

## Effectiveness of Aquaflor (50% Florfenicol) to Control Mortality Associated with *Streptococcus iniae* in Freshwater-Reared Subadult Sunshine Bass

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**Abstract.**—We conducted a field trial to evaluate the effectiveness of Aquaflor (50% florfenicol) for controlling mortality associated with *Streptococcus iniae* in freshwater-reared subadult sunshine bass (female white bass *Morone chrysops* × male striped bass *M. saxatilis*). Bacterial samples collected from moribund fish representing a reference population were presumptively identified microbiologically and were later confirmed to be *S. iniae* by biochemical characterization and polymerase chain reaction. The trial comprised a 1-d acclimation period, 10-d treatment period, and 14-d posttreatment period. During the treatment period, Aquaflor-medicated feed was administered to treated tanks ( $N = 3$ ) at a target dose of 10 mg of florfenicol·kg of fish<sup>-1</sup>·d<sup>-1</sup>, and nonmedicated feed was administered to control tanks ( $N = 3$ ). At the end of the posttreatment period, mean ( $\pm$ SD) cumulative mortality in treated tanks ( $9 \pm 11\%$ ) was significantly ( $P = 0.040$ ) less than that in control tanks ( $52 \pm 13\%$ ). Analysis of medicated feed samples revealed that treated tanks had received an actual dose of 8.3 mg florfenicol·kg fish<sup>-1</sup>·d<sup>-1</sup> (83% of target). No florfenicol was detected in control feed samples. Although the actual florfenicol dose administered to treated tanks was less than the target dose, the trial was accepted by the U.S. Food and Drug Administration Center for Veterinary Medicine as demonstrating the efficacy of Aquaflor to control mortality associated with *S. iniae* in cultured sunshine bass populations.

Intensification of both freshwater and marine aquaculture has led to the emergence of several bacterial diseases that have a broad host range. Well-known examples include diseases caused by the Gram-negative pathogens *Piscirickettsia salmonis*, *Aeromonas salmonicida*, *Flavobacterium columnare*, and *F. psychrophilum* and the Gram-positive pathogens *Mycobacterium* spp. and *Streptococcus iniae* (Winton 2001; AFS-FHS 2007). Consequently, health management goals for cultured fish populations usually include preventing disease and minimizing adverse effects of disease outbreaks when they occur (Plumb 1999; Winton 2001). Maintaining healthy rearing conditions (McCarthy and Roberts 1980; Munro and Roberts 1989), providing proper nutrition (Piper et al. 1982), routinely monitoring fish health, and administering

vaccines (Ellis 1989) can help prevent disease outbreaks. However, timely treatment with therapeutic drugs may be useful for controlling mortality and preventing epizootics when disease outbreaks occur (Klontz 1987; Alderman 1988; Plumb 1999).

In the USA, the Food and Drug Administration (FDA) Center for Veterinary Medicine has approved four antimicrobial compounds (sulfamerazine, oxytetracycline dihydrate, sulfadimethoxine–ormetoprim, and florfenicol) for use in medicated feed to control mortality due to systemic bacterial diseases in cultured food fish populations (USFDA 2008). Such approvals depend on meeting FDA requirements for demonstrating efficacy and human, environmental, and target animal safety (Greenlees 1997). Commercial products currently available are Terramycin 200 for Fish (active ingredient: oxytetracycline dihydrate; Phibro Animal Health, Ridgefield Park, New Jersey); Romet 30 and Romet TC (active ingredients: sulfadimethoxine–ormetoprim; manufactured by PHARMAQ Ltd., Fordingbridge, Hampshire, UK; distributed in the USA by Aquatic Health Resources LLC, Brainerd, Minnesota);

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and Aquaflor and Aquaflor CA-1 (active ingredient: florfenicol; Intervet Schering-Plough Animal Health Corp., Roseland, New Jersey). Approved uses (label claims) for these products are species specific and disease specific (USOFR 2007a), but none of these products is approved for use in controlling fish mortality associated with *S. iniae* infections.

Aquaflor and Aquaflor CA-1 are 50% florfenicol ([R-(R\*,S\*)]-2,2-dichloro-N-[1-(fluoromethyl)-2-hydroxy-2-[4-(methylsulfonyl)phenyl]ethyl]acetamide). Florfenicol is a broad-spectrum antimicrobial compound with both bacteriostatic and bactericidal properties and is active against a variety of Gram-positive and Gram-negative bacteria (Horsberg et al. 1996). Aquaflor has been approved in more than 20 countries (e.g., Norway, Japan, Chile, Canada, and USA) for the control of mortality in a variety of cultured fishes due to a variety of diseases and infectious fish pathogens. In the USA, Aquaflor is approved for the control of mortality due to (1) enteric septicemia associated with *Edwardsiella ictaluri* in channel catfish *Ictalurus punctatus* (Gaunt et al. 2003, 2004), (2) coldwater disease associated with *F. psychrophilum* in freshwater-reared salmonids, and (3) furunculosis associated with *Aeromonas salmonicida* in freshwater-reared salmonids (USFDA 2008). Currently, however, Aquaflor CA-1 can only be legally used for the control of channel catfish mortality due to columnaris disease associated with *F. columnare*. Under FDA regulations, Aquaflor and Aquaflor CA-1 must be (1) used under a Veterinary Feed Directive (VFD; veterinary prescription), (2) purchased from a feed mill licensed to manufacture VFD-medicated feeds, and (3) administered at a dose of 10 mg of florfenicol·kg of fish<sup>-1</sup>·d<sup>-1</sup> for 10 d (USOFR 2007b).

