MOSQUITO PRODUCTION AND MICROBIAL DIVERSITY IN CONTAINER HABITATS

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ABSTRACT

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Container-breeding mosquitoes comprise approximately 40% of known mosquito species. In addition to man-made containers, including tires and cemetery vases, many of these mosquitoes reside in natural container habitats, such as water-filled tree holes. Detritus is a key component of larval nutrition and its availability and quality directly relate to the production of adult mosquitoes. Microbial metabolism incorporates nutrients from detritus, which mosquitoes then procure via direct consumption of microorganism in biofilms and in the water column. The successful emergence of adults depends on the consumption of these microbial communities; therefore, I have examined the interaction of several container dwelling mosquito species, *Ochlerotatus triseriatus*, *Aedes albopictus* and *Aedes aegypti* in order to evaluate the contributions of microbial community dynamics to mosquito development. The studies in this dissertation were designed to integrate microbial community level dynamics with broader ecological processes associated with tree hole communities, including decomposition, competition, and facilitation. Using terminal restriction fragment polymorphism (T-RFLP) analysis and sequencing, I describe herein changes in the structure of bacterial and fungal communities in response to container type, mosquito density, and macroinvertebrate community composition.

ACKNOWLEDGMENTS

My journey towards a doctoral degree has been an experience that although frustrating or difficult at times, has served to enhance my sense of wonder about the natural world. I have been fortunate in that my research program has required that I acquire a more than passing familiarity with a variety of biological disciplines that are often separated under the traditional structure imposed by not only a university departmental structure, but the field of science as a whole. I have had an uncommonly integrated experience that has strengthened my desire to be a lifetime student of biology.

The pursuit of this degree has been a personal challenge that would not have been possible without the continuous support of my parents, who have provided love and unconditional support throughout my life. I have been most fortunate as well in having the encouragement, love, and support of my husband, Lukasz. I do not know how others survive a doctoral program, but I am certain that I could not have done so without having him in my life as both a wise colleague and a best friend.

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CHAPTER 1

INTRODUCTION

Container-dwelling forms represent approximately 40% of mosquito species, many of which are important arbovirus vectors, including West Nile Virus, yellow fever, and LaCrosse encephalitis (Laird 1988). Water-filled containers may be man-made (e.g. tires, cemetery vases, flower pots) or plant-based. The latter, called phytotelmata (phyto = plant, telmata = container), commonly occur as tree holes found either along tree trunks or at the tree base formed by root outcroppings. Tree holes are an excellent subject for studies of trophic interactions, due to the self-contained nature of the communities residing therein. Many insects with aquatic larval stages make their home in tree holes. Although typically dominated by mosquito larvae, non-mosquito representatives like *Helodes pulchella, Prionocyphon discoideus* (Coleoptera: Scirtidae), and *Culicoides guttipennis* (Diptera: Ceratapogonidae) are also common (Barrara 1988, Paradise 2000). The mosquito species in tree holes varies geographically. In Michigan, the primary mosquito species residing in tree holes is the Eastern treehole mosquito, *Ochlerotatus triseriatus* (Say). Recently, *Oc. japonicus* has begun to invade Michigan tree holes following its introduction into the United States in 1998 (Peyton et al. 1999).

Systematics

The subgenus *Ochlerotatus* was elevated to genus level by Reinert in 2000 based on morphological evidence. Prior to this re-classification, the genus *Aedes* was divided into two subgenera, *Aedes* and *Ochlerotatus*. It has subsequently been suggested that additional Aedini subgenera be elevated, including the subgenus Stegomyia (Reinert 2004). The most visible ramification arising from this change is the subgera elevation of two medically-important species, the Yellow Fever mosquito, *Aedes aegypti*, and the Asian Tiger mosquito, *Aedes albopictus* (Reinert 2004). As a result, elevation of these

genera has been a controversial subject and the complete adoption of Reinert's classifications by entomologists remains undetermined.

Medical Significance.

In addition being a nuisance to humans and animals, female mosquitoes often transmit diseases. The range of maladies vectored by mosquitoes is extensive and the importance of mosquitoes as disease vectors cannot be over-stated. For the purpose of providing a background for this project, however, the discussion of mosquito-borne diseases will be limited to those associated with *A. albopictus, A. aegypti,* and *Oc. triseriatus* in their North American range, specifically arthropod-vectored viruses, or arboviruses.

Of the arboviruses, yellow fever and dengue are the most serious and have had the greatest historical impact on the field of medical entomology in the United States. Both are associated with the Yellow Fever mosquito, *A. aegypti,* but *A. albopictus* is also a competent vector of these arboviruses (Gubler and Rosen 1976). Outbreaks of dengue and yellow fever were frequent in the United States from approximately the mid-seventeenth century until the mid-twentieth century. (Bryan et al. 2004). Eradication of larval habitats (i.e. standing water-filled containers), window-screening, and increased pesticide use contributed to the near-eradication of U.S. dengue and Yellow Fever outbreaks.

West Nile Virus (WNV) is the most visible mosquito-borne arbovirus, particularly in recent years. Initially occurring during late summer 1999 in New York City, WNV has subsequently spread across the continental U.S. to California (CDC 1999 a,b; Lanciotti 1999). Although mosquitoes in the genus *Culex* are the primary vector of

WNV, many *Aedes/Ocherotatus* species also transmit the disease, including *Oc. triseriatus* and *Oc. japonicus* (Sardelis et al. 2001,2002; Turrell et al. 2005).

Mosquitoes that act as bridge vectors between birds and humans, such as *C. pipiens* and *C. restuans*, are most likely to transmit WNV as birds serve as the reservoir. Mammals, including humans, are generally unable to infect mosquitoes, due to low viremia level in these hosts. Hence, *Aedes/Ochlerotatus* mosquitoes feeding on mammals are less likely to account for a large percentage of positive mosquito pools. *Culex* mosquitoes more strongly prefer bird hosts than do Aedes mosquitoes; therefore, *Culex* are more likely to serve as a bridge vector of WNV between vertebrate and avian disease hosts (Turrell et al. 2005).

Although not a major vector of WNV, *Oc. triseriatus* is the primary vector of La Crosse Virus (LaCV), a California serogroup bunyavirus (Centers for Disease Control and Prevention 2007, Watts et al. 1972). Although less common, *A. albopictus* has also vectors LaCV in North Carolina and Tennessee (Gerhardt et al. 2001). LaCV is distributed throughout the Eastern United States; it cycles between the mosquito vector and vertebrate host (primarily chipmunks). Symptoms include encephalitis and, rarely seizures and coma (Calisher 1983). Fatalities resulting from this disease occur in less than 1% of all clinical cases. Children are the primary risk group for LaCV.

Distribution and Life History

Oc. triseriatus are opportunistic; they lay eggs in both natural and artificial containers. In North America, *Ochlerotatus triseriatus* (Say) is the predominant mosquito species in basal pan-type tree holes, but may also occur in man-made containers such as tires, flower pots, and cemetery vases. The range of *Oc. triseriatus* encompasses much of

the Eastern United States and the southernmost sections of Eastern Canada, extending from New Brunswick, Canada in the north to Florida in the south.

In northern latitudes such as Michigan, *Oc. triseriatus* overwinter as eggs within their container habitat and emerge as larvae the following May, or earlier under warm temperatures. Adult females lay individual eggs along the water line of natural and artificial containers, where they may later be exposed to flooding during rain events. Eggs are responsive to decreases in CO_2 and elevated moisture, utilizing these stimuli as hatching cues (Clements 1992). The species is multivoltine contingent upon how early adults emerge and whether late-season weather conditions remain favorable. Eggs generally enter diapause by late September or early October.

Larval Habitat and Nutrition

In heterotrophic environments such as tree holes, nutrient inputs occur in three main forms: as stemflow (water run-off from trees associated with rain events), animal detritus, and allochthonous plant detritus. Leaves appear to be the most abundant source of dissolved organic matter (DOM) and fine particulate organic matter (FPOM) to the tree hole system. Animal detritus provides a richer source of nutrients to tree holes (Yee and Juliano 2006), although it represents a comparatively smaller portion of the total and the mechanism for its effect of increasing mosquito productivity has not been determined. Leaf quality and quantity are important determinants of adult mosquito production (Fish and Carpenter 1982, Leonard and Juliano 1995, Walker 1997). Leaf quality varies with leaf type. Specifically, leaves with high tannin content, such as oak leaves, contain a larger proportion of refractile (insoluable) to labile (soluble) material compared with those lower in tannins, such as beech or maple (Walker 1997). For all leaf types,

senescent leaves are inferior to fresh leaves, possessing comparatively reduced concentrations of soluble protein, nitrogen, and soluble carbohydrate. The quantity of leaf detritus also conditionally influences the mosquito productivity (Leonard and Juliano 1995). In addition to determining adult size, the ration of leaf litter available per larva is critical for larvae to enter the pupal stage. Available leaf rations interacted with several variables in the tree hole, including larval density (interspecific and intraspecific), presence of other macroinvertebrates, and stemflow.

Adult mosquito production in tree hole habitats is dependent on larval nutrition derived from allochthonous nutrient inputs including leaf and animal detritus and stemflow runoff. Microbial processing of these inputs increases the availability of detrital nutrients to higher trophic levels in tree hole communities, which may also include a diversity of macroinvertebrates in addition to mosquitoes (Carpenter 1983, Kitching 2001). Although mosquito larvae may ingest these detrital inputs in the form of fine particulate organic matter (FPOM), it is more evident that the nutrients and physical substrates provided by these inputs promote microbial colonization. Larval mosquitoes obtain nutrition by browsing the microbial biofilm associated with leaf and container wall surfaces or filter feed on small particles and planktonic bacteria in the water column (Merritt et al. 1992). While bacteria are a critical resource for larval maintenance, it is apparent that fungi supply larvae with additional nutrients required for growth (Kaufman et al. 2001, 2002, Kaufman and Walker 2006).

Stemflow and detritus quality drive the production of adult mosquitoes, as these factors directly impact microbial growth. Water running down tree trunks collects in tree holes during rain events thus introducing critical soluble nutrients (e.g. nitrogen and

phosphorus and other inorganic nutrients) and carbon to the nutrient-poor ecosystem (Carpenter 1982b). Stemflow provides critical nutrients need to promote decomposition of detrital inputs. Microbial processing releases nutrients contained in leaf material to the environment. The labile portion of leaf material (= leachate), containing carbon and nutrients (e.g. N and P) stored in leaf material, is released early in the decay process approximately within three days of entering the system (Carpenter 1982a). The refractile material, high in carbon but low in N and P, is largely unavailable to mosquito larvae. In addition to solublizing the labile component of leaf detritus, stemflow promotes microbial growth through introduction of limited nutrients. Microbes facilitate leaf decay by softening tissues so they become available for direct ingestion by larvae. More importantly, the microbial milieu, consisting of a diverse community of bacteria, fungi, and protozoans, incorporate the nutrients from the organic inputs into their biomass. Water column-associated materials as stated above are limited, but contain a higher density of protozoans, if present, than leaf and container wall surfaces. While a superior resource in terms of overall biomass for larvae compared with bacteria and fungi, these organisms are rapidly depleted under larval feeding pressure (Kaufman 1999, 2001).

Ecological Factors Contributing to Adult Mosquito Production

In addition to the direct effect of stemflow, detrital inputs and microbial processing, larval productivity is also affected by at least two other important indirect factors: competition and facilitation. Although effects of these interactions on the dynamics of tree hole macroinvertabrates has been the subject of several studies, the effect of such interactions on the microbial community has remained relatively unexplored. Given that the microbial community is the food resource driving competitive

interactions, one can predict that changes in the microbial community are likely to result as changes occur in the structure of the community exerting feeding pressure on it. Such interactions are likely to alter the microbial community, subsequently affecting adult mosquito production. This "bottom-up" effect would result if changes in the microbial community wrought by one species could reduce the relevant portion of the microbial community to the lowest level necessary for the own survival but below the equilibrium resource abundance (R*) threshold required for the competing species to maintain the its own population density, that is, where birth rate is equivalent to death rate (1980, 1990). This theory of competition has been described as a mechanism for organisms under competition, but has remained unexplored as a mechanism directing the outcome of competition among mosquito larvae. Rather, previous studies have focused on ration and quality of detrital inputs experienced by competing species while omitting a description of any explicit mechanism that would account for superior resource utilization. Alterations in the structure or composition of tree hole associated microbiota are important to our understanding of tree hole ecology, as they may translate into a positive or negative effect on mosquito production.

Research Objectives and Rationale

The goal of this dissertation was to increase our understanding of the microbial community resource base present in tree hole containers utilizing molecular genetic techniques that promote analysis of community structure. Although the importance of resource quality and quantity to mosquito development has been demonstrated, our understanding how detrital resource inputs interact with microorganisms is limited; therefore, in addition to understanding how resources contribute to mosquito success,

these studies seek to integrate microbial community level dynamics with broader ecological processes associated with tree hole communities, including decomposition, macroinvertebrate competition, and facilitation. Specifically, my dissertation objectives were to:

1. Determine the effects of detrital leaching on mosquito productivity, including a) whether the nutrients in leached senescent leaves are sufficient for growth; b) if the positive effect of unleached leaves on mosquito development can be restored by returning labile leaf components to microcosms; c) whether fresh leachate stimulates bacterial abundance and/or productivity; and d) whether leachate alone can support larval development.
2. Analyze the structure and diversity of microbial communities associated with container habitats via terminal restriction fragment length polymorphism (T-RFLP) analysis and sequencing of 16S and 18S rRNA genes. Included in this objective was to: a) assess differences in the microbial community structure of natural (tree hole) and artificial (tire) container habitats; and b) identify the contribution of container substrates to mosquito production.
3. Investigate the microbial resource relationship with facilitation of *Oc. triseriatus* populations by scirtid beetles using T-RFLP analysis of bacterial and fungal communities.
4. Investigate the microbial dynamics underlying competition between *Oc. triseriatus* and *A. albopictus* and *A. aegypti and A. albopictus* using T-RFLP analysis of bacterial and fungal communities.

CHAPTER 2

SENESCENT LEAF EXUDATE DECREASES MOSQUITO SURVIVAL IN TREE HOLE HABITATS

Introduction

Tree holes, a type of phytotelmata (plant-based water-filled container) (Frank and Lounibos, 1983), are small heterotrophic habitats harboring a diverse community of macroinvertebrates and microorganisms (Carpenter, 1983; Kitching, 2001). In Eastern North America, *Ochlerotatus triseriatus* (Say) is the predominant mosquito species and primary macroinvertebrate consumer in basal pan-type tree holes (Craig, 1983). Mosquito production in tree hole habitats depends on larval nutrition derived from allochthonous detrital inputs. These inputs consist primarily of plant material, with leaf litter as the major source of coarse particulate organic matter (CPOM), fine particulate organic matter (FPOM), and dissolved organic matter (DOM) in tree hole systems. In addition to leaf detritus, stemflow and animal detritus also contribute to allochthonous nutrient pools. Water runoff during rain events collects in tree holes, introducing critical soluble nutrients (e.g. nitrogen, phosphorus and other inorganic nutrients) and carbon to the nutrient-poor ecosystem (Kitching, 1971; Fish 1983; Carpenter, 1982b; Walker and Merritt, 1991). Macroinvertebrate carcasses and fecal material also contribute to the detrital pools (Daugherty et al., 2000; Yee and Juliano, 2006), although the presence of these materials is comparatively ephemeral.

Although mosquito larvae may ingest detrital inputs in the form of FPOM, it is more evident that the nutrients and physical substrates provided by these inputs promote microbial colonization; thus, adult production is indirectly linked to detrital inputs (Kaufman and Walker, 2007). Analyses of larval gut content and feeding behavior corroboratively indicate mosquitoes obtain food by browsing the microbial biofilm associated with leaf and container wall surfaces or by filtering small particles such as

planktonic bacteria from the water column (Cummins and Klug, 1979; Fish and Carpenter, 1982; Merritt et al., 1992; Walker and Merritt, 1991). The tree hole-associated microbial milieu consists of a diverse community of heterotrophic bacteria, fungi, and protozoans (Kaufman et al., 2001, 2002; Kaufman and Walker 2007). These microorganisms play an important role in tree hole ecosystems, contributing to carbon and nutrient cycles through the secondary production of microbial biomass and the recycling of organic carbon and nutrients thus increasing the availability of detritus-derived nutrients to higher trophic levels in tree hole communities (Kaufman and Walker 2006).

Adult mosquito production is conditionally dependent on the available per larva leaf ration (Fish and Carpenter, 1982; Hard et al., 1989 Leonard and Juliano, 1995; Walker et al., 1997) and the interaction thereof with habitat variables, including larval density, presence of other macroinvertebrates, and stemflow. The quality of leave material is also of critical importance, indicated in part by observations of greater mosquito production from microcosms stocked with fresh leaves compared with senescent leaves (Walker et al., 1997). Additionally, qualitative differences occur among leaf litter types such that species with faster decomposition rates are generally superior, supporting greater mosquito growth and survival than those with slow decomposition rates (Fish and Carpenter 1982). Decomposition of leaf material is associated with its availability for microbial degradation and its palatability for macroinvertebrates, as both processes are governed by the presence of lignin and nitrogen content (Bärlacher 1985, Moorehead and Sinsabaugh, 2006). Indeed, the presence of carbohydrates and other nutrients, particularly N, has a positive effect on the fungal productivity and mosquito

growth parameters. (Kaufman and Walker 2006) For all leaf types, senescent leaves are inferior to fresh leaves, possessing comparatively reduced concentrations of soluble protein, nitrogen, and soluble carbohydrate.

Leaf decomposition occurs in two distinct stages: initial leaching of labile components over a short time period and a long-term breakdown of refractory components (Carpenter, 1982a), Release of soluble leaf components occurs early in the decay process, approximately within 24 hours of entering the system (Gessner and Schwoerbel 1989, Webster and Benfield 1986). C:N ratios vary among leaf species, resulting in relative differences in the portions of labile and refractile leaf components and subsequent, differential breakdown among leaf types. The labile material, or leachate, is rich in nutrients that are critical for microbial and mosquito productivity. Refractile material, on the other hand, has a high C:N ratio, low phosphorus content and is likely unavailable directly to developing larvae (Carpenter, 1982a; Webster and Benfield, 1986). Microorganisms afford a bridge between the nutrients trapped in the leaf matrix and mosquito larvae by utilizing leaf material as substrates for growth while contributing to leaf breakdown.

The soluble leaf fraction represents the most important contribution of nutrients to larval productivity, yet this material disappears rapidly from the leaf matrix. Thus, leaves that are subjected to leaching prior to entering the tree hole may be of lower quality than unleached leaves and may physically impede inputs of higher quality leaves. Leaching has dampening effects on the growth responses of mosquitoes due to the consequent reduction in nutrient content (Walker et al., 1997). It follows that re-addition of leached contents should restore the required nutrients to systems stocked with leached

leaves. Furthermore, the effect of high nutrient concentration should manifest itself via the increased production of microorganisms. This prediction follows from the results of previous studies showing enhanced mosquito growth in response to nutrient supplementation via the microbial loop (Kaufman et al., 2002; Kaufman and Walker, 2006).; therefore, additions of leachate material obtained from an equivalent leaf pack mass should mitigate the effects of poor leaf quality on the development and productivity of *Oc. triseriatus.*

We describe here three experiments designed to elucidate the individual effects of labile and refractile leaf components on tree hole community dynamics. The purpose of this study was to determine: 1) whether the nutrients in leached senescent leaves are sufficient for growth; 2) if the positive effect of unleached leaves on mosquito development can be restored by returning labile leaf components to microcosms; 3) whether fresh leachate stimulates bacterial abundance and/or productivity; and 4) whether leachate alone can support larval development. We postulate that leaf litter present in tree holes contributes relatively little to mosquito growth compared to fresh inputs of leaf material; therefore, leached leaves alone should be insufficient for larval growth. A corollary of this hypothesis is that the leached fraction, containing labile substrates and nutrients to initiate high microbial activity, will support larval mosquito growth and development comparable to that observed in response to fresh leaf packs. Additions of leachate material obtained from an equivalent leaf pack mass should therefore "rescue" larvae from the effects of poor leaf quality. Further, we predict that bacterial populations will be enhanced in response to leachate due to the high nutrient content (Kaufman et al.,

2002; Kaufman and Walker, 2006) compared with populations that experience reduced leachate.

Materials and Methods

Microcosm construction

For each of the following experiments, tree hole-based microcosms were stocked with senescent red oak leaves (*Quercus rubra* L.) collected at Michigan State University's Kellogg Forest (Augusta, MI). Leaves were dried at 45°C for 48 h and added as 1-g leaf packs to microcosms constructed as in (Kaufman and Walker, 2006; Walker et al., 1991). Additionally, microcosms received a microbial inoculum, consisting of 3 ml homogenized natural tree hole water and particulates. Each contained a final volume of 500 ml, composed of deionized, distilled water and, if applicable, leachate in the amounts described below. Water levels were maintained throughout the experiment to account for evaporative losses. Microcosms were loosely covered with window screen and incubated under indirect lighting at 21 °C and 16:8 (L:D) photocycle (Percival Scientific, Inc., Perry, IA). Prior to the addition of 20 or 40 newly-hatched first instar *Oc. triseriatus* larvae (Day 0 for all experiments), microcosms were incubated for 3 days to allow time for microbial colonization of leaf surfaces and water column. The larvae used in the following experiments were hatched from eggs collected from our colonies at the Insect Microbiology Laboratory at Michigan State University.

Experiment 1.

The purpose of this study was to elucidate the contribution of the labile fraction of senescent leaves as detrital inputs into microcosms that model tree hole habitats. Leaf quality and leachate effects were assessed in a 2 x 4 multifactorial design with six

replicates per treatment. Unleached senescent red oak leaf (*Quercus rubra*) packs (1 g) were compared to similar leaves subjected to leaching for three days. This period is sufficient to account for the leaching of the labile components into the water column as the majority of this fraction is lost from the leaf matrix within three days of introduction to an aquatic environment (Carpenter, 1982a). Microcosms containing newly hatched first instar *Oc. triseriatus* larvae, described above, received the resulting leachate in amounts equivalent to 0, 25, 50, or 100% of that obtained from a 1 g leaf pack. To account for the effect of labile nutrients alone on mosquito performance, all leachate was filter-sterilized using a 0.2μm vacuum filter before addition to microcosms. Microcosms were checked daily for adult mosquitoes, which were collected and stored at -80°C. At the end of the experiment (day 70), adults and any remaining larvae and pupae were lyophilized and massed. The remaining leaf mass was also determined after drying leaves for three days at 50°C.

Experiment 2.

We tested the hypothesis that soluble nutrients and microbial biota present in the leachate fraction of leaf material increase mosquito performance in microcosms similar to those described above. Two levels of leaf quality, unleached or leached for three days, were applied to replicate microcosms. In contrast to the previous experiment, leachate was added to microcosms equivalent to 100% of the amount obtained from a 1 g leaf pack in two forms: unfiltered or pre-filtered through a 0.2μm vacuum filter. Additionally, a control treatment of deionized, distilled water was applied to replicate microcosms for each leaf quality treatment. The resulting 2x2x2 factorial design was replicated seven times to permit the replacement of leaf material sampled from six replicate microcosms

on day 0 (prior to the addition of larvae). At day -3, filtered and unfiltered bulk leachate were sampled for bacterial productivity, bacterial abundance, and nutrient analysis. Microcosm sampling was done at the onset and at the termination of the experiment (days 0 and 70, respectively). Additional nutrient samples were taken from microcosms several weeks into the experiment (day 12). Water samples were collected for bacterial productivity, bacterial abundance, and nutrient analysis (1-10 ml). Productivity subsamples (1 ml) were maintained at 20°C and abundance subsamples (5 ml) were preserved with formaldehyde at a final concentration of 3.7% until measurements could be taken. Leaf material was subsampled using a cork borer (10 mm diam) for estimates of bacterial abundance. Two discs were aseptically removed from leaf packs into 5 ml filter-sterilized phosphate buffer and preserved with formaldehyde (3.7% final concentration). Larvae, pupae, adults, and leaf packs were treated as described above to obtain dry weights.

Experiment 3.

In this experiment, we compared the relative contributions of labile and refractile leaf components to mosquito productivity. Senescent red oak leaf packs (1.0g) were leached for three days in 12 replicate microcosms containing 500 ml deionized, distilled water. The water from six microcosms, now containing labile leaf components, was poured into new microcosms and replaced with fresh deionized, distilled water. The resulting treatment design was thus: leached leaf + water, leached leaf + leachate, no leaf + leachate. As in the previous experiments, mosquitoes at all stages of development were collected and processed to obtain dry mass measurements.

Chemical analyses

Total nitrogen (N) and phosphorus (P) present in the samples were quantified via spectroscopy of unfiltered water samples (Kaufman and Walker 2006). For each analysis, persulfate oxidation techniques were used to convert all forms of phosphorus and nitrogen to phosphate and nitrate, respectively (Menzel and Corwin 1965, Crumpton et al. 1992, Bachmann and Canfield 1996). A colorimetric assay was used to enumerate total P (Murphy and Riley 1962), while second derivative spectroscopy was employed for enumeration of total N (Crumpton et al. 1992, Bachmann and Canfield 1996).

Bacterial abundance.

Bacterial abundance on the leaf surface and in the water column sub-samples was quantified via direct microscopic counts using the DAPI (49,6-diamidino-2-phenylindole) fluorescent staining procedure (Porter and Feig 1980, Walker et al. 1988, Kaufman et al. 2001). Water column and leaf disc samples were sonicated (Aquasonic model 50T, Westchester, PA) for 12 min to reduced cell clumping and/or dislodge cells (Velgi and Albright, 1993). Samples were vortexed and diluted as necessary with filtered-sterilized Milli-Q water (Millipore, Bedford, MA). After the staining material at a final concentration of 20 ug/ml for 15 min, samples were filtered onto black filters (0.2-mm pore size; Nucleopore, Costar, Cambridge, Mass). For each subsample, filters were counted (600 cells per filter minimum) at 1000X.

Bacterial productivity

Direct measurements of microbial biomass accumulation were conducted using a ^{3}H-leucine incorporation assay (Kirchman 2001). This technique measures of the incorporation of amino acids into protein in a bacteria-specific manner, through the use of short incubation periods and nanomolar leucine concentrations (Riemann and Azam

1992). A 5.85 ratio of labeled:unlabeled leucine was added to water subsamples at a concentration of 25 nM to achieve saturation of uptake kinetics (Kirchman 2001, Kaufman et al. 2001). Water samples were incubated with labeled leucine (L-leucine (4,5-^{3}H), 50 Ci/mmol- NEN, Life Science, Boston, MA) in the dark at room temperature for 30 min in 2 ml microcentrifuge tubes (Smith and Azam 1992, Kirchman 2001). Trichloroacetate [TCA, final concentration 10% (vol:vol)] was added to terminate reactions and precipitate protein. Two rinses of the TCA-protein precipitates were conducted with 10% TCA, followed by a single rinse with 5°C, 80% (vol:vol) ethanol. Standard liquid scintillation counting techniques were used to quantify the amount of radioactivity present in the samples.

Statistical Analysis

Within each experiment, multivariate analysis of variance (MANOVA) techniques were used to analyze groups of related variables (Proc GLM, SAS Institute). Specifically, all mosquito parameters measured, (total survival, male and female mass, and male and female development time) were grouped within a single MANOVA. Dependent variables with significant MANOVA results were subjected to individual univariate analysis of variance (ANOVA) followed by a Bonferroni correction to reduce the chance of Type 1 error (Rice, 1989). Bacterial parameters and nutrient concentrations measured in experiment 2 were analyzed using separate repeated measures MANOVAs (also called doubly-multivariate repeated measures MANOVA) to account for the effect of time on measurements. These variables were grouped separately from mosquito variables because they respond differently to treatment combinations (Kaufman et al., 2002). For the same reason, remaining leaf mass was analyzed independently of other

dependent variables in a separate ANOVA for each experiment. When necessary, data were square-root $[(x + 0.5)^{1/2}]$ or arcsine transformed prior to analysis to meet normality criteria. All values reported are non-transformed. Following univariate analysis, means separation was performed for significant independent variables in experiment 3 using Fisher's protected least significant difference (LSD) test.

Results

Experiment 1.

The main effects of leaf type and leachate had significant effects on mosquito production parameters. A significant interaction effect between leaf and leachate in this multivariate analysis of variance suggests that leaf effects changed with increasing amounts of leachate added to microcosms (Table 2.1). Mosquitoes in microcosms containing unleached leaves were characterized by significantly increased survival, reduced development time, and increased mass of male adult *Oc. triseriatus* (univariate analyses, Figure 2.1, Table 2.1), indicating that unleached leaves were a better resource for mosquitoes. In contrast, univariate analyses showed leachate additions were associated with significant changes in development time and body mass parameters for male mosquitoes only, with leachate additions of 100% producing increases in the former condition and reductions in the latter compared with the other treatments (Figure 2.1, Table 2.1). In all cases, mosquito parameters associated with additions of 100% leachate to leached leaves did not recover the level of those parameters associated with additions of 0% leachate to unleached leaves such that survival and adult body mass obtained were comparatively lower and development time was slower. Furthermore, additions of leachate to unleached leaves generally did not affect mosquito growth. Female mass was

the single exception to this observation; greater mass accrued in females provided with unleached leaves and 100% leachate compared with all other treatments. Finally, a significantly greater amount of leaf mass was lost over the course of the experiment from the leaves that were not subjected to leaching prior to their addition in microcosms ($F=5.13$; $df=7,39$; $p=0.0003$; Figure 2).

Table 2.1. Multivariate (MANOVA) and univariate (ANOVA) hypothesis test results for all factors and their interactions for mosquito production variables in experiment 1. ANOVA results for each variable are shown for factors with significant MANOVA *(P*<0.05).

Source	Wilks' Lamda	Response variable tested with ANOVA	*F*	df	*P*
Leaf	0.117		27.17	5,18	<0.0001*
		Female mass	3.00	1,22	0.097
		Male mass	4.41	1,22	0.047
		Female development time	13.27	1,22	0.001*
		Male development time	58.89	1,22	<0.0001*
		Survival	11.2	1,22	0.003*
Leachate	0.108		4.15	15,50	<0.0001*
		Female mass	1.99	3,22	0.145
		Male mass	3.67	3,22	0.028
		Female development time	1.29	3,22	0.301
		Male development time	6.92	3,22	<0.0001*
		Survival	0.47	3,22	0.706
Leaf*Leachate	0.179		4.91	10,36	0.002*
		Female mass	2.62	2,22	0.095
		Male mass	4.09	2,22	0.031
		Female development time	0.26	2,22	0.772
		Male development time	15.58	2,22	<0.0001*
		Survival	5.57	2,22	0.011

* Indicates significance at α-value < 0.05 after sequential Bonferroni correction.

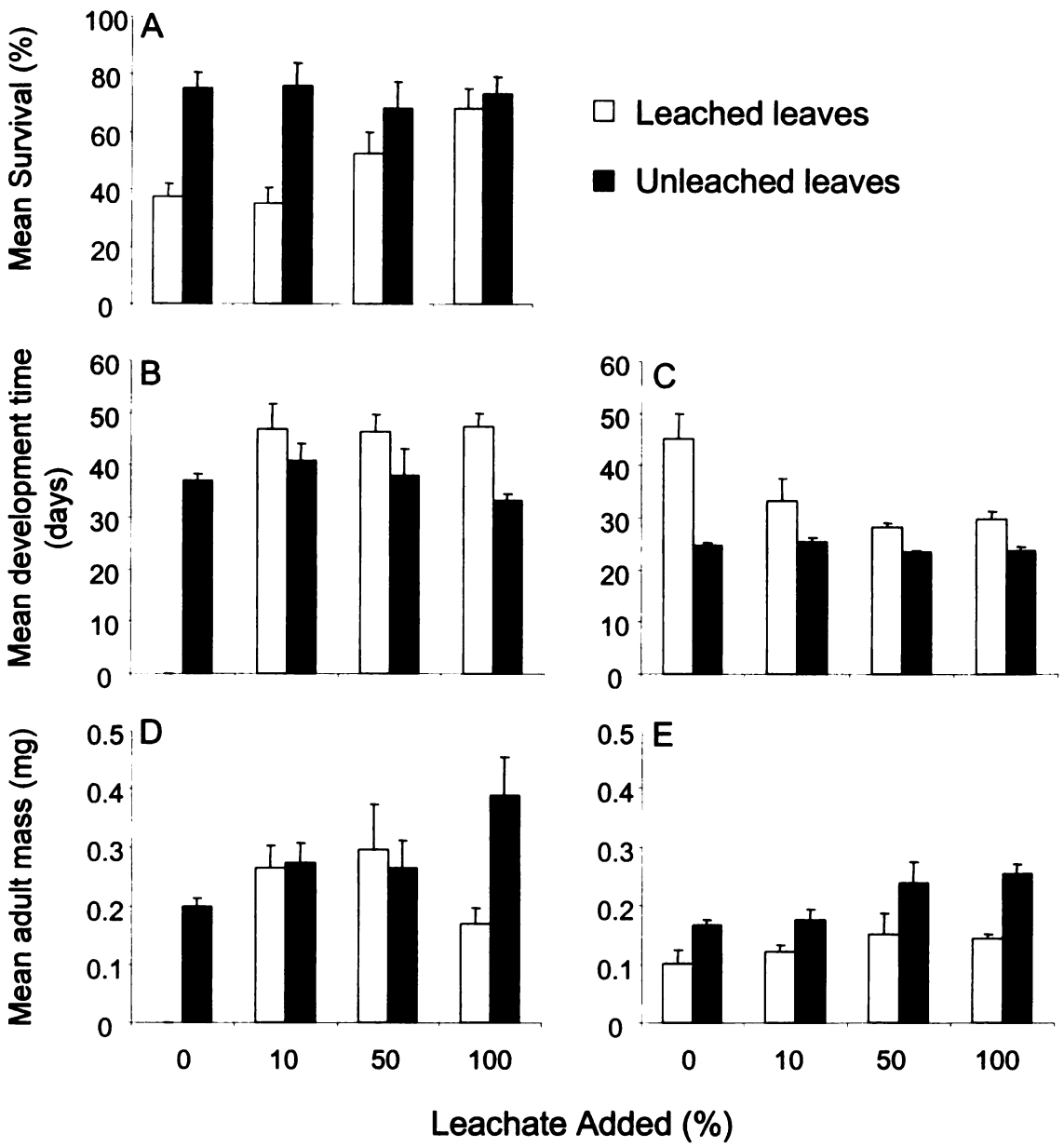

Figure 2.1. Mosquito production variables from experiment 1. (A) Survival (20 initial larvae). (B) Average female development time. (C) Average male development time. (D) Average female weight. (E) Average male weight. Values are means ± SE (n = 6 for all variables in A, C, and E. n = 2-6 for variables in B and D).

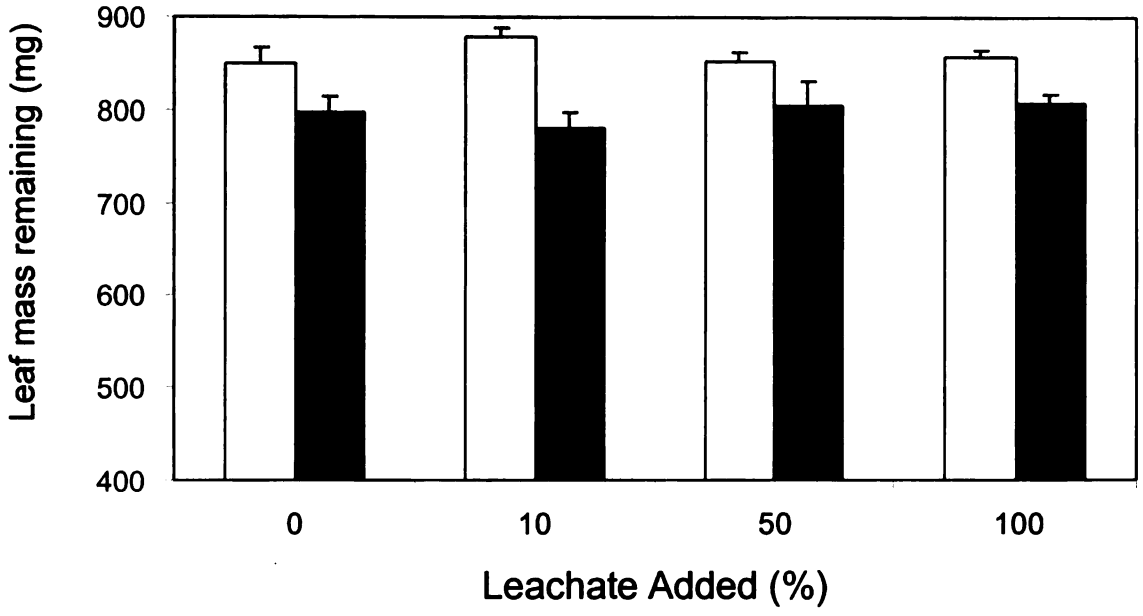

Figure 2.2. Leaf mass lost in experiment 1. Values are means ± SE (n = 6).

Experiment 2.

As in the first experiment, unleached leaves were a better resource for mosquitoes, supporting significantly greater adult production in these microcosms compared with microcosms containing unleached leaves (Table 2.2, Figure 2.3). Adult mass was greater and development time reduced by the presence of unleached leaves. Similarly, additions of leachate to microcosms had a significantly positive affect on mosquito growth. Unlike the first experiment, the effect of leached leaves on mosquito production was mitigated by the addition of leachate such that adult emergence in microcosms with this treatment combination was equal to or greater than emergence in microcosms with unleached leaves and 0% leachate. The production of adult mosquitoes was not effected by the condition of the leachate added (e.g. filter-sterilized or non-filtered) (Figure 2.3). Furthermore, filtration did not significantly interact with other treatment combinations to affect mosquito growth parameters under any condition.

