Diversity of Fish-Associated Flavobacteria of Michigan

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Diversity of Fish-Associated Flavobacteria of Michigan

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Abstract

Flavobacteriosis poses a serious threat to wild and propagated fish stocks alike, accounting for more fish mortality in Michigan and its associated state fish hatcheries than all other pathogens combined. Although this consortium of fish diseases has primarily been attributed to Flavobacterium psychrophilum, F. columnare, and F. branchiophilum, herein we describe a diverse assemblage of Flavobacterium and Chryseobacterium spp. isolates recovered from diseased as well as apparently healthy wild, feral, and farmed fish of Michigan. Among 254 fish-associated flavobacterial isolates recovered from 21 fish species during 2003–2010, 211 were identified as Flavobacterium spp., whereas 43 were identified as Chryseobacterium spp. according to ribosomal RNA partial gene sequencing and phylogenetic analysis. Although F. psychrophilum and F. columnare were indeed associated with multiple fish mortality events, many previously uncharacterized flavobacteria were recovered from systemically infected fish showing overt signs of disease, and in vitro protease assays demonstrated that these isolates were highly proteolytic to multiple substrates that comprise host tissues. Indeed, the majority of the isolates either (1) were most similar to recently described fish-associated Flavobacterium and Chryseobacterium spp. that have never before been reported in North America (e.g., F. oncorhynchi, F. araucananum, C. viscerum, C. piscicola, and C. chaponense) or (2) did not cluster with any described species and most likely represent novel flavobacterial taxa. This study highlights the extreme diversity of flavobacteria that are potentially associated with flavobacteriosis in Michigan.

Over the past two decades, the taxonomy of the family Flavobacteriaceae (Reichenbach 1992) was amended on multiple occasions (Bernardet et al. 1996, 2002), and advances in molecular techniques have rapidly expanded the number of genera and species within the family (Bernardet et al. 2011). Bacteria within this family occupy a wide range of ecological niches (Bernardet and Nakagawa 2006) and are associated with disease in an array of organisms, including invertebrates (Li et al. 2010), amphibians (Xie et al. 2009), reptiles (Hernandez-Divers et al. 2009), birds (Segers et al. 1993), and mammals (Haburjak and Schubert 1997), including humans (Benedetti et al. 2011). Serious diseases that pose threats to wild and propagated fish are caused by multiple genera within the family Flavobacteriaceae, including Flavobacterium spp. (Starliper 2011), Tenacibaculum

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spp. (Suzuki et al. 2001), and Chryseobacterium spp. (Muddarris and Austin 1989).

Most flavobacterial disease outbreaks in freshwater fishes are attributed to three Flavobacterium spp., namely F. psychrophilum, F. columnare, and F. branchiophilum (Austin and Austin 2007). In addition, a number of other fish-associated Flavobacterium spp. have been reported in association with fish disease, such as F. johnsoniae (Suebsing and Kim 2012), F. succinicans (Anderson and Ordal 1961), and F. hydatis (Strohl and Tait 1978), as well as a number of uncharacterized yellow-pigmented bacteria (Austin and Austin 2007). Most recently, a number of novel Flavobacterium spp. were isolated from diseased fish in Europe and South America, including F. oncorhynchi (Zamora et al. 2012a), F. chilense, and F. araucanum (Kämpfer et al. 2012).

Similarly, the genus Chryseobacterium (Vandamme et al. 1994) contains a number of fish-pathogenic species that have recently emerged as a serious problem in Europe and Asia (Bernardet et al. 2005; Michel et al. 2005). Indeed, numerous novel fish-associated Chryseobacterium spp. have been recently described, such as C. piscium (de Beer et al. 2006), C. piscicola (Ilardi et al. 2009), C. arothrii (syn. C. hominis; Kämpfer et al. 2009), C. chaponense (Kämpfer et al. 2011), and C. viscerum (Zamora et al. 2012b). Currently, there are no published reports of Chryseobacterium-caused diseases in fish in the continental USA.

In Michigan and its associated state fish hatcheries, Flavobacterium spp. have been associated with more fish mortality than all other fish pathogens combined (Faisal and Hnath 1994) contains a number of fish-pathogenic species that have recently emerged as a serious problem in Europe and Asia (Bernardet et al. 2005; Michel et al. 2005). Indeed, numerous novel fish-associated Chryseobacterium spp. have been recently described, such as C. piscium (de Beer et al. 2006), C. piscicola (Ilardi et al. 2009), C. arothrii (syn. C. hominis; Kämpfer et al. 2009), C. chaponense (Kämpfer et al. 2011), and C. viscerum (Zamora et al. 2012b). Currently, there are no published reports of Chryseobacterium-caused diseases in fish in the continental USA.

In Michigan and its associated state fish hatcheries, Flavobacterium spp. have been associated with more fish mortality than all other fish pathogens combined (Faisal and Hnath 1994). The 50-nL loops were used for fish ≤6 cm in length. Kidney or gill tissues (or both) for bacterial isolation were collected during fish health surveillance; other tissues (e.g., gills, fins, swim bladder fluid, external ulcers, or combination of these) were also bacteriologically analyzed when disease signs were observed. Collected samples were inoculated directly onto Hsu–Shotts medium (HSM; Bullock et al. 1986) and cytophaga agar (CA; Anacker and Ordal 1955), both of which were supplemented with neomycin sulfate at 4 mg/L of medium; plates were incubated at 22°C for up to 7 d (HSM) or at 15°C for up to 14 d (CA). Bacterial growth was then recorded, and individual colonies were subcultured for phenotypic and molecular analyses. For cryopreservation, an individual colony was inoculated into HSM or cytophaga broth and incubated for 3–5 d; 20% glycerol (volume/volume) was then added, and the sample was frozen at −80°C.

Characterization of recovered isolates.—Bacteria that grew on neomycin-supplemented HSM or CA were visually inspected for nondiffusible yellow pigment; 24–48-h-old cultures on HSM (at 22°C) and 48–96-h-old cultures on CA (at 15°C) were assayed via the Gram reaction, the string test, or both (AFS-FHS 2010). Representative isolates were tested for their ability to degrade (1) hemoglobin (0.1% weight/volume [w/v]; n = 118 isolates) using HSM as the basal medium, as modified from Shotts et al. (1985); (2) casein (5% w/v; n = 91) and elastin (0.5% w/v; n = 116) using HSM as the basal medium, as modified from Shotts et al. (1985); and (3) gelatin (n = 123), as detailed by Whitman (2004). Enzymatic assays were incubated by using the temperature at which bacteria were initially isolated, and results were read up to 7 d postinoculation. Gram-negative rods that grew on HSM, CA, or both and possessed a nondiffusible yellow pigment (n = 254 isolates) were suspected to be members of the family Flavobacteriaceae and were then subjected to molecular analyses.

16S ribosomal RNA gene amplification.—The DNA from each of the 254 isolates was extracted by using a Qiagen DNeasy Tissue Extraction Kit (Qiagen, Valencia, California) according to the manufacturer’s protocol. Quantification of extracted DNA was performed using the Quant-iT DS DNA Assay Kit in conjunction with a Qubit fluorometer (Life Technologies, Grand Island, New York). Amplification of partial 16S ribosomal RNA (rRNA) gene was conducted via PCR using the universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′, Marchesi et al. 1998; numbering is based on the 16S rRNA gene of Escherichia coli: Brosius et al. 1978). The 50-µL PCR for each sample contained a final concentration of 200 nM for each primer, 25 µL of 2× Go-Taq Green Master Mix (Promega, Madison, Wisconsin), and 20 ng of DNA template, with DNase-free water comprising the remainder of the reaction mixture. The DNA amplification was carried out in a Mastercycler Pro Thermal Cycler (Eppendorf, Hauppauge, New York) with an initial denaturation step at 95°C for 5 min, followed by 32 cycles of amplification that included denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 60 s. A final extension step
TABLE 1. Gross signs of disease observed among fish infected with flavobacteria from the 42 clusters identified in this study. Clinical signs were not observed in fish infected with flavobacteria from clusters XVI, XXI, XXIV, XXV, XXVI, XXX, XXXI, XXXII, XXXVI, and XLI. It should also be noted that the reported disease signs cannot be solely attributed to the flavobacteria that were recovered from the infected fish, as the role of other fish pathogens in disease progression was not investigated in this study (* = a portion of the infected fish did not present with any clinical signs of disease).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Disease signs in infected fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Melanosis, abdominal distension, fin erosion, hemorrhagic enteritis, renal and hepatic pallor, and generalized visceral edema</td>
</tr>
<tr>
<td>II</td>
<td>Melanosis; gill pallor; external focal ecchymotic hemorrhage; fin erosion or necrosis; hepatic, splenic, and renal pallor; generalized visceral edema; fluid within the swim bladder lumen; and friable and congested kidney*</td>
</tr>
<tr>
<td>III</td>
<td>Melanosis, gill clubbing, mild gill pallor, fin erosion, unilateral exophthalmia, renal and hepatic pallor, splenomegaly, and renal and hepatic congestion*</td>
</tr>
<tr>
<td>IV</td>
<td>Unilateral exophthalmia, fluid within the swim bladder lumen, friable spleen, and renal and hepatic congestion*</td>
</tr>
<tr>
<td>V</td>
<td>Erosion, necrosis, and hemorrhage of fins; clubbed gills; melanosis; splenomegaly; and renal and hepatic pallor*</td>
</tr>
<tr>
<td>VI</td>
<td>Enophthalmia, dermal ulceration, and splenomegaly</td>
</tr>
<tr>
<td>VII</td>
<td>Erratic swimming or spinning and mottled liver*</td>
</tr>
<tr>
<td>VIII</td>
<td>Melanosis; gill pallor; fin erosion; and hepatic, splenic, and renal pallor</td>
</tr>
<tr>
<td>IX</td>
<td>Melanosis, lordosis, unilateral exophthalmia, renal and hepatic pallor, and congested and swollen kidney</td>
</tr>
<tr>
<td>X</td>
<td>Gill pallor, lamellar erosion, unilateral exophthalmia, congestion at base of fins, hemorrhagic fins, hepatic and splenic pallor, and congested and swollen kidney*</td>
</tr>
<tr>
<td>XI</td>
<td>Enophthalmia; deep muscular ulceration; gill pallor; splenomegaly; and swollen, pale, and mottled liver*</td>
</tr>
<tr>
<td>XII</td>
<td>Melanosis, erratic swimming or spinning, fluid within the swim bladder lumen, splenomegaly, hepatic and splenic pallor, and mottled liver*</td>
</tr>
<tr>
<td>XIII</td>
<td>Muscle ulceration; fin erosion; gill pallor; splenomegaly; swollen liver, spleen, and kidney; hepatic and renal pallor; mottled liver; congested liver and kidney; multifocal ecchymotic hemorrhage of the liver; excessive amount of ovarian fluid in gravid spawning females; and edematous kidney*</td>
</tr>
<tr>
<td>XIV</td>
<td>Hemorrhagic fins, external ecchymotic hemorrhage, clubbed gills, and splenomegaly</td>
</tr>
<tr>
<td>XV</td>
<td>Fin erosion, external hemorrhage, hepatic pallor, congested liver and kidney, and splenomegaly*</td>
</tr>
<tr>
<td>XVII</td>
<td>Periocular hemorrhage; gill necrosis; external petechial hemorrhage; fin erosion; muscular ulceration; hepatic, splenic, and renal pallor; mottled liver; hepatomegaly; swollen kidney; friable and swollen spleen; and splenomegaly*</td>
</tr>
<tr>
<td>XVIII</td>
<td>Unilateral exophthalmia, congestion at base of fins, gill pallor and necrosis, muscular ulceration, erosion of dermis overlying the jaw, erythema isthmus, splenomegaly, hepatic pallor, and friable kidney*</td>
</tr>
<tr>
<td>XIX</td>
<td>Melanosis, enophthalmia, fin erosion, congestion at base of fins, and friable kidney</td>
</tr>
<tr>
<td>XX</td>
<td>Erratic swimming or spinning</td>
</tr>
<tr>
<td>XXII</td>
<td>Melanosis; gill pallor; fin erosion; and hepatic, splenic, and renal pallor*</td>
</tr>
<tr>
<td>XXIII</td>
<td>Gill pallor*</td>
</tr>
<tr>
<td>XXVII</td>
<td>Petechial hemorrhage of the ventrum, gill pallor, and congestion at base of fins</td>
</tr>
<tr>
<td>XXVIII</td>
<td>Unilateral exophthalmia; splenomegaly; friable, congested kidney; and pale, mottled liver</td>
</tr>
<tr>
<td>XXIX</td>
<td>Fin erosion, hepatic and renal pallor, and friable and/or swollen kidney</td>
</tr>
<tr>
<td>XXXIII</td>
<td>Dermal erosion, fin erosion, mildly swollen spleen, hepatic and renal pallor, and visceral edema</td>
</tr>
<tr>
<td>XXXIV</td>
<td>Fin erosion; melanosis; lordosis; muscle ulceration; bilateral exophthalmia; hepatic, splenic, and renal pallor; and swollen spleen</td>
</tr>
<tr>
<td>XXXV</td>
<td>Erratic swimming behavior and irregular opercular movement</td>
</tr>
<tr>
<td>XXXVII</td>
<td>Visceral edema, hepatic pallor, fluid within the swim bladder lumen, and petechial hemorrhage at base of fins</td>
</tr>
<tr>
<td>XXXVIII</td>
<td>Swollen, friable spleen*</td>
</tr>
<tr>
<td>XXXIX</td>
<td>Fin erosion, multifocal dermal ulceration, swollen spleen, and congested liver and kidney</td>
</tr>
<tr>
<td>XL</td>
<td>Gill pallor, unilateral exophthalmia, fin erosion, hemorrhagic nares, and swollen spleen</td>
</tr>
<tr>
<td>XLI</td>
<td>Enophthalmia, melanosis, fin erosion, splenic pallor, and swollen kidney</td>
</tr>
</tbody>
</table>

was performed at 72°C for 7 min. Amplicons were combined with SYBR Green gel stain (Cambrex Bio Science Rockland, Inc., Rockland, Maine), run on a 1.5% agarose gel at 50 V for 30 min, and visualized under ultraviolet exposure. A 1-kbp ladder (Roche Applied Science, Indianapolis, Indiana) was used as a molecular marker.

Phylogenetic analyses.—Amplicons were purified by using the QIAquick Purification Kit (Qiagen) according to the
manufacturer’s protocol except that the same 35 μL of elution buffer preheated to 56°C were used for both elution steps. Amplified DNA was then sequenced by use of the 27F primer at the MSU Genomics Technology Support Facility. Generated sequences were initially analyzed with the nucleotide Basic Local Alignment Search Tool (BLASTN) from the National Center for Biotechnology Information (NCBI, USA). Sequences for all formally described and “candidate” Flavobacterium spp. and Chryseobacterium spp., as well as for the outgroups Elizabethkingia miricola, Elizabethkingia meningosepticum, and Capnocytophaga ochracea, were downloaded from NCBI and the EzTaxon-e database (Kim et al. 2012). The percent similarity in 16S ribosomal DNA (rDNA) between the 254 Michigan isolates and the closest type strains was determined by using the alignment function in BLAST. Type strains were also aligned with the 254 isolates from Michigan fishes by using Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 to assess phylogenetic relationships. Neighbor-joining analysis was then performed (Saitou and Nei 1987) in MEGA, with evolutionary distances calculated by using the maximum composite likelihood method (Tamura et al. 2004). Topology robustness was evaluated by bootstrap analysis based upon 10,000 resamplings of the sequences, and a total of 711 characters was examined. Alignment gaps or missing data were deleted only in pairwise sequence comparisons, and the tree was rooted with Capnocytophaga ochracea as the outgroup. Only bootstrap values of 70% or greater were displayed on the resultant dendrograms and were interpreted as indicating strong support for the topology present at that respective node.

RESULTS
Bacterial cultures yielding Gram-negative, yellow-pigmented bacteria on HSM, CA, or both were obtained from 21 fish species, including the Channel Catfish Ictalurus punctatus, Coho Salmon Oncorhyncus kisutch, Chinook Salmon O. tsawytscha, Rainbow Trout O. mykiss, steelhead (anadromous Rainbow Trout), Bluegill Lepomis macrochirus, Sea Lamprey Petromyzon marinus, Walleye Sander vitreus, Lake Whitefish Coregonus clupeaformis, Cisco Coregonus artedi, Brown Trout Salmo trutta, Atlantic Salmon Salmo salar, Brook Trout Salvelinus fontinalis, Lake Trout Salvelinus namaycush, Mottled Sculpin Cottus bairdii, Northern Brook Lamprey Ichthyomyzon fos sor, Yellow Perch Perca flavescens, Smallmouth Bass Micropterus dolomieu, Largemouth Bass Micropterus salmoides, Muskellunge Esox masquinongy, and Northern Pike Esox lucius during 101 sampling events that took place between 2003 and 2010. Information on each of the isolates, including fish species, site, purpose of sampling, date, and organ of recovery, is detailed in Supp. Table 1. Of the 254 Gram-negative, yellow-pigmented flavobacterial isolates analyzed in this study, 211 were identified as Flavobacterium spp. (retrieved from 88 sampling events and 21 fish species) and 43 were identified as Chryseobacterium (retrieved from 26 sampling events and 12 fish species; Supp. Table 1) according to partial 16S rRNA gene sequences and BLASTN analysis.

The 211 Flavobacterium spp. isolates were 96.5–100% similar to 21 formally described and candidate Flavobacterium spp. Of those, 123 isolates were recovered from wild and feral Michigan fish, while 88 isolates were recovered from fish reared within hatcheries. The majority of the isolates were retrieved during routine health surveys (n = 155), whereas 56 isolates were associated with mortality episodes. Organs of recovery included the kidneys (n = 92), gills (n = 88), brain (n = 16), fins (n = 9), fluid within the swim bladder lumen (n = 4), and ulcers of the skin or muscle (n = 2; Supp. Table 1).

Forty-three of the yellow-pigmented bacteria recovered from Michigan fishes were most similar to members of the genus Chryseobacterium, ranging from 96.7% to 99.9% similarity with described and candidate Chryseobacterium spp. The isolates were recovered from wild or feral fish (n = 17) and from fish reared within hatcheries (n = 26); they were obtained during health surveys (n = 27) and mortality episodes (n = 16). Organs of recovery included the gills (n = 21), kidneys (n = 11), fins (n = 7), skin or muscle ulcers (n = 2), and brains (n = 2) of infected fish (Supp. Table 1).

Michigan Flavobacterium spp. were most similar to F. anhuiense (n = 2), F. aquidurens e (n = 15), F. araucanum (n = 20), F. chilense (n = 2), F. chungbukense (n = 2), F. columnare (n = 15), F. degelarchei (n = 2), F. frigidimar is (n = 12), F. glacei (n = 2), F. hircynium (n = 33), F. hibernum (n = 2), F. hydatis (n = 3), F. oncorthynchi (n = 20), F. pectinovorum (n = 28), F. psychrolimnae (n = 1), F. psychrophilum (n = 19), F. reichenbachii (n = 3), F. resistens (n = 2), F. succinicans (n = 16), and F. tiangeerense (n = 3). Phylogenetic analysis of Michigan fish-associated Flavobacterium spp. placed the isolates into 32 distinct clusters (bootstrap value ≥70%; Figure 1; Supp. Table 1); however, the topology of 32 isolates was unresolved (bootstrap value <70%). On the other hand, Michigan Chryseobacterium spp. were most similar to C. aquaticum (n = 1), C. chaponense (n = 2), C. ginsenosidum (n = 2), C. ginsenosidum (n = 6), C. greenlandense (n = 2), C. indologenes (n = 1), C. indoetheticum (n = 6), C. piscicola (n = 2), C. piscium (n = 5), C. scophthalmum (n = 1), C. shigense (n = 1), C. viscerum (n = 14), and C. vrystaatense (n = 2). Phylogenetic analysis of the 43 Michigan fish-associated Chryseobacterium spp. yielded 10 distinct clusters (bootstrap value ≥70%; Figure 2; Supp. Table 1), but the topology for 16 isolates could not be resolved (bootstrap value <70%). Disease signs in fish infected with bacteria belonging to each cluster are presented in Table 1. It must be noted, however, that some bacteria other than flavobacteria may have contributed to the observed lesions.

Flavobacterium spp. Isolates
The 16S rRNA gene percent similarity of the 33 Michigan isolates that were most similar to F. hircynium ranged from 97.0% to 98.8% (Supp. Table 1). Phylogenetic analysis yielded five distinct clusters (Figure 1): cluster I contained 6 isolates...
(Supp. Figure 1), cluster II included 13 isolates (Supp. Figure 1), cluster III included 6 isolates (Supp. Figure 2), cluster IV consisted of 4 isolates (Supp. Figure 3), and cluster V contained 3 isolates (Supp. Figure 4). The topology of three additional isolates (T65, S53, and T132) was unresolved (Figure 1). Within cluster I, isolates S113 and S114, both of which were recovered from the necrotic fins of hatchery-reared Brown Trout fingerlings undergoing a mortality episode, formed a well-supported group that was distinct from the other four isolates (Supp. Figure 1). It is also of interest that isolate T129 was most similar to F. succinicans based on 16S rDNA percent similarity and yet also fell within cluster I. Cluster II comprised the largest number of isolates that were most similar to F. hercynium, within which distinct subclusters were present (Supp. Figure 1). For example, isolates T101 and T102, which were recovered from the kidneys of Chinook Salmon and Brook Trout fingerlings raised at two different Michigan hatcheries, were quite distinct from the other members of cluster II (bootstrap value = 99%). With the exception of isolate S148, all of the isolates belonging to cluster II were recovered from wild and hatchery-reared salmonids (Supp. Table 1). Five of the six isolates belonging to cluster III were recovered from mortality events involving hatchery-reared salmonid fingerlings, and the remaining isolate originated from the kidney of a feral Chinook Salmon returning to the Swan River weir (Presque Isle County, Lake Huron watershed). The isolates comprising cluster IV, including isolate S15 that was most similar to F. chungangense based on 16S rDNA percent similarity (Supp. Figure 3), were all recovered from the kidneys and swim bladders of spawning Brown Trout and steelhead. Cluster V isolates all originated from hatchery-reared Brown Trout fingerlings (Supp. Table 1), with one of the three isolates (isolate S86) being associated with mortalities. Enzymatic activities for isolates within clusters I–V varied (Table 2). Cluster I was uniformly negative for gelatinase but varied in hemoglobin hydrolysis and caseinase and elastase activities. Cluster II was variable for all four proteases. Protease activities for clusters III–V are presented in Table 2.

The 16S rRNA gene percent similarity of the 28 Michigan isolates that were most similar to F. pectinovorum ranged from 97.1% to 98.4% (Supp. Table 1). Phylogenetic analysis yielded three distinct clusters (Figure 1): cluster VI contained 3 isolates (Supp. Figure 2), cluster VII included 11 isolates (Supp. Figure 1), and cluster VIII consisted of 4 isolates (Supp. Figure 1). The topology of the other 12 isolates was unresolved (Figure 1; Supp. Figure 2). Interestingly, isolates S31 (most similar to F. aquidurense based on 16S rDNA percent similarity), S37 (most similar to F. aquidurense), and S164 (most similar to F. frigidimaris) also fell within cluster VII (Supp. Figure 2). Eight isolates belonging to cluster VII (S40, S29, S35, S34, S38, S37, S31, and S41) were all recovered from the brains of hatchery-reared Coho Salmon fry undergoing a single mortality episode (Faisal et al. 2011). Isolates belonging to cluster VIII were recovered exclusively from hatchery-reared Brook Trout and Rainbow Trout, while the other two clusters (VI and VII) were recovered from both wild and hatchery-reared fish (Supp. Table 1). Among the three cluster VII isolates that were assayed for proteolytic activity, all degraded casein, hemoglobin, and elastin, but they did not proteolyze gelatin (Table 2). The three tested isolates within cluster VIII proteolyzed elastin and casein but varied in gelatinase activity (one of the three isolates was positive) and hemoglobin hydrolysis (one isolate was positive; Table 2).

The Michigan isolates (n = 20) that were most similar to newly described F. oncorhynchi ranged from 97.4% to 100% similarity in 16S rDNA. Phylogenetic analysis yielded two distinct clusters (Figure 1): cluster IX, which contained 8 isolates (Supp. Figure 4); and cluster X, which contained 12 isolates (Supp. Figure 2). Isolates belonging to cluster IX were nearly identical to the F. oncorhynchi reference sequence (99.3–100% similar), originated from six different fish species, and were predominately recovered from the gills, although three of the isolates were also recovered from kidneys (Supp. Table 1). Despite the high percent similarity, there was evidence of a distinct subclade within cluster IX (Supp. Figure 4). Three of the eight isolates were recovered from two mortality events in hatchery-reared Brown Trout and Brook Trout fingerlings raised at two Michigan hatcheries. Cluster X formed a distinct clade (Supp. Figure 2). Similar to cluster IX, the majority of the isolates within cluster X were recovered from the gills (9 of 12) and the remainder were recovered from kidneys (Supp. Table 1). Cluster X isolates were recovered from hatchery-reared salmonids, with the exception of isolate T103, which originated from a wild Sea Lamprey (Supp. Table 1). Proteolytic activities varied in both clusters (Table 2), but none of the isolates degraded hemoglobin.

