INTRODUCTION

We have previously shown that there is a distinct habitat diversification among individual anopheline species (Rejmánková et al. 1993, 1998; Manguin et al. 1996). The temporal and spatial distribution of larval habitats is defined by hydrology and vegetation. Four anophelines are of special interest as malaria vectors in Belize and other countries of Central America: *An. darlingi* Root, *An. vestitipennis* Dyar and Knab, and *An. pseudopunctipennis* Theobald, using a direct count method and DAPI staining technique. Bacterial counts from larval habitats were compared to those from adjacent open water. Several additional variables such as total suspended solids (TSS), particulate organic carbon (POC), and dissolved organic carbon (DOC) were also recorded in order to establish possible relationships with bacterial characteristics. Our results showed that the waters from larval habitats were enriched with bacteria as well as POC and DOC compared to open water. The major component of all samples consisted of cocci, the proportion of rods was similar and there were significantly more attached rods in habitat samples than in open water samples. *Anopheles vestitipennis* habitats had the highest values of each of the categories of bacteria as well as of POC and DOC. *Journal of Vector Ecology* 25(2): 229-238. 2000.

Keyword Index: *Anopheles albimanus*, *An. darlingi*, *An. pseudopunctipennis*, *An. vestitipennis*, bacteria, larval habitats, POC, DOC.
Figure 1. Map of Belize indicating the sampling locations.
al. 1996). During feeding, the anopheline larval body often orients with the posterior end to a plant-water interface. This specialized feeding mode at the air-water interface is termed “interfacial feeding” by Renn (1941). In the process of feeding, anopheline larvae use lateral palatal brushes (mouth brushes) to collect particles from surface microlayers from a wide distance around themselves (Merritt et al. 1992b). The surface microlayers are considerably enriched in organic materials compared to the rest of the water column (Walker and Merritt 1993). The comparison of particle sizes from surface microlayers and gut contents of 4th instar An. quadrimaculatus Say showed that larvae ingested mainly small particles in the range of 1.5 to 4.5 µm in diameter. Merritt and co-authors (1992a) pointed out that an understanding of the spatial and temporal distribution of the dietary resources available to larval mosquitoes in their natural habitats is needed in order to clarify the relationship among food availability, vector competence, and mosquito fitness. The knowledge of bacterial community structure and the significance of its components to larval nutrition of the anopheline mosquito is limited to studies on Anopheles quadrimaculatus from a freshwater marsh in Michigan (Walker et al. 1988; Walker and Merritt 1993; Merritt et al. 1996; Smith et al. 1998; Wallace and Merritt 1999) and a laboratory feeding study on An. quadrimaculatus and An. gambiae Giles (Wotton et al. 1997). The results of these studies clearly indicate that the heterotrophic microbial community plays a significant role in anopheline larval feeding.

Our goal in this study was to compare available food resources (in terms of bacterial counts) among larval habitats for the four anopheline species. Several additional variables such as total suspended solids (TSS), particulate organic carbon (POC), and dissolved organic carbon (DOC) as well as pH and specific conductivity were also recorded in order to establish relationships with bacterial characteristics.

METHODS

Study Site and Field Sampling

The country of Belize, with an area of 23,000 km² that includes both lowland and montane regions, is characterized by remarkable geomorphological and hydrological diversity (for details see Wright et al. 1959, King et al. 1992, Rejmánková et al. 1993, 1996). This diversity produces a wide variety of mosquito larval habitats. We collected samples from larval habitats in northern Belize (Corozal, Orange Walk, and Belize Districts), central Belize (Cayo District), and southern Belize (Toledo District) (Figure 1). The sampling covered a wide range of habitats of all four anopheline species including: (1) marshes with sparse macrophytes (Eleocharis cellulosa Torr.) and floating mats of cyanobacteria, (2) rivers with both submerged macrophytes (Vallisneria americana Michx., Cabomba spp.) and detritus, (3) creeks and ditches with filamentous algae, emergent macrophytes, and detritus, and (4) swamp forests with detritus in forest pools. We define a larval habitat as a water body or its part with a distinct vegetation and/or detritus assemblage (Rejmankanova et al. 1992). Twenty-six sampling sites were selected based on our previous knowledge of the distribution of the four species of interest throughout the region. Only 19 sites were used for the final analysis because data for the remaining seven were incomplete for various reasons. Sampling was conducted in August, i.e., the first half of the rainy season. At this time of the year, conditions in larval habitats in terms of water level, aquatic plant development and larval populations are generally quite representative of a “typical” situation.

