negative impact of mycolactone on oviposition as the laboratory trials conducted in Michigan, USA (KW, $\chi^2 = 8.7$, $P < 0.01$). Oviposition traps with a 0.8 µg/ml dose of mycolactone toxin had a mean of 38.9 (± 9.4) eggs on and control traps had 86.3 (± 9.2) eggs on average (Figure 3.8a). All species collected were identified as from the genus *Aedes* but were not identified to species level (due to permitting issues with returning samples to MI for identification).

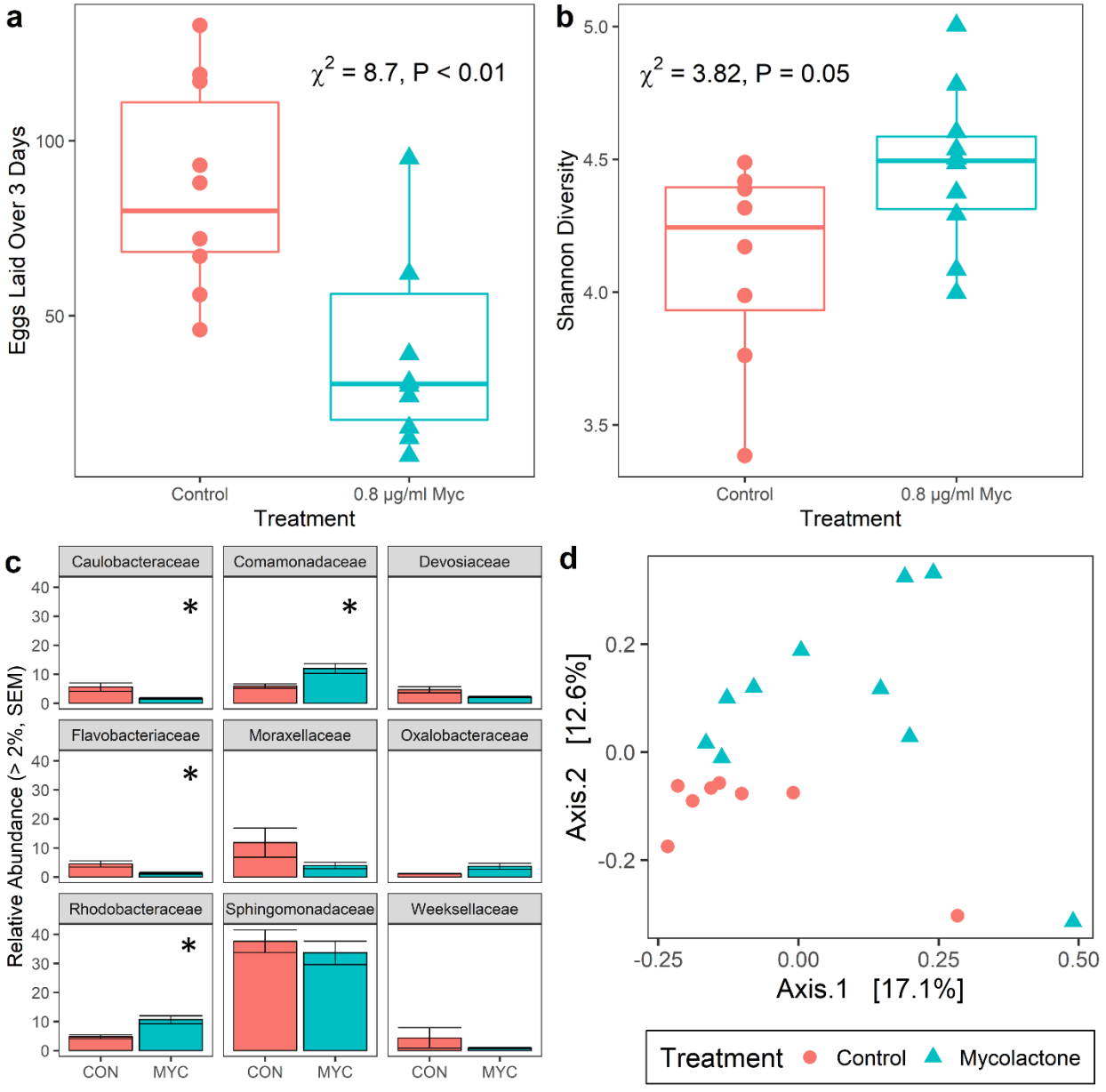
Field bacterial communities

A total of 1,540,771 bacterial sequencing reads (85,598 average reads per sample) were obtained from 18 water samples collected three days after the mycolactone toxin was added (8 control, 10 treatment, where DNA from two control samples did not amplify). After filtering, removal of singletons, and rarefaction to 15,000 reads (Figure S3.4), 794 Amplicon Sequence Variants (ASVs) and all samples remained. There was no difference in the number of ASVs between treatments (KW, $\chi^2 = 0.57$, $P = 0.45$). Shannon diversity was slightly higher in samples treated with mycolactone (0.8 µg/ml) compared to control samples (4.31 ± 0.12 vs. 4.02 ± 0.13) but the difference was not significant at alpha = 0.05 (KW, $\chi^2 = 3.82$, $P = 0.05$, Figure 3.8b).

Phylum level bacterial communities were similar between treatments (KW, P-adj > 0.05) with the majority of bacterial relative abundance coming from taxa in the phylum Proteobacteria (82.3% ± 3.4 of relative abundance in control samples, 80.1 ± 5.8 in mycolactone samples). At

lower taxonomic levels there were several families which differed in relative abundance. Four of the nine bacterial families which comprised greater than 2% of the total relative abundance significantly different between treatments (KW, P-adj < 0.05). Comamonadaceae (Betaproteobacteria) and Rhodobacteraceae (Alphaproteobacteria) were higher in abundance in the mycolactone treatment with Flavobacteriaceae (Bacteroidetes) and Caulobacteraceae (Alphaproteobacteria) higher in control samples (Figure 3.8c). A random forest model using genus level taxonomy differentiated treatments with a 22% error rate (4 of 18 samples misclassified) however, none of the top ten genus level indicators for the random forest model (chosen by Mean Decrease Accuracy) were significantly different between treatment (KW, P-adj > 0.05). Bacterial reads assigned to the genus *Mycobacterium* were present in eight of ten samples treated with mycolactone and four of eight controls, but no abundance was greater than 0.30% of the total sample relative abundance and there was no different between treatments (KW, $\chi^2 = 2.91$, P = 0.09, Figure S3.5). Control and mycolactone samples had similar levels of variability in bacterial beta diversity (Vegan: Betadispr: Jaccard distance, F = 0.23, P = 0.64) with treatment accounted for 11% (R^2) of the variation in community beta diversity (PERMANOVA, $F_{1,16} = 2.06$, P = 0.002, Figure 3.8d).

Figure 3.8. Field trial of mycolactone impacts on mosquito oviposition (Ouidah, Benin). a) Number of eggs laid over three days. Test for comparison between groups was a Kruskal-Wallis test. b) Differences in Shannon bacterial diversity between water samples (KW test). c) Family level relative abundance between treatments (KW test). * = P-adj < 0.05. PCoA plot of differences in bacterial beta diversity between treatment (Jaccard distance).



Discussion

In this study, we investigated the effect of the toxin mycolactone on aquatic bacterial and fungal communities and subsequent mosquito oviposition behavior. The influence of toxins produced by opportunistic environmental pathogens on other environmental microbes is largely unknown relative to our understanding of how these toxins influence humans and other animal hosts. The primary role of many of these toxins associated with environmental microbes is likely to improve the ability of its producer to survive and reproduce in its environment and only coincidently causes virulence in humans (Brown et al. 2012). As such, a better understanding of how these toxins influence their environmental habitats will improve our knowledge of opportunistic environmental microbes and the conditions which lead to human disease.

The addition of mycolactone had the opposite effect on mosquito oviposition as seen previously. Sanders et al. (2017) observed that similar doses of mycolactone (without other microbes present) was a potential cue for enhancing both host seeking and oviposition behavior. Our conflicting observation of mycolactone reducing oviposition in both laboratory and field experiments is likely a result of interactions between mycolactone and other microbes and not a direct mechanism of mycolactone. With mosquitoes using volatiles given off by bacteria as common cues for oviposition (Day 2016), and mycolactone hypothesized to act as an antagonist of quorum sensing machinery in other environmental microbes (Mashlawi et al. 2020, Dhungel et al. 2021), a reduction in Volatile Organic Compounds (VOCs) given off by aquatic communities treated with mycolactone is a plausible explanation for the observed oviposition behavior. Previous investigation of the interactions between mosquito oviposition (*Ae. aegypti*) and bacterial quorum sensing (strains of *Staphylococcous epidermidis* with an inhibited and wild-type regulator for

quorum sensing) suggest that reductions in bacterial quorum sensing activity leads to a reduction in attractiveness (Zhang et al. 2015).

With mechanical transmission from mosquitoes after contacting *M. ulcerans* in an aquatic environment one of the proposed transmission mechanisms (Wallace et al. 2017), the reduced oviposition response in containers containing aquatic microbes and mycolactone may have implications for the frequency of female mosquitoes encountering MU-containing aquatic habitats. Future work directly quantifying VOCs given off by naturally occurring microbial communities, and their effects on oviposition behavior, will be needed to better understand potential indirect impacts of mycolactone on insect behavior. Mashlawi (2020) found that the presence of mycolactone may contribute to a slight legacy effect on oviposition (i.e., mosquitoes raised in habitats containing mycolactone prefer to lay eggs in habitats containing mycolactone). Gravid female mosquitoes used in this study were either naive to mycolactone (colony strains) or from field populations where the presence/absence of MU in larval habitats was not assayed.

Aquatic microbes in both laboratory and field assays were impacted by the addition of mycolactone. Overall, water samples collected from field trials showed higher richness than water samples derived from leaf leachates. Though leaf materials used in the four laboratory trials were broadly similar, there was considerable variation between trials in community composition and diversity. Water collected during field trials showed greater impacts of the addition of mycolactone than laboratory trials. The lower impacts observed in the laboratory assays may be due to the shorter incubation time. Water samples in the laboratory trial were collected one day after mycolactone was added compared to the three days for field trials, giving the communities in the field trial more time to change in response. Additional investigations of incubation time will be needed to determine if these differential impacts are due to incubation period (see Preface).

The expression levels of aquatic microbes treated with mycolactone differed between high and low doses. Small sample sizes and a confounding effect of different preparations of leachates (trials) limited our ability to identify differentially expressed functional groups and organisms (i.e., using a cutoff of $P < 0.01$ rather than an adjusted P value). Previous work examining the effect of mycolactone on gene expression of a single bacterial species (*S. aureus*) identified 150 genes differentially expressed between treatment (FDR-adj P-value < 0.05, Dhungel et al. 2021), while we observed thirteen differentially abundant functional groups and organisms between treatment. Our findings along with previous work suggest that mycolactone alters the expression levels of other microbes in aquatic communities but additional investigations are needed to further explore differences due to treatment as well as dose-dependent effects.

The interactions between mycolactone, other aquatic microbes, and mosquitoes highlight the need for study of environmental toxins in complex communities to better understand the ecology and transmission of opportunistic pathogens. Following with the Coincidental Evolution of Virulence (CEV) hypothesis, the characteristics of *M. ulcerans* and its associated toxin mycolactone suggest mycolactone's primary role is in interacting with other environmental microbes and providing a fitness advantage (Brown et al. 2012, Dhungel et al. 2021). This idea is supported by our observed impacts of mycolactone on other microbes in polymicrobial communities. With mosquitoes identified as a potential mechanism in the transmission or dispersal of *M. ulcerans* (Sanders et al. 2017, Wallace et al. 2017), whether the effects of mycolactone on mosquito oviposition are intentional or are a byproduct of influencing other microbes (potentially through suppression of quorum sensing activity), remains a question for future study.

Conclusion

The addition of mycolactone to aquatic microbial communities altered community composition and gene expression in addition to dose dependent impacts on mosquito oviposition behavior. Our findings highlight a need to explore the effects of mycolactone and other microbial produced toxins in conditions closer to those found in nature as interactions between toxins and other organisms (e.g., microbes) may alter responses, compared to investigations of the toxins in isolation. While there remains much work to be done to fully characterize how the mycolactone toxin interacts with the environment, this study provides new information about the interactions between mycolactone, aquatic environments, and insect species. A better understanding of these interactions may shed light on the cryptic dispersal and transmission of *M. ulcerans* to humans.

CHAPTER FOUR:

INSECT-ASSOCIATED BACTERIAL COMMUNITIES IN AN ALPINE STREAM

Receveur et al. 2020 Hydrobiologia https://doi.org/10.1007/s10750-019-04097-w

Preface

While this already published article does not focus on mosquito microbiomes or oviposition, it explores a similar question to those investigated in previous chapters: How do environmental conditions and leaf-litter inputs alter the microbial communities of macroinvertebrates? Supplemental figures and tables from this appendix can be found at https://link.springer.com/article/10.1007/s10750-019-04097-w.