Streptococcal disease in fish is caused by Gram-positive bacteria, such as *Streptococcus* spp., *Lactococcus* spp., *Enterococcus* spp., and *Vagococcus* spp. The first confirmed streptococcal infection in cultured fish was reported in 1958 in rainbow trout *Oncorhynchus mykiss* cultured in Japan (Agnew and Barnes 2007). Streptococcal infections in fish, particularly those infections produced by *S. iniae*, have increased markedly with intensification of aquaculture practices and annually produce significant economic losses to the world aquaculture industry (Shoemaker and Klesius 1997). Agnew and Barnes (2007) listed 27 marine and freshwater fishes in which infection with *S. iniae* has been documented, including hybrids of striped bass *Morone saxatilis* (Stoffregen et al. 1996; Shoemaker et al. 2001), tilapias *Oreochromis* spp. (Kitao et al. 1981; Perera et al. 1994; Shoemaker and Klesius 1997), Japanese yellowtail *Seriola quinqueradiata* (Kaige et al. 1984), and rainbow trout (Kitao et al. 1981).

Streptococcal disease is one of the most common bacterial diseases in hybrid striped bass (Klesius et al. 2006).

*Streptococcus iniae* has emerged as an important fish pathogen over the past few decades (Agnew and Barnes 2007). Stoffregen et al. (1996) reported what they believed was the first outbreak of systemic streptococcal infection caused by *S. iniae* in sunshine bass (female white bass *Morone chrysops* × male striped bass) reared in a freshwater recirculation system. In fish, the disease state caused by infection with *S. iniae* generally results in meningitis and panophthalmitis, ultimately producing high levels of morbidity and mortality (Bromage and Owens 2002). Disease progression in fish is somewhat variable and dependent on such factors as virulence of the pathogen, route of infection, fish age, and environmental rearing conditions (Agnew and Barnes 2007). Clinical signs typically include pigment darkening, lethargy, erratic and spiral swimming, ulcers, and exophthalmia (Inglis et al. 1993; Plumb 1999; Fuller et al. 2001; Agnew and Barnes 2007; Buchanan et al. 2008). Although the pathogenesis of *Streptococcus* spp. may not be fully understood (Inglis et al. 1993), it is thought to be facilitated by exotoxins (Plumb 1999).

Relatively few laboratory or field studies have been conducted to evaluate the effectiveness of chemotherapeutants (e.g., enrofloxacin: Stoffregen et al. 1996; amoxicillin: Darwish and Ismaiel 2003) in controlling sunshine bass mortality associated with *S. iniae*. Darwish (2007) conducted a controlled laboratory study in which he experimentally induced *S. iniae* infection in sunshine bass and treated the fish with Aquaflor-medicated feed. Darwish (2007) concluded that the optimum treatment dose for controlling mortality was 10–15 mg florfenicol·kg fish<sup>-1</sup>·d<sup>-1</sup> for 10 d. However, approval by FDA requires field efficacy studies in which test fish are not experimentally infected with a pathogen. Therefore, we conducted a field trial to evaluate the efficacy of Aquaflor administered in feed at a dose of 10 mg florfenicol·kg fish<sup>-1</sup>·d<sup>-1</sup> for 10 d to control mortality associated with *S. iniae* in freshwater-reared subadult sunshine bass.

## Methods

*Test facility and test fish.*—The field trial was conducted September 16–October 10, 2003, at the Kent SeaTech Corporation (KST) aquaculture facility near Mecca, California. *Streptococcus iniae* was inadvertently introduced into the facility in the early 1990s with fingerling sunshine bass purchased from a commercial supplier. As was pointed out by Russo et al. (2006), once a species of *Streptococcus* is introduced into a fish farm, it can be difficult to

eradicate. This inadvertent introduction and the semi-recirculating freshwater reuse system at KST have resulted in annual outbreaks of *S. iniae*. The most severe outbreaks have occurred when water temperatures reach 23–28°C in summer and early fall, and all age-classes of naïve sunshine bass reared outdoors are susceptible. Facility records indicate that no other *Streptococcus* spp. have been identified there.

Test fish were obtained as small fingerlings from Keo Fish Farm, Inc. (Keo, Arkansas), and were reared at KST to a subadult size (approximate weight = 377 g) in outdoor concrete production tanks. Production tanks were monitored daily for mortalities and moribund fish exhibiting clinical signs of streptococcal septicemia associated with *S. iniae* infection. Such monitoring allowed us to identify a potential reference population of fish for use in the trial and to determine an appropriate time to begin treatment. Clinical signs included darkened pigmentation, unilateral or bilateral exophthalmia, lethargy, absence of a fright response, labored breathing, and loss of interest in feed.

*Test and control articles.*—The test article, Aquaflo, was administered in feed at a target dosage of 10 mg florfenicol•kg fish<sup>-1</sup>•d<sup>-1</sup> for 10 d. The medicated feed was prepared by mixing Aquaflo, commercial fish feed (Silver Cup 0.09375-in [0.238125-cm] trout pellets; Nelson and Sons, Inc., Murray, Utah), and menhaden fish oil in a laboratory mixer (Model SPS-1244; Marion Mixers, Inc., Marion, Iowa) at a concentration of 1.0 g florfenicol/kg feed. The control article consisted of nonmedicated Silver Cup trout pellets (0.238125 cm) that were not top-dressed with menhaden oil. Three 200-g samples of medicated feed and one 200-g sample of control feed were collected and analyzed to verify florfenicol concentrations in both feeds. Florfenicol concentrations were determined via high-performance liquid chromatography (Hayes 2005) by Eurofins AvTech Laboratories, Inc. (Portage, Michigan).

