STUDY PERFORMANCE REPORT

State: Michigan **Project No.:** F-80-R-6

Study No.: 230728 **Title:** Effects of *Piscirickettsia* infection on the muskellunge population of Lake St. Clair

Period Covered: October 1, 2004 to September 30, 2005

Study Objective: The objectives of this study are (1) to determine the infection rate in Lake St. Clair muskellunge and whether the rate varies spatially or temporally, (2) to determine if other fish species in the St. Clair System are infected by the bacteria, particularly migratory salmonids, (3) to identify the impacts of the organism on the health of individual muskellunge, and (4) to determine if the disease can be vertically transmitted.

Summary: Samples of fish and invertebrates were collected from Lake St. Clair in 2004 and 2005 for analysis. Seventy-nine percent of the 132 muskellunge captured in trap nets in Anchor Bay during 2004 and 2005 exhibited external symptoms considered consistent with *Piscirickettsia* infection. In 2004, a voluntary muskellunge lesion diary provided observations for 501 angler caught muskellunge. Red sores were noted on only 4% of those muskellunge.

A *Piscirickettsia* sp was isolated from muskellunge collected in 2004 (all samples) and 2005 (7 out of 9 samples. Titers ranged from 10^4–10^{10} $TCID_{50}$/gram tissues. The diagnosis was confirmed by electron microscopy Laboratory6 and molecular analysis using the ribosomal RNA gene sequences. Additional characterization revealed that the muskellunge *Piscirickettsia* isolates are tolerant to a wide range of environmental conditions, and are pathogenic to muskellunge but not to rainbow trout. The organism causes enlargement of internal organs and the formation of fluid-filled vesicles budding out of the internal swim bladder membrane. The cause of morbidity and mortalities seem to be caused by destruction of kidney glomeruli. Based on analysis of the gametes, there are no indications that this bacterium is vertically transmitted. *Piscirickettsia* sp. was also found in 57% of yellow perch samples. It is evident that this emerging infection is more widespread than originally thought.

Findings: Jobs 1–6 were scheduled for 2004-05, and progress is reported below.

Job 1. Title: Collect muskellunge samples from Anchor Bay.–During May 2004 and 2005, trap nets were fished in Anchor Bay, Lake St. Clair under Study 488 (Job 9). In 2004, 76 muskellunge were captured during the survey. Seven muskellunge were sacrificed and delivered to the MSU containment facility. Eighty-five percent of the muskellunge captured exhibited external symptoms considered characteristic of *Piscirickettsia* infection including puffy scales, red sores, hemorrhaging fins, and/or sunken eyes. In 2005, 56 muskellunge were captured in the trap nets and 70% exhibited the external symptoms. Nine muskellunge, including four with no external symptoms, were sacrificed and delivered to the MSU containment facility.

Job 2. Title: Collect other species of fish and macroparasites during spring, summer, and fall.–Other fish species (number in parentheses) collected from the Anchor Bay trap nets and delivered to the MSU containment facility in 2004 included: northern pike (19), smallmouth bass (2), largemouth bass (3), freshwater drum (3), shorthead redhorse (8), yellow perch (54), silver lamprey (1), crayfish (2), and snails (20). An additional 35 yellow perch (7 live and 28 fresh dead) were sampled with gill nets in late September and delivered to MSU.

Job 3. Title: Collect gametes, fertilize eggs, incubate and hatch eggs, culture fry to fingerling size, and collect samples from all stages for bacterial analysis.–On May 14, 2004 eggs were stripped from a ripe female muskellunge and fertilized with gametes from a male muskellunge. Approximately 1 liter of fertilized eggs was delivered to the MSU containment facility.

Job 4. Title: Analyze samples for bacterial infection (including various life stages and various species of fish and macroparasites).–

1. *Piscirickettsia* sp. Infection in the Muskellunge: There was no growth on any of the conventional cell-free bacterial media. Therefore, isolation was attempted in CHSE and FHM cell lines as per the international and national guidelines for aquatic animal disease diagnosis. The cell lines were inoculated with homogenates of muskellunge kidneys, spleens, and skin lesions. The cells were incubated for 21 days at 15° C in the case of CHSE and at 20° C in the case of FHM with daily microscopical observation. Following 10 days of incubation, cytopathic effects (CPE) in the form of cell rounding appeared in the inoculated cell lines, but not in the negative controls, followed by detachment and lysis (Figure 1). CPE appeared in all cultures inoculated with 2004 samples and seven out of nine 2005 samples. Titers ranged from 10^4–10^{10} $TCID_{50}$/gram tissues. In order to confirm diagnosis, samples of tissues and inoculated cultures were fixed for electron microscopy which was performed at the Michigan State University, Electron Microscopy Laboratory. Inoculated cultured cells and swabs from skin lesions were fixed in methanol and stained with Giemsa. Supernatants of inoculated cells were collected individually, filtered (0.45 µm), centrifuged into a glass slide, and then stained with Gram stain.

Giemsa-stained slides demonstrated the presence of intracellular coccoidal bacteria that ranged in size from 0.43 to 1.61 µm (Figure 2). These bacteria were Gram negative in Gram stained preparations. At the ultrastructural level, bacteria exhibited the typical Gram negative membrane and cell wall and were found exclusively intracellular within membrane-bound vacuoles in inoculated cell lines, in skin lesions, and in internal organs of affected fish (Figure 3). The morphological criteria noticed are in accordance with those described for *Piscirickettsia salmonis* by Fryer et al. (1990; 1992) and Fryer and Mauel (1997).