Abstract

The roles of macroinvertebrate and microbial communities in stream ecosystems are recognized to be important to energy flow and nutrient cycling. While the linkages of these major groups of aquatic organisms has not been thoroughly investigated, determining how these they interact is particularly important for understanding the mechanisms and potential evolutionary relationships that contribute to ecosystem processes, such as organic matter decomposition. We evaluated the microbiomes of aquatic insect species differing in trophic ecology and functional group designations at two sites along an Italian Alpine river with different elevation and environmental characteristics, one located above the tree-line and the other in a forested environment. We found that the internal microbiota of insects from different functional feeding guilds significantly varied in their taxonomic and functional composition and could be used to classify samples to both species and environment. We demonstrated that functional differences existed between the microbiota of insect species with different feeding behaviors, and that belonging to the same species was more important, in this context, than environmental or habitat conditions. These results provide new information on how the microbiomes of macroinvertebrates are influenced by the ecology of their hosts and habitat conditions in Alpine streams.

Introduction

There has been limited study of entire microbial communities associated with aquatic macroinvertebrates, and this is especially true in high altitude and high gradient mountain streams. Evidence from other systems suggests that the internal microbial communities, or microbiota, of insects and other invertebrates have important functional effects on both their biology and ecology (Douglas 2015; Henry et al., 2015; Moran & Telang 1998). These studies also show that the internal microbiomes may have co-evolved with certain species (e.g., ants) (Hooper et al., 2012; Moran & Telang 1998; Russell et al., 2009), providing important functional roles to the fitness and dispersal of many species. In addition, research has shown that the microbes of decaying organic matter (Benbow et al., 2019), in the form of plant (Cummins et al., 1973; Eggert & Wallace 2007; Moore et al., 2004) and animal (Pechal & Benbow 2016; Pechal et al., 2013) detritus, are acquired through feeding activities and may be transferred through insect developmental stages (e.g., larvae to pupae to adults) (Hocking & Reimchen 2006; Pechal et al., 2019; Weatherbee et al., 2017). In freshwater ecosystems microbial communities contribute to the decomposition of autochthonous and allochthonous organic matter (Baldy et al., 1995; Webster & Benfield 1986), and are known to vary along the watershed continuum (Savio et al., 2015), likely responding to riparian forest conditions, hydrological regimes and biotic interactions (Besemer et al., 2013; Widder et al., 2014) in ways that mediate the quality and quantity of organic matter that is transported downstream. However, how the internal microbial communities of aquatic insects contribute to these processes remains largely unknown.

Organic matter subsidies vary in quantity and typology along the length of watersheds (Cummins 1974; Vannote et al., 1980), intimately linked to the structure and diversity of riparian and basin vegetation. Small order streams draining forested watersheds have significantly higher

allochthonous organic matter inputs (i.e., litterfall) than streams draining unforested areas (Golladay 1997; Tank et al., 2010). Since the formulation of the River Continuum Concept (Vannote et al., 1980), stream ecology has adopted, modified and refined (Sedell et al., 1989; Statzner & Higler 1985; Winterbourn et al., 1981) a theoretic framework in which terrestrial and aquatic ecosystems are intrinsically linked as are downstream and upstream reaches, so that biological, physical, and chemical changes can be predicted along a longitudinal gradient. Under this framework, mountainous lotic systems assume great interest in understanding how organic matter is processed at the upper elevations of high gradient watersheds which harbor unique sets of ecological processes and specialized taxa (Ward & Saltz 1994).

Mountainous, low-order streams are distinctive systems characterized by cold, highly oxygenated and turbulent water, steep gradients, coarse substrata, low channel stability and limited nutrient availability (Hieber et al., 2005). Among these systems, the tree line defines separate areas of the riparian zone with different limiting factors for plant growth and thus species composition (Figure 4.1A). This abrupt change in riparian conditions is important (but largely uninvestigated in Alpine regions) in influencing solar radiation and quantity and quality of terrestrial organic matter subsidies. For example, below the tree line most energy inputs are derived from allochthonous non-living coarse particulate organic matter (CPOM), mainly terrestrial leaves (Tank et al., 2010), while above catchments have scarce terrestrial vegetation, and consequently reduced input of allochthonous organic matter. Aquatic macroinvertebrate communities are known to respond both taxonomically and functionally to changes in allochthonous CPOM from the riparian forests (Cummins & Klug 1979; Doretto et al., 2016; Merritt & Cummins 2006; Vannote et al., 1980).

One approach to investigate the linkages between allochthonous subsidies and associated biotic processing has been to evaluate functional groups of aquatic macroinvertebrates that process and consume organic matter differently (Cummins 2016; Cummins & Klug 1979; Merritt & Cummins 2006; Straka et al., 2012). Aquatic macroinvertebrates use a range of feeding strategies to obtain nutrients, and as such, display morphological and behavioral traits which can be used to classify their feeding behaviors into broad groups. These groups range from shredders, which feed directly on allochthonous inputs such as leaf materials (and microbes associated with these resources), to scrapers, which feed predominantly on biofilms present on substrates, and predators. It has been shown that the gut bacterial communities of aquatic invertebrates change in response to different food sources, with most studies using culture-based microbial survey approaches (Kaufman et al., 2000; Lawson et al., 1984); however, more comprehensive descriptions of how the entire gut microbial community, using recent genomic sequencing technologies, responds to changes in allochthonous CPOM inputs has been less studied (Pechal & Benbow 2016; Yun et al., 2014).

The overall goal of this study was to describe the internal microbiota of five aquatic insect species from four functional feeding groups (i.e., scrapers, shredders, filterers, and predators) at two elevations associated with distinct environmental characteristics (mainly related to elevation and the presence or absence of riparian forest cover) along an Alpine stream in Italy. We predicted that the internal microbiota of aquatic insects would differ, in part based on their elevational locations, with lower microbial diversity at the higher elevation site receiving less diverse CPOM, but that this difference would be mediated by species.

Materials and Methods

Study location

Aquatic insects were collected at two sampling locations along the Po River, the longest Italian lotic system, which originates from a spring below the northwest side of the Monviso mountain, in the Cottian Alps of north-western Italy (Figure 4.1B). Pian della Regina (Alpine prairie) was the high elevation location at 1750 m above sea level (m.a.s.l.) and above the tree line of the drainage basin. Here the stream was an open system flowing across a plain of glacial origin, characterized by extensive Alpine meadows pointed by large erratic boulders and very few, scattered *Larix decidua* Mill., 1768. Riparian vegetation was composed almost exclusively by herbaceous species, Poaeceae and Ericaceae. Within stream substrata were homogeneous and composed mainly by coarse elements (approximately 50% boulders, 40 % cobbles, 10% gravel/sand) (Figure 4.2A). Ostana (Forest) was the downstream, lower elevation location (971 m.a.s.l.) with forested riparian zones and slopes dominated by a mixed broadleaf forest containing *Fagus sylvatica*, *Acer* sp., *Fraxinus excelsior*, and *Alnus glutinosa*. Within stream substrata was similar to the Alpine prairie location and composed mainly of coarse elements (approximately 30% boulders, 50% cobble, 20% gravel/sand) (Figure 4.2B). These locations were selected by *a priori* knowledge of the aquatic macroinvertebrate taxa previously reported (Doretto et al., 2017; Fenoglio et al., 2015) where we could sample species from four functional feeding groups: scrapers, shredders, filterers, and predators.

Figure. 4.1 Study location. a) Representative photo of tree line in Ostana. b) Study drainage basin in Italy showing the elevation/altitude for the higher elevation Alpine prairie location (Pian della Regina) and lower elevation forest location (Ostana). Altitude is displayed in meters above sea level.

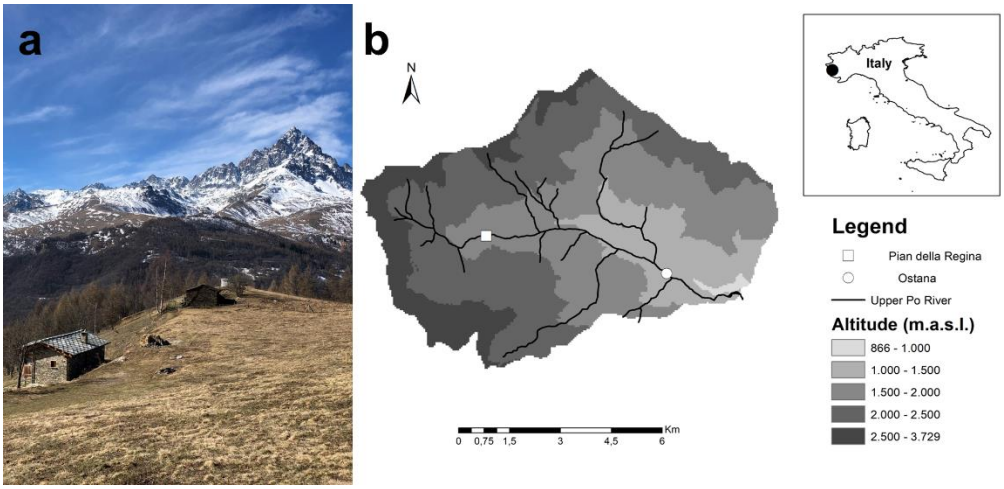
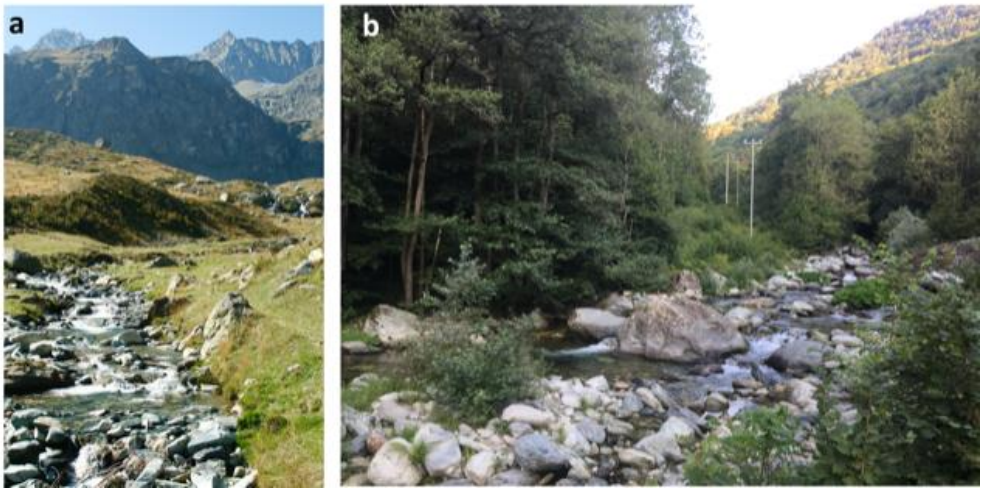


Figure. 4.2. Riparian vegetation cover a) high elevation Alpine prairie station (Pian della Regina) and b) lower elevation forested station (Ostana)



Sample collections

At each location, selected aquatic insect taxa were collected on a single occasion, to minimize temporal variation in gut contents and environmental variables. On 23 November 2017,

a season in which biodiversity and invertebrate abundance are generally highest in these ecosystems (Doretto et al., 2017; Fenoglio et al., 2015), benthic insects were hand collected by visually searching and turning over stream substrata. Five species were selected as representatives of the four most important FFG in mountain systems: for scrapers the mayfly *Epeorus alpicola* (Eaton, 1871) (Ephemeroptera: Heptageniidae), and for shredders the cranefly *Tipula* (*Acutitipula*) *maxima* Poda, 1761 (Diptera; Tipulidae). However, we could not find the same species of predator at both elevation locations, so two species of Systellognathan Plecoptera predators were used for microbiome characterizations: *Dictyogenus alpinus* (Pictet, 1841) (Perlodidae) for the forested location and *Perla grandis* Rambur, 1842 (Perlidae) for the Alpine prairie location. No filterers were collected at the alpine prairie location, while *Hydropsyche* sp. (Trichoptera: Hydropsychidae) were found at the forested location.

All specimens were immediately preserved in 95% molecular grade ethanol within sealed glass vials for laboratory identification under a stereomicroscope using regional dichotomous keys (Belfiore 1983; Fochetti & Tiernod e Figueroa 2008; Moretti 1983; Rivosecchi 1984) and then weighed. This preservation approach was used based on our previous success with describing the microbiomes of aquatic and terrestrial insects (Benbow et al., 2017; Pechal & Benbow 2016; Pechal et al., 2019; Receveur et al., 2018; Weatherbee et al., 2017). At the time of sampling, physical and chemical parameters were measured using multiparametric probes (physio-chemical properties [Quanta, Hydrolab] current [Mod RHCM, Idromar]) as well as organic matter and nutrients according to Italian standard methods of the Agenzia per la Protezione dell'Ambiente e per i servizi Tecnici - Istituto di Ricerca sulle Acque Consiglio Nazionale delle Ricerche (APAT-IRSA 2003).

Nucleic acid isolation and bioinformatic data processing

To limit the influence of microbes present on the external surfaces of the insects, after preservation and immediately prior to nucleic acid isolation, all samples were surface decontaminated using a 10% hypochlorite wash followed by a triple rinse in sterile water as previously described (Ridley et al., 2012), as we have done in previous work with aquatic insects (Pechal & Benbow 2016; Receveur et al., 2018). Following surface decontamination, samples were homogenized using sterile pestles as previously described (Pechal & Benbow 2016; Receveur et al., 2018). Briefly, DNA extraction was performed using the Blood and Tissue DNA kit (Qiagen®) with the addition of lysozyme (15 mg ml^{-1}, Invitrogen) during the lysis step before being quantified fluorometrically using a Qubit 2.0 (Grand Island, NY, USA) and a dsDNA High Sensitivity Assay Kit (Invitrogen). All DNA preparations were stored at -20°C until library preparation. Library preparation and sequencing (2 x 250 bp paired-end reads) was performed by the Michigan State University Research Technology Support Facility on an Illumina MiSeq platform following previously described methods (Caporaso et al., 2011). Variable region 4 (V4) of the 16S rRNA gene was amplified using indexed primers 515f and 806r (5′-GTGCCAGCMGCCGCGGTAA -3′, 5′- GGACTACHVGGGTWTCTAAT -3′) as described previously (Caporaso et al., 2011; Claesson et al., 2010; Kozich et al., 2013). Demultiplexing and base calling were performed using Bcl2fastq (v 2.19.1, Illumina) and RTA (v 1.18.54, Illumina).