The 16S rRNA gene percent similarity for the 20 Michigan isolates that were most similar to the F. araucananum reference strain ranged from 96.9% to 98.8%. When analyzed phylogenetically, two clusters were evident (Figure 1): cluster XI, which included nine isolates (Supp. Figure 5); and cluster XII, which also included nine isolates (Supp. Figure 4). Two of the 20 isolates (T157 and S162) were unresolved (Figure 1). Isolates belonging to cluster XI were recovered from nine different species of wild or feral (n = 6) and hatchery-reared (n = 3) fish (Supp. Table 1). Isolate T17, which was most similar to F. aquidurense based on 16S rDNA similarity, was one of the nine isolates in this cluster. All three isolates that originated from hatchery-reared fish were associated with mortality events in fingerlings of Chinook Salmon, Northern Pike, and Rainbow Trout (Supp. Table 1). Among these, isolate S21 was recovered from deep necrotic ulcers present on the dorsum of a Rainbow Trout (Figure 3A). Flavobacterium spp. isolates that belonged to cluster XII were primarily recovered from the gills of wild fish that were sampled during fish health surveillance (Supp. Table 1); however, isolate S43 was recovered from the brain of a hatchery-reared Coho Salmon fry during a mortality event (Faisal et al. 2011). Interestingly, of the wild fish from which the cluster XII isolates were recovered, the majority were collected in (1) the creek supplying water to the hatchery where...
FIGURE 1. Dendrogram generated using the neighbor-joining method in Molecular Evolutionary Genetics Analysis version 4, depicting the phylogenetic relationships between Michigan fish-associated *Flavobacterium* spp. and other described and candidate *Flavobacterium* spp. Bootstrap values greater than 70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.
the outbreak occurred in Coho Salmon (represented by isolates S126, S130, and S149) and (2) the hatchery effluent pond (represented by isolates S163, S161, and S166; data not shown), despite being sampled 4 years apart. Within cluster XI, all of the tested isolates were positive for caseinase and elastase activity, but they varied in gelatinase and hemoglobin hydrolysis activities (Table 2). The seven tested isolates within cluster XII were all positive for caseinase and hemoglobin hydrolysis and...
FIGURE 2. Dendrogram generated using the neighbor-joining method in Molecular Evolutionary Genetics Analysis version 4, depicting the phylogenetic relationships between Michigan fish-associated Chryseobacterium spp. and other described and candidate Chryseobacterium spp. Bootstrap values greater than 70% (expressed as percentages of 10,000 replicates) are presented at branch nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Elizabethkingia meningosepticum and Elizabethkingia miricola served as the outgroup.

were all gelatinase negative, but the isolates varied in elastase activity (Table 2).

Nineteen isolates examined in this study were most similar to F. psychrophilum according to 16S rDNA percent similarity (98.6–99.9%). Phylogenetic analysis performed on the isolates recovered from Michigan fishes yielded two well-supported clusters within the species (Figure 1): cluster XIIIa (Supp. Figure 6) comprised 15 isolates that were recovered predominantly from feral-spawning (n = 13) Oncorhynchus spp. (Supp. Table 1); and cluster XIIIb (Supp. Figure 6) consisted of the F. psychrophilum reference strain, one F. psychrophilum isolate that was recovered from feral-spawning Chinook Salmon, and three F. psychrophilum isolates that were recovered from hatchery-reared Atlantic Salmon fingerlings (Supp. Table 1). All 19 Michigan F. psychrophilum isolates were recovered from the kidneys of infected fish.

Isolates that were most similar to F. succinicans (n = 16) were the next most numerous group and ranged from 96.6% to 98.0% similarity in 16S rDNA relative to the reference strain. Upon phylogenetic analysis, three well-supported clusters were evident (Figure 1): cluster XIV with 2 isolates (Supp. Figure 5), cluster XV with 10 isolates (Supp. Figure 7), and cluster XVI with 3 isolates (Supp. Figure 7). However, isolate T129 was included in cluster I (described above; Supp. Figure 1). Interestingly, isolates belonging to these three clades were exclusively recovered from wild fish (Supp. Table 1) and were rarely
TABLE 2. Percentage of Michigan fish-associated Flavobacterium spp. and Chryseobacterium spp. isolates exhibiting gelatinase, caseinase, and elastase activities as well as the ability to degrade hemoglobin. Clusters VI, XIII, XIV, XVI, XXIII, XXIV, and XXXV were not tested for protease activity. The number of tested isolates is given in parentheses.

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associated with disease. The two Flavobacterium spp. isolates comprising cluster XIV, which was quite distinct from clusters XV and XVI, were both recovered from the kidneys of Lake Whitefish collected at Naubinway, Lake Michigan. Although clusters XV and XVI were close relatives, phylogenetic analysis provided evidence for their divergence (bootstrap value = 77%; Supp. Figure 7). Cluster XV isolates were recovered from both coldwater and warmwater fishes and were predominantly isolated from the gills (7 of 10 isolates). Cluster XVI isolates originated from the gills of Brown Trout yearlings residing in
Gross lesions present in Michigan fishes infected with *Flavobacterium* spp. and *Chryseobacterium* spp.: (A) necrotic ulceration (arrow) present on the dorsum of a Rainbow Trout from which *Flavobacterium* sp. isolate S21 was recovered (note the complete erosion of the dorsal fin and penetration into the underlying musculature); (B) severe necrosis and hemorrhage of the left pectoral fin (arrow) of a Brown Trout fingerling from which *Flavobacterium* sp. isolates belonging to cluster XIX were recovered; (C) erosion and necrosis of the caudal fin and caudal peduncle (arrow) of a Brook Trout fingerling from which *Chryseobacterium* sp. isolate T72 was recovered; (E) left pectoral fin of a yearling Brown Trout from which *Chryseobacterium* sp. isolate T62 was recovered (note severe necrosis and hemorrhage of the fin, with concurrent exposure of the eroded fin rays: arrow); and (F) multifocal dermal ulcerations (arrows) present on the trunk of a feral-spawning steelhead from which *Chryseobacterium* sp. isolate S25 was recovered. [Figure available online in color.]

Cherry Creek, which was sampled during 2008 and 2010 (Supp. Table 1). Representative isolates belonging to cluster XV did not degrade gelatin or hemoglobin and were variable for caseinase and elastase activities (Table 2).

Fifteen isolates that were most similar to *F. columnare* (16S rDNA similarity = 98.7–100%) were also analyzed in this study. Fourteen of the 15 isolates were nearly identical to the *F. columnare* reference sequence (99.7–100% similar), while isolate S81 was more distinct at 98.7% similarity. Phylogenetic analysis also reflected this difference (Figure 1), whereby the *F. columnare* reference sequence and 14 of the Michigan *F. columnare* isolates formed a distinct and homogeneous cluster (cluster XVII; Supp. Figure 8), while *F. columnare* strain S81 formed a separate branch, termed cluster XVIIa (Supp. Figure 8). Four Michigan *F. columnare* isolates were recovered from three mortality events involving hatchery-reared Yellow Perch (isolate S19), hatchery-reared Muskellunge fingerlings (isolates T89 and T90), and wild Smallmouth Bass from Lake St. Clair (isolate T79; Supp. Table 1). The remaining 13 *F. columnare* isolates were recovered from the kidneys of feral Coho Salmon and Chinook Salmon returning to the Platte River weir (Benzie County, Lake Michigan watershed), the Little Manistee River weir (Manistee County, Lake Michigan watershed), and the Swan River weir. Representative isolates (n = 4) belonging to cluster XVII uniformly degraded gelatin, casein, and elastin, but only one of the four isolates hydrolyzed hemoglobin (Table 2).

Fifteen isolates examined in this study were most similar to *F. aquidurense* according to 16S rDNA percent similarity (97.3–98.2%). Among these, two isolates (S31 and S37) belonged to cluster VII (described above; Supp. Figure 2), one isolate (T17) was included in cluster XI (Supp. Figure 5), one isolate (S30) was included in cluster XX (described below; Supp. Figure 9), one isolate (S107) was unresolved (Figure 1), and the remaining 10 isolates formed cluster XVIII (Supp. Figure 9). Isolates belonging to cluster XVIII, which displayed varying degrees of genetic heterogeneity (Supp. Figure 9), were recovered from the kidneys of feral Chinook Salmon (n = 6) during 2005 and 2007 (Supp. Table 1) and from the kidneys of hatchery-reared...
salmonid fingerlings \( n = 3 \) and wild larval Sea Lampreys \( n = 1 \). Among the cluster XVIII isolates, T16 was associated with a mortality event in cultured Chinook Salmon fingerlings. Protease assays performed on representative cluster XVIII isolates demonstrated that this group uniformly degraded casein and elastin but varied in gelatin and hemoglobin degradation (Table 2).

Isolates that were most similar to \( F. \) frigidimaris \( n = 12 \) ranged from 97.4% to 100% in 16S rDNA similarity to the \( F. \) frigidimaris reference strain. Phylogenetic analysis revealed the formation of cluster XIX, which contained four isolates and the \( F. \) frigidimaris reference sequence (Figure 1; Supp. Figure 9); and cluster XX (Supp. Figure 9), which contained isolates S5 and S30 (most similar to \( F. \) aquidurense according to 16S rDNA percent similarity). The remaining six isolates were unresolved (Figure 1; Supp. Table 1); however, among these unresolved isolates, five isolates were close to \( F. \) frigidimaris despite having a bootstrap value less than 70%, while one isolate was similarly close to \( F. \) hercynium (Figure 1). In addition, isolate S164 was included in cluster VII (Supp. Figure 2). Isolates belonging to cluster XIX were recovered from the kidneys, fins (Figure 3B), and brains of hatchery-reared salmonid fingerlings undergoing mortality (Supp. Table 1). Isolates within cluster XIX did not degrade gelatin or elastin, and they varied in caseinase activity and hemoglobin hydrolysis (Table 2). One isolate belonging to cluster XX was positive for hemoglobin degradation only (Table 2).

The 16S rDNA gene percent similarity for the 10 Michigan isolates that were most similar to the \( F. \) chunganense reference strain ranged from 96.5% to 98.0%. When phylogenetically analyzed, two clusters were evident (Figure 1): cluster XXI, which consisted of three isolates (Supp. Figure 2); and cluster XXII, which included five isolates (Supp. Figure 3). Isolate S129 was unresolved (Figure 1), and isolate S15 belonged to cluster XXIV (Supp. Figure 2); and cluster XX (Supp. Figure 4). The two isolates that were most similar to \( F. \) glycinum (97.4–98.0%) were recovered from the kidneys of hatchery-reared Channel Catfish (Supp. Table 1) and formed cluster XXVIII (Supp. Figure 4). The two isolates that were most similar to \( F. \) hibernum (97.9%) were recovered from the gills of wild Mottled Sculpin and Brown Trout (Supp. Table 1); both of these isolates were unresolved phylogenetically (Figure 1). The two \( F. \) psychrolimnae-like isolates (96.7–96.9%) formed cluster XXIX (Supp. Figure 5) and were both recovered from a hatchery mortality event among Brown Trout fingerlings (Supp. Table 1). Isolates that were most similar to \( F. \) glaciei \( n = 2 \) isolates; 98.5–98.6%) formed cluster XXX (Supp. Figure 1); one isolate (S42) was recovered from the brain of a moribund Coho Salmon fry in a hatchery stock undergoing a mortality episode (Faisal et al. 2011), and the other was recovered from the kidney of a wild Walleye (Supp. Table 1). The two isolates that were most similar to \( F. \) hibernum (97.7%) were distinct in that isolates S118 belonged to cluster XXV (described above; Supp. Figure 1), while isolate S140 was unresolved but shared a most recent ancestry with \( F. \) hibernum (Supp. Figure 5). Flavobacterium resistans-like isolates \( n = 2 \), 97.1–97.3), which were recovered from the kidneys of wild Largemouth Bass, formed cluster XXXI and shared a most recent ancestry with \( F. \) resistans (Supp. Figure 4). Isolate S2, which was most similar to \( F. \) psychrolimnae (99.6%), formed cluster XXXII with the \( F. \) psychrolimnae reference strain (Supp. Figure 6) and was originally recovered from the kidney of a wild Walleye.

**Chryseobacterium spp. Isolates**

Among the Michigan isolates within the genus Chryseobacterium, 14 were most similar to the recently described \( C. \) viscerum, ranging from 98.8% to 99.7% similarity in 16S rDNA (Supp. Table 1). Phylogenetic analysis yielded the formation of one cluster (cluster XXXIII; Supp. Figure 10) that contained isolates T86, T87, and T88; the remaining 11 isolates were unresolved, although they were close in proximity to the...
C. viscerum reference sequence (unresolved group 2, upper portion of Supp. Figure 10). Isolates within cluster XXXIII were recovered from the gills and kidneys of hatchery-reared Muskellunge fingerlings that were undergoing a single mortality event (Supp. Table 1) and that were also infected with F. columnare; all of these isolates uniformly degraded gelatin, casein, hemoglobin, and elastin (Table 2). The 11 unresolved C. viscerum isolates were recovered from the gills of wild Mottled Sculpin, Brook Trout, and Brown Trout (Supp. Table 1) residing in four different Michigan creeks. All of the unresolved C. viscerum isolates displayed gelatinase, caseinase, and elastase activities as well as hemoglobin hydrolysis (Table 2).

Six Michigan isolates were most similar to C. ginsenosidimutans (97.7–98.4%), all of which composed cluster XXXIV (Supp. Figure 11). However, some genetic heterogeneity was observed within this cluster, whereby isolates T107 and T130 diverged from their most recent common ancestor and that of isolates T62, S104, T68, and S110 (Supp. Figure 11). The six isolates within this cluster were recovered from hatchery-reared fish; four of the six were recovered from four different morbidity or mortality events in Brook Trout and Brown Trout fingerlings (Supp. Table 1) and were recovered from infected gills and necrotic and hemorrhagic fins (Figure 3E). Moreover, isolates T68 and T130 were recovered from the kidneys of infected salmonid fingerlings (Supp. Table 1). Protease assays found that two of the six isolates were positive for gelatinase and four of the six were positive for hemoglobin hydrolysis, but all of the tested isolates were positive for elastase and caseinase activities (Table 2). Among the six isolates that were most similar to C. indoltheticum (97.4–99.1% similarity in 16S rDNA), three formed cluster XXXV (Supp. Figure 11) and three were unresolved, despite clustering near C. indoltheticum and cluster XXXV (Supp. Figure 11). The isolates of cluster XXXV were recovered from the gills and brains of hatchery-reared steelhead fingerlings during a single mortality event (Supp. Table 1), whereas isolate S63 was recovered from a necrotic ulcer in the musculature of hatchery-reared Coho Salmon fingerlings and isolate T72 was recovered from the kidneys of hatchery-reared Ciscos with septicemia (Figure 3D).

The 16S rRNA gene percent similarity of the five Michigan isolates that were most similar to C. piscium ranged from 98.3% to 98.4% (Supp. Table 1). Phylogenetic analysis placed the five isolates, along with one isolate (S56) that was most similar to C. scopophilum, into two clusters (Figure 2). Cluster XXXVI contained isolate T24 and the C. balustinum, C. piscium, and C. scopophilum reference strains (Supp. Figure 11), while cluster XXXVII contained the other five isolates (Supp. Figure 11). Isolate T24 of cluster XXXVI was recovered from the gills of a hatchery-reared steelhead fingerling (Supp. Table 1) displaying signs similar to those of bacterial gill disease, and this isolate degraded gelatin, casein, hemoglobin, and elastin (Table 2). Three of the isolates within cluster XXXVII were recovered from hatchery mortality events in Cisco and Brown Trout fingerlings. Representative cluster XXXVII isolates degraded casein and elastin but were variable in gelatinase activity and hemoglobin degradation (Table 2).

The two isolates that were most similar to C. chaponense (99.1%) formed cluster XXXVIII, which also included the C. chaponense reference sequence (Supp. Figure 12). Isolate T115 was recovered from the kidneys of feral Chinook Salmon, whereas isolate T60 was recovered from the kidney of a hatchery-reared Rainbow Trout fingerling (Supp. Table 1). Chryseobacterium greenlandense-like isolates (n = 2; 98.1% similarity), which composed cluster XXXIX (Supp. Figure 11), were recovered from the kidneys of wild Walleyes (isolate S4) and from dermal ulcers on fetal steelhead (isolate S25; Figure 3F). The two isolates that were most similar to C. piscicola (96.7–99.7%) were isolate T63, which formed cluster XL with the C. piscicola reference sequence (Supp. Figure 11); and isolate T85, which was unresolved but shared a most recent ancestry with cluster XL (Supp. Figure 11). The two isolates that were most similar to C. vrystaatense (99.5–99.9%) formed cluster XLI along with the C. vrystaatense reference sequence (Supp. Figure 10). These isolates were recovered from the fins of wild Sea Lampreys and the gills of wild Mottled Sculpin (Supp. Table 1). The remaining three isolates from Michigan fishes were most similar to C. aquaticum (isolate S105; 99.7%), C. indoltheticum (isolate S7; 97.4%), and C. shigense (isolate S108; 98.6%). Isolate S105 was grouped in cluster XLI with C. aquaticum (Supp. Figure 11); isolate S108 shared a most recent ancestry with C. shigense (Supp. Figure 10); and isolate S7 was unresolved (Supp. Figure 11).

**DISCUSSION**

As expected, F. psychrophilum and F. columnare were associated with serious losses in an array of Michigan fish stocks. However, this study clearly demonstrated the multitude of other Flavobacterium spp. and Chryseobacterium spp. that are also associated with diseased fish, as evidenced by the formation of 42 distinct clusters upon phylogenetic analysis of 254 Michigan flavobacterial isolates (Figures 1, 2). Among the isolates that were identified as described Flavobacterium spp., F. columnare was recovered from multiple mass-mortality events involving wild and cultured fishes of Michigan, during which thousands of fish died (MSU-AAHL, unpublished records). In addition, F. columnare was isolated from numerous feral salmonid stocks returning to spawn at Michigan’s gamete collection facilities from 2006 to 2010, where the prevalence of systemic columnaris disease can exceed 50% in some locations on an annual basis (MSU-AAHL, unpublished records). While the majority of the F. columnare isolates examined in this study were homogeneous and nearly identical to the F. columnare reference sequence (GenBank accession number AB078047.1), isolate S81, which was recovered from a feral returning adult Coho Salmon at the Platte River weir in 2006, was distinct. It is well established that there are at least three F. columnare genomovars (Triyanto and Wakabayashi 1999), and isolates belonging to
distinct genomovars differ in pathogenicity (Shoemaker et al. 2008). Although a comprehensive examination of intraspecies genetic heterogeneity for Michigan \textit{F. columnare} isolates was not undertaken in this study, 16S rDNA sequencing results suggested that more than one \textit{F. columnare} genomovar was present in Michigan salmonids. Because of this, further studies are underway to investigate the genetic profiles of Michigan \textit{F. columnare} isolates according to the methods of Arias et al. (2004).

\textit{Flavobacterium psychrophilum} isolates were also recovered in this study. Phylogenetic analysis demonstrated two distinct and well-supported \textit{F. psychrophilum} genotypes; cluster XIIIa included \textit{F. psychrophilum} isolates that were recovered from \textit{Oncorhynchus} spp. returning to the Little Manistee River weir and Swan River weir and from hatchery-reared Brown Trout, while cluster XIIIb contained isolates that were recovered from hatchery-reared Atlantic Salmon and one isolate from Chinook Salmon returning to the Swan River weir. Numerous studies have demonstrated the genetic heterogeneity of \textit{F. psychrophilum} by various molecular methods (i.e., Madsen and Dalsgaard 2000; Izumi et al. 2003; Soule et al. 2005; Ramsrud et al. 2007; Chen et al. 2008; Del Cerro et al. 2010), and Chakroun et al. (1998) found strong correlations between \textit{F. psychrophilum} ribotype and host of origin, which may explain the predominance of isolates recovered from Atlantic Salmon within cluster XIIIb.

It is also noteworthy that \textit{F. branchiophilum}, the purported agent of bacterial gill disease (Wakabayashi et al. 1989), was not recovered throughout the course of this study despite the fact that a number of the examined fish displayed disease signs that are often associated with bacterial gill disease (i.e., gill clubbing, gill pallor, etc.; Table 1). Indeed, the original \textit{F. branchiophilum} isolates upon which the species’ description was based were recovered on cytophaga medium (Wakabayashi et al. 1989), which was one of the medium types utilized in this study. Thus, based on the present study, it appears that multiple flavobacterial species can be associated with what would often be diagnosed as bacterial gill disease. As such, the potential role of these less-well-known flavobacteria in bacterial gill disease deserves to be further investigated.

While \textit{F. columnare} and \textit{F. psychrophilum} were indeed recovered in this study, they comprised only about 26% of the flavobacteria that were recovered from the internal organs of diseased or systemically infected fish. Other described \textit{Flavobacterium} spp. that were identified in association with Michigan fishes included the cluster IX isolates, which were recovered from three hatcheries and four creeks or lakes; this cluster also contained the \textit{F. oncorhynchi} reference sequence. \textit{Flavobacterium oncorhynchi} was recently described from diseased juvenile Rainbow Trout in Spain (Zamora et al. 2012a); this study represents the first report of its presence in North America. Although the type strain was originally recovered from the liver of a trout exhibiting signs of an \textit{F. psychrophilum} infection (Zamora et al. 2012a), disease signs in the present study included congestion of the fins; unilateral exophthalmia; hepatic, splenic, and renal pallor; and occasionally necrosis and epithelial hyperplasia of the gills. The present study also provides evidence for the wide host range of \textit{F. oncorhynchi}, as it was recovered from (1) four genera within the family Salmonidae (i.e., \textit{Salmo}, \textit{Salvelinus}, \textit{Oncorhynchus}, and \textit{Coregonus}); (2) an important prey species, the Mottled Sculpin; and (3) the invasive, fish-parasitic Sea Lamprey. Previous studies in our laboratory have highlighted the potential for Sea Lampreys to act as a vector for important fish pathogens, such as \textit{Aeromonas salmonicida} (Faisal et al. 2007) and \textit{F. psychrophilum} (Elsayed et al. 2006).

Four Michigan isolates (cluster XIX) were definitively identified as \textit{F. frigidimarisi}, a species that was originally isolated from arctic seawater (Nogi et al. 2005). Although we are unaware of any other published reports of this bacterium being associated with fish, a sequence within GenBank that is 99% similar to the \textit{F. frigidimarisi} type strain (HE612101.1) indicates that similar bacteria were recovered from the kidneys of Rainbow Trout in Spain. One Michigan isolate was also identified as \textit{F. psychrolimnae}, a bacterium that was first isolated from microbial mats in antarctic lakes (Van Trappen et al. 2005). GenBank sequences given the title “\textit{F. psychrolimnae}” by depositories indicated that this bacterium was associated with the gastrointestinal tracts of fish, but closer inspection shows that these sequences are distinct from the \textit{F. psychrolimnae} reference sequence (data not shown). \textit{Flavobacterium} isolate S171 was 98.9% similar to \textit{F. hydatis} and formed a well-supported cluster with the \textit{F. hydatis} reference strain (cluster XXVI). \textit{Flavobacterium hydatis} was first isolated from the gills of diseased salmonids reared at the Platte River State Fish Hatchery, Michigan (Strohl and Tait 1978); over 40 years later, this same bacterium was recovered from the gills of a Brook Trout inhabiting Kinney Creek, which serves as a water supply for that same hatchery. However, signs of disease were not observed in the fish from which isolate S171 was recovered. \textit{Flavobacterium} isolate T105 was also identified as \textit{F. tiangeerense}, which was originally isolated from a glacier in China (Xin et al. 2009). Again, no published reports have linked this bacterium to fish, and a search within GenBank indicates that similar sequences are predominantly associated with glaciers.

A number of described \textit{Chryseobacterium} spp. were identified among Michigan fishes, and some of these isolates were associated with disease. For instance, 11 isolates (unresolved group 2) were 99.1–99.7% similar to \textit{C. viscerum}, a species that was just described as occurring among septicemic Rainbow Trout in Spain (Zamora et al. 2012b). The specific signs in \textit{C. viscerum}-infected fish were not reported in the original description (Zamora et al. 2012b); however, infected fish examined in the present study showed mild melanosis, mild unilateral exophthalmia, hepatic pallor, and congestion of the kidney and liver, although the role that other fish pathogens may have played in the development of these disease signs was not assessed. It is worth noting that all 11 isolates with high similarity to \textit{C. viscerum} were recovered from the gills of infected
fish, and six of the fish that were infected with this bacterium were apparently healthy. Thus, experimental challenges verifying the pathogenicity of this bacterium would be prudent, as it may have an opportunistic relationship in fish. Two isolates that were identified as *C. vrystaatense* (cluster XLI) were recovered from the eroded fins of a Sea Lamprey and from the gills of Mottled Sculpin in this study. Although *C. vrystaatense* was originally recovered from raw chicken in a South African processing plant (de Beer et al. 2006), searches within GenBank indicated that a similar bacterium was recovered from aquaculture systems in South Africa (EU598811). Thus, the role that this bacterium may play in association with fish warrants attention. *Chryseobacterium* isolate T63 (cluster XL) was identified as *C. piscicola*, a species that was described in association with ulcerative skin and muscle lesions of Atlantic Salmon in Chile (Ilardi et al. 2009) and that was subsequently identified as occurring in diseased Atlantic Salmon from Finland (Ilardi et al. 2010). In the present study, *C. piscicola* was recovered from severely eroded and necrotic fins of hatchery-reared Brown Trout, indicating either a facultative or secondary pathogenic nature. Indeed, Ilardi et al. (2010) assessed the ability of *C. piscicola* to cause disease under laboratory conditions and found that it was moderately virulent to salmon. Once again, we are unaware of any other reports of this bacterium in association with diseased fish in North America.

Yet another *Chryseobacterium* sp. recovered from Michigan fish was *C. aquaticum*, which was originally described from a Korean water reservoir (Kim et al. 2008). It was also recovered from Siberian Sturgeon *Acipenser baeri* fry in France (AY468465; Bernardet et al. 2005). Interestingly, a strain of *C. aquaticum* was shown to produce a novel antifungal protease (Gandhi Pragash et al. 2009), possibly indicating a mutualistic relationship between this bacterium and its host. Two Michigan fish-associated isolates (cluster XXXVIII) were also identified as *C. chaponense*, which was recently reported in Chilean farmed Atlantic Salmon that were co-infected with *F. psychrophilum* (Kämpfer et al. 2011) and from skin ulcers in Rainbow Trout in France (AY468464; Bernardet et al. 2005). In the original description by Kämpfer et al. (2011), *C. chaponense* was recovered from external lesions, fins, and gills of infected fish; in the present study, both isolates were recovered from the kidneys of systemically infected Chinook Salmon and Rainbow Trout. Disease signs in these fish included mildly swollen and friable spleens in the Chinook Salmon, whereas the Rainbow Trout were apparently healthy. Nevertheless, to our knowledge, this is the first report of *C. chaponense* systemically infecting fish in North America and thereby illustrates the widespread presence of this bacterium in North America and South America as well as in Europe.

In addition to recovery of the seven aforementioned *Flavobacterium* spp. and five *Chryseobacterium* spp., the vast majority of isolates recovered from Michigan fishes did not cluster with any formally described *Flavobacterium* or *Chryseobacterium* sp. despite clearly belonging to one of the two genera. In fact, the majority of isolates in this study (*n* = 170) were less than 98.7% similar to described members of the family Flavobacteriaceae—a similarity value that can be seen between distinct *Flavobacterium* spp. (Bernardet and Bowman 2006 and references therein). However, to definitively delineate novel flavobacterial taxa, polyphasic characterization must be carried out, as recommended by Bernardet et al. (2002). As such, ongoing studies to elucidate this diverse assemblage of fish-associated flavobacteria are underway in our laboratory.