The discrimination between Muskellunge isolate from *P. salmonis* was performed by molecular assays using the 16S ribosomal rDNA gene sequences. The following primers were used.

EubB (27F): AGAGTTTGATCMTGGCTCAG
EubA (1518R): AAGGAGGTGATCCANCCRCA

Amplicons (470 bp) were sequenced bidirectionally at the MSU Genomic Facility. A phylogenetic tree was then constructed by the neighbor-joining method, using the obtained sequence of Michigan Isolate and published sequences of a number of related and distant bacteria (Figure 4). The analysis demonstrated that Michigan isolates sequences conformed to the secondary structure models for the gamma subdivision of *Proteobacteria*, a member of the family *Rickettsiaceae*, and are closer to the Genus *Piscirickettsia* than to other bacterial genera. Additional phylogenetic analyses were then performed with expanded sequencing that involved the 16S, ITS, and 23S rDNA genes. These analyses confirmed that Muskellunge isolates were identical to each other but not identical to *P. salmonis* that devastates salmonid fish species populations (Figure 5).

Characterization of Muskellunge *Piscirickettsia* sp was performed on CHSA and FHM cell lines. Results demonstrated that optimum growth temperature is 20° C. The bacterium retained infectivity up to 14 days at 32° C, 7 days at 34° C, 6 hr at 37° C, and 5 minutes at 56° C. The

organism tolerated salt concentrations up to 30 ppt for 7 days. These criteria suggest a high tolerance of the organism to a wide range of environmental conditions.

Pathogenicity testing was performed at the MSU Research Containment Facility by intraperitoneally injecting non-infected fish fingerlings with 100 µl containing 10^4 bacteria. The bacteria caused high mortalities (87%) in muskellunge fingerlings within 28 days postinfection. There were mortalities in rainbow trout or largemouth bass fingerlings (Figure 6).

In naturally or experimentally infected muskellunge, *Piscirickettsia* sp caused enlargement of internal organs and the formation of fluid filled vesicles budding out of the internal swim bladder membrane (Figure 7). In hematoxylene and eosin stained tissue section, kidney glomeruli seems to have been destroyed and replaced multiplying bacteria (Figure 8).

2. *Piscirickettsia* sp. Infection in Muskellunge Gametes: No *Piscirickettsia* sp. or other bacterial species were retrieved from muskellunge ovarian fluids, eggs, or testicular tissues.

3. Other Pathogens of Importance Found in Muskellunge Samples: From two fish of the 2004 samples and one fish of the 2005 samples a rhabdovirus was isolated from kidneys and spleen homogenates (Figure 9). The virus replicated in FHM cell lines at 25° C. Its role in inducing these or other lesions remains to be elucidated.

4. *Piscirickettsia* sp. Infection in Other Fish and Invertebrate Species: From all other species examined, *Piscirickettsia* sp. was only present in the yellow perch with an infection rate of 57%. The yellow perch organism morphologically resembled the muskellunge's isolates and molecular analysis is being carried out to determine the relatedness between the two isolates (Figure 10).

Job 5. Title: Collect data on rate of external marks with muskellunge with voluntary muskellunge lesion diary program.–In 2003, a total of nine muskellunge anglers returned lesion diaries that contained records of the presence or absence of lamprey wounds, red sores, or other abnormal wounds on the muskellunge they boated in 2003. A total of 501 muskellunge observations were recorded. The fish ranged in length from 20 inches to 54 inches. Red sores or piscirickettsia lesions were observed on 20 fish (4%), while lamprey marks were observed on 38 fish (7.6%). Both red sores and lamprey marks were absent from fish less than 32 inches in length. Only two fish were recorded with both red sores and lamprey marks (0.3%). The number of muskellunge observations recorded by anglers varied by month, with nearly 65% of all observations recorded in July and August. Numbers of fish observations recorded in September, October, and November were much lower. Red sore observations were recorded from June to October, with the highest numbers of fish exhibiting sores in July and August. However, the highest proportion of fish observed with red sores was noted in October and June.

In May 2004, diaries were returned to eight of the cooperating anglers for their use during the 2004 muskellunge fishing season. One angler had moved and could not be contacted by mail or phone. A total of three lesion diaries were returned by cooperating anglers in the fall of 2004. A total of 76 muskellunge observations were recorded. No red sores or piscirickettsia lesions were observed on any of the fish, while lamprey marks were observed on 13 fish (17%). Due to reduced number of lesion diaries returned and low number of fish observations recorded, no summary of lesions and lamprey marks by season or fish size was attempted. Due to waning cooperation, lesion diaries were not distributed in 2005.

Job 6. Title: Prepare annual performance report.–This report was prepared.

Literature cited:

Fryer, J.L., C.N. Lannan, L.H. Garces, J.J. Larenas, and P.A. Smith. 1990. Isolation of a rickettsiaies-like organism from diseased coho salmon *Oncorhynchus kisutch* in Chile. Fish Pathology 25:107–114.

Fryer, J.L., C.N. Lannan, S.J. Giovannoni, and N.D. Wood. 1992. *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. Internatioal Journal of Systematic Bacteriology 42:120–126.

Fryer, J.L., and M.J. Mauel. 1997 the rickettsia: an emerging group of pathogens in fish. Emerging Infectious Diseases 3:137–144.