The raw sequencing reads were quality filtered using QIIME 2 (v 2018.11) using default settings (Bolyen et al., 2018). DADA2 was used to filter samples and remove low quality reads as well as chimeric sequences and other artifacts commonly present in Illumina data (Callahan et al., 2016). After singletons and amplicon sequencing variants with an abundance lower than 0.0005% were removed, a Naïve Bayes classifier was trained using the region amplified by the primers

(515f, 806r, 250 bp) and the Greengenes database (v 13.8) at a 99% confidence level before being used to assign taxonomy using default settings in QIIME 2 (Bokulich et al., 2018). Reads mapped to mitochondria or chloroplast were removed. A rooted phylogenetic tree, created using all remaining sequence variants and default settings in QIIME 2, FastTree (v 2)(Price et al., 2010), and MAFFT (v 7)(Katoh & Standley 2013) was used in calculating a phylogenetic diversity metric [Faith's phylogenetic distance (Faith's PD)]. Both Faith's PD and Shannon diversity were calculated using defaults settings in QIIME 2 (Faith & Baker 2006). To evaluate the functional community differences, PICRUSt 2 (https://github.com/picrust/picrust2) (Langille et al., 2013) was used to assign filtered sequencing reads to functional orthologs [Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO)] using the mp hidden-state prediction method (Louca & Doebeli 2017). Sequences files for this study have been deposited in the NCBI database under the accession number PRJNA547724.

Statistical analyses

Differences in macroinvertebrates mass between sampling location met the assumptions of normality and were tested with t-tests in R (v 3.5.2) (2013). Differences in the relative abundance of bacterial taxa between groups at the phylum and family level were tested using Kruskal-Wallis and Mann-Whitney tests in R with FDR corrections to account for multiple comparisons. To identify taxa which were differentially abundant at the genus level and test how well a model was able to classify samples to group (species or location), a machine learning algorithm (Random Forest) was used. Rather than test for differential abundances in all bacterial genera present (> 100), only the top indicators for each comparison were evaluated to limit the potential for spurious conclusions. The importance of an indicator in a Random Forest model is determined by how much removing that taxa from a model decreases the overall accuracy. The ten most important genus

level indicators (determined by mean decrease GINI score and mean decrease accuracy) used by the models to classify samples to group were tested for differences using Kruskal-Wallis and Mann-Whitney tests in R. The random forest model was implemented using default settings in the RandomForest package (1000 trees, v 4.6-14) (Liaw & Wiener 2002). Alpha diversity metrics (Faith's PD and Shannon diversity) were compared using Kruskal-Wallis tests with a FDR correction for multiple comparisons. Beta diversity and dispersion (taxonomic and functional) were compared between site and species using PERmutational Multivariate Analysis Of Variance (PERMANOVA, 999 iterations) tests using Jaccard distance implemented in the vegan package (v 2.5-4) (Oksanen et al., 2015). Differences in beta diversity were visualized using Principle Coordinate Analysis (PCoA) plots and shown with ellipses representing 95% CIs for the mean of each group. Data were visualized using a combination of ggplot2, ggpubr, and phyloseq packages (Kassambara 2017; McMurdie & Holmes 2013; Wickham 2016) with all code used in analysis available at https://github.com/BenbowLab/AlpineStreamMicrobiome.

Results

Stream conditions and macroinvertebrate communities

The forest sampling location had a lower mean temperature (10.5 vs 12.8 °C), higher conductivity (132 vs 98 μS cm^{-1}) and lower dissolved oxygen (9.38 vs 10.2 mg L^{-1}) than the Alpine prairie location while other parameters measured were similar. A total of 26 insect samples were used for sequencing analysis (Table 4.1). No *Hydropsyche sp.* were collected at the Alpine prairie and only two *Perla grandis* (Plecoptera:Perlidae) were collected at the forest location (provided in the results but no group with less than three individuals was included in statistical analyses). The average mass of *Epeorus alpicola* (Ephemeroptera:Heptageniidae) was nearly double (22.7 ± 3.86 [SE] mg vs 11.6 ± 4.0 mg) at the forest location (t =4.22, P = 0.005), while there was not a significant difference in *Tipula maxima* (Diptera: Tipulidae) mass between locations (t = 1.81, P = 0.21; Figure 4.3A).

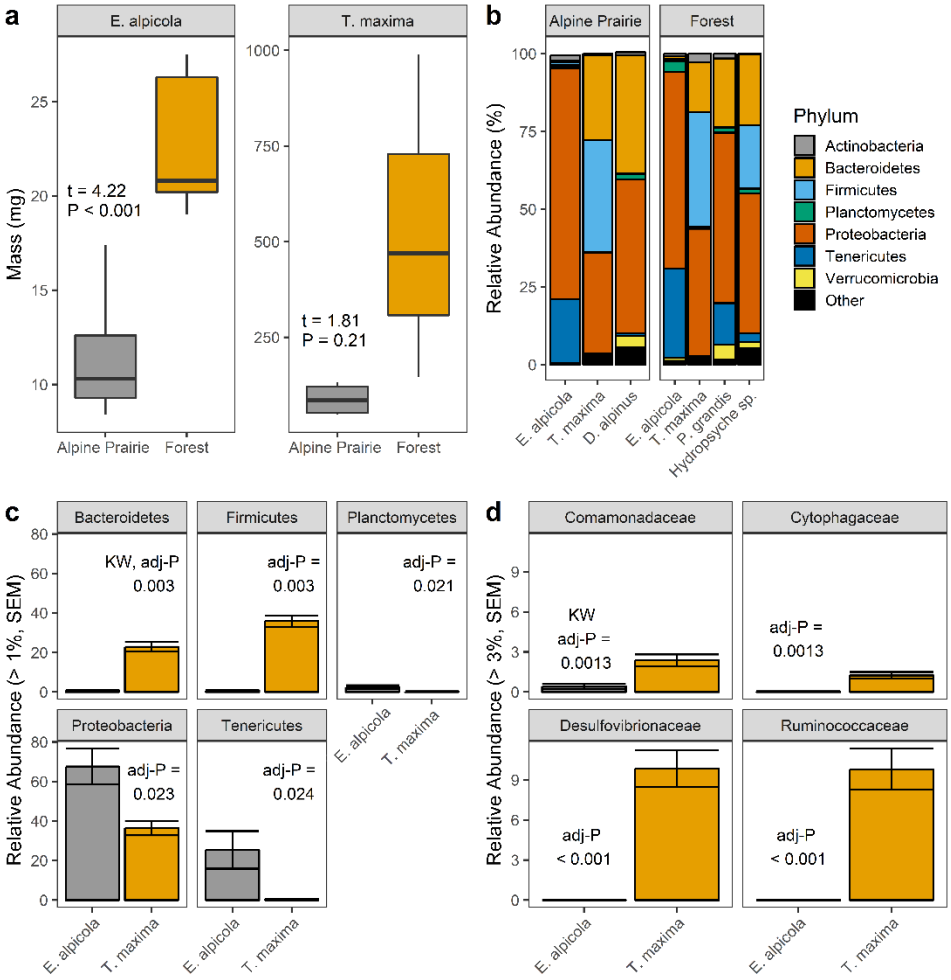
Table 4.1. Macroinvertebrate samples used for microbial sequencing.

Location	**Taxon Name**	**N**	**Mean Weight (mg)**	**SE**
Forest	*Hydropsyche* sp.	4	41.5	16.9
	Perla grandis	2	190.5	123.4
	Epeorus alpicola	5	22.8	1.7
	Tipula maxima	3	534.9	245.6
Alpine prairie	*Dictyogenus alpinus*	4	84	8.1
	Epeorus alpicola	4	11.6	2
	Tipula maxima	4	87.7	21.4

Internal microbial communities

From the 26 samples used for sequencing, a total of 809,647 reads were obtained after filtering representing 2,420 exact sequence variants. To limit bias due to differing read sizes, samples were rarefied to 3,000 reads per sample (Figure S1). The three most abundant phyla across all samples were Proteobacteria (51.9% ± [SE] 4.3), Bacteroidetes (17.5% ± 2.8), and Firmicutes (13.3% ± 3.4), representing 83% of the total communities. As no statistically significant differences in relative bacterial abundance due to environment (i.e., location) were observed at the phylum or family level between samples of the same species (Kruskal-Wallis, $P > 0.05$, Figure 4.3B), the two locations were combined to investigate what taxonomic differences between *Epeorus alpicola* and *Tipula maxima* were conserved at both sites. The relative abundances of Proteobacteria, Tenericutes, and Planctomycetes were significantly higher in *E. alpicola* than *T. maxima* while Bacteroidetes and Firmicutes were significantly more abundant in *E. alpicola* (KW, $P > 0.05$, Figure 4.3C) At the family level, there were thirteen bacterial families (greater than 1% of total abundance) which were differentially abundant between *E. alpicola and T. maxima* (KW, $P < 0.05$, Figure 4.3D).

Figure 4.3. Internal bacterial communities of macroinvertebrates from different species. a) Differences in mass (mg) for *E. alpicola* and *T. maxima* at the two sites. Significance was determined by t-tests. b) Phylum level bacterial relative abundance between species. c) Differences in phylum level relative bacterial abundance between *E. alpicola and T. maxima*. Samples from the two sites were combined and only phyla with a relative abundance greater than 1% of the total relative abundance are shown. Samples were compared with Kruskal-Wallis tests with FDR correction. d) Differences in family level relative bacterial abundance between *E.* alpicola and *T.* maxima. Samples from the two locations were combined and only families which made up greater than 3% of the total relative abundance are shown, for lower abundance families see Figure S2. Significance between species were compared with Kruskal-Wallis tests and FDR correction. Error bars are SEM.

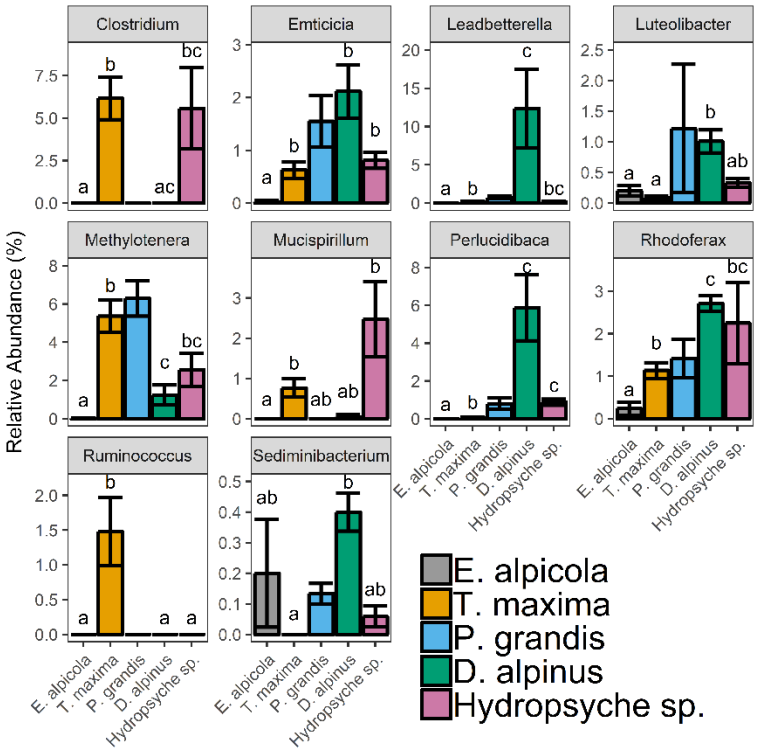


To identify important genera that were differentially abundant, rather than testing for differences in every genus (N = 170), a random forest modeling approach was used to determine the top ten predictors of each group, which would then be tested further. Modeling was able to

correctly classify which location a sample came from, regardless of species, with an Out Of Bag (OOB) error rate of 3.85% at the genus level (one sample from the Alpine prairie site misclassified as forest). The ten most important genera for classification (determined by Mean Decrease Gini score and Mean Decrease Accuracy) were then tested using Kruskal-Wallis tests to determine if they were differentially abundant between locations. None of the top ten indicators using either ranking method were significantly different between location (KW, $P > 0.05$).

To determine if modeling could predict species, regardless of location, and identify differentially abundant genera, samples from the two locations were combined and tested similarly as above. The random forest model was able to predict species (*P. grandis* not included in model, $N = 2$) with an error rate of 8.33% (two *Hydropsyche* sp. misclassified). All of the top ten predictors using both ranking methods were significantly different between species (KW, $P < 0.001$) with multiple comparisons (Mann-Whitney, $P < 0.05$) shown in Figure 4.4.

Figure 4.4. Top ten genus level predictors (determined by mean decrease GINI) for a random forest model predicting species (*P. grandis* was not included in the model, N = 2). Error bars represent SEM. Significance between pairwise comparisons (Mann-Whitney, FDR correction) are denoted by lowercase letters.