Table 2.2. Multivariate (MANOVA) and univariate (ANOVA) hypothesis test results for all factors and their interactions for mosquito production variables in experiment 2. ANOVA results for each variable are shown for factors with significant MANOVA *(P*<0.05).

Sources	Wilks' Lamda	Response variable tested with ANOVA	*F*	df	*P*
Leaf	0.326		7.85	5,19	0.0004*
		Female mass	0.15	1,23	0.704
		Male mass	0.66	1,23	0.425
		Female development time	0.05	1,23	0.819
		Male development time	0.76	1,23	0.392
		Survival	6.76	1,23	0.016*
Leachate	0.16		19.93	5,19	<0.0001*
		Female mass	4.37	1,23	0.048*
		Male mass	11.97	1,23	0.002*
		Female development time	3.13	1,23	0.09
		Male development time	15.33	1,23	0.001*
		Survival	18.52	1,23	0.0003*
Filter	0.66		1.95	5,19	0.132
Leaf x Leachate	0.588		2.66	5,19	0.055
Leaf x Filter	0.805		0.92	5,19	0.488
Leachate x Filter	0.853		2.54	5,19	0.063
Leaf x Leachate x Filter	0.853		0.66	5,19	0.66

* Indicates significance at α-value < 0.05 after sequential Bonferroni correction.

Table 2.3. Experiment 2 multivariate (MANOVA) and univariate (ANOVA) hypothesis test results for all factors and their interactions for productivity and abundance of bacteria on leaf surfaces and in the water column. ANOVAs were performed for significant ANOVA factors ($P<0.05$).

A) Between subjects				
Sources	MS	*F*	df	*P*
Leaf	18.4	68.3	1,23	<0.001*
Leachate	2.1	7.6	1,23	0.01*
Leaf x Leachate	0.6	2.4	1,23	0.14
Filter	2.0	7.3	1,23	0.01*
Leaf x Filter	5.2	19.3	1,23	<0.001*
Leachate x Filter	2.7	9.9	1,23	0.01*
Leaf x Leachate x Filter	2.3	8.5	1,23	0.01*
B) Within subjects				
Sources	Wilks' Lamda	*F*	df	*P*
Time	0.1	108.3	3,21	<0.001*
Leaf x Time	0.1	43.4	3,21	<0.001*
Leachate x Time	0.4	11.8	3,21	<0.001*
Leaf x Leachate x Time	0.5	8.4	3,21	<0.001*
Filter x Time	0.5	8.4	3,21	<0.001*
Leaf x Filter x Time	0.4	8.6	3,21	<0.001*
Leachate x Filter x Time	0.4	9.6	3,21	<0.001*
Leaf x Leachate x Filter x Time	0.5	8.0	3,21	0.001*

* Indicates significance at α-value < 0.05 after sequential Bonferroni correction.

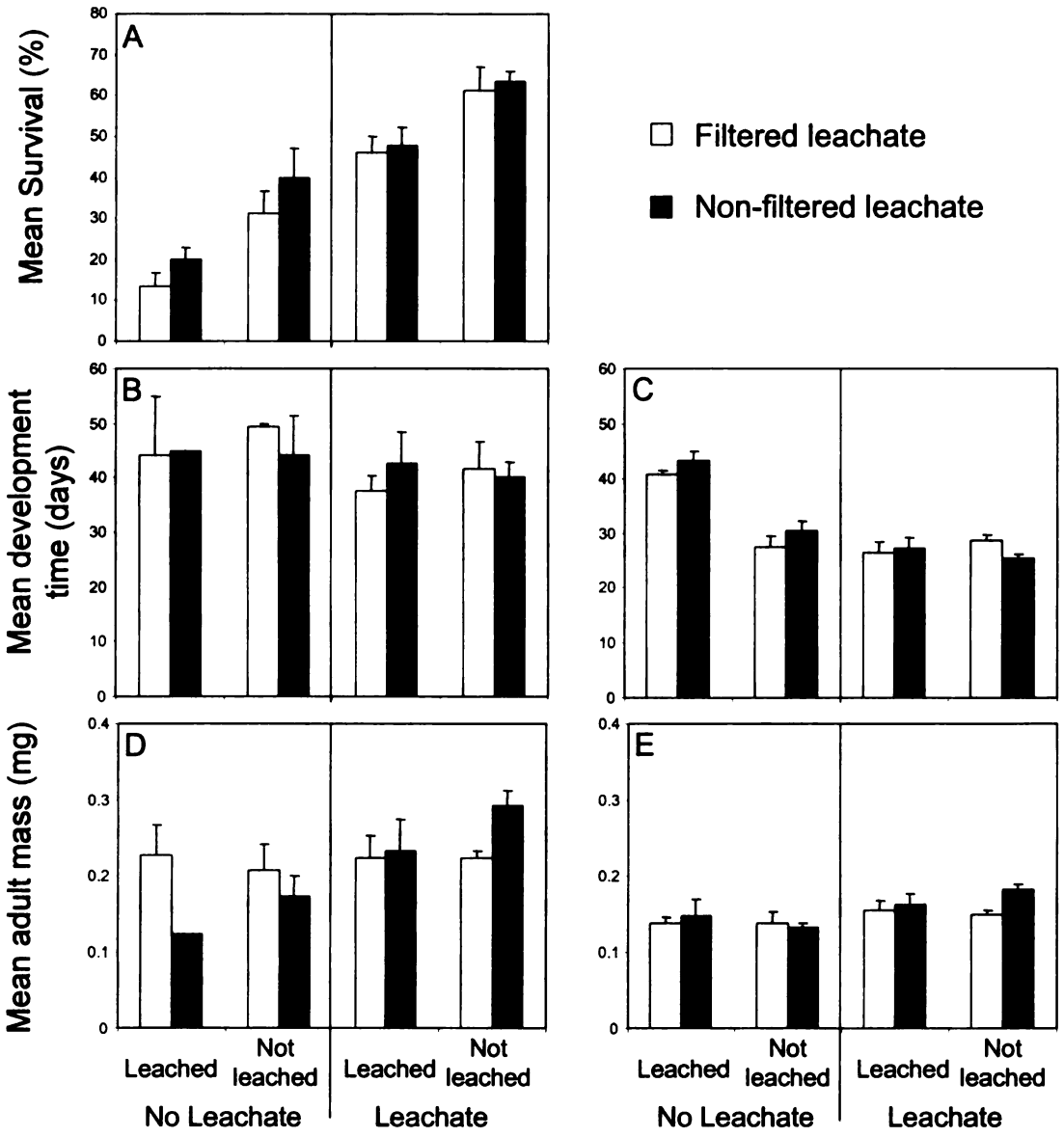

Figure 2.3. Mosquito production variables from experiment 2. (A) Survival (40 initial larvae). (B) Average female development time. (C) Average male development time. (D) Average female weight. (E) Average male weight. Values are means ± SE (n = 6 for all variables in A, C, and E. n = 1-6 for variables in B and D).

Microbial parameters. Bacterial abundance and productivity were significantly affected by leaf condition, leachate type, and filtration of the leachate, as indicated by

MANOVA with repeated measures on these parameters (Figures. 2.4 and 2.5; Table 2.3). Significant interactions were detected among all main effects, with the exception of leaf and leachate. Productivity of bacteria in the water column was greatest in response to unleached leaves at day 0 (prior to larval addition) when unleached leaves were present. Moreover, productivity was higher for this treatment combination in the presence of filtered leachate.

In addition, time had a significant affect on bacterial productivity and abundance. This trend disappeared by day 70 of the experiment, however. In general, bacterial productivity dropped below 1 x 10-7 pmol/ml for all treatment combinations at this time (Figure 2.4), although direct microscopic counts indicate that bacteria remained present at a similar abundance over the course of the experiment (Figure 2.5). Bacterial abundance in the water column responded in a similar manner to treatment combinations, with significantly higher concentrations of cells/ml at day 0 evident in microcosms receiving unleached leaves and filtered leachate (Figure 2.5; Table 2.3). By day 70, water column productivity had dropped to statistically similar lows for all treatments.

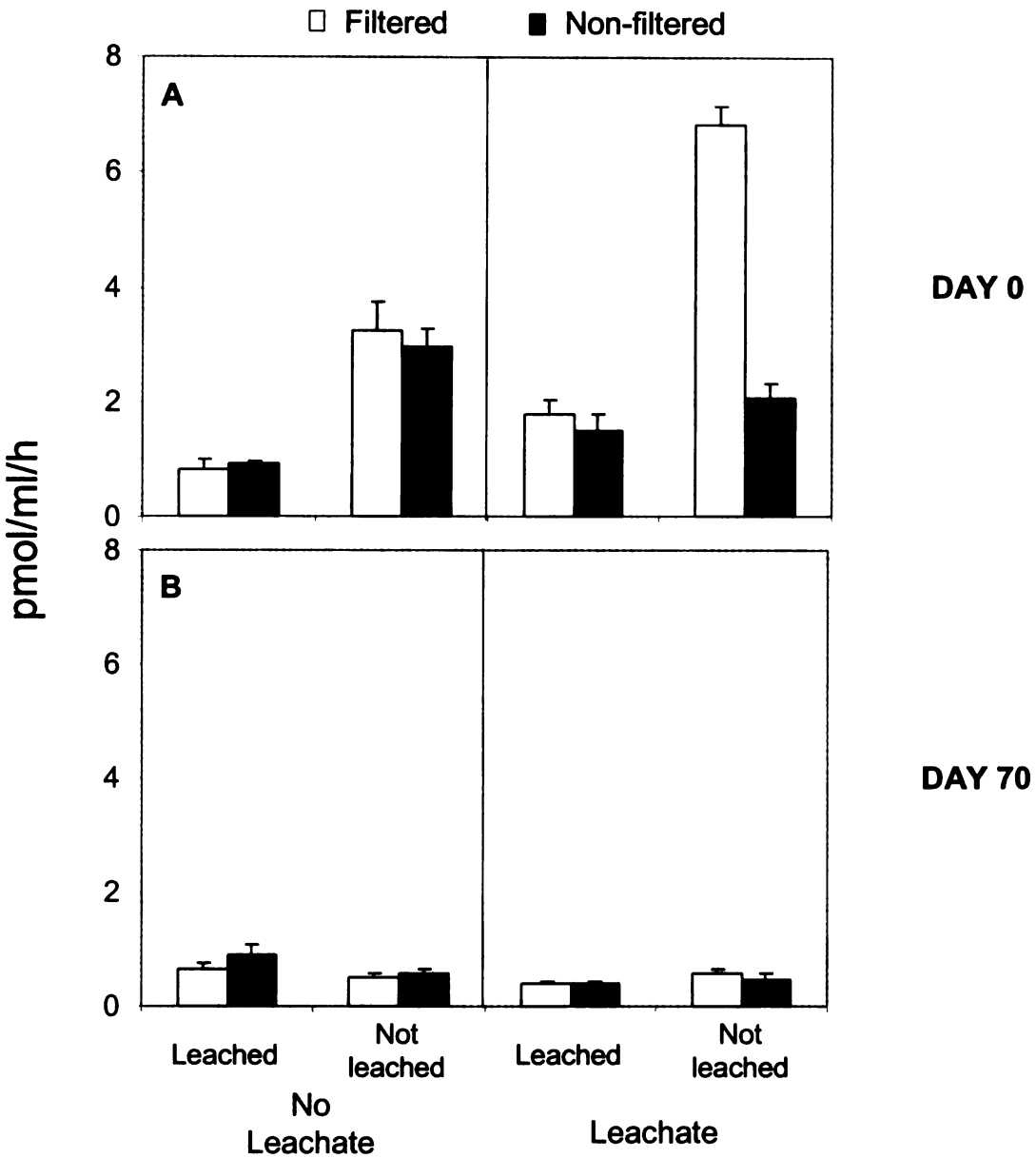

Figure 2.4. Water column bacterial productivity (leucine incorporation rate) response to leachate, filtration, and leaf condition in experiment 2. (A) day 0 and (B) day 70. Values are means ± SE (n = 6). Values are means ± SE (n = 6).

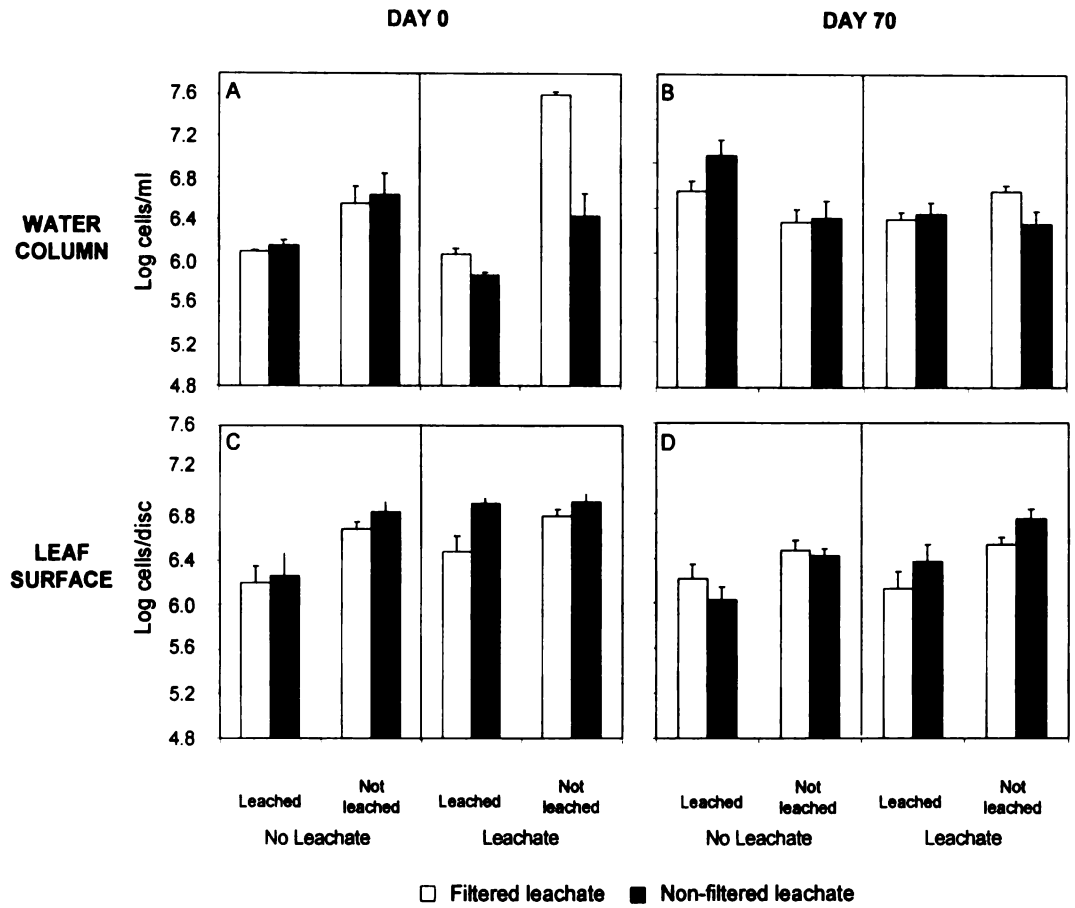

Figure 2.5. Bacterial abundance (direct microscopic counts) response to leachate, filtration, and leaf condition in experiment 2. Water column: (A) day 0 and (B) day 70. Leaf surface: (C) day 0 and (D) day 70. Values are means ± SE (n = 6).

Nutrient Analysis. Total N and P concentrations (Figure 2.6) were analyzed using a 2 (leaf) x 2 (leachate) x 2 (filtration) doubly- multivariate repeated measures MANOVA. This showed a significant main effect for leaf type and leachate, but no significant main effect for filtration (Table 2.4). Microcosms containing leached leaves contained greater amounts of N and P relative to those with unleached leaves. Similarly, microcosms with leachate contained higher N and P levels compared with microcosms lacking leachate (Figure 2.6).

Both leaf and leachate significantly interacted with filter, and interactions were evident among leaf, leachate, and leaf x leachate with time (Table 2.4). Specifically, total N and P concentrations decreased over time; at days 0 and 12, the content of N and P in microcosms containing leached leaves and no additional leachate was lower than in microcosms stocked with leached leaves and leachate. Follow-up univariate analyses indicate both N and P contributed to these results.

Table 2.4. Multivariate (MANOVA) and univariate (ANOVA) hypothesis test results for all factors and their interactions for nitrogen and phosphorus concentration in experiment 2. ANOVA's were performed for significant ANOVA factors ($P<0.05$).

A) Between subjects				
Sources	MS	*F*	df	*P*
Leaf	13.6		1,40	<0.001*
Leachate	8.3		1,40	<0.001*
Leaf x Leachate	0.03		1,40	0.22
Filter	0.0002		1,40	0.92
Leaf x Filter	0.3		1,40	<0.001*
Leachate x Filter	0.1		1,40	0.02
Leaf x Leachate x Filter	0.01		1,40	0.47
B) Within subjects				
Sources	Wilks' Lamda	*F*	df	*P*
Time	0.1	79.9	4,37	<0.001*
Leaf x Time	0.1	93.0	4,37	<0.001*
Leachate x Time	0.3	24.3	4,37	<0.001*
Leaf x Leachate x Time	0.5	7.7	4,37	<0.001*
Filter x Time	0.8	1.7	4,37	0.17
Leaf x Filter x Time	0.9	0.6	4,37	0.66
Leachate x Filter x Time	0.9	0.8	4,37	0.52
Leaf x Leachate x Filter x Time	0.9	0.6	4,37	0.65

* Indicates significance at α-value < 0.05 after sequential Bonferroni correction.

Figure 2.6. Water column chemistry for experiment 2 at days 0 (A,B), 12 (C,D), and 70 (E,F). Shown are total nitrogen (A,C,E) and total phosphorous (B,D,F).

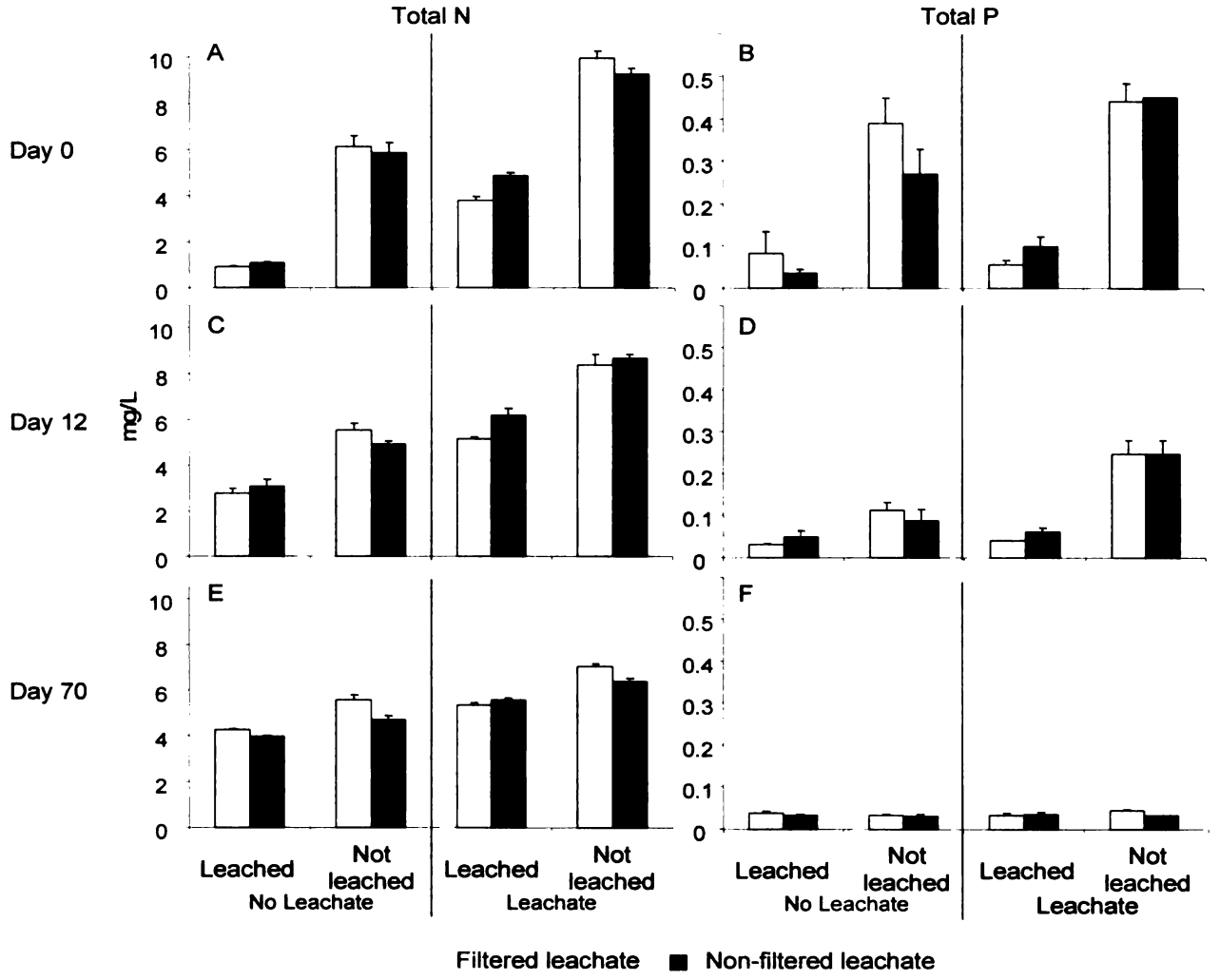
Total N
Total P
A
B
C
D
E
F
Day 0
Day 12
Day 70
mg/L
Leached
Not leached
No Leachate
Leachate
Filtered leachate
Non-filtered leachate

Figure 2.6.

Experiment 3.

Mosquito parameters were significantly affected by the type of input provided in microcosms (Table 2.5). Compared with microcosms receiving leachate only, microcosms containing leached and unleached leaf treatments produced significantly more adult mosquitoes, and these developed faster and attained greater mass (Table 2.5, Figure 2.7). Mosquito performance in leachate only microcosms was poor, with only two males and zero females produced with this treatment.

Table 2.5. MANOVA results for all factors and their interactions for mosquito production variables in experiment 3. ANOVA results for each variable are shown for factors with significant MANOVA *(P*<0.05).

		Response variable tested			
Source	Wilks' Lamda	with ANOVA	*F*	df	*P*
Treatment	0.304		3.53	6,26	0.01*
		Female mass	1.66	2,15	0.22
		Male mass	5.03	2,15	0.021*
		Female development time	21.21	1,3	0.019*
		Male development time	5.29	2,7	0.04*
		Survival	8.37	2,15	0.004*

* Indicates significance at α-value < 0.05 after sequential Bonferroni correction.

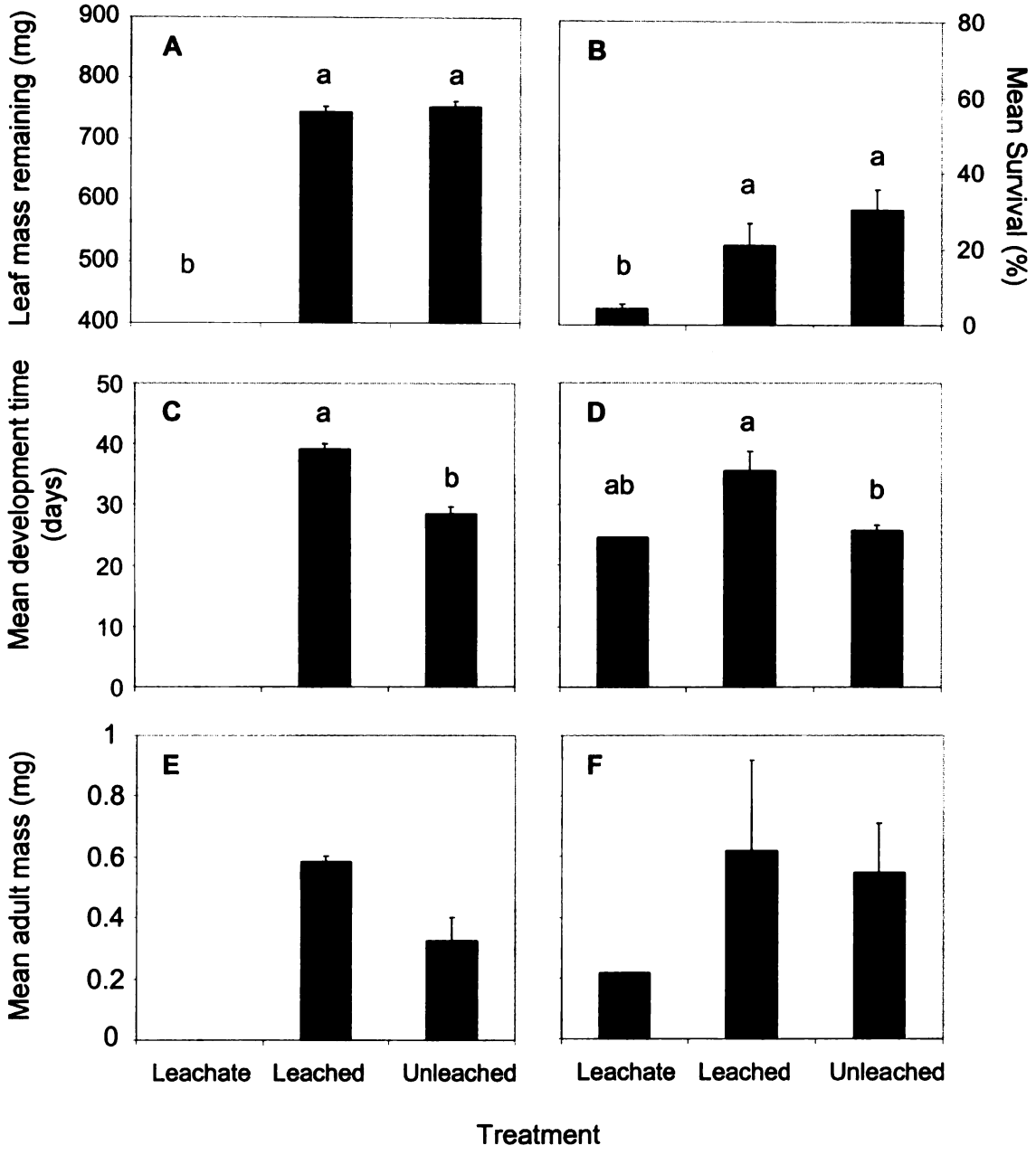

Figure 2.7. Mosquito production variables from experiment 3. (A) Average leaf mass remaining. (B) Survival (40 initial larvae). (C) Average female development time. (D) Average male development time. (E) Average female weight. (F) Average male weight. Values are means ± SE (n = 6 for all variables in A, B, E, and F. n = 0-6 for variables in C and D). Means within each column followed by the same letter are not significantly different, ($P > 0.05$, Fisher's Protected LSD Test).

Discussion

Senescent leaves are the most abundant allochthonous input in tree holes. Previous work suggests that the labile fraction of leaf material is critical to mosquito growth (Walker et al., 1997). In this experiment, mosquito production fell as much as 38% in microcosms treated with leached leaves. Thus, we expected that the positive effect of labile components on population growth could be reclaimed by the introduction of labile leaf components to microcosms containing leached leaves. In our first experiment, mosquito survival (larvae and adults) in microcosms containing leached leaves and 100% of the leachate produced by a an equivalent leaf pack was not significantly different from survival in those containing unleached leaves and no additional leachate, suggesting that for this parameter the effect of leachate could be restored. In contrast, development time and adult mass for males and females did not exhibit a positive response to the reintroduction of leachate. We postulate that this divergent effect occurred because leached leaves were adequate for maintenance of mosquitoes at the larval stage, but insufficient for driving adult production. Apparently, the failure of leachate restoration to stimulate equivalent adult production was due to the effect of filtering the leachate. A plausible explanation is that the available nutrients present in the leached leaf material were assimilated by microorganisms prior to filter-sterilization during the leaching period. Filtering the leachate before adding it to microcosms would, therefore, remove two critical components from the microcosms: incorporated nutrients and established populations of microorganisms.

Surprisingly, the results of Experiment 2 showed that filtration of leachate did not influence mosquito production parameters despite an initial surge in bacterial abundance

and productivity in microcosms receiving filtered leachate and unleached leaves. Evidently, microbial dynamics were affected by the treatment of leachate prior to its addition in microcosms. Greater productivity was observed following filtration, presumably due to the removal of established bacterial populations, allowing freshly inoculated populations to enter into an exponential growth stage. That bacterial populations were present prior to filtration is evidenced in experiment 1, wherein DMCs of bacterial cells in leachate prior to filtration were 7.2 log cells/ml. In addition, removal of protozoan predators may contribute to greater productivity following filtration.

The success of mosquitoes in the unleached leaf treatments compared with leached leaf treatments (no leachate added) in experiments 1 and 2 and in Walker et al. (1997) was presumably associated with corresponding high bacterial production and abundance at day 0. These high levels were not maintained throughout experiment 2, with differences among treatments becoming negligible by day 70. This result is not surprising, given previous work which indicates that grazing pressure from predators reduces bacterial productivity (Kaufman, 1999, Findlay, 1986). It should, however, be noted that the microbial analyses herein do not account for compositional changes in the microbial community. Indeed, microbial communities exposed to invertebrate feeding pressure are known to undergo a structural shift, potentially resulting in the dominance of indigestible forms (Jürgens and Matz, 2002)). Hence, microbial abundance may remain relatively unchanged throughout an experiment despite underlying changes in bacterial susceptibility to foraging mosquito larvae. That declines in bacterial productivity do not correspond with drops in abundance of the same magnitude also show that neither community composition nor metabolic activity were altered. The absence of changes in

bacterial abundance also suggests sufficient nutritive material is available to maintain microbial communities in the absence of stemflow events (which would bring in pulses of limiting nutrients such as N and P).

The failure of leachate alone to support larval development (experiment 3) suggests either the importance of surfaces, insufficient nutrient supply, or negative effects associated with high tannin content in the leachate . Surfaces are important to microorganisms, as they support the formation of biofilms, promote production of fungi, and have been shown to be grazed by larvae. Nutrients and DOC continue to leach from the leaf matrix, although at a greatly reduced rate.

In nature, rapid degradation of leaves may occur upon entering tree holes, particularly under high nutrient conditions (Macia and Bradshaw, 2000). Nutrients (N, P) promote decomposition because, despite available carbon pools, the production of microorganisms is limited by nutrients. Once the initial flush of nutrients from the leaf matrix has been exhausted, heterotrophic microorganisms are nutrient limited until replenishing stemflow or detritus enter the system. Although these initial fluxes of nutrient-rich leachate are critical to developing larvae, they may not be the norm. More likely the situation larvae experience is one supported by low quality leaf material. As *Oc. triseriatus* larvae hatch from mid- to late spring, leaf inputs are primarily in the form of senescent leaves that have been subjected to some degree of leaching throughout the winter.

CHAPTER 3

HABITAT-SPECIFIC CHANGES IN MICROBIAL COMMUNITY DIVERSITY ASSOCIATED WITH CONTAINER-BREEDING MOSQUITOES.

Introduction

Assessments of the microbial community composition and diversity are required if we are to understand the function and relative importance of individual microbial populations in tree holes and other container habitats, and to determine how these populations may contribute to overall mosquito productivity. Biotic and abiotic factors may impact the levels of richness and evenness in the microbial communities associated with tree holes (Bell 2005). Conversely, the relative productivity of mosquitoes in tree holes may vary directly in response to changes in the microbial community composition. Biotic factors may include the presence/absence or abundance of macroinvertebrates within tree holes, while abiotic factors include, but are not limited to detrital and nutrient inputs, stemflow events, pH, temperature dissolved oxygen concentration, and habitat size. The composition of leaf surface and water column-associated microbial communities of tree holes has been previously assessed in our laboratory and others via 16S rDNA sequence analysis and other molecular genetic approaches, fatty acid methyl ester (FAME) profile analysis (Kaufman et al. 1999, 2008; Xu et al. 2008), and DGGE (Bell 2005); however, container wall surfaces, an important component of the tree hole habitat, have not been examined. Furthermore, the microbial community associated with any component (water column, detrital surface, container surface) has remained unexamined in artificial container habitats such as tires, in which many medically important mosquito species commonly breed. Understanding the microbial dynamics of these habitats is critical if we wish to compare tree hole and tire systems, an important area of study given the preference of many medically important mosquito species (e.g. *A. albopictus* and *Oc. japonicus*) for these habitats.

There are a number of molecular genetic approaches available to assess microbial community composition that are culture independent (Dorigo et al. 2005, Talbot et al. 2008). Among these, analysis of terminal restriction fragment length polymorphisms (T-RFLPs) obtained from 16S and 18S rRNA genes is a relatively inexpensive method for analyzing microbial communities (Liu et al. 1997, Marsh 1999). This method provides an attractive alternative to traditional cloning and sequencing of microbial DNA due to the reduced cost and high throughput associated with sample processing. The decreases in time and expenditure result in microbial community analysis of replicated, manipulative experiments being a feasible option. The method has been employed previously in studies of bacterial and fungal communities in diverse habitats, but has not yet been used in published studies of larval mosquito habitats (Maknojia 2006) Bacterial diversity and nutritional significance of the surface microlayer in *Anopheles gambiae* (Diptera: Culicidae) larval habitats.

Our objective in the current study was to assess differences in the microbial community structure of tire and tree hole habitats. Specifically, we answered the following questions: 1) How does microbial community diversity vary among associated tree holes and tire habitats?; 2) does the microbial community of a container habitat change throughout the season i.e. does community succession occur?; 3) do the microbial diversity measurements obtained using t-RFLPs correspond to the measurements obtained through direct sequencing of microbes from these habitats?; and 4) within habitats, do container wall-associated microbial communities reflect the communities observed on leaves and in the water column?

Materials and Methods

Field. Two locations with tree holes containing populations of *Oc. triseriatus* were used for all field studies. The ysites, Toumey and Hudson woodlots, are located on the Michigan State University campus (East Lansing, MI) and have been utilized for previous studies of *Oc. triseriatus* (Kaufman et al. 2001, 2008). Tree holes in these woodlots are primarily associated with American beech *Fagus grandifolia* Ehrh.

An array of 18 tires was placed in the woodlots in the fall prior to the study year and filled with locally-collected rainwater and senescent leaves. Also added was a composite inoculum of microorganisms, consisting of water and particulates obtained from nearby tires used in previous years and known to harbor *Oc. triseriatus*. In addition, 12 tree holes were randomly selected from the woodlots for comparison with tire habitats. Upon hatching of natal populations of *Oc. triseriatus* larvae in tree holes the following spring (mid-April), we tethered two oven-dried senescent oak leaves obtained from Kellogg Biological Forest (Augusta, MI) into tires and tree holes using fishing line. Six 2 cm^2 tiles simulating container wall material, consisting of rubber drive belt material or tree bark, were also placed into tires and tree holes, respectively, for assessments of the container wall microbiota. Tree bark (beech tree) was obtained from tree fall within the woodlots and cut into tiles with a standard band saw. Leaf and container wall substrates were allowed to condition with microbiota for three days prior to the onset of the experiment on day 0. After conditioning, the tire array was seeded with newly-hatched first instar *Oc. triseriatus* taken from our laboratory colony, propagated the previous summer from the woodlots described herein. Larvae were added at densities of 0, 60, or 300 per tire, roughly approximating densities of 0, 40, and 80 larvae per liter. Each mosquito density was represented six times per treatment. The density of native *Oc.*

triseriatus populations in tree holes was estimated on day 0 by subsampling larvae with a syringe for counting in an enamel pan. In each container type, this procedure was repeated biweekly to assess mosquito densities and larval development until the majority of the initial hatch has emerged as adults. All larvae were returned to their respective containers after being counted and scored by instar.

Leaf, container wall, and water column samples were taken on day 15 and day 30 for analysis of microbial community structure. Leaf samples were procured aseptically using an 11 mm diameter cork borer. Additional water column and leaf samples were taken also on day 0 to establish baseline microbial communities prior to larval feeding. These dates were selected for microbial community sampling according to the development time of *Oc. triseriatus*, which typically undergo pupation two weeks after hatching. Leaf and container wall samples were placed in sterile phosphate buffer upon collection, and then sonicated for 12 min. on ice to remove loosely attached microorganisms. This method has been used in previous tree hole studies to obtain the fraction of microorganisms available to foraging mosquito larvae (Kaufman et al. 2008). DNA from samples was extracted for use in t-RFLP analysis and sequencing, as described below.

Laboratory. Microcosms were constructed concurrently with the field experiment to evaluate microbial communities at constant mosquito densities. Two independent experiments, each consisting of six replicates, were conducted to evaluate tire and tree hole container wall microbial communities. For each experiment, we stocked microcosms with 500 ml deionized water and a 1 g senescent red oak leaf pack obtained from Kellogg Forest. This method of microcosm construction has been described for previous studies

of tree hole dynamics (Kaufman et al. 2001, 2002, 2006). Two container wall tiles (described above), consisting of either tree bark or tire rubber, were added to tree hole and tire-simulating microcosms, respectively. Finally, each microcosm received a microbial inoculum (3 ml) of field-collected water and particulates obtained from a composite of tree hole or tire habitats. After a three day incubation period microcosms received either 0 or 40 newly-hatched first instar *Oc. triseriatus* larvae. Water column, leaf, and container wall samples for microbial community analysis were taken, as described above, on days 15 and 30. Additional water column and leaf samples were taken on day 0 prior to the addition of larvae to assess the affects of larval feeding on microbial communities.

