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Bacterial Florae in Larvae of the Lake Fly *Chironomus plumosus*

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Chironomus plumosus **midge larvae were collected from Lake Winnebago, Wisconsin, for 10 weeks in the summer of 1985 and 10 weeks in the summer of 1986 in order to determine their bacterial floras. Altogether, 18 genera and 29 species of bacteria were found and identified. Some spirillum-like bacteria which were not isolated in culture were found by electron microscopy. Scanning electron micrographs of sectioned larvae revealed bacteria throughout the intestines. Gram-positive organisms were most prevalent in the larvae in the early part of the summer, and gram-negative organisms were most prevalent during the later part. Larvae accumulate bacteria within their intestines.**

Chironomus plumosus, the midge, known as the lake fly by the residents of Oshkosh, Wisconsin, has been a nuisance to the population of that city since 1908. Hordes of these insects emerge like clouds from Lake Winnebago, Wisconsin, twice a year to inundate the lakeshore residents of the city of Oshkosh and other communities with their effluvia, exuviae, and dead and dying bodies. During this time, there has been no discernible health problem, other than allergies, which could be directly attributed to these insects (5).

To our knowledge, as of 1992, there is no published report on the presence of bacteria in *C. plumosus*. A protozoan (microsporidian) parasite was found in the midge and was studied in hopes of controlling the *Chironomus* larvae, but this was not successful (3). Bacteria in the ectoperitrophic space of the posterior midgut of *Xylotopus par* midge larvae have been observed by scanning electron micrography. These bacteria could not be isolated (6).

The adult *C. plumosus* does not have functional mouthparts and does not feed (2), and all feeding takes place during the larval stage. Therefore, the microflora of the midge is probably established at this stage of the life cycle.

Since microbes other than bacteria were reported to be present in midge larvae and no one knew whether the *C. plumosus* larvae carried a bacterial flora, it seemed prudent to undertake a study to determine the number and the types of bacteria present in the midge larvae.

Three sampling sites in Lake Winnebago (Fig. 1) were selected on the basis of different expected pollution levels. Site 3 was near the shore and downstream from the waste treatment plant outlet and was shallow (depth, 3 m). Site 2 was in the middle of the lake along a transect line (depth, 6 m), and site 1 was nearer the shore (depth, 4 m) and on the same transect line as site 2.

Each week from May to July 1985 and 1986, mud samples were collected with an Ekman dredge. Samples from each site were washed in a standard 60-mesh-sieve pail, placed in labelled individual containers, and transported to the laboratory, where the larvae were isolated and identified (4). Larvae collected from the mud were carefully separated from pupae. The larvae from all three sites were then thoroughly washed six times by repeatedly suspending the larvae in sterile 0.01 M phosphate buffer, pH 7, to remove adherent bacteria. The first-wash water contained about 4×10^3 to 5×10^3 bacteria per ml, and the last-wash water usually contained less than 10 to 20 bacteria per ml.

The bacteria in the washed and homogenized larva samples were enumerated by standard plate counts. Some direct microscopic counts were initially made on the homogenized samples for total-count comparison. Since a large mixed population of microbes was expected, various media such as MacConkey, KF, Hektoen, V-J, yersinia selective, Pseudosel, Rimler-Shotts, campylobacter BAP, and RCM agars