Still, it is clear from this study that not only do many of the clusters likely represent novel bacterial species, but some are likely to be pathogenic to fish. For example, cluster XVIII consisted of *Flavobacterium* spp. isolates that were recovered exclusively from systemically infected fish during 2005–2010, and one isolate (S12) was associated with a large mortality event in Chinook Salmon fingerlings at Thompson State Fish Hatchery in 2005. Similarly, *Flavobacterium* spp. isolates within cluster XI were recovered from kidneys, gills, and ulcers of infected fish, some of which had signs that mimicked those typical of bacterial coldwater disease (e.g., isolate S21 recovered from the ulcer in Figure 3A). Within the genus *Chryseobacterium*, cluster XXXVII isolates were associated with morbidity and mortality in aquacultured Cisco fingerlings and were also recovered from the kidneys and brains of systemically infected steelhead and Walleye fry, respectively. Moreover, cluster XXXIV isolates were recovered from multiple mortality events that occurred at three different hatcheries during three different years and likely represent a novel taxon. Many of the other clusters may also represent novel taxa. Clearly, there is a dire need to discern the role that these previously uncharacterized flavobacteria play in the health and diseases of fish.
the Laurentian Great Lakes and can serve as a platform for numerous studies to understand the role that these uncharacterized flavobacteria play in the health of Great Lakes fishes.

ACKNOWLEDGMENTS

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A Simple Reagent-Free Spectrophotometric Assay for Monitoring Metronidazole Therapy in Aquarium Water

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PLEASE SCROLL DOWN FOR ARTICLE
A Simple Reagent-Free Spectrophotometric Assay for Monitoring Metronidazole Therapy in Aquarium Water

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Abstract
A reagent-free spectrophotometric assay was developed to measure the concentration of metronidazole (a 5-nitroimidazole) in both freshwater and seawater matrices. This assay is simple, repeatable, sensitive, and precise and is ideal for use when a rapid, selective test to determine metronidazole concentration in aqueous matrices is necessary. The assay was practically tested on a South American fishes display during treatment with metronidazole for an outbreak of the flagellated parasite *Spironucleus* in a mixed cichlid (family Cichlidae) and tetra (family Characidae) community. The assay clearly illustrated the course of treatment for the system during a real clinical application. The assay is not without limitations, as interferences can occur from other drugs in the matrix with similar absorbance spectra. Nonetheless, this type of assay illustrates the potential for use of native absorbance assays in aqueous matrices for this and other therapeutic compounds.

Metronidazole (2-(2-methyl- 5-nitro- 1H- imidazol- 1-yl) ethanol) is a member of the family of antibiotic pharmaceutical compounds called 5-nitroimidazoles and is sold under the brand name Flagyl (Pfizer, New York). It is commonly used to treat bacterial gastrointestinal infections associated with enteric flagellates (Harms 1996). Metronidazole degrades DNA structure, resulting in inhibition of protein synthesis and cell death in the susceptible organism (Knight et al. 1978). In aquaria, metronidazole is used to treat infections caused by diplomonad flagellates, particularly *Spironucleus* (Sangmaneedet 1999). Depending on the target organism, treatment can be delivered by loading metronidazole into food or by bath immersion (Wildgoose 2001). As with all treatments, in order for treatment to be effective it is important to maintain an effective dose throughout the treatment regimen. This need has been hampered in the past by lack of a simple, robust assay for metronidazole in aqueous solutions.

For an assay to be used practically at a public aquarium it must satisfy the following criteria: it should be quick so that treatment can be adjusted in a timely manner, reproducible to show that the assay is selective and accurate, and require few reagents or complicated extraction and reaction steps due to budget and time constraints placed on the laboratory. Metronidazole has previously been determined via a HPLC method (Mishal et al. 2005), which does not meet these requirements due to the use of expensive reagents and extraction protocols. It has also been determined spectrophotometrically (Nagaraja et al. 2002), but this method also uses reagents and requires a reaction step that is time consuming and tedious. One method that satisfies the requirements necessary to be practical for aquarium use is a spectrophotometric assay of the therapeutic agent using its native absorbance. We conducted a pilot study to determine if some of the most commonly used drugs in ornamental fish medicine (Noga 2010) had a distinct native absorbance when
dissolved in freshwater and saltwater matrices and therefore could be measured spectrophotometrically. The study evaluated UV and visible light spectra using a wavelength scan, looking for unique spectral maxima that were not coincident with other naturally occurring peaks and were therefore good candidates for a native absorbance assay. During the pilot study, we determined that metronidazole could successfully be measured this way so we proceeded to develop a selective, repeatable, quick, and inexpensive assay to determine the concentration of metronidazole in both freshwater and saltwater matrices. We then confirmed the usefulness of the assay developed by measuring metronidazole concentration in a South American fishes display at the Georgia Aquarium during the course of a treatment for *Spiroplasma* infection.

**METHODS**

*Pilot study.*—The therapeutic drugs evaluated in the pilot study were methylene blue, trimethoprim sulfamethoxazole (TMS), oxolinic acid, dimilin, furazone green, and metronidazole. A good candidate for a native absorbance assay should have a clear maximum absorbance peak that will give a linear response for the calibration range (Owen 2000). We prepared 10-mg/L solutions of each drug in both freshwater and saltwater matrices. Then 10 mLs of each solution were placed into a clean 25-mL glass vial with a 25.4-mm light path, and then a wavelength scan was performed on each sample using a Hach DR5000 spectrophotometer. The wavelength scan measured absorbance from 190 to 900 nm using both a visible (tungsten lamp) and UV (deuterium lamp) light source with a spectral bandwidth of 0.5 nm.

![Figure 1](image-url)

**FIGURE 1.** Scatter plot of percent absorbance against wavelength of metronidazole, trimethoprim sulfamethoxazole (TMS), and oxolinic acid tested in a freshwater matrix.
2 nm. The unique absorbance maximum observed at 319 nm for metronidazole (Figures 1, 2) made it an ideal candidate for developing an assay to measure its concentration in saltwater and freshwater matrices spectrophotometrically. Although another peak absorbance was clearly seen at 332 nm, it proved to be a poor peak absorbance for the assay, as it failed to provide the linear response necessary to generate a calibration curve. There was no absorbance observed for any drug measured beyond the 450-nm region of the scan so the area from 450 to 900 nm was not considered in this assay. The other drugs evaluated in the pilot study were not considered for development due to poor absorbance maxima observed during the wavelength scans.

**Metronidazole assay.**—To determine the wavelength(s) at which peak absorbance occurred, solutions of metronidazole were prepared at different concentrations. Two separate standard curves were made: one for saltwater and the other for freshwater. The spectrophotometer was set to measure absorption at the 319-nm absorbance maximum for both saltwater and freshwater matrices, with a spectral bandwidth of 2 nm. Stock solutions were made in 1000-mL glass volumetric flasks using metronidazole (U.S. Pharmaceutical grade; Medesca) and the necessary stock waters, which for the assay were reverse-osmosis-treated freshwater and saltwater made with Instant Ocean (Marineland). Both the fresh and saltwater solutions were then filtered through a 0.2-μm vacuum filter flask. Standard curves were prepared using a rack of 24 25-mL round glass vials filled with ascending concentrations of the stock solution prepared by weighing out 2–20 mg aliquots of metronidazole and placing them in each
of two separate 1-L volumetric flasks. From these stock solutions, an eight-point curve with concentrations ranging from 0 to 20 mg/L was prepared for each matrix (Figure 3). For each reading, the spectrophotometer was zeroed with appropriate stock water. After reading, the start, maximum, and end of the absorbance peak was marked on each spectrum.

RESULTS

Assay Validation

The standard curves for both the freshwater and saltwater matrices demonstrated a relationship between absorbance and concentration in accordance with Beer’s law (Owen 2000) and showed linearity up to 20 mg/L (Figure 2) with $R^2$ values of 0.9961 for freshwater and 0.9978 for saltwater. For robust results, test solutions should be diluted until they produce readings below 20 mg/L. To determine the precision of the assay, 20 samples with a known metronidazole concentration of 5 mg/L were prepared and analyzed. The results were recorded for statistical analysis to determine the standard deviation, limit of detection, and percent relative standard deviation (%RSD; a measure of the spread of values). Using the calculated standard deviation as a measure of precision, this assay is capable of determining metronidazole levels with a precision of ± 0.50 mg/L for
freshwater and ± 0.57 mg/L for saltwater. The limit(s) of detection for freshwater and saltwater were determined by multiplying the standard deviation obtained earlier by 3. The limit(s) of detection were determined to be 1.48 mg/L for freshwater and 1.71 mg/L for saltwater. The %RSD was determined by dividing the standard deviation by the mean value obtained from the 20 samples; this gave a %RSD for freshwater of 9.56% and for saltwater of 9.66%. The assay also showed excellent spike recoveries of greater than 95%.

**Practical Application of Assay**

We applied the assay to determine if effective therapeutic dosing of metronidazole was being achieved in a South American fishes exhibit experiencing an outbreak of a flagellated parasite. The system was approximately 10,200 L and stocked with several species of South American fishes including Red Discus *Symphysodon aequifasciatus*, cichlids *Mesonauta* spp., Cardinal Tetras *Paracheirodon axelrodi*, and catfish *Corydoras* spp. The system was heavily planted and the temperature maintained at 29–30°C. The pH was maintained between 6.8 and 7.0 by computer-controlled additions of CO₂. The fish in the exhibit were displaying lesions and stringy feces that are commonly associated with *Spironucleus* infection. A blood smear was prepared from a postmortem cardiac puncture of a Red Discus to determine if the fish were systemically infected with *Spironucleus*, which is a diplomonad flagellate known to occur in the intestinal lining of fish and cause disease (Noga 2010) that may be lethal. Looking at the smear under the light microscope revealed a flagellated parasite with three sets of flagella with one pair located anterior, which is consistent with the appearance of *Spironucleus* (Poynton et al. 2002). The diagnosis resulted in a prescription that the exhibit be treated with an initial 25-mg/L dose of metronidazole, with maintenance doses to be added daily depending on the reading obtained using the metronidazole assay. The results were recorded and trended over the course of the 40-d metronidazole treatment in the South American fishes exhibit (Figure 4). The initial result showed 0 mg/L concentration on September 11, 2009, prior to treatment, followed by an increase.

![FIGURE 4](image-url). Measured metronidazole levels over the course of treatment in 2009.
to 20 mg/L on September 15, 2009, after the first dose was added to the system. A decline in concentration beginning October 5, 2009, was observed after the treatment period was over, and the removal of the drug managed through a series of water changes to the exhibit was also captured in the assay results. The testing concluded with a final reading of 0 mg/L on October 20, 2009.

**DISCUSSION**

A new simple method was developed for measuring the concentration of metronidazole in both seawater and freshwater matrices. Prior methods used to determine metronidazole were time consuming, expensive, and often involved complicated extraction steps. By contrast, this method is simple, quick, and ideal for use where a rapid, selective test to determine dissolved metronidazole levels in aqueous matrices is needed.

Using the assay, we successfully measured metronidazole levels applied to a South American fishes display, after a course of metronidazole was prescribed to treat an outbreak of *Spironucleus* in the exhibit. The assay was able to show clearly the course of the treatment in the system beginning with a zero measurement initially, followed by an increase after dosing, and the declining concentration at the conclusion of the treatment.

One of the limitations of the assay was the upper limit of measurement at 20 mg/L. To overcome this limitation, 2:1 dilutions were performed on samples that were determined to be over the measuring range, to bring the values within the linear measuring range. A 2:1 dilution was chosen to minimize the variability that could result from using dilutions of a higher factor. The specificity of this assay can be hampered by other drugs sometimes applied to aquarium systems. If a system has been treated with other drugs, a native absorbance assay of any drug previously administered should be performed to understand the possible effect on the matrix to be tested. A similar absorbance spectrum was observed for TMS (Figures 1, 2), which could potentially result in falsely high readings due to an additive effect on measured spectra. Before performing this assay, a system must not have been treated with TMS to ensure that there are no interfering absorption spectra near the 319-nm wavelength.

This study has illustrated the potential for native absorbance assays to be of use in certain situations where drug absorbance maxima are unique in the aqueous matrix. The potential use of this approach to develop new assays is limited only by the ability to generate and observe wavelength scans and the number of compounds to be tested that show sufficiently unique absorbance peaks. In aquaria, a number of compounds are used to treat a variety of illnesses and ailments via immersion baths. This technique could become a powerful tool to analyze the effectiveness of treatments and would be useful in studies of half-lives, drug persistence, and other related research, contributing to a more enlightened approach to chemotherapies in aquarium systems.

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Edwardsiellosis Caused by Edwardsiella ictaluri in Laboratory Populations of Zebrafish Danio rerio

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Edwardsielllosis Caused by *Edwardsiella ictaluri* in Laboratory Populations of Zebrafish *Danio rerio*

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**Abstract**

We report the first cases of *Edwardsiella ictaluri* causing epizootics in laboratory populations of Zebrafish *Danio rerio*. *Edwardsiella ictaluri* is primarily recognized as a disease of catfish species and is known to cause an economically significant disease in aquaculture.
In recent years there has been a dramatic increase in the use of Zebrafish *Danio rerio* in biomedical research. Zebrafish are raised indoors in research laboratories, in either recirculating or flow-through water systems with ultraviolet (UV) sterilizers (Harper and Lawrence 2011). Fish stocks are generally housed in separate tanks according to genetic background (wild-type, mutant, and transgenic lines) and by generation. The closed nature of these systems facilitates tracking of morbidities and mortalities as well as disease monitoring. The source of Zebrafish for research laboratories ranges from pond-reared fish for the aquarium trade to laboratories such as the Zebrafish International Resource Center (ZIRC), University of Oregon, in which pathogens are documented and controlled. Most research facilities introduce new fish into their main facilities as second generations derived from eggs that are surface disinfected with chlorine (Westerfield 2007; Kent et al. 2009).

Two of us (M. Kent and K. Murray) have been providing diagnostic services through the ZIRC to the Zebrafish community since 1999, and the diseases of laboratory Zebrafish have been documented and described (http://zebrafish.org/zirc/health/diseaseManual.php). The most common bacterial infections diagnosed in Zebrafish are chronic or asymptomatic infections by *Mycobacterium* spp., most often *M. chelonae* (Whipp et al. 2008; Murray et al. 2011). Although species like *M. marinum* and *M. haemophilum* have been associated with outbreaks of morbidity and mortality, these outbreaks are generally protracted and the onset of mortalities is not acute (Watral and Kent 2007; Whipp et al. 2007). Acute mortalities in Zebrafish facilities are more likely to be associated with minimal or uncompensated physiologic stress, often manifested as an acute stress response, due to perturbations in environmental (water) quality. Opportunistic pathogens may then be responsible for environmental gill disease or septicemia caused by secondary Gram-negative bacterial infections (e.g., *Aeromonas* spp., *Pseudomonas* spp., *Plesiomonas* spp.). Here we describe the unique presentation of acute mortalities associated with *Edwardsiella ictaluri* infections in laboratory Zebrafish facilities (Table 1).

Enteric septicemia of catfish (ESC) (Hawke 1979) is considered the most economically important cause of disease on catfish farms in the USA and is responsible for the majority of disease-related mortality annually (USDA 2003a, 2003b). The causative agent of ESC was identified and described as a new species named *E. ictaluri* (Hawke et al. 1981) and is a member of the Class Gammaproteobacteria, Order Enterobacteriales, and Family Enterobacteriaceae. Mortality rates in pond-raised Channel Catfish *Ictalurus punctatus* can range from 10% to 50% depending on the age and immune status of susceptible fish in the population (Hawke and Khoo 2004). Initially *E. ictaluri* was considered to be a host-specific pathogen of catfish species in the United States such as the Channel Catfish, the White Catfish *Ameiurus catus*, and the Brown Bullhead *A. nebulosus* (Hawke 1979; Hawke et al. 1981; Waltman et al. 1985); however, in recent years the bacterium has been identified as the cause of disease in other species of catfish internationally including Walking Catfish *Clarias batrachus* in Thailand (Kasornchandra et al. 1987), the Vietnamese freshwater catfish *Pangasius hypophthalmus* (Crumlish et al. 2002), Chinese Yellow Catfish *Pelteobagrus fulvidraco* (Liu et al. 2010) and the Japanese Ayu *Plecoglossus altivelis* (Sakai et al. 2008). Other susceptible catfish species in the United States are the Blue Catfish *I. furcatus* (Wolters and Johnson 1994) and the Tadpole Madtom *Noturus gyrinus* (Klesius et al. 2003). Infections have also been observed in noncatfish species, including the Green Knifefish *Eigemmina virescens* (Kent and Lyons 1982), the Devario Devario (*Danio*) devario (Waltman et al. 1985), the Rosy Barb *Puntius conchonius* (Humphrey et al. 1986), and the Nile Tilapia *Oreochromis niloticus* (Soto et al. 2012). In addition, experimental infection of noncatfish species has been achieved in Rainbow Trout *Oncorhynchus mykiss*, Chinook Salmon *O. tsawytscha* (Baxa et al. 1990), and Blue Tilapia *Oreochromis aureus* (Plumb and Sanchez 1983). Experimental infection of Zebrafish with a catfish isolate was achieved by injection and immersion (Petrie-Hanson et al. 2007), and recently this protocol was used to experimentally infect Zebrafish in studies.
on adhesion protection by probiotic bacteria (Rendueles et al. 2012). Prior to our account, epizootics caused by *Edwardsiella ictaluri* have not been reported in pond populations or research laboratory populations of Zebrafish.

*Edwardsiella ictaluri* is most closely related to *E. tarda* (56–62% DNA homology) and is typically identified using a combination of staining characteristics, cell morphology, and biochemical and physiologic tests (Hawke et al. 1981). The bacterium is a Gram-negative rod, motile by peritrichous flagella (0.75 µm in width × 1.25 µm in length), oxidase negative, and fermentative in O/F glucose or glucose motility deeps (GMD). The triple sugar iron (TSI) slant reaction is K/A with negative H₂S and the indole test is negative. Catfish strains of *E. ictaluri* consistently produce the code number 4004000 in the API 20E system (bioMérieux, Durham, North Carolina). More recently, molecular methods of identification have been utilized by diagnostic laboratories such as species-specific real-time PCR (Bloudeau et al. 2003), PCR amplification of the 16S rRNA gene with universal primers and sequencing of the amplicons followed by basic local alignment search tool (BLAST) analysis (Janda and Abbott 2007), and detection of the organism in water by real-time PCR (Griffin et al. 2011). The species has traditionally been regarded as homogeneous with catfish strains from different geographic locations being practically identical in biochemical phenotype (Plumb and Vinitnantharat 1989), isozymes (Starliper et al. 1988), plasmids (Newton et al. 1998), and serology (Chen and Light 1994). By means of genomic fingerprinting, as many as four different patterns were identified using arbitrarily primed PCR (AP-PCR) (Bader et al. 1998); however, Griffin (2011) found a high level of homogeneity in the electrophoretic profiles generated from 19 U.S. catfish isolates of *E. ictaluri* when they were analyzed by repetitive-element PCR amplification utilizing ERIC and BOX primer sets.

### METHODS

**Necropsy and bacterial isolation.**—Standard necropsy procedures included the following: observation of fish externally and internally for gross clinical signs, observation of gill clippings and skin scrapings microscopically in wet mounts for bacteria and parasites, aseptic dissection and collection of tissues (spleen, anterior kidney, and liver), and streaking tissue homogenates on trypticase soy agar with 5% sheep blood (SBA) (Remel, Lenexa, Kansas) for bacterial isolation. Primary isolation plates were incubated at 28°C for 48 h. Isolated colonies were subcultured to fresh SBA plates for maintenance of stock cultures prior to inoculation of test media.

**Biochemical identification of bacterial isolates.**—For presumptive identification, bacteria were evaluated by the Gram stain, cytochrome oxidase test, catalase test, glucose fermentation in GMD, esculin hydrolysis on bile esculin slants, H₂S production in TSI slants, and citrate utilization using standard bacteriological test tube methods (Hawke et al. 1981). Motility was assessed in wet mounts and in GMD. Inoculation of API 20E and API 50 CH strips (bioMérieux) and interpretation of results was done according to manufacturer’s instructions for Enterobacteriaceae; however, incubation of the strips was at 28°C for 48 h.

**DNA extraction.**—Representative isolates recovered from fish in the different cases or submitted to the Louisiana Aquatic Diagnostic Laboratory (LADL) for identification were subjected to molecular identification procedures. For each isolate, the bacteria were suspended in 500 µL of sterile phosphate-buffered saline (PBS) at a density equivalent to a McFarland 4 equivalence turbidity standard (Remel) (Lorian 1986). The cells were centrifuged at 3,000 g for 5 min, washed 2×, and resuspended in 200 µL of PBS. Bacterial suspensions were subjected to DNA extraction and purification as per the manufacturer’s protocol using the High Pure PCR Template Preparation Kit (Roche). The DNA was stored at 4°C until further use.

**PCR and 16S rRNA sequencing.**—The 16S rRNA encoding gene was amplified by PCR using the primers SSU27 (5’-AGAGTTTGATCMTGGCTCAG-3’) and SSU1492 (5’-TACGGYTACCTTGTACAGCTTT-3’) and Phusion high fidelity (HF) DNA polymerase (Finnzymes, Thermo Scientific,Vantaa, Finland) by the method of Pontier et al. (2009). The 50 µL universal eubacterial 16S rRNA PCR reaction was composed of 0.5 µm of each primer, 0.25 mm of dNTPs, 1.0 U of Phusion DNA polymerase, 5× Phusion HF buffer (Finnzymes), and approximately 50–250 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 30 s at 98°C, followed by 35 cycles of 10 s at 98°C, 30 s at 56.2°C, and 30 s at 72°C, with a final extension step of 10 min at 72°C in a Perkin Elmer GeneAmp PCR System 2400. The PCR products were subjected to electrophoresis on a 1% agarose gel and stained with SYBR Safe DNA gel stain (Invitrogen). Amplicons for

<table>
<thead>
<tr>
<th>Location</th>
<th>Case number</th>
<th>Date</th>
<th>Facility</th>
<th>Source</th>
<th>Histology/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Louisiana State University</td>
<td>LADL11-100</td>
<td>Jun 7</td>
<td>Quarantine</td>
<td>Commercial</td>
<td>+/+</td>
</tr>
<tr>
<td>Louisiana State University</td>
<td>LADL11-104</td>
<td>Jun 14</td>
<td>Quarantine</td>
<td>Commercial</td>
<td>+/+</td>
</tr>
<tr>
<td>University of Pittsburg</td>
<td>ZIRC D11-13</td>
<td>Feb 11</td>
<td>Quarantine</td>
<td>Commercial</td>
<td>+/+</td>
</tr>
<tr>
<td>University of Pittsburg</td>
<td>ZIRC D11-81</td>
<td>Aug 1</td>
<td>Quarantine</td>
<td>Commercial</td>
<td>+/+</td>
</tr>
<tr>
<td>University of Massachusetts Amherst</td>
<td>ZIRC D11-112</td>
<td>Nov 17</td>
<td>Main facility</td>
<td>In house</td>
<td>+/+</td>
</tr>
</tbody>
</table>
sequencing were purified with the QiaQuick PCR Cleanup Kit (Qiagen) as directed by the manufacturer and were sequenced on an Applied Biosystems 3130 Genetic Analyzer using PCR primers (F11–F5) and (F1–R13). The sequences were compared with those stored in GenBank using the BLASTN program from the National Center for Biotechnology Information (NCBI).

**Histopathology.**—Whole fish were fixed in neutral-buffered 10% formalin or Dietrich’s fixative, dehydrated in ethanol, and embedded in paraffin using standard procedures (Luna 1968). Tissues were sectioned at 5 µm thickness, mounted on slides, stained with hematoxylin and eosin, and examined by light microscopy.

**Auto-aggregation.**—Auto-aggregation was examined by comparing isolates from Channel Catfish with isolates from Zebrafish following growth in brain–heart infusion (BHI) broth. Auto-aggregation was demonstrated by inoculating 5 mL of BHI broth in a test tube with 50 µL of a bacterial suspension in PBS equivalent to a McFarland 0.5 turbidity standard. The broth culture was incubated for 18 h in a roller apparatus (Cell-Gro Tissue Culture Rotator, Barnstead/Lab Line Products, Dubuque, Iowa) at 80 rpm overnight to a final cell density of approximately 10^9 CFU/mL. The tubes were then placed vertically in a rack and the top at room temperature (21 °C) for 6 h with no shaking and examined for auto-aggregation hourly. A tube was scored positive for auto-aggregation if bacteria in suspension began clumping and settling to the bottom of the tube leaving a clear liquid in 90% of the tube within 1–2 h. Strains of *E. ictaluri* tested from Channel Catfish were LADL93-146, ATCC 33202, LADL88-108, LADL91-581, S07-794, and S05-518. Strains from Zebrafish were LADL11-100, LADL11-104, 11TAL197, 11TAL204, LADL11-194, and 11TAL232.

**Plasmid analysis.**—Plasmids were isolated from cultures using the Spin Miniprep kit (Qiagen, Valencia, California). Supercoiled plasmids were separated by 0.6% agarose gel electrophoresis with supercoiled ladder (New England Biolabs, Ipswich, Massachusetts) as the size standard. Plasmids were digested with either EcoRI or BstZ17I and were separated by 0.6% agarose gel electrophoresis with 1-kb ladder (New England Biolabs) as the size standard.

**Bacterial LPS recognition with Mab Ed9.**—Bacterial cultures were grown to late log phase and were pelleted by centrifugation, followed by two washes with PBS at pH 7.4. Pellets were resuspended in 1× lithium dodecyl sulfate (LDS) sample buffer (Life Technologies, Grand Island, New York) and boiled for 10 min. The lysate was cleared by centrifugation at 16,100 × g for 20 min, the lysate was diluted 1:100 in LDS buffer, and separated on a NuPAGE Novex 12% bis-tris gel (Life Technologies). Separated samples were transferred to a polyvinylidene fluoride membrane using the iBlot Dry Transfer System (Life Technologies). The membrane was incubated with the Ed9 lipopolysaccharide (LPS) monoclonal antibody raised against the ATCC 33202 isolate of *E. ictaluri*, donated by Dr. Jerold Ainsworth, Mississippi State University (Ainsworth et al. 1986), diluted 1:8 in tris-buffered saline containing 0.1% Tween-20 (Bio-Rad Laboratories, Hercules, California). Horseradish peroxidase-conjugated goat anti-mouse antibody (Thermo-Fisher Scientific, × Rockford, Illinois) was used to label the Ed9 antibody with SuperSignal West Pico chemiluminescent substrate (Thermo-Fisher Scientific).