Molecular analysis. From each experimental array (field tires and trees and laboratory microcosms simulating the same), we created composite samples for each treatment. Composites samples consisted of ca. 4 ng of DNA from individual treatment replicates. Separate composites were made for leaf, water, and container wall samples. Bacterial rRNA gene sequences were obtained using primers (63F: 5'-CAG GCC TAA CAC ATG CAA GTC-3' and 1387R: 5'-GGG CGG WGT GTA CAA GGC-3') targeting a 1,300 bp consensus region (Marchesi et al. 1998). PCR reactions consisted of ca. 10 ng composite DNA, 4 µl each of forward and reverse primers, 50 µl Failsafe™ PCR PreMix buffer E (Epicentre, Madison, WI), and 1 µl Failsafe™ PCR enzyme (*Taq*). PCR-grade water was added to bring the reaction to a final volume of 100 µl. Reactions were subjected through one cycle of 94 °C for 2 min, followed by 30 cycles of 94 °C for 45 s, 68 °C for 30s, and 72 °C for 1 min 30 s and a final cycle of 72°C for 7 min. Fungal rRNA gene sequences were amplified with the primers nu-SSU-0817-59F (5'-TTAGCATGGATAATR

RAATAGGA-3') and nu-SSU-1536-39R (5'-TTGCAATG CYCTATCCCCA-3'). Amplification of fungal sequences was carried out as described above for bacterial samples, except the annealing temperature was lowered to 58 °C, and extension at 72 °C was reduced to 1 min per cycle. Amplicons were purified using the QIAquick® PCR purification kit (Qiagen, Valencia, CA) and quantified using the Quant-iT spectrophotometric assay (Invitrogen, Carlsbad, CA).

Bacterial and Fungal rRNA gene sequences. For field and laboratory experiments, bacterial and fungal clone libraries were constructed by sequencing approximately 94 randomly chosen clones per mosquito density and container substrate. For economic and practical purposes, composite samples were created from individual treatment replicates. For field and laboratory tire and tree hole substrates, 16S and 18S rDNA was amplified as described above. In the case of tire microcosms, sufficient DNA was not available for the creation of 18S rRNA gene sequence libraries following T-RFLPs; therefore, these samples do not appear in subsequent analyses.

Clones were obtained by ligating the template DNA into pGEM-T easy vectors and transforming the vectors into competent *Escherichia coli* JM109 cells (Promega, Madison, WI). After 24 h, transformants were screened by plating on S-Gal/ampicillin (100 μg/ml) agar (Sigma, St. Louis, MO) and randomly selecting white colonies for isolated, overnight incubation at 37°C in ampicillin-treated (100 μg/ml) Luria-Bertani media. Plasmids were purified (and sequenced at the Michigan State University Research Technology Support Facility (RTSF).

Nonchimeric 16S and 18S rRNA gene sequences were classified using, respectively, the Ribosomal Database Project classifier program

(http://www.rdp.cme.msu.edu, release 9.59) and the Basic Local Alignment Search Tool (BLAST) available from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

T-RFLP analysis. The PCR-based T-RFLP method was used to examine community shifts among bacterial 16S rRNA and fungal 18S rRNA gene abundances. Shifts in the abundance of particular sequences are indicated by differences in the relative peak areas in the T-RFLP profiles obtained for each treatment replicate as the same concentration of PCR product was digested for each reaction. For bacterial and fungal genes, triplicate 100 µl PCR reactions were amplified under the conditions described above, except the forward primers for each gene region were fluorescently labeled with FAM (carboxyfluorescein) for detection by capillary electrophoresis (IDT). For each sample, amplification products from the three reactions were pooled during purification then digested (ca. 10 ng) with 20 U of *Msp*I (New England Biolabs, Cambridge, MA) in a 20 µl reactions for 3 h at 37°C. Reactions were stopped with a 20 min incubation at 65°C, followed by an ethanol precipitation to remove excess salts from the reaction prior to capillary electrophoresis. Samples were submitted to the Michigan State University research technology support facility (RTSF) for fragment identification with a PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at). Peak sizes and integrated areas under T-RFLP peaks were determined using Genescan Analysis software (version 3.7, Applied Biosystems). Binning of T -RFLP fragments was conducted using the T-RFLP Stats tools (Abdo et al. 2006) and R (R Development Core Team 2004).

Statistical analyses. Microbial communities present in field containers and laboratory microcosms were analyzed using principal component analysis (PCA) of the transformed

(logratio) percentage of peak area represented by T-RFLP fragments (JMP® Statistical Discovery Software, V5.1 (SAS Institute, Cary, NC.). The first two principal components (PCs) from field tires and microcosms community were used as variables for analysis of variance (ANOVA) to explore the effects of mosquito density and time on the structure of bacterial and fungal communities. Because mosquito density was a continuous variable in tree holes, PCs obtained from tree hole TRFLP fragments were subjected to logistic regression to determine whether microbial community changes correlated with mosquito density (Proc Reg, SAS V9.1, SAS Institute).

Results

Tree holes

T-RFLPs. Within the water column, significant changes in the bacterial community occurred over time (PC2), but not on leaf or container wall substrates (Table 3.1, Figure 3.1). Samples taken on day 0 exhibited significant shifts in composition by day 30 (Tukey's HSD: $p = 0.04$). In contrast, significant changes in fungal communities associated with leaves, container wall, and water column were evident over time (Table 3.1, Figure 3.3). Fungal communities changed significantly in response to time along PC1 (all substrates) and along PC 2 (water column). Shifts in fungal composition occurred between day 0 and day 15 (Tukey's HSD: water column, $p= 0.0005$; leaf surface, $p=0.01$), and between day 15 and 30 ($p=0.002$). On day 15 of the experiment, there were no significant correlations between mosquito density and PC1 scores from leaf, tile, or in water column samples ($p > 0.05$; $R^2 = 0.32, 0.12, 0.27$, respectively).

Clone libraries. For each composite tree hole sample, an average of 83 16S rRNA gene sequences (over 1000 total) were used for classification. The distribution of class

level bacterial taxa obtained from 16S rRNA gene libraries appeared to differ among the substrates sampled from field tree hole communities on day 15. Leaf and container wall samples were dominated by high percentages of sequences from Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes (Figure 3.2). Water column samples were dominated by the former two classes, although members of Bacteroidetes were noticeably absent from most water samples. Mosquito density appeared to have the largest impact on leaf-associated group, as indicated by an increase in the percentage of Bacteriodetes with mosquitoes and concurrent reductions in the percentage of Alphaproteobacteria.

An average of 81 sequences from tree hole composites from day 15 (over 1400 total) were used for 18S rRNA gene classification. Fungal communities on leaves and in water were characterized by a lower richness compared with container wall samples, though all substrates were generally dominated by the presence of Sodariomycetes (Figure 3.4). Water column fungal communities were not evaluated in response to low mosquito densities in this experiment, as insufficient template DNA was available for amplification; however compared to the medium larval density treatment, water samples in high larval densities treatments had a relatively greater abundance of Dothideomycete et Chaetothyriomycetes. A similar trend occurred in samples taken from container wall substrates. In addition, Leotiomycetes on both leaf and container wall surfaces also declined with increasing mosquito density.

Table 3.1. Analysis of variance results (ANOVA) for principle component (PC) values obtained from relative abundance of T-RFLP fragments in bacterial (16S rRNA) and fungal (18S rRNA) communities in tree holes. Shown are F values (*F*), degrees of freedom (df) and p values (*P*) for the main effect of sampling time for each substrate.

		PC1			PC2		
	Substrate	*F*	df	*P*	*F*	df	*P*
Bacteria							
	Leaf	1.19	2,24	0.32	0.61	2,24	0.55
	Water	2.71	2,24	0.09	4.76	2,24	0.02
	Tile	0.21	1,16	0.65	0.76	1,16	0.4
Fungi							
	Leaf	5.3	2,21	0.01	0.86	2,21	0.44
	Water	5.23	2,22	0.01	10.31	2,22	0.0007
	Tile	14.69	1,16	0.002	0.5	1,16	0.49

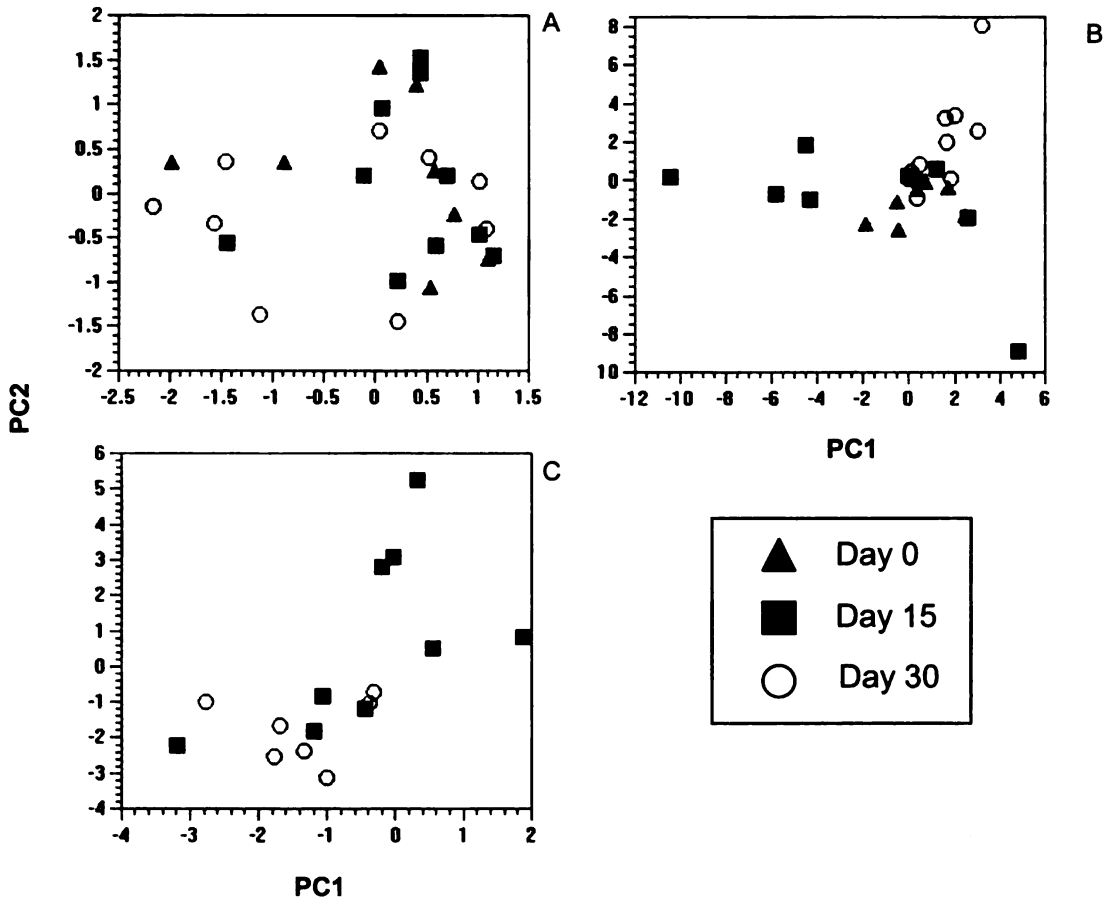

Figure 3.1. Principal component analysis of bacterial 16S rRNA gene communities from tree hole habitats. Panel A: Leaf , B: Water, C: Tile. PC axes 1 and 2 explained 24, 17, and 26% of the variation for the respective substrates.

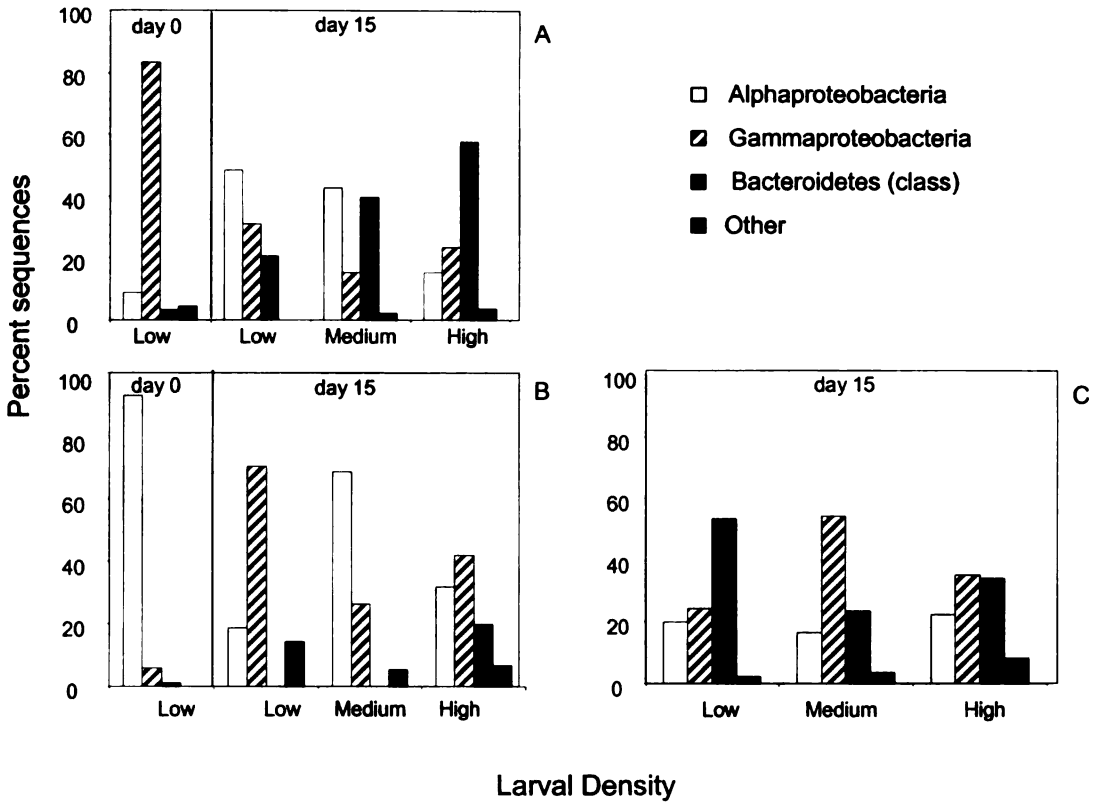

Figure 3.2. Percentage of bacterial 16S rRNA gene sequences in the class taxonomic level for composite samples taken from substrates in tree holes. A Leaf, B Water, C Tile (n = 4 per composite).

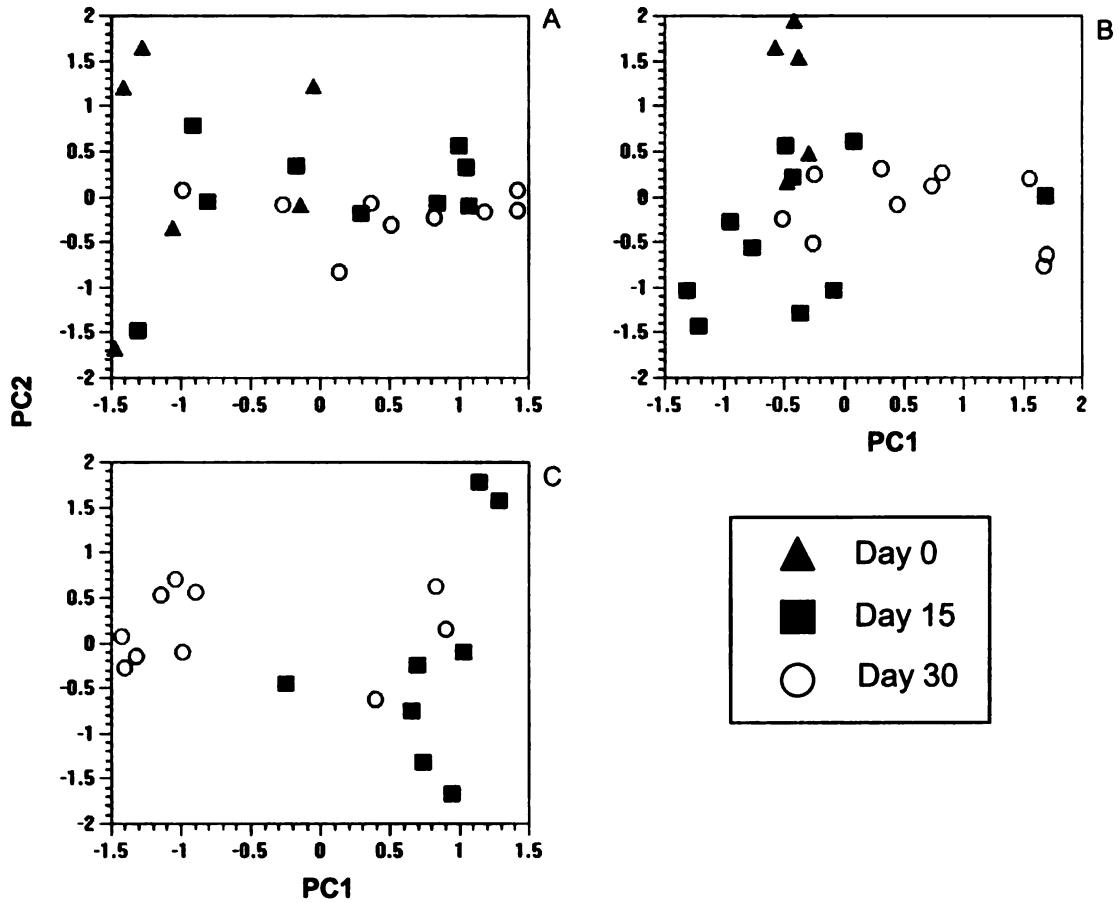

Figure 3.3. Principal component analysis of fungal 18S rRNA gene communities from tree hole habitats. Panel A: Leaf, B: Water, C: Tile. PC axes 1 and 2 explained 20, 19, and 28% of the variation for the respective substrates.

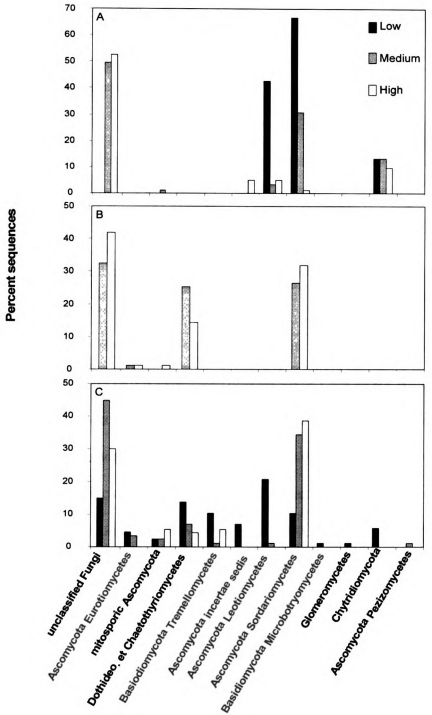

Figure 3.4. Percentage of fungal 18S rRNA gene sequences in the class taxonomic level (or above) for composite samples taken from substrates in tree hole habitats on day 15. A Leaf, B Water, C Tile (n = 4 per composite). Inset legend refers to mosquito density.

Tree hole microcosms

T-RFLPs. Unlike field tree holes, bacterial communities in the microcosms exhibited significant shifts over time on leaf and container wall surfaces in addition to significant shifts in the water column (Table 3.2, Figure 3.5). For each substrate, PC1 was associated with community changes in response to time. Samples taken at 30 days were significantly different from samples taken at the onset of the experiment (Tukey's HSD: leaf, $p= 0.0006$; water, $p<0.0001$; wall, $p<0.0001$). In addition, mosquito presence also had a significant effect on the structure of bacterial communities on leaves (PC1) and in the water column (PC2).

Clone libraries. An average of 92 sequences from tree hole microcosm samples was used for 16S rRNA gene classification (over 1300 total). Although no effect of mosquito presence was evident in the distribution of bacterial taxa across the substrates, a shift in dominance was apparent from day 0 to day 30 in leaf and water samples (Figure 3.6). The relative abundance of Alphaproteobacteria increased from a range of 47-57% of total sequences on day 0 to 69-90% by day 30. Concurrently, the relative percentage of Gammaproteobacteria declined from 22-40% of total sequences on day 0 to 0-15% on day 30.

For 18S rRNA gene classification, we used an average of 73 sequences from tree hole microcosm samples (over 1000 total). The abundance and richness of fungal taxa from microcosms simulating tree holes was relatively greater in samples taken from leaf surfaces compared with those taken from container wall or water column (Figure 3.7). As observed for field tree holes, Sodariomycetes were a dominant member of the fungal community from each of the substrates analyzed, although the relative abundance of this

group fluctuated differentially among the substrates with mosquito density. The relative abundance of mitosporic Ascomycota tended to be greater in leaf, water, and container wall samples from microcosms with no larvae, despite an absence of any temporal trend for these treatments.

Table 3.2. Analysis of variance results (ANOVA) for principle component (PC) values obtained from relative abundance of 16s rRNA gene T-RFLP fragments in tree hole microcosm communities and showing F values (*F*), degrees of freedom (df) and p values (*P*) for the main effects of sampling time and mosquito density on each substrate.

			PC1		PC2	
	Substrate	df	*F*	*P*	*F*	*P*
Leaf						
	Date	2,30	10.3	0.0004	1.21	0.31
	Density	1,30	6.91	0.01	1.83	0.19
	Date*Density	2,30	0.68	0.51	4.55	0.02
Water						
	Date	1,20	24.05	<0.0001	2.41	0.14
	Density	1,20	1.39	0.25	6.13	0.02
	Date*Density	1,20	0.75	0.4	6.24	0.02
Tile						
	Date	1,20	36.7	<0.0001	0.58	0.45
	Density	1,20	0.04	0.85	0.04	0.84
	Date*Density	1,20	0.73	0.4	0.02	0.9

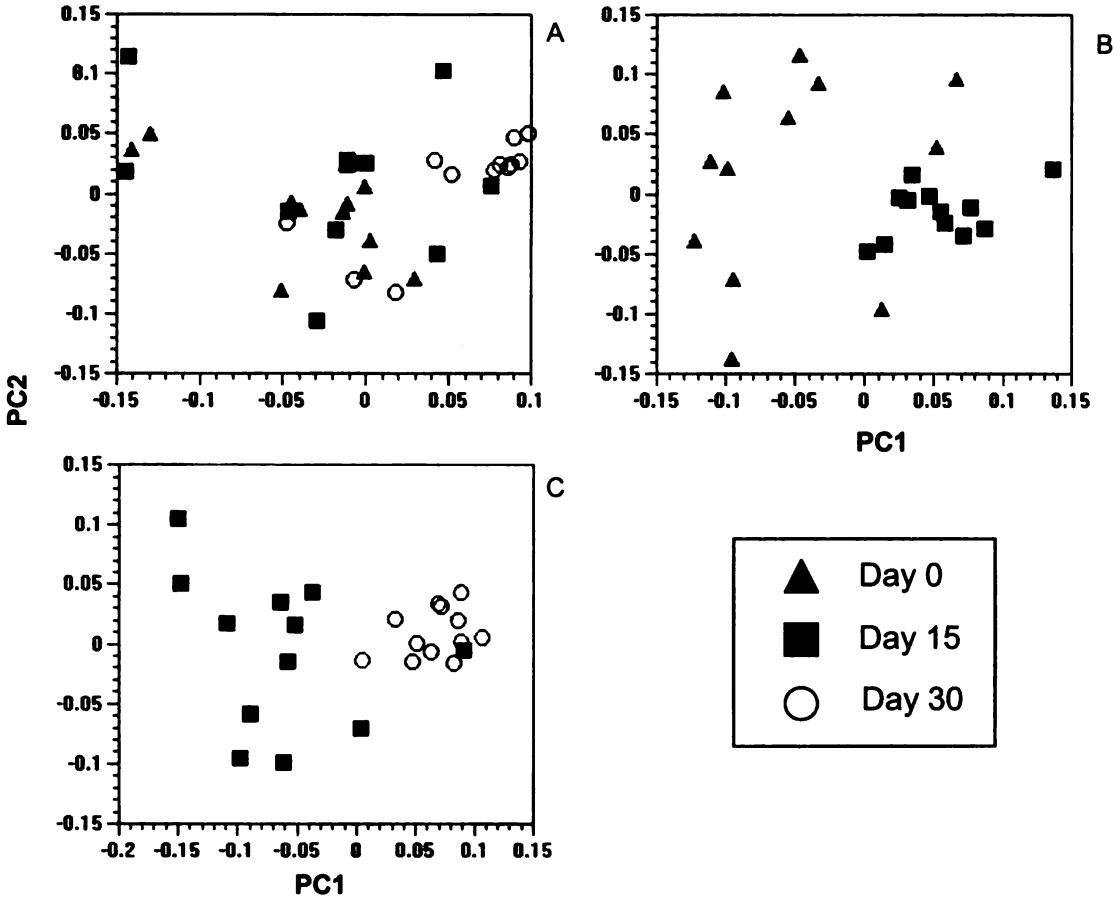

Figure 3.5. Principal component analysis of bacterial 16S rRNA gene communities from tree hole microcosms. Panel A: Leaf, B: Water, C: Tile. PC axes 1 and 2 explained 45, 50, and 69% of the variation for the respective substrates.

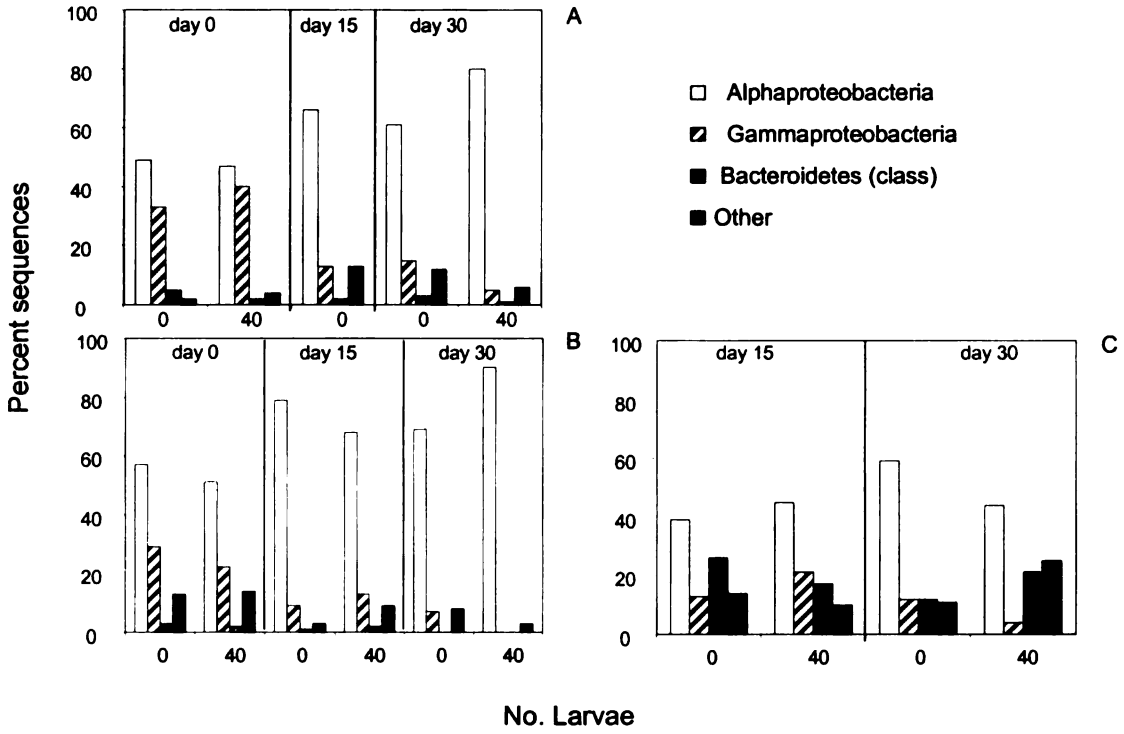

Figure 3.6. Percentage of bacterial 16S rRNA gene sequences in the class taxonomic level for composite samples taken from substrates in microcosms simulating tree holes. A Leaf, B Water, C Tile (n = 6 per composite).

Figure 3.7. Percentage of fungal 18S rRNA gene sequences in the class taxonomic level (or above) for composite samples taken from substrates microcosms simulating tree holes on days 0, 15, and 30. A, B Leaf, C, D Water, E,F Tile (n = 6 per composite). Panels A, C, and E are microcosms with 0 larvae, B, D, and F are microcosms with 40 larvae.

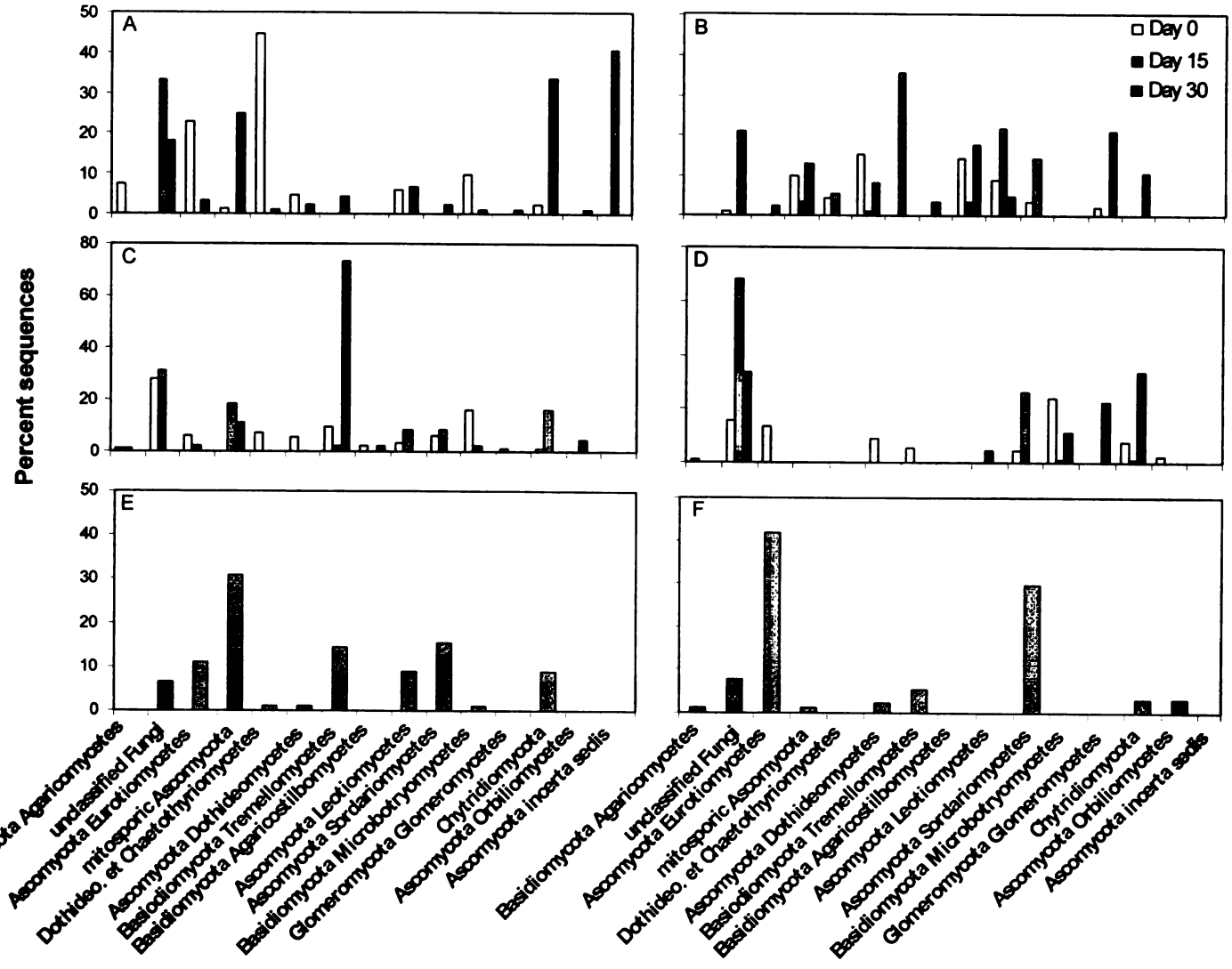
A
B
C
D
E
F
Day 0
Day 15
Day 30
Percent sequences
Basidiomycota Agaricomycetes
unclassified Fungi
Ascomycota Eurotiomycetes
mitosporic Ascomycota
Dothideo. et Chaetothyriomycetes
Ascomycota Dothideomycetes
Basiodiomycota Tremellomycetes
Basidiomycota Agaricostilbomycetes
Ascomycota Leotiomycetes
Ascomycota Sordariomycetes
Basidiomycota Microbotryomycetes
Glomeromycota Glomeromycetes
Chytridiomycota
Ascomycota Orbiliomycetes
Ascomycota inserta sedis

Figure 3.7.

Tires

T-RFLPs. Along PC1, bacterial communities fluctuated, reflecting significant changes between day 15 and day 30 on leaves, container walls, and in the water column (Tukey's HSD: p=0.05, <0.0001, and0.01, respectively). Leaf surface communities also shifted over time along PC2, between day 0 and day 15 (p=0.01) and between day 15 and day 30 (p=0.02) (Table 3, Figure 8).

Fluxes in the fungal community structure were evident on leaves along PC2, with significant shifts occurring from day 0 to day 15 (p<0.0001). Container wall surfaces did not reflect temporal variation in the fungal taxa present (Table 3.4, Figure 3.10). As in tree holes, correlations between mosquito density and PC1 scores from leaf, water, and tile samples were not significant ($p > 0.05$; R^2= 0.03, 0.01, 0.26, respectively).

Clone libraries. An average of 87 sequences from composite tire samples was used for 16S rRNA gene classification. In comparison to tree hole communities, the overall diversity of class level bacterial sequences appears less diverse across the substrates sampled from tire communities (Table 3.4, Figure 3.9). Alphaproteobacteria and Betaproteobacteria were conspicuously absent from field tire communities, although Flavobacteria were apparent. Other major bacterial groups seen in tire samples were Gammaproteobacteria and Bacteroidetes.

An average of 69 sequences from composite tire samples was used for 18S rRNA gene classifications. Fungal communities were generally dominated by Sodariomycetes across each substrate, although the relative abundance of this group varied with mosquito density within substrates (Fig 3.11). Tremellomycetes and Cryptomonads appeared in water samples, but comprised $\leq$7% of fungi associated with leaf surfaces or container

walls. In contrast to the other tire substrates, fungal communities associated with container walls were dominated by unclassified fungi. Finally, in contrast to tree hole communities (field and microcosm), Chytridomycota was not represented in the tire fungal communities.

Table 3.3. Analysis of variance results (ANOVA) for principle component (PC) values obtained from relative abundance of T-RFLP fragments in bacterial (16S rRNA) communities in tires. Shown are F values (*F*), degrees of freedom (df) and p values (*P*) for the main effect of sampling time and mosquito density for each substrate.

			PC1		PC2	
	Substrate	df	*F*	*P*	*F*	*P*
Leaf						
	Date	2,28	3.09	0.06	13.26	<0.0001
	Density	2,28	0.96	0.40	0.93	0.41
	Date*Density	4,28	1.61	0.20	0.54	0.71
Water						
	Date	2,36	4.15	0.02	2.37	0.11
	Density	2,36	6.07	0.005	0.37	0.69
	Date*Density	4,36	0.58	0.68	0.26	0.90
Tile						
	Date	2,24	111.3	<0.0001	0.01	0.90
	Density	1,24	0.75	0.48	1.80	0.19
	Date*Density	2,24	2.58	0.10	1.77	0.19

Table 3.4. Analysis of variance results (ANOVA) for principle component (PC) values obtained from relative abundance of T-RFLP fragments in fungal (18S rRNA) communities in tires. Shown are F values (*F*), degrees of freedom (df) and p values (*P*) for the main effect of sampling time and mosquito density for each substrate.

