



U.S. Fish & Wildlife Service

The Efficacy of AQUI-S[®] as an Anesthetic for Use on Juvenile and Adult Largemouth Bass *Micropterus salmoides*

Drug Research Report

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Illustration/USFWS Largemouth Bass

For additional information on the AADAP Program and comprehensive information on aquatic species drug approval efforts, visit the AADAP Website at: <http://www.fws.gov/fisheries/aadap/>

* Use of trade names does not constitute endorsement by the authors or by the U.S. Government *

Introduction

The use of anesthetics is an important tool with broad application to fisheries management programs. Anesthetics are physical or chemical agents that act on an animal by initially inducing a calming effect and subsequently inducing loss of equilibrium, mobility, consciousness, and reflex action (Summerfelt and Smith 1990). Most often, anesthetics are used to reduce stress associated with the handling or transportation of fish. Anesthetics are widely used both in the culture of captive populations and in field situations that involve the management of wild fish populations. As such, fish anesthetic research has been conducted on many compounds, including carbonic acid (Gelwicks et al. 1998), sodium bicarbonate (Peake 1998), quinaldine, benzocaine, and 2-phenoxyethanol (Gilderhus and Marking 1987; Iwama et al. 1989; Munday and Wilson 1997). Although several of these compounds are effective fish anesthetics, the only products currently approved by the U.S. Food and Drug Administration (FDA) for use as anesthetics on fish are two 3-aminobenzoic acid ethyl ester methanesulfonate products: (1) FINQUEL[®], which is registered by Fort Dodge Laboratories, Fort Dodge, IA, and sold by Argent Chemical Laboratories, Redmond, WA, and (2) Tricaine-S[®], which is manufactured and sold by Western Chemical, Inc., Ferndale, WA. Both FINQUEL[®] and Tricaine-S[®] are effective fish anesthetics (Schoettger and Julin 1967; Shoettger et al. 1967); however, use of either requires a 21-d post-treatment “withdrawal” period before harvestable-size fish can be slaughtered for market, released to be caught and consumed by humans, or otherwise used by humans for food. Consequently, the 21-d withdrawal period restricts approved use of FINQUEL[®] and Tricaine-S[®] in many cultured populations and in virtually all wild populations.

Fisheries professionals in the U.S. have long needed a FDA-approved fish anesthetic for which no withdrawal period is required. The need for such a “zero-withdrawal” anesthetic has led to research on clove oil, a naturally derived compound that contains 85 - 95% eugenol and 5 - 15% isoeugenol and methyleugenol as its “significant” active ingredients (USDHHS 2002). Clove oil is an effective fish anesthetic (Soto and Burhanuddin 1995; Anderson et al. 1997; Cho and Heath 2000; Prince and Powell 2000); however, FDA is unlikely to approve it as such because of concerns about the safety of eugenol (an equivocal carcinogen) and methyleugenol (carcinogenic in rodents) to humans (USDHHS 2002). Currently, we know of no individual, company, or organization willing to sponsor clove oil for use as an FDA-approved fish anesthetic.

A commercial product, AQUI-S[®], has recently emerged as a candidate for FDA approval for use in the U.S. as a zero-withdrawal fish anesthetic. The active ingredient in AQUI-S[®] is isoeugenol, a compound that can be legally used in the U.S. as a food flavor-enhancer (21-CFR 172.515). AQUI-S[®] was developed as a fish anesthetic by the New Zealand Institute of Crop and Food Research and is currently sponsored by AQUI-S New Zealand, Ltd (ANZL). AQUI-S[®] is approved for use as a food-safe, zero-withdrawal fish anesthetic in New Zealand, Australia, the Faroe Islands, and Chile. In these places, AQUI-S[®] is primarily used to achieve pre-slaughter “rested harvesting” (i.e., calming) of market-bound fish. In the U.S., Stehly and Gingerich (1999) have

demonstrated that AQUI-S[®], when used at concentrations of 20 - 50 mg/L, will anesthetize a variety of life-stages of fishes to the “handleable stage.”