**Antibiotic-susceptibility testing.**—Antibiotic susceptibility was determined by both disk diffusion and minimal inhibitory concentration (MIC) by broth microdilution using quality control methods outlined in the M42 and M49 documents of the Clinical Laboratory Standards Institute (CLSI 2006a, 2006b). For disk diffusion susceptibility tests, suspensions equivalent to a McFarland 0.5 turbidity standard were made of each culture to be tested in 5 mL of sterile 0.85% saline. Using a cotton-tipped applicator, Mueller-Hinton agar plates with 5% sheep blood were inoculated by streaking the entire surface with the applicator saturated with the bacterial suspension. Paper disks impregnated with the antibiotics: oxytetracycline (T30), enrofloxacin (ENO15), florfenicol (FFC 10), and Romet (25), were spaced evenly on the surface of the plate immediately after it was inoculated. A control plate inoculated with *Escherichia coli* ATCC 25922 served as the quality control standard. Plates were incubated at 28 °C and zones of diffusion were measured at 24 and 48 h. Broth microdilution tests were performed using the Sensititre Avian 1F plates (Trek Diagnostic Systems, West Sussex, UK). The plates were inoculated according to the manufacturer’s instructions but were incubated at 28 °C for 24 and 48 h. A control plate was inoculated with *E. coli* ATCC 25922.

**Fulfillment of Koch’s postulates.**—*Edwardsiella ictaluri* strain LADL11-100 was selected as a representative from Louisiana State University (LSU) outbreaks and was used in an immersion challenge to fulfill Koch’s postulates. Four 20-L tanks were each stocked with 10 Zebrafish (average weight, 0.5 g) and challenged by immersion in 10^7 CFU/mL for 2 h. After immersion of fish, the bacteria were flushed out of the tank by gradual water replacement in a flow-through system, and flow-through conditions were maintained for the duration of the 10-d trial. Mortality was recorded daily and moribund or dead fish were necropsied and bacterial cultures, taken from the liver and brain, were grown on tryptic soy agar with 5% sheep blood (TSAB) (Remel).

**RESULTS**

**Disease Outbreaks in Zebrafish**

**LSU Department of Biological Sciences.**—Two populations of Zebrafish (approximately 1 year of age) were obtained from a commercial source on June 7, 2011, (group 1, 468 fish) and on June 14, 2011, (group 2, 281 fish) and held in separate groups of quarantine tanks in the fish holding facilities of the Life Sciences Building, Department of Biological Sciences, LSU, for use in unrelated experiments. Upon arrival the fish exhibited no signs of transport stress or mortality. At 24 h after arrival, the fish in the first group began to exhibit clinical signs that
included hemorrhage in the skin near the eyes and opercula, base of fins, and ventral surface of the abdomen. By day 3, mortalities were occurring in the population and fish exhibiting clinical signs were submitted to the LADL at the LSU School of Veterinary Medicine where fish were subjected to a standard necropsy. Specimens showing clinical signs and others showing lethargic behavior were negative for parasites on the gills or skin. The gills were pale and internally the liver was pale and the spleen was swollen. Skin ulceration was noted on a few fish (Figure 1A). Other specimens exhibited clinical signs that included abdominal swelling due to accumulation of ascites (Figure 1B) and hemorrhaging in the skin near the eyes and opercula, base of fins, or ventral surface of the abdomen (Figure 1C, D). Mortality continued for 10 d until cumulative mortality had reached 280 fish and the decision was made to euthanize the remainder of the population. A second group of fish received from the same source was also diagnosed as positive for a Gram-negative bacterial septicemia and were euthanized after quarantine. In total, 750 Zebrafish were lost to the infection or euthanasia. Pure cultures recovered from the internal organs and brains of the moribund specimens were identified as E. ictaluri. A third group of Zebrafish was received and quarantined by LSU Department of Biological Sciences on June 17, 2011, from a different vendor. Fish in this group cultured negative for bacterial disease, postshipping mortality was minimal, and the fish performed well in the experiments.

University of Pittsburgh.—The University of Pittsburgh purchased 610 fish from a commercial source on January 18, 2011. The fish (approximately 1 year of age) were quarantined in 9-L tanks at a density of approximately 10 fish/L. A few mortalities (approximately 10 fish) occurred in the first week and were believed to be related to transport stress. After 2 weeks of acclimation in quarantine, 11 fish died in one tank in 1 d and in the following days more mortality occurred in this tank. Clinical signs included lethargy, raised scales, and hemorrhage in the skin of the operculum and abdomen. Preliminary diagnostic tests were negative for internal and external parasites and only one of four fish yielded a positive kidney culture of Plesiomonas shigelloides after 48 h incubation at 28°C. At this time, a total of 40 fish had died and the decision was made to treat the remaining fish in the tank with medicated feed containing the antibiotic florfenicol based on its efficacy in treating bacterial infections.
infections in Channel Catfish (Gaunt et al. 2004). The individual affected tank was separated from the rack on February 9, 2011, but on February 10 two fish had died in two other tanks. The principal investigator was asked to sacrifice all the remaining fish on the rack. The total number of fish sacrificed was 630: 550 fish from the new batch, 20 spinalized fish (fish with experimental spinal lesions), and 60 fish from a previous shipment. Normally fish in quarantine that show an outbreak of an infectious disease are euthanized and not treated to avoid putting the primary fish facility at risk, but in this case the high value of the current fish population prompted an attempt to reduce the mortality rate of affected fish. A 660-mg dosage of florfenicol (Intervet/Schering-Plow Animal Health, Roseland, New Jersey) was incorporated into 880 g of feed and fed to the fish ad libitum for 7 d. In the following days no additional fish died in the treated tank. The fish were sacrificed 3 d after treatment ended when the experimental endpoint was reached.

Three moribund specimens submitted to the ZIRC on August 1, 2011, for histopathological examination were reported to have “severe encephalitis, nephritis, and splenitis associated with acute bacterial disease.” Acid-fast stains were negative and a viral assay (Ambrose and Clewley 2006) performed by Dr. M. J. Crim, University of Missouri, designed to amplify viral DNA from a wide variety of viruses by PCR, yielded no positives. The brains of sentinel fish from the rack system in the first outbreak that were previously frozen and maintained at the University of Pittsburgh and brains of fish in the present outbreak were cultured on SBA at 25°C. The cultures were examined by the ZIRC and forwarded to the LADL at LSU for identification.

The two bacteria identified from the cultures were identified as *E. ictaluri* and *Pseudomonas fluorescens*; however, the *Pseudomonas* was deemed to be secondary because the bacterium seen in histology slides and histopathology was indicative of *E. ictaluri*.

**Bacterial Isolation and Identification**

Following necropsy, pure cultures of a Gram-negative bacterium were isolated from the organs and tissues of most of the fish submitted to diagnostic laboratories. Cultures from the liver, kidney, spleen, and brain of moribund specimens on TSAB produced predominant, slow-growing, grey, nonhemolytic colonies after 48 h incubation at 28°C. Occasionally secondary bacteria such as *Aeromonas hydrophila*, *Pseudomonas fluorescens*, and *Plesiomonas shigelloides* were isolated but their occurrence was not consistent. When these organisms were present they would overgrow the *E. ictaluri* due to their faster growth rate and larger colonies. *Edwardsiella ictaluri* was determined to be the primary causative agent of the disease in the Zebrasfish based on histopathology, consistency of isolation from multiple tissues and organs, and presumptive identification by biochemical tests and API 20E. The isolates were confirmed as *E. ictaluri* by conducting PCR, sequencing the 16S rRNA amplicons, and performing a search for matches in GenBank using the BLASTN program from the NCBI.

The causative bacterium in all cases was shown to be a Gram-negative rod, 0.75 × 1.5 μm in size, oxidase negative, fermentative in glucose, and negative for bile esculin hydrolysis. The TSI reaction was K/A with no gas and no H2S produced. The isolates from Zebrasfish differed from typical *E. ictaluri* isolates from catfish by being very weakly motile at 28°C, as determined in wet mount and GMD, and were positive for citrate in the API 20E system. Citrate was negative when tested in the tube test (Simmon’s citrate test). The resulting code in the API 20E was 4204000 which varies from the typical code of 4004000 of catfish isolates. In the API 50CH system Zebrasfish and Channel Catfish isolates give identical results in producing acid from carbohydrates, glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, D-maltose, and potassium gluconate. Zebrasfish strains of *E. ictaluri* are differentiated from Channel Catfish strains by being very weakly motile at 35°C and having weaker motility and autoagglutination. *Edwardsiella ictaluri* is differentiated from *E. tarda* by failing to grow at 35°C and having weaker motility, weaker gas production in carbohydrate media, negative H2S production, negative indole reaction, slower growth rate, and smaller colony morphology.

Four additional cultures from Zebrasfish isolated by Dr. Roy Yanong at the University of Florida, Tropical Aquaculture Laboratory, were submitted to the LADL and were identified as *E. ictaluri*. These isolates—11TAL132, 11TAL197, 11TAL193, and 11TAL232—were identical biochemically and gave the same API 20E code 4204000 as the other Zebrasfish isolates. Sequencing of the 16S rRNA amplicons and analysis using the BLASTN program identified all isolates from Zebrasfish to be *E. ictaluri* with 99% maximum identity using both forward and reverse primers.
TABLE 2. Bacterial strains used for biochemical identification, molecular identification, plasmid profiles, auto-aggregation, LPS specificity with Mab, and antibiotic-susceptibility testing.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>Source or location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 33202</td>
<td>Channel Catfish</td>
<td>ATCC (Georgia)</td>
</tr>
<tr>
<td>LADL93-146</td>
<td>Channel Catfish</td>
<td>Louisiana</td>
</tr>
<tr>
<td>LADL88-108</td>
<td>Channel Catfish</td>
<td>Louisiana</td>
</tr>
<tr>
<td>LADL91-581</td>
<td>Channel Catfish</td>
<td>Louisiana</td>
</tr>
<tr>
<td>S05-518</td>
<td>Channel Catfish</td>
<td>Mississippi</td>
</tr>
<tr>
<td>S07-794</td>
<td>Channel Catfish</td>
<td>Mississippi</td>
</tr>
<tr>
<td>LADL11-100</td>
<td>Zebrafish</td>
<td>Louisiana (LSU)</td>
</tr>
<tr>
<td>LADL11-142</td>
<td>Zebrafish</td>
<td>University of Pittsburg</td>
</tr>
<tr>
<td>11TAL132(LADL11-149)</td>
<td>Zebrafish</td>
<td>Florida</td>
</tr>
<tr>
<td>11TAL197(LADL11-184)</td>
<td>Zebrafish</td>
<td>Florida</td>
</tr>
<tr>
<td>11TAL204(LADL11-193)</td>
<td>Zebrafish</td>
<td>Florida</td>
</tr>
<tr>
<td>LADL11-194</td>
<td>Zebrafish</td>
<td>University of Massachussets Amherst</td>
</tr>
<tr>
<td>11TAL232(LADL11-203)</td>
<td>Zebrafish</td>
<td>Florida</td>
</tr>
</tbody>
</table>

Histopathology

In general, edwardsiellosis in the Zebrafish from all four cases described in this paper presented as a severe, multifocally extensive to diffuse, systemic disease characterized by tissue necrosis and large numbers of bacteria often within macrophages (Figure 2). The kidneys (pronephros and mesonephros) and spleen were most commonly and severely affected. Within affected tissues, notably the kidney, spleen, and olfactory rosettes, there were florid inflammatory cell infiltrates composed of macrophages, neutrophils, eosinophilic granular cells, and fewer lymphocytes (Figure 3B). In specimens from multiple laboratories there was diffuse and severe inflammation and necrosis of the nasal pits. In the area of the nares, locally extensive sheets of necrotizing chronic inflammation extended through the olfactory rosettes, obliterating them. The inflammation continued along the olfactory nerve into the olfactory bulbs, telencephalic ventricle, and telencephalon (Figure 3A, C). Spleens were enlarged, and more than 95% of the parenchyma was necrotic with numerous aponecrotic macrophages and innumerable rod-shaped bacteria (1 × 5 µm) within and between cells (Figure 4A). In kidneys, areas of inflammation and necrosis were evident as discrete expansive foci, which with progression of severity would preferentially affect hematopoietic cords prior to nephron necrosis (Figure 4B). The liver often contained small foci of necrosis and inflammation, often comprising less than 5% of the organ (Figure 4C). The nares, brain, and endomeninges were often affected, in some cases with marked expansion of the mesencephalic ventriciles by necrotic leukocytes and bacteria (Figure 4D). Other less common lesions included skeletal muscle necrosis with liquefaction and dermatitis with epidermal ulceration. Lesions were consistent with those described by Petrie-Hanson et al. (2007) in Zebrafish challenged by immersion with a catfish isolate of E. ictaluri. Infection of the olfactory sac, olfactory nerve, and olfactory lobe of the brain, leading to meningoencephalitis, has been described in the chronic form of the disease in Channel Catfish (Shotts et al. 1986; Newton et al. 1989; Morrison and Plumb 1994).

Auto-aggregation

Auto-aggregation occurred within 1 h with strains from Zebrafish: LADL11-100, LADL11-142, 11TAL197, 11TAL204, LADL11-194, and 11TAL232, but not with strains from Channel Catfish: ATCC 33202, LADL88-108, LADL91-581, S07-794, and S05-518.
Plasmid Profiles

The Zebrafish isolates of *E. ictaluri* present a different plasmid profile than is commonly seen in *E. ictaluri* isolated from Channel Catfish (Figure 5) (Lobb and Rhoades 1987; Speyerer and Boyle 1987; Newton et al. 1988; Fernandez et al. 2001). Channel Catfish isolates typically carry two plasmids, pEI1 and pEI2, that are approximately 4.8 and 5.6 kb, respectively (Fernandez et al. 2001). Six of the Zebrafish isolates carry two plasmids of approximately 3.5 and 4 kb, while the seventh isolate, LADL11-193 (11TAL204), carries two plasmids that are
FIGURE 4. (A) The spleen exhibits necrosis in more than 95% of the parenchyma with numerous necrotic macrophages and innumerable rod-shaped bacteria within and between cells. Pancreatic exocrine tissue is present at lower right. (B) Areas of inflammation and necrosis were evident in the kidney as discrete expansive foci with progression of severity preferentially affecting hematopoietic cords prior to nephron necrosis. In the left of the image many small shrunken pyknotic cells and karyorrhectic debris (necrosis) are evident admixed with many bacteria. (C) The liver contains several foci of necrosis with numerous bacteria. (D) The endomeninges are expanded two to three times their normal size by aponecrotic leukocytes and bacteria. The brain is at lower left of the image, pericephalic adipose at upper right (400x). [Figure available online in color.]

both about 4–4.5 kb and appear as a single band when supercoiled. Restriction enzyme digestion with EcoRI linearizes pEI1 and pEI2 (Figure 6), but only cuts the 4-kb band of the Zebrafish isolates (data not shown). Digestion with BstZ17I, however, linearizes both of the plasmids from Zebrafish isolates, but only cuts pEI1 of Channel Catfish E. ictaluri (data not shown). The BstZ17I-digested plasmids are approximately 3.5 and 4 kb for the six isolates, matching the size observed in supercoiled samples. Digestion of the LADL11-193 plasmids with BstZ17I linearizes a 4-kb plasmid that is possibly similar to that size of plasmid from the other Zebrafish isolates. The second plasmid of LADL11-193 cuts at least twice by BstZ17I, resulting in two visible fragments at approximately 1.7 and 2.8 kb. It is suspected that the 4-kb plasmids carried by the Zebrafish isolates are genetically related to pEI1 of Channel Catfish E. ictaluri based on previous hybridization data between 4- and 4.8-kb plasmids (Reid and Boyle 1989). It is unknown if the 3.5- or 4.5-kb plasmids carried by Zebrafish isolates contain similar DNA sequences as those carried by Channel Catfish isolates. Further work is required to sequence plasmids carried by the Zebrafish E. ictaluri isolates.

LPS recognition with MabEd9

The monoclonal antibody Ed9 raised against the ATCC 33202 strain of E. ictaluri from Channel Catfish (Ainsworth et al. 1986) is believed to be specific for E. ictaluri LPS (Lawrence et al. 2001), but failed to react with the LPS of the Zebrafish isolates (Figure 7). This demonstrates a difference in the structure of LPS between the Channel Catfish and Zebrafish isolates. The importance of this difference, however, is unknown due to the single epitope recognition of the monoclonal antibody. Early serological studies found little heterogeneity between E. ictaluri isolates (Plumb and Klesius 1988; Bertolini et al. 1990). However, Lobb et al. (1993) found that E. ictaluri isolated from Channel Catfish was serologically different from the strain isolated from the Green Knifefish, indicating...
serological heterogeneity may exist among *E. ictaluri* isolates from different species. Further analysis of the LPS moieties is required to determine the differences between the LPS of Zebrafish and Channel Catfish *E. ictaluri* isolates and whether these differences influence relative virulence.

**Antibiotic Susceptibility**

Antibiotic susceptibility as disk diffusion zones are listed in Table 3. The Sensititre Avian 1F plates were found to be useful in determining the MIC for tetracycline and oxytetracycline for Zebrafish *E. ictaluri* strains, but failed to show endpoints for florfenicol (<1.0 µg), enrofloxacin (<0.12 µg), and Romet as measured by SXT (<0.5/9.5 µg). In general, from these results the Zebrafish isolates were considered susceptible to antibiotics Romet, oxytetracycline, florfenicol, and enrofloxacin based on data available for human and veterinary bacterial isolates; however, clinical breakpoints for these drugs have not been determined for Zebrafish.

**Fulfillment of Koch’s Postulates**

Exposure to the challenge dose of 10^7 CFU/mL by immersion for 30 min resulted in 100% mortality of Zebrafish at 10 d postexposure. Clinical signs and pathology were consistent with fish in naturally occurring infections.
TABLE 3. Antibiotic susceptibility of Zebrafish isolates of *Edwardsiella ictaluri* by disk diffusion. Values in table are diffusion zone diameters in millimeters.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oxytetracycline T30</th>
<th>Romet SOR25</th>
<th>Florfenicol FFC30</th>
<th>Enrofloxacin ENO5</th>
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<tbody>
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<td>LADL11-100</td>
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<td>26</td>
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<td>11TAL232</td>
<td>30</td>
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</table>

DISCUSSION

*Edwardsiella ictaluri* is known as the causative agent of an economically important bacterial disease of farm-raised catfish in the USA and abroad; however, the bacterium has only rarely been reported from hosts other than catfish (Kent and Lyons 1982; Waltman et al. 1985; Humphrey et al. 1986; Soto et al. 2012). In this report we described the first cases of *E. ictaluri* causing naturally occurring epizootics in laboratory populations of Zebrafish. We also proposed the utility of quarantine in preventing the introduction and spread of this contagious pathogen into a laboratory with valuable Zebrafish colonies. Three different populations of Zebrafish, obtained from commercial sources and held in quarantine at three different research laboratories in three different states, experienced acute disease resulting from *E. ictaluri* infection, and a fourth case was presumptively identified by similar histopathological lesions. In most cases the decision was made to euthanize the remaining fish in the quarantined population. Euthanasia was chosen over antibiotic therapy because, although the bacteria were considered to be sensitive to several antibiotics, it was uncertain whether antibiotic therapy would eradicate the pathogen, and the risk of spread to other Zebrafish colonies in the laboratory was too great. Two laboratories did choose to use medicated feeds for short-term control of the infection until experiments could be completed and the remaining fish euthanized. Florfenicol was used as a medicated feed at the University of Pittsburgh and enrofloxacin was used as a medicated feed at the University of Massachusetts at Amherst and, in each case, mortality was curtailed. The carrier status of survivors was not assessed. In many research laboratories, the high value of the fish in the colonies makes it advantageous to develop management strategies that will make it possible to control or eradicate the pathogen.

After the diagnosis of *E. ictaluri* infection in the LSU groups, which resulted in isolation of two strains (LADL11-100 and LADL11-104), an additional sample of diseased fish (University of Massachusetts) was submitted for necropsy and resulted in isolation of a third strain, LADL11-194. Five additional cultures from Zebrafish in two other states, Pennsylvania and Florida, were submitted to the LADL for identification. In total, eight isolates from Zebrafish were identified as *E. ictaluri* by biochemical testing, API 20E and API 50 CH, and 16S rRNA sequencing. All Zebrafish *E. ictaluri* isolates were identical in biochemical and molecular tests for identification by partial amplification and sequencing of the 16S rRNA gene using specific primers. *Edwardsiella ictaluri* isolates from Zebrafish were differentiated from Channel Catfish isolates by exhibiting weaker motility and a different plasmid profile (two plasmids of 4.0 and 3.5 kb) with the exception of one isolate, 11TAL204, which had two plasmids of approximately 4 kb. The Zebrafish isolates also exhibited failure to react with Mab Ed9 in Western blot analysis, possibly indicating a different LPS O side-chain and a different serotype. Zebrafish isolates of *E. ictaluri* consistently
produced a different API 20E code (4204000) from Channel Catfish isolates, were nonmotile to weakly motile in GMD, and auto-aggregated in BHI broth. All Zebrafish isolates were considered to be susceptible to the antibiotics Romet, oxytetracycline, florfenicol, and enrofloxacin, although clinical breakpoints for these drugs have not been determined for Zebrafish.

Fish from all cases showed similar histological changes, characterized by numerous rod-shaped bacterial cells in macrophages and chronic necrotizing inflammation in various visceral organs. This was consistent with the findings of Petrie-Hanson et al. (2007) in experimentally infected Zebrafish. The occurrence of pathologic changes in the nares and forebrain was very characteristic of *E. ictaluri* infections in other species (Shotts et al. 1986).

A fourth Zebrafish research laboratory, with a history of morbidity and mortality in their fish, submitted fish to the ZIRC diagnostic service in September 2011. The facility operates a recirculating water system with UV sterilizers. Fish were purchased from a pet store and were moved into the system. Beginning in April 2011, the facility experienced 4 months of recurrent outbreaks of morbidity and mortality in the Zebrafish population. Signs of disease included color change, hemorrhage or redness, hydrocoelom, protruding scales, and skin ulcers. Morbidity and mortality recurred when new fish were added to the system. The facility had counted over 125 disease-associated mortalities when specimens were sent to the ZIRC. Five adult Zebrafish were fixed in preservative and submitted to the ZIRC for histopathology. The type of fixative used and age and genetic strain of the fish were not specified. Histopathology was consistent with *E. ictaluri* infections in other Zebrafish cases. Hence, we believe that fish from the fourth laboratory, in which bacterial culture was not conducted, were also infected with *E. ictaluri*. Because Zebrafish in this case were obtained from a pet store we reemphasize the need for routine quarantine procedures and screening of new fish for *E. ictaluri*.

*Edwardsiella ictaluri* is known to be an obligate pathogen that does not persist in the environment for very long and must be transmitted from fish to fish by close contact, via the water, by fecal shedding, or orally. We believe it is important for Zebrafish-rearing facilities and laboratories to be aware of this emerging disease problem and take proper precautionary measures to prevent contamination of valuable genetic stocks of this important laboratory fish.

ACKNOWLEDGMENTS

We thank Jerold Ainsworth of the College of Veterinary Medicine, Mississippi State University, for donating the Ed9 monoclonal antibodies used in the study, Patricia Gaunt of the College of Veterinary Medicine, Mississippi State University, for providing Romet antibiotic susceptibility disks, Roy Yangong of the University of Florida, Tropical Aquaculture Laboratory, Ruskin, Florida, for supplying additional Zebrafish isolates of *E. ictaluri*, and Judy Bennet of the University of Massachusetts at Amherst for additional Zebrafish specimens.

REFERENCES


EDWARDSIELLOSIS IN ZEBRAFISH


Salmonellae in Fish Feces Analyzed by In Situ Hybridization and Quantitative Polymerase Chain Reaction

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Salmonellae in Fish Feces Analyzed by In Situ Hybridization and Quantitative Polymerase Chain Reaction

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Abstract
The potential of fish to transfer salmonellae from heterogeneous aquatic biofilms into feces was assessed in controlled aquarium studies with Suckermouth Catfish \textit{Hypostomus plecostomus} and with biofilms inoculated with salmonellae. Neither the presence of catfish nor inoculation with salmonellae had detectable effects on the abundance of the microbial community. Densities of the microbial community were about 10^5 cells/mL in the water during a 1-week period, whereas densities of the microbial community increased 10-fold (10^6 to 10^7 cells/mg) in catfish feces during the same period. Salmonellae were detected by both quantitative polymerase chain reaction (qPCR) and situ hybridization in water samples immediately after inoculation, in numbers of about 10^4 cells/mL, representing up to 20\% of the cells of the microbial community. Numbers decreased by three orders of magnitude within the first 3 d of the study, which represented only 0.01\% of the community, and became undetectable after day 5. In catfish feces, numbers of \textit{Salmonella} initially increased to up to 6\% of the cells of the community but then declined. These results suggest that \textit{Salmonella} are not biomagnified during gut passage, and thus, fish only provide a means for the translocation of this pathogen.

Members of the genus \textit{Salmonella} represent important zoonotic pathogens (Humphrey 2000) that have been detected in a broad range of animal reservoirs including invertebrates, reptiles, birds, and mammals (Beach et al. 2002; Refsum et al. 2002; Hahn et al. 2007; Gaertner et al. 2011). Intestinal tracts of vertebrates are assumed to be the native habitat of salmonellae, and terrestrial or aquatic environments are contaminated by the release of salmonellae through feces (Woodward et al. 1997; Gopinath et al. 2011). Salmonellae persist not only in soil and water but also in plants and biofilms for extended periods (Murray 1991; Baloda et al. 2001; Côté and Quessy 2005; Ishii et al. 2006; Byappanahalli et al. 2009). In biofilms, for example, we detected salmonellae even in habitats of exceptional water quality, such as spring-fed Spring Lake and the upper reach of the San Marcos River, Texas (Hahn et al. 2007; Gaertner et al. 2008b, 2011; Sha et al. 2011). Salmonellae were present in natural biofilms in Spring Lake with a significant microheterogeneity and with differences in diversity of viable strains (Sha et al. 2011). In the laboratory, specific isolates remained pathogenic, persistent, and viable in biofilm and the water column for up to 28 d (Sha et al. 2013).