			PC1		PC2	
	Substrate	df	*F*	*P*	*F*	*P*
Leaf						
	Date	2,44	229.32	<0.0001	0.20	0.82
	Density	2,44	1.11	0.34	0.96	0.39
	Date*Density	4,44	0.89	0.47	0.76	0.56
Water						
	Date	2,28	3.13	0.06	2.76	0.08
	Density	2,28	3.07	0.06	0.35	0.70
	Date*Density	4,28	0.03	0.97	0.68	0.51
Tile						
	Date	2,27	1.11	0.30	2.10	0.16
	Density	1,27	3.08	0.06	0.79	0.46
	Date*Density	2,27	0.00	0.99	2.39	0.11

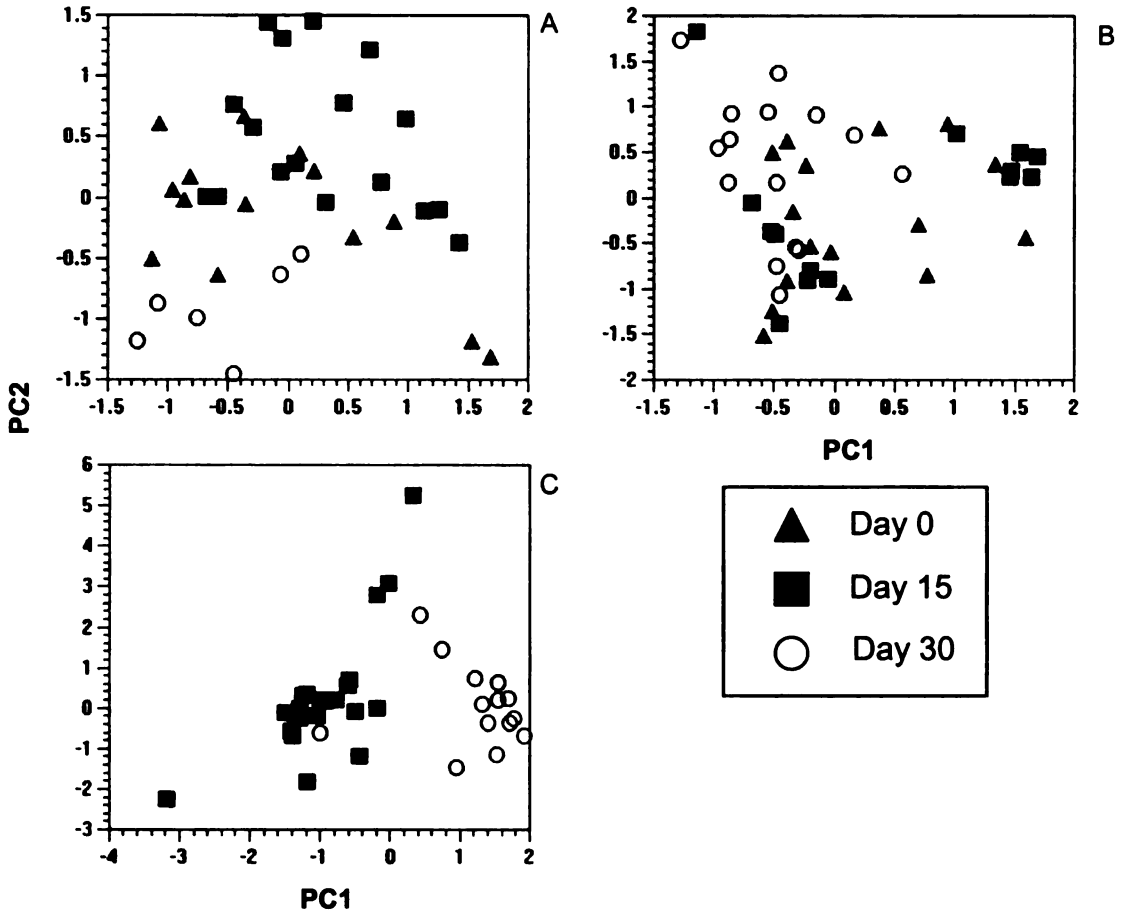

Figure 3.8. Principal component analysis of bacterial 16S rRNA gene communities from tire habitats. Panel A: Leaf , B: Water, C: Tile. PC axes 1 and 2 explained 20, 19, and 28% of the variation for the respective substrates.

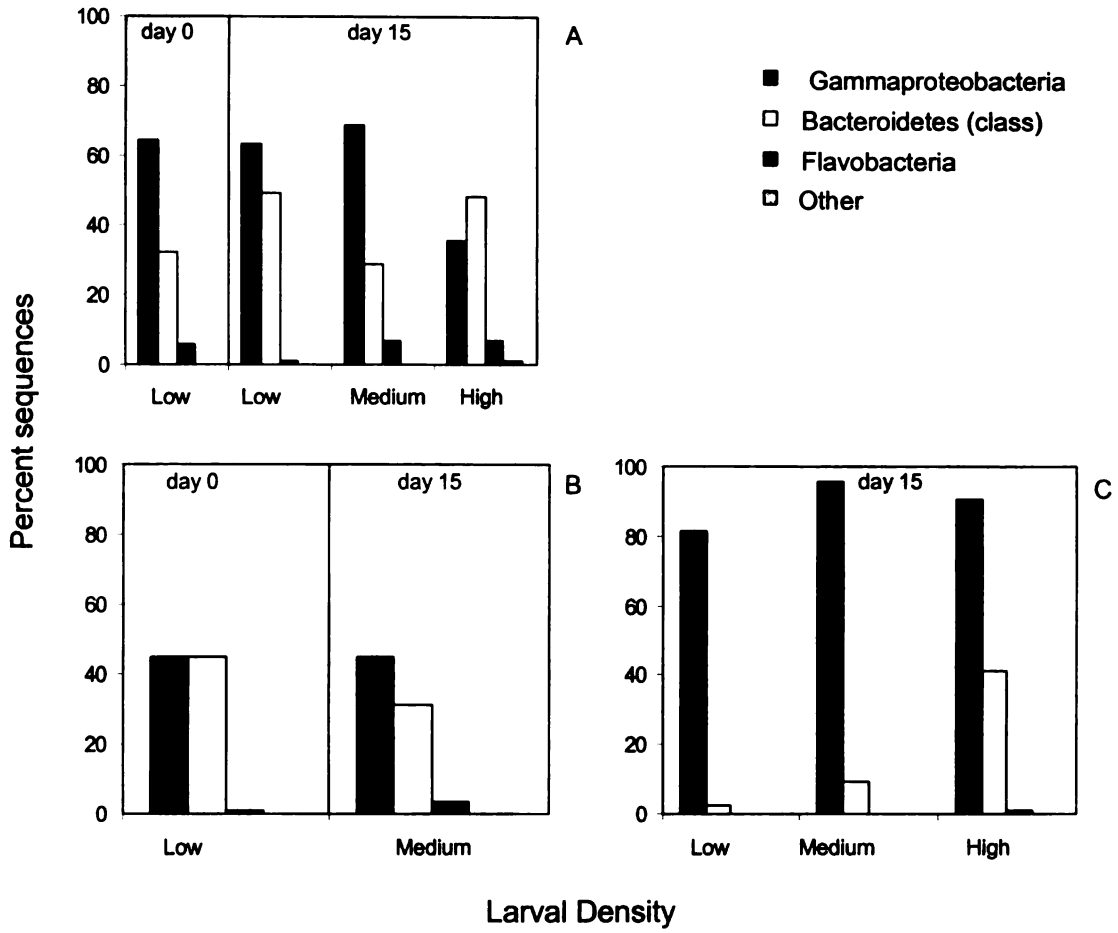

Figure 3.9. Percentage of bacterial 16S rRNA gene sequences in the class taxonomic level for composite samples taken from substrates in tire habitats. A Leaf, B Water, C Tile (n = 4 per composite).

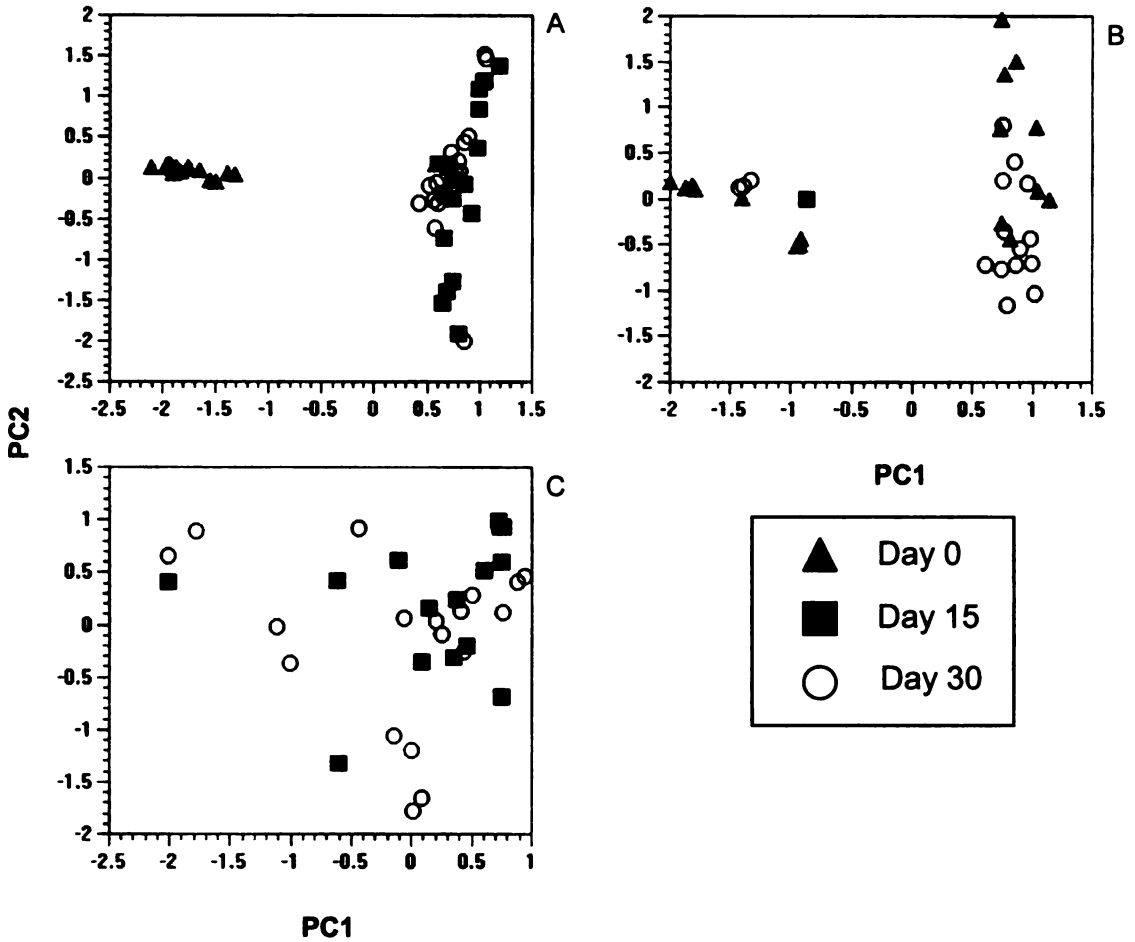

Figure 3.10. Principal component analysis of fungal 18S rRNA gene communities from tire habitats. Panel A: Leaf , B: Water, C: Tile. PC axes 1 and 2 explained 24, 23, and 14% of the variation for the respective substrates.

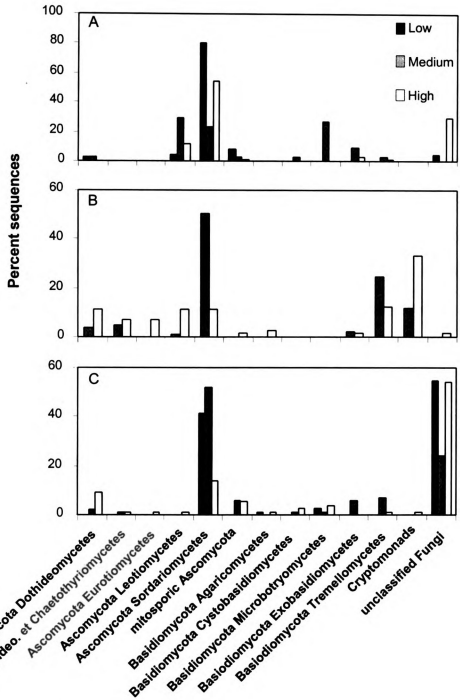

Figure 3.11. Percentage of fungal 18S rRNA gene sequences in the class taxonomic level (or above) for composite samples taken from substrates in tire habitats on day 15. A Leaf, B Water, C Tile (n = 4 per composite). Inset legend refers to mosquito density.

Tire microcosms

T-RFLPs. The presence of mosquitoes in microcosms significantly impacted bacterial communities in the water column along both principle component axes (Table 3.5, Figure 3.12). In addition, PC2 corresponded to temporal changes in water column bacterial communities, with significant shifts occurring between samples taken at the onset of the experiment and those taken on day 15 (Tukey's HSD: $p = 0.01$) and day 30 (Tukey's HSD: $p < 0.0001$). A significant change in the community also occurred from day 15 to day 30 (Tukey's HSD: $p<0.0001$). Bacterial communities associated with tiles simulating tire container walls were significantly affected by the presence of mosquitoes along PC; however, sampling date did not impart significant changes on tire communities along either axis.

Clone libraries. As in the field experiment, the bacterial sequences present in microcosms simulating tire habitats appeared to be less diverse than in tree hole microcosms (Figure 3.13). In contrast to field tire communities, however, Alphaproteobacteria were present in the tire microcosms despite exhibiting decreased abundance over time. In general, Alphaproteobacteria and Gammaproteobacteria dominated tire microcosms over time for each substrate.

Table 3.5. Analysis of variance results (ANOVA) for principle component (PC) values obtained from relative abundance of 16s rRNA gene T-RFLP fragments in tire microcosm communities and showing F values (*F*), degrees of freedom (df) and p values (*P*) for the main effects of sampling time and mosquito density on each substrate.

			PC1		PC2	
	Substrate	df	*F*	*P*	*F*	*P*
Leaf						
	Date	2,28	1.81	0.23	1.64	0.21
	Density	1,28	0.47	0.49	0.26	0.62
	Date*Density	2,28	1.36	0.27	0.5	0.61
Water						
	Date	2,45	3.78	0.03	52.7	<0.0001
	Density	1,45	52.1	<0.0001	10.7	0.002
	Date*Density	2,45	1.24	0.3	0.83	0.44
Tile						
	Date	1,16	0.11	0.74	0.15	0.71
	Density	1,16	6.14	0.02	0.54	0.47
	Date*Density	1,16	0.00	0.97	0.19	0.67

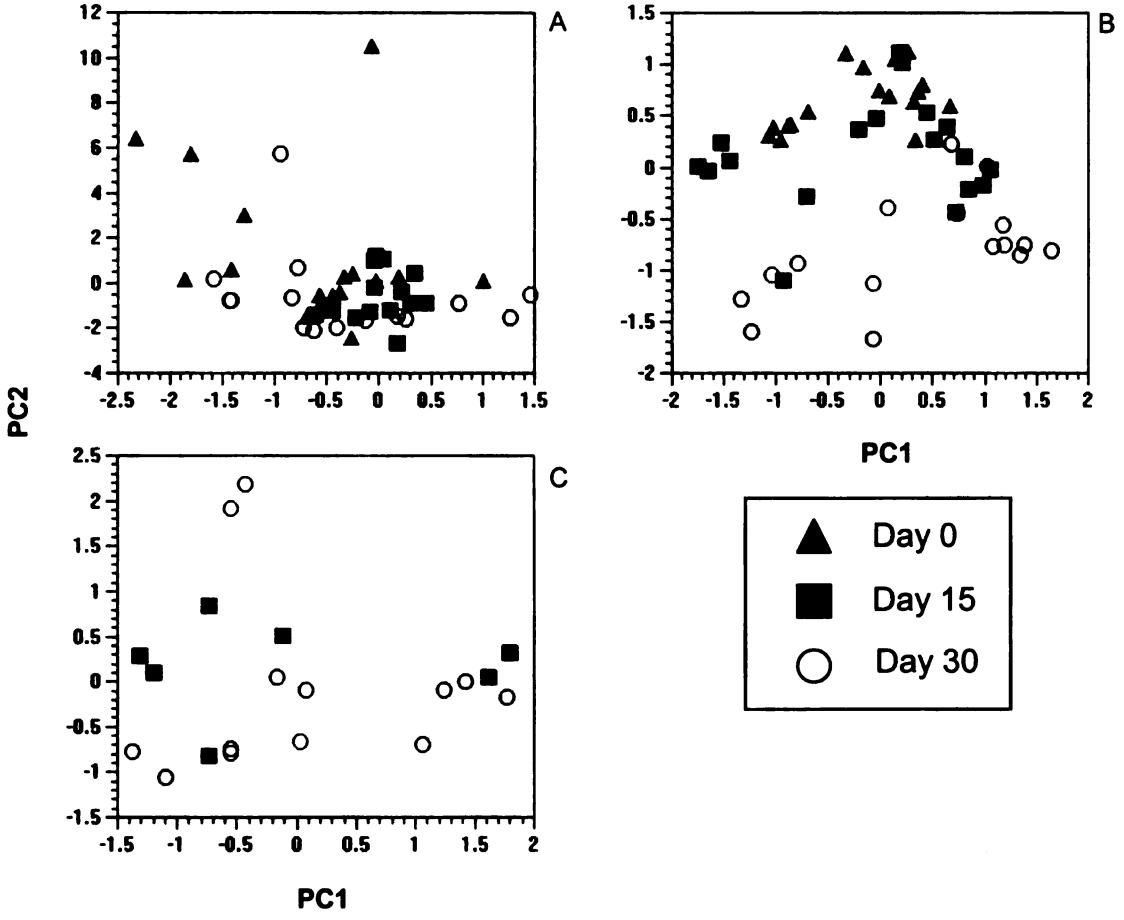

Figure 3.12. Principal component analysis of bacterial 16S rRNA gene communities from tire microcosms. Panel A: Leaf, B: Water, C: Tile. PC axes 1 and 2 explained 29, 42, and 58% of the variation for the respective substrates.

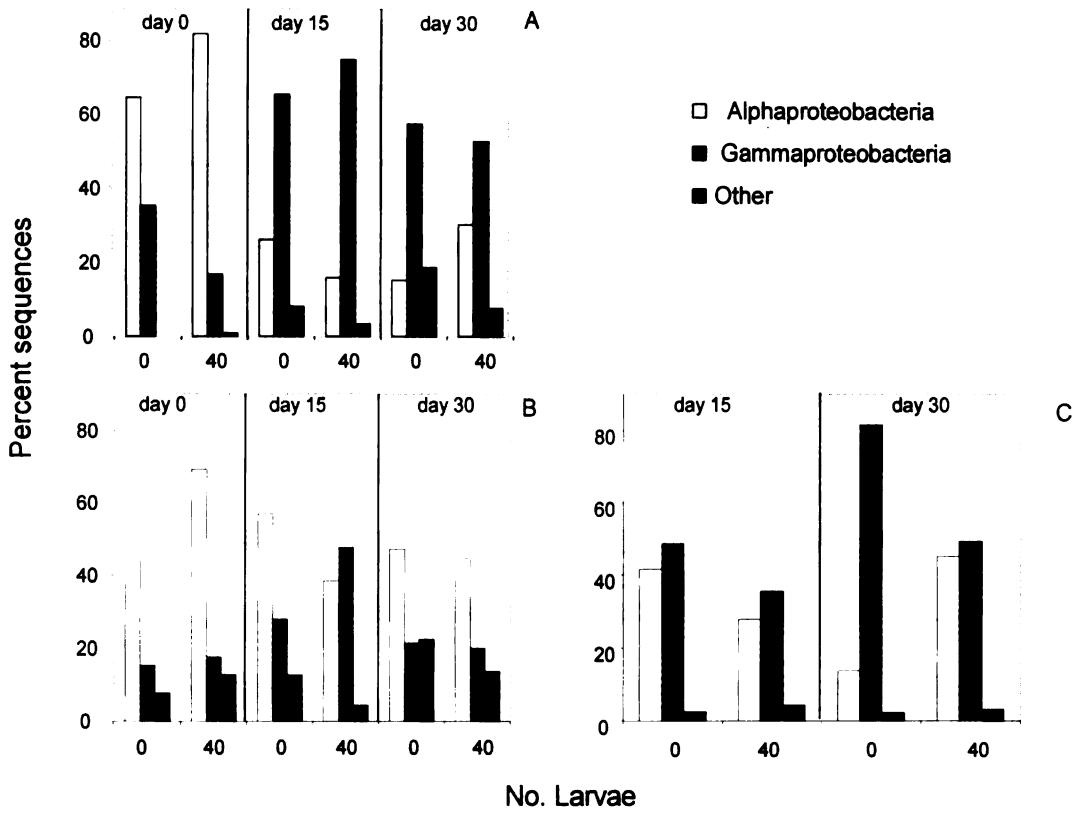

Figure 3.13. Percentage of bacterial 16S rRNA gene sequences in the class taxonomic level for composite samples taken from substrates in microcosms simulating tire habitats. A Leaf, B Water, C Tile (n = 6 per composite).

Discussion

To our knowledge, this is the first study to address the composition of microbial communities associated with tire container habitats. Evaluating the variation in tree hole versus tire habitats is an important consideration, as mosquito species often segregate on the basis of container type. The differential capacity among mosquitoes for vectoring diseases may be relevant, as competitive displacement among species may occur in specific habitats, resulting in a superior disease vector supplanting a mosquito species that is more innocuous from a public health perspective. For example, the invasive mosquito *Oc. japonicus,* which has spread through much of the Eastern United States in recent years (Andreadis 2001), has recently begun to encroach upon tire habitats in Michigan (personal observation). If time proves this species to be a superior competitor, native populations of *Oc. triseriatus* may be supplanted.

Temporal fluctuation in the structure of bacterial and fungal communities was the most evident effect observed in field and microcosm experiments for both container types. Bacterial communities obtained from leaf, water, and container wall samples shifted significantly from day 0 to day 30, with only one exception. Changes in leaf-associated bacterial communities were evident in tree hole microcosms, but not in field tree holes. Fungal communities also fluctuated among all three substrates in field tree holes; however, among tire substrates, only shifts in leaf-associated communities were evident. Temporal shifts in community structure are possibly related to changes in the surrounding environment, including disturbances in the habitats related to temperature, precipitation, and wind-borne introduction of new microorganisms to the communities. These factors are less likely to affect temporal changes in microcosms, as such

environmental variables are carefully controlled. Instead, it is possible that bottom-up effects, such as the build-up of waste products from developing larvae or by-products associated with leaf decomposition, may also be contributing to shifts in microorganism community structure over time.

Contrary to what we might have expected based on previous studies, larval density generally did not affect the communities of bacteria or fungi represented by T-RFLP fragments from field tree holes or tires. Indeed, significant larval affects on principal component variables representing bacterial communities were only observed in the water column of the latter. Mosquito density did influence bacterial communities in tree hole and tire microcosms, however. Effects in these habitats manifested in the water column and, for tree hole microcosms, in association with leaf material. Several factors may have contributed to the absence of mosquito effects in field sample, including overall variation in the field experiments. Variation not due to mosquitoes, including stemflow and the presence of alternative leaf and other detrital material, may have obscured the effect of larval feeding on microbial communities.

The communities of heterotrophic bacteria observed in this study were generally dominated by Alphaproteobacteria and Bacteroidetes, groups commonly associated with aquatic habitats (Kirchman 2002). As described by Kaufman (2008), leaf-associated Alphaproteobacteria in tree holes decreased with larval presence. Within this group, Caulobacteraceae decreased with larval density. The Porphyromonadaceae within the Bacteroidetes phylum, also fluctuated in response to mosquito density. As in Kaufman et al. (2008) the abundance of this group decreased with mosquito feeding pressure;

however, in the current study the effect was observed on container wall tiles rather than leaf surfaces.

In contrast to the variability of bacterial groups at the class level in tree holes, all substrates in tire communities and leaf material in tire microcosms were dominated by Gammaproteobacteria, with little apparent response to mosquito feeding. Unlike other Proteobacteria, Gammaproteobacteria tend to be less abundant in freshwater habitats (Kirchman 2002). This difference suggests that the two communities may be differentiated, from the mosquito point of view, with respect to the quality of microbial food resources available. It has been observed in microcosm studies that some Gammaproteobacteria, the Enterobacteriaceae, may increase in the presence of mosquito larvae, and that this group may be more resistant to digestion given its regular colonization of animal guts (Kaufman et al. 1999). Betaproteobacteria were absent from each of the habitats samples, which is unexpected given the preponderance of this group in aquatic habitats and previous tree hole studies (Kirchman 2002, Kaufman 2008).

The relatively high frequency of 18S rRNA gene sequence in tire habitats classified only to the level of Fungi suggests a preponderance of unique species compared with tree hole habitats. Sodariomycetes were highly represented among each container type. Within the Sodariomycetes, Diaporthales was the most represented order, a fact that is unsurprising due to the utilization of freshwater habitats by members of this group (Samuels and Blackwell 2001; Zhang and Sung 2008). Breakdown of plant detritus, particularly lignin, is a function ascribed to several members of this group; hence, their abundance in detritus-based habitats such as tires and tree holes is consistent with the function expected of microbial community members.

The cumulative variation explained by principle components 1-3 was much lower in our field community samples compared with those taken from microcosms. More variation is expected in natural systems than in controlled laboratory experiments; however, another factor that may have contributed to this difference is the addition of a single microbial inoculum in microcosms at the onset of the experiment. In contrast to field tree holes, new allochthonous sources of microbial groups were generally not available from the surrounding environment. Although large introductions were precluded by screen enclosures, it is assured that new microbial introductions into field containers (e.g. windborne or soil-associated) contributed to a higher overall variability in these systems that was independent of the imposed factors.

Although temporal flux was highly evident in fungal communities on tree hole container surfaces, the container walls of tire habitats did not exhibit similar community dynamics. The difference between materials comprising the container walls cannot be overlooked as a critical factor that may contribute to the species-specificity of container surfaces. Indeed, in the current study, we were unable to obtain sufficient fungal material from tiles in microcosms representing tire container wall surfaces to proceed with fungal community analyses on this substrate. Bark-lined tree holes represent an additional source of decaying organic matter for mosquitoes residing in these habitats. Although characterized by a high C:N ratio, and thus a relatively recalcitrant source of organic carbon compared with other forms of tree hole detritus, bark-lining in tree holes provides an important niche for fungal growth that is absent in tire habitats. Such inherent difference may explain the success of *Oc. triseriatus* in tree holes, or at least suggest a

possible basis for which they may be outcompeted by other mosquito species in tire habitats.

It has become increasingly clear from recent research that fungi play a critical role in driving *Oc. triseriatus* production (Kaufman et al. 2008, Xu et al. 2008, Pelz-Stelinski et al. unpublished). Scirtid beetle larvae, which often co-occur with *Oc. triseriatus* in tree holes, are known to facilitate the production of mosquito adults (Bradshaw and Holzapfel 1992; Paradise 1999, 2000; Daugherty and Juliano 2000, 2001, 2002). Recently, we have shown that changes in the structure of fungi on leaves and in the water column occur in the presence of scirtid beetles (Pelz-Stelinski et al. unpublished). Any contribution to fungal activity, be it from the presence of macroinvertebrate facilitators or increases in available fungal niche space, should result in enhanced mosquito success. In spite of this, we have not discovered any evidence that would indicate specific fungal groups are consistently selected by foraging larvae. Rather, fungi appear to be a general resource that is differentially available to mosquitoes with respect to substrate. Tire and tree hole habitats are distinct with respect to their the structure of fungal communities, but perhaps also in terms of the biomass they support. Although we did not quantify fungal biomass or activity in this study, it is apparent that fungi were less available on container walls associated with tire habitats. Future studies should address the relative contribution of fungal biomass to resource pools in tire habitats as a possible factor regulating mosquito populations.

CHAPTER 4

MULTITROPHIC INTERACTIONS IN TREE HOLE COMMUNITIES ARE FACILITATED BY SCIRTID BEETLES

Introduction

Tree holes are small, discrete ecosystems that contain heterotrophic communities driven by allochthonous inputs of soluble and particulate organic matter. In Eastern North American tree holes, larvae of the Eastern treehole mosquito, *Ochlerotatus triseriatus* (Say), are usually the dominant macroinvertebrate consumers. Leaves are a typical source of coarse particulate organic matter (CPOM), but animal-derived detritus, such as invertebrate carcasses and fecal material, also supply energy to these systems (Daugherty et al. 2000, Yee and Juliano 2006). In addition, stemflow runoff brings dissolved organic carbon and nutrients into the habitats (Carpenter 1982, Walker et al. 1991, Kaufman et al. 1999, 2002). The success of *Oc. triseriatus* and similar mosquito species is defined by high adult productivity and body weight at emergence, and this depends on the nutrition they receive while in the larval stage. Microorganisms are the main nutritional resource for mosquito larvae, which feed by browsing on container surface microbial biofilms and filtering FPOM and microorganisms from the water column (Merritt et al. 1992). Fungi associated with leaf detritus alone may account for around 10% of the detrital biomass in tree hole habitats (Kaufman et al. 2002, 2006). Hence, microbial degradation of detritus is a critical link between developing larvae and nutrients, as processing by microorganisms incorporates nutrients and otherwise inaccessible carbon from CPOM.

In the Midwestern and Eastern United States, larval scirtid beetles (*Helodes* and *Prionocyphon* spp.) are shredders which often co-occur with *Oc. triseriatus* in tree holes (Barrera 1996; Paradise 2004). Scirtid feeding activity results in the skeletonization of leaf detritus and the associated conversion of coarse particulate organic matter (CPOM) to fine particulate organic matter (FPOM) in the form of small leaf particles and feces.

Several studies indicate that the presence of these beetles in tree holes conditionally facilitates the survival and development of *Oc. triseriatus* under conditions of low leaf litter availability by improving the quality of resources available to *Oc. triseriatus* (Bradshaw and Holzapfel 1992; Paradise 1999, 2000; Daugherty and Juliano 2000, 2001, 2002). In addition to mosquito populations, processing chain benefits have been reported to facilitate populations of the ceratopogonid midge *Culicoides guttipennis* in the presence of Helodid beetles (Paradise and Dunson 1997). Similarly, processing chain commensalisms benefiting mosquitoes have been reported in pitcher plants. By increasing the conversion of coarse particulate plant material to particulates, the midge *Metriocnemus knabi* facilitate populations of the mosquito *Wyeomyia smithii* in pitcher plants (Heard 1994). Increased conversion of leaf material into FPOM is the main mechanism cited for any benefit experienced by mosquitoes in the presence of scirtids (Paradise 1999, 2000; Daugherty and Juliano 2002, 2003). Indeed, Daugherty and Juliano (2003) demonstrated that additions of scirtid fecal material and its associated microbiota to microcosms increased larval *Oc. triseriatus* development by providing additional nutrient resources; however, the source of this positive effect (feces, microbiota, or a combination) has not been determined. Furthermore, although the conversion of CPOM into FPOM may benefit foraging larvae by being ingested directly, it may also promote access to fungal material otherwise embedded in the leaf matrix.

Previous studies have addressed larval mosquito feeding on microorganisms in container habitats, but microbial food resource dynamics remain ill-defined. Water-column-associated bacteria exhibit negligible or inconsistent responses to mosquito presence and, thus far, there have not been any published studies of fungal communities

associated with tree hole water columns. Furthermore, larval feeding effects on the composition of water column microbial communities are limited to bacteria and protists (Kaufman et al. 1999, 2002, Cochran-Stafira and von Ende 1998, Kneitel and Miller 2002, Tryzcinski et al. 2005). In contrast, there is convincing support for the importance of microbial communities associated with detritus in previous assessments of tree holes. Increased fungal enzyme activity, and decreased bacterial productivity and abundance are associated with mosquito feeding (Kaufman et al. 1999, 2001, 2002, Kaufman and Walker 2006). Despite the apparent response of microorganisms to larvae, the composition of microbial communities in tree holes has been addressed only recently (Kaufman et al. 2008, Xu et al. 2008). In that study, larval feeding effects were evident on the taxa comprising fungal and bacterial communities associated with leaf detritus, with particular influence on Saccharomycetes, Dothideomycetes, and Chytridiomycota fungal taxa, and on Alpha- and Betaproteobacteria. It is apparent that any influence of scirtids on the microbial community may affect mosquitoes, given the direct utilization of microorganisms by larvae for nutrient acquisition. By altering the detritus, scirtid beetles modify the bottom-up influence of detritus on bacterial and fungal populations. Such blending of "bottom-up" influences and "top-down" trophic cascades are described for aquatic ecosystems, including tree hole container habitats, yet the ramifications of scirtid beetles on microorganisms is poorly understood (Carpenter and Kitchell, 1987,1988).

Although previous studies have postulated mechanisms for facilitation of mosquito larvae by scirtids, they are limited to measurements of macroscopic changes such as the abundance of FPOM, population densities of mosquitoes, and decomposition of scirtid carcasses (Paradise 1999, 2000; Daugherty and Juliano 2002, 2003). While all

of these factors play a role in mosquito growth, these indirect assessments lack the intermediate microbial step linking the trophic levels. Elucidating the effect of scirtid feeding activity on mosquito productivity requires an assessment of the changes such feeding renders on the microbial community. In an effort to understand the ecological influences driving mosquito production, we sought to describe microbial mechanisms underlying facilitation of larval *Oc. triseriatus* growth and development by scirtid beetles via assessments of the microbial community associated with scirtid presence in microcosms simulating natural tree holes. We postulated that detritus-associated fungal dynamics would be the most dramatically affected by scirtid presence as this location is the "center of activity" for foraging scirtid larvae (Dixon and Chapman 1980).

Materials and Methods

Experimental design. The mechanism regulating the facilitation of *Oc. triseriatus* larvae by scirtid beetle larvae was investigated by crossing beetle presence/absence with mosquito presence/absence in a multi-factorial design with the following scirtid: *Oc. triseriatus* ratios: 0:0, 10:0, 10:40, 0:40. Twelve replicates of each treatment combinations were constructed, with one set of six replicates destructively sampled midway through the experiment (ca. day 30). Because resource level mediates the interaction between these species such that facilitation is only apparent at low resources, low leaf litter rations were used for all replicate microcosms.

Individual microcosms simulating natural tree holes received a 1 g senescent oak leaf pack in 500 ml deionized water. Microcosms were constructed similarly to those described in previous tree hole studies (Walker et al. 1991, Kaufman et al. 2001, 2006). A

3 ml microbial inoculum, consisting of homogenized tree hole water and particulates, was added to microcosms three days prior to the addition of macroinvertebrates.

Oc. triseriatus larvae, obtained from our colony at Michigan State University, were added as newly-hatched first instar. Scirtid beetle larvae, ranging from 2nd to 4th instar, were obtained from local tree holes (Toumey Woodlot, E. Lansing, MI) and replaced semi-weekly as necessary to maintain a constant population in scirtid treatments. Dead beetles were removed to avoid confounding the effect of scirtids by providing additional resources to mosquito or microbial populations. Due to the difficulty of identifying live beetle larvae, scirtids were not identified to species (Paradise and Dunson 1997; Daugherty and Juliano 2001, 2003).

Sampling. Leaf condition was assessed by taking dry weight measurements of leaf discs on day 30 and day 50. Leaf disc samples (20 mm diam.) were procured from each microcosm on day 30 and day 50 using a cork borer. Two leaf samples apiece were taken for assessing bacterial abundance, bacterial productivity, fungal biomass, fungal degradation enzymes and microbial community structure. Prior to analyses, leaf disc samples were sonicated for 12 min in an ice bath to obtain the loosely attached, surface-associated microorganisms, as these better represent those encountered by foraging mosquito larvae (Kaufman 2008). In addition to leaf samples, water samples were also taken from microcosms on each sample date. After stirring microcosms to homogenize water and particulates, 15 ml samples were removed from microcosms for the analyses indicated above.

Bacterial Abundance and Productivity. Bacterial abundance on the leaf surface and in the water column was quantified from subsamples via direct microscopic counts

(DMCs) of bacteria using the DAPI (49,6-diamidino-2-phenylindole) fluorescent staining procedure (Porter and Feig 1980, Walker et al. 1988). Formalin (3% formaldehyde final concentration)-preserved samples were kept at under dark conditions at 4°C until analysis, then filtered onto black Nucleopore filters (0.2-mm pore size; Costar, Cambridge, Mass). After filtering, samples were stained at a final concentration of 20 ug/ml for 5 min. Two filters were counted (200 cells per filter) for each subsample at 1000X using a Nikon E800 fluorescent microscope (Nikon, Inc. Melville, NY).

Bacterial productivity was estimated by the incorporation of [^{3}H]- leucine (50 Ci/mmol, Life Science, Boston, MA) into bacterial biomass using the microcentrifuge tube method (Smith and Azam 1992, Kirchman 2001). Labeled leucine was added at a final concentration of 25 nM. It was determined previously that saturation of uptake kinetics occurred at 100 and 400 nM for water and leaf samples, respectively (Kaufman 2001, 2002, 2006). Samples were incubated for 30 min at 20°C to allow for the incorporation of labeled leucine into bacterial biomass. Following incubation, 5% TCA (trichloroacetic acid) was added to terminate the reaction and precipitate protein. Samples were subsequently rinsed and concentrated as described in prior microcosm studies (Kaufman 2001, 2006). Standard liquid scintillation counting of samples was conducted in a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Fungal Biomass. Fungal biomass was estimated using surrogate measurements of ergosterol, a sterol associated with fungal cell walls (Newell and Barlocher 1993, Suberkropp and Weyers 1996). Leaf disc subsamples sonicated as described above in sterile microcosm water were kept in the dark and stored in high performance liquid chromatography (HPLC) grade methanol prior to extraction and quantification of

ergosterol using HPLC and UV detection (Kaufman et al. 2001, Kaufman and Walker, 2006).