*Null hypothesis and experimental design.*—The null hypothesis was that mean cumulative mortality of sunshine bass in treated tanks was equal to mean cumulative mortality in control tanks. To test the null hypothesis, medicated and control feed treatments were allocated among six test tanks in a completely randomized design, with tanks serving as experimental units ( $N = 3$  tanks/treatment).

*Experimental procedures.*—The trial was conducted outdoors under a natural photoperiod. In one production tank (189,500 L; ~22,000 fish), mortality increased from near 0% to 0.1–0.2% per day during a 5-d period. This tank was designated as the reference tank after *S. iniae* was recovered from moribund fish and presumptively identified as the causal mortality

agent and after sensitivity of the *S. iniae* isolate to florfenicol was established (procedures described in next section). The reference tank was supplied with oxygenated water (75% reuse water and 25% geothermal well water) at a rate sufficient to achieve 0.42 water exchanges/h and maintain water quality attributes at levels suitable for sunshine bass culture (hardness [mean  $\pm$  SD] = 66  $\pm$  6 mg CaCO<sub>3</sub>/L; alkalinity = 92  $\pm$  6 mg CaCO<sub>3</sub>/L; pH = 6.7  $\pm$  0.3). Visibility was limited to a water depth of approximately 30–46 cm because of turbidity.

Six 157.5-L, flow-through test tanks with individual inflows and aeration stones were set up for the trial (Figure 1). Water from the reference tank was diverted to the test tanks at a rate sufficient to achieve 3.6 water exchanges/h. Inflow rate, water exchange rate, and supplemental in-tank aeration in the test tanks kept water quality variables at values suitable for sunshine bass culture. Each test tank was stocked with 50 fish from the reference population. Initial stocking density in each test tank (~120 g fish/L) was greater than that in the reference tank (~44 g fish/L) to exacerbate *S. iniae* infection among the test fish and to facilitate a robust evaluation of treatment efficacy.

The trial comprised a 1-d acclimation period, 10-d treatment period, and 14-d posttreatment period. Test fish were not fed during the acclimation period. During the treatment period, medicated and control feeds were hand-fed to assigned tanks twice daily at 1% of initial mean biomass per tank per day. Feed amounts were adjusted weekly for each tank to account for mortality; however, no adjustments were made for weight gain. During the posttreatment period, control feed was hand-fed to all tanks twice daily at 1% of biomass per tank per day. During this period, feed amounts were not adjusted for mortality or weight gain.

Mortality, general behavior, and feeding responses were monitored throughout the trial. All mortalities were recorded and removed from tanks daily. Experienced culturists recorded behavior as “normal” or “abnormal,” and all abnormal behavior was described (e.g., piping or swimming at the surface). Feeding response was recorded as “aggressive” (defined as most fish actively feeding at or near the water surface, with few feed pellets observed in the tank effluent), “semiaggressive” (few fish actively feeding at or near the surface, with approximately 50% of offered feed observed in the tank effluent), or “nonaggressive” (no fish actively feeding at or near the surface, with most of the offered feed pellets observed in the tank effluent). It was estimated that all offered feed pellets were either consumed within 15 s or discharged in the tank effluent. Water temperature and dissolved oxygen (DO) concentration were measured in each test tank

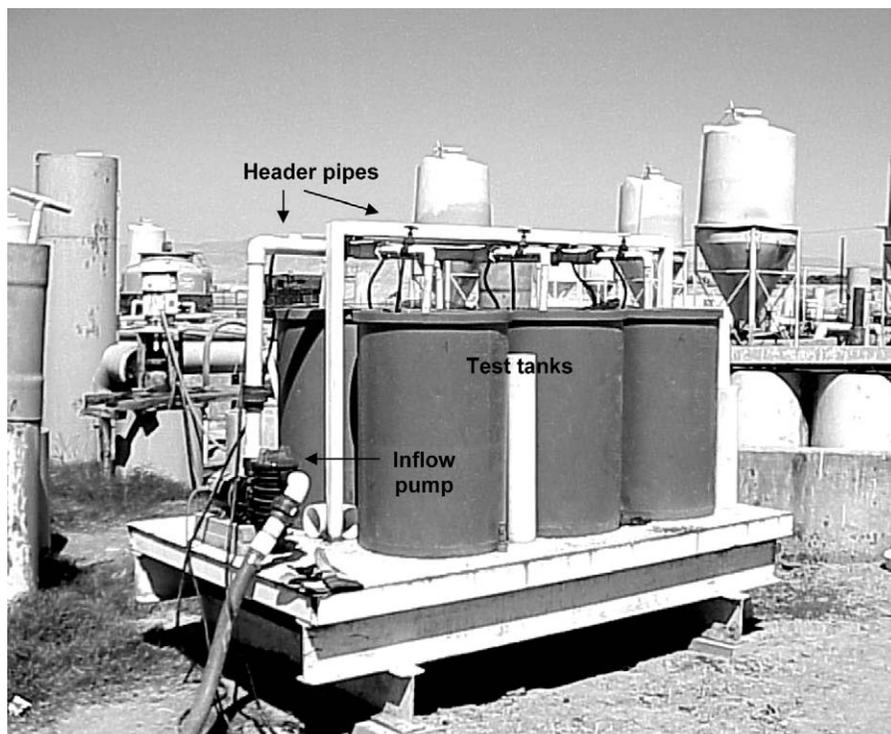


FIGURE 1.—Test tanks ( $N = 6$ ) used in a controlled field trial conducted in 2003 to evaluate the efficacy of Aquaflor (50% florfenicol; administered orally in feed at  $10 \text{ mg of florfenicol} \cdot \text{kg of fish}^{-1} \cdot \text{d}^{-1}$  for 10 d) in controlling sunshine bass mortality associated with *Streptococcus iniae*.