Currently, there is a multi-agency effort underway to generate data required for FDA approval of AQUI-S[®] for use as a zero-withdrawal fish anesthetic. The U.S. Fish and Wildlife Service’s Aquatic Animal Drug Approval Partnership (AADAP) Program is responsible for completing the efficacy technical section for all freshwater fish. As part of that effort, an AQUI-S[®] field efficacy study was conducted on largemouth bass (LMB; test fish) *Micropterus salmoides*. The objectives of the study were to (1) evaluate the efficacy of 20, 40, and 60 mg/L AQUI-S[®] to sedate LMB to the handleable stage of anesthesia and compare the results with that of an ideal anesthetic, and (2) determine if the pH of water used to prepare anesthetic solutions was affected by addition of AQUI-S[®] or the anesthetic used as a control (i.e., Tricaine-S[®]). Largemouth bass were selected as a test fish species because they are (1) a commonly cultured fish species, and (2) considered one of several representative coolwater fish species. To minimize variability in determining the sedation endpoint, we considered a fish to be handleable when it lost equilibrium, ceased swimming, easily hand-captured, held above the surface of the water, and measured for length with minimal fish movement. This level of sedation is comparable to Schoettger and Julin’s (1967) stage 3b level of anesthesia. A fish was considered “recovered” when it regained equilibrium and easily avoided objects in its path. We acknowledge that there are levels of anesthesia other than “handleable” that may be of interest to fish culturists and fisheries managers, such as shallow anesthetization for long-hauling or deep anesthetization for surgical procedures. However, ANZL and the core group of researchers working on the initial AQUI-S[®] approval believe that limiting the research to a claim for sedation to the handleable stage may be the quickest and easiest path for an new animal drug approval for AQUI-S[®].

Methods

Study Design

The study was conducted September 14 - 16, 2004, at Montana Fish, Wildlife and Parks’ Miles City State Fish Hatchery (Miles City, MT). The study consisted of four experiments (Table 1), in each of which 20, 40, and 60 mg/L AQUI-S[®] “treatments” and 80 mg/L Tricaine-S[®] “control” were used to sedate one of two life stages of LMB (test fish) at one of two different water temperatures. Life stages tested were juvenile (mean length, 18.0 cm) and adult (mean length, 36.5 cm). In Experiments 1 and 2, respectively, juvenile and adult fish were sedated to handleable at a median water temperature of 18°C (range, 17.9 - 18.1°C; well water). In Experiments 3 and 4, respectively, juvenile and adult fish were sedated to handleable at a median water temperature of 12.5°C (range, 12.3 - 12.9°C; water from the Tongue River, Custer County, MT). The order in which each combination of anesthetic and dose was tested in each experiment was determined randomly, and study participants were blinded to the treatment order to minimize data collection bias.

Study Conduct

Water chemistry—Water hardness and alkalinity of the well water and Tongue River water (i.e., source waters) used to prepare anesthetic solutions were measured using HACH (HACH, Co., Loveland, CO) digital titrators and reagents. Water temperature, dissolved oxygen concentration (DO), and pH of source waters and anesthetic solutions were measured with a YSI (Yellow Springs Instruments, Yellow Springs, OH) Model 95 DO meter and Model 60 pH meter, respectively. Mean hardness, alkalinity, pH, and DO of well water was 206 mg/L (as CaCO₃), 150 mg/L (as CaCO₃), 8.3, and 8.4 mg/L, respectively. Mean hardness, alkalinity, pH, and DO of Tongue River water was 8 mg/L (as CaCO₃), 754 mg/L (as CaCO₃), 8.8, and 10.0 mg/L, respectively. Water hardness, alkalinity, pH, and dissolved oxygen were adequate for rearing a wide variety of fish species (Piper 1982). In this study, the well water was classified as “hard, highly buffered,” and Tongue River water was classified as “soft, highly buffered” according to a scheme developed by Bain and Stevenson (1999). The latter is characteristic of some waters in arid zones (Cole 1979).

Table 1. Rainbow trout life stages and water temperatures tested in each of the four experiments.

Variable	Experiment number			
	1	2	3	4
Life-stage	Juvenile	Adult	Juvenile	Adult
Water temperature	18°C	18°C	12.5°C	12.5°C

Preparation and verification of anesthetic solutions—Bulk solutions of anesthetic were prepared by weighing out pre-determined amounts of AQUI-S[®] and Tricaine-S[®] and dispersing or dissolving each in separate 284-L tubs partly filled with source water. AQUI-S[®] solutions were prepared by first dispersing the pre-determined amount of AQUI-S[®] (4.54 , 9.07 , and 13.61 g to prepare 20, 40, and 60 mg/L AQUI-S[®], respectively) in at least a 10-fold dilution of source water in a capped 100-mL Nalgene[®] bottle. Bottles were shaken vigorously to form a white, milky solution, which was poured into the tubs. Bottles were rinsed repeatedly with source water until no visible trace of AQUI-S[®] was left in the Nalgene[®] bottle. Each rinse was added to the tub of anesthetic, which was brought up to a final volume of 227 L with source water. Finally, the solution was gently stirred to thoroughly disperse AQUI-S[®] throughout the tub. Solutions of Tricaine-S[®] (18.14 g to prepare 80 mg/L Tricaine-S[®]) were prepared in a similar manner.