Enzyme Activity. Leaf enzyme activity was assayed through incubations of leaf disc subsamples with two methylumbelliferyl (MUF)-labeled substrates, 4-methylumbelliferyl-β-D-cellobioside and 4- methylumbelliferyl- β-D-xyloside, which will respectively estimate cellobiohydrase, and xylosidase activity. These substrates are analogous to plant polymers and thus provide an estimate of leaf-associated carbohydrase activity (Kaufman and Walker 2006). MUF was liberated upon enzymatic cleavage of the substrates during a 1.5 h incubation at 22°C. Unbound MUF, which fluoresces at 360 nm, was measured using a 96-well Hoefer DyNA Quest 200 fluorometer (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Microbial Community Analysis. The compositions of leaf- and water column-associated bacterial and fungal communities in microcosms were assessed using terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu 1997, Marsh 1999). Leaf disc samples were sonicated as described above in sterile phosphsate buffered saline to dislodge surface- associated microorganisms. Thereafter, DNA was isolated from leaf sonicate and water samples using the MoBio UltraClean soil extraction kit (Carlsbad, CA). Independent amplification of DNA for T-RFLP analysis were performed with two primers sets respectively targeting small subunit (SSU) ribosomal DNA from bacteria (16S rDNA) and fungi (18S rDNA). A 1300 bp 16S rDNA fragment was amplified using universal eubacterial primers 63F and 1387R (5'-CAGGCCTAACACATGCAAGTC-3' and 5'-GGGCGGWGTGTACAAGGC-3', respectively) (Marchesi et al. 1998). A 762 bp 18S rDNA fragment was amplified using universal fungal primers nu-SSU-0817-59F

and nu-SSU-1536-39R (5'-TTAGCATGGA ATAATRRAATAGGA-3' and 5'-ATTGCAATGCYCTATCCCCA-3', respectively) (Borneman and Hartin 2000). Forward primers for each target sequence were fluorescently labeled (6-FAM) at the 5' end (IDT Technologies). Template DNA from fungal and bacterial communities was amplified in three 100 ul PCR reactions per primer set using the Failsafe™ PCR System (Epicentre Biotechnologies, Madison, WI). Each PCR reaction consisted of ~10 ng template DNA, 50 ul Failsafe™ PCR PreMix buffer E, 1 ul Failsafe™ enzyme, and 4 ul of each DNA primer. Reactions were held at 94°C for 2 min, then amplified under PCR cycle conditions optimized for each target sequence. For 16S rDNA, reaction mixtures were cycled 30 times through the following steps: denaturing for 45 s at 94°C, annealing for 30 s at 68°C, and extension for 1 min 30 s at 72°C. For 18S rDNA, reaction mixtures were cycled 30 times through the following steps: denaturing for 45 s at 94°C, annealing for 30 s at 58°C, and extension for 1 min at 72°C. Both reactions were subjected to a final extension step for 7 min at 72°C. PCR products from triplicate reactions were co-purified with the Qiaquick™ PCR purification kit (Qiagen, Valencia, CA) and then were subjected to independent digestions with two restriction enzymes, MspI and HhaI (New England Biolabs, Cambridge, MA) (200 ng product with unit of Hha or Msp for 3 h at 37°C) then incubated overnight with ethanol at -80°C to remove excess salts. The size and frequencies of each labeled fragment was determined by capillary electrophoresis of digested samples with the PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical Analysis. Multivariate analysis of variance (MANOVA) was used to analyze related mosquito productivity variables, including total adult mass, total survival,

and total number of adults emerged (SAS v. 9.1, SAS Institute, Inc., Cary NC, USA). Values were log transformed prior to analysis. Water chemistry values, bacterial abundance, bacterial productivity, leaf enzyme activity, ergosterol content, and leaf mass lost were analyzed using a standard analysis of variance (ANOVA) followed by Bonferroni correction to control the experiment-wide error (Rice 1989). In addition, means were square-root transformed as needed to meet the assumptions of ANOVA. In addition to the main treatment affect, the effect of experimental block on response variables was also assessed. Upon finding that blocking was significant, individual blocks were treated separately to determine the within-block treatment effects.

T-RFLPs were aligned and peak areas were determined using the GeneScan software package (Applied Biosystems). A sample was discarded if its summed peak area was less than 1000 or if a normality plot indicated the presence of significant outliers. In addition, peaks that comprised <3% of the community or appeared in fewer than three samples were also discarded. Common peaks were binned using the "T-RFLPs Stats" analysis tools (iBest http://www.ibest.uidaho.edu/tools/trflp_stats/index.php) REF. The resulting matrices were converted to log ratios of the relative abundance of peak area in each sample, then analyzed by principle component analysis (PCA) on the covariance matrix using JMP® Statistical Discovery Software (SAS Institute, Inc., Cary NC, USA).Scores from PC's 1-3 and transformed percentages of TRFLP fragments were subjected to ANOVA for treatment comparisons.

Results

Adult mass, emergence, development time, and total survival of mosquitoes did not differ significantly in microcosms containing scirtids compared with those in which

scirtid beetles were absent and the lack of effect was consistent from day 30 to day 50 (Figure 4.1; MANOVA: Wilks' Lambda = 0.78, df = 5,16, $p = 0.49$). In contrast, leaf decomposition was significantly affected by the presence of sciritids in microcosms ($F = 11.96$; df = 1, 23; $p = 0.003$), such that microcosms containing scirtids had a significantly lower proportion of leaf mass remaining than non-scirtid microcosm (Figure 4.2; Tukey's HSD, $P < 0.05$). Neither mosquito presence nor the interaction between the insects significantly affected leaf decomposition ($F = 0.69$; df = 1, 23; $p = 0.417$; and $F = 0.0$; df = 1, 23; p = 0.962, respectively).

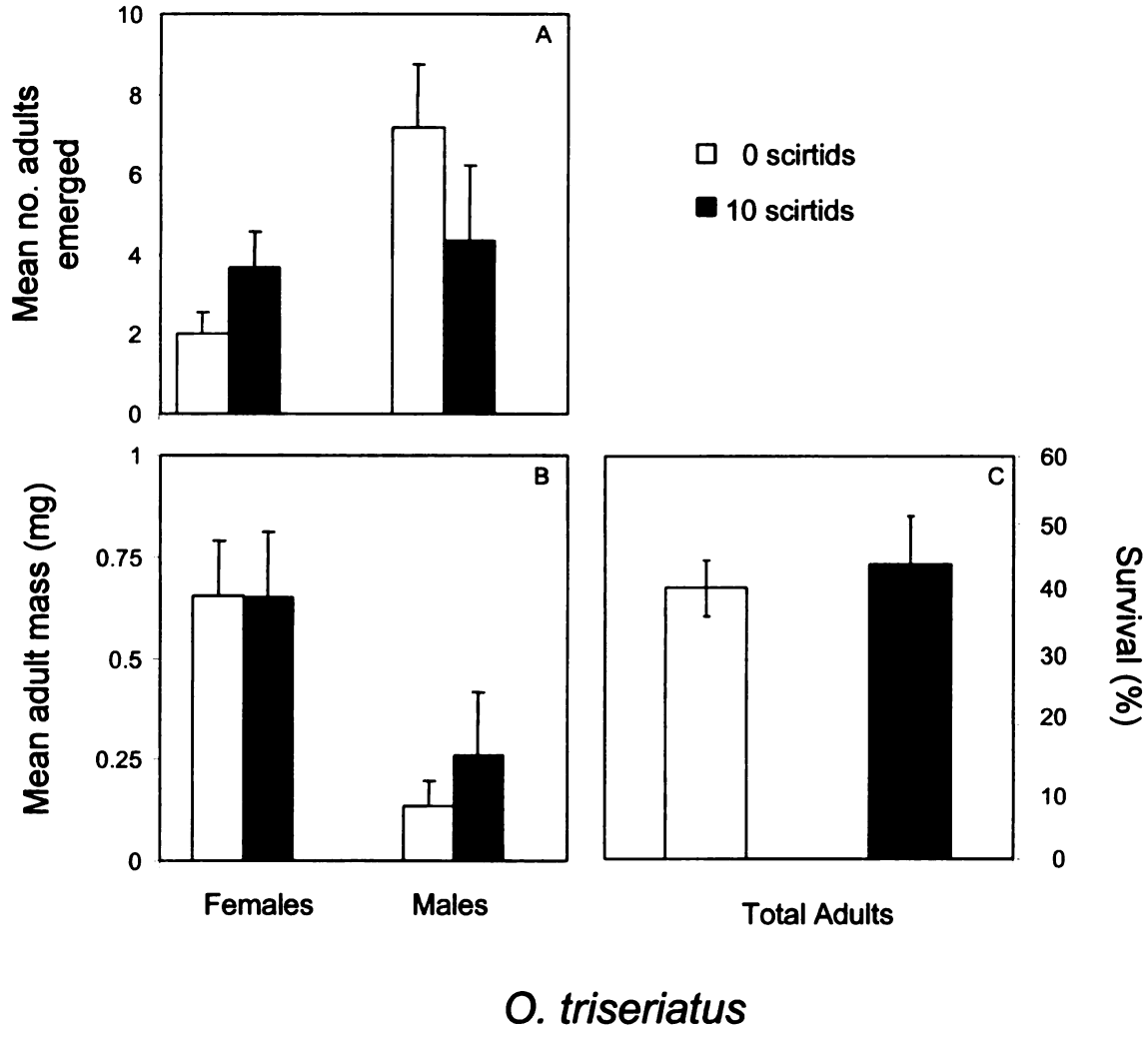

Figure 4.1. Mosquito production variables from microcosms containing 40 *Oc. triseriatus* larvae. (A) Number of adults (B) Total adult mass. (C). Survival (40 initial larvae). open characters represent microcosms with no scirtids filled characters represent 10 scirtids. Values are means ± SE; $n = 12$ all variables.

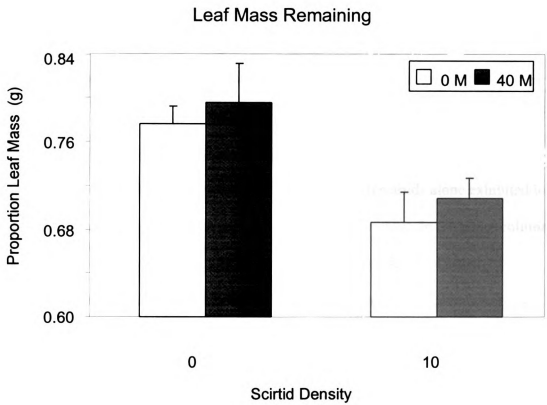

Figure 4.2. Proportion of leaf mass remaining in microcosms on day 50. S: Scirtid, M: Mosquito, white bars: 0 M, grey bars : 10 M. Values are means ± SE (N=24).

Bacterial abundance on leaf surfaces was depressed in the presence of macroinvertebrates (Table 4.1 and Figure 4.3). Under these reduced-resource conditions, mosquito larvae significantly lowered bacterial abundance compared with no larvae conditions; however, scirtid presence scirtid presence significantly interacted with mosquito presence at day 30 such that microcosms containing both insects had a greater abundance of leaf-associated bacteria than those containing mosquito larvae alone (Tukey's HSD test, $P < 0.05$). Although scirtid beetles did not significantly decrease bacterial abundance at day 30, by day 50 microcosms with scirtids alone exhibited lower abundance than microcosms with no macroinvertebrates present. In the water column, bacterial abundance was not significantly affected by the presence of either macroinvertebrate, although a significant decrease in abundance did occur from day 30 to day 50.

Leaf associated bacterial productivity was significantly lower in the presence of mosquito and scirtid beetle larvae compared with the no macroinvertebrate treatment (Table 4.1 and Figure 4.3). Productivity differences were not apparent among microcosms receiving macroinvertebrates, likely in response to the significant interaction between mosquito larvae and scirtid beetles. Significant reductions in water column bacterial productivity were evident in the presence of mosquito larvae and productivity remained unchanged whether scirtids were present or absent. In addition, water column productivity also declined significantly from day 30 to day 50 for microcosm treatments.

The activity of leaf decomposition enzymes for the substrates xylan and cellobiose significantly increased in microcosms containing scirtids compared with microcosms in which scirtid beetles were absent (Table 4.2 and Figure 4.4). This effect

was neither significantly influenced by time nor the presence of mosquito larvae in microcosms. In contrast, although fungal biomass (ergosterol concentration) in microcosm leaf samples was numerically greater in microcosms containing scirtids this effect was not statistically significant. Fungal biomass was not significantly influenced by the presence of either *Oc. triseriatus* or scirtid larvae; however, the concentration of ergosterol was significantly greater for all treatments on day 50 compared to day 30 (Table 4.2 and Figure 4.4).

Table 4.1. Summary of ANOVA results for bacterial abundance and bacterial productivity values from microcosm water column and leaf material.

Source	df	*F* Value	*P* Value
Water column abundance (cells/ml)			
Mosquito	1	0.15	0.699
Scirtid	1	0.69	0.410
Time	1	13.88	0.001*
Mosquito*Time	1	0.00	0.975
Scirtid*Time	1	0.01	0.907
Mosquito*Scirtid	1	0.79	0.379
Error	37		
Leaf surface abundance (cells/disc)			
Mosquito	1	23.62	<0.001*
Scirtid	1	0.83	0.368
Time	1	0.5	0.483
Mosquito*Time	1	1.7	0.200
Scirtid*Time	1	6.89	0.012*
Mosquito*Scirtid	1	6.61	0.014*
Error	40		
Water column productivity (nmol leucine /ml/h			
Mosquito	1	9.37	0.004*
Scirtid	1	0.14	0.712
Time	1	12.91	0.001*
Mosquito*Time	1	0.8	0.378
Scirtid*Time	1	0.7	0.406
Mosquito*Scirtid	1	0.25	0.617
Error	40		
Leaf surface productivity (nmol leucine /disc/h)			
Mosquito	1	14.77	0.001*
Scirtid	1	6.51	0.016
Time	1	2.35	0.135

Table 4.1 (cont'd).

Mosquito*Time	1	2.04	0.163
Scirtid*Time	1	1.39	0.247
Mosquito*Scirtid	1	17.41	<0.001*
Error	32		

*Indicates p-values that are significant following Bonferroni adjustment

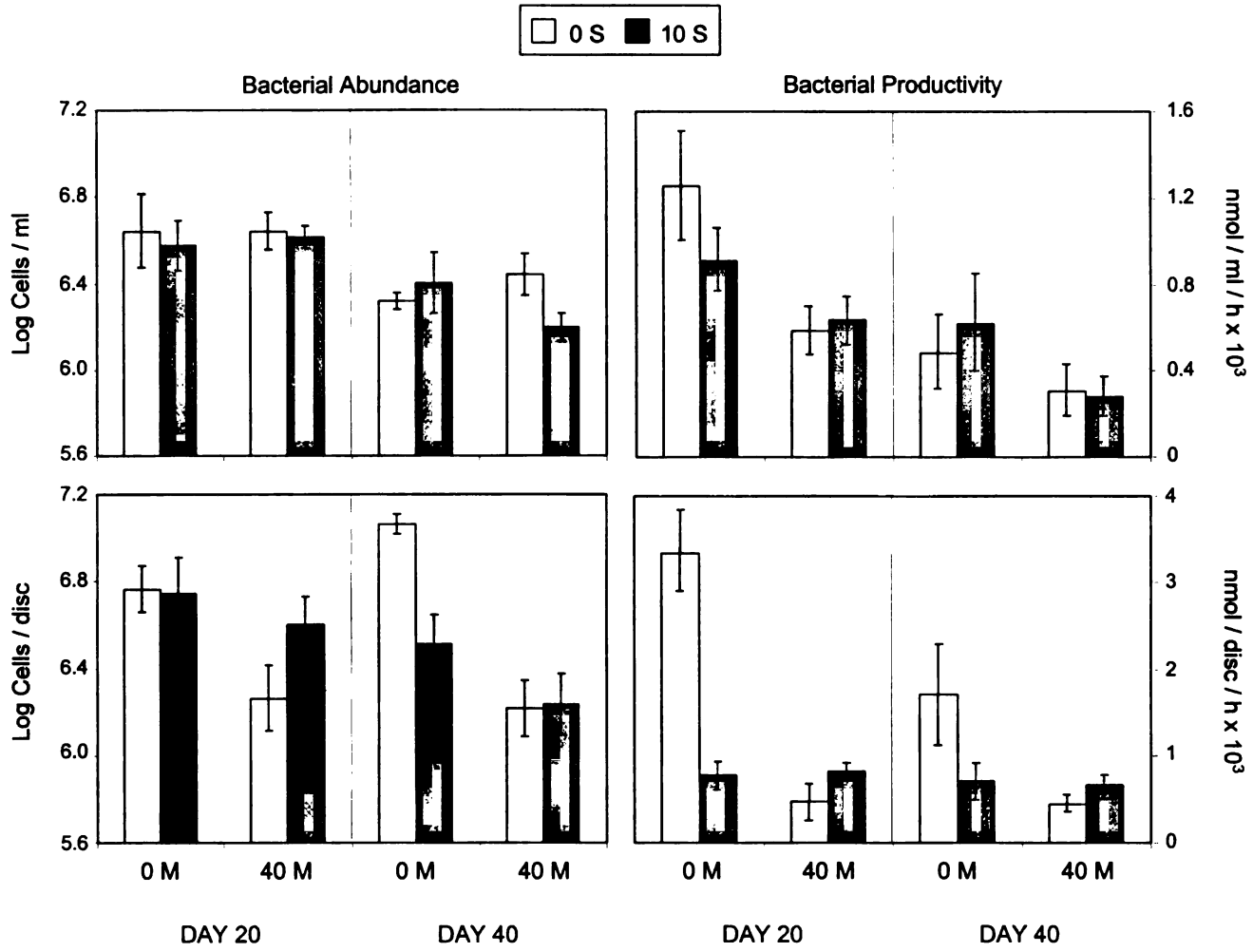

Figure 4.3. Bacterial production and abundance. S: Scirtid, M: Mosquito, white bars: 0 S, grey bars: 10 S. Values are means ± SE (N=24).

Table 4.2. Summary of ANOVA results for ergosterol and fungal degradation enzyme concentrations in microcosm leaf material.

Source	df	*F* Value	*P* Value
Ergosterol (ppm / leaf disc)			
Mosquito	1	2.04	0.162
Scirtid	1	0.61	0.439
Time	1	12.84	0.001*
Mosquito*Time	1	0.88	0.3558
Scirtid*Time	1	0.88	0.353
Mosquito*Scirtid	1	0.92	0.344
Error	36		
Xylose polymer activity (ppm/disc/h)			
Mosquito	1	0.40	0.533
Scirtid	1	9.96	0.003*
Time	1	0.16	0.691
Mosquito*Time	1	0.04	0.839
Scirtid*Time	1	0.38	0.542
Mosquito*Scirtid	1	0.02	0.884
Error	40		
Cellobioside polymer activity (ppm/disc/h)			
Mosquito	1	0.03	0.871
Scirtid	1	10.66	0.002*
Time	1	0.48	0.494
Mosquito*Time	1	0.0	0.969
Scirtid*Time	1	0.11	0.740
Mosquito*Scirtid	1	1.25	0.269
Error	40		

*Indicates p-values that are significant following Bonferroni adjustment

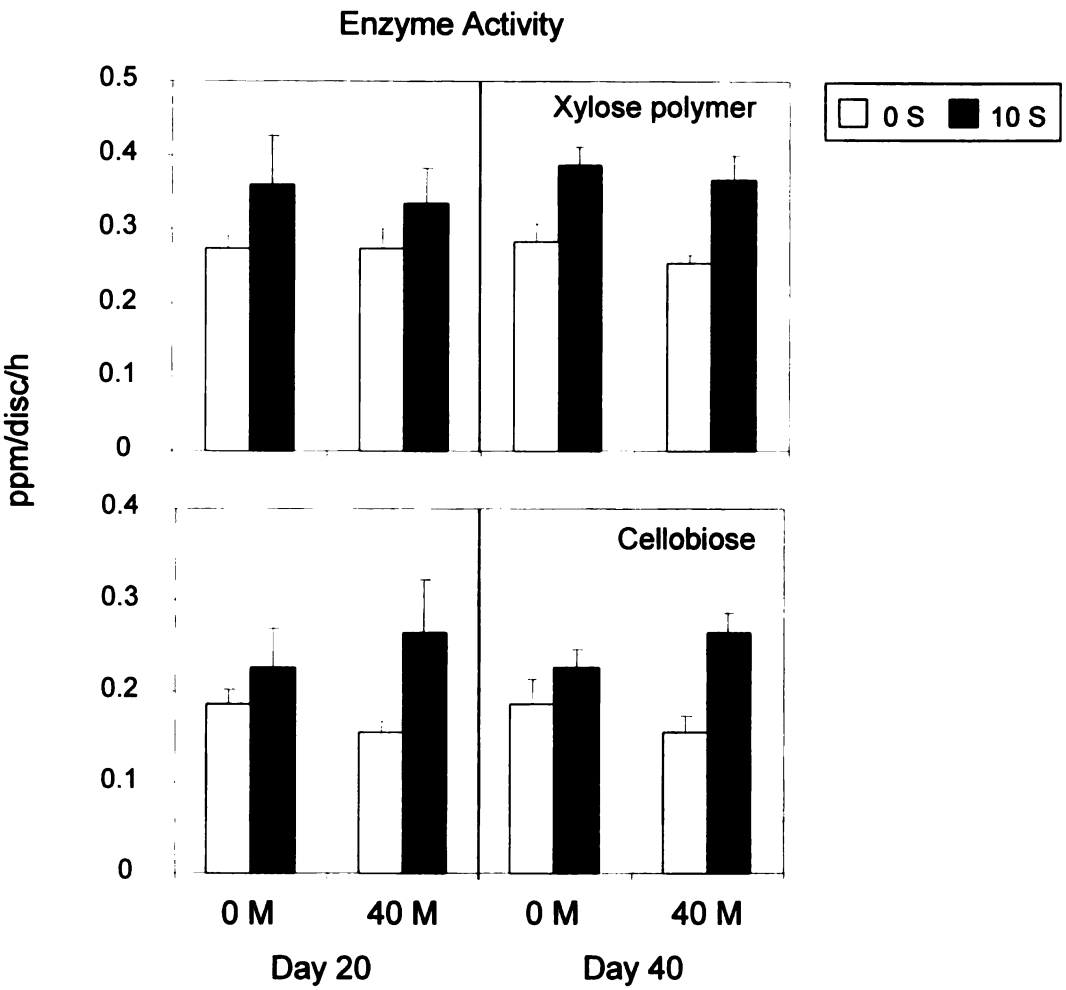

Figure 4.4. Fungal enzyme activity. S: Scirtid, M: Mosquito, white bars: 0 S, grey bars : 10 S. Values are means ± SE (N=24).

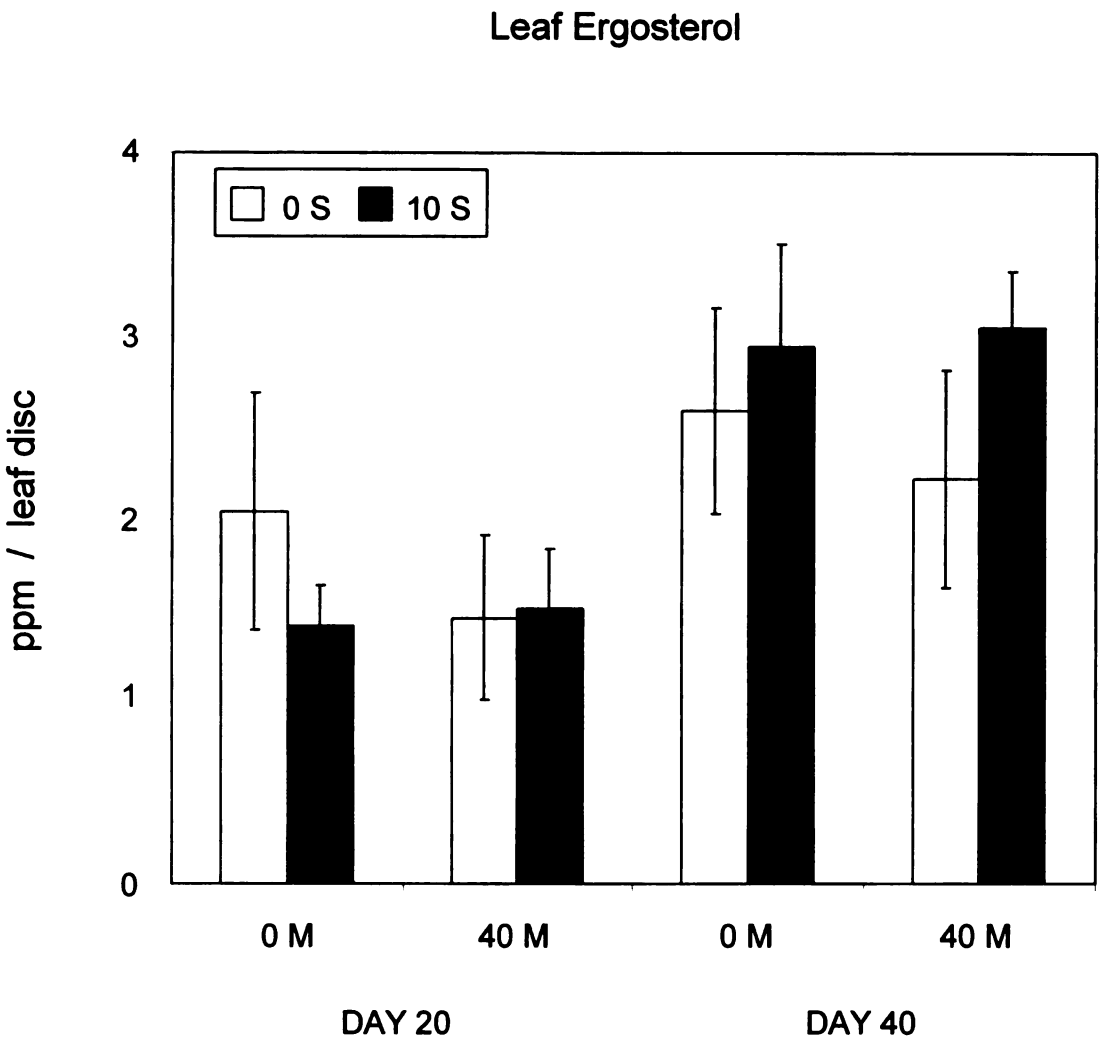

Figure 4.5. Fungal biomass and enzyme activity . S: Scirtid, M: Mosquito, white bars: 0 S, grey bars : 10 S. Values are means ± SE (N=24).

Total nitrogen in microcosms was significantly greater on day 50 than on day 30 (Tukey's HSD, $P = 0.004$), but was not significantly affected by either mosquito of scirtid presence ($F = 1.48$; df = 1, 40; $p = 0.23$; and $F = 1.53$; df = 1, 40; $p = 0.223$, respectively). Total phosphorus in microcosms was not significantly affected by the

presence of either macroinvertebrate in microcosm ($F = 1.27$; df = 4, 43; $p = 0.3$). In addition, the Block effects were not significant for N ($F = 0.67$; df = 1, 32; $p = 0.41$) or P ($F = 0.01$; df = 1, 32; $p = 0.94$)

T-RFLPs

MSP. PC1 scores for bacteria in the water column were significantly affected by time (Blocks I and II), exhibiting shifts in the community structure from day 20 to day 45 (F= 5.86, df = 1, 10, $P = 0.001$; F= 14.03, df = 1, 15, $P = 0.002$) (Figure 4.6; complete ANOVA tables are shown in Appendix). Scirtids also had a significant effect on bacterial communities along PC1 time in Block II (F= 16.93, df = 1, 15, $P < 0.001$, but not in Block I. PC2 scores were not significantly affected by treatments in Block I; however, in Block II, both time and mosquito presence significantly affected water column bacteria along PC2 ($F = 53.23$, df = 1, 15, $P < 0.001$; $F = 27.90$, df = 1, 15, $P < 0.001$). Both mosquito presence and time significantly affected PC1 scores for leaf surface bacterial communities in Block II ($F = 41.17$, df = 1, 15, $P < 0.001$; $F = 8.04$, df = 1, 15, $P = 0.012$), but the same effect was not observed in Block I. Neither insect presence nor time significantly affected on PC2 in either block.

Water column fungal communities exhibited significant shifts in response to mosquito presence and time along PC2 (Block II: $F = 37.95$, df = 1, 15, $P < 0.001$; F= 13.23, df = 1, 15, $P = 0.002$) (Figure 7). For both blocks, there were no significant effects of any factor on PC1. Similarly, a shift in leaf-associated fungal communities in response to mosquito presence was significant along PC2 in Block II of the experiment ($F = 6.19$, df = 1, 16, $P = 0.024$). In general, however, no significant shifts were evident in leaf fungal communities across the experiment in response to time or scirtid presence.

HHA. PC1 scores for bacteria in the water column were significantly affected by scirtids in Block I ($F = 21.06$, df = 1, 11, $P = 0.001$). PC 2 scores representing water bacterial communities were significantly affected by the interaction between mosquitoes and scirtids (Block I: $F = 12.94$, df = 1, 11, $P = 0.004$) or mosquitoes alone (Block II: $F =$ 6.19.67, df = 1, 15, $P = 0.01$). Leaf surface bacterial communities represented by principal component axes were significantly affected by the interaction of scirtids and time (PC2, Block I: $F = 6.63$, df = 1, 14, $P = 0.022$) or by scirtids alone (PC1, Block II: F = 5.09, df = 1, 15, $P = 0.039$).

Water column fungal communities changed in response to mosquito presence (PC1, Block I: $F = 10.02$, df = 1, 7, $P = 0.016$), and the interaction of mosquito presence and time (PC2, Block II: $F = 11.45$, df = 1, 16, $P = 0.004$). Scirtids did not significantly affect changes in leaf-associated fungal communities, although these communities changed in response to the presence of mosquitoes (PC2: $F = 45.52$, df = 1, 15, $P <$ 0.001) and time (PC1: $F = 24.29$, df = 1, 15, $P < 0.001$; PC2: $F = 8.65$, df = 1, 15, $P =$ 0.010) in Block II.

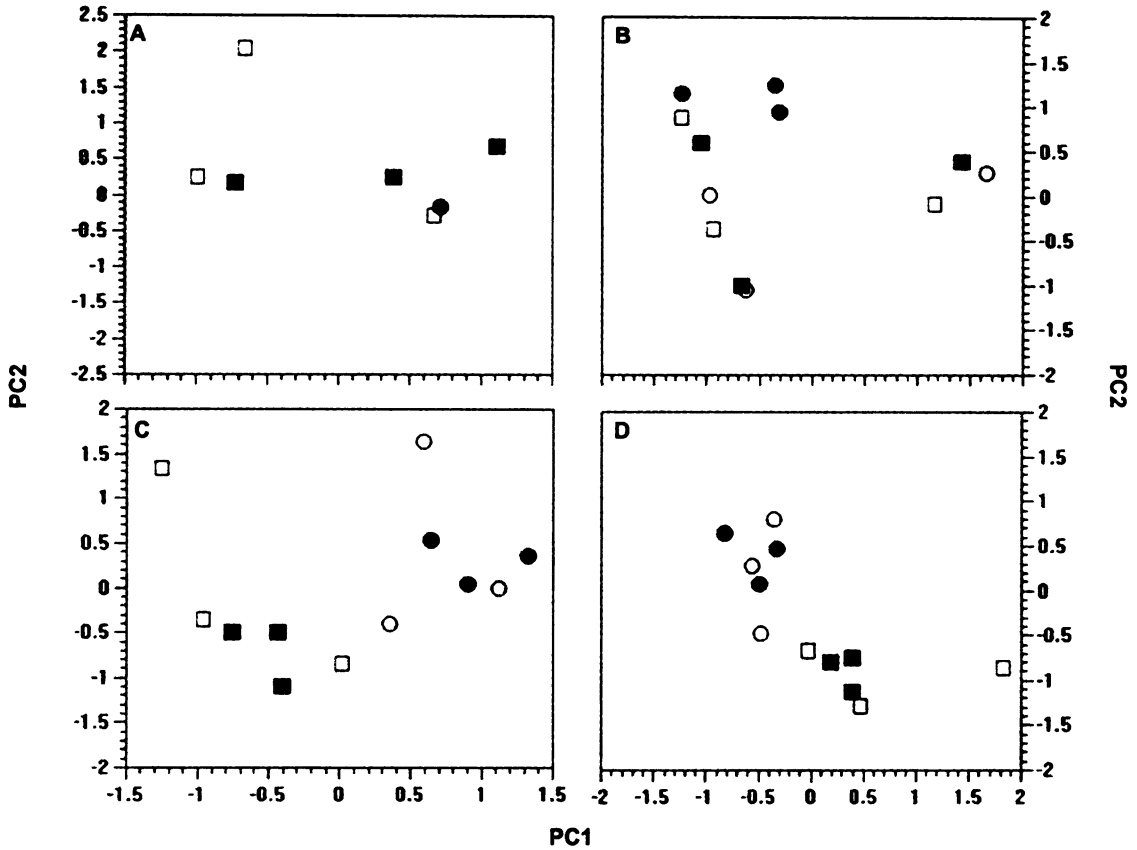

Figure 4.6. PCA ordination of bacteria peak area data for leaf (A and B) and water (C and D) samples taken from Block I (A and C) and Block II (B and D) microcosms on day 20. The amount of variation explained by each PC is indicted on the axes for each panel. Treatments are represented by the following symbols: open, 0 scirtids; filled, 10 scirtids; squares, 0 mosquitoes; circles, 40 mosquitoes.

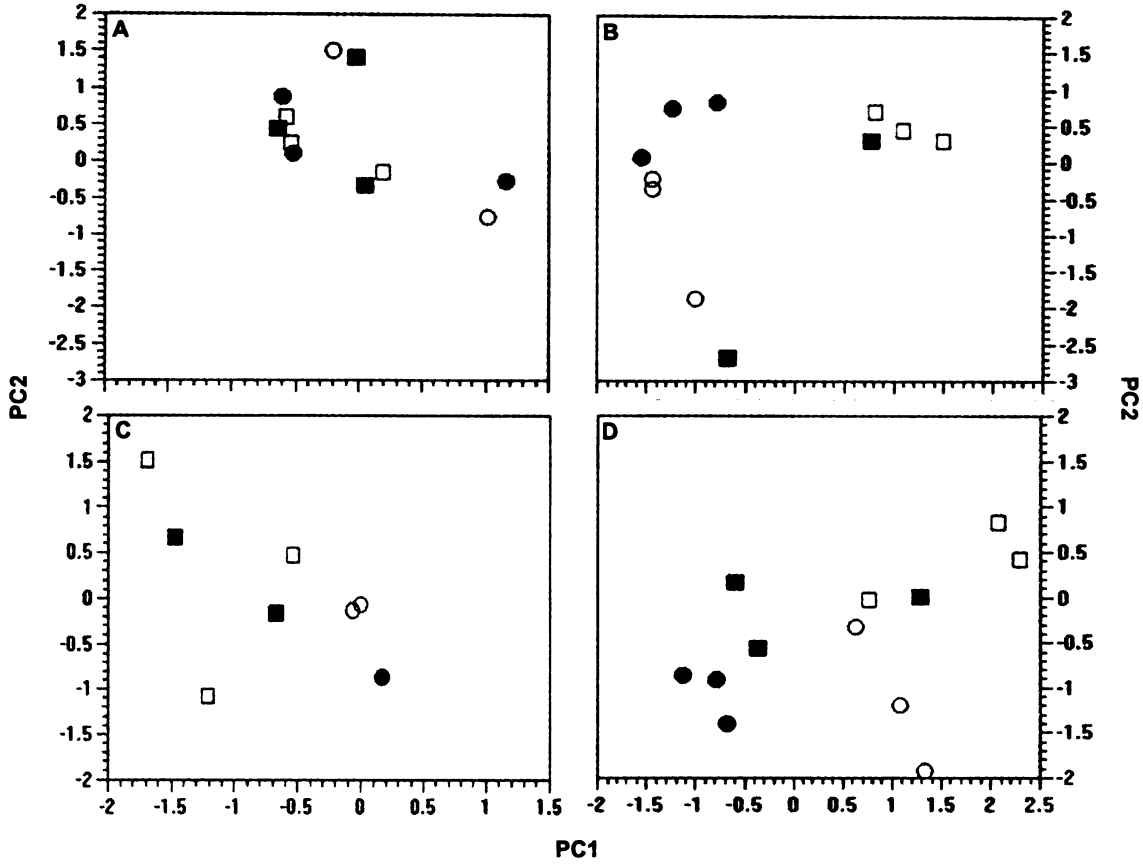

Figure 4.7. PCA ordination of fungal peak area data for leaf (A and B) and water (C and D) samples taken from Block I (A and C) and Block II (B and D) microcosms on day 20. The amount of variation explained by each PC is indicted on the axes for each panel. Treatments are represented by the following symbols: open, 0 scirtids; filled, 10 scirtids; squares, 0 mosquitoes; circles, 40 mosquitoes.

Discussion

Although scirtids had major effects on microbial community parameters, this did not translate into positive affects on mosquito populations expected if facilitation is occurring. While it is possible that the lack of scirtid-mediated facilitation in this study may be an artifact of our experiment, it is more likely that any positive scirtid impact may have been negated by competition among the macroinvertebrates for the same resources.

Other studies have indicated that scirtid beetles may exert negative effects on mosquito populations, such as decreased body mass at adulthood and reduced survival, when resource levels are high (Paradise 1999, 2000). This switch from commensalism to amensalism under different nutrient regimes, also described by Heard (1994) in another processing chain commensalism, may manifest as a continuum of affects on mosquito performance, ranging from highly facilitative to negative. Thus, we postulate that our current findings represent a condition under which resources were sufficiently available to both macroinvertebrates, resulting in a neutral affect of scirtids on mosquito populations.

This study evaluated the impact of scirtid beetle presence on microbial community dynamics in tree holes. We demonstrated that even at low densities, scirtid beetles increased the rate of decay of leaf detritus and altered the community structure of microorganisms in water and on leaves. Although leaf fungal biomass was slightly higher but not significantly so in the presence of scirtids, lower remaining leaf mass in these treatments means that on a per mass basis, a greater percentage of fungal biomass was contained in leaf material in the presence of scirtids. This conclusion is supported by corresponding increases in the activity of the leaf polymer degradation enzymes, xylanase and cellobiosidase, in association with scirtid beetles. Taken together, these results indicate accelerated leaf decay associated with beetle feeding activity, supporting the hypothesis that increased processing of leaf detritus drives facilitation. Although scirtid-induced reductions in leaf mass have been documented previously (Paradise 2000), the current study is the first to indicate a microbial mechanism for leaf processing other than physical conversion of coarse particulate organic matter (CPOM) to fine particulate

organic matter (FPOM). In addition, although the latter contributes to the pool of resources utilized by *Oc. triseriatus*, transitions between filtering (consuming FPOM and microorganisms) and browsing (consuming microbial biofilms) feeding modes are known to occur (Merritt 1992). If browsing behavior is indeed more commonly exhibited by late-instar *Oc. triseriatus*, then we would expect that late-instar mosquito larvae would benefit from the increase in leaf-associated fungal biomass conferred by scirtid beetles.