twice daily with a YSI Model 95 DO meter (Yellow Springs Instruments, Inc., Yellow Springs, Ohio). During the trial, mean ( $\pm$ SD) water temperature and DO concentration were  $26.6 \pm 0.8^\circ\text{C}$  and  $13.3 \pm 1.6 \text{ mg/L}$ , respectively. Mean daily water temperature decreased from  $28.5^\circ\text{C}$  to  $26.1^\circ\text{C}$  during the first 3 d of the treatment period but was relatively stable thereafter. Mean daily DO concentration was maintained at or above  $10.0 \text{ mg/L}$ . Based on the elevation of KST (0 m above mean sea level), oxygen saturation was maintained at or above 100%. Source water was moderately hard and slightly acidic and was considered suitable for the rearing of sunshine bass (Kohler 2000).

*Fish health, bacteriology, and histopathology procedures.*—Before the trial started, four moribund fish exhibiting the clinical signs previously described were collected from the reference tank to verify infection with *S. iniae*. These fish were euthanized in a solution of Tricaine-S (Western Chemical, Ferndale, Washington) and were subjected to gross internal and external examinations and bacteriological assays. Bacteriological assays consisted of aseptically plating brain and head kidney tissues from each fish onto blood agar (BA; tryptic soy agar [TSA] plus 5% sheep's blood;

Hardy Diagnostics, Santa Maria, California), incubating at  $28^\circ\text{C}$  for 48 h before initial interpretation, and incubating for another 7 d to ensure recovery of more fastidious microorganisms.

Antimicrobial sensitivity to florfenicol ( $30 \mu\text{g}$ ) was tested with sensitivity discs supplied by Intervet Schering-Plough Animal Health Corp. and under standard disk diffusion procedures (CLSI 2003; Miller et al. 2003). Briefly, two bacterial isolates recovered from reference fish were cultured overnight at  $28^\circ\text{C}$  on BA plates, single colonies were removed, and the inoculum was prepared and adjusted to an optical density standard of 0.5 McFarland units. The inoculum was streaked over the surface of a single Mueller-Hinton + BA plate (150-mm diameter; Hardy Diagnostics) with a sterile cotton applicator. Using sterile forceps, a sensitivity disk was placed near the center of each inoculated agar plate, and each plate was incubated at  $28^\circ\text{C}$  in a humid chamber for 24 h. Zones of inhibition were detected with the unaided eye, the diameter was measured to the nearest 1 mm, and 23–24-mm zones of inhibition were obtained.

Bacterial colonies were presumptively identified as *Streptococcus* spp. by using phenotypic traits (Holt et

al. 1994). A single bacterial colony derived from one of the reference fish was selected as representative for the disease outbreak. Each colony was subcultured onto either TSA or BA for further biochemical characterization. Unless otherwise indicated, all bacteriological media were purchased from Hardy Diagnostics. The following characteristics were examined: Gram reaction and cell morphology, oxidase (enzyme code 1.9.3.1; IUBMB 1992) and catalase (1.11.1.6) activity (oxistrips and 3% catalase reagent, respectively), motility (hanging drop) after overnight static incubation in Todd-Hewitt broth (THB), hemolysis of sheep erythrocytes (TSA + 5% sheep's blood), hydrolysis of esculin (esculin agar slant), and growth in THB containing 6.5% NaCl (Sigma Aldrich, St. Louis, Missouri) or 40% bile salts (Sigma Aldrich). All reactions were incubated under static conditions at 28°C for up to 7 d before interpretation.

Definitive identification of the presumptive *Streptococcus* spp. isolate to species was performed by polymerase chain reaction (PCR) using *S. iniae*-specific primers designed to anneal to unique regions of the 16S–23S ribosomal DNA intergenic transcribed spacer (ITS) region (Berridge et al. 1998). The *S. iniae* reference strain (ATCC [American Type Culture Collection] 29178) was included as a positive control. Five additional bacterial isolates served as negative controls to verify the specificity of the PCR assay. These isolates included *Streptococcus agalactiae* (ATCC 13813), *Streptococcus pyogenes* (ATCC 19615), *Yersinia ruckeri* (ATCC 29473), *Aeromonas salmonicida* (ATCC 33658), and *Aeromonas hydrophila* (ATCC 7965). Briefly, a single colony derived from each representative isolate was used to inoculate 5 mL of THB, and the cultures were grown static overnight at 28°C. Bacterial cells were concentrated by centrifugation (14,000 revolutions/min for 5 min), and total genomic DNA was isolated with a Bactozol DNA Isolation Kit (MRC, Inc., Cincinnati, Ohio) according to the manufacturer's protocol for Gram-positive bacteria. The DNA was resuspended in sterile tris-EDTA buffer, quantified by spectrophotometry (260-nm wavelength), and adjusted to 0.1 µg of DNA/µL in sterile deionized water. Reactions were performed in 25-µL volumes containing 0.1 µg of total genomic DNA. The following PCR cycle profile was used: 2 min at 90°C followed by 36 cycles of 30 s at 94°C, 30 s at 55°C, and a 45-s extension at 72°C. The molecular weights of the resulting PCR products were verified against 100-base-pair (bp) standards on 1.5% agarose gels stained with ethidium bromide. Polymerase chain reaction products were then purified with a QIAquick PCR Cleanup Kit (QIAGEN, Inc., Valencia, California) according to the manufacturer's protocol and were

sequenced in both directions by using Applied Biosystems, Inc. (ABI; Foster City, California) PRISM BigDye Terminator cycle sequencing chemistry on an ABI Model 3700 automated sequencer. Approximately 370 bp of complimentary strand sequence data were obtained from the PCR products of the isolate and the *S. iniae* reference isolate (ATCC 29178). Sequence data were edited and aligned and found to be identical (0% sequence divergence) over this 370-bp ITS region. The 370-bp consensus sequence was subjected to a search using the Basic Local Alignment Search Tool (BLAST; BLASTN version 2.2.1; National Center for Biotechnology Information, GenBank, Bethesda, Maryland).