Bacterial community diversity

Forest *E. alpicola* microbial communities were more phylogenetically diverse (9.05 ± [SE] 0.7, Faith's PD: KW, $\chi^2 = 6$, P = 0.014, Figure 4.5A) than *E. alpicola* from the Alpine location (4.73 ± 0.26), while Shannon diversity was not significantly different between location (KW, $\chi^2 = 2.94$, P = 0.086, Figure 4.5B). For *T. maxima*, diversity was not different between locations for either alpha diversity metric (KW, P > 0.05). Comparing between species at the forest location, *T. maxima* and *Hydropsyche sp.* displayed similar levels of bacterial diversity while having significantly higher Shannon (Mann-Whitney, P < 0.05) and phylogenetic diversity (MW, P < 0.05) than *E. alpicola*. A similar pattern was observed at the prairie location with *T. maxima* and

D. alpinus having significantly higher diversity than *E. alpicola* according to both metrics (MW, $P < 0.05$).

Differences in beta diversity was visualized using PCoA plots and compared with PERMANOVA tests using Jaccard distance. Species, sampling location, as well as their interaction impacted beta diversity, with species having the largest effect size (PERMANOVA, $P < 0.01$, Table 4.2, Figure 4.5C). As the interaction between location and species significantly influenced beta diversity, the effects of species were investigated separately for each location. At both the forest (*E. Alpicola, T. maxima, and Hydropsyche sp.*) and prairie (*E. alpicola T. maxima, and D. alpinus*) sites, all species within a location had significantly different microbial communities from each other (PERMANOVA, $P < 0.05$).

Table 4.2. Differences in taxonomic beta diversity (Jaccard, PERANOVA) between species (*P. grandis* not included, N = 2) and location. SS = Sums of Squares, MS= Mean Squares.

	Df	**SS**	**MS**	**F**	**R^2**	**P (<)**
Taxa	3	3.10	1.03	3.10	0.30	0.001
Location	1	0.64	0.64	1.93	0.06	0.01
Taxa:Location	1	0.67	0.67	2.01	0.07	0.001
Residuals	18	6.01	0.33			
Total	23	10.43				

Functional community composition

Due to the reliance on high-quality gene annotations for predicting individual functional pathways, which are lacking for many poorly characterized environmental sample types (Langille et al., 2013; Radivojac et al., 2013), predicted bacterial functional differences between location and species were explored using community diversity metrics rather than individual pathway abundances. While species had a significant (PERMANOVA, $F = 4.81$, $P > 0.001$) effect on

functional community diversity and accounted for 41% of the variation (R^2), location did not have a significant effect (P > 0.05, Table 4.3). As there was not a significant effect of location, samples from both locations were combined to determine how community functional diversity differed between species. When pairwise comparisons were run between insect species (*P. grandis* not tested, N = 2), all had significantly different functional communities (PERMANOVA, P < 0.05, Figure 4.5d).

Figure 4.5. Differences in community diversity between species a) Differences in Faith's phylogenetic diversity (Faith's PD) between species. Comparisons between species were tested with Kruskal-Wallis tests with pairwise significance (Mann-Whitney) denoted by lowercase letters. b) Shannon diversity differences between species. c) PCoA plot (Jaccard distance) showing community differences due to taxonomic composition. d) PCoA plot (Jaccard) showing predicted functional differences in beta diversity between species. Ellipses represent 95% CI for the mean of each group.

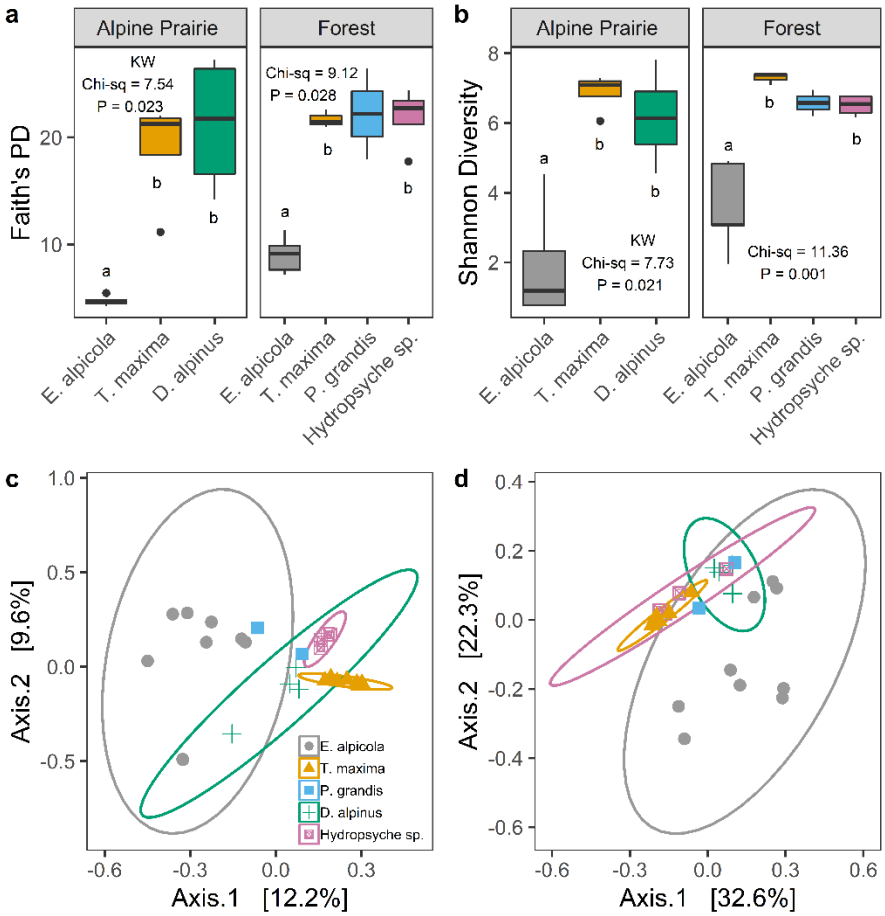


Table 4.3. Differences in predicted functional beta diversity (Jaccard, PERMANOVA) between species (*P. grandis* not included, N = 2) and location. SS = Sums of Squares, MS= Mean Squares.

	Df	**SS**	**MS**	**F**	**R^2**	**P**
Taxa	3	0.87	0.29	4.81	0.41	0.001
Location	1	0.09	0.09	1.45	0.04	0.18
Taxa:Location	1	0.06	0.06	1.04	0.03	0.39
Residuals	18	1.09	0.06			
Total	23	2.11				

Discussion

In this study, we examined how the internal microbiota of aquatic macroinvertebrates differed among species belonging to four functional feeding groups in two Alpine stream habitats with different riparian conditions. While invertebrate-microbe interactions have long been recognized as an essential component of understanding food web interactions in aquatic systems (Cummins & Klug 1979; Kaufman et al., 2000), this study represents the first comparison between the internal microbial communities of different macroinvertebrate species using high throughput genomic sequencing in Alpine stream communities. Two other recent studies using high throughput sequencing to compare the microbiota of aquatic invertebrate functional groups occurred in Midwest streams (USA) (Ayayee et al., 2018), and associated with salmon decomposition (Pechal & Benbow 2016) in Alaska (USA).

While invertebrate feeding groups differ in their predominant method of feeding, most aquatic macroinvertebrates are omnivorous and readily uptake food from a variety of sources (e.g., scrapers ingesting particulate organic matter or eukaryotes during feeding on surfaces) though their ability to digest certain foods can vary due to multiple factors, including differences in their internal microbiota (Pechal and Benbow 2016), pH, and oxygen conditions (Cummins and Klug 1979). For example, it has been shown that craneflies (shredder, Diptera: Tipulidae) require microbial conditioning of leaf surfaces for development and use gut bacteria to help break down ingested food (Klug & Kotarski 1980; Lawson & Klug 1989). While in terrestrial systems, the functional roles of microbes are widely documented (e.g., nitrogen fixation or cellulose degradation) (Alonso-Pernas et al., 2017; Alonso-Pernas et al., 2018; Ayayee et al., 2018; Gupta et al., 2012), in aquatic systems comparatively little research exists, but it is hypothesized that there are similar relationships (Ayayee et al., 2018). Although we chose not to examine individual

functional pathways due to limitations of using gene amplicon data for this purpose in understudied systems (Langille 2018; Langille et al., 2013; Radivojac et al., 2013), we found that the internal bacterial functional diversity and composition was distinctly different with species explaining close to half (41%) of the variation in the communities. As macroinvertebrates from different feeding groups ingest and process predominantly distinct forms of organic matter (Ayayee et al., 2018; Cummins & Klug 1979), it would be expected that their gut community assemblages would be adapted to different functional roles, similar to terrestrial insects (Larsen et al., 2016; Mason et al., 2016). While further research will be needed to elucidate exactly what functional roles these bacteria play, we demonstrate both structural and functional differences among microbial communities of insect species with different feeding behaviors.

We observed several differentially abundant bacterial taxa and were able to successfully classify internal communities to both species and site with a high degree of accuracy. At all taxonomic levels (phylum, family, and genus) there were significantly different bacterial abundances between species, but no significant effects of location. In contrast to previous studies (Ayayee et al., 2018; Pechal & Benbow 2016), which observed that predatory feeding groups had lower phylogenetic diversity than grazers/scrapers and filterers, we observed that the predatory *D. alpinus* had higher bacterial diversity than *E. Alpicola* (scraper), regardless of location. Predators (Perlodidae: *D. alpinus*) showed similar levels of bacterial diversity (Shannon and Faith's PD) to both filterers (Hydropsychidae: *Hydropsyche* sp.) and shredders (Tipulidae: *T. maxima*) while the scraper *E. alpicola* (Heptageniidae) had lower diversity than all other groups. The lower phylogenetic diversity of *E. alpicola* microbiota at the upstream prairie site, may be related to the lower diversity of benthic algae, and in particular diatoms, that has been shown in the upper sections of Alpine rivers compared with downstream reaches (Falasco & Bona 2011). These results

highlight that additional studies are needed to identify factors that shape macroinvertebrate gut communities.

While we expected the diversity of the microbial communities within *T. maxima* (shredder) to be higher at the forested site due to the presence of more diverse allochthonous and autochthonous resources below Alpine tree lines (Wilhelm et al., 2015) we observed no significant differences in alpha diversity or taxa composition (phylum, family or genus level), suggesting *T. maxima* at these sites are acquiring gut communities with limited colonization from microbes associated with their food. Though location significantly impacted the beta diversity of the taxonomic bacterial communities, it had a smaller effect than species. When comparing bacterial community function, location no longer had significant impacts on diversity, suggesting that although taxonomic differences exist between sites, the taxa present are playing similar functional roles within the insect guts, similar to functional redundancy in other systems (Rosenfeld 2002).

Although differences in beta diversity were observed between sites, there were surprisingly no other significant taxonomic or functional differences between site for either *E. alpicola* (scraper) or *T. maxima* (shredder). As solar radiation and the presence of riparian vegetation significantly alters the taxonomic composition and function of biofilm communities (Wagner et al., 2015; Wilhelm et al., 2015), it would be expected to see differences between scrapers at the two sites, who use biofilms as their predominant food source, if their microbiota simply reflected their diet. That no differences in taxonomic composition or functional diversity were observed between scrapers or shredders at the two sites suggests their gut microbiota may be a result of selective colonization by bacterial taxa and not simply a reflection of their food. This similarity of bacterial communities within insect species from different locations has previously been reported and may be a result of similar nutritional components and microbial species sorting due to similarities in

the gut environment (e.g. morphology, pH, and oxygen conditions) (Anderson & Cargill 1987; Ayayee et al., 2018; Pechal & Benbow 2016).

Conclusion

Under the theoretical framework of the River Continuum Concept (RCC) and its derivatives (Junk et al., 1989; Vannote et al., 1980; Ward & Stanford 1995) high gradient, low order mountain streams provide a unique opportunity to investigate the impact of watershed conditions on bacterial community assembly within macroinvertebrate species from different feeding groups. While the tree line represents a drastic change in riparian conditions, we observed limited effects on internal bacteria community structure and function, compared to the effects of insect species from different feeding groups. While there were site-specific differences in bacterial taxonomic diversity, these changes were not reflected in community function suggesting although different bacterial communities are present at the two locations, they provide similar functions within the insects sampled. The observed dissimilarities between species of different functional feeding groups, regardless of riparian conditions, agrees with previous research in suggesting that conditions within their digestive system allows for selective colonization of microbes, with distinct functional roles, and do not simply reflect their environment/diet (Ayayee et al., 2018). That large differences in CPOM inputs (e.g., leaf material) and light conditions did not lead to differences in individual bacterial taxa or functional changes was surprising, particularly for shredders and scrapers as leaf material and autotrophic organisms represent their predominant food source. As algal and fungal communities comprise important roles in primary production and organic matter processing in stream systems (Cummins 1974; Danger et al., 2013; Kuehn 2016) how these taxa differ between functional feeding guilds will require further investigation. While this study provides initial data on how species and habitat may be linked with insect gut bacterial communities in alpine systems, additional studies are needed to expand on this evidence and test multiple representatives of each functional feeding groups in several catchments. Our study was

limited to one species for each functional group (except for predators), making the distinction between the effect of species and functional group difficult to untangle. Larger, more comprehensive surveys and manipulation experiments are warranted to differentiate species and functional feeding group gut microbial community structure.