In the upper reach of the San Marcos River, salmonellae were detected in the intestine of four trophically diverse fishes, i.e., piscivorous Largemouth Bass \textit{Micropterus salmoides}, omnivorous Channel Catfish \textit{Ictalurus punctatus}, invertivorous and detritivorous Common Carp \textit{Cyprinus carpio}, and algivorous and detritivorous Suckermouth Catfish \textit{Hypostomus plecostomus}, and up to 33\% of the fish analyzed were positive for salmonellae, and serovars were highly variable among individuals (Gaertner et al. 2008c). Salmonellae are not considered to be part of the normal intestinal flora of fish (Janssen and Meyers 1968; Pal and Dasgupta 1991), even though they were detectable for up to 30 d in Channel Catfish artificially exposed to salmonellae (Lewis 1975). Thus, fish exposed to salmonellae could become asymptomatic carriers of this pathogen (Heuschmann-Brunner 1974; Bocek et al. 1992).
Consequently, fish potentially constitute an important factor in the dissemination and persistence of salmonellae in aquatic environments (Lawton and Morse 1980).

The aim of our study was to determine whether fish would consume salmonellae from natural biofilms and return them to the environment through fecal matter, ultimately enhancing the abundance or persistence of salmonellae in aquatic environments. In this study, we used the same design as in our previous studies on the fate of salmonellae in biofilms, which were conducted as controlled aquarium studies using biofilms on tiles inoculated with salmonellae (Sha et al. 2013). Suckermouth Catfish was selected to assess the role of fish in the transfer of salmonellae from biofilms into feces, because they consume algae and amorphous detritus from benthos of the San Marcos River (Pound et al. 2011). Quantification of salmonellae was achieved at selected sampling times during 1 week using quantitative polymerase chain reaction (qPCR) and in situ hybridization, and data were related to shifts in abundance of the entire microbial communities in time.

METHODS

Heterogeneous aquatic biofilms were grown on ceramic tiles (2.2 × 2.2 cm, nonglazed) in a stream channel adjacent to the Freeman Aquatic Biology Building at Texas State University–San Marcos with running spring water for 12 months. Previous studies using more than 120 tiles with biofilms demonstrated the absence of salmonellae (Sha et al. 2013), and therefore biofilms from only 10 haphazardly selected tiles were checked for salmonellae by PCR after semiselective enrichment in Rappaport–Vassiliadis enrichment broth (RVS) (Gaertner et al. 2009; Sha et al. 2011). Since these controls remained negative for salmonellae, all remaining biofilms were assumed to be free of salmonellae as well. Tiles with biofilms were then used in three treatments with three replicates each and established in 36-L aquaria in the laboratory. Treatments 1 and 2 each contained 200 tiles with biofilms free of salmonellae that were placed on the bottom of each aquarium. For treatment 3, tiles with biofilms were covered with 10 L of water in aquaria. This water was inoculated with Salmonella strain S11 serovar Thompson with 1.4 ± 0.2 × 10<sup>8</sup> cells/mL, estimated from the reading at an optical density of 564 nm. At 16 h after inoculation, tiles were transferred to three Salmonella-free aquaria. Biofilms on these tiles harbored approximately 6.0 ± 1.4 × 10<sup>8</sup> (mean ± SE) Salmonella cells per tile as demonstrated by qPCR analysis for nine haphazardly selected tiles (Sha et al. 2013). All aquaria were then filled with spring water and aerated through air stones (3 cm<sup>3</sup>). Aquaria for treatments 2 and 3 received one large or up to six small Suckermouth Catfish, taken from Spring Lake by grappling. All treatments were kept at room temperature (i.e., 25°C) and artificial light conditions at a 16 h light : 8 h dark photoperiod for 7 d.

Water samples were collected directly after setup, whereas additional water samples and fish feces samples were obtained in 12-h intervals (i.e., 12, 24, 36, 48, 60, and 72 h after setup), followed by 24-h intervals (i.e., 4, 5, 6, and 7 d after setup). Water samples (500 mL) were filtered through 0.2-μm Whatman Nuclepore Track-Etched membranes, and the filter placed into 50-ML graduated plastic tubes (Falcon) containing 20 mL of phosphate-buffered saline (PBS) (0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2). Cells were released from filters by sonication in a Fisher sonic cleaner (2QT; Fisher Scientific, Pittsburgh, Pennsylvania) for 10 min. Filters were removed afterwards, and released cells were collected after centrifugation at 4,400 × g for 15 min (Sha et al. 2013). Fish feces (40 mL) were collected with a syringe from the bottom of each aquarium and concentrated by centrifugation at 4,400 × g for 15 min. Cell pellets from water and feces were resuspended in 1 mL of sterile distilled water, and each three subsamples of 100 μL were then used for quantification by in situ hybridization or qPCR. After 3 and 7 d, additional 100-μL samples were used for semisselective enrichment and characterization of Salmonella isolates by repetitive sequence-based PCR (rep-PCR) (Hahn et al. 2007).

At the end of the study after 7 d, fish were euthanized by pithing, intestines were removed, and intestinal lining and contents were exposed by a longitudinal incision. Intestines from fish of the same treatment were pooled and transferred to 1 mL of distilled water in an Eppendorf microcentrifuge tube, which was then shaken by hand for 20 s to release and disperse the contents of the intestines. The intestines were then removed from the tube, and distilled water was added to the remaining liquid to final volume of 1 mL. Each three 100-μL subsamples were then used for quantification of salmonellae by in situ hybridization and qPCR, and for semisselective enrichment and subsequent analysis by end-point PCR.

For quantification of salmonellae by in situ hybridization, the subsamples of water, feces, and fish intestine content were fixed in 4% paraformaldehyde in PBS at 4°C for 16 h (Amann et al. 1990). Afterwards, samples were washed in PBS and stored in a final volume of 500 μL of 50% ethanol in PBS at −20°C until further use (Amann et al. 1990). Samples were spotted on gelatin-coated slides [0.1% gelatin, 0.01% KCr(SO<sub>4</sub>)<sub>2</sub>], dried at 42°C for 15 min, and subsequently dehydrated in 50, 70, and finally 95% ethanol for 3 min each. Hybridizations were carried out with probe Sal3 (5′AAT CAC TTC ACC TAC GTG, Escherichia coli position 1713–1730) (Nordentoft et al. 1997) that binds to 23S rRNA of all Salmonella enterica subspecies tested so far (excepting only subspecies IIIa), but should not detect S. bongori (Fang et al. 2003). Reactions were performed in 9 μL of hybridization buffer (0.9 M NaCl, 20 mM tris/HCl, 5 mM EDTA, 0.01% sodium dodecyl sulfate [SDS], pH 7.2)
containing 10% formamide, to which 1 µL of probe (25 ng/µL) that included 4′-6-diamidino-2-phenylindole (DAPI) at a final concentration of 200 ng/µL was added, at 42°C for 2 h. After hybridization, the slides were washed with hybridization buffer at room temperature for 15 min, rinsed with distilled water, and air-dried. Slides were mounted with Citifluor AF1 solution (Citifluor, London, UK) and examined with an Eclipse 80i microscope (Nikon, Lewisville, Texas), fitted for epifluorescence microscopy with a mercury lamp (X-Cite 120; Nikon) and filter cubes UV-2E/C (EX340–380, DM400, BA4435-485, for DAPI detection; Nikon) and Cy3 HYQ (EX535/50, DM565, BA610/75, for Cy3 detection; Nikon), respectively. Bacteria were counted at 1,000 × magnification in 25 fields, selected at random, covering an area of 0.01 mm². Detection of DAPI and Cy3 were determined from the same image (using the respective filter cubes) taken with a cooled CCD camera (CoolSNAP ES²; Photometrics, Tucson, Arizona), and Nikon’s NIS Elements imaging software (version 3). Treatment effects in the number of DAPI-stained cells in water and feces across time intervals were tested with a one-factor ANOVA (α = 0.05), and Tukey’s honestly significant difference (HSD) was used to test differences between treatments. Analyses were conducted in the software package R, version 2.11.1 (www.R-project.org).

For the quantification of salmonellae by qPCR, cells in the subsamples of water, feces, and fish intestine content were lysed in a final volume of 200 µL of 50-mM NaOH at 65°C for 30 min. Detection and quantification of salmonellae was achieved using lysates or 10-fold dilutions as a template in a final volume of 200 µL of Quanta Mix (Quanta BioSciences, Gaithersburg, Maryland), 0.2 µL of each primer 139 (5′ GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 (5′ TCA TCG CAC CGT CAA AGG AAC C) (100 ng/µL) and 1 µL of DNA template in an Eppendorf Mastercycler (ep realplex2; Eppendorf, Hauppauge, New York) (Sha et al. 2013). Conditions included an initial denaturation at 96°C for 3 min, and 35 cycles of denaturation at 96°C, annealing at 64°C, and extension at 72°C, each for 30 s. Amplification was followed by a melting curve analysis. Quantification was based on a standard curve generated from serial dilutions of ethanol-fixed cells of Salmonella typhimurium ATCC 14028 quantified by epifluorescence microscopy (Eclipse 80i; Nikon) after DAPI staining.

Semiselective enrichment of salmonellae was used for their detection in intestine samples by end-point PCR, and for the characterization of isolates in intestine samples, and in water and feces samples collected on days 3 and 7 by rep-PCR. For enrichment, each 100-µL subsample was transferred to a 2-mL cryotube containing 1 mL of buffered peptone water (BPW) (10 g/L peptone, 5 g/L NaCl, 9 g/L Na₂HPO₄, 1.5 g/L KH₂PO₄, pH 7.2) (ISO 1993) and incubated at 37°C for 24 h. Subsamples (100 µL) of this semispecific enrichment for salmonellae were transferred to new tubes with RVS, and salmonellae were enriched a second time as described above (Gaertner et al. 2008a).

For end-point PCR analyses of intestine contents, 100-µL samples of this second enrichment were transferred to a sterile, 1.5-mL Eppendorf microcentrifuge tube, and cells were pelleted by centrifugation at 14,000 × g for 2 min. The cell pellet was washed with 500 µL of sterile distilled water once and subsequently lysed in 100 µL of 50-mM NaOH by incubation at 65°C for 15 min with shaking. Lysed cells were kept at −20°C until use. End-point PCR was performed in a PTC-200 thermocycler (MJ Research, Waltham, Massachusetts) in a total volume of 50 µL containing 10 µL of PCR buffer (500 mM KCl, 25 mM MgCl₂, 200 mM tris/HCl, 0.1% Triton 100, pH 8.4), 1 µL of each primer (100 ng/µL), 0.2 µL Taq polymerase (5 U/µL), and 10 µL of the cell lysates (Hahn et al. 2007), with an initial denaturation at 96°C for 2 min, followed by 35 rounds of temperature cycling with denaturation at 96°C, primer annealing at 64°C, and elongation at 72°C, each for 30 s (Malorny et al. 2003). Salmonella typhimurium ATCC 14028 was used as a positive control. The PCR products were analyzed by gel electrophoresis on 2% agarose gels in tris-acetate–EDTA buffer after staining with ethidium bromide (0.5 µg/mL) (Sambrook et al. 1989).

For the characterization of salmonellae in water, feces, and the intestine samples, 100-µL subsamples of the second enrichments were plated on RVS agar (RVS solidified with 15 g agar/L). After incubation at 37°C for 16 h, 10 colonies were chosen haphazardly from each sample and incubated in LB broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37°C for 7 h (Sha et al. 2011). Cells from 100-µL subsamples as well as from a culture of the inoculated Salmonella strain S11 were pelleted by centrifugation and lysed in 100 µL of 50-mM NaOH as described above. End-point PCR as described above was used to identify isolates representing salmonellae, which were then further characterized by rep-PCR. Rep-PCR was performed in a total volume of 25 µL with primer BoxA1R (5′ CTA CGG CAA GGC GAC GCT GAC G) and 2 µL of lysate as described in Hahn et al. (2007). Banding profiles were screened visually by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook et al. 1989) and compared with that obtained with lysed cells of Salmonella strain S11.

All chemicals used in this study were purchased from Fisher Scientific (Pittsburgh, Pennsylvania) if not indicated otherwise.

RESULTS AND DISCUSSION

The number of DAPI-stained cells did not differ among treatments in water (F₂,₆ = 4.7, P = 0.06) or in feces (F₁,₄ = 7.0, P = 0.94). Across treatments, the number of DAPI-stained cells ranged between 0.4 and 3.8 × 10⁵ cells/mL in water...
TABLE 1. Mean (±SE in parentheses) number of DAPI-stained cells (×10^2) in 1 mL of water or 1 mg of Suckermouth Catfish feces (dry weight).

<table>
<thead>
<tr>
<th>Time</th>
<th>Medium</th>
<th>Hours</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>12</td>
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<tr>
<td></td>
<td>Water</td>
<td>1,541</td>
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<tr>
<td></td>
<td>(1,370)</td>
<td></td>
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<tr>
<td></td>
<td>Treatment 1(biofilm)</td>
<td>2,865</td>
<td>913</td>
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<tr>
<td></td>
<td>Feces</td>
<td>23,376</td>
<td>31,627</td>
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<tr>
<td></td>
<td>Treatment 2(biofilm, fish)</td>
<td>2,400</td>
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<tr>
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<td>Water</td>
<td>2,228</td>
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<tr>
<td></td>
<td>(770)</td>
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<tr>
<td></td>
<td>Feces</td>
<td>25,407</td>
<td>(22,492)</td>
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<td></td>
<td>Treatment 3(biofilm, fish, salmonellae)</td>
<td>16,061</td>
<td>21,240</td>
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<tr>
<td></td>
<td>Water</td>
<td>979</td>
<td>3,813</td>
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<td></td>
<td>(140)</td>
<td></td>
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<tr>
<td></td>
<td>Feces</td>
<td>15,746</td>
<td>(10,029)</td>
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</table>

Values obtained from two aquariums instead of three (the aquariums with dead fish were excluded).

and between 1.6 and 10.6 × 10^6 cells/mg in feces (Table 1). While results for water samples demonstrated that neither fish nor inoculation noticeably affected the microbial community during the experiment, the interpretation of results for feces was more ambiguous since the accuracy of the results is affected by several methodological issues. Since samples from water and feces were not dispersed before application to slides (e.g., in 0.1% pyrophosphate buffer by sonication: Zarda et al. 1997) to avoid dilution of low numbers of salmonellae, accumulations of large numbers of cells on particulate material were observed (Figure 1). These affected within-sample variability during enumeration and thus resulted in large standard errors. This issue was more pronounced in feces samples where accurate enumeration was also affected by the small amounts of feces collected and the associated difficulties to accurately determine dry weights at different times, potentially resulting in an overestimation of cell numbers towards the end of the study. We were also unable to completely remove feces at each sampling, which could have resulted in growth of organisms in aging feces and thus in the detection of higher cell numbers of microbes towards the end of the study. As a consequence, we are unable to state whether the increase in numbers in feces through time is accurate or affected by our experimental setup and analyses.

In situ hybridization with probe Sal3 allowed us to visualize salmonellae in both water and feces samples from treatment 3 where Salmonella strain S11 was inoculated (Figure 1). Salmonellae could not be detected in intestine samples from fish harvested at the end of the study from treatment 3, and also not in any samples from treatments 1 and 2 that did not receive salmonellae (data not shown). Detection of salmonellae in samples from treatment 3 was achieved without any pretreatments to enhance cell permeability for probes (Zarda et al. 1997) or the addition of blocking reagents to reduce potential interference of background material (Hahn et al. 1997). However, due to the small number of Salmonella cells present, the analyses depended on our ability to concentrate cells from the original samples (i.e., cells from 500 mL of water concentrated in 1 mL of sample) and to avoid any further dilution during sample preparation for

FIGURE 1. Detection of microbes (i.e., DAPI-stained cells) (left panel) and salmonellae (right panel) in (A and B) water and (C and D) Suckermouth Catfish feces samples by epifluorescence microscopy.
hybridization. Salmonellae were detected in water samples directly after setup, in numbers of about 10^4 cells/mL. Numbers decreased by two orders of magnitude within the first 72 h of the study and became undetectable after day 5 (Table 2). In feces samples, numbers of salmonellae increased 10-fold during the first 36 h of the experiment from 2 to 26 × 10^4 cells/mg feces and then decreased gradually to about 100-fold at day 7 (Table 2). These findings were similar to our previous studies (Sha et al. 2013) and studies of others (Liang et al. 1982; Klein and Alexander 1986). Basic Salmonella population dynamic profiles obtained by in situ hybridization were similar to profiles obtained by qPCR analysis (Table 2) in water (r = 0.92) and feces (r = 0.89). Thus, while cell numbers of the entire microbial community were either stable or slightly increased during the experiment in water and feces, respectively, numbers of Salmonella decreased rapidly in time. In water samples, Salmonella cells were initially abundant, comprising up to 20% of the microbial community, but decreased in relative abundance by one order of magnitude within 12 and 24 h (Figure 2). In feces, Salmonella cells comprised up to 6% of the microbial community within the first 48 h before decreasing within 60 h (Figure 2). These results suggest the selective removal of Salmonella from these samples, which may be a function of predation as indicated in previous studies on salmonellae inoculated into natural or sterilized lake water (Liang et al. 1982) or other bacteria such as E. coli, Pseudomonas, or Klebsiella pneumoniae (Scheuerman et al. 1988).

Neither in situ hybridization nor qPCR analysis did detect salmonellae in intestine samples of fish harvested at the end of the study. However, end-point PCR after semiselective enrichment of salmonellae detected them in intestine samples of all fish from treatment 3 that had received tiles with biofilms inoculated with Salmonella strain S11. Rep-PCR patterns of all isolates obtained from these intestines, and also from water and feces samples collected at days 3 and 7, resembled that of strain S11 indicating that this strain was consumed and shed by the catfish. Intestine contents from fish of treatment 2, which had received tiles with biofilms free of salmonellae, were all negative for the invA gene. In addition, salmonellae

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### TABLE 2. Mean (±SE in parentheses) number of salmonellae (×10^2) in 1 mL of water or 1 mg of Suckermouth Catfish feces (dry weight). A dash (–) indicates cell number <100 cells.

<table>
<thead>
<tr>
<th>Time</th>
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<td>Water</td>
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<td></td>
<td>107 (59)</td>
<td>52 (30)</td>
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<td>Feces</td>
<td>221 (199)</td>
<td>543 (575)</td>
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<tr>
<td></td>
<td>Feces</td>
<td>364 (120)</td>
<td>741 (845)</td>
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*Not sampled.

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### FIGURE 2. Change in abundance of salmonellae, as percentage of all organisms (i.e., DAPI-stained cells), in water and Suckermouth Catfish feces samples from treatment 3, analyzed by in situ hybridization (i.e., FISH-based detection) and by qPCR (i.e., qPCR-based detection) in time.
could not be isolated from the intestines, or from water and feces samples. These results are in agreement with those of our previous study (Gaertner et al. 2008b), where we had shown that salmonellae in the intestine of fish were normally associated with particulate material in highly variable numbers. This suggests that salmonellae are not components of the indigenous microbial community in fish intestines but are taken up with particulate material including biofilms. While our experimental setup demonstrates that *Salmonella* can be taken up with food sources such as biofilms and be released into feces, it does not allow us to make strong statements about quantitative aspects of this transfer. *Salmonella* in feces could also originate in part from biofilms with cells becoming detached from the biofilms as demonstrated in other studies (Ksoll et al. 2007), released into water, and then adsorbed onto fecal material.

Fish and other aquatic organisms have been documented as potential vectors for human pathogens for many years (Metz 1980; Minette 1986; Chattopadhyay 2000; Fell et al. 2000; Hansen et al. 2008). Infections with salmonellae were generally related to the consumption of fish (Novotny et al. 2004), but they could also come from the environment contaminated by fish. Aquarium water, for example, was the source of salmonellosis in a child (Senayake et al. 2004). Persistence and dissemination of salmonellae in fish were dependent on the number of salmonellae administered to the fish with high numbers required for their detection in intestines or muscles of the fish 4 weeks after administration (Buras et al. 1985; Nesse et al. 2005). In our previous study (Sha et al. 2013), we demonstrated a fast decline of salmonellae in biofilms in time, which could be the basis for low percentages of salmonellae in both water and feces samples towards the end of the study, and also could explain the necessity to enrich for salmonellae cells so that they can be detected in low numbers in the intestine. Although fish seem to be able to take up salmonellae through their food resources and shed them through their feces into the environment, numbers of salmonellae after gut passage depend on their abundance in the original food resources and are not biomagnified during passage, and thus, fish only provide a means for translocation of this pathogen. Interestingly, our findings are consistent with another pathogen (i.e., *E. coli*) that is consumed and transferred by fish but not biomagnified within the intestine (Hansen et al. 2008). Consequently, fish are vectors for some microbial pathogens but are not contributing to biomagnification of pathogens within the aquatic communities.

**ACKNOWLEDGMENTS**

The authors are grateful for support from Texas State University, Department of Biology, Alexander–Stone Endowment, National Science Foundation (IOB-0615762; administered by D. Garcia, Texas State University), and the state of Texas through the American Recovery and Reinvestment Act. The study was performed in compliance to the rules overseen by the Texas State Institutional Animal Care and Use Committee (permits 0721-0530-7 and 05-05C38ADFB) and Texas Parks and Wildlife Department (permit SPR-0601-159).

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Identification of Largemouth Bass Virus in the Introduced Northern Snakehead Inhabiting the Chesapeake Bay Watershed

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COMMUNICATION

Identification of Largemouth Bass Virus in the Introduced Northern Snakehead Inhabiting the Chesapeake Bay Watershed

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Abstract

The Northern Snakehead Channa argus is an introduced species that now inhabits the Chesapeake Bay. During a preliminary survey for introduced pathogens possibly harbored by these fish in Virginia waters, a filterable agent was isolated from five specimens that produced cytopathic effects in BF-2 cells. Based on PCR amplification and partial sequencing of the major capsid protein (MCP), DNA polymerase (DNAPol), and DNA methyltransferase (Mtase) genes, the isolates were identified as Largemouth Bass virus (LMBV). Nucleotide sequences of the MCP (492 bp) and DNAPol (419 pb) genes were 100% identical to those of LMBV. The nucleotide sequence of the Mtase (206 bp) gene was 99.5% identical to that of LMBV, and the single nucleotide substitution did not lead to a predicted amino acid coding change. This is the first report of LMBV from the Northern Snakehead, and provides evidence that noncentrarchid fishes may be susceptible to this virus.

The Northern Snakehead Channa argus is a piscivorous fish native to eastern Asia. It is the most important snakehead cultured in China and has been purposely introduced to a number of Asian countries owing to its culinary attributes. For decades this fish has been imported to the United States as a live product to meet food market and aquarium trade demands. It is believed that accidental or purposeful introductions of this species into open waters were artifacts of this trade. Northern Snakeheads have now been observed in Florida, Maryland, Virginia, North Carolina, and the New England states (Courtenay and Williams 2004). The presence of Northern Snakeheads in the mid-Atlantic region has garnered attention and, coupled by evidence that these populations are expanding (Odenkirk and Owens 2007), has been given sensationalized media coverage. Strategies to eradicate or control the spread of this invasive fish have thus far failed, and it is predicted that the Northern Snakehead is likely to increase its present range (Odenkirk and Owens 2005; Herborg et al. 2007; Jiao et al. 2009).

The introduction of nonnative, invasive species poses the risk of introducing exotic pathogens. Emerging infectious diseases are those that have recently increased in incidence, geographic range, or host range. The introduction of diseases into new regions is a significant cause of ecosystem degradation and species extinction and is now recognized as an important source of emerging diseases (Dobson et al. 1986; Vitousek et al. 1997; Daszak et al. 2000). Little is known about pathogens of the Northern Snakehead that inhabit waters of the United States.

METHODS

As part of a preliminary pathogen survey, Northern Snakeheads (n = 15) were collected via electroshocking from multiple sites within Dogue Creek (near 38° 41′ 48.97″N, 77° 6′ 58.50″W) and Little Hunting Creek (38° 42′ 35.00″N, 77° 4′ 38.00″W) during September 2006. Both are tidal tributaries of the Potomac River and are located in Fairfax County, Virginia. After capture and measurement, fish were euthanized with a lethal aqueous dose of tricaine methanesulfonate followed by cervical dislocation. A gross necropsy, which included external and internal examinations, was performed on each specimen. Tissue samples

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were collected from skin and muscle, gill, liver, kidney, spleen, heart, gonad, gut, and mesentery and preserved in 10% neutral buffered formalin for histological processing and analysis via standard laboratory methodology. Additional samples of liver, kidney, and spleen were extracted aseptically and pooled for each individual fish for virus assessment via cell culture. All fresh tissue samples were maintained on ice for transport and processed within 16 h of collection. Tissues were homogenized with a mortar and pestle using alundum as an abrasive and suspended in 0.1-M phosphate buffered saline (PBS, pH 7.0–7.2). Tissue homogenates were then centrifuged at 1,500 × g for 20 min at 4°C, diluted in PBS, and passed through a 0.45-µm (average pore diameter), low-protein-binding syringe filter. Tissue dilutions of 1:50, 1:100, 1:500, and 1:1,000 (w/v) were prepared for cell line inoculation. The BF-2, CHSE-214 and EPC cell lines were inoculated with each dilution of sterile filtered tissue homogenate and incubated at 15, 20, or 25°C. Tissue culture medium was Opti-MEM containing 8% fetal bovine serum, 100 µg/mL of gentamicin, and 5 mM tris-HCl. A blind passage was performed for all samples for which a cytopathic effect (CPE) was not evident after 14 d in culture at dilutions of 1:50 or 1:100. Other dilutions for samples experiencing CPE were also passaged. Combined medium was passed through a 0.45-µm membrane filter and diluted 1:10, 1:100, and 1:1,000. Dilutions were plated onto the host cell line of origin and incubated at 20°C or 25°C. Cultures were considered negative if CPE was not observed after an additional 30 d of culture.