To support bacteriological findings and determine whether there was a mixed infection, selected tissues were collected from each of the four reference fish for histopathology. Brain, heart, kidney (head and trunk regions), liver, gastrointestinal tract, and gills were fixed in 10% neutral buffered formalin and processed with standard procedures into paraffin wax; 5-µm sections were cut, stained with hematoxylin and eosin, and examined microscopically.

On the last day of the treatment period, six test fish were collected to assess fish health midway through the trial. No live fish exhibited clinical signs of streptococcal disease. Therefore, one dead fish was collected from each of two control tanks, and one live fish was collected from each of the other four test tanks because they contained no dead fish. All six fish were subjected to gross internal and external examinations and bacteriological assays as described previously. No other fish were sampled for fish health assessments.

*Statistical analysis.*—At the end of the trial, mean cumulative mortality in treated tanks was compared with mean cumulative mortality in control tanks via a mixed-effects logistic model fitted with the GLIMMIX procedure in the Statistical Analysis System (Wolfinger and O'Connell 1993; SAS 1997). The random effect of tank was modeled with an *R*-side covariance structure. Mean cumulative mortality in the two treatment groups was judged to be statistically different if the *P*-value was less than 0.05.

## Results

### *Mortality and Behavior*

At the end of the trial, mean ( $\pm$ SD) cumulative mortality of sunshine bass in treated tanks ( $19 \pm 11\%$ ) was significantly ( $P = 0.040$ ) less than that in control tanks ( $52 \pm 13\%$ ; Figure 2a). Highest mean daily mortality occurred on treatment day 5 in treated tanks and on treatment day 4 in control tanks (Figure 2b). Throughout the trial, general behavior in treated and control tanks was characterized as normal because fish