Prepared by: Michael V. Thomas and Dr. Mohamed Faisal
Date: September 30, 2005

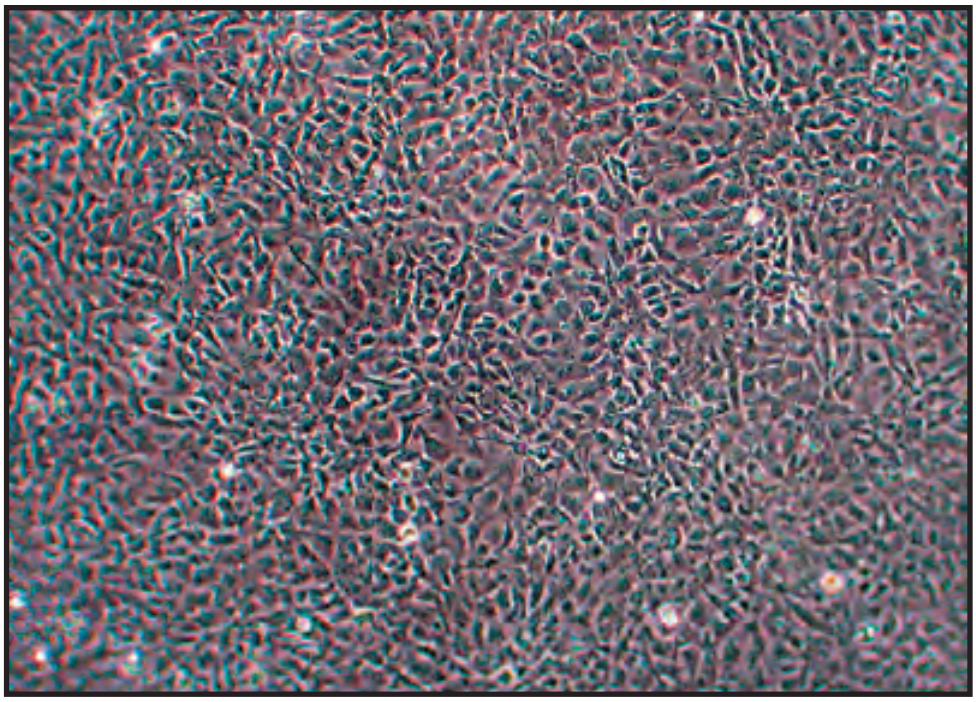

Figure 1a.−Healthy FHM cell line.

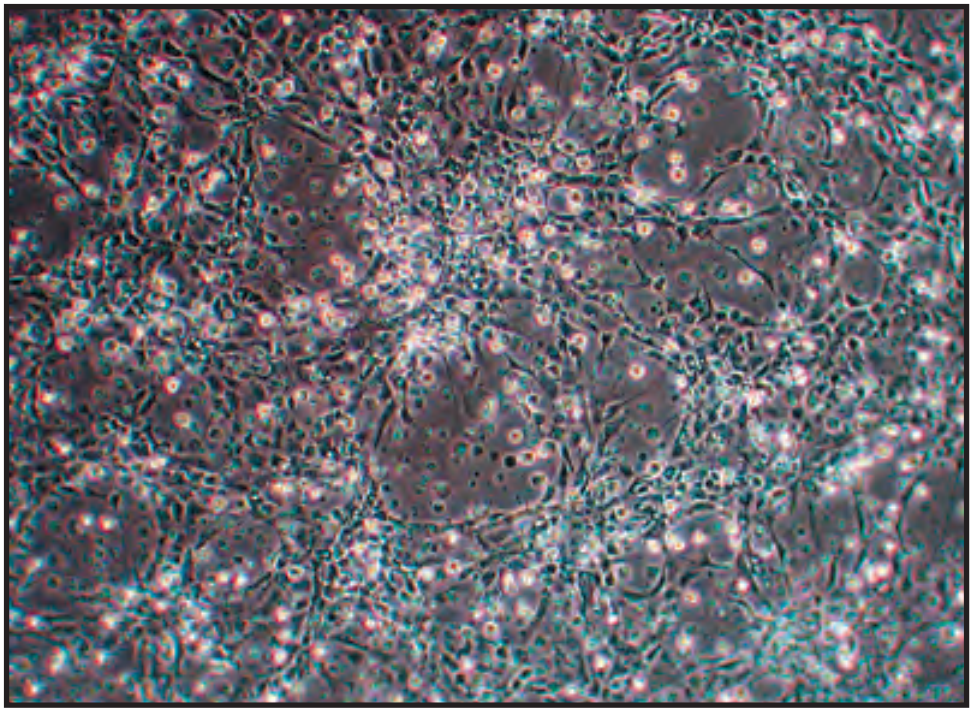

Figure 1b.−Cytopathic effects of FHM cell lines caused by Muskellunge *Piscirickettsia* sp.

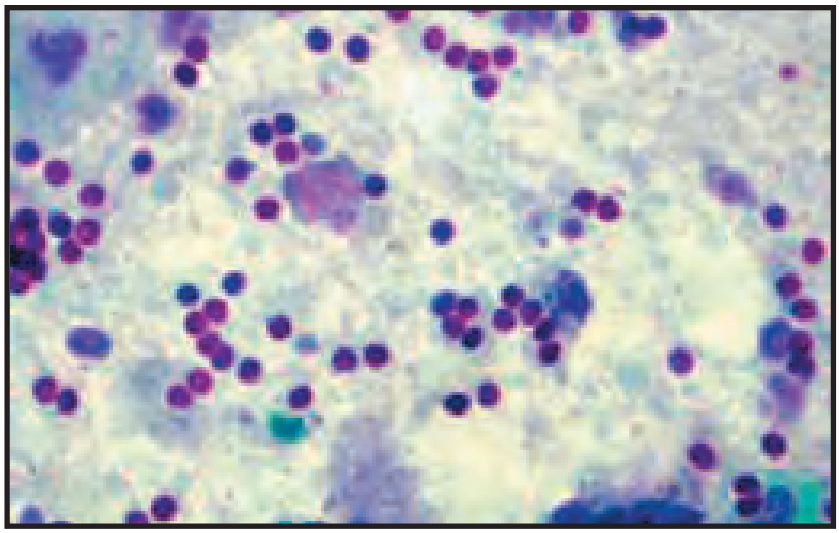

Figure 2.−Muskellunge *Piscirickettsia* sp. in affected skin lesions stained with Giemsa.