CHAPTER FIVE:

CAPACITY BUILDING FOR ANALYSIS OF NEXT GENERATION SEQUENCING DATA

Abstract

The growing availability and cost-effectiveness of genomic sequencing continues to revolutionize how many scientific studies are conducted. However, a major limiting factor on the use of this technology is training in data analysis, especially in regions which have historically limited access to sequencing infrastructure. Towards the goal of mitigating that issue, this chapter focuses on the development of workshop and tutorial materials for the analysis of two common types of sequencing data, gene amplicon and whole genome sequencing. The main goals of this chapter were to: i) Conduct data collection and analysis case studies (two in Benin, one in Italy) with the goals of expanding the skills of international partners towards collection and analysis of high-throughput sequencing data and ii) Use these projects to develop online and in person workshops and tutorials. Outreach conducted as part of this chapter has reached over 300 individuals (approximately 70 during in person training and > 250 through online accesses). Genomic tools provide great promise for improving our understanding of insect-microbe interactions but training in data analysis remains a major part of making these methods more accessible.

Introduction

Vector borne diseases such as malaria, yellow fever and dengue account for significant morbidity and mortality around the world with reporting of over 1 billion cases every year (WHO 2020). These diseases disproportionately impact developing countries and while research and the development of novel control strategies are essential to improve public health, few institutions in these countries have the training and experience to conduct cutting-edge research in these fields. In Benin, vector borne diseases are a major public health challenge with an estimated 1.5 million cases of malaria annually (WHO 2020). Due to the complex interaction of environmental, biological, and sociological factors on vector borne disease transmission, conducting locally and regionally relevant research while also developing and enhancing technological capacity is essential for effective disease control strategies.

Growing levels of insecticide resistance are globally recognized as a threat to human health, particularly regarding vector borne diseases including malaria, yellow fever, and dengue. In numerous cases, insecticide resistance has been shown to stem from long-term or heavy use of insecticides, both of which lead to selection pressure towards insecticide resistance. Mutations in the acetylcholinesterase gene (ACE-1^R strain) provide resistance to organophosphate insecticides while the kdr^R strain contains a mutation in sodium channel genes, providing resistance to pyrethroid insecticides (Djogbénou et al. 2008, Perrier et al. 2021). These mutations represent a major threat to the effectiveness of insecticide applications for disease control, with both being present in Benin (Corbel et al. 2007, Djogbénou et al. 2008). Recently, insecticide resistance, to both organophosphate and pyrethroid based insecticides, has been shown to be mediated by bacterial symbionts (Kikuchi et al. 2012, Nkya et al. 2013, Xia et al. 2013) but additional research is needed.

Most high-throughput technologies used to study microbiomes fall into two broad categories, amplicon-based approaches or whole genome based approaches (WGS) (Preidis and Hotez 2015). Amplicon based approaches target a specific region of DNA, such as the 16S ribosomal RNA gene and then compare the sequences obtained to a database of known sequences to determine the taxonomic composition of samples. (Dave et al. 2012, Cole et al. 2014). In contrast, whole genome methods use random sequencing of DNA to infer taxonomic composition and function (Preidis and Hotez 2015). The benefits and drawbacks of each of these methods has been reviewed numerous times and continues to change as new techniques and technologies are developed (Poretsky et al. 2014, Jovel et al. 2016, Ranjan et al. 2016).

Several current and future technologies show great promise for increasing the accessibility and decreasing the cost of microbiome research. With the constant development and refinement of new techniques, the cost of sequencing has fallen strikingly since 2001, from a cost of $5,292 (USD) per megabase (mb) of genomic DNA, to less than $0.02 in 2017 (Wetterstrand 2017). While sequencing platforms such as the Illumina HiSeq and Ion Torrent PGM are staples of many genomics facilities, the machine costs alone can total upwards of $100,000, which represents a major barrier towards the use of these platforms. However, the current trend of developing smaller sequencing platforms such as the MiniSeq (Illumina) and MinION (Oxford Nanopore), with initial costs as low as $1,000, may lead to greater adoption of NGS as a research tool for microbiome research.

Regardless of the techniques or platforms used, a major consideration for studies of microbial communities is the analysis that occurs after the data is obtained. In many cases, data analysis for these projects is computationally intensive and can quickly become impossible without access to specialized computing resources (Schadt et al. 2010). The advent of cloud computing,

which allows users easy access to computing resources on an as-needed basis, provides a way to conduct computationally intensive analyses without needing dedicated on-site resources. One area that could have rapid, measurable impacts on vector borne disease research efforts is training in incorporating large publicly available genomic datasets into research and surveillance efforts (highlighted by the importance of genomic surveillance in the ongoing COVID-19 pandemic). In the last decade, the development, and increasing cost-effectiveness, of high throughput sequencing technologies has fundamentally changed how studies are conducted across numerous fields. The interest in and usefulness of projects such as The Human Genome Project have led to numerous similar projects, including the Human Microbiome Project, and of great interest to public health in Africa, the *Anopheles gambiae* 1000 genome project, which provides genetic variation data from different populations of *A. gambiae*, the principal vector of malaria in Africa, as well as variation within malaria strains themselves. One of the hallmarks of these projects, that make them so useful, is the public availability of the large amount of data produced. While the data from these projects, as well as the software used for analysis are both freely available, training in using these techniques remains a major barrier to their, an issue highlighted multiple times, including a Nature news article titled, “Don’t let useful data go to waste” (Denk 2017)

Three case studies were conducted with international collaborators to provide experience with hands-on collection and analysis of sequencing data. Two case studies were conducted in collaboration with the Institut Régional de Santé Publique (IRSP), part of the Université d’Abomey–Calavi in Benin, while a third case study was conducted with the Università del Piemonte Orientale in Turin, Italy and the Centro per lo Studio dei Fiumi Alpini (AlpStream) in Ostana, Italy. The two case studies in Benin were related to associations between *An. gambiae* and insecticide resistance. While the third case study, (conducted in Italy) was not directly related to

mosquito microbiomes, it explored a similar question to chapter two above: how different leaf litter inputs (and other environmental differences) alter the internal microbiome of aquatic insects. Sampling schemes for the two gene amplicon case studies (Benin and Italy) were designed to build a dataset to be used in future workshops as well as collect preliminary data to explore a relationship of interest to collaborators in those locations. As part of these case studies, electronic and in person workshop materials/tutorials were created and presented specifically related to gene amplicon and whole genome sequencing data (Figure 5.1, see Table 5.1 for the location of examples workshop materials).

Figure 5.1 Example of online resources created for case study two (https://benbowlab.github.io/Phylogeny.html).

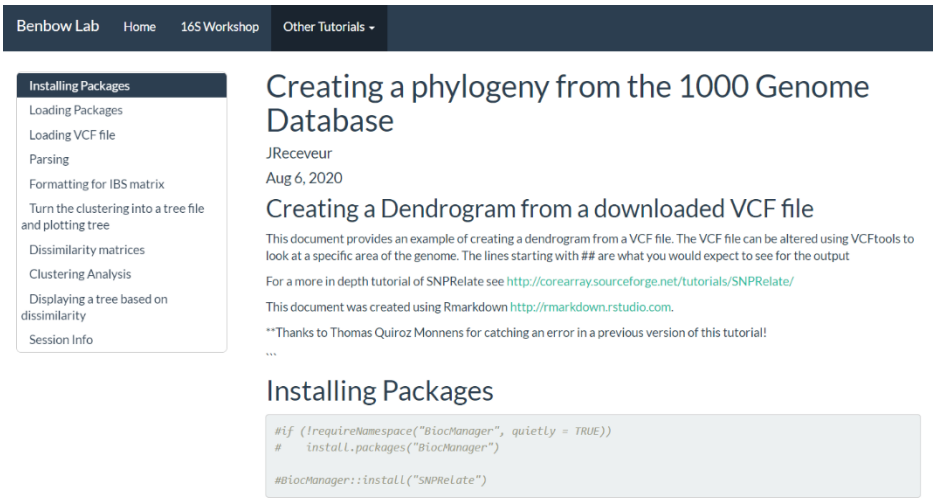


Table 5.1. Overview of materials available for each of the three case studies. For case studies two and three, the html files provide an easily accessible tutorial format where formatted code and explanations can be viewed in an internet browser.

Case study	Goal	Packages (Environment)	Example Materials
1: Microbiome and insecticide resistance traits	Initial filtering, taxonomic assignment, and rarefaction of raw gene amplicon data	QIIME2, DADA2, etc (Python)	https://github.com/BenbowLab/BenbowLab.github.io/blob/master/BMoz16SExampleCode.sb
	Comparing between groups of samples (differences due to mosquito strain)	QIIME2, Emperor, etc (Python)	
2: Building a phylogenetic tree using publicly available data	Initial filtering, and processing/subsetting around a gene of interest	VCFtools (command line)	Appendix III
	Building a phylogenetic tree from a processed VCF file	SNPRelate (R Studio)	https://benbowlab.github.io/Phylogeny.html
3: Environmental impacts on aquatic macroinvertebrate microbiomes	Comparing between groups (Diversity, taxonomic composition, machine learning, etc.)	Various (R studio)	https://benbowlab.github.io/ItalyWorkshop.html

Case study 1: Microbiome and insecticide resistance traits

The internal adult microbiome of two strains of resistant *Anopheles gambiae* mosquitoes (Ace-1^R and kdr^R) were compared to a susceptible strain (Kisumu). All strains had been maintained in colony (Ouidah, Benin, temperature and humidity similar to outside conditions) for a number of generations prior to the experiment. For each of the strains tested (Kisumu, Kisumu-Kdr^R,

Kisumu-Ace-1^R, Kisumu-Ace-1^R-Kdr^R), adult mosquitoes (approximately five days post emergence and prior to blood-feeding) were collected and preserved in 96% EtOH. Each replicate sample within a strain (N = 7-8 depending on strain) came from a separate rearing cage of mosquitoes. Samples came from two trials (using the same methods but collected, extracted, and sequenced at different times). Mosquitoes were surface decontaminated using a 10% bleach rinse before a triple rinse in sterile water. DNA extractions were performed using the DNeasy Blood and Tissue kit (Qiagen) with the addition of lysozyme (15 µg/ml) during the lysis step. Library preparation, sequencing, and analysis was performed similarly to chapter two.

From all samples, 1,411,417 sequencing reads were obtained from 30 samples (mean reads = 47,047). After filtering and rarefaction to 1,000 reads, 25 samples remained. There was no difference in Shannon diversity between strains (Kruskal-Wallis, $\chi^2 = 0.13$, $P = 0.98$), however there was a strong effect of trial ($\chi^2 = 15.07$, $P < 0.001$, Figure 5.2). Similarly, beta diversity (Jaccard) was not strongly influenced by strain ($F_{3,17} = 1.61$, $R^2 = 0.07$, $P = 0.17$) but trial had a large effect on the bacterial communities ($F_{1,17} = 37.1$, $R^2 = 0.54$, $P = 0.015$, Figure 5.3, Table 5.2). In both trials, bacterial communities were predominantly Bacteroidota and Proteobacteria but there were not any significantly different taxonomic groups at the phylum, family or genus levels between strains (KW, P-adj > 0.05). While limited to a few samples and largely overshadowed by impacts of trial, our observations suggest the presence of these mutations does not result in major shifts to the internal microbial communities of *An. gambiae* maintained in colonies.

Figure 5.2 Impact of trial (set) on bacterial Shannon diversity.

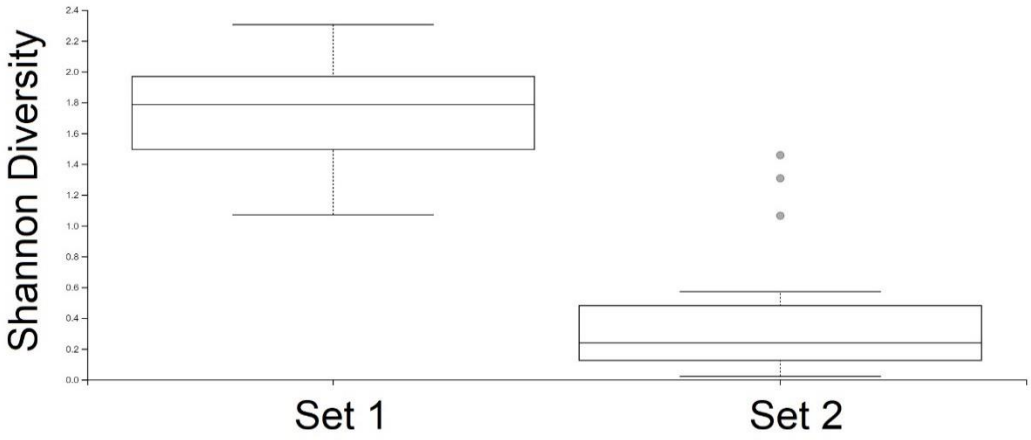


Figure 5.3. PCoA of effect of trial and strain on bacterial beta diversity (Jaccard distance). AcerKis= Kisumu with ACE-1^{R} mutation, AcerKisKdr= Kisumu with ACE-1^{R} and KDR^{R} mutations, KisKdr = Kisumu with KDR^{R} mutation, Kisumu = no mutant phenotype.