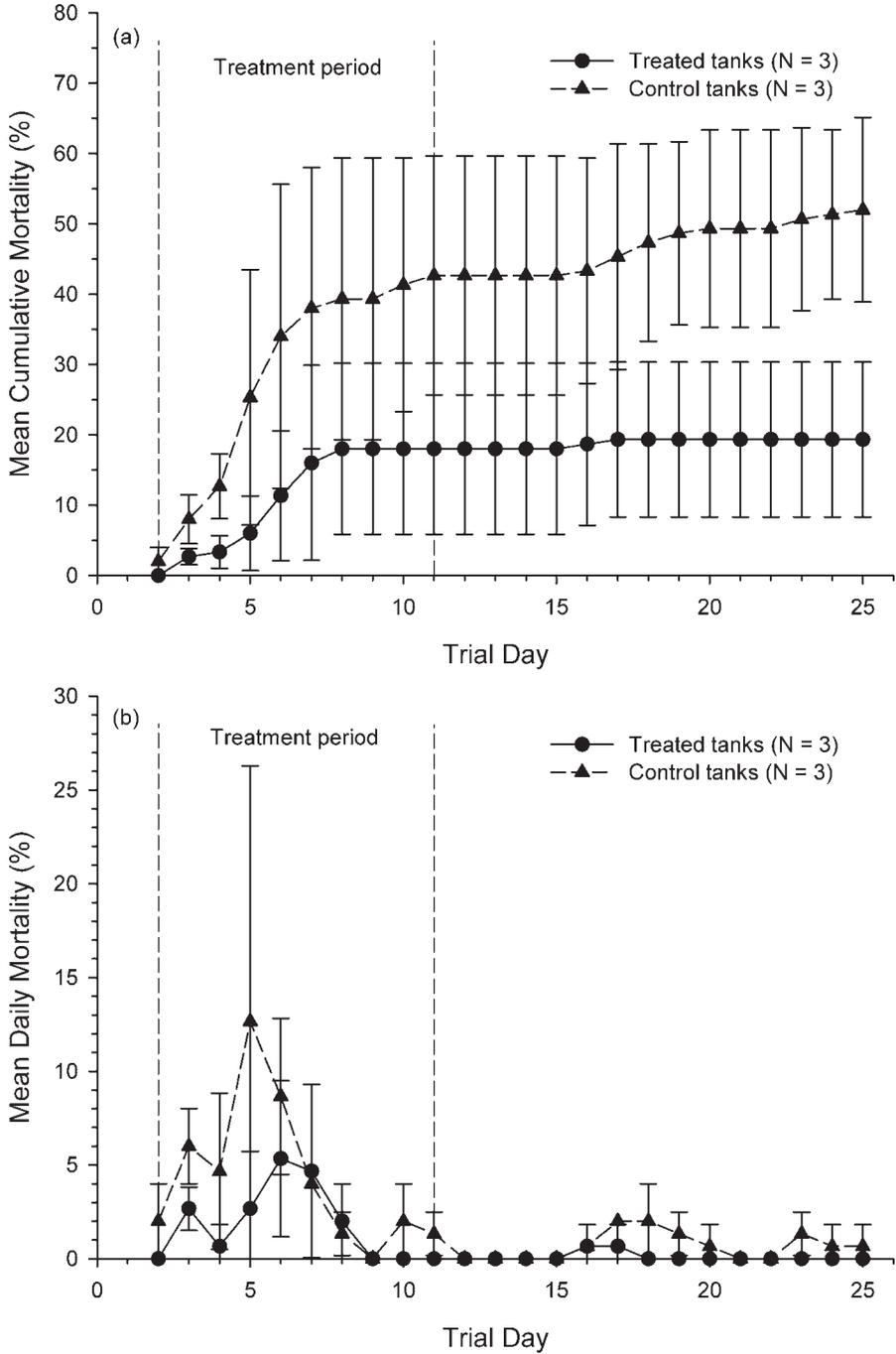


FIGURE 2.—Mean ( $\pm$ SD) (a) percent cumulative mortality and (b) percent daily mortality of sunshine bass in treated and control tanks during a controlled field trial conducted in 2003 to evaluate the efficacy of Aquaflo<sup>®</sup> (50% florfenicol; administered orally in feed at 10 mg of florfenicol  $\cdot$  kg of fish<sup>-1</sup>  $\cdot$  d<sup>-1</sup> for 10 d) in controlling fish mortality associated with *Streptococcus iniae*.

exhibited subsurface activity. Both treated and control fish were characterized as feeding nonaggressively on the first treatment day; however, fish in both treatment groups fed semiaggressively on virtually every day during the treatment period and aggressively on every day of the posttreatment period.

#### *Florfenicol Doses Administered*

Mean florfenicol concentration in the medicated feed samples was 830 mg florfenicol/kg feed (range = 728–984 mg/kg). Based on feeding at 1% of biomass per tank per day, the florfenicol dose administered to the treated tanks was 8.3 mg florfenicol·kg fish<sup>-1</sup>·d<sup>-1</sup> (83% of target). Florfenicol was not detected in the control feed.

#### *Fish Health, Bacteriology, and Histopathology*

In fish collected from the reference population before the start of the trial, large numbers of an axenic culture of white, circular, raised, strongly beta-hemolytic bacterial colonies were recovered from the brain and head kidney. Preliminary biochemical identification demonstrated that the isolates were aerobic, nonmotile, oxidase- and catalase-negative, Gram-positive cocci occurring in pairs and chains. Isolates were able to hydrolyze esculin but failed to grow in the presence of 6.5% NaCl or 40% bile salts. These biochemical characteristics were consistent with pyogenic isolates of the genus *Streptococcus*, including *S. iniae* (Holt et al. 1994).

A predicted single PCR amplicon of approximately 380 bp was generated from the trial isolate and from *S. iniae* reference strain ATCC 29178. Polymerase chain reaction products were not detected for any of the negative control reference isolates, including two closely related species of *Streptococcus* (*S. agalactiae* and *S. pyogenes*). Together, these data strongly support the genetic identity of the isolate as *S. iniae*. The BLAST search indicated that PCR products were most closely related to the 16S–23S ITS sequences listed for *Streptococcus* spp. The only highly homologous database match (>95% identical nucleotides) was with *S. iniae*, providing further support for identification of the isolate recovered from the reference population as *S. iniae*. These molecular results were consistent with past molecular diagnostics performed at KST when sunshine bass were infected with *S. iniae*.

Moderate to large numbers of intra- and extracellular bacterial cocci (in pairs and chains) were detected histologically in the brain, heart, head kidney, and spleen of all fish examined from the reference population. The most substantive finding was a moderate to marked granulomatous inflammation associated with the presence of the bacteria in the

spleen, kidney, and heart. Single granulomatous foci were not uncommon throughout the parenchyma of the head kidney and spleen. In the latter instance, this observation appeared to involve the splenic ellipsoids. A marked granulomatous inflammation was also evident on the meninges of the optic and olfactory lobes of the brain; low to moderate numbers of bacterial cocci (in pairs and chains) were associated with these changes.

Each of the four reference fish collected before initiation of treatment displayed a similar mixed bacterial microflora on the gill surface; however, there was an absence of overt lamellar epithelial change in response to the presence of the bacteria. Occasionally, low numbers of ciliated protozoa (morphologically resembling *Ambiphyra* spp. and *Trichodina* spp.) were seen in association with these changes. Except for gill-associated bacteria and parasites, there were no histological changes suggesting an underlying mixed systemic bacterial infection in the reference population.

Bacteria cultured from brain tissue from the two dead control fish collected on the last day of the treatment period were presumptively identified as *S. iniae*. *Streptococcus iniae* was not recovered from any of the four live fish collected on the last day of the treatment period. No other bacterial fish pathogens were found.

#### **Discussion**

In our study, Aquaflo (50% florfenicol) medicated feed effectively controlled mortality associated with *S. iniae* in freshwater-reared subadult sunshine bass. These findings further support the effectiveness of Aquaflo for treating bacterial infections because mortality was significantly reduced in the treated tanks even though the actual florfenicol dosage administered (8.3 mg·kg fish<sup>-1</sup>·d<sup>-1</sup> for 10 d) was only 83% of the target dosage (10 mg·kg fish<sup>-1</sup>·d<sup>-1</sup> for 10 d). Considerable florfenicol can be lost from uneaten feed if it remains in water for 5 min (Yanong et al. 2005); however, in this trial, feed was either consumed or discharged in tank effluent within approximately 15 s. Florfenicol-medicated feed treatment (10 mg·kg fish<sup>-1</sup>·d<sup>-1</sup> for 10 d) was also shown to effectively control mortality in fingerling sunshine bass (3–4 g) that were experimentally infected with *S. iniae* (Darwish 2007). Mean cumulative mortality observed in treated fish was greater in our study (19%) than in Darwish's (2007) study (13%). Although the two studies are not directly comparable, adult and subadult fish are typically more susceptible to streptococcosis than are younger life stages (Agnew and Barnes 2007). Based on our results and those of Darwish (2007), we speculate that florfenicol-medicated feed treatment will

prove efficacious for controlling *S. iniae*-associated mortality in all sunshine bass life stages. Florfenicol-medicated feed treatment has also been shown to be effective for controlling fish mortality associated with *Aeromonas salmonicida* (Nordmo et al. 1994; Shepard et al. 1994; Samuelsen et al. 1998), *Vibrio anguillarum* (Samuelsen and Bergh 2004), and *Edwardsiella ictaluri* (Gaunt et al. 2003, 2004) infections.