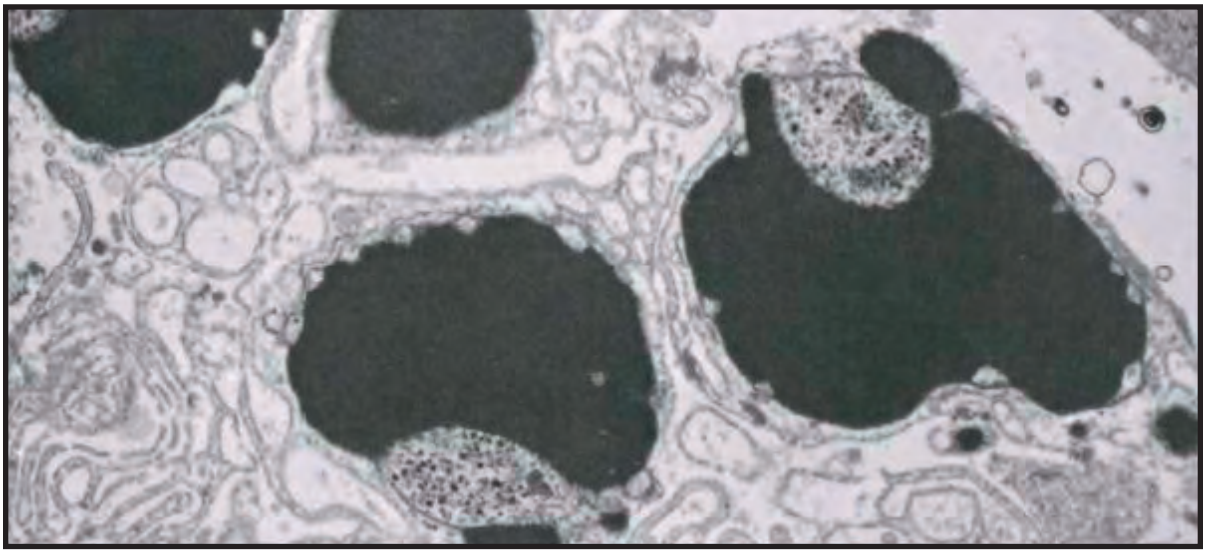

Figure 3.−Electron microscopy photograph of muskellunge *Piscirickettsia* sp. Notice the intracellular enclose in membrane-bound vesicle.