At each site we collected larvae that were either identified at our field lab (3rd and 4th instars) or preserved in alcohol and identified at a later date. We were not able to differentiate 2nd instars of An. vestitipennis from An. punctimacula Dyar and Knab. Because our previous study showed that habitats of these two species often overlap (Rejmánková et al. 1998), all these habitats are included under the An. vestitipennis category. We also recorded the hydrology type and vegetation present, and measured pH and water conductivity. Water samples were collected from each of the larval mosquito habitats and from adjacent open water with an eyedropper (throughout the text we will refer to these as “habitat” and “water” samples, respectively). Eyedropper collected samples included water from approximately the upper <5 mm water layer. We chose the eyedropper for sampling rather than the Harvey-Burzell glass plate method (Walker and Merritt 1993) because in some types of habitats (detritus, dense filamentous algae) the glass plate method would not work. Our samples thus represent a mixture of true surface layer (<1 mm) with a subsurface layer. Two replicate samples were collected from each habitat and one from the open water. Nine ml of water were collected into an autoclaved vial and filtered formaldehyde was immediately added to preserve bacteria and yield a final volume of 10 ml and final concentration of 2 % formaldehyde. Samples were stored in the refrigerator for two weeks until they could be analyzed at the University of California, Davis.

In addition to samples for bacterial counts, we collected water samples from each habitat and adjacent
Figure 2. Relative proportions of different types of bacteria in larval habitats and adjacent open water.

Figure 3. Comparison of bacterial counts ($x \times 10^6 \text{ml}^{-1}$), POC and DOC (mg l$^{-1}$) among Anopheles albimanus (A), An. vestitipennis (V), An. darlingi (D), and An. pseudopunctipennis habitats (P). Vertical bars indicate the standard error. Number of replicates: A – 6; V – 7; D – 3; P – 3. Differences of means between measured variables (after log transformation) were evaluated with Scheffe’s test. Means sharing the same letter are not statistically different ($P > 0.05$).
open water for total suspended solids (TSS), particulate organic matter (POM), and dissolved organic carbon (DOC) analysis. TSS and POM were measured following the standard procedures (American Public Health Association 1985). One hundred ml of water was filtered through a pre-weighed glass-fiber filter, dried at 100°C and weighed to obtain TSS. TSS filters were then combusted at 550°C for 1 h and the difference between dry and combusted filter represented POM. Samples for DOC were filtered through a 0.2 µm Millipore filter, stored in the refrigerator and analyzed on the Shimadzu Total Organic Carbon Analyzer (TOC 500). Specific conductivity was measured using the Hanna conductivity meter and pH was measured with the Hanna pH-meter.

**Slide Preparation and Bacteria Enumeration**

Samples were diluted to a countable concentration of 1:10 with autoclaved water (Walker and Merritt 1993). Bacteria from each sample were enumerated using a direct count method after being stained with the fluorochrome 4', 6 diamidino-2-phenylindole (DAPI) (Porter and Feig 1980, Walker et al. 1988). Samples were stained with DAPI solution (2 µg ml⁻¹ final concentration) in the dark at 4°C for 20-30 min. We followed the filtration and slide preparation methods of Walker et al. (1996) using a 17 mm diameter glass chimney and 0.2 µm pore size (25 mm diam.), black, polycarbonate Millipore filter backed by a 0.45 µm pore size, HA-type Millipore filter. Two slides were prepared from each sample.

For bacterial enumeration, we used a Leitz Laborlux microscope with epifluorescence light fittings, HBO 100-W-2 mercury lamp, Leitz wide-band UV filter set A (excitation filter 360 nm; dichoric filter 400 nm; barrier filter 430 nm), 100 x oil immersion objective, and 10 x oculars. On each slide, bacteria were counted by category (rods, cocci, and attached rods) within a grid. At least 15 different fields were randomly chosen until at least 200 bacteria were counted per morphological group in habitat samples and a minimum of 200 total bacteria were counted in water samples (Walker and Merritt 1993, Walker et al. 1996). Total density of bacteria for each sample and each category is expressed as number of bacteria per milliliter and was calculated using a standard formula (Walker et al. 1988) that takes into account the total bacteria count, sample volume filtered, number of fields counted, grid area, filter area, formaldehyde factor, and dilution factor.

