Date of Approval: August 25, 2026

FREEDOM OF INFORMATION (FOI) SUMMARY

ORIGINAL NEW ANIMAL DRUG APPLICATION (NADA)

NADA 141-630

AQUI-S® E10%

(eugenol immersion solution)

Freshwater finfish (field-use only)

For sedation to a handleable condition in freshwater finfish (field-use only).

Sponsored by:

AQUI-S New Zealand Limited

Executive Summary[1]

AQUI-S® E10% (eugenol immersion solution) is approved for sedation to a handleable condition in freshwater finfish (field-use only). Field-use is the use of AQUI-S® E10% to sedate wild populations of freshwater finfish for resource management for spawning, tagging, weighing and measuring, and other activities. Fish may be released back into the natural water body immediately after recovery from sedation.

The term "freshwater" refers to fish that are living in freshwater at the time they are treated.

Effectiveness

The U.S. Fish & Wildlife Service conducted clinical effectiveness field studies on multiple species of freshwater fish, including rainbow trout, brown trout, cutthroat trout, lake trout, walleye, yellow perch, Nile tilapia, blue catfish, hybrid striped bass, channel catfish, common carp, and fathead minnows. Fish were exposed as individuals or small groups (10 or 15 fish) in static immersion baths, and the time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length were recorded. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were monitored for survival for 24 hours.

The sedation and recovery time results across all freshwater finfish tested were:
Freshwater salmonid finfish treated at 25 mg eugenol/L:
Mean sedation times ranged from 1.3 to 3.2 minutes.
Mean recovery times ranged from 3.6 to 10.8 minutes.

Freshwater non-salmonid finfish treated at 40 to 72 mg eugenol/L:
Mean sedation times ranged from 0.9 to 3.1 minutes.
Mean recovery times ranged from 1.9 to 14.4 minutes.

Temporary behavioral observations such as headshaking, coughing, or slight agitation were noted in some fish during initial exposure but resolved quickly, no adverse reactions were reported, and all fish survived the 24-hour post-treatment monitoring period. Collectively, all the studies provide substantial evidence that AQUI-S® E10% administered as a static immersion bath is safe and effective for sedating freshwater finfish to a handleable condition under species-appropriate concentrations and conditions.

Target Animal Safety

The U.S. Fish & Wildlife Service conducted three margin of safety studies in healthy fingerling rainbow trout (15 °C), yellow perch (19 °C), and channel catfish (26 °C) to establish safe exposure doses and durations. Each study employed the same experimental design, in which groups of 15 fish were exposed to 0 (control), 1X, or 1.5X

[1] The effectiveness and target animal safety sections of the executive summary were generated using artificial intelligence-powered writing tools and has been reviewed and edited for clarity and accuracy.

eugenol concentrations for 4 different durations selected to approximate 50 to 100% survival. Each concentration-duration combination was replicated three times. The 1X concentrations were 40 mg eugenol/L for rainbow trout, 100 mg eugenol/L for channel catfish, and 80 mg eugenol/L for yellow perch; the corresponding 1.5X concentrations were 60, 150, and 120 mg eugenol/L, respectively.

Results demonstrated an adequate margin of safety for all three species. The margin of safety — defined as the time difference between the maximum safe exposure duration (≥95% survival) and the time required to sedate 80% of fish to a handleable condition — was 5.5 and 2.9 minutes at the 1X and 1.5X doses for rainbow trout, 4.1 and 2.2 minutes for channel catfish, and 9.6 and 5.2 minutes for yellow perch. Across all three studies, no gross lesions were observed in any fish. Histopathological evaluations, conducted for rainbow trout and channel catfish but not yellow perch, revealed no significant treatment-related changes. The only consistent behavioral finding was transient headshaking in ≥75% of eugenol-exposed rainbow trout and yellow perch during the first 15 to 30 seconds of exposure; channel catfish did not exhibit this behavior. Collectively, all three studies support the conclusion that AQUI-S® E10% is safe for use at 1X concentrations for sedation to a handleable condition, with adequate margins of safety based on both concentration and duration of exposure.

Human Food Safety
The Food and Drug Administration (FDA) conducted a human food safety assessment of eugenol to ensure that any residues of eugenol in the edible tissues of treated animals do not exceed the concentration that presents a reasonable certainty of no harm to the human consumer when AQUI-S® E10% is used according to the label. The human food safety evaluation is conducted from the perspectives of microbial food safety, toxicology, and residue chemistry.

This drug is not an antimicrobial. Therefore, FDA determined that a microbial food safety assessment is not required.

After review of the publicly available information, FDA established the acute reference dose (ARfD) for eugenol as 2 mg/kg body weight (bw) and the safe concentration for fish muscle with skin in natural proportions as 400 ppm.

The data provided from metabolism and residue depletion studies were used to assess the quantity and nature of the residues in tissues derived from animals treated with AQUI-S® E10% (eugenol immersion solution). The target tissue is muscle with skin in natural proportions because that is the edible tissue for all fish (except those where the skin is inedible, i.e., catfish and eel). The marker residue is eugenol because that is the major compound found in the edible tissues after exposure. A tolerance is not assigned for eugenol used for field use because the total residue concentrations are substantially below the safe concentration for acute exposure to eugenol (400 ppm). FDA evaluated the validated analytical method and found its use acceptable.

User Safety
The product labeling contains precautions for people handling or administering AQUI-S® E10%. This includes wearing personal protective equipment (e.g., gloves, lab coats, eyewear), avoiding skin/eye contact and ingestion, and washing hands after use. The label states that accidental exposure may cause an allergic skin reaction in some people.

Conclusions
Based on data submitted by the U.S. Fish & Wildlife Service and the sponsor, FDA determined that AQUI-S® E10% is safe and effective when used according to the label.