Other than the study by Darwish (2007), there is little published information demonstrating the potency of florfenicol against *Streptococcus* spp. or that florfenicol effectively controls fish mortality due to streptococcosis. However, minimum inhibitory concentrations (MICs) of florfenicol against *S. iniae* strains obtained from diseased fish have been reported by several authors. Fukui et al. (1987) reported an MIC of 3.1 µg/mL for two *S. iniae* strains, Yanong et al. (2005) reported MICs ranging from less than 1 to 2 µg/mL for five strains, and Darwish (2007) reported MICs ranging from 2 to 4 µg/mL for 13 strains. It was not our objective to establish a florfenicol MIC for the *S. iniae* strain isolated in the current trial; however, such information would have supplemented published data.

At present, disk diffusion susceptibility cutoff values have not been developed and validated for florfenicol and *S. iniae*, and we know of no published reports describing wild-type and non-wild-type inhibition zone diameters. In our study, *S. iniae* did not grow up to the disk border. We characterized this result as an inhibitory response and inferred that in this case, *S. iniae* did not display a marked resistance to florfenicol in vitro. Because there are no published data to show that the KST strain of *S. iniae* isolated in this trial is sensitive to florfenicol, we assumed that any supposed sensitivity would be specific at this locale because florfenicol treatment controlled the mortality of fish in the treated tanks. Also, we assumed that the bioavailability (*F*) of florfenicol was high in sunshine bass. High *F*-values have been reported for other fishes, including Atlantic salmon *Salmo salar* (*F* = 99%; Horsberg et al. 1996), Atlantic cod *Gadus morhua* (*F* = 91%; Samuelsen et al. 2003), and koi (ornamental variant of common carp *Cyprinus carpio*; *F* = 99%). In addition, Yanong et al. (2005) estimated an *F*-value of 65% in three-spot gourami *Trichogaster trichopterus* based on intramuscular injection. Conversely, Kosoff et al. (2009) found relatively lower serum concentrations of florfenicol in hybrid striped bass and suggested that lower treatment efficacy could result. Although our study objective did not include measurement of florfenicol plasma levels, our mortality results indirectly support the supposition that the *F*-value of florfenicol in sunshine bass is relatively high. Estab-

lishment of optimal florfenicol dosing regimens to control sunshine bass mortality caused by various pathogens would benefit from additional pharmacokinetics studies.

The presumptive and confirmatory diagnostic assays performed in our study indicated that *S. iniae* was the suspected causative agent of mortality in sunshine bass. Moreover, the clinical signs exhibited by the reference and test fish—including darkening of the skin, exophthalmia, lethargy, and reduced feeding response—were consistent with documented effects of streptococcal infection in fishes (Bromage and Owens 2002; Russo et al. 2006; Agnew and Barnes 2007; Darwish 2007). Test fish fed nonaggressively on the first day of the treatment period, perhaps because they were not yet acclimated to the test tanks. Fish appeared to acclimate shortly thereafter because they fed more aggressively throughout the remainder of the treatment period. A longer acclimation period was considered; however, delaying treatment in effectiveness trials incurs a risk of excessive mortality. Our histological observations, including meningitis (Eldar and Ghittino 1999; Bromage and Owens 2002), large areas of cellular hyperplasia, and numerous foci of infection in the spleen and kidney tissue (Ferguson et al. 1994; Perera et al. 1998; Eldar et al. 1999; Russo et al. 2006; Chen et al. 2007), are also consistent with previously reported histopathology of streptococcal disease in sunshine bass (Stoffregen et al. 1996), rainbow trout (Eldar and Ghittino 1999), zebrafish *Danio rerio* (Ferguson et al. 1994; Neely et al. 2002), tilapias (Miyazaki et al. 1984; Perera et al. 1998; Chen et al. 2007), and red drum *Sciaenops ocellatus* (Eldar et al. 1999). Collectively, our behavioral, histological, and microbiological results described a classical example of *S. iniae* infection and provided the opportunity to conduct a field efficacy trial with naturally infected fish. Efficacy trials conducted on naturally infected populations can be confounded by concomitant pathogens. However, the ectoparasite infestation we encountered was at subclinical levels and thus did not unduly influence mortality. For the sake of comprehensive diagnostics, it would have been beneficial to sample more fish during the course of the study for confirmatory fish health examinations.