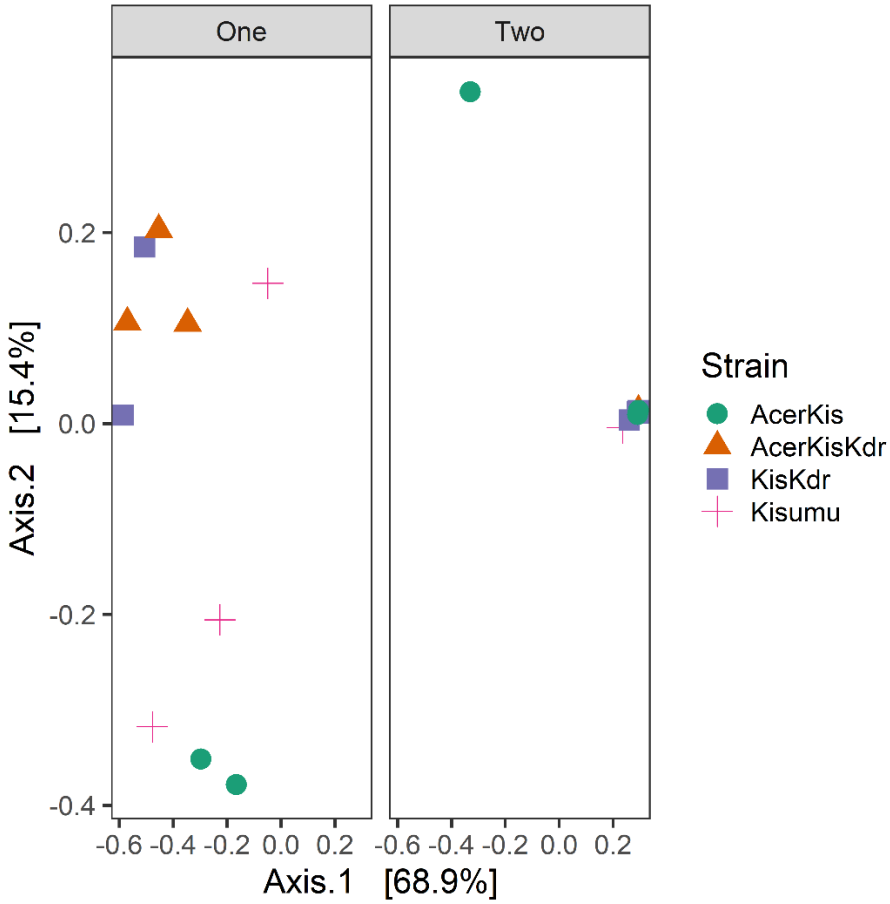


Table 5.2. PERMANOVA of strain and trial impact on bacterial beta diversity. SS =Sum of Squares, MS = Mean Squares. PERMANOVA tests used Jaccard distance and 999 permutations.

	Df	SS	MS	F.Model	R2	Pr(>F)
Strain	3	0.2996	0.09988	1.621	0.07078	0.172
Trial	1	2.2864	2.28636	37.103	0.54008	0.001
Strain:Trial	3	0.5998	0.19992	3.244	0.14168	0.015
Residuals	17	1.0476	0.06162	0.24746		
Total	24	4.2333	1			

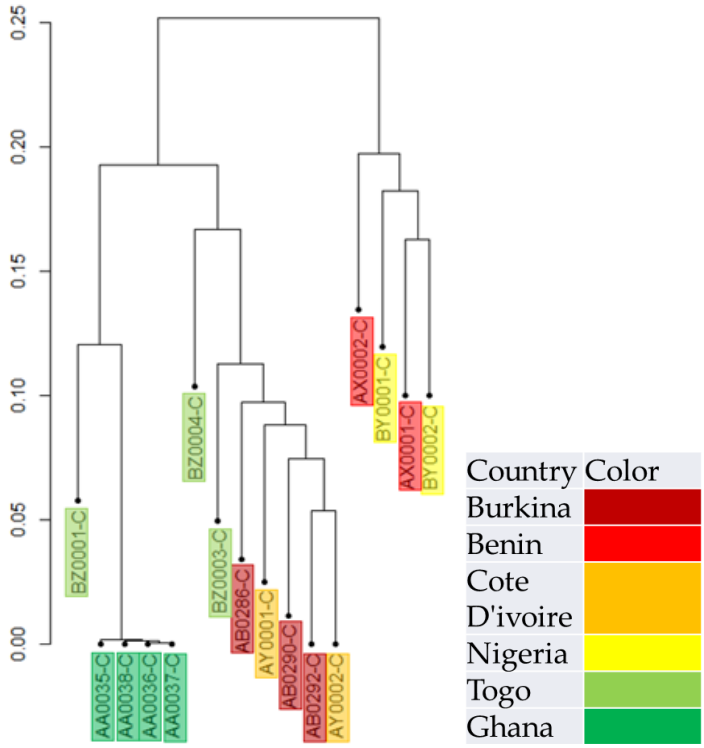
Case study 2: Building a phylogenetic tree using publicly available data

For this case study, a subset of the *An. gambiae* 1000 genome database (An. gambiae Consortium, 2017) was used to create in-person and online workshop materials related to the accessing and analysis of genomic sequencing data. Sixteen samples from across West Africa (Burkina-Faso, Benin, Cote D'ivoire, Nigeria, Togo, and Ghana) were used with the goals of providing tutorials/code for: i) Accessing and initial processing of data around a gene of interest (ACE-1), ii) Calculating of Weir and Cockerham Fst estimates, and iii) Building a phylogenetic tree in R based on Single Nucleotide Polymorphisms (SNPs, Figure 5.4). In contrast to case studies one and three, detailed results of data analysis are not presented in this section as the data used was not collected as part of this dissertation but from publicly available sources (An. gambiae Consortium, 2017).

Case study two was conducted using only subset of the total data available for several reasons. While a larger dataset would provide a greater geographic area and increased inference about study questions, the focus of this work was more to provide instruction in the accessing and processing of public available datasets and less on the answering of a particular question related to genomic variation. Additionally, limiting the sample data to a relatively small dataset (approx. 18.7 Gb without any filtering), eliminated the need for an established high performance computing infrastructure or cloud computing architecture (e.g., Amazon Web Service), and allowing all

analysis to be conducted on personal laptops with freely available open-source software. Modules used in this case study are built around VCFtools (Danecek et al. 2011) and the R packages gdsfmt and SNPRelate (Zheng et al. 2012, R core development team 2019).

Figure 5.4. Example phylogenetic tree created using Identity By State (IBS). Tree is built using SNP polymorphisms withing the acetylcholinesterase gene (ACE-1).



Case study 3: Environmental impacts on macroinvertebrate microbiota

Case study three explored differences in the internal microbiome of aquatic macroinvertebrates at two locations along the Po River that differed substantially in their riparian and environmental conditions (Figure 5.5). The alpine prairie site was located above the tree line with minimal inputs of leaf litter (scattered *Larix decidua* present) while the forested site had substantial inputs of leaf material into the river. Twenty-six samples were sequenced from five macroinvertebrate species (*Epeorus alpicola*, *Tipula maxima*, *Hydropsyche sp*, and *Perla grandis*).

The mass of *E. alpicola* (scraper functional feeding group) individuals at the forested site was higher than individuals at the alpine prairie site (Figure 5.6a). Bacterial communities differed significantly between both species and site with several phylum differing between species (Figure 5.6b-c). Similarly, species influenced alpha and beta diversity measures for bacterial and functional communities ($P > 0.05$, Figure 5.7). Species had a strong impact (PERMANOVA, $F = 4.81$, $P < 0.001$), on functional community diversity and accounted for 41% (R^2) of the variation in functional traits while location did not have a significant effect ($P > 0.05$). The observed dissimilarities between species of different functional feeding groups, regardless of riparian conditions, suggesting that conditions within their digestive system allows for selective colonization of microbes, with distinct functional roles, and do not simply reflect their environment/diet (Ayayee et al. 2018). For a more in depth investigation of these samples, see chapter four for the published version of this study (Receveur et al. 2020).

Figure 5.5. Macroinvertebrate sampling locations along the Po River. a) Alpine prairie location. b) Forested location.

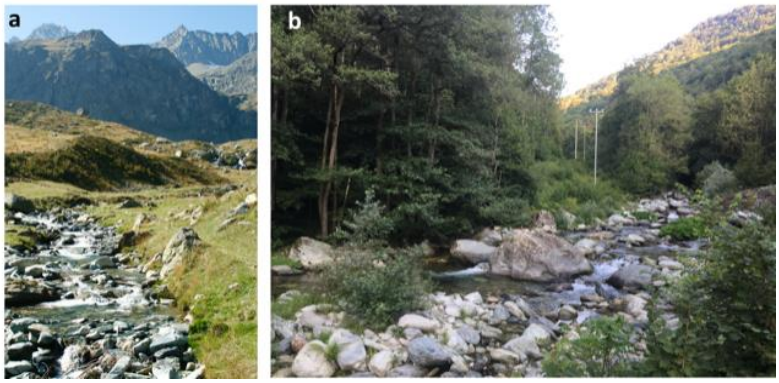


Figure 5.6. Internal bacterial communities of macroinvertebrates from different species. a) Differences in mass (mg) for *E. alpicola* and *T. maxima* at the two sites. Significance was determined by t-tests. b) Phylum level bacterial relative abundance between species. c) Differences in phylum level relative bacterial abundance between *E. alpicola and T. maxima*. Samples from the two sites were combined and only phyla with a relative abundance greater than 1% of the total relative abundance are shown. Samples were compared with Kruskal-Wallis tests with FDR correction. d) Differences in family level relative bacterial abundance between *E. alpicola* and *T. maxima*. Samples from the two locations were combined and only families which made up greater than 3% of the total relative abundance are shown. Significance between species were compared with Kruskal-Wallis tests and FDR correction. Error bars are SEM.

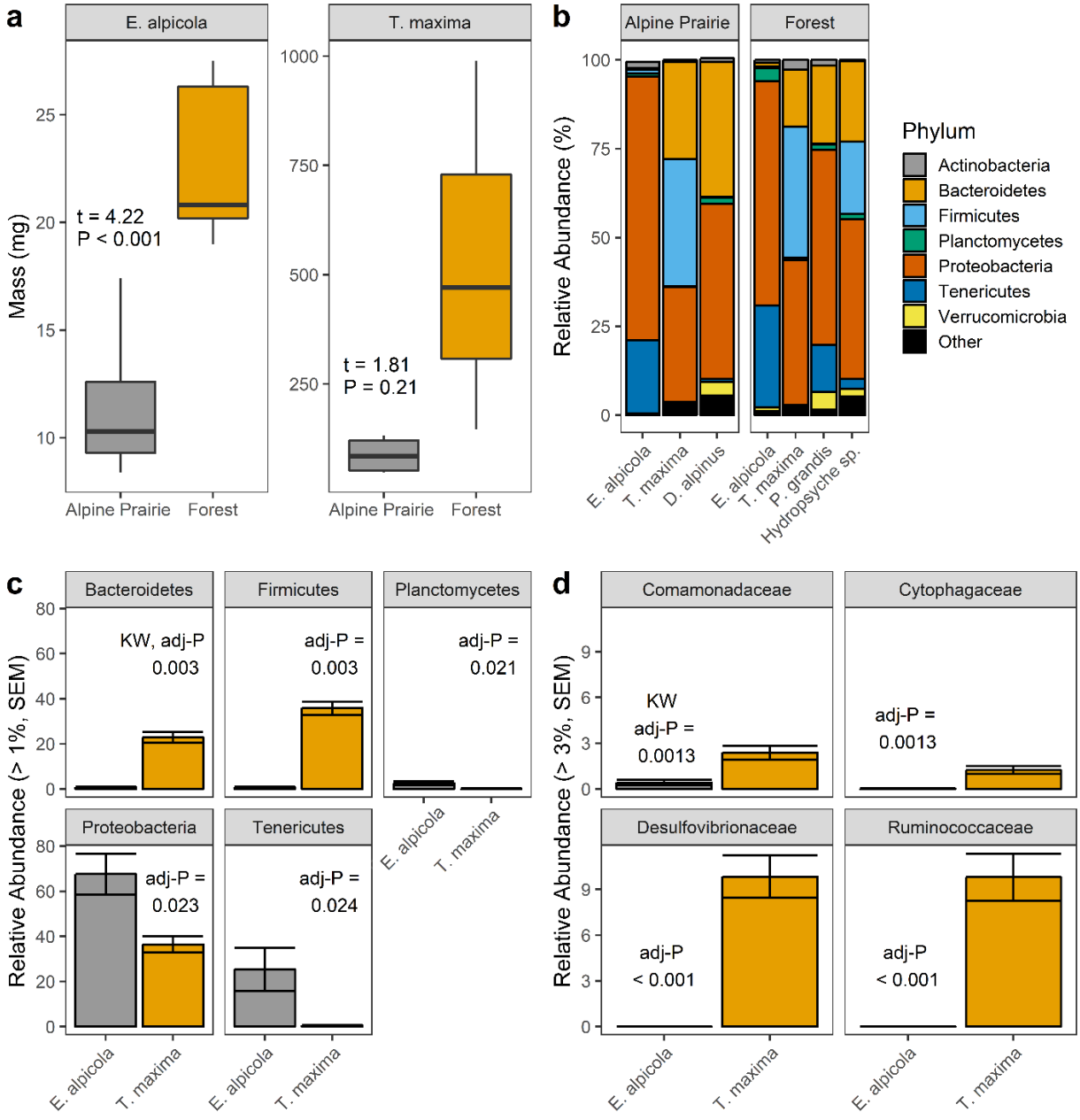
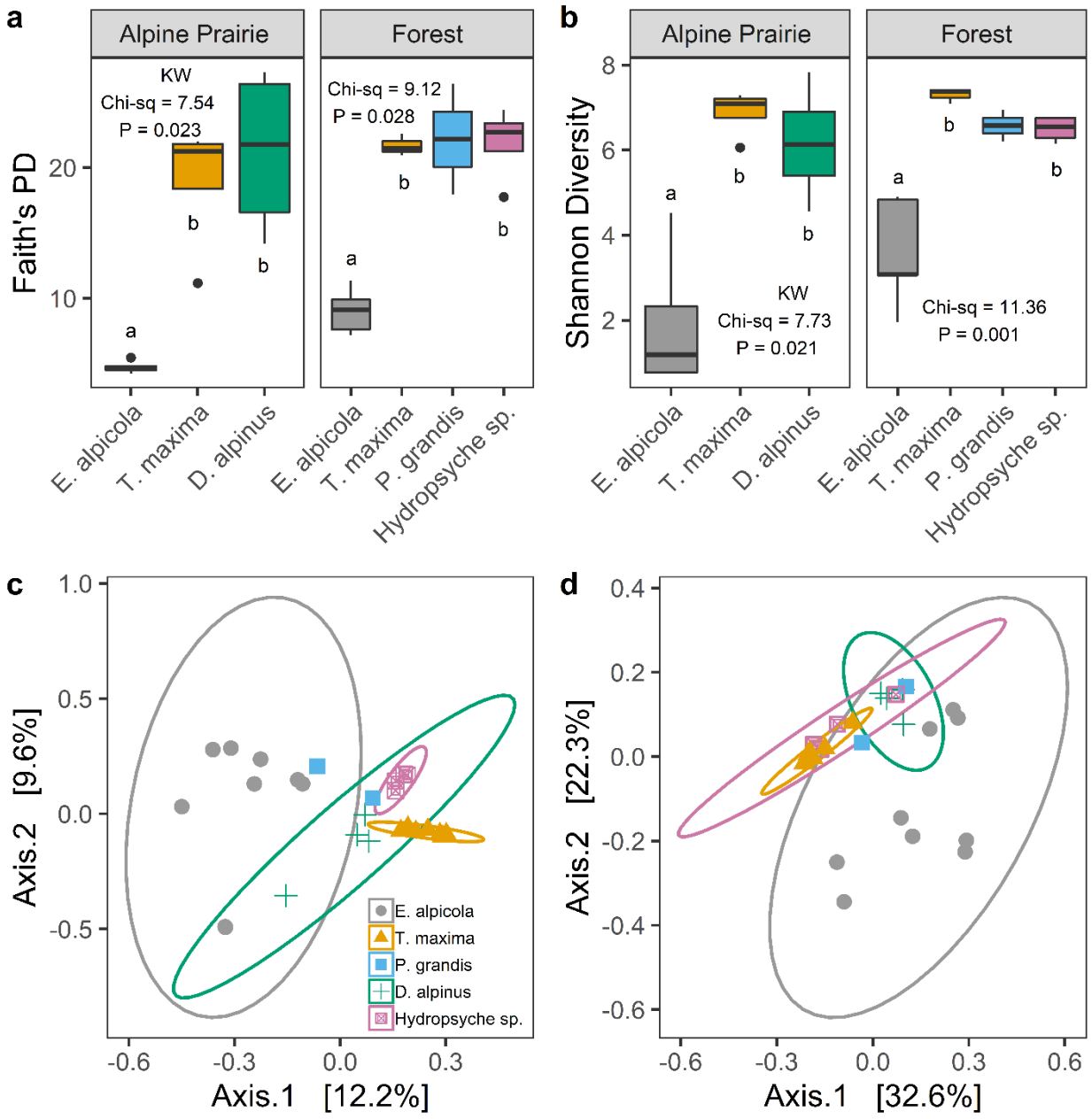