Based on electron microscopy (data not shown), we suspected that the viral agent was a member of the family Iridoviridae or a morphologically similar group. Tissue culture supernatants from cells exhibiting CPE were extracted with a High Pure Viral Nucleic Acid Kit (Roche Diagnostics). Extractions were performed as per manufacturer supplied protocols. End-point PCR was performed on all DNA preparations using 2 µL of unquantified DNA extract, 400 nM of each primer in a 25-µL reaction of GoTaqGreen PCR master mix (Promega). Primer pairs and cycling conditions for the reactions are listed in Table 1. Largemouth bass virus (PB02-30)

<table>
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<tr>
<th>Primer set (PS)</th>
<th>Primer pair</th>
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<th>Target</th>
<th>Expected product size (bp)</th>
<th>Amplification</th>
<th>Reference</th>
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<td>GACTTGGCCACTTATGAC</td>
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<td>DNApolR</td>
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<td>CCTTGTGCTGTCGCTGCGGAG</td>
<td></td>
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</table>

*aDenaturation (94°C, 60 s), annealing (48°C, 30 s), extension (72°C, 60 s), cycles (30).*  
*bDenaturation (94°C, 60 s), annealing (50°C, 30 s), extension (72°C, 60 s), cycles (30).*  
*cDenaturation (94°C, 60 s), annealing (55°C, 30 s), extension (72°C, 60 s), cycles (30).*  
*dDenaturation (94°C, 60 s), annealing (60°C, 30 s), extension (72°C, 60 s), cycles (30).*  
*eY = C or T in the sequence.*
was used as a positive control. Extracted DNA from uninfected cells was used as a negative control. Target genes included the major capsid protein (MCP), DNA polymerase (DNApol), and DNA methyltransferase (Mtase), as well as the DNA-dependent RNA polymerase and others (Table 1). The PCR products were resolved in 2% agarose matrices by electrophoresis at 90 V for 90 min and stained with GelRed (Biotium). All PCR products were purified using DNeasy PCR purification kits (QIAGEN) and prepared for direct sequencing.

RESULTS
Based on gross and histological observations, all 15 snakehead specimens were apparently healthy. Fish ranged in age

![Phylogram of fish and amphibian iridoviruses](image)
from 1 to 5 years with a median of 4 years as determined from otolith annuli. Average weight was 1,692 ± 148 g (mean ± SD) and TL was 548 ± 24 mm. An external open lesion to the dorsal surface of the head, consistent with a bird strike lesion, was noted in one specimen, but no other gross lesions were observed. Only a few histological lesions were noted among tissue specimens of all 15 fish, and these were largely consistent with the presence of the intracoelomic helminths or monogenetic trematodes in the gill.

Cell culture of tissue homogenates from the clinically normal Northern Snakehead yielded CPE from 5 of 15 individual fish that was best described as cell rounding and multifocal detachment of the monolayer (not shown). The primer sets PS1, PS2, PS3, PS4, and PS5 amplified product of expected size from the LMBV PB02-30 isolate and all snakehead isolates (not shown). The PS7 and PS8, which amplify sequence from a Largemouth Bass iridovirus isolated in China (Deng et al. 2011), did not amplify product from these isolates. Amplification and sequencing of viral DNA verified the isolates as a member of the genus Ranavirus. Partial gene sequences were submitted to the National Center for Biotechnology Information (NCBI) database for MCP (JQ178328), DNApol (JQ178329), and Mtase (JQ178328). Nucleotide sequences from all snakehead isolates were identical; however, only partial sequences from one isolate were submitted. Amplicons from the positive control LMBV PB02-30 were sequenced as well (MCP [JQ178325], DNApol [JQ178326], and Mtase [JQ178327]). Sequence identity of the three partial gene sequences between the snakehead isolates and the LMBV PB02-30 was 100% for all but the DNA methyltransferase gene. A single synonymous mutation was noted in this sequence from all snakehead isolates, but this nucleotide substitution is not predicted to affect protein coding.

DISCUSSION

While LMBV is typically associated with Largemouth Bass, it has been isolated from other fish species including noncentrarchids. Ranaviruses are recognized as multihost pathogens (Schock et al. 2008). Peer-reviewed documentation of this virus isolated from other hosts is scarce; however, isolations are documented in the U.S. Fish and Wildlife Service National Wild Fish Health Survey database (USFWS 2011). Between April 18, 1996 and April 5, 2013, USFWS scientists sampled 252 different species in the contiguous United States. A query of this database identified 16 species of fish from which LMBV had been isolated (Table 2). Identification of LMBV for this survey included primary isolation via cell culture and confirmation with PCR (Heil 2009). While this virus is primarily isolated from species of the family Centrarchidae, species from other families are also apparent hosts. Of note, this virus is commonly isolated from clinically healthy fish. The isolates reported in the present survey were from apparently healthy snakehead, and it is unknown if this virus causes disease in the Northern Snakehead.

Here we present the first documented isolation of LMBV from Northern Snakehead. It is unknown whether the LMBV isolated from these fish originated overseas. It is suspected that LMBV is of southeastern Asian origin and was introduced by the tropical fish trade. This possibility is supported by sequence data of the doctor fish iridovirus and guppy virus 6, or was

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Number positive</th>
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<tbody>
<tr>
<td>Largemouth Bass Micropterus salmoides</td>
<td>Centrarchidae</td>
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<tr>
<td>Smallmouth Bass Micropterus dolomieu</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Bluegill Lepomis macrochirus</td>
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</tr>
<tr>
<td>Suwannee Bass Micropterus notius</td>
<td>Centrarchidae</td>
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</tr>
<tr>
<td>Redbreast Sunfish Lepomis auritus</td>
<td>Centrarchidae</td>
<td>2</td>
</tr>
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<td>Longear Sunfish Lepomis megalotis</td>
<td>Centrarchidae</td>
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</tr>
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<td>Rock Bass Ambloplites rupestris</td>
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<td>1</td>
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<td>Black Crappie Pomoxis nigromaculatus</td>
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</tr>
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<td>Yellow Bass Morone mississippiensis</td>
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</tr>
<tr>
<td>Muskellunge Esox masquinongy</td>
<td>Esocidae</td>
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<tr>
<td>Bluehead Chub Nocomis leptocephalus</td>
<td>Cyprinidae</td>
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<tr>
<td>Freshwater Drum Aplodinotus grunniens</td>
<td>Sciaenidae</td>
<td>2</td>
</tr>
<tr>
<td>Spotted Sucker Minotrema melanops</td>
<td>Catostomidae</td>
<td>1</td>
</tr>
</tbody>
</table>
transmitted while fish inhabited waters of the United States (Mao et al. 1999). The latter is the most likely scenario and is suggested by the phylogeny constructed here (Figure 1); however, a more extensive interrogation of the viral genome would be necessary. Centrarchids constitute a portion of Northern Snakehead diet, and exposure to LMBV per os is likely. Of note, the nucleotide sequences of these isolates are not identical to LMBV based on the limited sequencing performed. An iridovirus similar to LMBV has been documented in China that causes an ulcerative syndrome in Largemouth Bass (Deng et al. 2011), but that virus is considerably different than the U.S. LMBV and our isolates (Figure 1; Table 3). Publically available gene sequence in the NCBI database is limited for LMBV and closely related ranaviruses. The significance of LMBV in snakeheads is unknown; however, the fact that they are a host indicates that they are a component of pathogen ecology in the Chesapeake Bay watershed.

In order to predict or mitigate the effects that such a multispecies pathogen may have on a community of host species, it is critical to understand the dynamic interactions between pathogen and hosts. Largemouth Bass and Northern Snakehead exploit overlapping habitats, which may pose risk in regards to pathogen transmission. In recent years, LMBV has become a pathogen of increased interest in Virginia where Largemouth Bass mortality associated with LMBV has been reported in John H. Kerr (Buggs Island) Reservoir and Briery Lake. Given the importance of Largemouth Bass sportfishing, it is critical to better understand the relationships between LMBV and potential host species, including the Northern Snakehead.

### ACKNOWLEDGMENTS

The authors thank Andrew Goodwin (University of Arkansas at Pine Bluff) for contributing the largemouth bass virus control (PB02-30). We also thank Jim Winton and Carla Conway at the U.S. Geological Survey, Western Fisheries Research Center, and Bill Granath and Jim Driver at the University of Montana for performing the electron microscopy evaluation of tissue culture supernatants. The authors thank Gavin Glenney (US-FWS, Northeast Fishery Center, Fish Health Center, Lamar, Pennsylvania) for reviewing this manuscript. Jim Winton provided a review as well for which we are thankful.

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### TABLE 3. Percent identity of the nucleotide (DNA) and protein (AA) between partial sequences of the Northern Snakehead isolates and related iridoviruses. Viral genes included the 492 nucleotides of the major capsid protein (MCP; 492 bp), 519 nucleotides of DNA polymerase (DNApol; 519 bp), and DNA methyltransferase (Mtase; 206 bp). Identity was denoted as not available (NA) if homologous sequence data were not present in the NCBI database. Nucleotide sequences for these comparisons (MCP, DNA pol and Mtase) included (1) snakehead isolate: JQ178328, JQ178329 and JQ178330; (2) largemouth bass virus (LMBV; PB02-30, USA): JQ178325, JQ178326, and JQ178327; (3) LMBV (USA): AF080250, DQ159940, and AF100199; (4) LMBV (China): GU256635, NA, and GU256634; (5) doctor fish virus (DFV): FR677324, FJ374281, and AF100202; (6) guppy virus 6 (GV6): FR677325, FJ374282, and NA; (7) epizootic hematopoietic necrosis virus (EHNV; complete genome): FJ433873; (8) frog virus 3 (FV3; complete genome): AY548484; (9) tiger frog virus (TFV; complete genome): AF389451; (10) European catfish virus (ECV; complete genome): JQ724856; (11) Ambystoma tigrinum stebbensi virus (EHNV; complete genome): FJ433873; (12) frog virus 3 (FV3; complete genome): AY548484; (9) tiger frog virus (TFV; complete genome): AF389451; (10) European catfish virus (ECV; complete genome): JQ724856; (11) Ambystoma tigrinum stebbensi virus (EHNV; complete genome): FJ433873; (12) lymphocytosis disease virus (LCDV; complete genome): AY380826.

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<th>Mtase</th>
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<tr>
<td></td>
<td>Nucleotide (%)</td>
<td>AA (%)</td>
<td>Nucleotide (%)</td>
</tr>
<tr>
<td>LMBV (PB02-30, USA)</td>
<td>100</td>
<td>100</td>
<td>99.5</td>
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A Strain of Siniperca chuatsi Rhabdovirus Causes High Mortality among Cultured Largemouth Bass in South China

Dongmei Ma a, Guocheng Deng a, Junjie Bai a, Shengjie Li a, Lingyun Yu a, Yingchun Quan a, Xiaojing Yang a, Xiaoyan Jiang a, Zemin Zhu a,b & Xing Ye a

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A Strain of *Siniperca chuatsi* Rhabdovirus Causes High Mortality among Cultured Largemouth Bass in South China

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Abstract

In April 2011, 40% mortality of Largemouth Bass *Micropterus salmoides* juveniles occurred at a farm of Zhongshan City, Guangdong Province, China. Infected fish became lethargic, exhibited corkscrew and irregular swimming, and developed a distended abdomen and crooked body. Fish began to die within 2 d after the appearance of clinical signs. 

In order to analyze the pathogeny and diagnose the disease earlier, observation of clinical signs, cell infection, titer calculation, electron microscopy, immersion infection assay for fish, and nucleotide sequence analysis were carried out. Fathead minnow (FHM) cell cultures, inoculated with filtrate of liver and spleen homogenates from the diseased fish, developed the obvious cytopathic effect 46 h after inoculation in the primary culture and 24 h at the first passage. Typical rhabdovirus particles, 115–143 nm in length and 62–78 nm in diameter, were observed in infected FHM cells by direct transmission electron microscopy. The isolated virus produced a titer of $10^{7.15}$ TCID50/mL. Immersion-Fish infected with the virus had similar clinical signs and 80% mortality with $10^{2.5}$ LD50/mL. The data indicated that the rhabdovirus was the lethal pathogeny of the current disease. Based on nucleoprotein-gene nucleotide sequence multiple alignment analysis, the newly isolated virus is a strain of *Siniperca chuatsi* rhabdovirus (SCRV) under family *Rhabdoviridae*, which was initially isolated from Mandarin Fish *Siniperca chuatsi*. Up to the present, at least four virus strains have been isolated from diseased Largemouth Bass, which have had different clinical signs. Comparison of the clinical signs can help in an early diagnosis of the disease.

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Largemouth Bass *Micropterus salmoides* is an economically important freshwater fish in China and has an annual production of 100,000 metric tons (Bai et al. 2008). In recent years, the outbreaks of epizootic viral diseases at Largemouth Bass farms have become more frequent. A ranavirus strain and a megalocytivirus strain were isolated from diseased cultured Largemouth Bass in China in 2008 and 2009, respectively (Deng et al. 2011; Ma et al. 2011). In both cases, only adult individuals were naturally infected. In April 2011, a new epidemic broke out in cultured Largemouth Bass (2.5–4.5 cm in length) in Guangdong Province of China, and about 200,000 fish died in five 4-ha ponds.

Rhabdoviruses are virulent causative agents of serious aquatic viral diseases. Fish rhabdoviruses have broad host ranges. For viral hemorrhagic septicaemia virus (VHSV) alone, a virus of the family *Rhabdoviridae* and genus *Novirhabdovirus*, more than 80 species of fish have been identified as its hosts (Olesen and Skall 2012). The family *Rhabdoviridae* was partitioned into the genera *Nucleorhabdovirus*, *Cytorhabdovirus*, *Novirhabdovirus*, *Vesiculovirus*, *Ephemerovirus*, and *Lyssavirus* (Assenberg et al. 2010). Fish can be infected by certain viruses of the genera *Novirhabdovirus* and *Vesiculovirus* (Amend et al. 1969; Ahne et al. 2002). Members of the family are negative-stranded RNA viruses surrounded by a lipid envelope, which is generated from the plasma membrane of the infected host cell. Rhabdovirus virions are rod- or bullet-shaped particles about 100–430 nm in length and 45–100 nm in diameter (Assenberg et al. 2010). The antigenome of the virus encodes five common genes: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large major protein (L) (Assenberg et al. 2010).

In this study, a rhabdovirus was isolated from diseased Largemouth Bass fingerlings. The clinical signs of infected fish were described, and comparison of the nucleotide sequences of the N genes between the current virus and other rhabdoviruses indicated that the current isolate is a strain of *Siniperca chuatsi* rhabdovirus (SCRV).

**METHODS**

*Fish sample collections.*—Moribund Largemouth Bass (2.5–4.5 cm TL, n = 25) were collected from the aquaculture farm in Dansha Village of Zhongshan City. Fish were placed in plastic bags containing crushed ice and shipped to Pearl River Fisheries Research Institute within 2.5 h for necropsy. Fish were dissected to check for pathological signs.

*Preparation of virus isolates.*—Livers and spleens of five individuals were pooled (1 g) and homogenized in 10 mL of medium 199 (Gibco) without fetal bovine serum (FBS). After being centrifuged and filtered through a sterile 0.22-µm filter, the viral filtrate was inoculated onto a monolayer of fathead minnow (FHM) cells (Gravell and Malsberger 1965) and then incubated in fresh culture medium containing 2% FBS at 25°C. Cell cultures then were examined daily for the cytopathic effect (CPE). The viruses were titrated using a limiting dilution assay and expressed as the tissue culture infective dose with 50% endpoint (TCID50) according to the method of Reed and Muench (1938).

*Electron microscopic analysis of virus isolates.*—The virus-infected cells were fixed, dehydrated, embedded, sectioned, stained, and observed under a Philips-CM10 transmission electron microscope (FEI, Eindhoven, The Netherlands).

*Immersion infection of Largemouth Bass.*—One hundred healthy Largemouth Bass (3.5–4.0 cm TL) were randomly divided into four experimental groups and one control group and transferred into 150-L tanks with recirculating water at 24°C. The fish were left unfed for 48 h and then exposed to the virus. A series of dilutions from $10^{-3}$ to $10^{-4}$ was performed from the initial propagated virus isolates ($10^{7.15}$ TCID50/mL) with normal saline (0.65% NaCl in double-distilled H2O) within 4-L containers. The fish in the treatment group were placed in the containers for 60 min. The control fish received no treatment. All groups were then placed into separate 150-L tanks. The mortality was recorded 20 d after infection. Re-isolation and re-infection assays were also carried out.

*Genome sequence analysis.*—Total RNA was extracted from the virus-infected FHM cells and diseased fish liver using the Trizol reagent (Invitrogen). Total RNA was reversely transcribed to produce first-strand cDNA using the ReverTra Ace-α reverse transcription kit (Toyobo, Osaka, Japan). Primers targeting five known fish rhabdoviruses (SCRV, VHSV, infectious hematopoietic necrosis virus [IHNV], spring viremia of carp virus [SVCV], and snakehead rhabdovirus [SHRV]) genomic sequences were designed (Table 1). Negative control PCRs were performed by using the uninfected cell culture and the healthy fish as templates. The PCR products were sequenced using an ABI-3730 auto-sequencer (Applied Biosystems, Foster, California). Basic local alignment search tool (BLAST) was used to search for similar sequences. Another nine pairs of primers were designed to amplify the potential genes in the whole genome (Table 1; see Figure 5).

In order to analyze the taxonomic status of the current virus isolate, the nucleotide sequence of the N gene in the virus was aligned with the corresponding sequences of other rhabdoviruses in a previous description (Talbi et al. 2011), including the various perch rhabdoviruses, as well as SCRV, pike fry rhabdovirus (PFRV), eel virus European X (EVEX), Stratford virus (STRV), and lake trout rhabdovirus (LTRV). Multiple alignments were performed using the program Clustal X 1.81, and a neighbor-joining (NJ) analysis based on a Kimura 2-parameter model was carried out using the program MEGA 5 with 1,000 data bootstrap replications.

**RESULTS**

*Symptoms of Diseased Largemouth Bass.*—At the time of the disease outbreak in Largemouth Bass fingerlings in April 2011 at the fish farm, the water temperature
TABLE 1. Primers and primer sequences. The primers were designed according to the genome sequence (shown as the GenBank accession) of the rhabdoviruses.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′−3′)</th>
<th>Rhabdovirus</th>
<th>GenBank accession</th>
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<td>GAATGGAACACAAATCATC</td>
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<td>NC_008514</td>
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<td>GCCTGTCACGAGGATCG</td>
<td>rhabdovirus (SCRV)</td>
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<tr>
<td>IHNVF</td>
<td>CACCGTACTTTGCTGCTAC</td>
<td>Infectious hematopoietic necrosis virus (IHNV)</td>
<td>HM461966</td>
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<td>IHNVR</td>
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<td>Viral hemorrhagic septicemia virus (VHSV)</td>
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<td>VHSVR</td>
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<tr>
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<tr>
<td>SVCVR</td>
<td>TCTTGAGGCCAAATAGCTC</td>
<td>(SHRV)</td>
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<td>TTATTCGCCATCGAGCCTC</td>
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ranged from 18°C to 25°C. Affected fingerlings became lethargic, exhibited corkscrew and irregular swimming behavior, and had emaciated and crooked bodies and distended abdomens. Early symptoms of the disease included muscle hemorrhage near the anus and a long, semitransparent, fecal cast often trailing from the anus. Some of the diseased fish had severely distended abdomens, bulging eyes, petechial hemorrhages from mandible to abdomen, pale gills, and swollen and hemorrhaged livers (Figure 1). Mortality reached about 40%. Subsequently, a similar disease broke out in 2–6-cm largemouth bass and killed 30–50% of the fish in several aquaculture farms in Xingtan Village, Shunde City, in the vicinity of Zhongshan.

Virus Isolation and Reproduction

The virus filtrate isolated from the liver and the spleen of diseased fish was inoculated onto a FHM cell monolayer. A CPE was first observed 46 h after inoculation in the primary culture. In the first passage, CPE was detected at 24 h after inoculation, and numerous round cells in small foci appeared in the cell monolayer (Figure 2A). At 48 h, more round cells were apparent (Figure 2B). As CPE progressed, the cells in the center of the foci became detached (Figure 2C). By 96 h, the entire monolayer of cells was affected and detached cells aggregated to form a network-like monolayer (Figure 2D). Uninfected control cells exhibited normal morphogenesis (Figure 2E). The virus titer reached $10^{7.15}$ TCID50/mL at the third passage.

Electron Microscopy Analysis of the Virus

Electron microscopy analysis revealed large amounts of virus particles in the cytoplasm and the cell gap of infected FHM cells. The intact particles had a typical bullet-shaped structure that had a rounded end and a flat-based end. They range from 115 to 143 nm in length and from 62 to 78 nm in diameter ($n = 50$) (Figure 3).

Immersion Infection of Largemouth Bass

Serial dilutions ($10^{-1}$ to $10^{-4}$) of the virus isolations ($10^{7.15}$ TCID50/mL) were used for immersion infection. The mortality was positively related to virus titers. The highest mortality reached 85% with a virus titer of $10^{6.15}$ TCID50/mL, and
the lowest mortality was 10% with a titer of $10^{3.15}$ TCID50/mL. The detailed data are shown in Table 2. Challenged fish began to exhibit clinical signs at 48 h in two virus titers ($10^{6.15}$ and $10^{5.15}$ TCID50/mL) after infection; the clinical signs were similar to those of a natural infection and included lethargy, corkscrew and irregular swimming, an emaciated and crooked body, distended abdomen, exophthalmia, swollen and hemorrhaged liver, and a fecal cast trailing from the anus (Figure 4). In contrast, control fish exhibited no mortality and no clinical signs of infection. After re-isolation, re-immersion infection with $10^{-1}$ and $10^{-2}$ dilutions of the virus isolates caused Largemouth Bass mortalities up to 90% and 50%, respectively. Affected fish had clinical signs similar to those from natural infection.

### PCR Amplification and Sequence Analysis

A specific DNA fragment (about 850 bp) was successfully amplified with the primers for SCRV, and no fragment was detected in the negative controls. The PCR products were purified and sequenced. Results using BLAST indicated that the nucleotide sequence was closely identified to the N gene of SCRV. Subsequently, another nine pairs of primers were designed to amplify the whole genome. A sequence of 11,367 bp including a 3′ untranslated region and five genes was obtained, which encoded N, P, M, G, and L genes, and an accessory gene, the small hydrophobic (SH) protein, between M and G genes (Figure 5). But the 5′ untranslated region was not identified.

To determine the taxonomic status of the virus isolate, the nucleotide sequence of the N gene was aligned with the published sequences of other rhabdoviruses in the phylogenetic tree (Talbi et al. 2011). As shown in Figure 6, the rhabdoviruses in the
cluster I was divided into eight distinct genogroups (A–H), which was supported by high bootstrap values (76–100%) (Talbi et al. 2011). The current virus exhibits a high genetic similarity (97% identity) to SCRV isolated in 1999 and belongs to genogroup H of cluster I in the tree. These characteristics indicate that the present virus isolate is a strain of SCRV.

**DISCUSSION**

Up to the present, at least four virus strains have been isolated from Largemouth Bass and proven to be responsible for significant morbidity and mortality. Largemouth Bass virus (LMBV) was isolated from diseased individuals in South Carolina. Fish naturally infected with LMBV appeared normal and lacked external lesions, but they floated at the water’s surface (Plumb et al. 1996). Another ranavirus strain was isolated in China in 2008 (Deng et al. 2011). Although the predicted amino acid sequence of the major capsid protein was 98% identical to that of LMBV, the fish infected by the virus exhibited the distinct clinical signs, including extensive ulceration on the body surface, local hemorrhages, necrosis of naked muscle, and tumefaction and ulceration of the fin base (Deng et al. 2011). The third kind of virus, a megalocytivirus, was isolated from diseased Largemouth Bass in 2009. Affected fish had swollen livers, spleens,
and kidneys (Ma et al. 2011). The above three DNA viruses belong to the family Iridoviridae and infect adult Largemouth Bass naturally.

In this study, a rhabdovirus with nonsegmented (−) ssRNA was isolated from infected Largemouth Bass fingerlings (2.0–6.0 cm in length). The water temperature for the rhabdovirus natural infection ranged from 18°C to 25°C, which is lower than those for ranavirus and megalocytivirus infections (Deng et al. 2011; Ma et al. 2011). The clinical signs of the current virus-infected fish were different from those of the fish infected with the current strain of SCRV and included hemorrhaging of large areas of body surface (head, the periphery of the oral cavity, fin base, and tail), darkened red skin with black stripes above lateral line, exophthalmia, and pale gills (Zhang and Li 1999).

The host range of fish rhabdoviruses is broad. More than 80 species of fish comprising 19 orders and 45 families have been identified for VHSV alone. In this study, a rhabdovirus was isolated from Largemouth Bass. Sequence analysis based on the phylogenetic tree (Talbi et al. 2011) showed that both the current isolate and SCRV belonged to genogroup H in cluster I. These two strains were isolated from two distant regions that were not connected by waterways in 2011 and 1999, respectively, and exhibited a high identity, which was greater than 97% for N gene sequences. Similarly, the close genetic relationships were observed between the perch rhabdovirus isolate R6146 from France and two brown trout virus isolates (LTRV and STRV) from northern Europe (Talbi et al. 2011). These observations suggest that SCRV is expected to be transmitted to and cause disease in other species of fish by the transportation of infected fish. Moreover, SCRV represents a threat to cultured and wild Largemouth Bass globally and highlights the necessity of preventing the spread of both known and emerging diseases in aquatic animals generally.
FIGURE 5. The predicted viral genome structure. The positions and the sizes of open reading frames (ORFs) are indicated (N, nucleoprotein; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, large major proteins) and the SH gene encoding accessory protein is shaded. The short lines below show the PCR amplified fragments for assembling the complete genome, and the numerals show the primer numbers.