Reducing exposure to environmental stressors helps minimize the risk of *Streptococcus* spp. infections in cultured Nile tilapia *Oreochromis niloticus* (Chang and Plumb 1996) and presumably in all finfish. Water temperature (Agnew and Barnes 2007) and changes in water temperature (Ndong et al. 2007) are potential environmental stressors that have been shown to affect *S. iniae* infections in fish. Historically, the highest mortality of sunshine bass at KST has occurred during

the late summer and has been mostly attributed to streptococcal disease. The average high air temperature in the Palm Springs, California, area (about 48.3 km [30 mi] from the facility) is about 33°C in May and October and 40°C during June–September. In a study modeling the effect of water temperature on the progression of *S. iniae* infection in tilapia, optimum growth of *S. iniae* was predicted to occur at 37°C (Zhou et al. 2008). Accordingly, *S. iniae*-induced mortality of Nile tilapia was found to be greater at 25°C than at 15°C (Perera et al. 1997) and greater at 30°C than at 25°C (Chang and Plumb 1996). Mukhi et al. (2001) noted that mortality among pond-raised Mozambique tilapia *Oreochromis mossambicus* intensified towards the end of summer when water temperature was  $30 \pm 2^\circ\text{C}$ . Similarly, when investigating *S. iniae*-induced mass mortality of white-spotted rabbitfish *Siganus canaliculatus*, Yuasa et al. (1999) showed that chronic infections occurred at approximately 25°C, while acute infections with higher mortality occurred at 28–32°C. Conversely, Perera et al. (1997) reported higher mortality of tilapia at 20°C than at 15, 25, 30, or 35°C. Similar to results reported by Perera et al. (1997), Bromage and Owens (2009) found that *S. iniae*-associated mortality of barramundi *Lates calcarifer* peaked at moderate temperatures (25–28°C). Bromage and Owens (2009) suggested that the upper limits of a fish species' temperature range would support optimal performance of an individual's immune system. Therefore, because barramundi grow best at 27–36°C, Bromage and Owens (2009) speculated that mortality as a result of streptococcal infection was reduced at temperatures greater than 28°C. Ultimately, the extent to which water temperature affects *S. iniae*-related mortality may be species-specific and is likely influenced by other characteristics of a fish's external and internal environment. More severe infections with *S. iniae* and greater mortality rates have also been associated with osmotic stress (Morgan and Iwama 1991; cited by Chang and Plumb 1996), suboptimal pH (Perera et al. 1997), low DO concentrations and elevated nitrite concentrations (Bunch and Bejerano 1997), high stocking densities (Shoemaker et al. 2000), and crowding stress (Stoffregen et al. 1996). The constant interaction among fish, pathogen, and environment is well documented (Plumb 1999); thus, disease expression and the extent of disease-associated mortality will continually vary over time and space.

We considered the target florfenicol dose administered in this trial to be efficacious. There are, however, reports that higher florfenicol doses might enhance efficacy. Darwish (2007) recommended a dose of 10–15 mg florfenicol·kg fish<sup>-1</sup>·d<sup>-1</sup> for 10 d to obtain optimum control of mortality in sunshine bass that

were experimentally infected with *S. iniae*. Other researchers have also reported 10–15 mg florfenicol·kg fish<sup>-1</sup>·d<sup>-1</sup> as being efficacious against enteric septicemia in channel catfish (Gaunt et al. 2003, 2004), furunculosis in Atlantic salmon (Nordmo et al. 1994; Sheppard et al. 1994; Samuelsen et al. 1998), and vibriosis in Atlantic cod (Samuelsen and Bergh 2004). However, comparisons among investigations can be problematic because many factors (e.g., timing of treatment, level of disease, fish feeding behavior, and natural versus experimental infections) can affect trial outcomes. Before treating fish with a high dose of florfenicol (e.g., 15 mg·kg fish<sup>-1</sup>·d<sup>-1</sup>), a cost–benefit analysis could determine whether the potential increase in survival warrants the increased cost of purchasing the antibiotic and manufacturing the medicated feed. For example, administering a 10-d Aquaflor-medicated feed treatment to 100,000 sunshine bass fingerlings fed at 1% of mean body weight per day would cost approximately US\$500 for a florfenicol dose of 10 mg·kg fish<sup>-1</sup>·d<sup>-1</sup> and \$650 for a dose of 15 mg·kg fish<sup>-1</sup>·d<sup>-1</sup> (C. Nelson, Nelson and Sons, personal communication). In addition, end users should consider that administering less-than-effective doses of an antibiotic may increase the probability that bacterial resistance will develop.

Currently, there are no U.S. Department of Agriculture-approved vaccines or FDA-approved antibiotics commercially available to prevent *S. iniae* infections or control *S. iniae*-associated mortality in striped bass or their hybrids. Although approved aquaculture drugs can be administered “extra-label” via veterinary prescription (USOFR 2007c; e.g., Terramycin 200 for Fish, Romet 30, and Romet TC), it would be advantageous to have an FDA-approved antibiotic specific for *S. iniae*. Other antibiotics (e.g., enrofloxacin and amoxicillin) shown to be efficacious for combating *S. iniae* infections in sunshine bass (Stoffregen et al. 1996; Darwish and Ismaiel 2003) are also used in human medicine; consequently, they are not likely to be approved by FDA for use in aquaculture. Currently, Aquaflor appears to be the only product for which FDA approval to control *S. iniae*-associated mortality in finfish is likely in the near future, and results from this study have been submitted to FDA in support of a new approval for this claim. However, because there is little published literature about the effectiveness of florfenicol in controlling mortality associated with *S. iniae* in warmwater finfish, additional studies should be conducted to more thoroughly evaluate the effectiveness of Aquaflor for this indication. Laboratory- and field-based studies should be conducted on several representative species of freshwater-reared warmwater fishes at a variety of

life stages, stocking densities, water temperatures, and florfenicol concentrations. In addition, efforts should be made to (1) sample more fish for health assessments, (2) culture and presumptively identify the pathogen suspected of causing mortality based on a greater sample size of dead and moribund fish, (3) definitively confirm pathogen species from more isolates, and (4) generate data to help establish MICs and disk diffusion cutoff values.

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