![Figure 5. The relationship between dissolved organic carbon (DOC) and density of different types of bacteria (number x 10⁶ ml⁻¹).](image-url)
Figure 4. Canonical correspondence ordination diagram for mosquito larvae and environmental variables measured in their habitats. Arrows indicate the direction and relative importance of the environmental variables (COC – cocci, ROD – rods, ATR – attached rods, TOT – total number of bacteria, DOC – dissolved organic carbon, IC – dissolved inorganic carbon, TC – total dissolved carbon, POC – particulate organic carbon, TSS – total suspended solids). Full squares indicate the position of mosquito species: A = *Anopheles albimanus*, V = *An. vestitipennis*, D = *An. darlingi*, and P = *An. pseudopunctipennis*. Open circles with numbers indicate sampling locations. *An. albimanus* sampled at locations: 6, 10, 16, 17, 18, 19; *An. vestitipennis* sampled at locations: 1, 2, 7, 8, 9, 14, 15; *An. darlingi* sampled at locations: 11, 12, 13; *An. pseudopunctipennis* sampled at locations 3, 4, 5.
Data Analysis
A two-way ANOVA with habitat vs. open water as one factor, and a mosquito species as a second factor was used to compare the group means of log-transformed variables. Differences in variables among the habitats of the individual species were evaluated using ANOVA with Scheffe’s multiple-comparison test. Relationships among environmental variables and the occurrence of the individual anopheline species were investigated using the canonical correspondence analysis (CANOCO) (Ter Braak, 1987a, b). CANOCO is a constrained ordination technique, the results of which are based on species presence and values of environmental variables simultaneously. The ordination axes are constrained to optimize their relationship with a set of environmental variables, the direction of which can be indicated in the ordination diagram by arrows with length proportional to their importance. Species are represented by points in the ordination diagram. These points represent approximate values of weighed averages of the species with respect to environmental variables. Statistical validity of resulting environmental axes is evaluated by an unrestricted Monte Carlo permutation test.

RESULTS

Anopheles albimanus was the most common species, occurring consistently in cyanobacterial mats in marshes of northern Belize, but also in detritus in rivers and roadside ditches with macrophytes. Anopheles vestitipennis was found in swamp forest pools with detritus and in habitats with tall dense macrophytes. Detritus assemblages in rivers contained An. darlingi while filamentous green algae were typical habitats for An. pseudopunctipennis. There were no significant differences among larval habitats and open water in pH and water conductivity. In most cases, the bacterial characteristics (total bacteria, rods, cocci and attached rods), POC and DOC were significantly or marginally significantly higher in larval habitats than in adjacent open water (Table 1). The differences were most distinct for An. vestitipennis habitats.

Figure 2 shows that the major component of all bacterial samples were cocci, the proportion of rods was similar in both the larval habitats and open water, and there were significantly more attached rods in habitat samples than in open water samples. Anopheles vestitipennis habitats were characterized by the highest values of each of the categories of bacteria as well as of POC and DOC (Figure 3). However, because of the low number of replicates, statistically significant differences were found only between An. albimanus and An. vestitipennis habitats for total number of bacteria, rods, cocci, attached rods and POC. Anopheles darlingi differed significantly from An. vestitipennis in number of cocci, POC and DOC. The only significant difference between An. albimanus and An. darlingi was found in the concentration of DOC. There was no significant difference between An. darlingi and An. pseudopunctipennis in any of the measured characteristics. The habitats of An. vestitipennis are clearly characterized by the largest amount of bacteria, POC, and DOC. This result is confirmed by the results of the CANOCO analysis (Figure 4). When the species and environmental variables were analyzed with CANOCO, we found all of the variables included in the analysis pointed to the same ordination space where the analysis placed An. vestitipennis. The relative importance of individual environmental variables in the CANOCO ordination diagram is indicated by length of the arrows. The relationship between mosquito species data and environmental data was significant only for POC and DOC (p<0.02 and p<0.05 respectively; Monte Carlo test).

There was a significant positive correlation between DOC and densities of all bacteria types (Figure 4). There was an indication of positive relationship between POC and densities of bacteria, but the correlations were not statistically significant (data not shown).