The concentration of AQUI-S[®] in each tub of AQUI-S[®] solution was verified analytically to confirm that it was within an acceptable range (i.e., ±25%; as per FDA-accepted protocol guidelines) of the target concentration. From each tub of bulk anesthetic solution, one 100-mL sample of anesthetic solution was collected

immediately after preparing the anesthetic bath, and another 100-mL sample was collected after all fish had been tested. Standards (0, 5, 10, 20, 40, and 80 mg/L) were prepared from a 1,000-mg/L stock solution by using the same procedures as those used to prepare samples. Standards and samples were prepared for analysis by using a HEPES/Gibbs reagent method and analyzed spectrophotometrically at 352 nm. Overall mean doses administered to test fish in the four experiments were 20.6, 41.8, and 64.8 mg/L AQUI-S[®] and differed from target doses by no more than 8%.

Sedating and recovering test fish—In each experiment, the three AQUI-S[®] treatments and one Tricaine-S[®] control were each administered to 15 test fish. The time to handleable and recovery from handleable was timed for each fish. In Experiments 1 and 3, juvenile test fish were sedated and allowed to recover in 19-L buckets filled with approximately 11 - 15 L of anesthetic solution or fresh source water. In Experiments 2 and 4, adult test fish were sedated and allowed to recover in 66-L buckets filled with approximately 27 - 30 L of anesthetic solution or fresh source water. After each fish was sedated and removed from a bucket of anesthetic, the contents of the bucket were discarded and refilled for the next test fish. For logistic reasons, only 5 of the 15 individual fish were tested at a time. Water temperature and DO were measured in each exposure and recovery bucket. General behavior was observed when fish were initially placed in the anesthetic solution. Total length (to the nearest 0.5 cm) of each test fish was measured as it was transferred from anesthetic solution to the freshwater recovery bucket. General behavior was observed again after a fish had been moved to fresh water and had fully recovered from sedation. After recovery, all 15 fish were moved at once to fish rearing-tanks and monitored for survival for 24 h post-treatment. No mortalities occurred during the treatment, recovery, or post-treatment observation periods.

Measuring pH of anesthetic solutions—In each experiment, pH of each anesthetic solution was measured to determine whether addition of AQUI-S[®] or Tricaine-S[®] affected the pH of the source water. The pH in one of every five buckets of anesthetic solution used to sedate test fish was measured in each experiment (i.e., a total of six pH measurements were made to address pH changes in the two water sources).

Data analysis

Times to sedate test fish to handleable and times to recovery were analyzed with Kaplan-Meier (K-M) survival analysis (Borkowski 2002; Glantz 2002; SYSTAT 2004a, 2004b). K-M analysis was used to calculate the medians and 95% confidence intervals for measured times. Comparisons of K-M survival curves were made with Mantel Log-rank ($P = 0.05$) and Holm-Sidak tests (P -value depends upon the number of comparison's being made). Mean pH values from each anesthetic treatment or control were qualitatively compared with the pH of unaltered source water.

Results

In all four experiments, 100% of test fish in each AQUI-S[®] treatment group became handleable within 6.8 min (Table 2). Based on data collected at 20, 40, and 60 mg/L AQUI-S[®], median times for juveniles to become handleable and recover ranged from 1.4 to 6.8 min and from 2.5 to 5.5 min, respectively. Median times for adults to become handleable and recover ranged from 2.6 to 6.6 min and from 2.6 to 8.5 min, respectively (Table 2). In all four experiments, median time to handleable decreased

Table 2. Median time (minutes) to handleable and recovery from handleable for juvenile and adult largemouth bass exposed to AQUI-S[®] and Tricaine-S[®] at water temperatures of approximately 12.5 and 18°C.