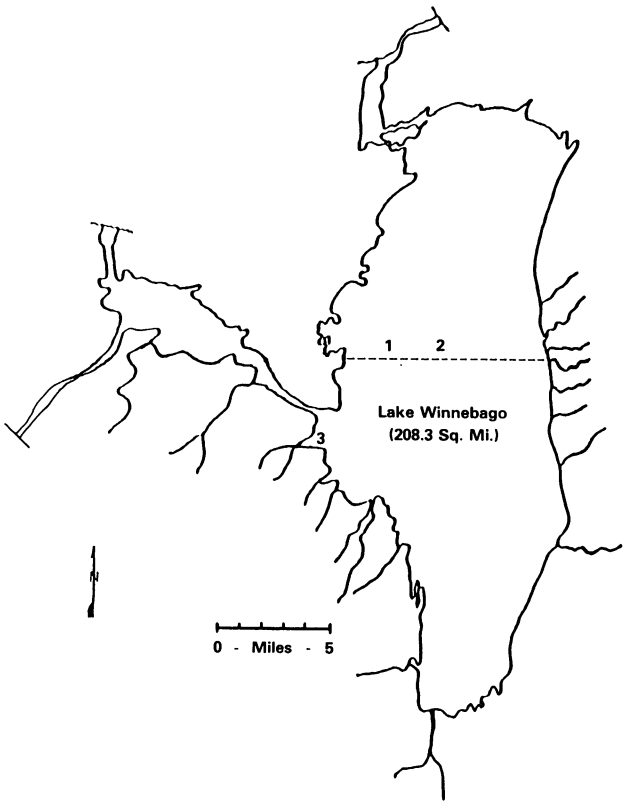

FIG. 1. Map of Lake Winnebago, Wisconsin, showing the three sampling sites.

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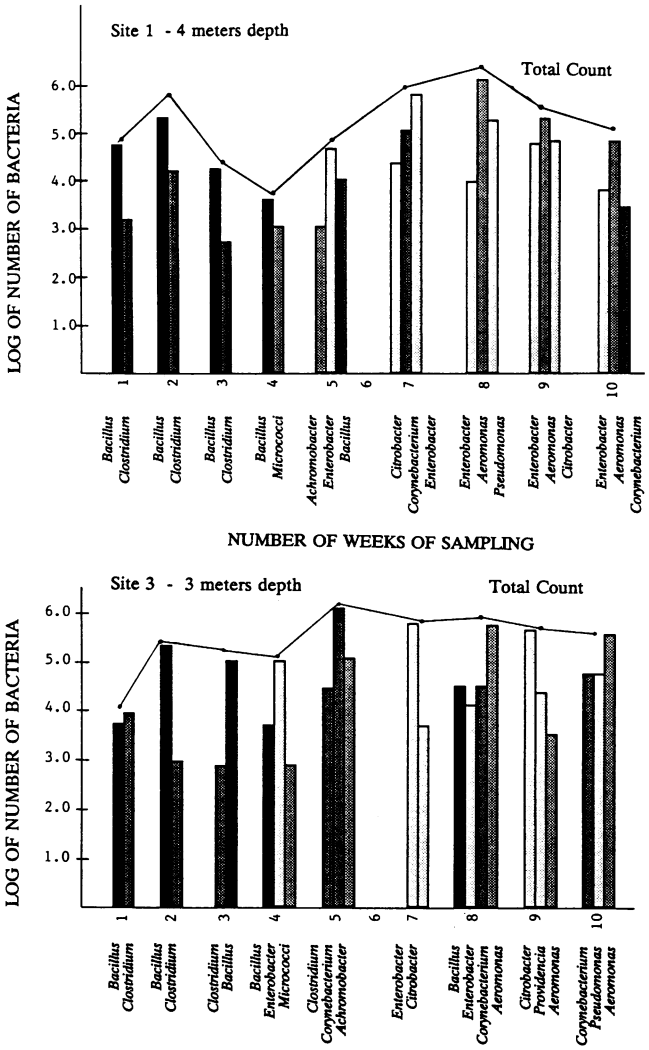