DISCUSSION
Numerous larval surveys over the years of research in Belize and other tropical regions convinced us that the driving variables determining the presence or absence of larvae of specific anopheline species are the hydrology and habitat composition in terms of live aquatic micro and macrophytes and detritus. Other properties, especially physical and chemical water characteristics, of larval habitats do not seem so important and their ranges often overlap for several different species (Rejmankova et al. 1993, 1998). This is not surprising since the importance of aquatic vegetation to mosquito larvae has been stressed by many authors in the past (Hess and Hall 1943, Orr and Resh 1989). Because the macrophytic components of larval habitats of the malaria vectors in Belize are so different among each other we wanted to find out whether these differences will be reflected in differences in types of bacteria found in these habitats. Numbers of bacteria found in our samples were similar or slightly higher than bacterial counts from similar environments (littoral zone with bulrushes: 1.4-5.3 x 10^9 ml^-1, Wetzel...
Table 1. Comparison of group means of variables from larval habitats and adjacent open water. Means were calculated from original data. Data within individual columns, after log transformation, were evaluated using a two-way ANOVA. All bacterial counts are in $\times 10^6$ ml$^{-1}$; DOC=dissolved organic carbon, POC=particulate organic carbon, TSS=total suspended solids, all in ppm. Values in parenthesis represent the standard error of mean.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>HABITAT/</th>
<th>n</th>
<th>TOTAL</th>
<th>RODS</th>
<th>COCCI</th>
<th>AT. RODS</th>
<th>DOC</th>
<th>POC</th>
</tr>
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<tr>
<td>An. albimanus</td>
<td>HABITAT</td>
<td>6</td>
<td>4.08 (0.48)</td>
<td>1.2 (0.10)</td>
<td>2.1 (0.35)</td>
<td>0.82 (0.24)</td>
<td>20.3 (2.6)</td>
<td>10.8 (3.6)</td>
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<td>OPEN WATER</td>
<td>5</td>
<td>2.4 (0.6)</td>
<td>0.8 (0.21)</td>
<td>1.5 (0.5)</td>
<td>0.02 (0.01)</td>
<td>11.0 (3.5)</td>
<td>4.6 (3.1)</td>
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<tr>
<td>An. vestitipennis</td>
<td>HABITAT</td>
<td>7</td>
<td>11.5 (2.5)</td>
<td>2.4 (0.4)</td>
<td>5.9 (0.9)</td>
<td>3.1 (1.2)</td>
<td>29.4 (5.2)</td>
<td>41.2 (9.1)</td>
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<tr>
<td></td>
<td>OPEN WATER</td>
<td>7</td>
<td>3.8 (1.4)</td>
<td>1.2 (0.4)</td>
<td>2.7 (0.9)</td>
<td>0.2 (0.05)</td>
<td>12.6 (3.3)</td>
<td>11.4 (6.4)</td>
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<td>An. darlingi</td>
<td>HABITAT</td>
<td>3</td>
<td>5.52 (0.77)</td>
<td>2.26 (0.40)</td>
<td>2.29 (0.55)</td>
<td>0.97 (0.21)</td>
<td>6.14 (2.5)</td>
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<td>0.51 (0.23)</td>
<td>0.89 (0.22)</td>
<td>0.08 (0.06)</td>
<td>4.79 (2.15)</td>
<td>4.4 (4.3)</td>
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<tr>
<td>An. pseudopunct.</td>
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<td>7.4 (3.5)</td>
<td>1.6 (0.47)</td>
<td>3.91 (1.69)</td>
<td>1.90 (1.1)</td>
<td>10.0 (2.4)</td>
<td>9.7 (5.4)</td>
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<td>0.94 (0.56)</td>
<td>1.23 (0.53)</td>
<td>0.44 (0.42)</td>
<td>3.3 (2.3)</td>
<td>7.0 (3.7)</td>
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</table>

Two-way ANOVA, P-values:
- Habitat vs. open water factor (A): <0.0001 <0.0001 <0.0001 <0.0001 0.0010 0.0532
- Species factor (B): 0.0377 0.1200 0.0071 0.2505 0.0001 0.0034
- Interaction (A X B): 0.2880 0.1278 0.3272 0.3191 0.1990 0.3929
of attached rods than samples from water, 21% and 5% of the total, respectively. Habitats have higher abundance of detritus particles for attached bacteria (see higher POC and TSS). This is in agreement with Torrington et al. (1994) who found the abundance of attached bacteria to be regulated by particle abundance for attached cells. Walker and Merritt (1993) found similar proportions in their surface microlayer (23%) and subsurface samples (7.6%). It has been suggested that food value of detritus particles is enhanced when they are colonized with bacteria (Walker and Merritt 1993; Wotton 1994).

The ecological importance of bacterial processes in tropical shallow water ecosystems has been stressed by many authors (Hamilton et al. 1995, Waichman 1996), especially the role of bacteria in carbon transformations and aquatic food webs. This paper presents the first descriptive data on carbon sources and bacterial counts in larval habitats of several species of anopheline mosquitoes in tropical freshwater. More detailed studies on the structure of microbial communities of these habitats and significance of their components to larval nutrition are needed.

Acknowledgments

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