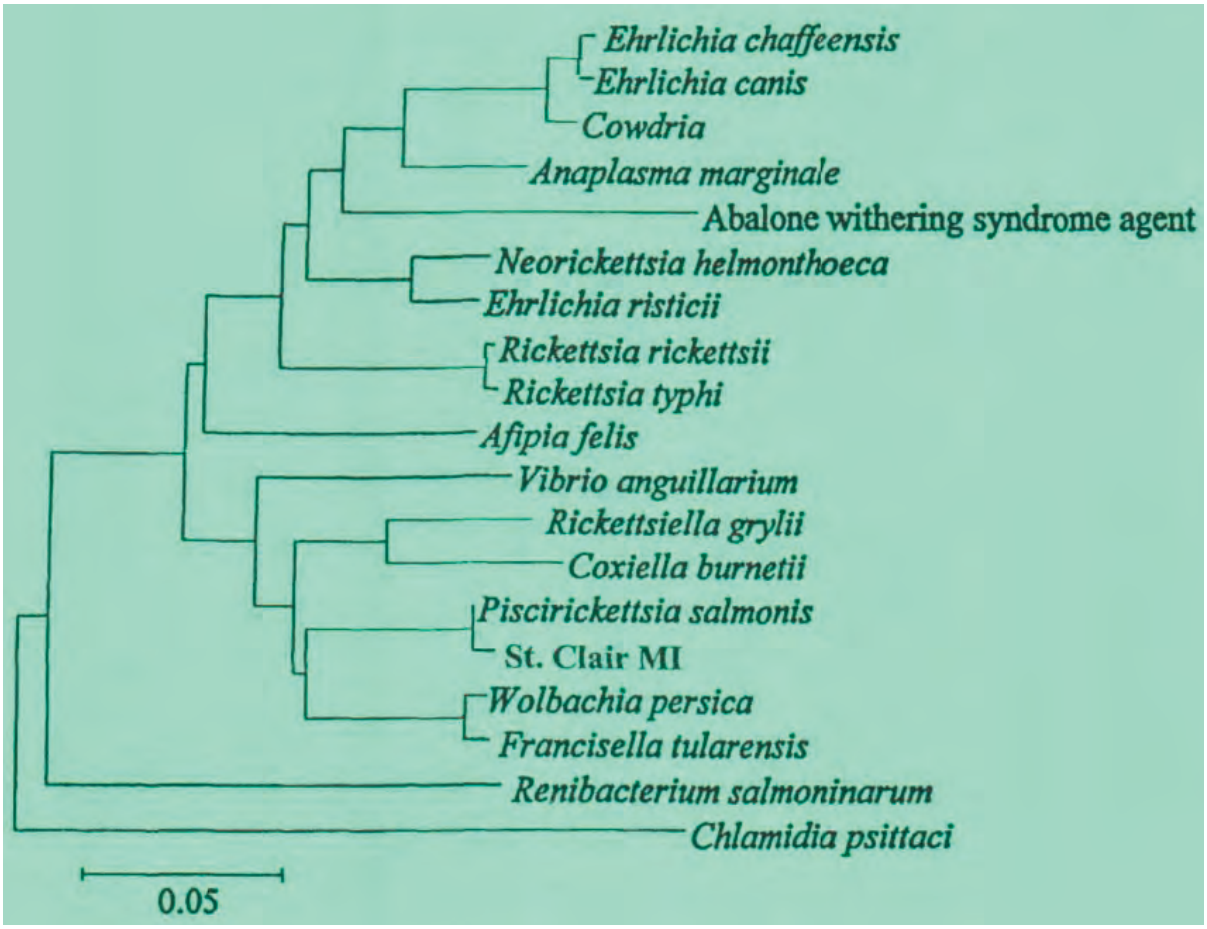

Figure 4.−Phylogenetic tree, based on 16 S rDNA sequences only, constructed by the neighbor-joining method the obtained sequence of Michigan Isolate and published sequences of related and distant bacteria.

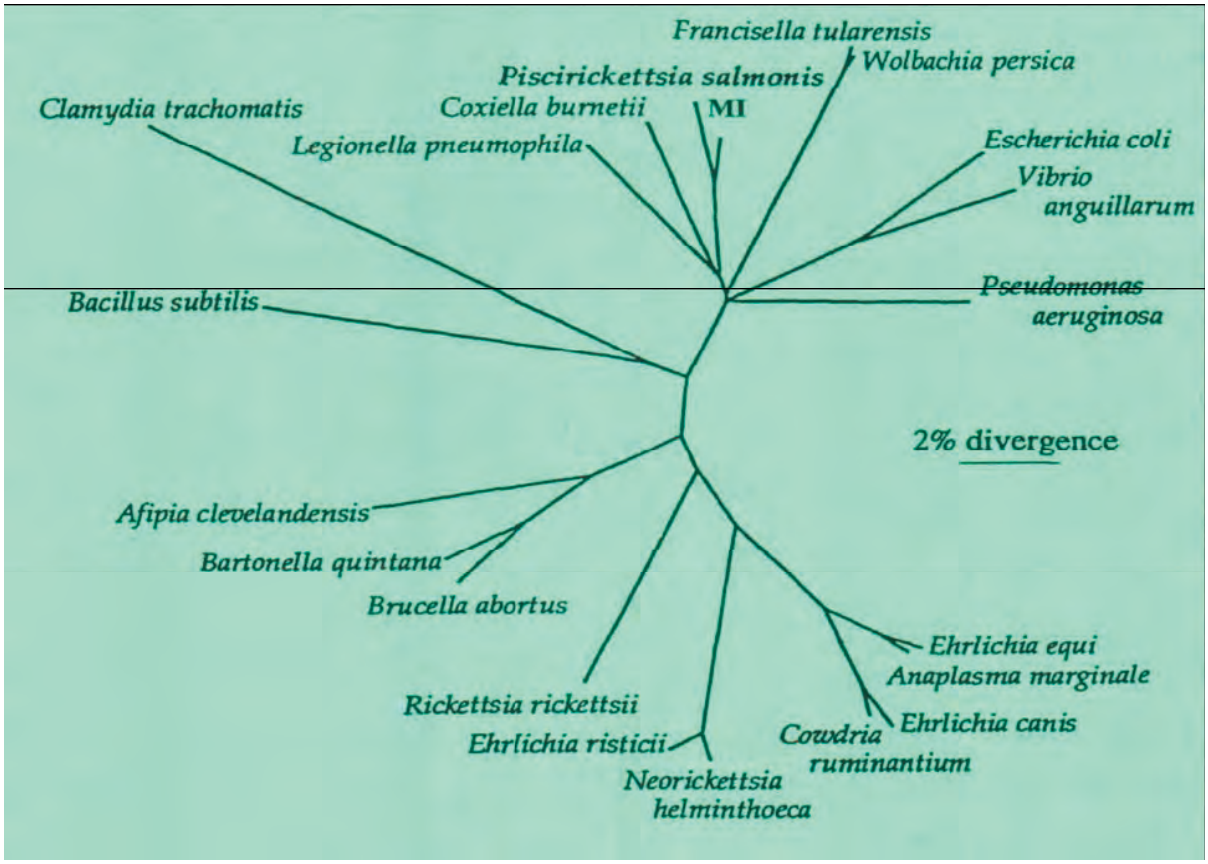

Figure 5.−Phylogenetic analyses involving sequeneces of the 16S, ITS, and 23S rDNA genes. These analyses confirmed that muskellunge isolates were identical to each other, but not identical to *P. salmonis*.