FIGURE 6. The neighbor-joining phylogenetic tree of the N gene nucleotide sequences of the current isolate and 28 rhabdovirus isolates in the previous report (Talbi et al. 2011); the eight genogroups A–H are indicated. Branch lengths are drawn to scale, and bootstrap-support values (>75%) are shown for key nodes. The current isolate is closely related to SCRV in genogroup H within cluster I.
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REFERENCES


Reduced Myxobolus cerebralis Actinospore Production in a Colorado Reservoir May Be Linked to Changes in Tubifex tubifex Population Structure

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Reduced Myxobolus cerebralis Actinospore Production in a Colorado Reservoir May Be Linked to Changes in Tubifex tubifex Population Structure

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Abstract

Elucidating the dynamics of a parasitic infection requiring two hosts in a natural ecosystem can be a daunting task. Myxobolus cerebralis (Mc), the myxozoan parasite that causes whirling disease in some salmonids, was detected in the Colorado River upstream of Windy Gap Reservoir (WGR) in 1988. Subsequently, whirling disease was implicated in the decline of wild Rainbow Trout Oncorhynchus mykiss in the river when WGR was identified as a point source of Mc triactinomyxon (TAMs). Between 1997 and 2004, numerous investigations began to elucidate the etiology of Mc in WGR. During this period, Mc TAM production in WGR declined more than 90%. Explanations for the decline have included differences in stream discharge between years, changes in the thermal regime of the lake, severe drought, changes in the fish population structure in WGR, and reductions in the prevalence and severity of Mc infection in salmonids in the Colorado and Fraser rivers upstream of WGR. All of these have been discredited as explanations for the reduced TAM production. In 2005, a new study was conducted to replicate the studies completed in 1998. In this paper, the results of a new real-time polymerase chain reaction assay utilized to quantify the mitochondrial 16S rDNA specific to each of four lineages of Tubifex tubifex in pooled samples of 50 oligochaetes are presented. These results suggest that compared with 1998, the densities of aquatic oligochaetes and T. tubifex have increased, TAM production has been greatly reduced, and the decline is congruent with the dominance of lineages I, V, and VI of T. tubifex—three lineages that are refractory or highly resistant to Mc infection—in the oligochaete population. While it is possible that the resistant lineages function as biofilters that deactivate Mc myxospores, the reason for the decline in TAM production in WGR remains an enigma.

FRAMEWORK

Historical Background

Myxobolus cerebralis (Mc) is a myxosporean parasite that can cause whirling disease in trout and salmon (Markiw and Wolf 1983; Wolf and Markiw 1984). The parasite requires two hosts for completion of its life cycle. Triactinomyxon (TAM) actinospores released from infected, Mc-susceptible Tubifex tubifex oligochaetes are infective to many species of salmonids. Infected fish, in turn, produce myxospores that are infectious to Mc-receptive tubificid worms (Wolf and Markiw 1984). The parasite was first detected in Rainbow Trout Oncorhynchus mykiss at public and private fish hatcheries in Colorado during November and December 1987. Commercially...
produced Rainbow Trout that were stocked into three private ponds in Grand County, Colorado, tested positive for \( Mc \) in 1988. Two of the stocked ponds were in the Colorado River basin upstream of Windy Gap Reservoir (WGR), the focal point of this study. Water from both ponds flows into the Colorado River and then into WGR, a small, shallow, 38-ha impoundment near Granby, Colorado. The average depth of the lake is approximately 1 m and the maximum depth is only about 6 m (excluding the small forebay area for intake pumps at the northwestern corner of the dam). The confluence of the Colorado and Fraser rivers is located approximately 1 km upstream of the inlets to WGR (Figure 1).

Annual fish health inspections of wild Rainbow Trout in the Colorado River 22 km downstream of WGR began in April 1983 in conjunction with a spring spawning operation. Myxospores of the \( Mc \) parasite were first detected in spawning adult wild Rainbow Trout in April 1992. Empirical evidence gathered from intensive field efforts and extensive testing of trout for \( Mc \) infection throughout the upper Colorado River basin during 1993 and 1994 indicated that loss of wild Rainbow Trout year-classes in the river below WGR began in 1991 (Walker and Nehring 1995; Nehring and Walker 1996). Recruitment failure among wild Rainbow Trout fry in the river continued unabated through 2008. In contrast, wild Brown Trout \( Salmo trutta \) fry recruitment and survival has been excellent every year of this long-term investigation (1993–2008).

Water filtration studies (Thompson and Nehring 2000), aquatic oligochaete studies (Zendt and Bergersen 2000; Nehring et al. 2003), and sentinel fish exposure experiments (Thompson et al. 2002) completed in the 1990s clearly demonstrated that WGR was a major point source of TAM actinospores of \( Mc \) and a primary factor in the whirling disease epizootic in Rainbow Trout downstream of the lake. The total annual numbers of TAMs in the discharge from WGR in the periods April 1997–March 1998 and April 1998–March 1999 were conservatively estimated to be \( 960 \times 10^9 \) and \( 1.8 \times 10^{12} \), respectively (Nehring et al. 2002). Very high TAM densities continued to be observed in the water flowing out of WGR through December 2000. Monthly water filtration studies (Thompson and Nehring 2000) conducted between April 1997 and December 1998 revealed that the average TAM densities in the discharge from WGR were 30 times as great as the densities observed at
TABLE 1. Estimated density (number/L) and frequency of detection of triactinomyxon (TAM) actinospores of Myxobolus cerebralis with respect to the highest average 10-d discharge ($Q$ [m$^3$/s]) in the Colorado River 50 m downstream of Windy Gap Reservoir from April 1997 through June 2006.

<table>
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<th>Year</th>
<th>Highest average 10-d $Q$</th>
<th>Density</th>
<th>Filtration occasions (number positive/total)</th>
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<td>2004</td>
<td>8.13</td>
<td>0.414</td>
<td>0</td>
</tr>
<tr>
<td>2005</td>
<td>23.2</td>
<td>0.853</td>
<td>0</td>
</tr>
<tr>
<td>2006b</td>
<td>14.9</td>
<td>0.491</td>
<td>0</td>
</tr>
</tbody>
</table>

*Water filtrations were conducted from April through December 1997.
*Water filtrations were conducted from January through June 2006.

filtration sites on the Fraser and Colorado rivers 1 km upstream of WGR. This was the case even though WGR has never been stocked with trout and supports a very sparse trout population.

The Conundrum

Compared with the levels observed between April 1997 and December 2000, the monthly estimates of TAM density emanating from WGR declined sharply in 2001 and remained even lower through June 2006 (Table 1). Initially, two hypotheses (Nehring and Thompson 2003) were postulated for the decline in TAM production: a decrease in the wetted surface area in the reservoir in 2001 compared with 1997–2000 and record drought in 2001 and 2002. First, Mc-infected salmonids in the Fraser River and Colorado River basins upstream of WGR were thought to be the source of myxospores that sustained the high levels of TAM production in the lake from 1997 through 2000 and that elevated stream discharge from melting snow mobilized myxospores in the sediments upstream of the lake and deposited them in WGR. A core sampling study of the lake in 1998 clearly demonstrated that the areas of WGR receiving direct inflow from the Colorado and Fraser rivers were producing most of the TAMs (Nehring et al. 2003). Second, the extremely low levels of spring runoff in 2001 and 2002 stemming from severe drought conditions were considered inadequate to mobilize the myxospores in sediment-laden areas upstream and transport them to the lake. Linear regression analysis of the total estimated acre-feet of discharge into WGR from the Fraser and Colorado rivers from May through September 1998–2003 (independent variable) on the estimated total TAMs in the effluent of WGR for July through June those same years (dependent variable) yielded a significant correlation ($P = 0.006; r = 0.9755$), suggesting that the myxospore input into WGR was linked to the total spring–summer discharge levels in the Fraser and Colorado rivers. Snowfall in the Colorado River basin, however, was significantly above average during the winter of 2002 and 2003 and peak spring–summer discharge levels in 2003 equaled or exceeded those observed between 1998 and 2000 (Figure 2A). Yet, estimated TAM densities remained very low in 2003, 2004, and 2005 compared with the period from 1997–2000 (Table 1).

Another possible explanation for the dramatic decline in TAM production is a change in the fish community in WGR. However, gill-net survey results spanning a period of 12 years (1992–2004) do not support that hypothesis (Table 2). White Suckers Catostomus commersoni and Longnose Suckers C. catostomus were the dominant fish in gill-net catches in every survey, both before and after the decline in TAM production in WGR. Brown Trout catch ranged from 6% to 16% during the same time period, while Rainbow Trout and kokanee Oncorhynchus nerka were the least abundant species netted, comprising only 1–5% of the total catch in years when they were captured.

Finally, a decrease in the prevalence and severity of Mc infection in salmonids in the Colorado River upstream of WGR could have led to a decrease in the number of myxospores being transported into WGR. The results of disease-testing between 1994 and 2005 using the methodology of Markiw and Wolf (1974)


<table>
<thead>
<tr>
<th>Species</th>
<th>1992</th>
<th>1999</th>
<th>2001</th>
<th>2004</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catostomids</td>
<td>305 (87.9)</td>
<td>114 (82.0)</td>
<td>155 (88.6)</td>
<td>172 (94.0)</td>
</tr>
<tr>
<td>Brown Trout</td>
<td>24 (6.9)</td>
<td>22 (15.8)</td>
<td>14 (8.0)</td>
<td>11 (6.0)</td>
</tr>
<tr>
<td>Rainbow Trout</td>
<td>18 (5.2)</td>
<td>2 (1.5)</td>
<td>3 (1.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Kokanee</td>
<td>0 (0)</td>
<td>1 (0.7)</td>
<td>3 (1.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>All species</td>
<td>347 (100)</td>
<td>139 (100)</td>
<td>175 (100)</td>
<td>183 (100)</td>
</tr>
</tbody>
</table>
FIGURE 2.  (A) Mean daily flow (m$^3$/s [cms]) and (B) instantaneous water temperature (°C) versus the estimated density (number/L) of triactinomyxon (TAM) actinospores of *Myxobolus cerebralis* in the Colorado River discharge from Windy Gap Reservoir, April 1997–June 2006.
TABLE 3. Average *Myxobolus cerebralis* myxospore concentrations in cranial tissues of Brown and Rainbow Trout collected from the Colorado River 1 km upstream of Windy Gap Reservoir at various times from 1994 to 2005.

<table>
<thead>
<tr>
<th>Period</th>
<th>Brown Trout</th>
<th>Rainbow Trout</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apr 1994</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>Nov 1998</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Apr 1999</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Oct 1999</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>May 2000</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td><strong>Period of reduced TAM production (2001–2005)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sep 2001</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>May 2004</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>Oct 2005</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

to determine the prevalence and severity of infection in age-1 Rainbow and Brown Trout collected from the Colorado River just upstream of the lake are summarized in Table 3. Cranial myxospore concentrations in both Brown and Rainbow Trout collected from the Colorado River 1 km upstream from WGR between 2001 and 2005 were not much different from the levels observed in these species between 1994 and 2000 (Table 3).

All of the preceding lines of evidence lead to the hypothesis that a shift in the tubificid worm population structure in WGR is responsible for the decline in TAM production in the lake since 2001. Application of molecular tools and polymerase chain reaction (PCR) techniques to the *Tubifex* genome (Sturmbauer et al. 1999; Beauchamp et al. 2001, 2002) have shown that the aquatic oligochaete *T. tubifex* is comprised of several different clades, strains, or lineages (or that there are a number of cryptic species within this genus; Beauchamp et al. 2001, 2002, 2005, 2006).

Numerous laboratory exposure studies have repeatedly demonstrated that the different clades, strains, or lineages of tubificid oligochaetes vary in susceptibility to *Mc* infection (Beauchamp et al. 2002, 2005, 2006; DuBey and Caldwell 2004; Kerans et al. 2004; DuBey et al. 2005; Arsân et al. 2007; Hallett et al. 2009; Zielinski et al. 2011). Hereafter, the term “lineage” will be used in reference to genetically distinct groups of *T. tubifex* that differ in susceptibility to the *Mc* parasite.

**Historical Data: 1998–2004**

Data from three separate studies conducted between 1998 and 2001 (Zendt and Bergersen 2000; Nehring et al. 2003; Beauchamp et al. 2005) and one in 2004 provide a basis for comparison with the results from a new study in 2005. The studies in 1998 (Nehring et al. 2003), 2001 (Beauchamp et al. 2005), and 2004 utilized core sampling to collect aquatic oligochaetes and estimate the density of *T. tubifex* in WGR. Aquatic oligochaete samples collected from WGR in 1998 were tested by PCR for DNA of *Mc* to determine parasite prevalence (Zendt and Bergersen 2000). Cryogenically archived oligochaete samples from 1998 were tested retrospectively in early 2004 using real-time polymerase chain reaction (qPCR) technology and primers designed to detect and quantify DNA belonging to different mitochondrial 16S lineages of *T. tubifex* (Beauchamp et al. 2001, 2002). These analyses provided information regarding the structure of the *T. tubifex* population along a single transect in WGR during 1998. Additional oligochaete samples were collected along the same transect during the summer of 2004 and analyzed using the same protocol, primers, and qPCR techniques. These tests provided empirical evidence that a shift in the relative abundance of the various lineages (I, III, V, and VI) of worms occurred in WGR that might explain the decline in TAM production since 2000. The data suggested that there was a decline in the relative abundance of lineage III worms between 1998 and 2004 and concomitant increases among lineage I, V, and VI oligochaetes. Lineage III *T. tubifex* are known to be highly susceptible to infection by *Mc*, while lineages I, V, and VI are known to be highly resistant to infection (Beauchamp et al. 2002, 2005; DuBey and Caldwell 2004; DuBey et al. 2005; Hallett et al. 2009; Zielinski et al. 2011).

A second study in 1998 focused on the temporal and spatial distribution of TAM production in WGR (Nehring et al. 2003). That study demonstrated that the zone of highest TAM production in WGR was congruent with the area of the lake through which water from the Colorado and Fraser rivers flows into and out of the reservoir. These findings and insights provide the baseline for comparison with the results from the 2005 study.

The objectives of our study were threefold. The first objective was to determine whether the dynamics and spatial distribution of TAM production in WGR had changed from 1998 to 2005. The second objective was to develop a spatial distribution of the various lineages of *T. tubifex* in WGR during the open-water period of 2005. The third objective was to determine whether the
relative abundance of the Mc-susceptible lineage III oligochaete was inversely proportional to the relative abundance of resistant *T. tubifex* lineages (I, V, and VI).

**METHODS**

**Field Protocols**

The field portion of the study was completed between May and October 2005. When Windy Gap Dam was completed in 1983, the surface area of the lake at full pool was approximately 42 ha, according to the Northern Colorado Water Conservancy District. In 2000, when the perimeter of the lake was circumscribed with Global Positioning System (GPS) mapping technology and the surface area of the four islands in the lake was subtracted, the wetted surface area of the lake was estimated to be 38 ha. Geographical information systems (ArcGIS) mapping techniques were used to develop a quadrat map of 38 1-ha sampling units. Twenty-three of the 38 sampling units were approximately square. The boundaries of the 15 remaining sampling units, which intersected the edge of the lake, were irregular in shape but approximately 1 ha in area (Figure 1). This quadrat map sampling grid was superimposed upon a satellite image of WGR obtained from the Internet (Global Explorer. Windy Gap Reservoir [Landsat 7]; 1:24,000; Walnut Creek, California; October 10, 1999).

The boundaries of each sampling unit were loaded into a handheld GPS unit with a visual screen that allowed the field crew to collect one core sample in each quadrat on each sampling occasion. Each sample location was marked by GPS so that all collection sites could be superimposed on the aerial photograph, allowing for spatial and temporal evaluation of the oligochaete population and TAM spore production in the lake during the 2005 open-water period.

Four core samplers, each with a cross-sectional area of 53.52 cm² and handles that were 1, 2, 3.5, or 7 m, were used to extract substrate cores containing aquatic oligochaetes at any depth up to 7 m. Substrate cores varied in thickness from 5 to 20 cm depending upon the softness of the substrate. Each sample was placed into a benthic invertebrate sampling kick net (250-µm-mesh screen), rinsed liberally with water from the lake to flush unneeded sediment and fine organic debris from the sample, and then placed into a 3.9-L plastic bucket filled with filtered lake water for transport to the laboratory. One core sample was collected per month from each of the 38 1-ha sampling units. Sampling took place in May, June, July, September, and October.

**Laboratory Protocols**

*Triactinomyxon estimation.*—After collection, all samples were allowed to stand for 24 h at approximately 15°C. The water from each bucket was then filtered through a 20-µm-mesh screen to capture and concentrate any *Mc* TAMs released by *T. tubifex* worms. The total volume (nearest mL) of filtrate concentrate was recorded. Ten 1-mL samples were drawn from the filtrate, and 60 µL of a saturated aqueous solution of crystal violet biological stain were added to each sample to stain all biological material in the sample. An 80-µL aliquot was drawn from each 1-mL sample with a micropipette, placed under a microscope cover slip on a gridded petri dish (2 mm/gridded square), and examined by stereozoom microscopy to identify and enumerate TAM spores. The results were standardized and reported as TAMs·m⁻²·d⁻¹ to facilitate a comparison with the results from a similar study conducted in 1998 (Nehring et al. 2003).

During the 1998 and 2005 studies, parallel water samples equivalent in volume to the filtrate screened for *Mc* TAMs were drawn and preserved for PCR analysis for detection of *Mc* DNA to confirm that the TAMs observed during microscopic screening were indeed *Mc*. During the 1998 study, the PCR screening methodology developed by Andree et al. (1998) (which utilizes a segment of the 18S rDNA gene of *Mc*) was used to assess the accuracy and precision of the microscope screening process. During the 2005 study, the qPCR technique utilizing the heat shock protein (Hsp 70) gene was used to test for the presence of *Mc* DNA (Cavender et al. 2004). Over the course of 7 years (1997–2004), more than 4,000 samples drawn from concentrated filtrates of 1,893-L water samples were tested for the presence of *Mc* DNA. The 18S test was used on 2,662 samples between April 1997 and March 2001. The Hsp 70 test was used on 1,498 samples between April 2001 and June 2003. In these exercises, the Hsp70 test was shown to have higher precision and accuracy as well as greater ability to detect low levels of *Mc* TAMs (Nehring and Thompson 2003).

Over the course of the study, five other actinospores were occasionally observed in the screened filtrates. However, all were easily distinguished from *Mc* TAM spores by clear differences in overall size, shape, and conformation of the caudal processes. The three caudal processes of *Mc* TAMs are curvilinear and average about 200 µm in length (range, 150–220 µm), as reported by El-Matbouli and Hoffmann (1998). Two of the five actinospores were similar in overall conformation to *Mc* TAMs, possessing a style and three caudal processes. However, the caudal processes of the smaller non-*Mc* TAMs were more robust, not curvilinear, and less than 100 µm in length. Moreover, the sporoplasm-enveloping cell of the smaller TAM is larger in diameter than the style, giving it an oblong, bulb-like appearance. The caudal processes of the larger non-*Mc* TAMs were 400–450 µm in length—almost twice as long as those of the *Mc* TAMs—and slightly curvilinear. Three other actinospores had the conformation(s) of *Raabeia*, *Echinactinomyxon*, and *Hexactinomyxon* spp., as illustrated in Kent et al. (2000), and could not be confused with TAMs of the *Mc* parasite. When any of these actinospores were observed in water samples screened by stereozoom microscopy, those 80-µL aliquots were preserved in 70% ethanol and submitted for PCR testing (1998) or Hsp 70 qPCR screening (2005). In cases in which there were no *Mc* TAMs concurrently present in the sample, all 18S PCR and Hsp 70 qPCR tests were negative for *Mc* DNA.
Oligochaete estimation.—All aquatic oligochaetes were picked from every substrate sample and examined by stereozoom microscopy for haired and pectinate chaetae. Hair and pectinate chaetae on tubificid worms are generally reliable phe-

Table 4. The qPCR reaction components for estimating the proportion and quantity of mitochondrial 16S rDNA sequence copies from each of four lineages (I, III, V, and VI) of T. tubifex present in a sample containing 1–50 oligochaetes. The thermal cycling parameters were as follows: 1 cycle at 95°C for 9 min; 45 cycles at 94°C for 30 s; and 45 cycles at 57°C for 30 s. Abbreviations are as follows: 3BHQ-2 = Black Hole Quencher-2 (Integrated DNA Technologies); Cy5 = the nonsystematic name of a cyanine dye belonging to the polymethine group; 6FAM = 6-carboxyfluorescein; HEX = hexachloro-fluorescein; MGBNFQ = Minor, Grove Binder, Nonfluorescent Quencher (Applied Biosystems); TxRd = sulforhodamine 101 acid chloride; and na = not applicable.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl2</td>
<td>6 mM</td>
<td>na</td>
</tr>
<tr>
<td>dNTP</td>
<td>1.6 mM</td>
<td>na</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 µM</td>
<td>5′-CAGGACAAGAGACCTATAGAG-3′</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 µM</td>
<td>5′-TTATCTCAAGGTGCTTGATC-3′</td>
</tr>
<tr>
<td>Lineage I probe</td>
<td>1 µM</td>
<td>5′-Cy5-CCCTAATAACTTAGACCACCGGTCAATTA-3BHQ-2-3′</td>
</tr>
<tr>
<td>Lineage III probe</td>
<td>1 µM</td>
<td>5′-HEX-CATTACCTCACCACAAAACGATATTAC-3BHQ-2-3′</td>
</tr>
<tr>
<td>Lineage V probe</td>
<td>1 µM</td>
<td>5′-6FAM-CAACTAATCTCATAG-MGBNFQ-3′</td>
</tr>
<tr>
<td>Lineage VI probe</td>
<td>1 µM</td>
<td>5′-TxRd-TCACCCTTAAATTATAGACACCGTC-3BHQ-2-3′</td>
</tr>
<tr>
<td>AmpliTaq Gold</td>
<td>4 U</td>
<td>na</td>
</tr>
<tr>
<td>10× Core buffer II (without Mg)</td>
<td>2 µL</td>
<td>na</td>
</tr>
<tr>
<td>H2O</td>
<td>2.4 µL</td>
<td>na</td>
</tr>
<tr>
<td>Sample DNA</td>
<td>2 µL</td>
<td>na</td>
</tr>
</tbody>
</table>

Lineage qPCR assay.—All preserved worm samples were analyzed by qPCR to determine the proportions and quantities of the four different T. tubifex lineages (I, III, V, and VI) present in each sample, as previously determined to be present in North America and in WGR (Beauchamp et al. 2001, 2002, 2005). DNA extractions were carried out on all samples using Qia-

gen DNeasy or DNeasy 96 spin columns kits, according to the manufacturer’s instructions (mouse tail protocol). DNA extracts were treated with Gene Releaser (BioVentures, Inc., Murfreesboro, Tennessee) to remove PCR inhibitors, according to the manufac-
turer’s instructions. The qPCR assay was developed as a single, multiplexed, and quantitative extension to the four in-
dividual lineage-specific PCR assays developed by Beauchamp et al. (2001, 2002). The qPCR assay used a single pair of primers to amplify a 166-bp fragment of the mitochondrial 16S rDNA in all four lineages, which were then distinguished and quantified using four different, lineage-specific fluorescently labeled Taq-
Man probes. The primer and probe oligonucleotide sequences as well as the qPCR reaction components and thermal cycling parameters are shown in Table 4.

All qPCR reactions were run singly on a Stratagene MX4000 real-time PCR instrument using filter sets for FAM (401270), HEX (401273), TxRd (401272) and Cy5 (401276). Baseline fluorescence was set by the instrument software (MX4000 version 4.20) using the default “adaptive baseline, analysis term settings” option. Each qPCR run included previously quantified positive qPCR standards consisting of a 10-fold serial dilution series of four quantified, linearized plasmid DNAs, each containing the 166-bp 16S rDNA fragment from one of the four T. tubifex lineages cloned into the pDrive cloning vector (Qiagen) mixed in equimolar ratios, and ranging from 5 molecules/µL to 5 × 107 molecules/µL. Each qPCR run also included a reaction with deionized water added instead of template DNA as a negative control. Calculated reaction efficiencies for each of the four lineage plasmid DNAs (mixed) were typically ≥ 95% and highly linear (R² ≥ 0.990) across the entire range of plasmid DNA di-
lutions (5 molecules/reaction to 5 × 107 molecules/reaction).
tested. All qPCR runs for which the reaction efficiency for any of the four lineage plasmid DNAs was lower than 85% were discarded or repeated. The qPCR reaction was tested for cross-reactivity using samples of other closely related, meristically identified oligochaetes. No cross-reactivity was observed (i.e., there was no amplification) for DNA extracted from samples of *Limnodrilus hoffmeisteri*, *L. claparedeianus*, and *Hyodrilus templetoni*, the three additional tubificid species known to occur in WGR (Zendt and Bergersen 2000).

Aliquots of DNA from single-worm samples of each of the four lineages were mixed in different ratios to determine the minimum sensitivity for detection of a minority-lineage DNA in the presence of an excess of a DNA from a different lineage. The results indicated that the minority-lineage DNA was still detectible (log linear increase in Δ fluorescence) at quantification thresholds (Cq; Bustin et al. 2009) up to 43 cycles in the presence of a 50-fold excess of majority-lineage DNA for all lineage combinations (data not shown). Therefore, DNA extraction on field-collected samples was limited to 50 worms or less total. Due to the wide variation in total extracted DNA concentrations from field samples (and thus in the Cq values observed for the DNA from different lineages), rather than using an arbitrary Cq cutoff value (beyond which a sample was scored as negative for a lineage), samples with Cq values as high as 43 were scored as positive if they exhibited a log-linear increase in fluorescence between at least three successive cycles and the result could be repeated in a second, independent qPCR reaction. Quantified copy number values for all positive samples were calculated from their Cq values using the standard curve developed from the Cq values and the known target copy numbers of the linearized plasmid dilution series positive controls included in each qPCR run, eliminating any intersample or interplate variation.

The relationship between the qPCR results (molecules of the rDNA target sequence for each of the four lineages of *T. tubifex*) and the number of worms in a sample was determined by qPCR testing of dozens of haired worm samples of varying sizes and numbers of worms (ranging from 1 to 100 per aliquot) prior to beginning field collections. The number of rDNA molecules of each lineage detected in these samples varied widely with the sizes and number of worms in the sample. We calculated, however, an average of 2.10 × 10^9 rDNA molecules per worm across all four lineages, so this value was defined as the average number of rDNA molecules per “worm equivalent” and applied to the results of the qPCR tests from the field collections to compute the average “*T. tubifex* equivalent” for each lineage in each sample. Each worm sample was also screened for the presence of *M. tam* DNA using the Hsp70 qPCR test described by Cavender et al. (2004). Estimates of the prevalence of *M. tam* infection in WGR were made for each month of sampling in 2005 as well as the season as a whole by applying the same mathematical formula used by Zendt and Bergersen (2000), as outlined in Boswell and Patil (1987). The estimates for 2005 were then compared with the estimates from the 1998 study (Zendt and Bergersen, 2000).