Presence of scirtid beetle larvae was insufficient to reduce bacterial production associated with leaf surfaces, although the effect of scirtids was marginally significant. In contrast, the mosquito effect on leaf productivity is pronounced. A significant interaction between mosquito and scirtid main effect indicates that mosquitoes drive down productivity even in the absence of scirtids. That mosquitoes drive down bacterial productivity on leaves is well-described by Kaufman et al. (1999, 2001, 2002, 2006); however, this is the first study to indicate that another tree hole invertebrate may also contribute to reduced productivity. Although a reduction in bacterial productivity in the presence of mosquitoes also occurred in the water column, a corresponding change in bacterial abundance did not occur in response to the presence of either mosquitoes or scirtids. This is unsurprising in light of previous findings from tree holes. Kaufman et al. (2001) suggested that such decoupling of bacterial abundance and growth rate may result from shifts in the structure of bacterial communities in response to macroinvertebrate presence.

While scirtids did not increase the abundance or productivity of water column bacteria, they did have an impact on water column bacterial community structure. Using T-RFLPs, we determined that when scirtid beetles are present shifts in community

structure occur involving a few key bacterial groups. Bacterial community shifts in response to mosquito larvae have been described for pitcher plant and tree hole communities (Cochran-Stafira and von Ende 1998, Kaufman et al. 1999, Kneitel 2002); however this is the first study to evaluate this response in the presence of scirtid beetles. In contrast to bacterial communities, leaf surface fungal communities changed in response to time and mosquito larvae, but not the presence of scirtid beetle larvae. Although communities were more productive and had greater biomass (ergosterol), neither scirtids nor mosquitoes changed the nature of this community.

That bacterial community changes evident on the leaf surface were influenced by scirtid as well as mosquito presence is unsurprising because scirtid feeding would necessarily result in the consumption of surface-associated bacterial biofilms along with leaf particulates. However, the effect of scirtids on the water column bacteria suggests their movements (feeding and locomotion) along leaves may dislodge surface bacteria and FPOM into the water, thus altering the bacterial community in both locations. Indeed, the same effect is likely caused our water-column fungal communities to shift in response to scirtids. Overall, mosquito presence had the most influence on changes in microbial community composition. Surface and water column –associated bacterial and fungal communities all changed significantly in response to mosquito presence, in correspondence with our previous observations (Kaufman et al. 1999, 2008, Xu et al. 2008) Although we did not assess water column fungal biomass in the present study, we suspect that mosquitoes change fungal composition in the water column by reducing the taxa susceptible to larval digestion (Graça 2001, Rossi 1985).

The community results obtained using T-RFLPs are limited to the level of classification conferred by fragment sizes. Although differences might have not been apparent based at the "species" levels, defined herein as fragments differing in size by at least one base pair, changes in community structure might be more apparent at higher orders of classification. Surprisingly, though scirtids may open additional niches for fungal colonization via shredding leaf material, scirtids did not appear to directly affect the structure of fungal communities through their feeding activity. The absence of scirtid-associated changes in the structure of fungal communities may indicate these macroinvertebrates are not affecting the structure at all, or at least not at the "species" level of resolution provided by T-RFLPs. Alternatively, changes may not be visible as a result of the technique used to harvest surface associated microorganisms from the leaf surfaces. Loosely attached fungi are represented in the sonicated leaf samples while fungi whose hyphae are enmeshed in the matrix of the decomposing leaf are not. Rather than accessing only loosely attached fungi, beetles consume whole leaf particles with the associated fungi; therefore, changes in the composition of fungal communities may be relatively uniform, in contrast to the compositional changes in response to mosquitoes observed in this study and others (Kaufman 2008). Thus, scirtids may very well be altering leaf-associated fungal communities and increasing fungal-derived nutrients in the system via feeding on embedded fungal hyphae, however, that question should be addressed in future experiments.

We found that the two enzymes used for TRFLP digestions in some cases produced slightly different results that, although not uncommon, underscore the necessity of using multiple enzymes to refine the "species" composition obtained for each

treatment. To our knowledge, this is the first study to utilize T-RFLP analysis for assessing microbial community changes in a replicated experimental study of mosquito habitats. While previous studies have had success utilizing cloning and other methods of microbial community structure analysis, these techniques are often laborious and limited by the number of replicate samples that may be reasonably, and economically, processed. In addition, differences among microbial community results in blocks I and II suggest variation in the initial inocula used in microcosms. Although composite inocula were used in both cases, the inocula were obtained at separate times, immediately preceding the onset of each experiment. The compositional differences in these communities underscore the heterogeneity present in tree hole communities and deserve consideration in future studies of scirtid and mosquito populations. Variation in the condition of available resources is postulated to underlie the stochastic distribution of scirtid beetles occupying nearby tree hole such that tree holes with high quality litter resources are likely to harbor scirtid populations (Paradise 1998; Paradise & Kuhn 1999; Paradise 1999).

As shredders, scirtid feeding in tree holes accelerates detrital processing by reducing leaf material into finer fragments. From a mosquito's perspective, scirtid feeding alters detrital surfaces and water column content, thus directly impacting the primary larval feeding zones. Leaf surfaces in particular are a tough, non-nutritive vehicle upon which resides a film of rich nutrients incorporated as microbial biomass. Although bacteria in this biofilm are severely grazed down by mosquito larvae, fungal members of this biofilm are less susceptible because they are anchored into the matrix of decaying tissue itself. Scirtid feeding may make this resource more available to larvae by exposing

hyphae and/or releasing small, ingestible fragments containing fungal hyphae into the filter feeding zone. The fact that scirtid feeding altered water column fungi supports the latter mechanism, but measurements of fungal biomass in the water column are necessary to further this hypothesis. Because resource availability is the most important contributing factor to mosquito growth, scirtid-associated changes in the underlying structure of microbial communities and the spatial rearrangement of these communities may provide mechanisms by which scirtid beetle presence in tree holes alters mosquito success under nutrient-limited conditions.

CHAPTER 5

COMPETITIVE INTERACTIONS BETWEEN *OCHLEROTATUS TRISERIATUS* (SAY) AND *AEDES ALBOPICTUS* (SKUSE) (DIPTERA: CULICIDAE) ARE INFLUENCED BY HABITAT-ASSOCIATED MICROBIAL COMMUNITY DYNAMICS

Introduction

Container-dwelling forms represent approximately 40% of mosquito species, many of which are important arbovirus vectors, including West Nile Virus, yellow fever and LaCrosse encephalitis (Laird 1988). The vectorial capacity of adult mosquitoes is directly related to the number of adults produced from the natal container habitat. Under conditions of high larval density, competition for food resources is intense and inherently changes the availability of such resources. Limited food resources at the larval stage results in the production of smaller adults with lower metabolic reserves, and leads to higher nutrient demands at this stage and thus increased incidence of host contacts. As a result, these mosquitoes may be more competent vectors of arboviruses (Alto et al. 2005). Several studies have shown that the Asian tiger mosquito, *Aedes albopictus*, is superior to the Eastern tree hole mosquito, *Ochlerotatus triseriatus*, under conditions of larval competition. Both species are major vectors of human pathogens (West Nile Virus and LaCrosse encephalitis, respectively).

Competition, both inter- and intraspecific, is one important factor mediating the production of adult mosquitoes within and among species sharing water-filled container habitats. Interspecific competition is common and occurs depending on the range overlap of container-breeding mosquitoes (Kitching 2001, Juliano and Lounibos 2005). In southern latitudes, *Aedes albopictus* and *A. aegypti* frequently share larval habitats (Rai 1991). *Ochlerotatus* (= *Aedes*) *triseriatus* may overlap with either of these species in the southernmost portion of their range, which extends to Florida in the Eastern United States. In its northern range, *Oc. triseriatus* has historically been free from intraspecific competition; however in recent years *Ochlerotatus japonicus* has begun to invade

container habitats in this region (Sardelis et al. 2001, Scott et al. 2001, Roppo et al. 2004).

Compared to larger water body mosquito habitats, density dependent mortality unrelated to predation appears to be a primary population regulator of container-dwelling larval mosquitoes as species compete directly for the unpredictable food resources that characterize container habitats (Juliano 2007). Container-dwelling mosquitoes belong to the grazing/filtering functional feeding group; larvae depend on the microbial biofilm associated with leaf and container wall surfaces, and on fine particulate organic matter (FPOM) and planktonic microbes present in the water column (Walker and Merritt 1991). Interspecific competition functions among cohorts of larvae through shared resource pools and shared food acquisition behaviors (Kitching 2001). Within a single species, we expect that food acquisition behaviors are similar in terms of the proportion of time spent browsing or grazing on surfaces verses filtering in the water column or at the water surface. Among competing species, these proportions may differ, as is the case between *A. aeqypti* and *A. albopictus*. For example, when leaves are the food substrate *A. albopictus* outcompetes *A. aegypti* (Barrera 1996, Juliano 1998, Daugherty et al. 2000, Braks et al. 2004, Yee et al. 2004), however the advantage shifts to *A. aegypti* when animal carcasses are utilized as a food resource (Daugherty et al. 2000). Yee et al. (2004) suggest that the dominance of *A. albopictus* over *A. aegypti* in all but the latter case can be attributed to differences in the relative time spent browsing on leaf substrates. In their study, *A. aegypti* spent more time filter-feeding in the water column, where microbes are less abundant.

Previous studies have examined interspecific competition among mosquito larvae by measuring adult productivity at different resource levels when larvae are alone or under interspecific competition. In addition to the above studies of A. *albopictus* and *A. aegypti*, differences in success at low resource levels are also seen in competition studies involving *Oc. triseriatus* and *A. albopictus* (Livdahl and Willey 1991, Novak et al. 1993, Teng and Apperson 2000). Although the proximate cause of this outcome is unknown, it is apparent that *A. albopictus* has the potential to replace *Oc. triseriatus* where the species co-occur. These studies suggest a clear competitive advantage for one species at a defined low resource level or as detritus sources vary (Yee et al. 2007). However, given our current understanding of how mosquito larvae utilize resource pools, the mechanism driving such outcomes remains ambiguous. The lack of a clearly-defined mechanism remains an important gap in our understanding of asymmetric success under competition.

Complicating the ambiguous mechanism is the ill-defined nature of the food resources in larval mosquito habitats such as tree holes. Allochthonous inputs, consisting of plant and animal detritus, and stemflow are the primary nutrient-supplying inputs into the system (Carpenter 1982, Daugherty et al. 2000, Kitching 2001). Plant material in the form of senescent leaves appears to supply the majority of the organic carbon to these systems and is thus the resource input typically used by researchers for laboratory-based microcosms simulating natural habitats. Leaf matter, as stated above, cannot be ingested directly by larvae but is accessed instead via intermediate microbial processing in which nutrients from leaf material and stemflow inputs become available to larvae as they are incorporated into microbial biomass. Softening of the leaf tissue through microbial processing may also contribute to an accessible pool of FPOM, but the extent to which

this may occur is unknown. Therefore, it is apparent that any study of interspecific larval competition must take into account the microbial community that comprises the actual resource for which species compete.

Studies suggest that larval feeding alters the microbial communities present in tree holes, resulting in bacterial forms that are indigestible, and thus unavailable, to the mosquito larvae (Kaufman et al. 1999, 2008, Xu et al. 2008). Furthermore, several groups of microorganisms, including protozoa, disappear or are severely depleted from the system under larval feeding pressure compared with larva-free conditions (Kaufman et al. 2002). It is therefore probable that the mechanism underlying competition among larvae is the depletion of available consumable microbial forms, rather than direct utilization of detrital resource pools. We tested the hypothesis that competing mosquito species differentially utilize the available microbial resources to produce the differential growth parameters observed under competition. We postulate that one of the following mechanisms are responsible for the result of interspecific competition among larvae: 1) species exhibit differences in their ability to utilize available microbial forms, 2) within-species feeding patterns result in differential harvesting of microbes from resource reservoirs i.e. water column verses leaf surfaces, and 3) one species may drive down the total available microbial resources to a level below which the competing species cannot maintain comparable productivity. The purpose of this study was to assess the structure of the microbial community associated with tree hole habitats to determine the viability thereof as a resource mediating interspecific competition among larvae. Specifically, our objective was to evaluate known competitive interactions between *A. albopictus* and the

Eastern tree hole mosquito, *Oc. triseriatus*, to determine whether the documented success of *A. albopictus* derives from one of the three mechanisms described above.

Materials and Methods

Microcosms. We constructed microcosms containing *Oc. triseriatus* and *A. albopictus* under interspecific and intraspecific competition at two mosquito densities. Senescent red oak leaves (*Quercus rubra* L.) collected at Michigan State University's Kellogg Forest (Augusta, MI) were dried at 45°C for 48 h. Treatments were applied to individual microcosms stocked with 1.0 g oak leaf packs, 500 ml deionized water, and a microbial inoculum consisting of 3 ml homogenized natural tree hole water and particulates.

Prior to the addition of newly-hatched first instar mosquito larvae, all microcosms were conditioned for 3 days to allow time for microbial colonization of leaf surfaces and water column. The larvae used in the following experiments were hatched from eggs collected from our colonies in the Insect Microbiology laboratory at Michigan State University.

To evaluate the dynamics of microbial resources underlying interspecific competition between *Oc. triseriatus* (T) and *A. albopictus* (A), microcosms were treated with two levels of two factors, competitor (intraspecific, or TT and AA, vs. interspecific, or AT) and density (high vs. low), for a total of a total of six treatment combinations. Intraspecific competition microcosms contained an equal ratio of individuals from each species i.e. 15:15 or 30:30.To evaluate the effect of density on mosquito performance, initial densities of 60 (high) or 30 (low) first instar larvae were added to microcosms.

Treatment combinations were replicated a total of twelve times to provide sufficient microcosms for destructive sampling.

Samples. Microcosms were sampled four times during the 60 day course of the experiment- at day 0 (prior to larval addition), day 15, day 30 and day 60. On each date, four replicate microcosms from each treatment were destructively sampled.
During each sampling period, leaf discs and of water samples were removed from microcosms for measuring productivity, abundance, and structure of microbial communities as described below. Although the data are not presented here, additional water samples were taken from microcosms on all sampling dates for nutrient (nitrogen and phosphorus) analysis. Leaf samples were aseptically procured with a cork borer (11 mm diameter), placed in filter-sterilized microcosm water (productivity – see below) or phosphate-buffered saline (abundance and community analysis – see below), and sonicated for 12 min to dislodge loosely-associated microorganisms.

Mosquito Measurements. Microcosms were checked daily for the presence of adult mosquitoes. Adults were collected and stored at 4°C until the microcosm was destructively samples. Larvae were gathered by decanting the remaining microcosm water through a fine mesh sieve and stored at 4°C. Where possible, we sexed and identified adults, larvae, and pupae to species before freeze-drying the mosquitoes to obtain dry mass measurements.

Bacterial Abundance. Leaf- and water column-associated bacterial abundance was assessed by removing a single leaf disc and 15 ml of water from microcosms per sampling period. Samples were preserved for later analysis with formalin at a final concentration of 3.4%. Bacterial abundance on the leaf surface and in the water column

subsamples was quantified via direct microscopic counts (DMCs) of bacteria and protozoa using the DAPI (49,6-diamidino-2-phenylindole) fluorescent staining procedure (Porter and Feig 1980, Walker et al. 1988). Samples were filtered onto two black Nucleopore filters (0.2-mm pore size; Costar, Cambridge, Mass), stained at 2 mg/ml for 5 min, and counted at 1000X.

Bacterial Productivity. A ^{3}H-leucine incorporation assay was used to directly measure accumulation of microbial biomass (Kirchman 2001). Quantifications of the amino acid incorporated into protein are achieved in this technique in a bacteria-specific manner using short incubation periods of samples with nanomolar concentrations of leucine (Riemann and Azam 1992). Labeled leucine (L-leucine (4,5-^{3}H), 50 Ci/mmol-NEN, Life Science, Boston, MA) was added to water and sonicated leaf samples at a concentration of 25 nM. To achieve previously reported saturation of uptake kinetics (Kirchman 2001, Kaufman et al. 2001), unlabeled leucine was added with labeled leucine to bring concentrations in water and leaf samples to 100 and 400 nM, respectively. Leucine incubations were done in the dark at 20°C for 20 min in 2 ml microcentrifuge tubes (Smith and Azam 1992, Kirchman 2001). Trichloroacetate [TCA, final concentration 10% (vol:vol)] was added to terminate reactions and precipitate protein. Two rinses of the TCA-protein precipitates were conducted with 10% TCA, followed by a single rinse with 5°C, 80% (vol:vol) ethanol. A Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA) was used to quantify the amount of radioactivity present in the samples.

T-RFLP Analysis. The structure of leaf- and water column-associated bacterial and fungal communities in microcosms was assessed using tagged restriction fragment

length polymorphism (T-RFLP) analysis (Marsh 1999). We extracted DNA from the water column and sonicated leaf samples described above using the MoBio UltraClean soil extraction kit (Carlsbad, CA). Universal primers targeting segments of the small subunit (SSU) ribosomal DNA and specific to bacteria and fungi (16S rDNA and18S rDNA, respectively) were used for amplifications. Bacterial DNA was amplified using universal eubacterial primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi et al. 1998). Fungal DNA was amplified using primers nu-SSU-0817-59F (5'-TTAGCATGGATAATRRAATAGGA-3') and nu-SSU-1536-39R (5'- TTGCAATGCYCTATCCCCA-3') (Borneman and Hartin 2000). The forward primers were fluorescently-labeled at the 5' end with FAM (carboxyfluorescein) for detection by capillary electrophoresis. Reaction mixtures (100 ul final volume) included ca. 10 ng DNA template, 50 ul Failsafe PreMix buffer E™ (Epicentre Biotechnologies, Madison, WI), 1 ul Failsafe™ enzyme, and 4 ul forward and reverse primers. The cycles used for 16S rDNA were as follows: 1 cycle at 94°C for 2 min; 30 cycles at 94°C for 45 s, 68°C for 30 s, and 72°C for 1 min 30 s; and1 cycle at 72°C for 7 min. Similarly, 18S rDNA was cycled through the following: 1 cycle at 94°C for 2 min; 30 cycles at 94°C for 45 s, 58°C for 30 s, and 72°C for 1 min; and1 cycle at 72°C for 7 min.Gel electrophoresis was performed to verify amplification of template DNA. Three PCR reactions per sample were pooled and purified (Qiaquick PCR purification Kit, Valencia, CA). PCR products were digested independently with two restriction enzymes, MspI and HhaI (New England Biolabs, Cambridge, MA) at 37°C for 3 h. Following electrophoresis of digested samples on a capillary electrophoresis genetic analyzer (PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA), the size

and frequency of each terminal restriction fragment was determined using Genescan Analysis software (Applied Biosystems).

Statistical Analysis. Multivariate analysis of variance (MANOVA) was used to analyze related variables: water chemistry values, mosquito productivity values, bacterial productivity, and bacterial production (SAS system fir Windows 9.1; SAS Institute, Cary, NC). Mosquito productivity values included the mass, survival, and total emerged adults (males and females) obtained per microcosm. Peaks in T-RF electropherograms were identified and binned using the T-RFLPS Stats tools (Abdo et al. 2006) and R (R Development Core Team 2004). The relative percent abundance of peak areas obtained from T-RFLP profiles obtained from bacterial and fungal communities were transformed (logratio) then subjected to principal component analysis (JMP® Statistical Discovery Software, V5.1 (http://www. jmpin.com, SAS Institute, Inc., Cary NC, USA).

Results

Mosquito production. Mosquito species and density significantly affected *Oc. triseriatus* populations, but not *A. albopictus* (Table 5.1), suggesting interspecific competition between the two species. The success of *Oc. triseriatus* (adult body mass, adult emergence, and overall survival) was lower under interspecific competition at high total mosquito densities, as indicated by a significant interaction between mosquito density and species (Table 5.1 and Figures 5.1-5.3). More *A. albopictus* adults were produced than *Oc. triseriatus* under every condition, although individual species performed better at low intraspecific densities (Figures 5.1-5.3). In addition, *A. albopictus* emerged sooner than did *Oc. triseriatus* for both density and competition levels. In low density treatments, pupation ranged from day 13 for intraspecific *A. albopictus* to day 34

for *Oc. triseriatus* (Figure 5.1). Microcosms were preserved until day 60, although additional pupation did not occur beyond day 34. For both species male mosquitoes developed earlier than females under inter- and intraspecific competition. In addition, the mean adult emergence was significantly earlier for *A. albopictus* that *Oc. triseriatus*. More *A. albopictus* adults were produced than *Oc. triseriatus* when the two species competed and under low density intraspecific competition (Figure 5.2, C and D). At high densities, fewer mosquitoes emerged under interspecific conditions compared with intraspecific conditions, regardless of species. The opposite trend occurred for *A. albopictus*, which exhibited increased adult emergence when competing with *Oc. triseriatus* at low density.

Under intraspecific competition, the mean body mass was similar among males of both species, however under interspecific competition, the mean body mass of *A. albopictus* males increased relative to *Oc. triseriatus* (Figure 5.3, A and B). In contrast, the body mass of male *Oc. triseriatus* under interspecific conditions was depressed or unchanged compared to intraspecific levels. Female *Oc. triseriatus* body mass remained the same under low density interspecific conditions, but could not be measured at high densities, as no adult females emerged from these microcosms Figure 5.3, C and D). Reductions in adult emergence and body size when individual species were at high densities suggest that intraspecific competition was more intense than at low densities.

Bacterial abundance and productivity. Bacterial abundance in the water column and on leaf surfaces did not differ significantly between mosquito densities or under inter- or intraspecific competition treatments (Figure 5.4; Table 5.2). Over time, the abundance of bacteria remained the same, although the number of cells present in the

water column were consistently lower than on leaf material (Scheffe′ a posteriori test, $P = 0.05$).

Productivity in the water column and on leaf surfaces decreased significantly from day 15 to day 30 (Figure 5.5; Table 5.3). In both homogenous and heterogeneous mosquito populations high larval densities were significantly associated with reductions in water column bacterial productivity compared with low density treatments, although this effect was mitigated by time. Despite the significant interaction of larval presence and sampling date, the species combination treatments did not exert a significant effect on the production of bacteria on any of the sampling dates examined. On days 30 and 45, there was no difference in productivity between the two larval densities, nor did productivity differ in the treatments between the two sampling dates (Scheffe′ a posteriori test, $P = 0.05$).

Table 5.1. Multivariate analysis of variance (MANOVA) results for emergence and mass of *A. albopictus* and *Oc. triseriatus* in microcosms. Total adults and females were analyzed as separate dependent variables.

	Source	*F statistic	df	P value
A. albopictus				
	Mosquito	1.4	5,6	0.34
	Density	2.65	5,6	0.13
	Mosquito*Density	1.89	5,5	0.25
Oc. triseriatus				
	Mosquito	0.13	5, 9	0.001
	Density	0.33	5,9	0.05
	Mosquito*Density	0.23	5, 8	0.02

*Wilkes' Lambda.

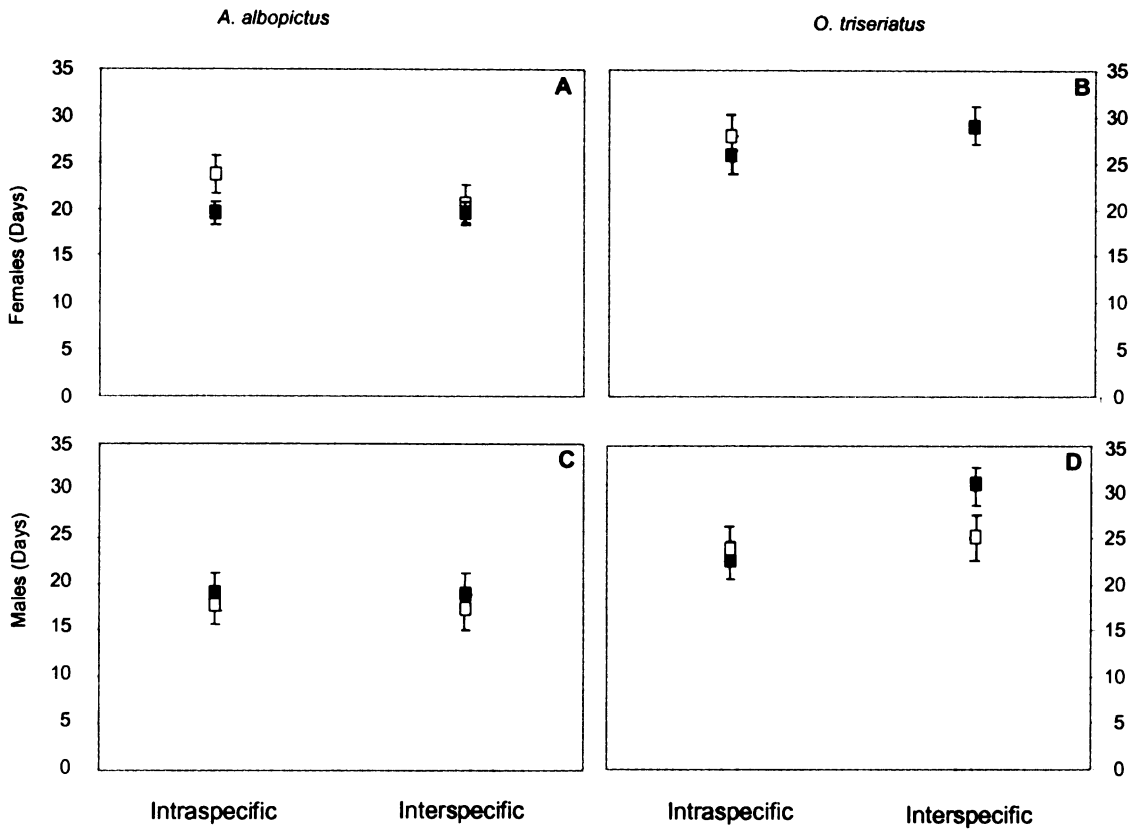

Figure 5.1. Development time for two densities of *A. albopictus* and *Oc. triseriatus* in microcosms. Low mosquito densities are represented by filled characters and high densities by open characters. Panels A and B are females, C and D are males. Values are means ± SE, n = 3-4 for each point.

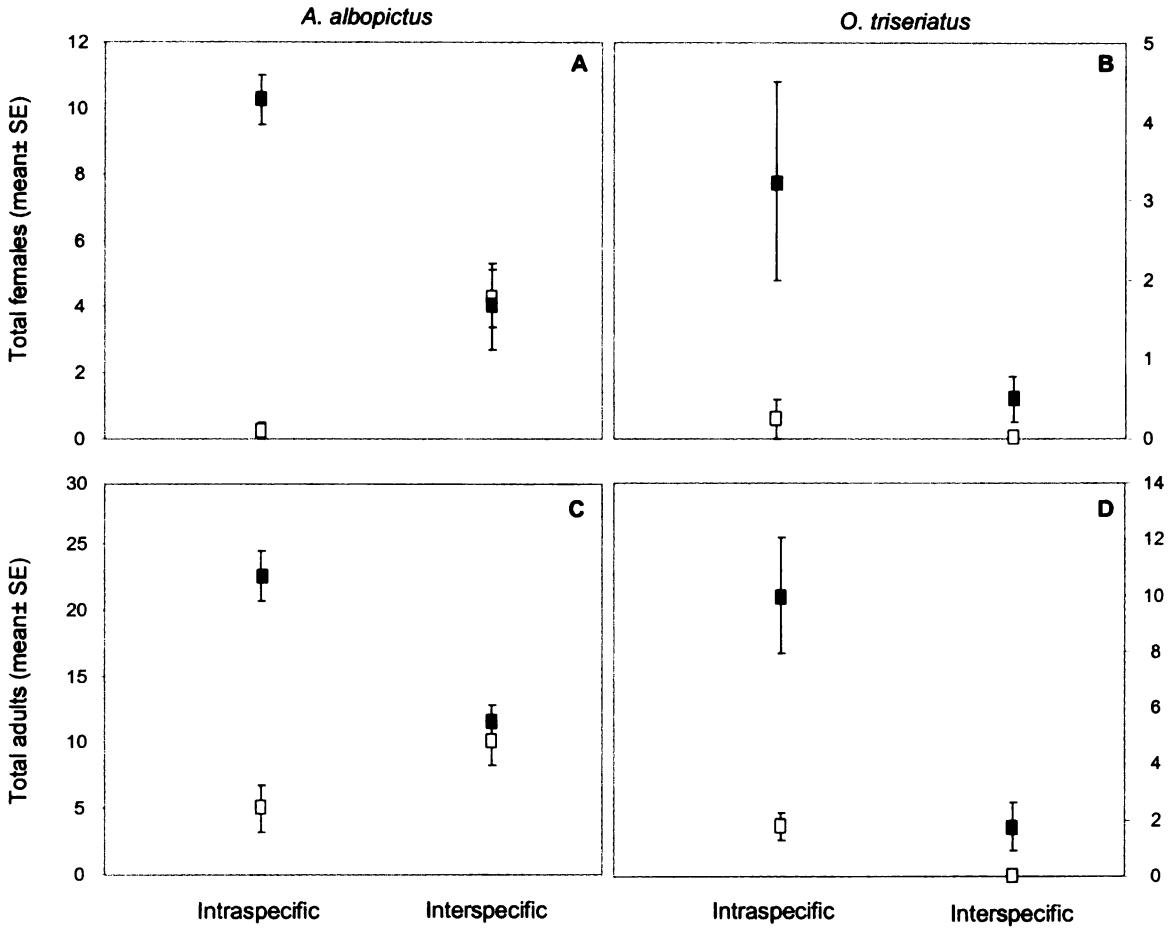

Figure 5.2. Number of adult *A. albopictus* and *Oc. triseriatus* emerged from microcosms. Low mosquito densities are represented by filled characters and high densities by open characters. Panels A and B are females, C and D are total number of adults. Values are means ± SE, n = 3-4 for each point.

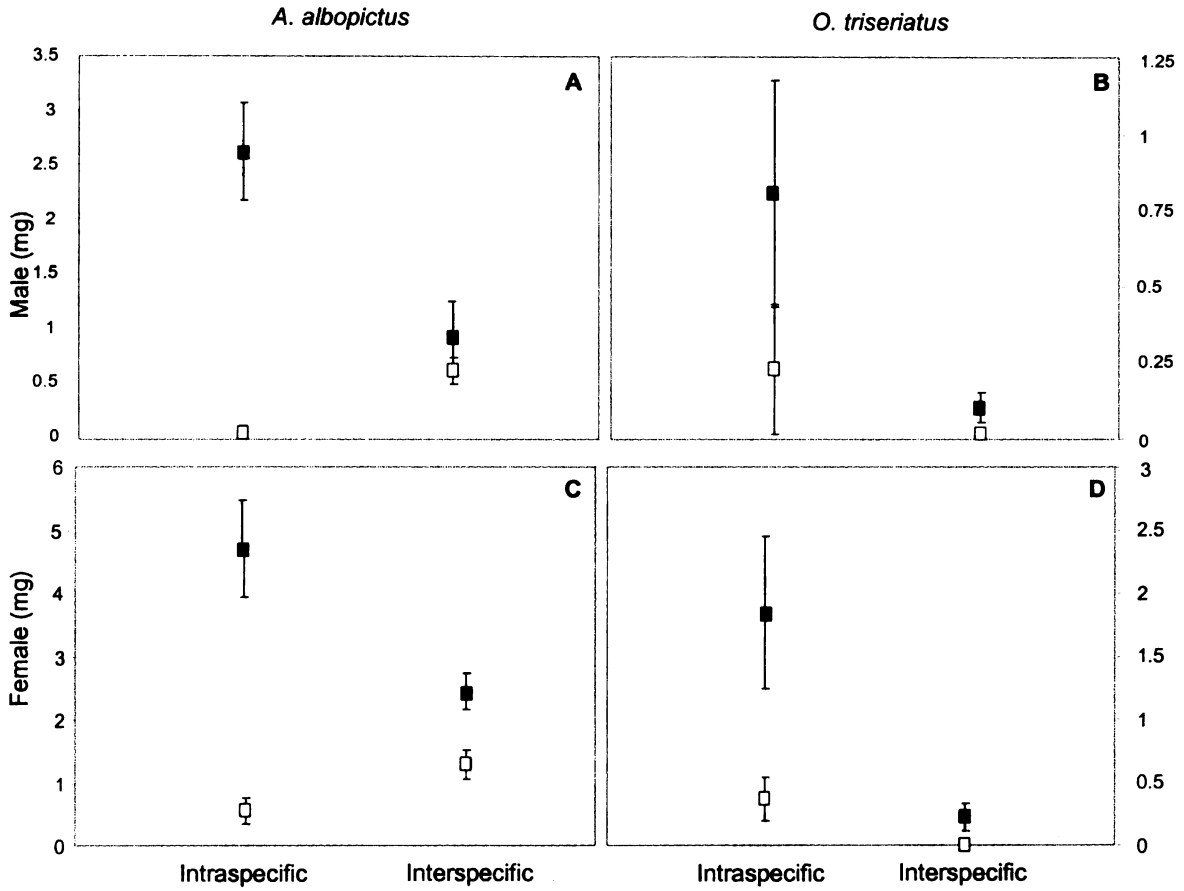

Figure 5.3. Mass of adult *A. albopictus* and *Oc. triseriatus* from microcosms. Low mosquito densities are represented by filled characters and high densities by open characters. Panels A and B are females, C and D are males. Values are means ± SE, n = 3-4 for each point.

Table 5.2. Multivariate analysis of variance (MANOVA) results for bacterial abundance in microcosms on days 15 and 30.

	F statistic	df	P value
Bacterial abundance			
Mosquito	0.84	8, 30	0.57
Density	1.67	4, 15	0.21
Mosquito*Density	1.05	8, 30	0.42

*Wilkes' Lambda.

Table 5.3. Multivariate analysis of variance (MANOVA) results for bacterial productivity in microcosms on days 15, 30 and 60.

Source	F statistic	df	P value
Time	1.6	8, 98	0.1358
Mosquito	0.6	4, 98	0.6627
Density	9.24	2, 49	0.0004
Mosquito*Time	6.86	4, 98	<.0001
Density*Time	1.34	8, 98	0.2322
Mosquito*Density	1.75	4, 98	0.1451
Mosquito*Density*Time	32.2	4, 98	<.0001

*Wilkes' Lambda.

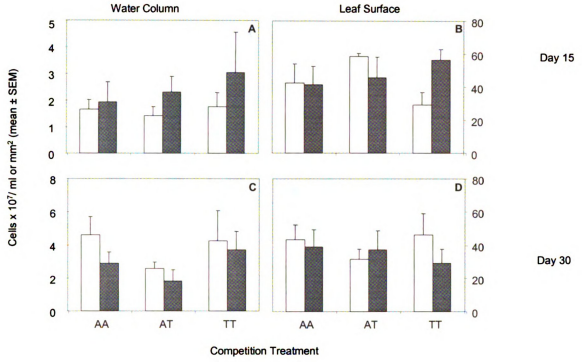

Figure 5.4. Bacterial abundance (direct microscopic counts) in microcosms containing two densities of *A. albopictus* (A) and *Oc. triseriatus* (T), alone or under interspecific competition. Low mosquito densities are represented by filled characters and high densities by open characters. Panels A and C are water column samples, B and D are leaf surface samples. Samples were taken on day 15 (A and B) and Day 30 (C and D). Values are means ±SE, n = 3-4 for each point.