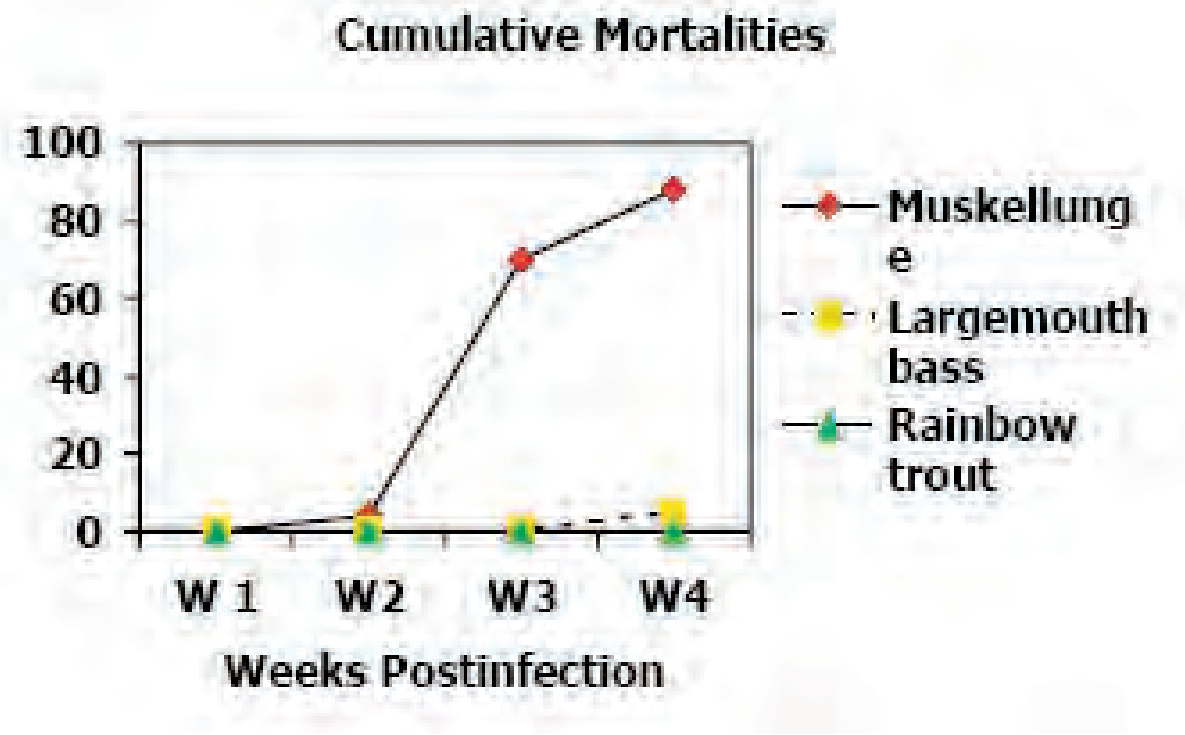

Figure 6.−Pathogenicity testing performed by intraperitoneal injection non-infected fish fingerlings with 100 µl of tissue culture supernatant containing 10^4 bacteria.

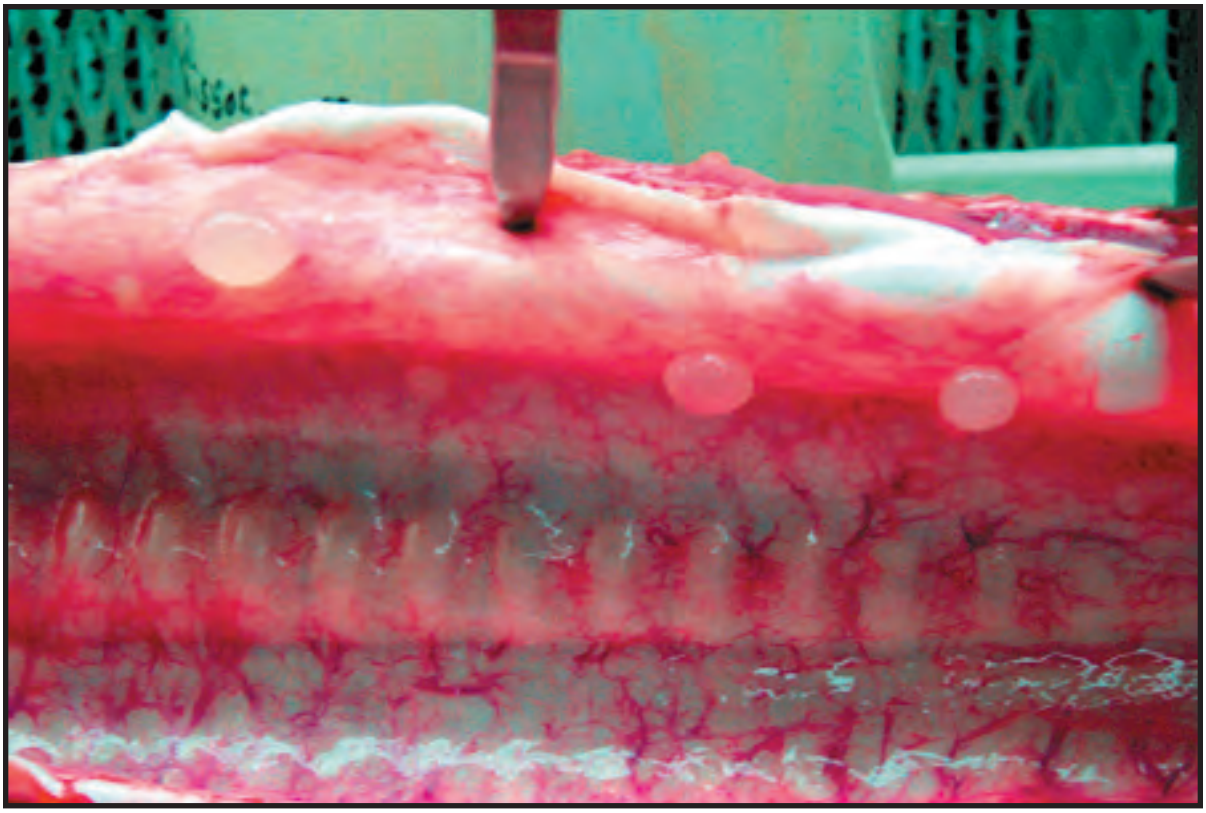

Figure 7.−Naturally infected muskellunge showing fluid-filled vesicles budding out of the internal swim bladder membrane.