Anesthetic concentration (mg/L)	Experiments 1 and 2 (water temperature = 18.0°C)				Experiments 3 and 4 (water temperature = 12.5°C)				
	Time to handleable (± 95% CI)		Time to recovery (± 95% CI)		Anesthetic concentration (mg/L)	Time to handleable (± 95% CI)		Time to recovery (± 95% CI)	
	Juv ^a	Adult	Juv	Adult		Juv	Adult	Juv	Adult
20 AQUI-S [®]	5.5 (1.0)	6.1 (1.1)	2.5 (0.3)	2.6 (0.5)	20 AQUI-S [®]	6.8 (0.5)	6.6 (0.3)	3.9 (0.6)	4.5 (0.2)
40 AQUI-S [®]	1.6 (0.1)	4.9 (0.8)	3.0 (0.8)	7.6 (0.8)	40 AQUI-S [®]	2.5 (0.4)	4.4 (0.2)	4.8 (1.2)	6.8 (2.9)
60 AQUI-S [®]	1.4 (0.2)	2.6 (0.1)	3.3 (0.2)	6.7 (0.6)	60 AQUI-S [®]	2.4 (0.1)	2.9 (0.3)	5.5 (1.2)	8.5 (1.4)
80 MS-222	2.0 (0.1)	3.1 (0.2)	1.5 (0.1)	2.0 (0.1)	80 MS-222	2.3 (0.1)	2.8 (0.8)	1.7 (0.3)	2.8 (0.6)

^a Juv = Juvenile

as AQUI-S[®] concentration increased, and K-M survival analysis confirmed that in all experiments the time to handleable decreased significantly as AQUI-S[®] concentration increased from 20 to 40 to 60 mg/L. No trend was observed with respect to the concentration of AQUI-S[®] used to sedate fish to the handleable stage and median time for fish to recover from handleable.

In addition, in all four experiments, 100% of the test fish in each Tricaine-S[®] group became handleable within 3.1 min (Table 2). Median times for juveniles to become handleable and recover ranged from 2.0 to 2.3 min and from 1.5 to 1.7 min, respectively. Median times for adults to become handleable and recover ranged from 2.8 to 3.1 min and from 2.0 to 2.8 min, respectively (Table 2). Qualitative comparison showed that times to handleable were most similar between 60 mg/L AQUI-S[®] and 80 mg/L Tricaine-S[®]. However, test fish consistently recovered more quickly following sedation to handleable with Tricaine-S[®].

Comparisons within AQUI-S[®] treatments were made between (a) Experiments 1 and 2 and between (b) Experiments 3 and 4 to investigate possible life-stage effects on

times to and from handleable when water temperature was held constant at either 18 or 12.5°C (Table 2). At both temperatures, median times for juveniles to become and recover from handleable were faster than those for adults. However, K-M analysis only confirmed significant differences in times to and from handleable for fish exposed to 40 and 60 mg/L.

Comparisons within AQUI-S[®] treatments were also made between (a) Experiments 1 and 3 and between (b) Experiments 2 and 4 to investigate possible water temperature effects (12.5°C vs 18°C) on times to and from handleable when life-stage was held constant at either juvenile or adult. Comparisons of median times indicated, and K-M analysis confirmed, that juveniles became handleable and recovered significantly faster at the warmer temperature than at the cooler temperature. No such trends were observed between median times to and from handleable for adult fish.

Comparisons of the pH of each anesthetic treatment or control showed that addition of AQUI-S[®] to well water (highly alkaline, hard water) increased the pH by 0.05 - 0.12 units, whereas the addition of Tricaine-S[®] decreased the pH by 0.8 units. The addition of AQUI-S[®] to Tongue River water (highly alkaline, soft water) had no effect on pH of the source water, whereas the addition of Tricaine-S[®] decreased the pH by 0.15 units.

Discussion and Conclusions

Although our results demonstrated that AQUI-S[®] effectively sedated juvenile and adult LMB to the handleable stage and that all fish recovered within a reasonable period of time, we used a more qualitative approach to evaluate the overall utility of AQUI-S[®] to fish culturists and fisheries managers. For example, the time-to-event, behavior, and mortality data generated in this study allowed us to compare the overall induction-recovery performance of AQUI-S[®] to that of an “ideal” fish anesthetic as it is currently described by the American Fisheries Society (AFS; Marking and Meyer 1985; Summerfelt and Smith 1990). According to AFS, an “ideal” fish anesthetic has an induction time of < 15 min (preferably < 3 min), a recovery time of < 5 min, is nontoxic to fish, and causes no persistent effects on fish behavior. In this study, induction and recovery times observed at 20 mg/L met the standards of an “ideal” fish anesthetic. At 40 and 60 mg/L, only induction times met these standards because median recovery times were 2 - 4 min longer than the preferred recovery time of < 5 min.