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I. GENERAL INFORMATION

A. File Number

NADA 141-630

B. Sponsor

AQUI-S New Zealand Limited
6 Aglionby Street
Lower Hutt, Wellington, 5010
New Zealand

Drug Labeler Code: 086168

U.S. Agent Name and Address:

Fatou Diop
knoell Animal Health, LLC
2 Christy Drive
Suite 102
Chadds Ford, PA 19317

C. Proprietary Name

AQUI-S® E10%

D. Drug Product Established Name

eugenol immersion solution

E. Pharmacological Category

Sedative

F. Dosage Form

Immersion Solution

G. Amount of Active Ingredient

112 mg/mL

H. How Supplied

1-Liter and 4-Liter containers

I. Dispensing Status

Over the Counter (OTC)

J. Dosage Regimen

Freshwater salmonid finfish (field-use only): 25 to 40 mg eugenol per L

Freshwater non-salmonid finfish (field-use only): 40 to 100 mg eugenol per L

K. Route of Administration

Immersion

L. Species

Freshwater finfish (field-use only)

M. Indication

For sedation to a handleable condition in freshwater finfish (field-use only).

II. EFFECTIVENESS

A. Dosage Characterization

The effect of the active drug is the function of the administered concentration and exposure period. Fisheries professionals routinely sedate fish for procedures such as collection of samples or morphometric data. Eugenol doses selected for the effectiveness trials were based on results from preliminary tests conducted to identify doses that would sedate a variety of fish to a handleable condition in less than 3 minutes. Based on that information, eugenol was administered at a dose of 25 mg eugenol/L to freshwater salmonids, and a dose of 40 to 72 mg eugenol/L to freshwater non-salmonids for sedation to a handleable condition.

B. Substantial Evidence

The following studies, conducted by the U.S. Fish and Wildlife Service, provide substantial evidence that AQUI-S® E10% (eugenol immersion solution) is effective for the sedation to a handleable condition of freshwater finfish. The studies below are ordered so that all salmonid studies are described first, followed by non-salmonid studies.

1. Clinical Field Study

   Title: The Efficacy of AQUI-S®20E[2] (10% Eugenol) to Sedate Rainbow Trout *Oncorhynchus mykiss* to the Handleable Stage of Anesthesia. (Study No. AQS20E-11-EFF-01)

   Study Dates: May 5, 2011, to August 10, 2011

   Study Location: Bozeman, MT

[2] AQUI-S®20E (also presented as AQUI-S20E, AQUI-S® 20E throughout this document) was a proprietary name used through drug development but was changed to AQUI-S® E10% for approval. All the proprietary names represent the same product which contains 112 mg eugenol per mL and is 10% eugenol weight per weight (w/w).

Study Design:

Objective: To evaluate the effectiveness of 25 mg eugenol/L administered in a static bath to sedate subadult rainbow trout to the handleable stage within 5 minutes.

Study Animals: 60 subadult rainbow trout; mean length: 22.4 cm

Experimental Design: The study included 60 fish equally allocated to 2 treatment groups, eugenol and tricaine methanesulfonate. To administer the treatments, the 60 fish were divided into 4 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. This study was conducted according to Good Clinical Practices (GCP).

Drug Administration: Eugenol was administered at 25 mg/L and tricaine methanesulfonate was administered at 80 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length were recorded. Water temperature and dissolved oxygen (DO) concentration were measured in exposure containers. Water alkalinity and hardness were measured in the source water, and pH was measured in the source water and the bulk containers of sedative solutions. The eugenol concentration was verified in 10 of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data set was statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed $\alpha=0.05$. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and standard deviation (SD) of time to sedation to a handleable condition and time to recovery for rainbow trout sedated with 25 mg eugenol/L were 1.76 minutes (0.21) and 3.96 minutes (0.89), respectively.

Water Quality: Water hardness, alkalinity, and pH of the source water were 244 mg/L $CaCO_3$, 169 mg/L $CaCO_3$, and 8.12, respectively. Mean water pH was 8.18

and 7.64 in the eugenol and tricaine methanesulfonate bulk working solutions, respectively. Mean water temperature was 13.6 °C and mean DO concentration was 8.4 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 25 mg eugenol/L.

Behavior: Headshaking was observed in 17 of 30 (57%) of the fish exposed to eugenol and 18 of 30 (60%) of the fish exposed to tricaine methanesulfonate. Headshaking lasted only a brief period of time after initial exposure to the sedative solution and all recovery behavior was normal.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study demonstrates the effectiveness of 25 mg eugenol/L administered as an immersion bath to sedate rainbow trout (*Oncorhynchus mykiss*) to a handleable condition.

2. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% Eugenol) to Sedate Adult Brown Trout *Salmo trutta* to Handleable. (Study No. AQS20E-11-EFF-05)

Study Dates: May 5, 2011, to February 28, 2012

Study Location: La Crosse, WI

Study Design:

Objective: To evaluate the effectiveness of 25 mg eugenol/L administered in a static bath to sedate adult brown trout to the handleable stage of anesthesia within 5 minutes.

Study Animals: 90 adult brown trout; mean length: 30.6 cm; mean weight: 310.9 g. (Note: mean length and weight were based on 60 fish in the eugenol treatment group and tricaine methanesulfonate treatment group.)

Experimental Design: The study was conducted according to GCP. The study included 90 fish equally allocated to 3 treatment groups: benzocaine, tricaine methanesulfonate, and eugenol. To administer the treatments, the 90 fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 25 mg/L and tricaine methanesulfonate was administered at 80 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were

measured in exposure containers. Water alkalinity and hardness were measured in the source water, and pH was measured in the source water and bulk working solutions. The eugenol concentration was verified in 10 out of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data set was statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed α=0.05. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD time to sedation to a handleable condition and time to recovery for adult brown trout sedated with 25 mg eugenol/L was 1.8 minutes (0.33) and 7.1 minutes (1.84), respectively.

Water Quality: Water hardness, alkalinity, and pH of the source water were 178 mg/L $CaCO_3$, 125 mg/L $CaCO_3$, and 8.0, respectively. Mean water pH was 8.1 and 7.3 in eugenol and tricaine methanesulfonate bulk working solutions, respectively. Mean water temperature was 13.0 °C and mean DO concentration was 9.4 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 25 mg eugenol/L.