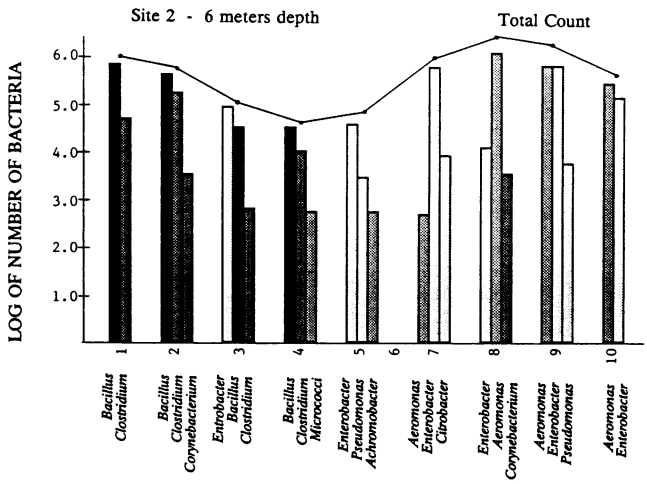

FIG. 2. Total counts of bacteria found in 1 g of the lake fly larvae collected at three sites and distribution of bacteria by genus.

were used to isolate and identify bacterial florae including pathogens. All media were incubated both at 25°C and at the respective optimum growth temperature under both aerobic and anaerobic conditions. Anaerobic growth conditions were obtained by using GasPak jars and/or a Coy anaerobic glove box. Definitive identification of the isolated colonies found on the various selective media was performed by using the API system for members of the family *Enterobacteriaceae* and *Clostridium* spp., Oxi-ferm tubes for gram-negative nonfermentative bacilli, and standard laboratory methods (1).

For localization of the bacteria within the larvae, thoroughly washed whole larvae were placed in formalin and Zenker's fixative, embedded in paraffin, and sectioned and stained with hematoxylin-eosin for tissue structure. For scanning electron microscopy, thoroughly washed larvae were placed in Bouin's fixative (in 30% ethanol) and stored in 70% ethanol. The larvae were cut into segments and were dehydrated in a graded ethanol series followed by an amyl acetate series in ethanol and three changes in absolute amyl acetate. The segments were critical-point dried in liquid CO_2 and mounted on aluminum stubs with silver printer's ink. They were given a 200-Å (20-nm) gold-palladium coating with a sputter coater and examined and photographed with a Hitachi HHS-2R scanning electron microscope.

Although the bacterial counts per gram of larvae were different among the three sites, between 1985 and 1986 the basic patterns of microbial succession and the total counts were essentially alike. For simplicity, data for only 1986 are presented here (Fig. 2). During the first three weeks, *Bacillus* and *Clostridium* spp. were the most predominant organisms within the larvae at all three sites, although a good number of *Enterobacter* bacteria were also found at site 2 (Fig. 2) during the third week. For both years, *Bacillus* and *Clostridium* spp. were the most dominant organisms within the larvae.

During the fourth and fifth weeks, we observed a shift in population within the larvae from spore-forming gram-positive to gram-negative organisms. For example, at all three sites micrococci began to appear in the larvae during the fourth week, and the gram-negative organisms started to predominate during the fifth week. *Corynebacterium* organisms were observed in large numbers at sites 1 and 2 during weeks 7 and 10 and week 8, respectively, and at site 3 during weeks 5, 8, and 10. In general, from the seventh through the tenth week, gram-negative bacteria were the predominant population. This was true for all three sites and for both years.

During the eighth, ninth, and tenth weeks, *Aeromonas* organisms were found to be the dominant population. Many other organisms which were present at levels less than 300/g of larvae were not plotted in the graph. There was also a considerable reduction in total count in the fourth week at all sites, which was usually the transition period for the shift in population from gram-positive to gram-negative organisms.