Figure 5.7. Differences in community diversity between species a) Differences in Faith's phylogenetic diversity (Faith's PD) between species. Comparisons between species were tested with Kruskal-Wallis tests with pairwise significance (Mann-Whitney) denoted by lowercase letters. b) Shannon diversity differences between species. c) PCoA plot (Jaccard distance) showing community differences due to taxonomic composition. d) PCoA plot (Jaccard) showing predicted functional differences in beta diversity between species. Ellipses represent 95% CI for the mean of each group.



Conclusion

Workshop and tutorial material developed as part of these three case studies were used for in person workshops and training in Turin, Italy and Ouidah, Benin as well as individual and small group instruction at Michigan State University. In total, approximately 70 individuals took part in in-person training using these materials while at least 250 individuals accessed online materials (determined by unique viewers on GitHub). The accessibility of high throughput sequencing to answer biological questions will continue to increase as costs decrease and new techniques are developed. However, with analysis skills a major bottleneck in the use of sequencing datasets, there remains a need to increase training opportunities, especially in areas which have historically been heavily impacted by vector-borne diseases.

APPENDICES

APPENDIX I:

SUPPLEMENTAL MATERIALS

Figure S2.1. Bacterial rarefaction curve for leachate samples.

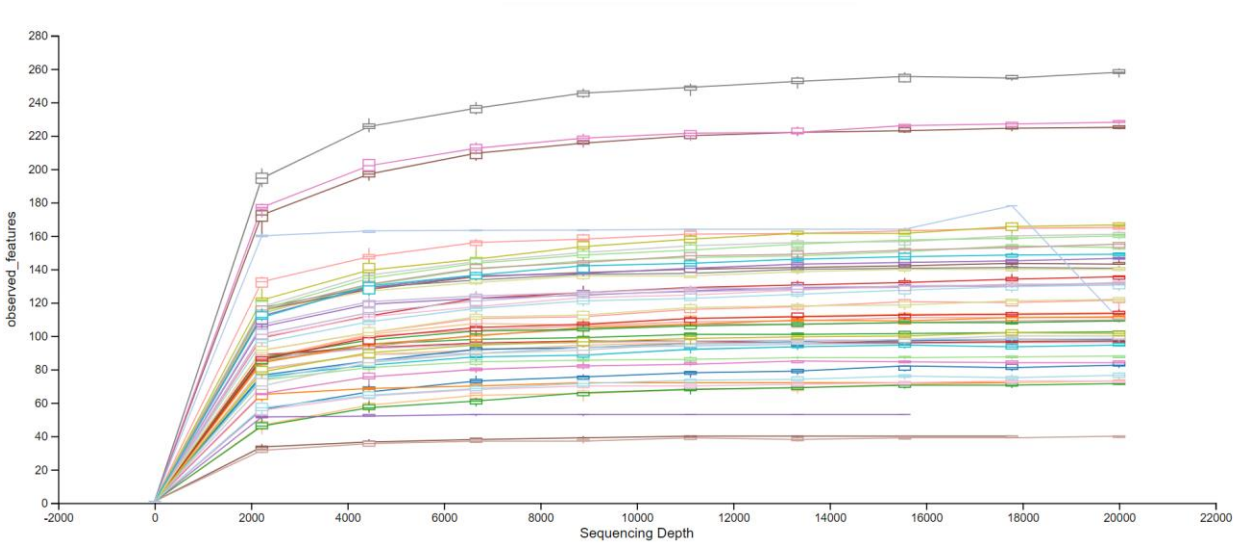


Figure S2.2. Fungal rarefaction curve for leachate samples .

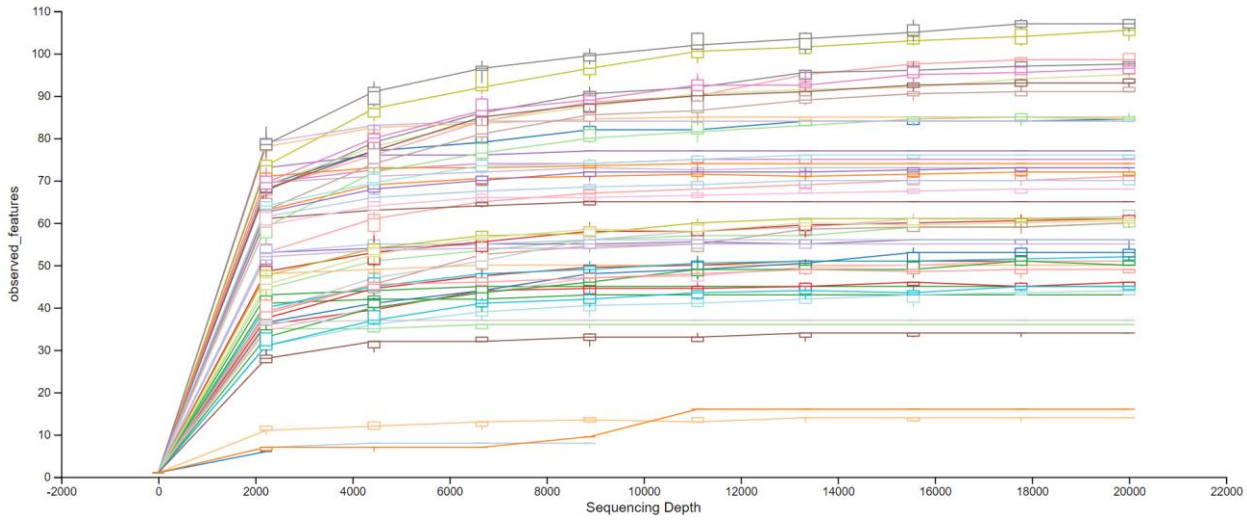


Figure S3.1. Bacterial alpha rarefaction for impact of mycolactone dosages on oak leachate.

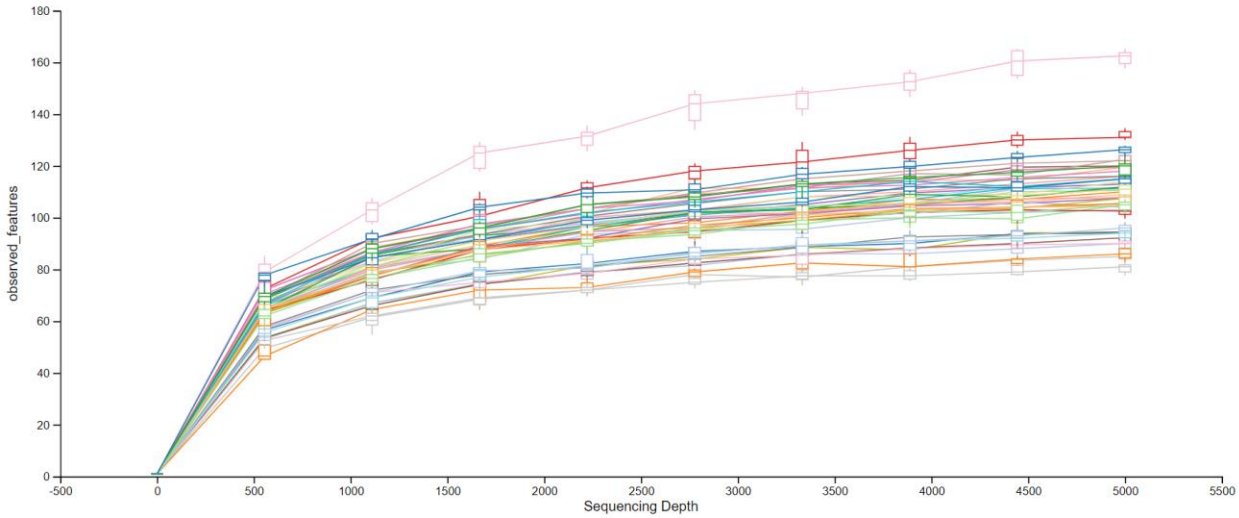


Figure S3.2. Fungal alpha rarefaction for impact of mycolactone dosages on oak leachate.

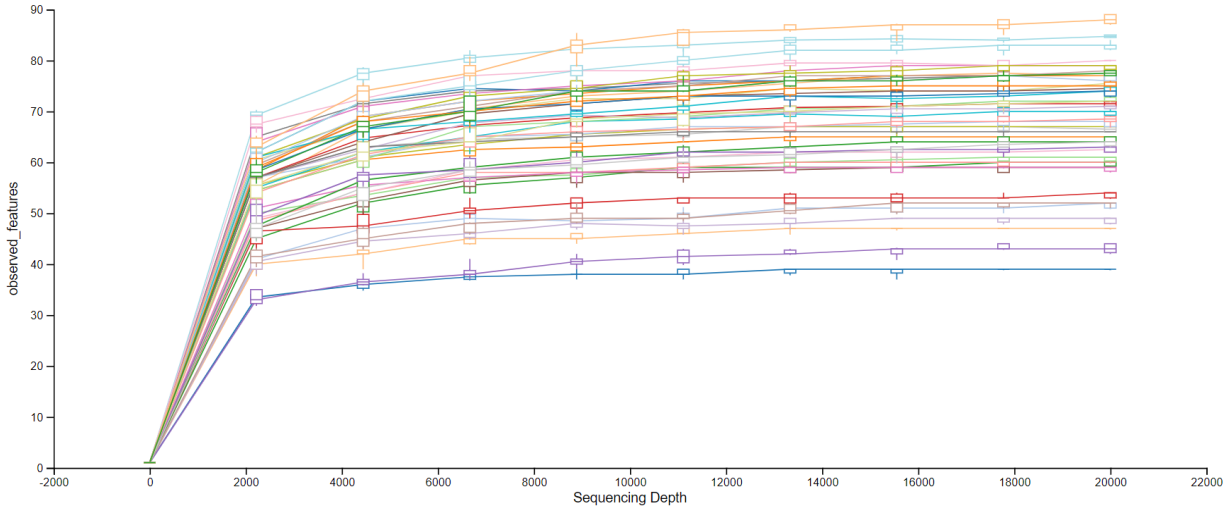


Figure S3.3. Genus level bacterial abundance by treatment and trial. Error bars are SEM, Numbers (1-4) indicate trials.