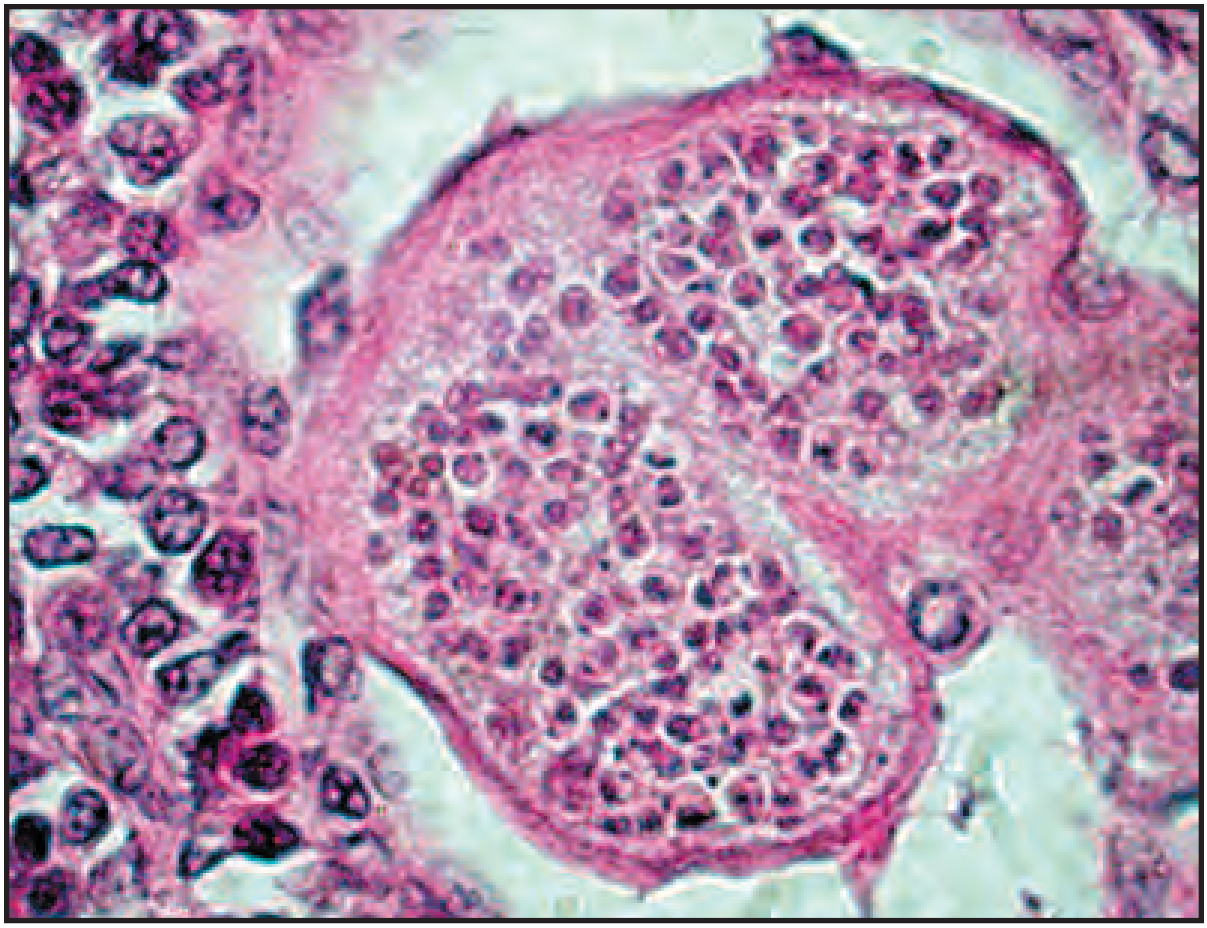

Figure 8.−Hematoxylene and eosin stained tissue section showing destroyed kidney glomeruli filled with multiplying bacteria.