The total number of organisms in the final-wash water was always 10 to 20/ml. Repeated total bacterial counts of the lake water revealed 1×10^3 to 8×10^3 organisms per ml (the higher numbers were obtained during the later part of the summer). Usually, the populations of bacteria found in 1 g of the larvae were about 10 times greater than those found in the lake water, indicating that the larvae accumulated the bacteria within their intestines. Species of bacteria found in lake water mimic those found within the intestines of lake fly larvae. Nevertheless, certain species of bacteria were found in much higher numbers within the larva intestines at certain

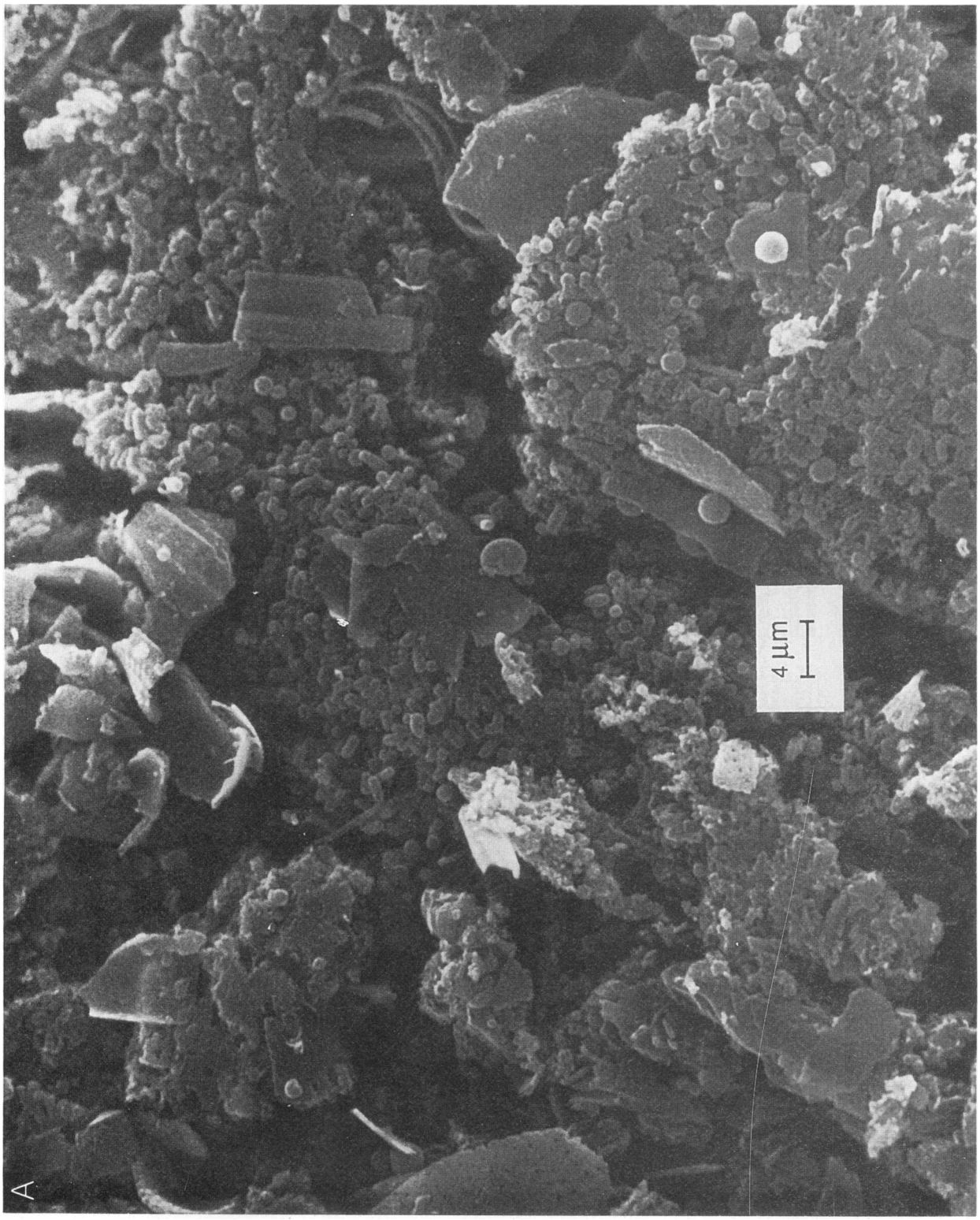

FIG. 3. Predominantly small rod-shaped bacteria (A) and diatoms, small rods, and filamentous bacteria (B) in the seventh segment of a larva intestine.