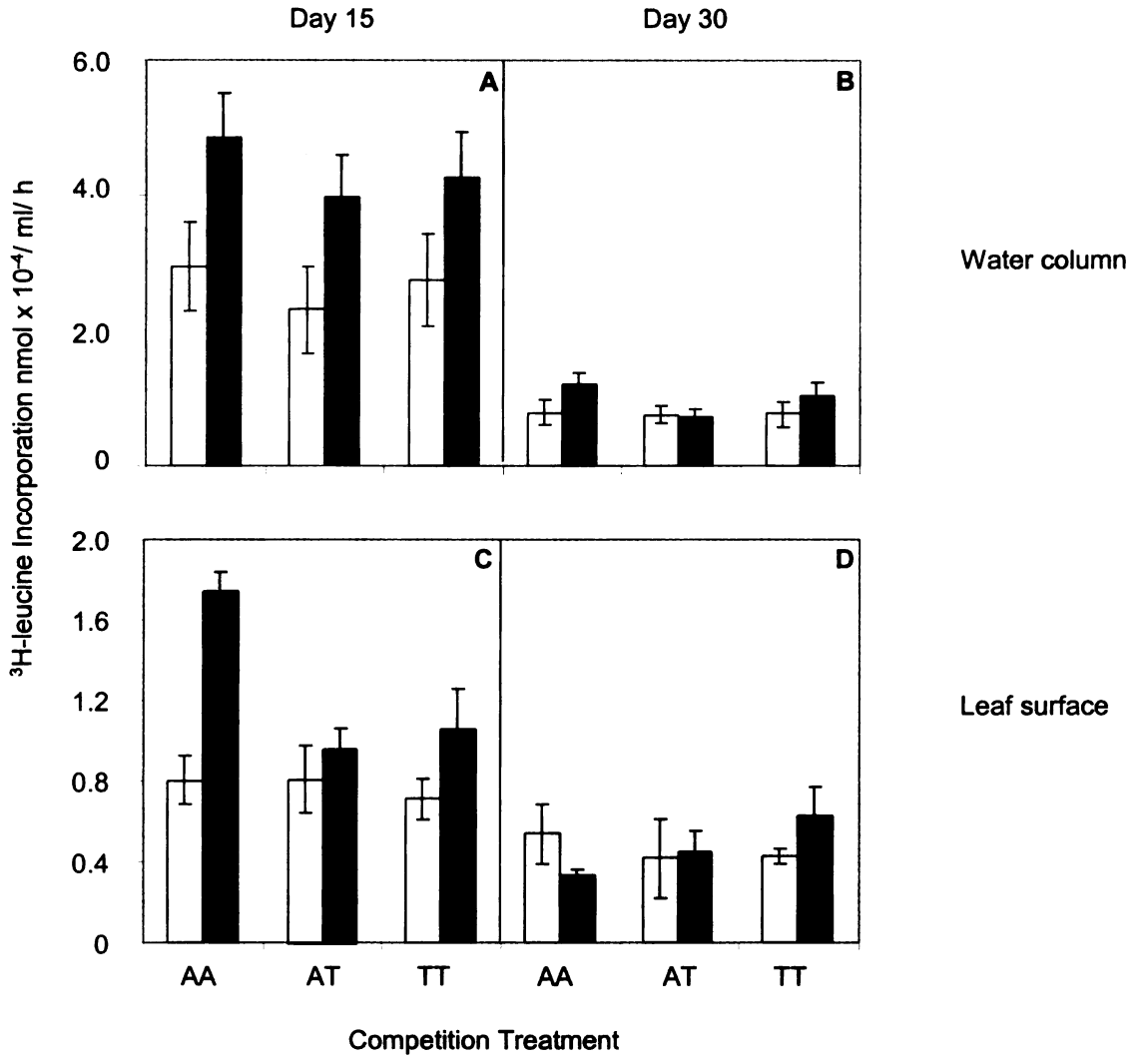

Figure 5.5. Bacterial productivity (leucine incorporation rates) in microcosms containing two densities of *A. albopictus* (A) and *Oc. triseriatus* (T), alone or under interspecific competition. Low mosquito densities are represented by filled characters and high densities by open characters. Panels A and B reflect samples taken from the water column, C and D are from leaf surfaces. Samples, taken on day 15 (A and C) and day 30 (B and D), are represented as means ± SE, n = 3-4 for each point.

T-RFLPs. Water-associated bacterial communities exhibited significant differences along principle component 1 on days 15 and 30 in response to mosquito density, but not in response to interspecific or intraspecific mosquito species combination (Figure 5.4; Table 5.2). Such differences were also apparent in leaf bacterial communities on day 15, but not on day 30. Neither mosquito nor density exerted a significant effect on PC2 (data not shown). Cumulatively, PC 1-3 explained 38-79% and 67-78% of the overall variance in leaf and water column associated bacterial communities, respectively (Figure 5.6). Restriction fragment 109 accounted for most of the variability in the water column bacterial community along PC1 and fragment 398 along PC2; however, the abundance of fragment 109 decreased from day 15 to day 30 (Figure 5.6; Table 5).

In contrast to bacterial communities, mosquito treatment significantly interacted with density to influence water column fungal communities on day 15 along PC1 (Figure 5.7; Table 5.3). This interaction effect was also slightly evident on 30 at $P = 0.1$. A significant affect of mosquito species combination on the structure of leaf-associated fungal communities was reflected by PC2 on days 15 and 30 (Figure 5.7; Table 5.4). Water column fungi were also influenced significantly by mosquito treatment on day 30, however this effect was not evident on day 15. The total variation leaf and water column fungal communities explained by PC1-3 ranged from 47-54% and 54-64%, respectively (Figure 5.7). Although not shown here, all differences in the structure of microcosm microbial communities associated with mosquito species competition and mosquito density had disappeared by day 60.

Table 5.4. Analysis of variance (ANOVA) results for PC1 values obtained from PCA analysis of bacterial (16S rDNA) T-RFLP peak area data.

	Water			Leaf		
Source	F statistic	df	P value	F statistic	df	P value
day 15						
Mosquito	0.50	2	0.62	0.58	2	0.57
Density	17.50	1	0.001	5.65	1	0.03
Mosquito*Density	1.29	2	0.31	1.36	2	0.29
Error		14			13	
day 30						
Mosquito	1.03	2	0.41	1.07	2	0.36
Density	8.66	1	0.02	1.88	1	0.19
Mosquito*Density	0.21	2	0.82	1.98	2	0.17
Error		6			17	

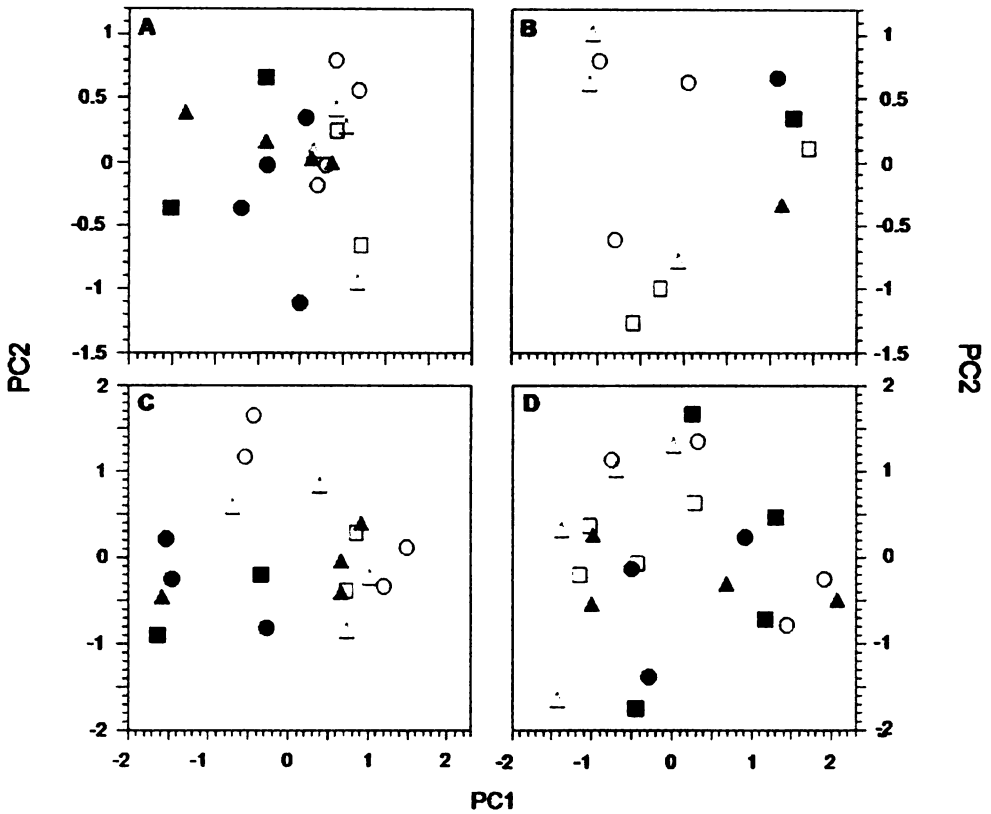

Figure 5.6. Principle component analysis ordination of bacterial T-RFLP peak area data for microcosms containing two densities of *A. albopictus* (squares) and *Oc. triseriatus* (circles), alone or under interspecific competition (triangles). High mosquito densities are represented by filled characters and low densities by open characters. Panels A and B are water column samples, C and D are leaf surface samples. Samples were taken on day 15 (A and C) and Day 30 (B and D). n = 3-4 for each point.

Table 5.5. Analysis of variance (ANOVA) results for PC1 values obtained from PCA analysis of fungal (18S rDNA) T-RFLP peak area data.

	Water			Leaf		
Source	F statistic	df	P value	F statistic	df	P value
day 15						
Mosquito	0.50	2	0.62	1.60	2	0.23
Density	0.30	1	0.59	4.21	1	0.06
Mosquito*Density	4.29	2	0.03	0.38	2	0.69
Error		14			17	
day 30						
Mosquito	3.51	2	0.05	3.05	2	0.08
Density	0.26	1	0.61	2.08	1	0.17
Mosquito*Density	2.92	2	0.08	0.38	2	0.69
Error		15			15	

Table 5.6. Analysis of variance (ANOVA) results for PC2 values obtained from PCA analysis of fungal (18S rDNA) T-RFLP peak area data.

	Water			Leaf		
Source	F statistic	df	P value	F statistic	df	P value
day 15						
Mosquito	0.61	2	0.56	6.08	2	0.01
Density	0.22	1	0.23	0.54	1	0.47
Mosquito*Density	0.63	2	0.63	0.04	2	0.96
Error		14			17	
day 30						
Mosquito	7.32	2	0.01	5.39	2	0.01
Density	0.60	1	0.45	0.42	1	0.53
Mosquito*Density	3.14	2	0.07	0.04	2	0.96
Error		15			15	

Table 5.7. Bacterial (16S) T-RFLP fragments with high factor loadings.

	Day 15		Day 30	
Substrate	PC1	PC2	PC1	PC2
Water	109, 451	365, 398	109	252, 398, 456
Leaf	456, 460	258, 456	118, 150, 394	150, 394, 597

Table 5.8. Fungal (18S) T-RFLP fragments with high factor loadings.

	Day 15		Day 30	
Substrate	PC1	PC2	PC1	PC2
Water	582, 767	582, 559, 767	211, 547, 707	559, 760
Leaf	212, 559, 767	527, 559, 592	53, 559, 584	53, 584, 716, 767

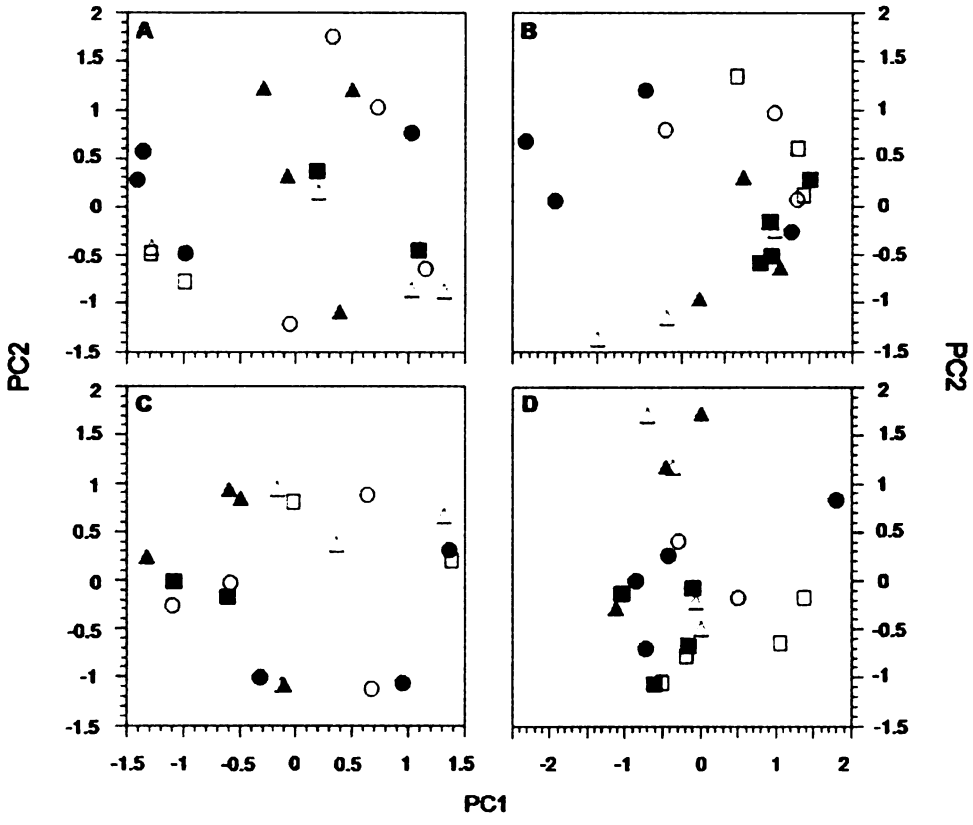

Figure 5.7. Principle component analysis ordination of fungal T-RFLP peak area data for microcosms containing two densities of *A. albopictus* (squares) and *Oc. triseriatus* (circles), alone or under interspecific competition (triangles). High mosquito densities are represented by filled characters and low densities by open characters. Panels A and B are water column samples, C and D are leaf surface samples. Samples were taken on day 15 (A and C) and Day 30 (B and D). n = 3-4 for each point.

Discussion

In agreement with other studies, we have shown here that under conditions of high larval density, competition for food resources in container habitats of larval mosquitoes is intense and inherently changes the availability of such resources. For both species, high densities negatively impacted larval success; however, interspecific competitive interactions proved to be adverse only for *Oc. triseriatus* populations. Larvae of these mosquitoes feed primarily by grazing on the microbial biofilm associated with the container wall and detrital leaf surfaces or filtering microorganisms in the water column; therefore, we postulated that measurable changes in the leaf and water column-associated microbial community structure or abundance should occur at high interspecific densities of two species. Our data do not support the hypothesis that *A. albopictus* outcompete *Oc. triseriatus* by depleting resources below a level at which the latter can survive if microbial abundance as a whole is the resource considered. We suggest instead that the competitive advantage exhibited by *A. albopictus* may derive from a lower resource-requirement in this species compared to *Oc. triseriatus* due to their comparably smaller body size.

Bacterial abundance and production rates did not differ among mosquito species combinations although both measures were lower in high density treatments on day 15 in response to higher grazing pressure. Bacterial abundance fell equally among the treatments by day 30 and remained unchanged for the remainder of the experiment, indicating that both mosquito species are depleting bacteria uniformly. Similar to the present study, prior tests of density-dependent larval impacts on bacterial abundance have not been supported (Kaufman et al. 2001), although decreases in leaf-associated bacterial

abundance and water column productivity occurred when larvae were present verses absent.

Although not evaluated in the present study, the effect of mosquito feeding is usually not associated with decreases in overall fungal abundance as indicated by ergosterol concentrations in leaf material (Kaufman et al. 2001, 2002). However, it remains possible that differential harvesting of fungi or protozoans by the mosquito species investigated here impacted competitive outcomes. Protists were not observed in DAPI preps, indicating that this group was heavily grazed by both species and therefore difficult to measure as a component of the competition mechanism.

Evaluation of terminal restriction fragments by principle component analysis indicates that the native bacterial and fungal communities exhibit mosquito-induced structural changes. Bacterial communities were influenced greatly by the density of mosquitoes present in microcosms. This effect is unsurprising, given that under high densities, feeding pressure increases and may in fact overwhelm the influence of individual mosquito species on community composition. Analysis of fatty acid methyl ester (FAME) profiles has shown that larval feeding pressure changes the structure of water column and leaf surface bacterial communities (Kaufman 1999). Subsequently, our research group has constructed bacterial (16S) clone libraries to identify groups associated with larval feeding (Kaufman et al. 2008, Xu et al. 2008). In particular, these findings indicate that bacterial diversity decreases with larval feeding and the abundance of certain groups, particularly the Flavobacteriaceae, increase in the absence of larvae during mosquito feeding.

Despite a slight interaction of mosquito species composition with overall mosquito density (significant at $P < 0.1$), the mosquito communities present in microcosms had the greatest influence on the structure of fungal communities. Mosquito species composition effected leaf –associated fungal communities on both day 15 and day 30. Previous reports have not indicated changes in overall fungal biomass, as estimated by ergosterol concentration in response to larval feeding (Kaufman et al. 2001, 2002), although Kaufman et al. (2008) recently reported that several leaf-associated fungal taxa exhibit shifts in response to larval feeding. When *Oc. triseriatus* were present in tree holes, increases in the abundance of Dothideomycetes and Saccharomycetes occurred concurrent with decreased in the abundance of the Chytridomycota.

We did not observe compositional differences in microbial communities on day 60 associated with any of our treatments likely due to cessation of the majority of feeding weeks earlier. Indeed, mosquito-associated changes in bacterial communities began to dampen by day 30 of the experiment. Pupation of mosquitoes ended by day 34 and although a small number of larvae remained in microcosms until day 60, their feeding was insufficient for pupation and thus unlikely to contribute to significant shifts in microbial community structure.

Of additional interest is the apparent temporal difference between bacterial and fungal in community changes. While changes in the bacterial community were less intense by day 30, changes in the fungal communities began to ramp up between day 15 and day 30, reflecting more significant influences of mosquito community and density on fungal structure late in the mosquito development. This suggests that mosquito feeding is having the greatest impact on fungi between days 15 and 30, unsurprising given that the

majority of mosquito adults emerged between day 15 and 25. The association of such a shift with earlier development and greater emergence of *A. albopictus* under interspecific competition with *Oc. triseriatus* suggests that the former mosquito species is more efficient at exploiting available fungal resources than *Oc. triseriatus*. Such differentially utilization of fungal resources may underlie the resulting differences in mosquito production observed when the species compete (Livdahl and Willey 1991, Novak et al. 1993, Teng and Apperson 2000). Resource-based competition has been described between *A. albopictus* and *Oc. triseriatus* using leaves as the available resource mediating competition. The role of fungi in mosquito production may be critical, allowing mosquitoes to access nutrients otherwise available in the leaf matrix as they are incorporated into fungal tissue (Kaufman and Walker 2006). Additionally, fungi would be sources of essential lipids typically not present in bacteria. Many of these lipids are necessary for emergence and flight (Dadd 1973), thus impacting directly on adult production rates from the larval habitat. Although fungi support mosquito development by increasing leaf decomposition, and thus the abundance of fine particulates and dissolved substances in the water column (Pelz-Stelinski, Chapter 3 unpublished data, Kaufman and Walker 2006), this study is the first to indicate that mosquitoes may differentially utilize fungi resulting in production differences among species. Such a disparity may be due to explicit differences in the preference of mosquitoes for particular fungal taxa, resulting in the community composition changes observed here. There is substantial evidence from studies of other insect detritivores that fungal composition influences feeding rates and that, conversely, feeding can influence fungal community composition (Graca 2001, Rossi, 1985).

More likely, however, is a difference in food acquisition, utilization, or a combination thereof inherent to the competing larvae. Within the context of our original postulates, we suggest that one or more of the following mechanisms are responsible for the result of significant interspecific competition among larvae: 1) species exhibit a differential ability to harvest and/or metabolize available fungal forms, 2) species exhibit differences in their overall feeding behavior such that microbes are harvested differentially from resource reservoirs i.e. water column verses leaf surfaces, and 3) *A. albopictus* may drive down the total available fungal resources to a level below which the competing species cannot maintain comparable productivity.

Understanding the mechanism driving competitive advantages is of particular importance when one species is capable of competitively displacing the other, as in the case of *A. aegypti*, displaced by *A. albopictus* in many habitats located in the southern United States (see in Lounibos et al. 2001, Juliano and Lounibos 2005). In situations of invading species, displacement of native mosquito populations may carry the risk increased dissemination of disease if the invasive species is a superior bridge vector. This is true of *A. albopictus* and *Oc. triseriatus*, where although the latter has the potential to serve as a bridge vector, its preference for feeding on mammalian hosts makes *Oc. triseriatus* a less competent vector of diseases such as West Nile Virus than the more opportunistic feeding of *A. albopictus* (Turell et al. 2005). Livdahl and Willey (1991) indicate displacement of *Oc. triseriatus* in man-made containers by *A. albopictus* is likely where the two co-occur. Because the range of *A. albopictus* does not extend to Michigan, *Oc. triseriatus* has remained relatively free from competition with the occasional exception of native *Culex* spp. This interaction has not, however, resulted in the

displacement of *Oc. triseriatus* from their habitat. In contrast, *Oc. japonicus* larvae have been concurrently found with *Oc. triseriatus* in Michigan during 2005 and 2006 (personal observation). If it were to occur, competitive displacement of *Oc. triseriatus* poses a threat to human health because *Oc. japonicus* is a superior vector of West Nile Virus and is the only mosquito in Michigan with the capacity to vector Japanese encephalitis (U. S. Centers for Disease Control and Prevention 2006). Evaluations of differential resource usage by container-dwelling mosquitoes under interspecific and intraspecific conditions may therefore provide not only a novel example of a complex trophic interaction, but would also open the possibility for a practical means of relieving displacement through the manipulation of specific microbial groups.

CHAPTER 6

INTERSPECIFIC MICROBIAL RESOURCE UTILIZATION IN *AEDES ALBOPICTUS* AND *AEDES AEGYPTI*

Introduction

Container habitats are complex communities containing a diversity of macroinvertebrate and microbial fauna. The dynamics of macroinvertebrate interactions have been the focus of a large body of research, particularly due to the association of such habitats with mosquito species. Nearly 40% of mosquito species utilize container habitats, which include tires, tree holes, and commentary vases (Laird 1988). Among these species are many important vectors of arboviruses, such as LaCrosse encephalitis, yellow fever, dengue, and West Nile Virus. The food webs within container habitats are generally driven by a variable milieu of allochthonous inputs, characterized by leaf and animal detritus, and stemflow (Kitching 2001). Historically, the contribution of such inputs has served as the basis for understanding the magnitude of mosquito production from containers. Previous studies have indicated that the quantity and quality of detrital resources, particularly leaf material, are of utmost importance to mosquito development (Lounibos et al. 1992, Walker et al. 1997). It has become increasingly clear, however, that the micrbiota supported by allochthonous inputs play an intermediate role in container food webs, ultimately driving mosquito production.

The community dynamics of microorganisms associated with the water column and detrital leaf surfaces have been assessed predominately via measurements of bacterial abundance and productivity and fungal biomass (Kaufman et al. 1999, 2001, 2002, 2006), although more recently bacterial and fungal taxonomic shifts have been identified t 16S and 18S rRNA gene sequence analyses (Kaufman et al. 2008, Xu et al. 2008). These studies demonstrated that larval feeding reduces the abundance and productivity of

bacterial leaf surfaces, while also changing the structure of both fungal and bacterial communities associated with leaf surfaces.

The response of microbial communities to the presence of mosquito larvae has been limited to studies of the Eastern tree hole mosquito, *Ochlerotatus triseriatus* (Say). Although *Oc. triseriatus* often occur as intraspecific populations in the Northern portion of their range, more often multiple mosquito species co-occur as a result of their overlapping ranges and container preferences. Such is the case with two important vector species, *Aedes albopictus* and *Aedes aegypti*. The competitive interactions underlying the distribution of these species have been the subject of much interest given the capacity of both species for exerting conditional displacement of the other under interspecific competition. Several studies have shown that declines in North American populations of *A. aegypti* correlate to the appearance of *A. albopictus* (O'Meara et al. 1993, 1995, Hobbs et al. 1991, Hornby et al. 1994, Mekuria and Hyatt 1995). In contrast to urban habitats wherein co-occurrence may take place, displacement of *A. aegypti* frequently occurs in suburban or rural habitats (Hornby et al. 1994, O'Meara et al. 1995). Juliano (1998) showed that the decline of *A. aegypti* is probably driven by interspecific resource competition among larvae. Under resource limited conditions, *A. albopictus* develop faster and attain a greater body size that do *A. aegypti* when the species co-occur (Barrera 1996, Juliano 2004); however, this effect was mitigated when interspecific populations were provided with abundant resources (Daugherty et al. 2000).

Apparent competition has been suggested as a mechanism driving the competitive outcomes and may occur via characteristics exhibited during non-competing life stages. For example, condition-specific responses to fluctuating abiotic conditions are

responsible for the outcome of competition between *Aedes aeqypti* and *A. albopictus*. The latter exhibits a competitive edge over *A. aegypti* under wet (normal) conditions, sustaining greater population growth at low resource levels (Costanzo et al. 2005). Under dry environmental conditions, however, the competitive advantage shifts to *A. aegypti* due to their comparatively higher tolerance of eggs to desiccation.

Differences in modes of feeding between the two species may be responsible for the competitive advantage exhibited by *A. albopictus*. Although we do not attempt to quantify such differences here, relative differences in amount of filter feeding versus surface grazing should be reflected in the microbial communities associated with these substrates under interspecific and intraspecific conditions. In addition, the capacity of *A. albopictus* to reduce populations of overall microbial abundance, or the abundance of individual microbial taxa, below a level that will sustain *A. aegypti* may also contribute to the outcome of interspecific competition.

In this paper, we describe the response of microbial communities to the presence of *A. albopictus* and *A. aegypti* alone or under interspecific competition. Specifically, the objectives were to determine: 1) whether shifts in the structure of microbial communities occur in response to overall mosquito density? or 2) if the composition of mosquito communities in containers has a specific impact on the dynamics of microorganisms that contributes to the displacement of *A. albopictus* by *A. aegypti?* If general resource abundance is responsible for the success of *A. albopictus*, we predict that microbial community composition would not respond differently when the species compete, whereas we predict that if larval competition is dependent on the structure of microbial

communities, changes in the relative abundance of microbial community members should be apparent under interspecific verses intraspecific conditions.

Materials and Methods

Microcosms. We investigated the microbial dynamics underlying interspecific competition between *A. albopictus* and *A. aegypti* in laboratory microcosms simulating natural container habitats. To construct microcosms, senescent red oak leaves (*Quercus rubra* L.) were obtained from Michigan State University's Kellogg Forest (Augusta, MI), dried at 45°C for 48 h, and added to containers as 1.0 g leaf packs. Three days prior to the addition of mosquitoes, 500 ml deionized water and a 3 ml of microbial inoculums were also added to the containers. The microbial inoculums consisted of composite water and particulates randomly sampled from tree holes located in woodlots near the Michigan State University campus (East Lansing, MI) and homogenized in a standard kitchen blender. After allowing microcosm microbiota to condition for three days, newly hatched first instar *A. albopictus* and *A. aegypti* were added to microcosms (day). Larvae were procured from our laboratory colony, originally obtained from the Malaria Research and Reference Reagent Resource Center (Manassas, VA). Larvae were added in total densities of 30 or 60 total larvae with the following rations of *A. aegypti*: A. *albopictus*: 0:30, 30:0, 15:15, 30:30, 0:60, and 60:0. In addition, no larvae microcosms were included to assess the overall affect of mosquitoes on microbial community dynamics. Twelve replicates were constructed per treatment combination, to allow for destructive sampling of six replicate microcosms on day 7 and day and day 14. These dates were chosen to obtain measurements of microbial activity during the time the majority of larval

development occurred. Throughout the experiment, microcosms were held in an environmental chamber at 28° C and 16L: 8D.

Sampling. Microcosms were monitored daily for mosquito adults. After eclosing, adults were collected, sexed and identified before undergoing liophilization for subsequent dry mass measurement. Larvae remaining in microcosms upon destructive sampling were collected and similarly identified, lyophilized and massed; identifications to species were not made below the third instar, nor were larvae distinguished by sex, due to the difficulty of observing relevant morphological characters. In addition to mosquitoes, samples of leaf material and microcosm water were taken on day 10 and day 17for analysis of microbial community structure and bacterial productivity (described below). Leaf samples were aseptically obtained using a sterilized cork borer (11 mm), placed in filter-sterized phosphate-buffered saline, and sonicated on ice for 12 min. to dislodge the loosely-attached fraction of microorganisms. Leaf sonicates were utilized rather than whole-leaf extracts because this fraction represents microorganisms physically available to grazing mosquito larvae.

Bacterial production. Accumulation of bacterial biomass in water column and leaf sonicate microcosm samples was directly measured via quantifications of ^{3}H-leucine incorporation (Kirchman 2001). Using bacteria-specific nanomolar leucine concentrations of 25 nM (Smith and Azam 1992), we measured the incorporation of the labeled amino acid following incubations of the samples with ^{3}H-leucine (L-leucine (4,5-^{3}H), 50 CI/mmol- NEN, Life Science, Boston, MA). Unlabeled leucine was added with labeled leucine, bringing the total leucine concentration in leaf and water samples to 400 and 100 nM, respectively, to account for saturation of uptake kinetics (Kirchman 2001,

Kaufman et al. 2001). Following incubation of samples at 20°C for 20 min, trichloroacetate (TCA; 10% vol:vol, final concentration) was added to precipitate protein and terminate incorporation of additional leucine. Two additional TCA rinses and a single ethanol (80%) rinse were used to precipitate additional protein. Finally, samples were suspended in scintillation cocktail for quantification of radioactivity with a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Fullerton,CA).

Microbial community analysis. *T-RFLPs.* The structure of leaf- and water column-associated bacterial and fungal communities in microcosms was assessed using tagged restriction fragment length polymorphism (T-RFLP) analysis (Marsh 1999). DNA was extracted from the samples described above with the MoBio UltraClean soil extraction kit (Carlsbad, CA). After ensuring the success of extractions via PCR and agarose gel electrophoresis, sample DNA was amplified using labeled (5'-FAM) fungal (18S rDNA) (Borneman and Hartin 2000) and bacterial (16S rDNA) (Marchesi et al. 1998) primers in independent PCR reactions. The primers used for fungal and bacterial amplifications were, respectively: nu-SSU-0817-59F (5'-TTAGCATGGATAATRRAATAGGA-3'), nu-SSU-1536-39R (5'- TTGCAATGCYCTATCCCCA-3'); and 63F (5'-CAGGCCTAACACATGCAAGTC-3'), 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Integrated DNA Technologies, Coralville, IA). Co-purifications of products from three 100 ul PCR reactions per primer set were conducted using the Qiaquick PCR purification Kit (Valencia, CA). Subsequently, PCR products were subjected to digestion with the restriction enzyme, MspI (New England Biolabs, Cambridge, MA) according to the methods described by Marsh (1999). Samples were then submitted to the Michigan State University Research and Technology Support Facility for electrophoresis of digested

samples on a capillary electrophoresis genetic analyzer (PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA). Size and frequencies determinations for each terminal restriction fragment were determined using Genescan Analysis software (version 3.7, Applied Biosystems). Only samples with combined peak areas of 10,000 or greater were retained for subsequent analyses.

Statistical Analysis. T-RFLP data from 16S and 18S rRNA gene communities were subjected to principal component analysis (PCA) using R (R Development Core Team 2004). The principal components (PCs) , derived from the linear combination of peak area abundances for individual fragments, were analyzed by standard analysis of variance (ANOVA) to evaluate the main effects of mosquito species (interspecific or intraspecific populations), sampling date (day 7 or day 14), and larval density (0, 30, or 60). Mosquito production values (mass and development time for males and females) and bacterial productivity (per microcosm) were also analyzed by ANOVA, followed by Bonferroni adjustment of experiment error from $\alpha = 0.05$. Following the ANOVA, Tukey's HSD post hoc test was used for separation of treatment means. Main effects contributing to mosquito production were limited to species and density, as only mosquitoes obtained from microcosms retained until the end of the experiment (day 24) were included in the analysis.

Results

Mosquito production. For both mosquito species, male adult mass was significantly affected by mosquito treatment (Table 6.1); however, in both cases male mass responded to the overall effect of density rather that species composition, as mosquitoes at low density (30 larvae per microcosm) attained greater average body mass that mosquitoes in

high density treatments (60 larvae per microcosm) (Figure 1 and 2). A similar trend is suggested for female mosquitoes of both species, although only changes in *A. aegypti* adult female body mass were significant following Bonferroni correction (Table 6.1, Figure 6.2). At high total mosquito densities (alone or with *A. albopictus*), females body mass was significantly reduced compared with low density treatments in that only microcosms containing low mosquito densities produced adult females.

The time for development of *A. albopictus* from larvae to adults was not significantly impacted by mosquito species, and this effect was consistent between males and females (Table 6.1, Figure 6.1). In contrast, male and female *A. aegypti* development time was significantly affected by the absence of adult emergence under high density treatments, when the species was alone or under interspecific competition (Table 6.1, Figure 6.2).

Table 6.1. ANOVA results for mosquito production variables.

Response Variable	Source	*Aedes albopictus*			*Aedes aegypti*		
		df	*F*	*P*	df	*F*	*P*
Male Mass	Species	1,26	3.21	0.08	1,21	1.15	0.3
	Density	1,26	46.07	<0.0001*	1,21	98.6	<0.0001*
Male development	Species	1,26	0.01	0.94	1,21	1.44	0.24
	Density	1,26	5.73	0.02	1,21	65.79	<0.0001*
Female Mass	Species	1,26	2.53	0.12	1,21	3.2	0.09
	Density	1,26	9.04	0.006*	1,21	13.87	0.001*
Female development	Species	1,26	0.53	0.47	1,21	2.79	0.11
	Density	1,26	4.75	0.04	1,21	15.16	0.0007*

*Indicates significance following Bonferroni adjustment. Interactions between species and density not shown in table were not significant ($p<0.05$).

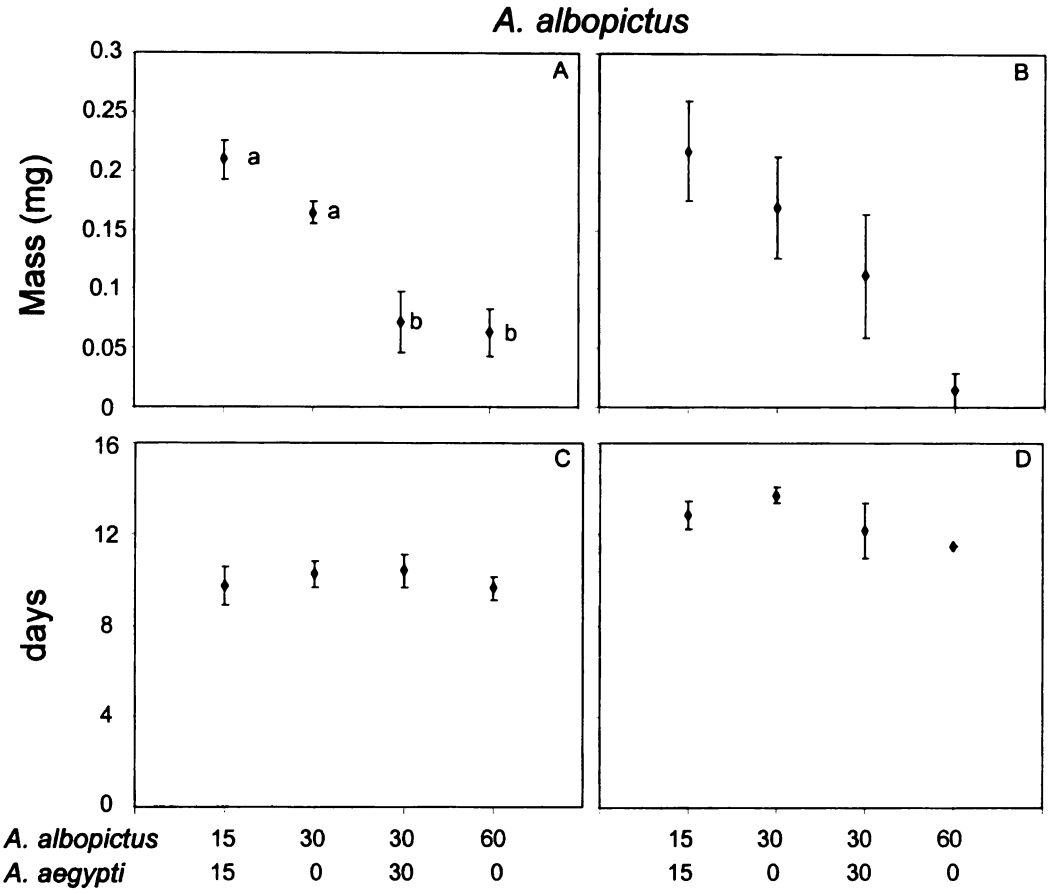

Figure 6.1. Mosquito production variables for *A. albopictus* males (A and C) and females (B and D). Average mosquito weight (A and B) and development time (C and D) are shown as means ± SE. n = 6 for variables in A and B, n = 2-6 for variables in C and D.

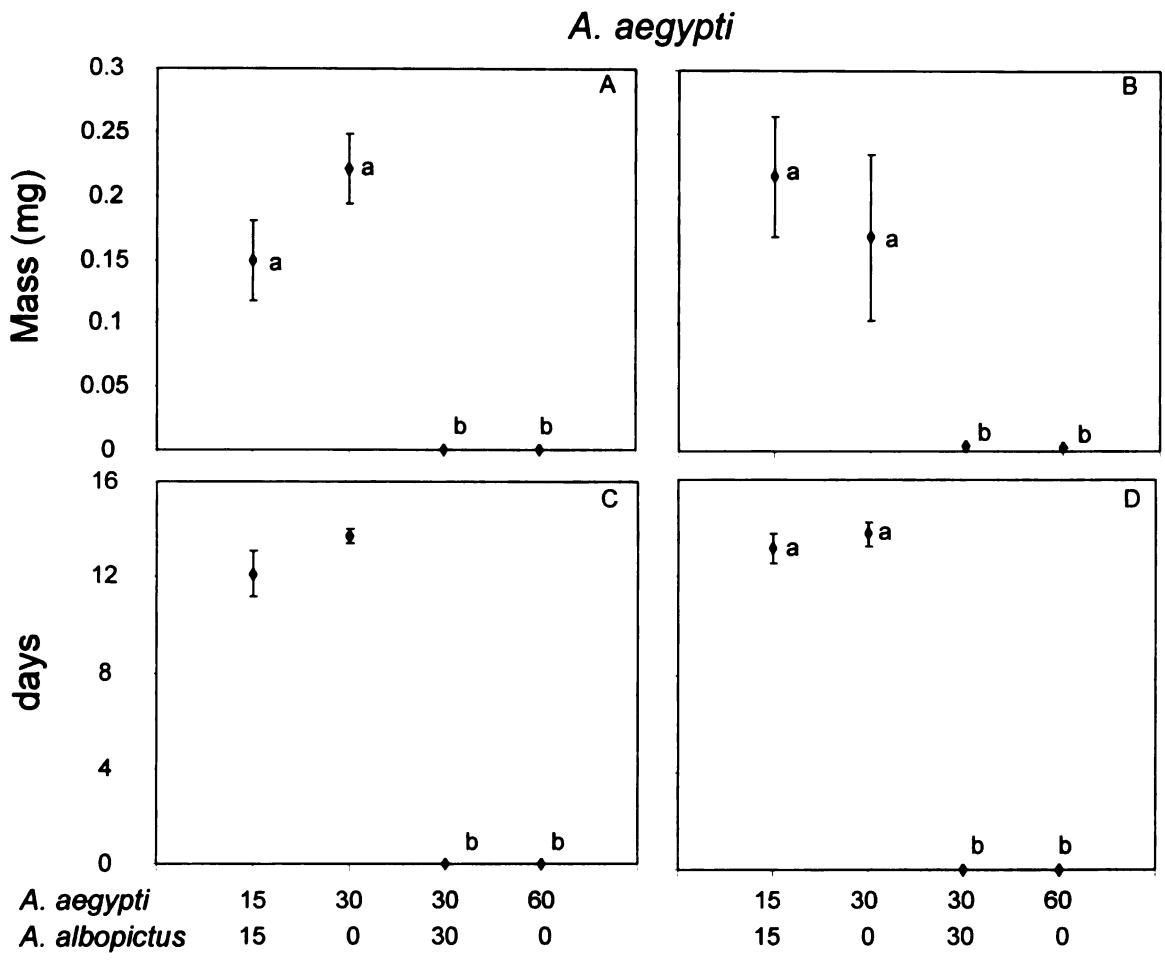

Figure 6.2. Mosquito production variables for *A. aegypti* males (A and C) and females (B and D). Average mosquito weight (A and B) and development time (C and D) are shown as means ± SE. n = 6 for variables in A and B, n = 0-6 for variables in C and D. Treatments means with different letters are significantly different following Bonferroni correction for experiment wide error.