## RESULTS

### Temporal and Spatial Dynamics of Mc TAM Production

The temporal relationship between the estimated densities of *M. tam* actinospores (TAMs/L) observed in the effluent of WGR and the mean daily discharge (m^3/s) from April 1997 through June 2006 is shown in Figure 2A. The production of *M. tam* was positively correlated with peak spring discharge levels for the first 6 years of the study (1997 through 2002); however, that correlation did not continue during the last 3.5 years of the study. This was most obvious during 2003, when the peak in mean daily discharge was similar to that occurring in 1999 and 2000 yet *M. tam* density remained near all-time-low levels (Table 1). The estimated densities of *M. tam* actinospores (TAMs/L) versus mean daily water temperature (°C) in the Colorado River at the outfall of WGR from April 1997 through June 2006 are shown in Figure 2B. Over the course of the study, there was little variation in the annual thermal regime of the Colorado River at the outlet of WGR despite the large decline in *M. tam* density that began in 2001 and continued through June 2006 (Table 1; Figure 2B).

The spatial distributions of estimated TAM production (TAMs·m^−2·d^−1) in WGR for 1998 and 2005 are shown in Figure 3A and 3B, respectively. During the 2005 study, *M. tam* were detected in 17.9% (34 of 190) of the concentrated filtrate water samples, compared with 82.5% (160 of 194) of the samples in 1998 (Nehring et al. 2003). In 1998, estimated TAM production exceeded 1,000,000·m^−2·d^−1 at 15 sampling sites, compared with only 1 site in 2005. Estimated TAM production exceeded 500,000·m^−2·d^−1 but was no more than 1,000,000·m^−2·d^−1 at 12 sampling sites in 1998, compared with 2 sites in 2005.

The aliquots of sample microscopically examined for actinospores in the two studies (2,100 in 1998 and 1,890 in 2005) were similar, but the number of *M. tam* observed and enumerated was not. During the 1998 study, 15,361 *M. tam* were enumerated, compared with 656 during 2005.

### Temporal and Spatial Dynamics of Non-Mc TAM Production

Non-*M. tam* actinospores were rarely observed during the microscopic screening of water samples. We observed and enumerated non-*M. tam* actinospores in 32 of 2,100 sample aliquots during the 1998 study, compared with 20 of 1,890 during the 2005 study, resulting in occurrence rates of 1.5% and 1.1%, respectively. In 1998, 520 non-*M. tam* actinospores were observed, compared with 15,361 *M. tam* TAMS. During the 2005 study, 293 non-*M. tam* actinospores were enumerated, compared with 656 *M. tam* TAMS. It is noteworthy that 191 of the 520 non-*M. tam* actinospores observed during the 1998 study came from one core sample, while 200 of the 293 non-*M. tam* actinospores seen in 2005 also came from a single core sample. Among the five types of non-*M. tam* actinospores observed during the two studies, the *Hexactinomyxon* form with
FIGURE 3. Spatial distributions of estimated triactinomyxon production (number $\cdot m^{-2} \cdot d^{-1}$) by *Tubifex tubifex* in Windy Gap Reservoir in May, June, July, September, and October of (A) 1998 and (B) 2005.
six caudal processes was the most abundant in 2005, accounting for 215 of the 293 non-Mc spores. More than 99% of the non-Mc actinospores enumerated were observed during June and July 2005. In contrast, a very large triactinomyxon-form TAM (approximately twice as large as Mc TAMs) accounted for 411 of the 520 non-Mc actinospores observed during the 1998 study, and the majority (408) were seen during September and October. A small triactinomyxon actinospore (approximately 50% smaller than an Mc TAM) as well as Raabeia- and Echinactinomyxon-form actinospores comprised the remainder of the non-Mc actinospores observed in the two studies (Kent et al. 2000). In those cases in which an 80-µL aliquot was screened by microscopy and there were no Mc TAMs present in the sample, the aliquot was preserved in 70% ethanol for analysis with the 18S PCR and Hsp 70 qPCR tests. The results were all negative for Mc DNA. No further PCR screening of the non-Mc spores was done because of the extra cost and time involved and the fact that it was beyond the scope of the study.

Spatial Distribution and Abundance of T. tubifex Lineages

Over the course of the 2005 study, more than 133,000 aquatic oligochaetes were separated from the sediment and detritus in the 190 core samples, an average of 697 worms per sample. There were no samples devoid of worms; however, the number per core varied from 3 to 7,087, suggesting a very patchy distribution. Among the haired worm subsamples preserved for screening with the lineage qPCR assay, lineage III T. tubifex DNA was detected in 141 of the 190 core samples, indicating that this Mc-susceptible tubificid is widely distributed in WGR. DNA specific for lineage VI T. tubifex was detected in the haired worm subsamples from all 190 core samples, indicating that this tubificid is distributed throughout the lake.

Mc TAMs were detected in the topwater filtrate(s) from 34 of the 190 core samples. Among those 34 samples, DNA for the Mc-susceptible lineage III T. tubifex was detected in 28 of the haired worm subsamples screened by the lineage qPCR assay. Among the haired worm subsamples preserved for lineage qPCR assay from the core samples, Mc DNA was detected in 67 of the 190 samples. Lineage III T. tubifex DNA was detected in 47 of those 67 samples. These results demonstrate a high level of congruence between the presence of lineage III T. tubifex DNA and Mc DNA (indicating the presence of Mc in the sample). However, perfect agreement would not be expected given that a maximum of 100 haired worms were preserved for screening in any core sample while the average number of oligochaetes/core sample was 697 (and more than 7,000 in one sample).

In 8 of the 190 core samples, only lineage VI DNA was detected in the haired-worm subsamples preserved for screening with the lineage qPCR assay. Among those eight samples, a single TAM was detected in the topwater filtrate from one sample and six TAMs were detected in another. However, the total numbers of haired oligochaetes in those two core samples were 978 and more than 1,200, making it highly probable that there were lineage III Mc TAM–producing tubificids among the large number of unscreened worms.

The Cq values and calculated T. tubifex worm equivalents for various sample sizes among the four lineages of worms from the 2005 study are summarized in Table 5. Among the samples with 50 T. tubifex worms, the average total T. tubifex equivalents ranged from 31.4 to 80.6 across the four lineages. The calculated average T. tubifex equivalents (and associated standard errors) compare well with the actual mean number of

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>T. tubifex/sample</th>
<th>Cq values</th>
<th>Total T. tubifex equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>SE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Samples with 100% lineage I DNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>17.1</td>
<td>0.173</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>22.0</td>
<td>1.50</td>
</tr>
<tr>
<td><strong>Samples with 100% lineage III DNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>18.5</td>
<td>0.142</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>21.3</td>
<td>0.314</td>
</tr>
<tr>
<td>20</td>
<td>2–10</td>
<td>21.7</td>
<td>0.660</td>
</tr>
<tr>
<td><strong>Samples with 100% lineage V DNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>19.4</td>
<td>0.178</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>21.1</td>
<td>0.502</td>
</tr>
<tr>
<td><strong>Samples with 100% lineage VI DNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>18.6</td>
<td>0.251</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>21.7</td>
<td>0.362</td>
</tr>
</tbody>
</table>

aThe actual mean number of worms in the 20 samples was 4.70 and the actual SE was 0.637.
T. tubifex worms (and associated standard error) for lineage III worms footnoted in Table 5. Among the 325 core samples for which the first 100 haired oligochaetes were preserved for qPCR testing, the average T. tubifex equivalents was 66.4 among the 50 haired T. tubifex worm aliquots analyzed, which is congruent with the probability that the largest worms are more visible and tend to be selectively separated and preserved at a higher rate. In contrast, among the 41 core samples for which the desired number of haired T. tubifex worms was not attained and all haired worms were preserved and analyzed for lineage determination, the average number of worms per test tube was 23.2 and the calculated average T. tubifex equivalents was 18.9, which is also congruent with the hypothesis that the larger worms tended to be separated and preserved first when large numbers of worms were present in the sample.

The Cq in the qPCR reactions almost always occurred between the 16th and 25th thermal cycles for all of the four lineages being tested, even when a sample contained only a single T. tubifex oligochaete. This was also the case for the dominant lineage among the samples containing DNA for two to four lineages. Among samples containing DNA for two or more lineages, the Cq values for the subdominant lineages could be 30 or more. However, when the number of molecular target copies for the dominant-lineage DNA in the sample was at least two orders of magnitude greater than that of the subdominant DNA, the T. tubifex worm equivalents for the subdominant oligochaete(s) was ≤ 1% of the total T. tubifex equivalents. Estimates for total T. tubifex equivalents among samples containing 50 T. tubifex oligochaetes varied from 2 to more than 250, depending upon the relative sizes of the worms included in the sample.

Spatial representations of the core sampling sites together with approximations of the ranges of the estimated densities (m²) of lineage I, III, V, and VI T. tubifex at the different sites are shown for 2005 in Figures 4A–D, respectively. The estimated densities of all oligochaetes, haired tubificids, and the four lineages of T. tubifex are shown in Table 6. Application of the four-probe multiplex qPCR assay to the haired worm samples collected during 1998, 2004, and 2005 allowed the parsing of the data into estimates of abundance for each of the four lineages of T. tubifex in WGR. During a separate study in September 2001, four substrate core samples (53.5 cm²/core in cross-sectional area) were collected at 10 sites distributed across WGR (Beauchamp et al. 2005). All haired oligochaetes were separated from the core samples for the 10 sites, and up to 50 T. tubifex per sample site were randomly selected for individual lineage typing according to the PCR protocols described by Beauchamp et al. (2001, 2002). Taken together, these data suggest that the oligochaete population density doubled between 1998 and 2005. The data also suggest that the densities of T. tubifex belonging to the resistant lineages (I, V, and VI) increased substantially over the same period. In contrast, the estimated density of the Mc-susceptible lineage III oligochaetes was much the same in 2005 and 1998. However, the relative abundance of this lineage declined from 39–45% of the total in 1998 and 2001 to 7–14.2% in 2004–2005.

The scale of the temporal and spatial core sampling effort in WGR during 2005 was much greater than that of the studies in 1998 (Zendt and Bergersen 2000), 2000, 2001 (Beauchamp et al. 2001, 2002, 2005), and 2004. For that reason, rigorous statistical analysis for differences in the mean density of T. tubifex between years is not warranted. Linear regression analysis of the 2005 data revealed that the estimated abundance of lineage III T. tubifex was significantly correlated with that of both lineage VI T. tubifex and total T. tubifex (P < 0.0001). Similarly, the estimated Mc TAM density (TAMs · m⁻² · d⁻¹) was also significantly correlated with the estimated density of lineage III and total T. tubifex for the 34 core samples for which TAMs were detected in the water filtrates (P < 0.0001).

The ambient levels of Mc infection among T. tubifex in WGR in 2005 were significantly lower than those observed in 1998, as the lower confidence limits for the 1998 estimates do not overlap the upper confidence limits for the monthly estimates in 2005 or for the average prevalence between the years (Tables 7, 8).

**DISCUSSION**

It is unclear whether the absolute abundance of the lineage III worms in WGR changed between 1998 and 2005. The problem

**TABLE 6.** Estimated relative abundances of aquatic oligochaetes, haired tubificids (see text), and Tubifex tubifex oligochaetes by lineage in Windy Gap Reservoir in 1998, 2001, 2004, and 2005. The estimates for 1998, 2004, and 2005 are standardized as number per square meter, while the data for 2001 are the actual numbers of worms individually typed for lineage determination.

<table>
<thead>
<tr>
<th>Year</th>
<th>Oligochaetes/m²</th>
<th>Core samples</th>
<th>Estimated densities by lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Haired</td>
<td></td>
</tr>
<tr>
<td>1998</td>
<td>45,537</td>
<td>34,702</td>
<td>1,012</td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td>379</td>
<td>40</td>
</tr>
<tr>
<td>2004</td>
<td>60,173</td>
<td>42,982</td>
<td>25</td>
</tr>
<tr>
<td>2005</td>
<td>97,373</td>
<td>88,657</td>
<td>190</td>
</tr>
</tbody>
</table>

**Notes:**

- a The actual number of worms that were microscopically screened and identified as T. tubifex, not the actual number of core samples.
- b No data for total oligochaetes.
- c The data for 2001 represent the numbers of haired oligochaetes randomly selected and individually typed for lineage determination; random selection was from 40 substrate core samples (53.5 cm²/core) collected from 10 sites in Windy Gap Reservoir during September, as shown in Table III and Figure 1 in Beauchamp et al. (2005).
is that the data for 1998 (and 2004) were derived from samples collected along a north–south transect across the middle of the reservoir and are not temporally or spatially comparable to the sampling protocol used in 2005. In 2005, the 190 core samples were collected at random from thirty-eight 1-ha sampling units covering the entire wetted surface of the lake. Because of this stratified random sampling design repeated across 5 months, the estimated densities of the various lineages of *T. tubifex* should

![Figure 4](image-url)

**FIGURE 4.** Spatial distributions of estimated densities (number/m$^2$) of (A)–(D) *Tubifex tubifex* lineages I, III, V, and VI in Windy Gap Reservoir in May, June, July, September, and October 2005.

<table>
<thead>
<tr>
<th>Year</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Sep</th>
<th>Oct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>0.0269 ± 0.0099</td>
<td>0.0118 ± 0.0040</td>
<td>0.0078 ± 0.0032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>0.0030 ± 0.0021</td>
<td>0.0067 ± 0.0030</td>
<td>0.0089 ± 0.0035</td>
<td>0.0013 ± 0.0011</td>
<td>0.0096 ± 0.0036</td>
</tr>
</tbody>
</table>

*These samples were collected during August 1998.
more accurately reflect the true relative abundances and distributions of the various lineages of *T. tubifex* throughout the entire reservoir in 2005 than the densities obtained for 1998 and 2004. The estimated abundance of lineages I, V, and VI of *T. tubifex* in WGR appear to have increased substantially in 2005 compared with 1998 (Table 6). These differences, however, may simply be an artifact of the much greater sampling intensity in 2005.

In 2005, the average seasonal abundance of lineage III worms was 14.2% of the combined abundance of the resistant *T. tubifex* lineages, ranging from 6.1% to 18.6% during the 5-month sampling period (Table 6). In 1998, the average seasonal abundance of the *Mc*-susceptible lineage III *T. tubifex* was 39.1% of the combined abundance of the resistant lineages. In September 2001, the abundance of the lineage III worms was estimated at 45.2% (Beauchamp et al. 2005). The estimates of the prevalence of *Mc* infection were 2.69% and 1.18% for May and June 1998, respectively, compared with 0.30% and 0.67% for the same months in 2005. The average prevalence of infection was 1.23% for 1998, compared with 0.53% in 2005 (Table 8). Zedt (1999) reported that the prevalence of infection among *T. tubifex* in the area of the old river channel in WGR in 1998 was significantly higher than that in *T. tubifex* collected from the nonchannel areas of the lake. Those results are consistent with the findings of a parallel study conducted in 1998 (Nehring et al. 2003) that demonstrated that the area of WGR receiving freshwater inflow from the Colorado and Fraser rivers was the zone of the lake where TAM production was extremely high (Figure 3A). While the primary zone of TAM production observed between May and October 2005 was still in the area of the lake receiving freshwater inflow, it was substantially reduced (both temporally and spatially) compared with 1998 (Figure 3B).

All of the foregoing supports the conclusion that the decline in ambient levels of TAM production that began in 2001 and continued through June 2006 (Figure 2) is real. In 2005, lineages of *T. tubifex* that were not susceptible to the *Mc* parasite comprised an estimated 86% of the population, while the estimated relative abundance of TAM-producing lineage III worms was as low as 6.1% during 1 month and was never higher than 18.6% for the 5-month sampling period. In 2005, *T. tubifex* lineages I, V, and VI (which have high resistance to *Mc* infection) dominated the oligochaete community in the portion of WGR where TAM production was highest in 1998 (Figure 4A–D).

Up until 2003, laboratory research studies had suggested that only lineage V *T. tubifex* oligochaetes were completely refractory for *Mc* infection but that there were differences in susceptibility among lineages I, III, and VI (Beauchamp et al. 2001, 2002). Subsequent research on the San Juan River in New Mexico revealed that of these three lineages only lineage III *T. tubifex* were infected by *Mc* (DuBey and Caldwell 2004). DuBey et al. (2005) demonstrated that lineage VI *T. tubifex* did not become infected after exposure to 500 *Mc* myxospores in a laboratory experiment at temperatures of 5, 17, and 27°C. Similarly, Arsan et al. (2007) did not detect infection among *T. tubifex* lineages I, IV, and VI from Alaska exposed to 500 *Mc* myxospores per worm under laboratory conditions. The same was true for similarly exposed lineage I, V, and VII *T. tubifex* from Oregon (Hallett et al. 2009). However, the results of laboratory exposures of lineage I *T. tubifex* from the Gallatin River in Montana to *Mc* myxospores were mixed. In two experiments, the lineage I worms released very low numbers of TAMs (Kerans et al. 2004; Rasmussen et al. 2008) while no TAMs were detected in a third experiment (Kerans et al. 2005).

Laboratory studies have often shown that *Mc* infection of susceptible lineage III *T. tubifex* can have negative bioenergetic consequences, resulting in poorer growth rates, decreased reproductive success, reduced fitness, and poorer survival than among uninfected lineage III worms or nonsusceptible lineage VI worms (Beauchamp et al. 2001, 2002; Stevens et al. 2001; Kerans et al. 2004; DuBey et al. 2005; Rasmussen et al. 2008; Shirakashi and El-Matbouli 2009; Zielinski et al. 2011). These studies suggest that exposure of lineage III *T. tubifex* to infection by *Mc* could negatively affect worms of this lineage in the natural environment and give a competitive advantage to the resistant lineages. In our 2005 WGR study, however, the estimated densities of *Mc*-susceptible lineage III *T. tubifex* were positively correlated with the estimated total densities of the resistant lineage I, V, and VI *T. tubifex*. Moreover, the estimated population densities of the lineage III *T. tubifex* in WGR did not appear to change appreciably between the periods of high and low TAM production in 1998 and 2005—findings that are not necessarily congruent with the results of some of the laboratory studies.

There are plausible explanations for the seeming incongruence. First, the infection prevalence among lineage III worms in WGR in both 1998 and 2005 may have been low enough that the bioenergetic cost of *Mc* infection was below the population-level

### Table 8. Yearly summary of estimated prevalence of *Myxobolus cerebralis* infection among *Tubifex tubifex* worms collected from Windy Gap Reservoir in 1998 and 2005 (also see Table 7).

<table>
<thead>
<tr>
<th>Year</th>
<th>Positive samples</th>
<th>Total samples</th>
<th>Worms sample</th>
<th>Prevalence</th>
<th>Variance</th>
<th>SE</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>88</td>
<td>489</td>
<td>16</td>
<td>0.0123</td>
<td>1.71 × 10⁻⁶</td>
<td>0.001308</td>
<td>± 0.0026</td>
</tr>
<tr>
<td>2005</td>
<td>92</td>
<td>393</td>
<td>50</td>
<td>0.0053</td>
<td>3.07791 × 10⁻⁷</td>
<td>0.000555</td>
<td>± 0.0011</td>
</tr>
<tr>
<td>2005</td>
<td>54</td>
<td>199</td>
<td>50</td>
<td>0.0063</td>
<td>7.39151 × 10⁻⁷</td>
<td>0.000860</td>
<td>± 0.0017</td>
</tr>
</tbody>
</table>

*These data are for May, June, and July 2005 to allow for comparison with the 1998 samples collected in May, June, and August.*
impact threshold for the lineage III worms. Second, habitat quality (at the microhabitat level) for individual colonies of worms at WGR may be of such overwhelming importance that it would mask any negative bioenergetic impacts for Mc-infected lineage III worms. The lake bottom is dominated by black mud that is constantly being enriched by the excrement of the hundreds of waterfowl that are continuously present at the lake during the 7-month open-water period. Month- and site-specific density estimates for all haired T. tubifex worms in WGR varied by more than four orders of magnitude in 2005, suggesting a very patchy distribution that may (in part) be due to localized differences in habitat quality. Laboratory studies have demonstrated that TAM production among Mc-infected lineage III T. tubifex was greater among worms held in mud or silt substrates than those held in sand (Arndt et al. 2002; Blazer et al. 2003; D. Baxa and R. P. Hedrick, University of California–Davis, personal communication). It is hypothesized that the bacterial fauna occurring in the organically enriched black mud that is often present in eutrophic lacustrine environments plays a critical role in maintaining healthy oligochaete populations that in turn may enhance TAM production (Wavre and Brinkhurst 1971; Blazer et al. 2003). Soft, enriched black muck characterizes most of the bottom of WGR. In high-quality microhabitats with higher densities of lineage I, V, and VI oligochaetes, resistant T. tubifex might consume and deactivate greater numbers of Mc myxospores, thereby functioning as biofiltering organisms.

Among the 394 individual samples containing haired oligochaetes in our 2005 study, lineage III DNA was detected in 64% of the samples containing DNA of one or more of the highly resistant lineages, indicating a high degree of sympatry among the Mc-susceptible and resistant lineages. Milbrink (1993) suggested that mutualism occurs among mixed cultures of oligochaetes existing in sympathy because they ingest more, respire less, and assimilate and grow more rapidly than the same species living in allopatry. Thus, lineage III T. tubifex living in sympathy with a tubificid population dominated by Mc-resistant oligochaetes should benefit from the reduced probability of encountering Mc myxospores, thus being able to feed, reproduce, and thrive in a microhabitat where the threat of Mc exposure has been down-regulated. The extensive sampling of WGR conducted in 2005 demonstrated that lineage III worms comprised approximately 14% of the oligochaete population (Table 6). The resistant lineages I, V, and VI comprised approximately 78% of the worm community. The remaining 9% of the oligochaete population was comprised of related species, primarily Limnodrilus hoffmeisteri, L. claparedieanus, and Hyodrilus templetoni (Zendt and Bergersen 2000), which are not compatible hosts for the Mc parasite (Kerans et al. 2004).

In laboratory trials, Beauchamp et al. (2006) found that TAM production was reduced 70% among mixed cultures of Mc-susceptible and -resistant worms compared with pure cultures of susceptible worms. Moreover, at the end of the cohabitation experiments the resistant T. tubifex (lineage V) worms were dominant, suggesting that resistant worms have a competitive advantage over Mc-susceptible worms in habitats where the parasite is highly enzootic. In WGR, an inverse relationship between the relative densities of lineage III T. tubifex and lineages I, V, and VI would be expected if there was overt competition between the susceptible and resistant T. tubifex lineages. But there is no evidence of a decline in lineage III worms. The estimated abundance of lineage III T. tubifex was positively correlated with that of both lineage VI and total T. tubifex, results that are congruent with the concept of mutualism suggested by Milbrink (1993).

Exposure of T. tubifex infected with the Mc parasite to water temperatures of 25°C or more for 3 d under laboratory conditions caused the infected worms to purge the infection, whereupon they remained parasite-free until they were reexposed to Mc myxospores at lower water temperatures (El-Matbouli et al. 1999). Blazer et al. (2003) observed significant TAM production among eastern T. tubifex held at temperatures of 9, 13, and 17°C and exposed to Mc myxospores but not among exposed worms held at 20°C. Water temperatures in WGR were monitored continuously from April 1997 to June 2006 (Figure 2B). Instantaneous water temperatures never reached 20°C during that 9-year period. The slight increases in water temperature observed in WGR during the severe drought years of 2001 and 2002 are an unlikely explanation for the decline in TAM production that began in 2001. That is especially true in light of the fact that the estimated abundance of Mc-susceptible lineage III T. tubifex did not change between 1998 and 2005.

Numerous studies have repeatedly demonstrated that TAM production capability can vary widely among mitochondrial 16S lineage III T. tubifex populations from different geographic regions (Stevens et al. 2001; Kerans et al. 2004; Arsan et al. 2007; Rasmussen et al. 2008; Hallett et al. 2009; Zielinski et al. 2011). To our knowledge, Baxa et al. (2008) is the only study to demonstrate that there was sufficient genetic variation within a population of TAM-producing lineage III worms to enable clonal lines of both TAM-producing and non-TAM-producing phenotypes to be derived. Although the presence of parasite stages among the Mc-exposed, non-TAM-producing phenotype was documented at the cellular level by both hematoxylin and eosin histology and in situ hybridization (Antonio et al. 1998), no release of TAMs was ever demonstrated, even at myxospore doses of 5,000 and 10,000/worm. Therefore, it is conceivable that the decline in TAM production in WGR observed in 2005 is the result of a shift in the population structure of lineage III T. tubifex towards a non-TAM-producing phenotype. Whether or not this is the case is unknown. However, the estimated Mc infection prevalence among T. tubifex in WGR during 1998 ranged between 0.78% and 2.69% (Zendt and Bergersen 2000) and never exceeded 0.96% in 2005 (Table 7). Rognlie and Knapp (1998) report that Mc infection prevalence among susceptible T. tubifex in aquatic ecosystems is typically less than 10%. At these low levels of parasite contact, selection pressure towards rapid development of host resistance would not be likely (Minchella 1985).
It is evident that the Mc-resistant *T. tubifex* lineages I, V, and VI were the dominant oligochaetes in WGR in 2005, together comprising approximately 86% of the haired oligochaete population. The sevenfold advantage in density among these worms suggests that the probability of a lineage III worm ingesting an Mc myxospore in WGR was much lower in 2005 than it was in 1998. Our results are congruent with the findings and conclusions of others (Kerans et al. 2004; Beauchamp et al. 2006; Baxa et al. 2008), suggesting that colonization of aquatic habitats where Mc is enzootic by lineage I, V, and VI *T. tubifex* could down-regulate the ambient levels of infection and reduce its impacts on wild trout populations. Whether or not this is the explanation for the decline in TAM production in WGR remains an enigma. The ability of lineage I, V, and VI *T. tubifex* to consume and deactivate Mc myxospores and thereby function as biological filters requires further study. It is noteworthy, however, that the lineage qPCR assay proved to be an efficient method for processing large numbers of worms to assess the spatial and temporal density, diversity, and distribution of *T. tubifex* in WGR. We believe this methodology can be applied wherever a deeper understanding of the role of tubificid worms in both lentic and lotic aquatic ecosystems is needed.

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