FIG. 3—*Continued*

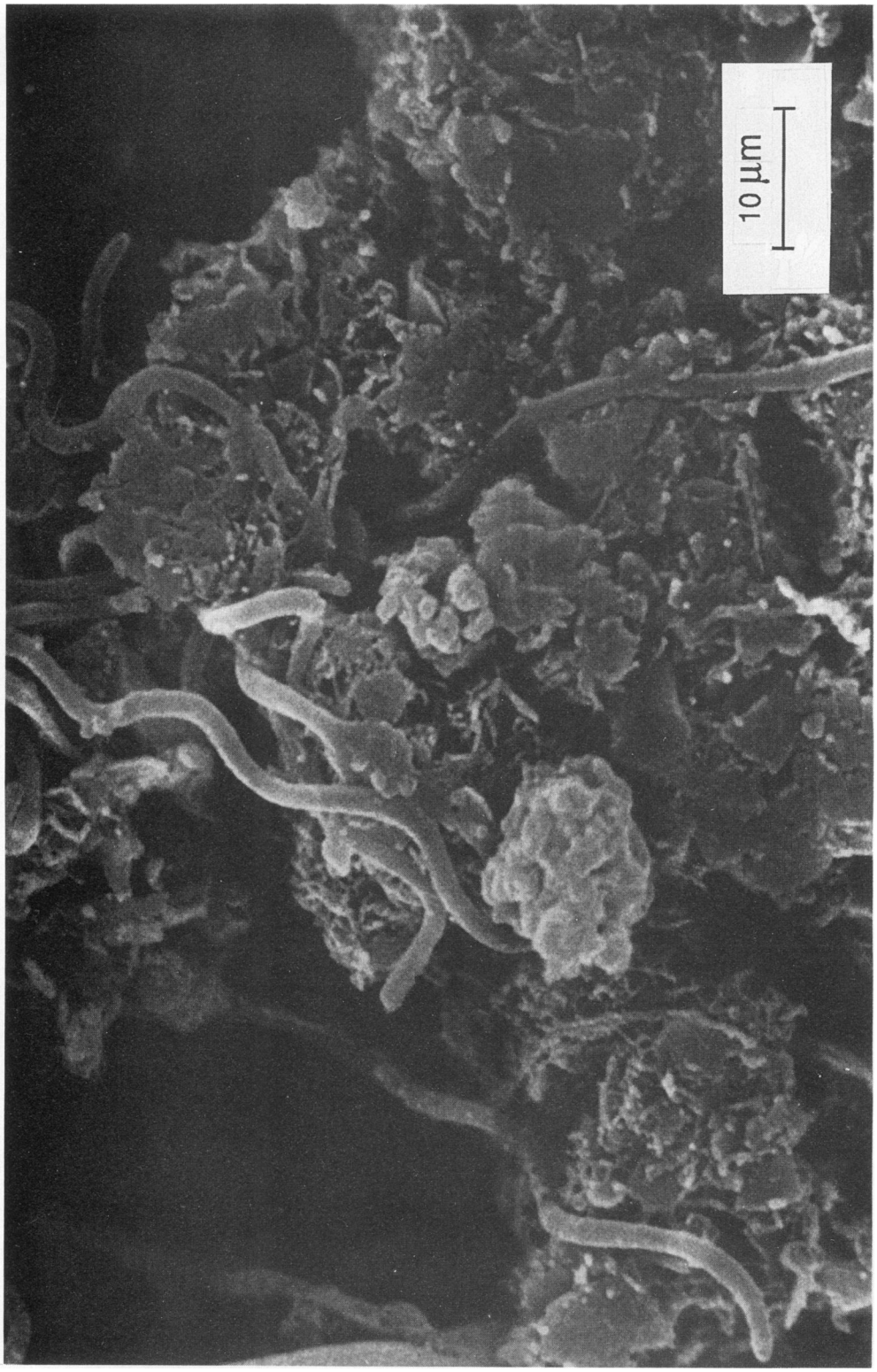

FIG. 4. Spirillum-like organisms within the sixth segment of a larval intestine.