Behavior: Twenty-five of 30 (84%) brown trout exposed to eugenol and 27 of 30 (90%) brown trout exposed to tricaine methanesulfonate appeared slightly agitated when first placed in the sedative. Behavior during recovery was normal. All abnormal behaviors only lasted a few seconds, and all fish recovered.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study demonstrates the effectiveness of 25 mg eugenol/L administered as an immersion bath to sedate brown trout (*Salmo trutta*) to a handleable condition.

3. Clinical Field Study

Title: The Efficacy of AQUI-S®20E (10% Eugenol) to Sedate Adult Cutthroat Trout *Oncorhynchus clarkii lewisi* to the Handleable Stage of Anesthesia. (Study No. AQUI-S®20E HQSS-03)

Study Dates: May 5, 2011, to October 19, 2011

Study Location: Bozeman, MT

Study Design:

Objective: To evaluate the effectiveness of 25 mg eugenol/L administered in a static bath to sedate adult cutthroat trout to the handleable stage of anesthesia within 5 minutes.

Study Animals: 90 adult cutthroat trout; mean length: 26.3 cm.
(Note: mean length was based on 60 fish in the eugenol treatment group and tricaine methanesulfonate treatment group.)

Experimental Design: The study included 90 fish equally allocated to 3 treatment groups: eugenol, tricaine methanesulfonate, and benzocaine. To administer the treatments, the 90 fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate group was included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 25 mg/L and tricaine methanesulfonate was administered at 80 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity and hardness were measured in the source water, and pH was measured in the source water and the bulk containers of sedative solution. The eugenol concentration was verified in 10 of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data were statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed $\alpha=0.05$. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD time to sedation to a handleable condition and time to recovery for adult cutthroat trout sedated with 25 mg eugenol/L was 2.12 minutes (0.35) and 10.84 minutes (3.54), respectively.

Water Quality: Water hardness, alkalinity, and pH of the source water were 212 mg/L $CaCO_3$, 168 mg/L $CaCO_3$, and 8.04, respectively. Mean water pH was 8.07 and 7.51 in the eugenol and tricaine methanesulfonate bulk working solutions, respectively. Mean water temperature was 13.8 °C and mean DO concentration was 8.5 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 25 mg eugenol/L.

Behavior: Headshaking was observed in 3 of 30 (10%) of the fish exposed to eugenol and 1 of 30 (3.3%) of the fish exposed to tricaine methanesulfonate. Gill coughing was observed in 5 of 30 (16.7%) of the fish exposed to eugenol. Headshaking and gill coughing lasted only a brief period of time after initial exposure to the sedative solution, and all recovery behavior was normal.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study demonstrates the effectiveness of 25 mg eugenol/L administered as an immersion bath to sedate cutthroat trout (*Oncorhynchus clarkii lewisi*) to a handleable condition.

4. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% Eugenol) to Sedate Fingerling and Adult Rainbow Trout *Oncorhynchus mykiss* to Handleable. (Study No. AQS20E-11-EFF-HQSS-09)

Study Dates: May 5, 2011, to May 3, 2012

Study Location: Bozeman, MT

Study Design:

Eugenol was administered as a static immersion bath at 25 mg/L at water temperatures of about 10 and 16 °C. Sixty juvenile rainbow trout (mean length: 17.0 cm; mean weight: 55.9 g) and 60 adult rainbow trout (mean length: 40.4 cm; mean weight: 931.7 g) were used. Thirty fish of each size were exposed at each water temperature. The order of treatments was not randomized, and no controls were used. Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length with minimal to no

movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were monitored for survival for 24 hours. Water temperature and DO were measured once in bulk sedative solution containers, in each exposure container before a fish was placed in the solution, and in each recovery container before a fish was placed in the recovery water. Water samples for dose verification were collected from the top, middle, and bottom of each bulk sedative solution container on each exposure day.

Results:

Data on time to a handleable condition were summarized but not statistically analyzed. The mean and SD for time to sedation to a handleable condition and time to recovery for juvenile rainbow trout at 10 °C was 1.8 minutes (0.41) and 4.7 minutes (1.23), respectively. The mean and SD for time to sedation to a handleable condition and time to recovery for juvenile rainbow trout at 16 °C was 1.3 minutes (0.38) and 3.6 minutes (0.75), respectively.

The mean and SD for time to sedation to a handleable condition and time to recovery for adult rainbow trout at 10 °C was 1.7 minutes (0.36) and 6.4 minutes (1.9), respectively. The mean and SD for time to sedation to a handleable condition and time to recovery for adult rainbow trout at 16 °C was 1.3 minutes (0.19) and 4.8 minutes (1.35), respectively.

Water Quality: The mean water temperature and DO of the sedative solutions for the two temperatures were 10.5 °C and 9.1 mg/L, and 16.4 °C and 7.8 mg/L, respectively.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 25 mg eugenol/L.

Behavior: At 10 °C for the adult fish, head shaking was observed in 1 of 30 (3%) fish. At 10 °C for the juvenile fish, head shaking was observed in 1 of 30 (3%) fish and gill coughing was observed in 1 of 30 (3%) fish. At 16 °C for the adult fish, no abnormal behaviors were noted during sedation. At 16 °C for the juvenile fish, head shaking was observed in 1 of 30 (3%) fish. Behavior during recovery was considered normal in all fish. All abnormal behaviors only lasted a few seconds, and all fish recovered.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports that eugenol administered as an immersion bath at 25 mg eugenol/L is effective for the sedation of juvenile and adult rainbow trout (*Oncorhynchus mykiss*) to a handleable condition.

5. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% Eugenol) to Sedate Individual and Groups of Fingerling Lake Trout *Salvelinus namaycush* to Handleable. (Study No. AQS20E-11-EFF-08)

Study Dates: May 5, 2011, to March 13, 2012

Study Location: La Crosse, WI

Study Design:

Eugenol was administered as a static immersion bath at 25 mg eugenol/L. Eighty fingerling lake trout (mean length: 12.1 cm and mean weight: 14.9 g; based on the 40 individually sedated fish) were used and were arbitrarily assigned to 8 groups of 10 fish each. Fish in the first 4 groups were individually sedated and fish in the last 4 groups were sedated in groups of 10. The order of treatment was not randomized, and no controls were used. For all individual fish exposures, time to sedation to a handleable condition, time to recovery, general behavior of fish during sedation, and fish length and weight were recorded. For group exposure, time to sedation to a handleable condition and general behavior during sedation were recorded. Water alkalinity and hardness were measured in the source water. Water temperature, DO concentration, and pH were measured in the source water and sedative solutions. The concentration of eugenol within two individual exposure containers and one group exposure container was also verified. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Recovery time for group sedated fish was not recorded. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Results:

Data on time to handleable were summarized but not statistically analyzed. The mean time for individually sedated fish to become handleable was 3.2 minutes with SD of 0.4 minutes. The mean time to recovery for individually sedated fish was 8.0 minutes with SD of 1.48 minutes. The mean time for group sedated fish to become handleable was 2.4 minutes with SD of 0.37 minutes.

Water Quality: Hardness, alkalinity, and pH of the source water were 180 mg/L $CaCO_3$, 124 mg/L $CaCO_3$, and 7.6, respectively. Mean water temperature and mean DO concentration in the exposure containers used for individually sedated fish was 13.7 °C and 10.1 mg/L, respectively. Mean water temperature and mean DO concentration in the exposure containers used for group sedated fish was 14.2 °C and 9.8 mg/L, respectively.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 25 mg eugenol/L.

Behavior: Among the 40 individually sedated fish, 3 fish (8%) displayed head-shaking and 9 fish (23%) displayed coughing when placed in exposure containers. Eleven (27%) of the 40 group sedated fish displayed coughing when placed in exposure containers. Fish behavior during recovery was not recorded.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports that eugenol administered as an immersion bath at 25 mg eugenol/L is effective for both individual and group sedation of lake trout (*Salvelinus namaycush*) to a handleable condition. This study shows that small groups of lake trout (10 fish) can be sedated to a handleable condition together.

6. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% Eugenol) to Sedate Juvenile Walleye *Sander vitreus* to Handleable. (Study No. AQS20E-11-EFF-06)

Study Dates: May 5, 2011, to March 29, 2012

Study Location: La Crosse, WI

Study Design:

Objective: To evaluate the effectiveness of 40 mg eugenol/L administered in a static bath to sedate juvenile walleye to a handleable condition within 5 minutes.

Study Animals: 90 juvenile walleye; mean length: 13.4 cm; mean weight: 20.0 g (Note: mean length and weight were based on 60 fish in the eugenol treatment group and tricaine methanesulfonate treatment group).

Experimental Design: This study was conducted according to GCP. The study included 90 fish equally allocated to 3 treatment groups: eugenol, tricaine methanesulfonate, and benzocaine. The fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate group was included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 40 mg/L and tricaine methanesulfonate was administered at 80 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity and hardness were measured in the source water, and pH was measured in the source water and the bulk containers of sedative solution. The eugenol concentration was verified in 10 of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol

data were statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed α=0.05. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD for time to sedation to a handleable condition and time to recovery for walleye sedated with 40 mg/L eugenol was 2.0 minutes (0.42) and 10.2 minutes (2.06), respectively.

Water Quality: Mean water temperature and DO concentration during sedation were 18.2 °C and 9.4 mg/L, respectively. Water hardness, alkalinity, and pH were 176 mg/L $CaCO_3$, 132 mg/L $CaCO_3$, and 7.82, respectively.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 40 mg eugenol/L.

Behavior: Twelve of 30 (40%) walleye exposed to eugenol exhibited head shaking and/or coughing during sedation. During recovery, 1 of 15 (7%) walleye exposed to eugenol exhibited piping. These behaviors were temporary. There were no abnormal behaviors during sedation and recovery in walleye exposed to tricaine methanesulfonate.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study demonstrates the effectiveness of 40 mg eugenol/L administered as an immersion bath to sedate walleye (*Sander vitreus*) to a handleable condition.

7. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% Eugenol) to Sedate Juvenile Yellow Perch *Perca flavescens* to Handleable. (Study No. AQS20E-11-EFF-07)

Study Dates: May 5, 2011, to March 29, 2012

Study Location: La Crosse, WI

Study Design:

Objective: To evaluate the effectiveness of 40 mg eugenol/L administered in a static bath to sedate juvenile yellow perch to a handleable condition within 5 minutes.

Study Animals: 90 juvenile yellow perch; mean length: 10.5 cm; mean weight: 10.8 g (Note: mean length and weight were based on 60 fish in the eugenol treatment group and tricaine methanesulfonate treatment group).

Experimental Design: This study was conducted according to GCP. The study included 90 fish equally allocated to 3 treatment groups: eugenol, tricaine methanesulfonate, and benzocaine. The fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate group was included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 40 mg/L and tricaine methanesulfonate was administered at 80 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity and hardness were measured in the source water, and pH was measured in the source water and the bulk containers of sedative solution. The eugenol concentration was verified in 10 of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data were statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed $\alpha=0.05$. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD time to sedation to a handleable condition and time to recovery for yellow perch sedated with 40 mg eugenol/L was 3.1 minutes (0.88) and 9.7 minutes (2.48), respectively.

Water Quality: Mean water temperature and DO concentration during sedation were 18.0 °C and 9.8 mg/L, respectively. Water hardness, alkalinity, and pH were 176 mg/L $CaCO_3$, 132 mg/L $CaCO_3$, and 8.0, respectively.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 40 mg eugenol/L.

Behavior: Eight of 30 (27%) yellow perch exposed to eugenol exhibited head shaking during sedation. One of 30 (3%) yellow perch exposed to eugenol exhibited coughing during sedation. One of 30 (3%) yellow perch exposed to eugenol exhibited slight agitation during sedation. Behavior during recovery was normal. All fish exposed to tricaine methanesulfonate were normal during sedation. One of 30 (3%) yellow perch exposed to tricaine methanesulfonate exhibited head shaking during recovery. All abnormal behavior was temporary, and all fish recovered.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study demonstrates the effectiveness of 40 mg eugenol/L administered as an immersion bath to sedate yellow perch (*Perca flavescens*) to a handleable condition.

8. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% Eugenol) to Sedate Fingerling Yellow Perch *Perca flavescens* to Handleable. (Study No. AQS20E-11-EFF-HQSS-08)

Study Dates: May 5, 2011, to April 3, 2012

Study Location: La Crosse, WI

Study Design:

Eugenol was administered as a static immersion bath at 40, 60, and 80 mg eugenol/L. Thirty fingerling yellow perch (mean length: 7.6 cm; mean weight: 4.3 g) were used. Ten fish were individually exposed to each eugenol dose. The order of treatment was not randomized, and no controls were used. Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature, DO, and pH were measured in the source water and bulk sedative solutions. Water alkalinity and hardness were measured in the source water. The concentration of eugenol was verified in the 60 and 80 mg/L concentration exposure buckets. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Results:
Data on time to sedation to a handleable condition were summarized but not statistically analyzed. Results are included in Table II.1.

Table II.1. Mean and standard deviation (SD) time to sedation to a handleable condition (min) and recovery (min) for fingerling yellow perch.

Eugenol (mg/L)	Time to Handleable (min ± SD)	Time to Recovery (min ± SD)
40	1.9 ± 0.40	8.1 ± 2.30
60	1.7 ± 0.39	8.6 ± 0.90
80	1.6 ± 0.16	10.1 ± 0.83

Water Quality: Mean water temperature and mean DO concentration in the exposure buckets containing 40 mg eugenol/L were 18.4 °C and 9.7 mg/L, respectively. Mean water temperature and mean DO concentration in the exposure buckets containing 60 mg eugenol/L were 18.1 °C and 9.6 mg/L, respectively. Mean water temperature and mean DO concentration in the exposure buckets containing 80 mg eugenol/L were 17.9 °C and 9.7 mg/L, respectively. The pH values of the bulk working solutions for 40 mg eugenol/L, 60 mg eugenol/L, and 80 mg eugenol/L were 8.1, 8.0, and 8.1, respectively. Hardness, alkalinity, and pH of the source water were 170 mg/L $CaCO_3$, 210 mg/L $CaCO_3$, and 8.1, respectively.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 60 and 80 mg eugenol/L. Dose verification was not done for 40 mg eugenol/L.

Behavior: Eight (80%) of 10 fish exposed to 40 mg eugenol/L, 7 (70%) of 10 fish exposed to 60 mg eugenol/L, and 8 (80%) of 10 fish exposed to 80 mg eugenol/L exhibited slight agitation during sedation. One (10%) of the 10 fish exposed to 40 mg eugenol/L displayed an abnormal behavior of 'whole body shaking'. Among the 10 fish exposed to 80 mg eugenol/L, 2 fish (20%) displayed head shaking and 1 fish (10%) displayed extreme agitation during sedation.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports that eugenol administered as an immersion bath at 40, 60, and 80 mg eugenol/L is effective for the sedation of yellow perch (*Perca flavescens*) to the handleable condition.

9. Clinical Field Study

Title: Use of AQUI-S®20E and BENZOAK® to Sedate Walleye *Sander vitreus* to the Handleable Stage of Anesthesia. (Study No. AQUI-S®20E HQSS-02)

Study Dates: March 2, 2010, to October 3, 2011

Study Location: Moravia, IA

Study Design:

Objective: To evaluate the effectiveness of 60 mg eugenol/L administered in a static bath to sedate juvenile walleye individually and in groups to a handleable condition.

Study Animals: 240 juvenile walleye; mean length: 22.5 cm.

Experimental Design: Eighty fish were arbitrarily allocated to each of 3 treatment groups: eugenol, tricaine methanesulfonate, and benzocaine. To administer the treatments, the 80 fish were divided so that 40 fish were individually exposed and 40 fish were divided into 4 groups of 10 and exposed as a group. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. Information on the benzocaine treatment groups is not reported here.

Drug Administration: Eugenol was administered at 60 mg/L and tricaine methanesulfonate was administered at 150 mg/L. Both drugs were administered as static immersion baths.

Measurements and Observations: Ten fish were used to determine the health of the reference population prior to the start of the study. Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length were recorded for all individual fish exposures. For group exposure, time to sedation to a handleable condition, general behavior during sedation and recovery, and fish length were recorded. Water temperature and DO concentration were measured in all exposure containers. Water alkalinity and hardness were measured in the source water, and pH was measured in the source water and the exposure containers for individual fish. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated and recovered under static conditions. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. The only data set that was statistically analyzed was the 40 fish individually sedated with eugenol. Treatment success was analyzed using an exact binomial test at two-tailed $\alpha=0.05$. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Descriptive statistics were computed for the groups that were sedated collectively to accomplish a secondary objective, which was to compare the mean time to a handleable condition between fish sedated individually and fish sedated in groups.

Results:

All 40 fish individually sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.00027).

The mean and SD of time to sedation to a handleable condition and mean time to recovery for juvenile walleye sedated individually with 60 mg eugenol/L was 0.9 minutes (0.196) and 1.86 minutes (0.581), respectively.

The mean time to sedation to a handleable condition and SD for group-exposed fish at 60 mg eugenol/L was 1.02 minutes (0.219).

Water Quality: Water hardness, alkalinity, and pH of the source water were 97 mg/L $CaCO_3$, 64 mg/L $CaCO_3$, and 9.89, respectively. The mean pH of the individual exposure containers was 10.45. Mean water temperature was 20.9 °C and mean DO concentration was 10.2 mg/L for sedative solutions prepared for individual sedation.

Behavior: One fish (individually exposed) experienced headshaking after initial exposure to eugenol. It lasted a brief time, and all recovery behavior was normal.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports that eugenol administered as an immersion bath at 60 mg eugenol/L is effective for both individual and group sedation of walleye (*Sander vitreus*) to a handleable condition. This study shows that small groups of walleye (10 fish) can be sedated to a handleable condition together.

10. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% eugenol) to Sedate Fingerling Nile Tilapia *Oreochromis niloticus* to Handleable. (Study No. AQS20E-11-EFF-11)

Study Dates: May 5, 2011, to February 9, 2012

Study Location: Carbondale, IL

Study Design:

Objective: To evaluate the effectiveness of 60 mg eugenol/L administered in a static bath to sedate fingerling Nile tilapia to a handleable condition within 5 minutes.

Study Animals: 90 fingerling Nile tilapia; mean length: 11.0 cm; mean weight: 25.9 g (Note: mean length and weight were based on 60 fish in the eugenol treatment and tricaine methanesulfonate groups.)

Experimental Design: The study was conducted according to GCP. The study included 90 fish equally allocated to 3 treatment groups: benzocaine, tricaine methanesulfonate, and eugenol. To administer the treatments, the 90 fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 60 mg/L and tricaine methanesulfonate was administered at 150 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity, hardness, ammonia, nitrite, and nitrate were measured in the source water, and pH was measured in the source water and the bulk containers of sedative solution. The eugenol concentration was verified in 10 out of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data set was statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed $\alpha=0.05$. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD time to sedation to a handleable condition and time to recovery for fingerling Nile tilapia sedated with 60 mg eugenol/L was 1.8 minutes (0.57) and 3.8 minutes (1.05), respectively.

Water Quality: Water hardness, alkalinity, and pH of the source water were 58 mg/L $CaCO_3$, 599 mg/L $CaCO_3$, and 8.9, respectively. Source water mean ammonia, nitrite, and nitrate were 0.08 mg/L, 0.008 mg/L, and 12.2 mg/L, respectively. Mean water pH was 8.8 and 8.1 in the eugenol and tricaine methanesulfonate bulk working solutions, respectively. Mean water temperature was 25.7 °C and mean DO concentration was 7.7 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 60 mg eugenol/L.

Behavior: Piping was observed in 1 of the 30 (3%) fish exposed to eugenol and in 3 of the 30 (10%) fish exposed to tricaine methanesulfonate when placed in the exposure bucket. Agitation was observed in 20 of the 30 (67%) exposed to eugenol, and 11 of the fish exposed to tricaine methanesulfonate when placed in

the exposure bucket. During recovery, whirling was observed in 2 of the 30 (7%) fish following exposure to eugenol and 7 of the 30 (23%) fish following exposure to tricaine methanesulfonate. One of the 30 (3%) fish was also slightly agitated following exposure to tricaine methanesulfonate. These behaviors were temporary and ceased as sedation or recovery progressed.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study demonstrates the effectiveness of 60 mg eugenol/L administered as an immersion bath to sedate Nile tilapia (*Oreochromis niloticus*) to a handleable condition.

11. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% eugenol) to Sedate Subadult Blue Catfish *Ictalurus furcatus* to Handleable. (Study No. AQS20E-11-EFF-09)

Study Dates: May 5, 2011, to February 9, 2012

Study Location: Carbondale, IL

Study Design:

Objective: To evaluate the effectiveness of 60 mg eugenol/L administered in a static bath to sedate subadult blue catfish to a handleable condition within 5 minutes.

Study Animals: 90 subadult blue catfish; mean length: 21.9 cm; mean weight: 77.3 g (Note: mean length and weight were based on 60 fish in the eugenol treatment and tricaine methanesulfonate groups.)

Experimental Design: This study was conducted according to GCP. The study included 90 fish equally allocated to 3 treatment groups: benzocaine, tricaine methanesulfonate, and eugenol. To administer the treatments, the 90 fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 60 mg/L and tricaine methanesulfonate was administered at 150 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity, hardness, ammonia, nitrite, and nitrate were measured in the source water. The eugenol concentration was verified in 10 out of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal

swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data set was statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed α=0.05. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

Time to sedation to a handleable condition and time to recovery were inadvertently not recorded for one fish. All remaining 29 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0031).

The mean and SD of time to sedation to a handleable condition and time to recovery for subadult blue catfish sedated with 60 mg eugenol/L was 1.4 minutes (0.39) and 5.4 minutes (1.85), respectively.

Water Quality: Water hardness and alkalinity of the source water were 138 mg/L $CaCO_3$ and 124 mg/L $CaCO_3$, respectively. Source water mean ammonia, nitrite, and nitrate were 0.20 mg/L, 0.02 mg/L, and 11.6 mg/L, respectively. Mean water temperature was 24.0 °C and mean DO concentration was 7.5 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 60 mg eugenol/L.

Behavior: Five out of 29 (17%) fish were slightly agitated when placed in the eugenol bucket. Piping was observed in 1 of 30 (3%) fish and slight agitation was observed in 8 of 30 (27%) fish exposed to tricaine methanesulfonate when placed in the exposure bucket. These behaviors lasted only a brief period of time after initial exposure to the sedative solution, and all recovery behavior was normal.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study demonstrates the effectiveness of 60 mg eugenol/L administered as an immersion bath to sedate blue catfish (*Ictalurus furcatus*) to a handleable condition.

12. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% eugenol) to Sedate Adult Hybrid Striped Bass *Morone chrysops* x *M. saxatilis* to Handleable. (Study No. AQS20E-11-EFF-10)

Study Dates: May 5, 2011, to February 9, 2012

Study Location: Carbondale, IL

Study Design:

Objective: To evaluate the effectiveness of 60 mg eugenol/L administered in a static bath to sedate adult hybrid striped bass to a handleable condition within 5 minutes.

Study Animals: 90 adult hybrid striped bass; mean length: 38.4 cm; mean weight: 723.1 g (Note: mean length and weight were based on 60 fish in the eugenol treatment and tricaine methanesulfonate groups.)

Experimental Design: The study was conducted according to GCP. The study included 90 fish equally allocated to 3 treatment groups: benzocaine, tricaine methanesulfonate, and eugenol. To administer the treatments, the 90 fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 60 mg/L and tricaine methanesulfonate was administered at 150 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity, hardness, ammonia, nitrite, and nitrate were measured in the source water. The eugenol concentration was verified in 10 out of 30 exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data set was statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed $\alpha=0.05$. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD time to sedation to a handleable condition and time to recovery for adult hybrid striped bass sedated with 60 mg eugenol/L was 0.9 minutes (0.19) and 5.2 minutes (1.08), respectively.