Bacterial productivity. Bacterial production on leaf surfaces responded significantly to the main effect of mosquito species composition on day 7 of the experiment; however, this effect was not apparent on day 14 (Table 6.2). In contrast, the affect of mosquito species composition on bacterial production in the water column was significant on day 14, but not day 7. The significance of species in this case likely a result of the significant interaction of species and density on bacterial productivity, as production of bacteria was remained high in microcosms without mosquitoes on day 14, but was comparably lower when mosquito larvae were present. A similar significant interaction occurred for bacterial production associated with leaf surfaces. For both substrates (leaf and water column), mosquito density did not significantly affect the production of bacteria on day 7 or day 14.

Table 6.2. ANOVA results for bacterial productivity.

	Source	Water			Leaf		
		df	*F*	*P*	df	*F*	*P*
Day 7	Species	3	0.53	0.08	3	12.79	<0.0001*
	Density	1	0.08	0.78	1	1.08	0.31
	Species*Density	3	2.95	0.07	3	0.04	0.96
	Error	32			32		
Day 14							
	Species	3	8.41	0.0003*	3	1.52	0.22
	Density	1	0.39	0.53	1	2.99	0.09
	Species*Density	3	5.45	0.008*	3	10.73	0.0002*
	Error	34			34		

*Indicates significance following Bonferroni adjustment. Species treatment refers to the effect of mosquito species composition on bacterial production. Interactions between species and density not shown in table were not significant ($p<0.05$).

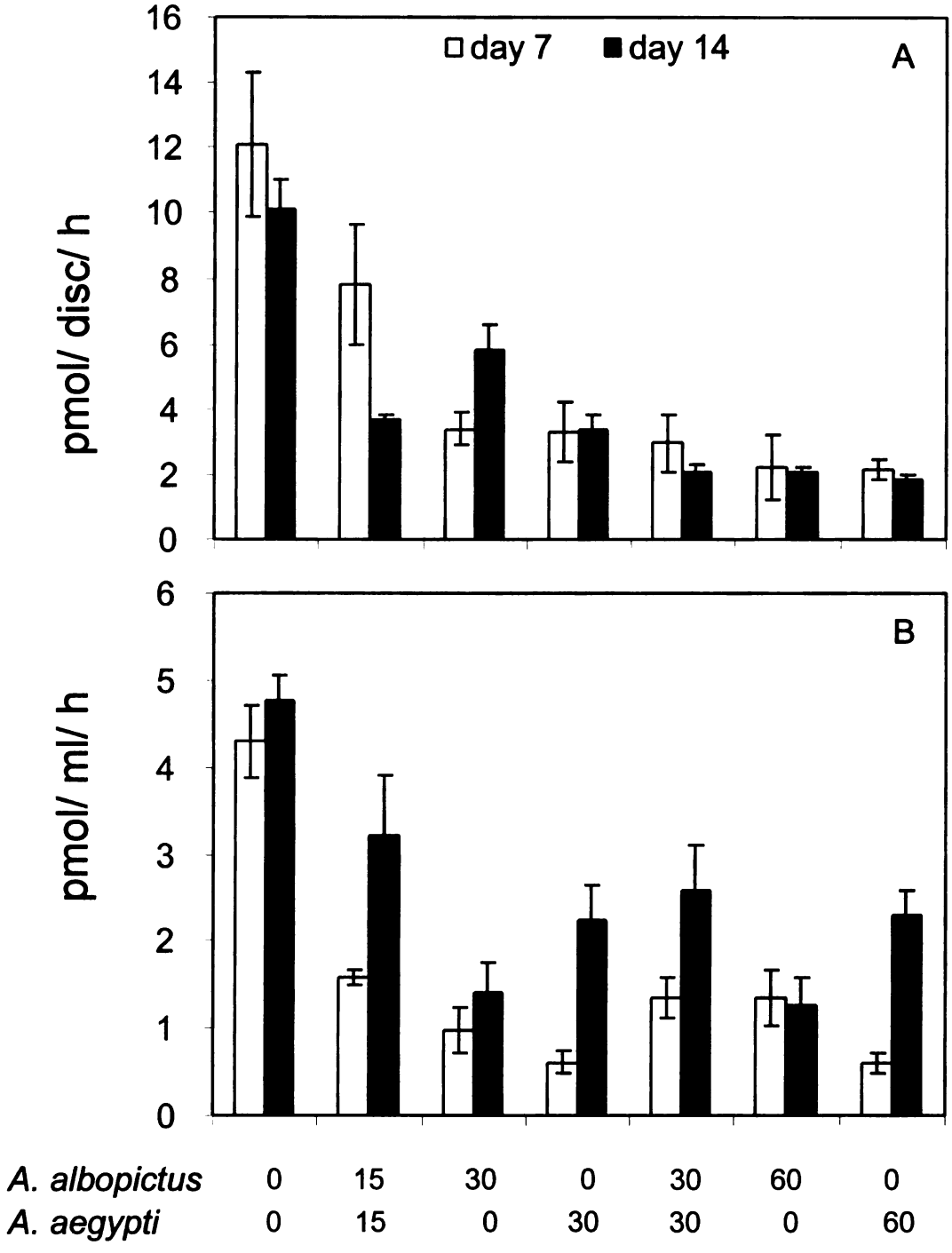

Figure 6.3. Bacterial productivity in microcosms on day 7 and day 14 following larval addition. (A) Leaf surface. (B) Water column. Values are means ± SE (n=5-6).

T-RFLPs. Using PCA to derive new variables representing linear combinations of individual microbial populations represented by T-RFLP fragments, we determined that microbial communities responded significantly to the main effects in this experiment. Bacterial communities on leaf surfaces changed significantly between days 15 and 30, as indicated by the significant affect of time on PC's 1 and 2 (Table 6.3, Figure 6.4). The main effect of competition type combination did not significantly affect the structure of bacterial communities along either PC axis; however, PC1 representing leaf-associated bacterial communities was significantly affected by the density of mosquitoes in microcosms (Table 6.3). Although bacteria in water column samples obtained from microcosms were not sufficient to permit robust univariate analysis of treatment effects on principal component axes, PCA revealed that the overall affect of larval presence was sufficient to alter the structure of bacterial communities (Figure 6.5).

Of the two mosquito species, only *A. aegypti* presence had a significant affect on leaf-associated fungal communities represented by PC1 (Table 6. 4, Figure 6.6). Density, on the other had, exerted a significant affect on fungal communities represented by PC2 in microcosm containing both species, and by PC1 in microcosm containing *A. aegypti*. In addition, the main effect of time on PC1 was significant in microcosms in which *A. albopictus* appeared, although a similar significant effect was not apparent for *A. aegypti*.

Table 6.3. Summary of ANOVA results for principal component (PC) scores from leaf surface bacterial (16S rRNA gene) T-RFLP profiles.

		PC1		PC2	
Source	df	*F*	*P*	*F*	*P*
Competition type	2	8.05	0.006	1.98	0.15
Density	1	1.04	0.36	0.71	0.40
Time	1	22.39	<0.0001	14.31	0.0004
Competition*Density	2	0.19	0.83	1.11	0.34
Competition*Time	2	0.33	0.72	0.14	0.71
Density*Time	1	0.14	0.71	0.42	0.66
Competition*Density*Time	2	0.41	0.67	1.38	0.26
Error	59				

Table 6.4. Summary of ANOVA results for principal component (PC) scores from leaf surface fungal (18S rRNA gene) T-RFLP profiles.

		PC1		PC2	
Source	df	*F*	*P*	*F*	*P*
Competition type	2	7.22	0.002	0.66	0.52
Density	1	5.97	0.02	17.24	0.0002
Time	1	0.00	0.98	12.93	0.001
Competition*Density	2	5.65	0.008	0.16	0.85
Competition*Time	2	0.92	0.41	4.29	0.02
Density*Time	1	0.64	0.43	0	0.96
Competition*Density*Time	2	0.88	0.36	5.52	0.02
Error	59				

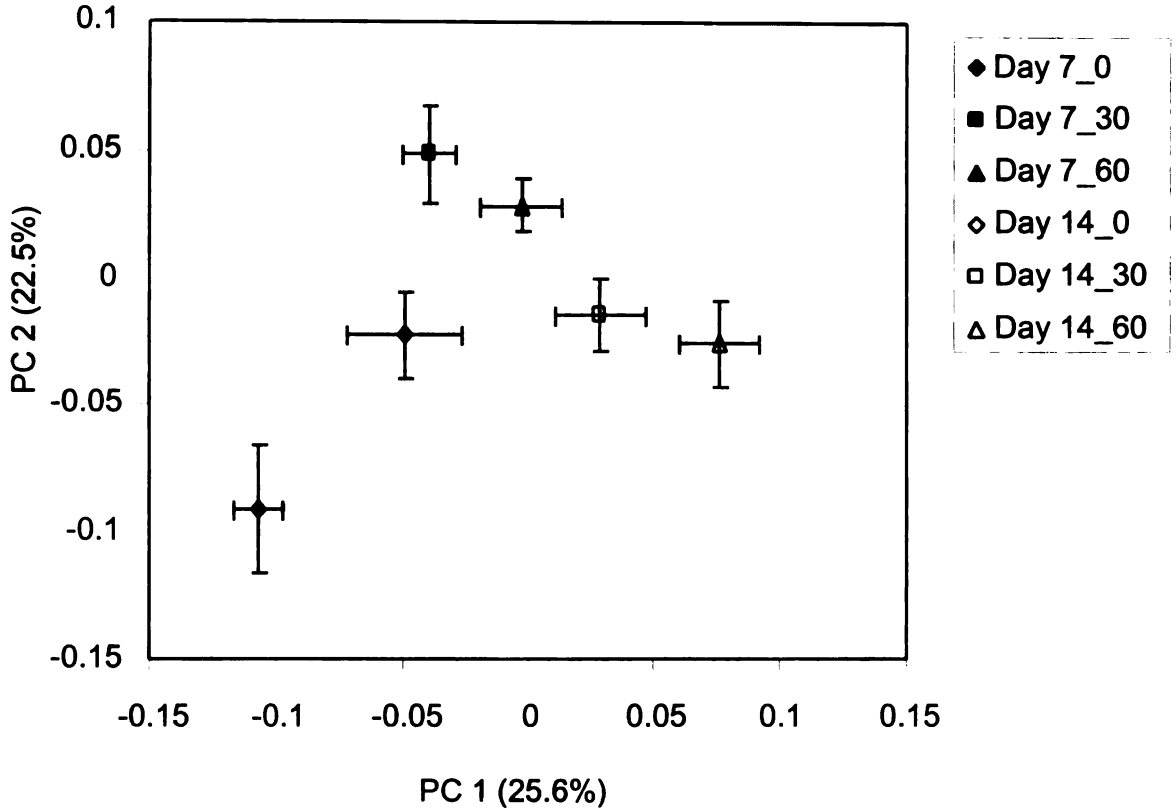

Figure 6.4. PCA of TRFLP fragment peak areas from leaf surface bacterial (16S rRNA gene) communities. Total density of mosquitoes (0, 30, or 60) are compared for each sampling date (Means ± SE). The two component axes explain 48.1% of the variation.

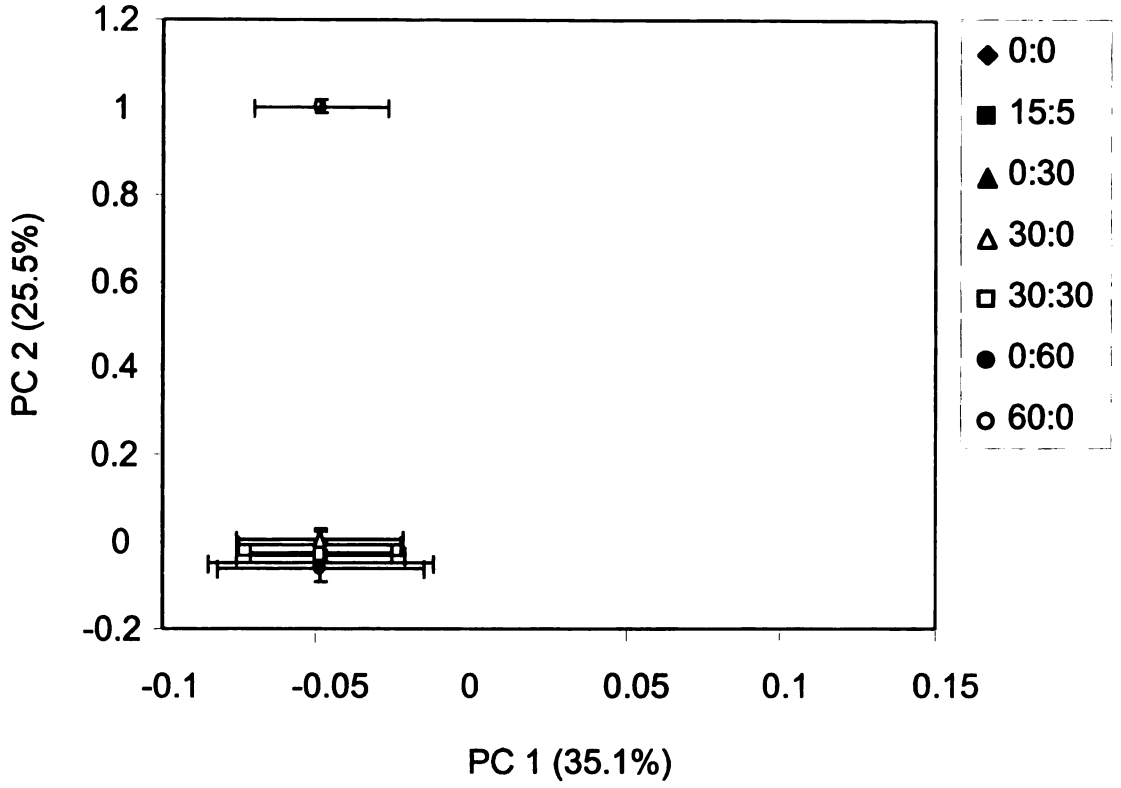

Figure 6.5. PCA of TRFLP fragment peak areas from water column bacterial (16S rRNA gene) communities. Individual *A. albopictus*:*A. aegypti* treatments are compared (Means ± SE). The two component axes explain 60.6% of the variation.

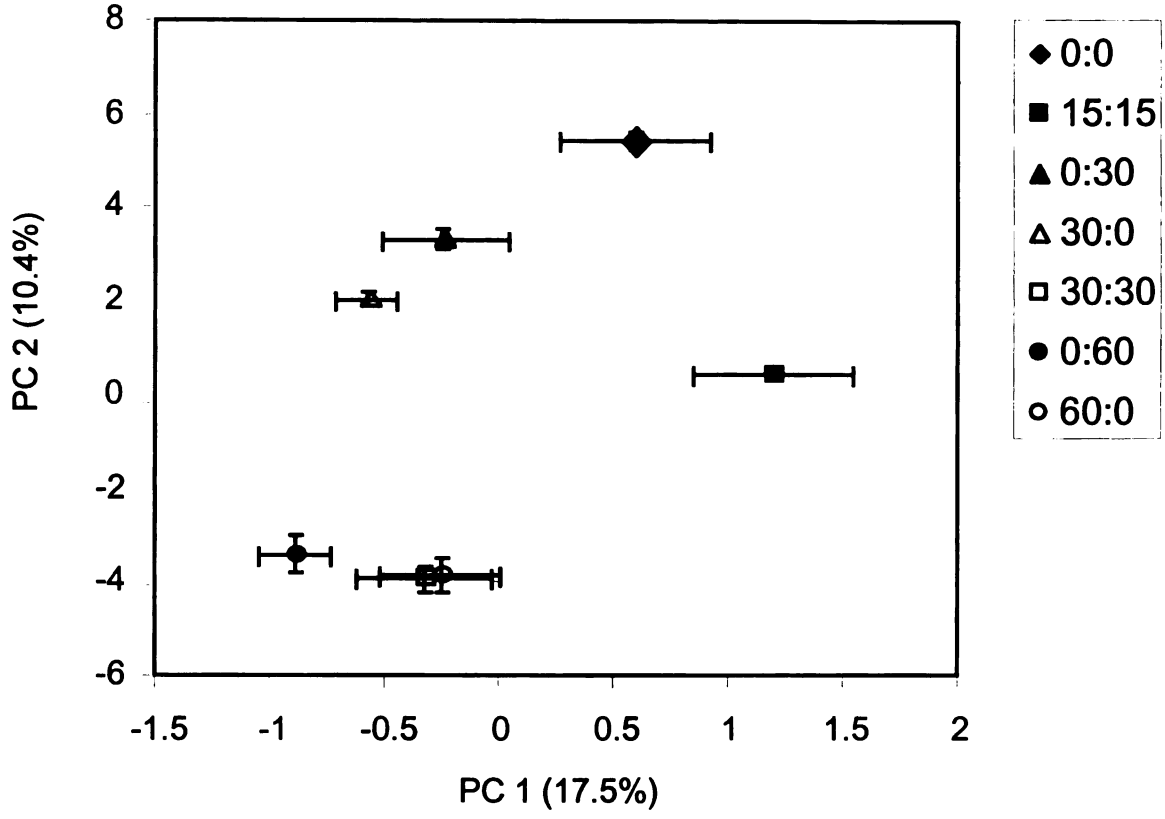

Figure 6.6. PCA of T-RFLP fragment peak areas from leaf surface fungal (18S rRNA gene) communities. Comparisons of individual *A. albopictus*: *A. aegypti* treatments are shown (Means ± SE). The two component axes explain 27.9% of the variation.

Discussion

Performance of mosquito populations in microcosms was consistent with findings from several key studies which defined the nature of competition between *A. albopictus* and *A. aegypti* (Barrera 1996, Juliano 1998). Although both species fared poorly under high density and hence, low resource treatments, adult *A. albopictus* production continued through the experiment. Male and female *A. aegypti*, in contrast, failed to emerge from each high density treatment. Thus, *A. aegypti* was more susceptible to negative effects conferred by high densities than *A. albopictus*.

Interestingly, despite prior studies to the same effect in nature, this is the first study to describe *A. albopictus* success over *A. aegypti* in a microcosm environment. Previous microcosm studies found *A. aegypti* to be a superior competitor in laboratory studies (Moore and Fisher 1969, Ho et al. 1989, Black et al. 1989); however, in these experiment, artificial diets were employed rather that leaf material microorganisms as the resource base. As in the current experiment, Juliano (1998) utilized plant detritus in field experiments evaluating competitive outcomes between the two species. Finding that *A. albopictus* was competitively superior to *A. aeqypti* at high densities, he postulated that the different resources used in these studies promoted differential outcomes when the two species compete. Hence, clearly defining the resource utilized by competing mosquitoes is essential to understanding competitive outcomes. While the important of resource type has been illustrated by the above cases, subsequent studies have not addressed mosquito-mediated changes at the level of microorganisms. If we consider that mosquitoes consume microorganisms as a food resource in order to access the nutritional reserves

provided by plant detritus, then we would expect that mosquito feeding may affect microbial communities differentially in a species-specific manner.

The microcosms in this study were designed to describe the dynamics underlying bacterial and fungal communities associated with mosquito species competition. Although the use of 16s rRNA gene sequence data has been used previously to describe microbial populations, the technique is of limited use in replicated experimental designs for economic and practical reasons. The T-RFLP technique, by comparison, permits a finer level of taxonomic resolution for a large number of samples, wherein each fragment defined can be construed as a unique "species." The results obtained from principal component analysis of variables derived from the relative abundance of microbial rRNA gene fragments, it is apparent that mosquito species indeed change the constitution of bacterial and fungal communities associated with leaf material and, in the case of bacterial communities, the water column.

Regardless of the mosquito species composition represented in microcosms (e.g. interspecific or intraspecific populations), density appeared to be the most important factor contributing to variation in bacterial communities. Both leaf-associated and fungal communities exhibited changes that are undoubtedly related to the effects of mosquitoes grazing down populations of microorganisms. Indeed, that a time effect was evident from day 7 to day 14 is consistent with the hypothesis that the microbial community decreases over time in the presence of larvae. That mosquito presence affects microorganisms has been clearly determined by previous microcosm studies which report not only reduction of biomass but also shifts in community composition (Kaufman et al. 1999, 2001, 2002, 2006, 2008).

In the current study, we have sought to determine whether mosquito feeding alters microbial communities in a species-specific manner. We found that the composition mosquitoes present only affected leaf-associated bacterial communities when intraspecific *A. aegypti* populations were compared with interspecific mosquito communities. Although a marginal effect of species composition was also found in comparisons of intraspecific and intraspecific *A. albopictus* communities, the effect was not significant following a Bonferroni adjustment for multiple comparisons. Thus, the shift in fungal structure only when *A. albopictus* are present suggests that they may differentially altering the fungal community on leaf surfaces compared with *A. aegypti*. The mechanism for this effect is unclear, although we postulate that it stems from differences in the development of the species under larval competition. Kaufman et al. (2008) suggested that *Oc. triseriatus* larvae at multiple stages of development may differentially alter fungal communities in loosely attached verses adherent fractions of leaf material. Also, in stream communities, use of diatom communities by different macroinvertebrate groups was in part a function of consumer size (Tall et al. 2006). Because overall mosquito feeding contributes to changes in microbial communities, we suspect that a faster developing species, such as *A. albopictus*, should affect such changes more rapidly than *A. aegypti*, resulting in the difference fungal communities when *A. albopictus* are present. A similar result would be expected in intraspecific mosquito populations as well if a comparison was made between a population comprised of a single cohort (that is, the same instar) and a mixed population of early and late instar larvae.

In the past, our research group has demonstrated that mosquito feeding impacts the microbial community in tree holes. The changes wrought by larval activity have manifest as decreases in the abundance of bacterial and fungal biomass, and bacterial growth rates associated with leaf material, although changes in fungal biomass have been less evident (21-24). In addition to these "big picture effects," changes in the structure of microbial communities have also been evident in response to larval feeding. Shifts in major taxonomic groups are reflected in both bacterial and fungal communities associated with leaf material in tree holes. Evidence from 16S rRNA gene sequence analysis suggests that larval presence selectively effects members of the Betaproteobacteria, Alphabroteobacteria, and Bacteriodetes bacterial classes, resulting in depression of abundance in the latter two groups and enhancement in the former (Kaufman et al.2008). Similarly, Dothideomycetes and Saccharomycete fungal taxa decreased in response to larval feeding, while the abundance of Chytridiomycota members increased. Recently, we have utilized T-RFLP analysis to evaluate larval feeding effects on microbial communities in a fashion that permits both replication and greater resolution of microbial taxa (Pelz-Stelinski, Chapter 2). Compared with the results obtained from sequence analysis, T-RFLP results reflected a considerable amount of variation among replicates such that treatment differences were difficult to resolve. Indeed, variation in among treatments is also characteristic of sequence analysis carried out at fine resolution (Pelz-Stelinski, Chapter 2 and Kaufman et al. 2008); however, as the taxonomic level of resolution chosen for community analysis becomes increasingly broad (i.e. genus level verses class level), larval feeding effects become apparent. This may suggest that the level of resolution provided by T-RFLPs, that is to say "species-

level" (Marsh 1999), may not be biologically relevant from the mosquito perspective. The current findings suggest that the effect of combined mosquito density drives shifts in the structure of microbial communities rather than individual species. We postulate that, in addition to other possible factors regulating resource-based competition between the two species, either or both of the following mechanisms might also be possible: 1) microbial groups shift in response to mosquito feeding, but at a higher level of taxonomic resolution not evident here; or 2) mosquito species differ in their physiological or behavioral capacity for assimilating/harvesting the nutritional resources provided by microorganisms.

GENERAL CONCLUSIONS

In a series of experiments designed to evaluate the effects of detrital leaching on *Oc. triseriatus* productivity, I determined that the soluble fraction of leaf detritus initially released into container habitats is significantly associated with higher bacterial production and greater mosquito success relative to containers containing leaves from which the soluble component was removed. In addition, mosquito performance was less robust when the leached fraction of leaves was provided in the absence of leaves, although mosquito productivity in response to this treatment was still better than when leached leaves alone were provided for developing larvae.

Several studies in this dissertation employed terminal restriction fragment polymorphism (T-RFLP) analysis to obtain profiles of the fungal and bacterial communities associated with container habitats. Comparisons of field and laboratory container habitats showed that, in addition to temporal fluctuations, the composition of microbial communities change in response container type and mosquito density. Shifts in the structure and dynamics of container-associated microbial communities were also evident in response to macroinvertebrate species composition. Although the composition of fungal communities exhibited a significant shift only in response to *Oc. triseriatus* density, more fungal decomposition enzyme activity was associated with presence of scirtid beetle larvae, which are known to facilitate the production of mosquitoes from tree holes. Data from terminal restriction fragment polymorphism (T-RFLP) analysis suggested that scirtid presence influenced bacterial communities associated with leaf material and the water column. Furthermore, I showed increased processing of leaf detritus, higher leaf-associated enzyme activity, and higher leaf-associated fungal biomass was due to scirtid presence. Such shifts suggest beetle feeding facilitates

mosquito production indirectly through the microbial community rather than directly through an increase in available fine particulate organic matter (FPOM).

Different species often utilize similar available food resources, but show differential growth under competitive conditions. Using experimental microcosms, I examined the effect on microbial community dynamics and community structure in response to competition between *A. albopictus* and *Oc. triseriatus*, and between *A. albopictus* and *A. aegypti*. I postulated that leaf- and water column-associated microbial community structure and abundance would differ in microcosms containing interspecific mosquito populations compared with intraspecific populations. Patterns of T-RFLPs obtained from microcosm studies suggested differences in the diversity microbial community under conditions of interspecific versus intraspecific competition. The hypothesis that mosquito species exhibit a differential ability to survive at low microbial-resource levels and/or to digest the microorganisms available was supported by changes in the leaf and water column-associated microbial community structure, productivity, and abundance under interspecific competition between *A. albopictus* and *Oc. triseriatus* and between *A. albopictus* and *A. aegypti*.

The studies described in this dissertation cumulatively illustrate changes in the structure of fungal communities associated with mosquito presence. Shifts in the fungal community were evident in response to mosquito species composition and overall mosquito density. Although the importance of these shifts to developing mosquitoes has not been determined, it is clear that In addition, these studies illustrate the dependence of microbial dynamics on the initial microbial inoculum present in container habitats. Differences among container habitat types varied between field and laboratory

experiments, suggesting the need for further research into the relevance of particular microbial assemblages to mosquito development. In addition, future work is should assess the response of protozoan communities to mosquito presence. Although not a component of these studies, it is likely that protozoans groups will also exhibited shifts under the regimes evaluated in this dissertation, as they are similar to bacterial and fungal communities in their role as a nutritional resource for developing mosquito larvae.

APPENDIX 1.

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

Appendix 1.1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2008-05

Title of thesis or dissertation (or other research projects):

MOSQUITO PRODUCTION AND MICROBIAL DIVERSITY IN CONTAINER HABITATS

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)
Kirsten Pelz-Stelinski

Date April 25, 2008

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America.
Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix 1.2

Voucher Specimen Data

Page_1_of_2_Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Ochlerotatus japonicus (Theobald)	Michigan Saginaw Co. Saginaw 15 September 2006 oviposition trap		10						MSU
Aedes albopictus (Skuse)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony		10						MSU
Aedes albopictus (Skuse)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony					10			MSU
Aedes albopictus (Skuse)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony						10		MSU
Aedes aegypti (Linnaeus)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony		10						MSU
Aedes aegypti (Linnaeus)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony					10			MSU
Aedes aegypti (Linnaeus)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony						10		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)
Kirsten Pelz-Stelinski

Date 25-Apr-08

Voucher No. 2008-05
Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date 4/25/2008

Appendix 1.2

Voucher Specimen Data

Page 2 of 2 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Ochlerotatus triseriatus (Say)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony		10						MSU
Ochlerotatus triseriatus (Say)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony					10			MSU
Ochlerotatus triseriatus (Say)	Michigan Ingham Co. East Lansing 29 March 2007 MSU campus lab colony						10		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)
Kirsten Pelz-Stelinski

Date 25-Apr-08

Voucher No. 2008-05
Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator Date

APPENDIX 2.

SUMMARY OF ANOVA RESULTS FOR PRINCIPAL COMPONENT ANALYSIS OF MICROBIAL COMMUNITIES ASSOCIATED WITH MICROCOSMS IN CHAPTER 3.

Appendix 2, Table 1. Summary of ANOVA results for PC1 scores from water column 16S rDNA T-RFs digests (Block I).

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Msp					
Mosquito	1	5.86	0.036	1.73	0.218
Scirtid	1	0.11	0.741	0.27	0.613
Time	1	21.06	0.001*	0.23	0.643
Mosquito x Time	1	2.58	0.139	0.33	0.577
Scirtid*Time	1	0.12	0.735	0.21	0.654
Mosquito*Scirtid	1	0.0	0.954	0.02	0.899
Error	10				
Hha					
Mosquito	1	1.05	0.328	0.04	0.838
Scirtid	1	10.69	0.008*	1.35	0.270
Time	1	0.01	0.941	0.01	0.930
Mosquito*Time	1	0.21	0.656	0.00	0.974
Scirtid*Time	1	0.05	0.827	1.68	0.222
Mosquito*Scirtid	1	0.08	0.786	12.94	0.004*
Error	11				

*Indicates p-values that are significant following Bonferroni adjustment

Appendix 2, Table 2. Summary of ANOVA results for PC1 scores from water column 16S rDNA t-RFs digests (Block II).

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Msp					
Mosquito	1	6.75	0.019	53.23	<0.001*
Scirtid	1	16.93	<0.001*	1.94	0.184
Time	1	14.03	0.002*	27.9	<0.001*
Mosquito x Time	1	0.50	0.489	0.42	0.529
Scirtid*Time	1	7.37	0.015	0.05	0.824
Mosquito*Scirtid	1	1.00	0.333	0.27	0.614
Error	15				
Hha					
Mosquito	1	0.90	0.358	8.67	0.01*
Scirtid	1	1.74	0.207	0.32	0.58
Time	1	0.00	0.978	0.00	0.966
Mosquito*Time	1	0.00	0.970	0.34	0.571
Scirtid*Time	1	0.56	0.465	0.54	0.473
Mosquito*Scirtid	1	0.95	0.354	0.62	0.444
Error	15				

*Indicates p-values that are significant following Bonferroni adjustment

Appendix 2, Table 3. Summary of ANOVA results for PC1 and PC2 scores from leaf surface 16S rDNA t-RFs digests (Block I)

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Msp					
Mosquito	1	2.94	0.106	0.51	0.486
Scirtid	1	1.92	0.185	0.02	0.895
Time	1	1.90	0.187	4.08	0.060
Mosquito x Time	1	1.65	0.217	0.41	0.532
Scirtid*Time	1	0.39	0.539	0.00	0.344
Mosquito*Scirtid	1	1.61	0.223	0.95	0.810
Error	16				
Hha					
Mosquito	1	3.23	0.094	0.43	0.521
Scirtid	1	0.61	0.450	1.47	0.245
Time	1	0.72	0.410	3.22	0.094
Mosquito*Time	1	0.02	0.896	0.00	0.953
Scirtid*Time	1	0.00	0.971	6.63	0.022
Mosquito*Scirtid	1	0.74	0.736	0.34	0.569
Error	14				

Appendix 2, Table 4. Summary of ANOVA results for PC1 and PC2 scores from leaf surface 16S rDNA t-RFs digests (Block II).

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Msp					
Mosquito	1	41.17	<0.001*	2.16	0.162
Scirtid	1	5.33	0.036	0.19	0.672
Time	1	8.04	0.012*	2.0	0.178
Mosquito x Time	1	1.39	0.257	0.60	0.449
Scirtid*Time	1	0.10	0.756	0.85	0.372
Mosquito*Scirtid	1	3.11	0.098	0.10	0.101
Error	15				
Hha					
Mosquito	1	1.85	0.194	3.12	0.098
Scirtid	1	5.09	0.039	3.10	0.099
Time	1	0.20	0.661	0.03	0.868
Mosquito*Time	1	3.03	0.102	0.54	0.475
Scirtid*Time	1	0.03	0.858	0.31	0.589
Mosquito*Scirtid	1	1.80	0.200	0.40	0.539
Error	15				

*Indicates p-values that are significant following Bonferroni adjustment

Appendix 2, Table 5. Summary of ANOVA results for PC1 and PC2 scores from water column 18S rDNA t-RFs digests (Block I).

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Hha					
Mosquito	1	10.02	0.016*	0.15	0.706
Scirtid	1	1.72	0.231	0.30	0.601
Time	1	1.45	0.267	8.05	0.025
Mosquito*Time	1	0.96	0.360	0.53	0.489
Scirtid*Time	1				
Mosquito*Scirtid	1	0.02	0.900	0.03	0.873
Error	7				

*Indicates p-values that are significant following Bonferroni adjustment

Appendix 2, Table 6. Summary of ANOVA results for PC1 and PC2 scores from water column 18S rDNA t-RFs digests (Block II).

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Msp					
Mosquito	1	1.46	0.246	37.95	<0.001*
Scirtid	1	3.09	0.099	0.86	0.368
Time	1	0.00	0.964	13.23	0.002*
Mosquito x Time	1	3.62	0.077	0.13	0.725
Scirtid*Time	1	1.01	0.331	0.07	0.798
Mosquito*Scirtid	1	1.76	0.204	2.27	0.153
Error	15				
Hha					
Mosquito	1	1.80	0.199	0.84	0.373
Scirtid	1	0.11	0.739	0.36	0.556
Time	1	0.10	0.758	6.37	0.023
Mosquito*Time	1	0.24	0.629	11.45	0.004*
Scirtid*Time	1	0.00	0.956	0.13	0.723
Mosquito*Scirtid	1	1.72	0.208	0.2	0.663
Error	16				

*Indicates p-values that are significant following Bonferroni adjustment

Appendix 2, Table 7. Summary of ANOVA results for PC1 and PC2 scores from leaf surface 18S rDNA t-RFs digests (Block I).

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Msp					
Mosquito	1	0.08	0.785	0.80	0.390
Scirtid	1	0.51	0.492	0.83	0.381
Time	1	0.02	0.879	1.98	0.187
Mosquito x Time	1	0.16	0.698	0.19	0.673
Scirtid*Time	1	0.73	0.412	0.00	0.994
Mosquito*Scirtid	1	0.44	0.521	0.00	0.955
Error	11				
Hha					
Mosquito	1	0.77	0.400	0.34	0.574
Scirtid	1	0.29	0.601	0.17	0.687
Time	1	10.1	0.340	0.57	0.466
Mosquito*Time	1	1.21	0.297	0.19	0.675
Scirtid*Time	1	0.08	0.782	0.20	0.665
Mosquito*Scirtid	1	2.13	0.175	0.06	0.809
Error	10				

*Indicates p-values that are significant following Bonferroni adjustment to

Appendix 2, Table 8. Summary of ANOVA results for PC1 and PC2 scores from leaf surface 18S rDNA t-RFs digests (Block II).

Source		PC1		PC2	
	df	*F* Value	*P* Value	*F* Value	*P* Value
Msp					
Mosquito	1	0.05	0.829	6.19	0.024
Scirtid	1	0.23	0.639	1.20	0.289
Time	1	1.02	0.329	3.21	0.092
Mosquito x Time	1	0.16	0.692	1.18	0.2931
Scirtid*Time	1	0.01	0.917	1.67	0.214
Mosquito*Scirtid	1	0.12	0.732	0.70	0.414
Error	16				
Hha					
Mosquito	1	1.58	0.226	4.19	0.058
Scirtid	1	0.35	0.560	0.01	0.922
Time	1	24.29	<0.001*	8.65	0.010*
Mosquito*Time	1	0.05	0.829	3.38	0.085
Scirtid*Time	1	0.68	0.421	1.29	0.272
Mosquito*Scirtid	1	1.40	0.254	0.02	0.867
Error	16				

*Indicates p-values that are significant following Bonferroni

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