Overall, the AQUI-S[®] efficacy data generated in this study demonstrated that AQUI-S[®] concentrations of 20, 40, and 60 mg/L are efficacious and safe for sedating juvenile and adult LMB to the handleable stage at water temperatures of about 12.5 and 18°C. Such a use of AQUI-S[®] will likely be acceptable to most fisheries professionals because (a) “handleable” induction and recovery times fall within, or are reasonably close to, the guidelines of an “ideal” fish anesthetic, and (b) no adverse effects were observed. We also observed that, regardless of water temperature, the time required for juvenile and adult LMB to become handleable will decrease as AQUI-S[®] concentration is increased from 20 to 40 to 60 mg/L.

Comparison of temperature-effect data showed that at a given water temperature and AQUI-S[®] concentration, juvenile LMB will likely become and recover from handleable faster than adult LMB. As pointed out by Stehly and Gingerich (1999), smaller fish of the same species have greater gill surface area (gill:body) than larger fish, and hence, in theory, more waterborne anesthetic should pass through the gills of smaller fish per unit volume of body size than through the gills of larger fish.

There was no consistent relationship between water temperature and times required to sedate fish to handleable and recovery from handleable. However, at different water temperatures and a given AQUI-S[®] concentration, juvenile LMB will likely become handleable and recover from handleable faster in warmer water than in cooler water. Although temperature-related changes in respiratory rate could account for the decreased anesthetic induction and recovery time described above, such a relationship was not observed for adult LMB.

Results from this study also showed that addition of AQUI-S[®] did not substantially change the pH in either the highly buffered, hard water or in the highly buffered, soft water. Addition of Tricaine-S[®] did not substantially change the pH in the soft, highly buffered water from the Tongue River. However, addition of Tricaine-S[®] did decrease the pH of the well water (alkalinity, 150 mg/L CaCO₃; hardness, 206 mg/L CaCO₃). The observed decrease in pH is consistent with the acidic nature of Tricaine-S[®] and the effect it has on the pH of weakly buffered fresh water (Schreck and Moyle 1990). However, we do not consider such a change in the pH of the Tongue River water as biologically significant with respect to LMB.

Comparison of our results to other studies that measured anesthetic induction times is problematic. We agree with Iverson et al. (2003), who concluded from a review of several studies that (a) there is no simple definition of “efficacy of anesthetics” in fish and that (b) determining the time interval for fish to become handleable is a highly subjective variable. At a minimum, determination of this variable is dependent upon the handler, the fish, and the specific procedure(s) carried out, as well as on environmental and biological factors. In this study, we sedated individual fish of a specific species and size under specific environmental conditions to a stage of anesthesia where, in our estimation, they could be easily handled and measured for length. We believe such a level of handle-ability is suitable for many fish management/husbandry procedures such as weighing, artificial spawning, and tagging. In addition, our standards for “recovery” included the observation that fish were swimming and avoiding obstacles; thus, impingement on tailscreens of tanks or raceways, or the likelihood of fish being swept down river, would be minimized. Therefore, results from other studies, such as the study conducted by Stehly and Gingerich (1999) on representative coolwater fishes cannot be easily compared to results from our study. Comparison difficulties are collectively due to differences in criteria that defined the induction and recovery endpoints, fish species, and life stages tested and the environmental testing conditions. In spite of the difficulties in comparing studies, their results and ours support the conclusion that AQUI-S[®] is an effective and safe anesthetic that meets (or at least approximates) AFS criteria for an ideal anesthetic.

The data generated in this study have been submitted to the FDA Center for Veterinary Medicine (CVM) Aquaculture Drugs Team and will contribute to the overall effort to gain FDA approval of AQUI-S® as a fish anesthetic. Specifically, acceptance of this data will contribute to completion of the effectiveness technical section for all coolwater fish. To complete the efficacy technical section for a coolwater fish claim, CVM requires (at a minimum) that studies similar to this one be conducted on two other representative coolwater fish species. As such, we have completed one study on smallmouth bass *M. dolomieu* (two life-stages, two water temperatures) and one study on walleye *Stizostedion vitreum* (two life-stages, two water temperatures) at 20, 40, and 60 mg/L AQUI-S®, both of which have submitted to CVM for review. In addition, we plan to collect supplemental/supportive efficacy data on other representative coolwater fish (e.g., shovelnose sturgeon *Scaphirhynchus platyrhynchus*, northern pike *Esox lucius*, and June suckers *Chasmistes liorus*). The results of our coolwater efficacy work will put us one step closer to obtaining approval of AQUI-S® as a FDA-approved zero-withdrawal fish anesthetic.

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