Water Quality: Water hardness and alkalinity of the source water were 142 mg/L $CaCO_3$ and 141 mg/L $CaCO_3$, respectively. Source water mean ammonia, nitrite, and nitrate were 0.17 mg/L, 0.01 mg/L, and 13.4 mg/L, respectively. Mean water temperature was 24.3 °C and mean DO concentration was 7.9 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 60 mg eugenol/L.

Behavior: Four of the 30 (13%) fish were slightly agitated during exposure to eugenol. Following exposure to eugenol, spitting was observed in 6 of the 30 (20%) fish during recovery. Seventeen of the 30 (57%) fish exposed to tricaine methanesulfonate were slightly agitated when placed in the exposure bucket. Following exposure to tricaine methanesulfonate, 27 of the 30 (90%) fish exhibited spitting and 12 of the 30 (40%) fish exhibited piping during recovery. The abnormal behaviors observed were temporary and ceased as sedation or recovery progressed.

Adverse Reaction: One (3%) of the 30 fish exposed to tricaine methanesulfonate died during the post-recovery period. The fish had no external signs of trauma or disease. The cause of death was unknown.

Conclusion: This study demonstrates the effectiveness of 60 mg eugenol/L administered as an immersion bath to sedate hybrid striped bass (*Morone chrysops* x *M. saxatilis*) to a handleable condition.

13. Clinical Field Study

Title: The Efficacy of AQUI-S®20E (10% eugenol) to Sedate Adult Hybrid Striped Bass *Morone chrysops* x *M. saxatilis* to Handleable. (Study No. AQS20E-11-EFF-02)

Study Dates: May 5, 2011, to January 20, 2012

Study Location: Carbondale, IL

Study Design:

Objective: To evaluate the effectiveness of 60 mg eugenol/L administered in a static bath to sedate adult hybrid striped bass to a handleable condition within 5 minutes.

Study Animals: 90 adult hybrid striped bass; mean length: 32.7 cm; mean weight: 447.6 g (Note: mean length and weight were based on 60 fish in the eugenol treatment and tricaine methanesulfonate groups.)

Experimental Design: The study included 90 fish equally allocated to 3 treatment groups: benzocaine, tricaine methanesulfonate, and eugenol. To administer the treatments, the 90 fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 60 mg/L and tricaine methanesulfonate was administered at 150 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity, hardness, ammonia, nitrite, and nitrate were measured in the source water, and pH was measured in the source water and the bulk working solutions. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data set was statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed $\alpha=0.05$. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD time to sedation to a handleable condition and time to recovery for adult hybrid striped bass sedated with 60 mg/L eugenol was 0.9 minutes (0.16) and 7.5 minutes (0.99), respectively.

Water Quality: Water hardness, alkalinity, and pH of the source water were 53.8 mg/L $CaCO_3$, 72 mg/L $CaCO_3$, and 8.3, respectively. Mean ammonia, nitrite, and nitrate were 0.19 mg/L, 0.03 mg/L, and 13.3 mg/L, respectively. Mean water pH was 8.2 and 7.2 in the eugenol and tricaine methanesulfonate bulk working solutions, respectively. Mean water temperature was 24.8 °C and mean DO concentration was 7.4 mg/L in the exposure containers.

Behavior: All behavior during sedation was considered normal. During recovery, piping of the fish at the surface of the water was observed in 19 of 30 (63%) hybrid striped bass following exposure to eugenol and 27 of 30 (97%) following exposure to tricaine methanesulfonate. Piping only lasted a few seconds, and all fish recovered.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports the effectiveness of 60 mg eugenol/L administered as an immersion bath to sedate hybrid striped bass (*M. chrysops* x *M. saxatilis*) to a handleable condition.

14. Clinical Field Study

Title: The Efficacy of AQUI-S®20E (10% eugenol) to Sedate Adult Blue Catfish *Ictalurus furcatus* to Handleable. (Study No. AQS20E-11-EFF-03)

Study Dates: May 5, 2011, to January 20, 2012

Study Location: Carbondale, IL

Study Design:

Objective: To evaluate the effectiveness of 60 mg eugenol/L administered in a static bath to sedate adult blue catfish to a handleable condition within 5 minutes.

Study Animals: 90 subadult blue catfish; mean length: 21.1 cm; mean weight: 62.4 g (Note: mean length and weight were based on 60 fish in the eugenol treatment and tricaine methanesulfonate groups.)

Experimental Design: The study included 90 fish equally allocated to 3 treatment groups: benzocaine, tricaine methanesulfonate, and eugenol. To administer the treatments, the 90 fish were divided into 6 groups of 15 fish each, and 2 groups were randomly allocated to each treatment. The order of treatments was randomized. The tricaine methanesulfonate groups were included to maintain masking in the study. Information on the benzocaine treatment group is not reported here.

Drug Administration: Eugenol was administered at 60 mg/L and tricaine methanesulfonate was administered at 150 mg/L. Both drugs were administered as static immersion baths. Fish were individually sedated and recovered.

Measurements and Observations: Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. Water temperature and DO concentration were measured in exposure containers. Water alkalinity, hardness, ammonia, nitrite, and nitrate were measured in the source water, and pH was measured in the source water and the bulk working solutions. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated

under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Statistical Methods: The primary response variable was treatment success or failure. An individual fish was classified as a treatment success if it took the fish 5 minutes or less to become sedated to a handleable condition. Only the eugenol data set was statistically analyzed. Treatment success was analyzed using an exact binomial test at two-tailed α=0.05. Eugenol was considered effective if the percentage of successes was significantly different from 80% and at least 80% of the fish became sedated within 5 minutes.

Results:

All 30 fish sedated with eugenol were treatment successes. This percentage was significantly different from 80% (P-value = 0.0025).

The mean and SD time to sedation to a handleable condition and time to recovery for adult blue catfish sedated with 60 mg/L eugenol was 1.6 minutes (0.29) and 7.7 minutes (1.23), respectively.