times of the year. For example, *Bacillus* spp. were found in greater numbers in the intestine relative to lake water during early summer, as was an *Aeromonas* sp. in late summer (Fig. 2).

Most of the dominant genera, both gram-positive and gram-negative, appeared in cycles during certain weeks and almost vanished at other times. Generally, the gram-positive organisms were most prevalent in the earlier part of the summer and the gram-negative organisms were more prevalent during the later part, as the ambient temperature started to increase. This general pattern was persistent in both 1985 and 1986. We were unable to isolate overt human bacterial pathogens from the larvae, except in one instance, although a variety of opportunistic pathogenic bacteria were found. Altogether, 29 species belonging to 18 genera were found in the lake fly larvae, isolated, and identified. They included the following: gram-positive rods, *Bacillus*, *Corynebacterium* and *Clostridium* spp.; gram-positive cocci, *Micrococcus* sp., *Micrococcus luteus*, and *Staphylococcus epidermidis*; gram-negative rods, *Enterobacter agglomerans*, *Serratia marcescens*, *Serratia*-like bacteria, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas*-like bacteria, *Pseudomonas putida*, *Pseudomonas putrefaciens*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, *Achromobacter* biotype 1, *Achromobacter* biotype 2, *Citrobacter freundii*, *Citrobacter amalonaticus*, *Aeromonas hydrophila*, *Acinetobacter anitratum*, *Yersinia enterocolitica*, *Edwardsiella* sp., *Providencia stuartii*, *Escherichia coli*, and *Flavobacterium* sp.

To determine the location of bacteria in larvae, plate counts of intact larvae, of intestines, and of the eviscerated carcasses were made. The averages of four different counts were 7.5×10^4/g for intact larvae, 4.4×10^5/g for intestines, 7×10^4/g for eviscerated carcasses, and 1.2×10^2/ml for holding water (sterile water in which the washed larvae were held for this and all other experiments throughout this study). Although we always noticed a large amount of defecated material and debris in the holding water, only a small number of culturable bacteria (1.2×10^2/ml) were present in the debris. In fact, lake water had many more organisms (1×10^3 to 8×10^3/ml) than this holding water with the excretory materials. This localization study indicates that *Chironomus* larvae accumulate bacteria primarily in the intestine.

The histological studies using the hematoxylin-eosin stain for tissue structure and the Gram-Weigert stain for bacteria also indicated the presence of bacteria only within the intestine. The scanning electron micrograph of the sectioned larvae further confirmed the presence of bacteria within the intestine. A large number of bacteria in all segments starting from the third segment behind the head were always observed. Usually, the first two segments were free of bacteria. Along with large numbers of bacteria (Fig. 3), some diatoms in the intestine were observed. A large number of spirillum-like organisms was also observed (Fig. 4). For the last several years, we made repeated attempts to isolate this spirillum-like bacterium by using midge larvae and various other enrichment and/or commonly used culture media including PSS broth, MPSS broth, and nutrient broth (7) without any success. Our failure to isolate the spirillum-like bacterium shown in the scanning electron micrograph perhaps points to the fastidious nutritional needs of this bacterium, which may be fulfilled by the live midge larvae.

In conclusion, a large number of bacteria are present in the intestines of *C. plumosus* larvae. Since no bacteria in the lake fly or its larva that are truly pathogenic for humans were isolated or identified, we concluded that lake flies are not a microbiological health hazard to residents of the area around Lake Winnebago.

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