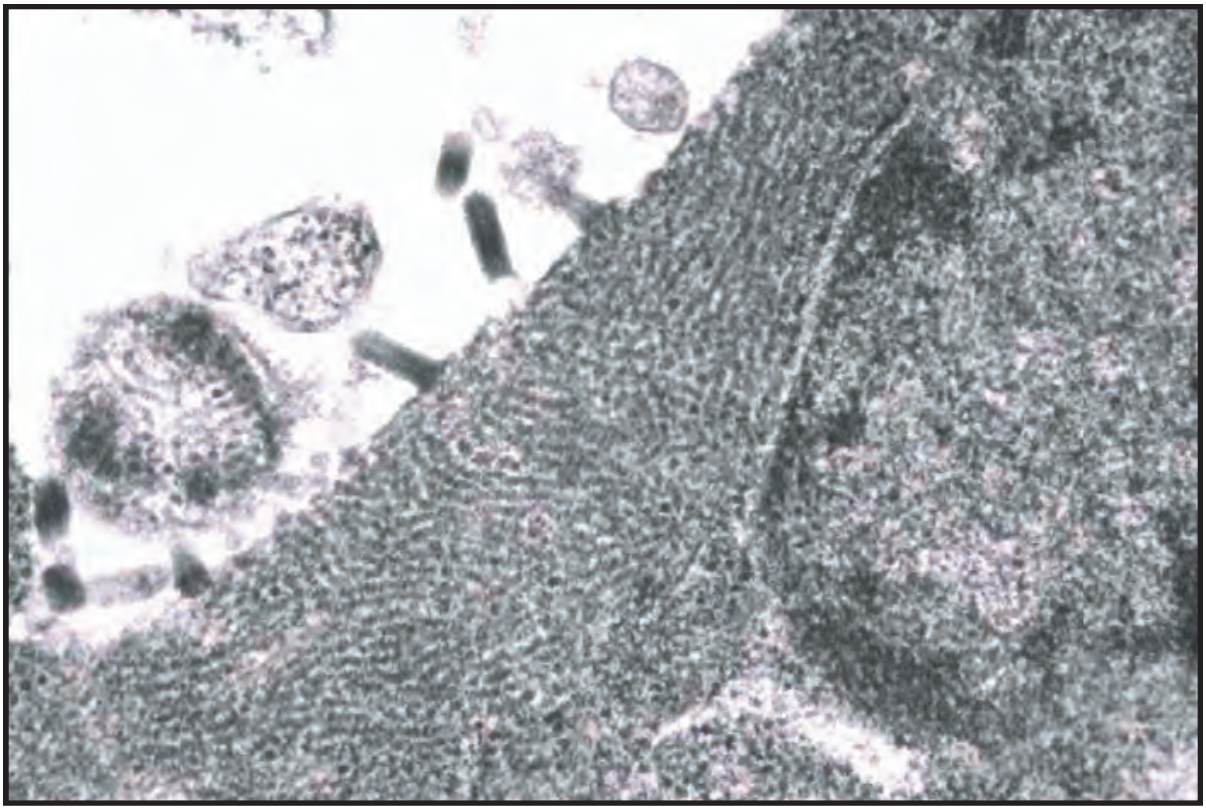

Figure 9.−Ultrastructural details of a rhabdovirus isolated from Lake St. Clair muskellunge on FHM cell line.

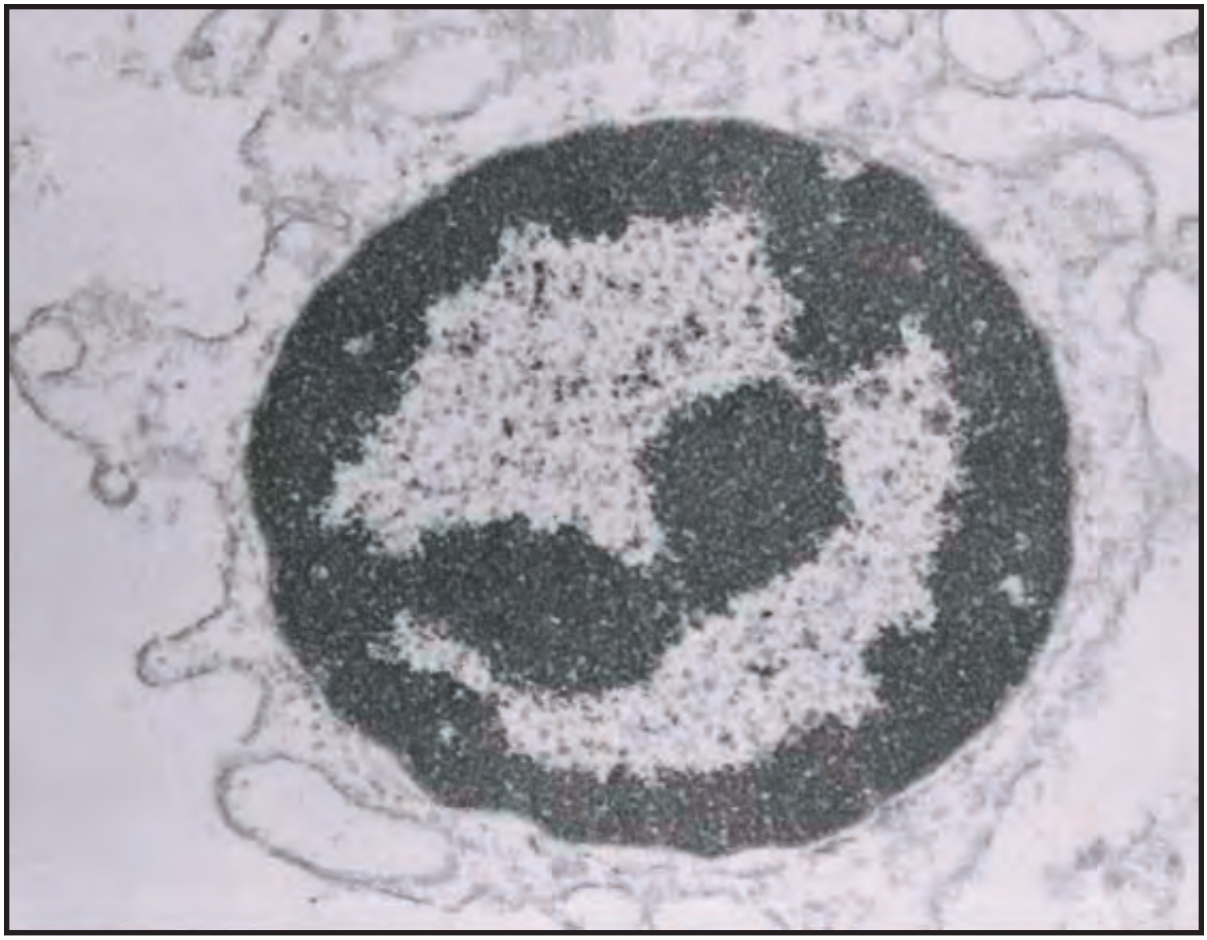

Figure 10.−A *Piscirikettsia* sp from the yellow perch.