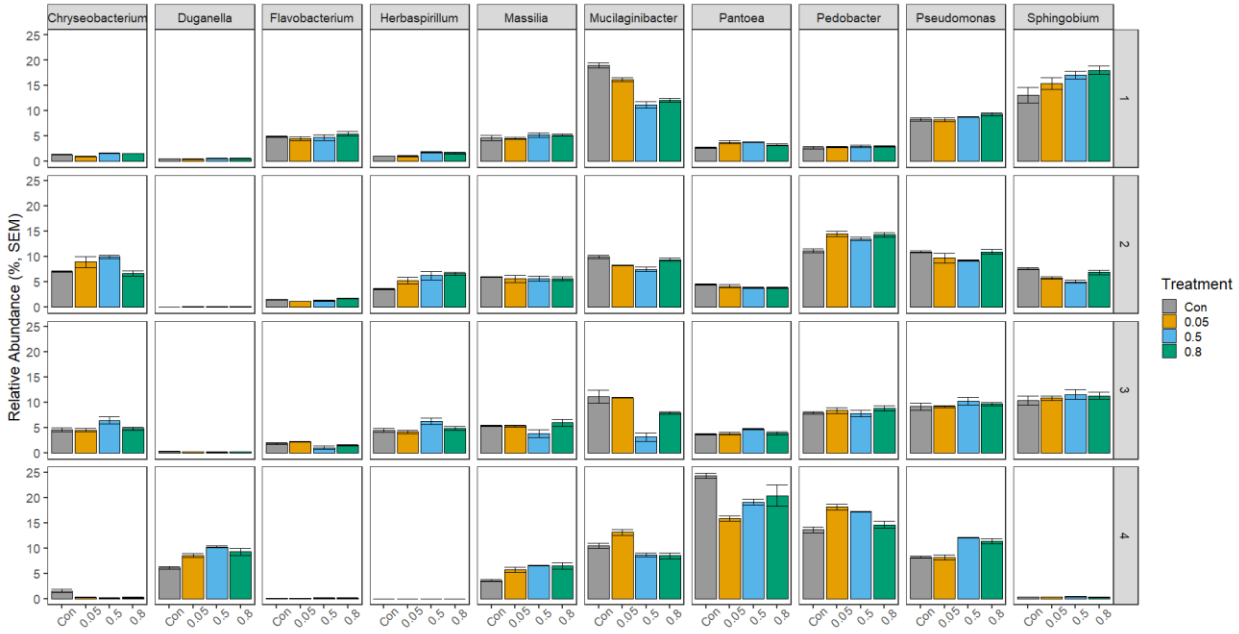


Figure S3.4. Bacterial alpha rarefaction curve (field collected samples, Ouidah Benin).

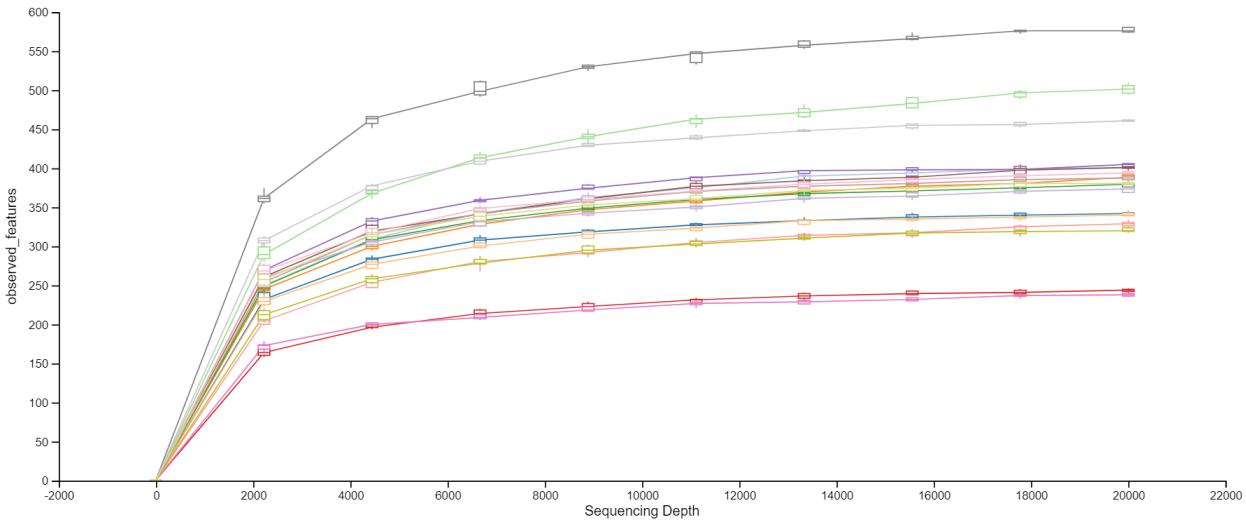


Figure S3.5. Relative abundance of *Mycobacterium* in water samples from field trial. Error bars are SEM. Mycolactone treatment was a 0.8 µg/ml dose of mycolactone.

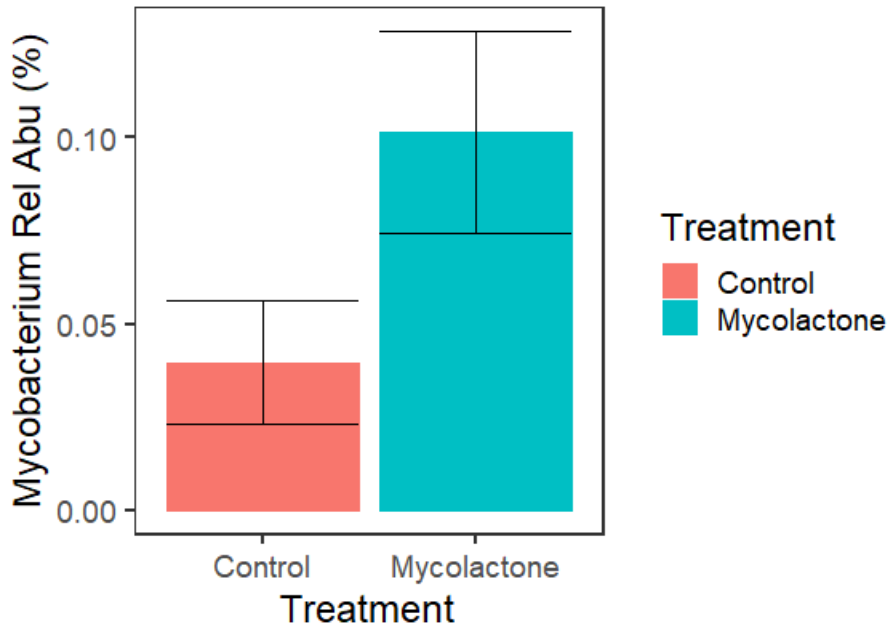


Table S3.1. Pairwise Tukey HSD comparisons of fungal variant richness between trials. ANOVA model: Variant Richness ~ Trial x Treatment. Diff = mean differences in number of observed fungal variants. Lwr = Lower band of 95% confidence intervals. Upr = Upper band for 95% confidence intervals.

Group	Comparison	Diff	Lwr	Upr	P-adj
Trial	2-1	6.00	0.80	11.20	0.019
	3-1	6.75	1.55	11.95	0.007
	4-1	-16.08	-21.28	-10.88	0
	3-2	0.75	-4.45	5.95	0.979
	4-2	-22.08	-27.28	-16.88	0
	4-3	-22.83	-28.03	-17.63	0
Treatment	0.05-Con	5.00	-0.20	10.20	0.063
	0.5-Con	0.00	-5.20	5.20	1.000
	0.8-Con	-0.33	-5.53	4.87	0.998
	0.5-0.05	-5.00	-10.20	0.20	0.063
	0.8-0.05	-5.33	-10.53	-0.13	0.043
	0.8-0.5	-0.33	-5.53	4.87	0.998

APPENDIX II:

REPRINT LICENSE: INSECT-ASSOCIATED BACTERIAL COMMUNITIES IN AN ALPINE STREAM

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APPENDIX III

EXAMPLE CODE FOR ACCESSING 1000 GENOME DATA WITH VCFTOOLS

- Installing VCFtools
  git clone https://github.com/vcftools/vcftools.git
  cd vcftools
  ./autogen.sh
  ./configure
  make
  make install
- Potential issues
  - If mac development tools are not installed it will tell you, if autoconfig is not installed you will get a not found error when running autogen.sh but make sure you are in the right directory because it will return the same error message if you try it in the wrong directory.
  - You may get an error if bgzip or tabix are not installed with some of the commands, if you do, see http://www.htslib.org/doc/tabix.html
- Navigate to folder with data using cd
  - Type ls to see what files are in the current folder (for windows the command is dir and linux !dir works as well)
- Type in vcftools and hit enter
  - If installed correctly, you should see a couple lines with general info about vcftools appear
- Commands in vcftools
  - Start each command with vcftools to specify that you want to use that program
  - next specify the data you want to use
    - This can either in a .vcf format where you would write --vcf filename.vcf
    - Or in a vcf.gz format which is a compressed version where you would write --vcf.gz filename.vcf.gz
  - The next part of the file is where you specify the action you want the program to do (a full list of options is below)
    - For example, if you want to filter out chromosome 3L you would now input --chr 3L
    - Or if you wanted to output the relatedness between samples using Yang's method from 2010 you would type --relatedness
    - Other commands may require additional inputs but the more complicated inputs are listed below
  - The next optional part is the --recode option
    - This will take the header/metadata and only include the metadata for samples that passed filtering

- For example, if you give it the command to filter chromosome 3L, adding the recode option will remove all the header/metadata associated with anything that is not on chromosome 3L
- For most of the filtering options, always add the --recode option, otherwise it interferes with analysis. There are exceptions but don't worry about them for now

    - The next part of the command is the output file, where you specify what you want to the output file name to be
        - Most of the commands will add their own file extension to the end regardless of what you write
            - For example if you name a file with -o filteredSamples.vcf the command will return a file name of filteredSamples.vcf.vcf
            - Some general formatting tips for naming files
                - Do not include any spaces, special characters (like $ % !)
                - Keep them as short as possible while still being informative
                    - It reduces the chance of making a spelling mistake while writing and saves time
                - Keep your use of capital letters consistent
                    - If you have a file named filteredSamples.vcf and wanted to use that in an input function if you inputted filteredsamples.vcf it wouldn't work and it would return an error message

- An example with all the parts put together, if you wanted to filter out the sequences for chromosome 3L and save it in a new file, while recoding the metadata you would write the following command
    - vcftools --vcf merged.vcf --chr 3L --recode --out chr3L
    - the command would create a new file named chr3L.vcf in the file directory that you are currently in.

Example of taking the mosquito genome data, filtering before testing for relatedness between samples

- Filtering
    - 1st step remove any SNPs that have a filter other than PASS from the previous filtering steps that were conducted by the *A.* gambiae genome project
        - Some examples of sites that would have a flag other than pass are SNPs of low quality or locations that were otherwise unsuitable for SNP analysis, for example SNPs in areas of long tandem repeats that the reference file has low mapping quality for
        - vcftools --vcf merged.vcf --remove-filtered-all --recode --out Pass
    - 2nd step, remove any SNPs that do not meet the quality that you want (in this example 40 but replace it with whatever number you want)
        - Sequencing Depth
            - vcftools vcf PASS.vcf --min-meanDP 40 --recode --out depth40

    - 3rd step Genotype filtering, removes all locations where the genotype quality is below a user defined number
        - vcftools --depth40.vcf --minGQ 30 --out filtered
    - Separating out the data by chromosomes
        - vcftools --vcf filtered.vcf --chr 1L --recode --out chr1L
        - the recode option is important for this step
        - For the rest of the chromosomes, replace 1L with the chromosome you want (the data from this project is named according to the NCBIs naming convention for mosquito chromosomes)
        - The files at this step will have to be divided into L and R arms but they can be put back together later
    - Separating out data by base pair
        - if you would like to subset a section of a .vcf file
        - vcftools --vcf filtered.vcf --from-bp 1 --to-bp 10000 --recode --out bp1.10000
        - The NCBI database will give the locations for genes using the same reference strain as used to create the vcf file.
- Calculate the Fst for each SNP in a group of samples
    - For this command, you will need to create two or more text files, each of them containing all the sample names for one population with no header, for each of the populations you would like to compare.
    - For example to compare the differences in SNPs between coluzzi and gambiae

    - vcftools --vcf InGene.vcf --weir-fst-pop AnColuzzi.txt --weir-fst-pop AnGambiae.txt out InGeneBySpecies

APPENDIX IV

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number:

2021-5

Author and Title of thesis:

Joseph Receveur

Influence of aquatic microbes on mosquito oviposition behavior and life history

Museum(s) where deposited:

Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Family	Genus-Species	Life Stage	Quantity	Preservation
Culicidae	*Aedes aegypti*	Adult	10	Pinned
Culicidae	*Aedes japonicus*	Adult	10	Pinned
Culicidae	*Aedes japonicus*	Larvae	10	Ethanol

LITERATURE CITED

LITERATURE CITED

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