Water Quality: Water hardness, alkalinity, and pH of the source water were 55.2 mg/L $CaCO_3$, 112 mg/L $CaCO_3$, and 8.6, respectively. Water ammonia, nitrite, and nitrate were 0.0 mg/L, 0.11 mg/L, and 5.5 mg/L, respectively. Mean water pH was 8.5 and 7.5 in the eugenol and tricaine methanesulfonate exposure containers, respectively. Mean water temperature was 24.4 °C and mean DO concentration was 7.4 mg/L in the sedative solutions.

Behavior: There were no abnormal behaviors noted in the blue catfish during sedation or recovery.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports the effectiveness of 60 mg eugenol/L administered as an immersion bath to sedate blue catfish (*Ictalurus furcatus*) to a handleable condition.

15. Clinical Field Study

Title: The Efficacy of AQUI-S®20E (10% Eugenol) to Sedate Subadult and Adult Hybrid Striped Bass *Morone chrysops* x *M. saxatilis* to Handleable. (Study Nos. AQUI-S®20E HQS-04 and AQUI-S®20E HQS-05)

Study Dates: May 5, 2011, to February 3, 2012

Study Location: Carbondale, IL

Study Design:

Eugenol was administered as a static immersion bath at 48, 60, and 72 mg/L at water temperatures of about 16 to 17 and 22 °C. Sixty subadult hybrid striped bass (mean length: 23.6 cm; mean weight: 161.4 g) and 50 adult hybrid striped

bass (mean length: 36.7 cm; mean weight: 714.7 g) were used. Ten fish of each size were exposed to each eugenol dose. Adult hybrid striped bass were not exposed to the 60 mg/L eugenol dose at the 22 °C water temperature. The order of treatments was not randomized, and no controls were used. Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours. Water temperature and DO were measured once in bulk sedative solution containers and in each exposure container before a fish was placed in the solution. Water hardness and alkalinity were measured in source water. pH was measured once in source water and once in each sedative tub immediately after preparation.

Results:

Data on time to sedation to a handleable condition were summarized but not statistically analyzed. Results are included in Tables II.2, II.3, II.4, and II.5.

Table II.2. Mean and standard deviation (SD) time to sedation to a handleable condition (min) and recovery (min) for adult hybrid striped bass at a water temperature of 17 °C.

Eugenol (mg/L)	Time to Handleable (min ± SD)	Time to Recovery (min ± SD)
48	1.5 ± 0.22	5.7 ± 1.03
60	1.7 ± 0.21	6.5 ± 0.89
72	1.5 ± 0.10	11.7 ± 4.85

Table II.3. Mean and standard deviation (SD) time to sedation to a handleable condition (min) and recovery (min) for adult hybrid striped bass at a water temperature of 22 °C.

Eugenol (mg/L)	Time to Handleable (min ± SD)	Time to Recovery (min ± SD)
48	0.9 ± 0.09	7.0 ± 1.11
72	0.9 ± 0.21	6.0 ± 1.47

Table II.4. Mean and standard deviation (SD) time to sedation to a handleable condition (min) and recovery (min) for subadult hybrid striped bass at a water temperature of 16 °C.

Eugenol (mg/L)	Time to Handleable (min ± SD)	Time to Recovery (min ± SD)
48	1.9 ± 0.39	13.5 ± 3.37
60	1.7 ± 0.25	12.9 ± 2.00
72	1.8 ± 0.22	14.4 ± 1.82

Table II.5. Mean and standard deviation (SD) time to sedation to a handleable condition (min) and recovery (min) for subadult hybrid striped bass at a water temperature of 22 °C.

Eugenol (mg/L)	Time to Handleable (min ± SD)	Time to Recovery (min ± SD)
48	0.8 ± 0.10	4.4 ± 1.16
60	1.0 ± 0.18	5.2 ± 0.8
72	1.0 ± 0.12	4.8 ± 0.98

Water Quality: For the adult fish, the mean water temperature and DO of the sedative solutions for the two temperatures were 17.1 °C and 9.5 mg/L, and 22.1 °C and 9.2 mg/L. For the subadult fish, the mean water temperature and DO of the sedative solutions for the two temperatures were 16.1 °C and 9.8 mg/L, and 22.1 °C and 8.5 mg/L. Source water pH ranged from 8.0 to 8.8. The pH of the sedative solution ranged from 7.8 to 8.9. Water hardness and alkalinity ranged from 54 to 70 mg/L $CaCO_3$ and 66 to 250 mg/L $CaCO_3$, respectively. Mean nitrite, nitrate, and ammonia levels ranged from 0.01 to 0.2 mg/L, 9.5 to 12.5 mg/L, and 0.08 to 0.88 mg/L, respectively.

Behavior: At 17 °C, 5 of 30 (16.7%) adult hybrid striped bass displayed agitated behavior during sedation, and 1 of 30 (3.3%) fish was observed piping during recovery. At 22 °C, 1 of 20 (5%) adult hybrid striped bass displayed agitated behavior during sedation and 7 of 20 (35%) were observed piping during recovery. At 16 °C, no subadult hybrid striped bass displayed agitated behavior during sedation and 1 of 30 (3.3%) fish was observed piping during recovery. At 22 °C, 1 of 30 (3.3%) subadult hybrid striped bass displayed agitated behavior during sedation and 1 of 30 (3.3%) was observed piping during recovery.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports that eugenol administered as an immersion bath at 48, 60, and 72 mg eugenol/L is effective for the sedation of subadult hybrid striped bass (*M. chrysops* x *M. saxatilis*) to a handleable condition. In addition, this study supports that eugenol administered as an immersion bath at 48 and 72 mg eugenol/L is effective for the sedation of adult hybrid striped bass (*M. chrysops* x *M. saxatilis*) to a handleable condition.

16. Clinical Field Study

Title: The Efficacy of AQUI-S®20E (10% Eugenol) to Sedate Individual and Groups of Subadult Channel Catfish *Ictalurus punctatus* to Handleable. (Study No. AQS20E-11-EFF-04)

Study Dates: May 5, 2011, to February 1, 2012

Study Location: Carbondale, IL

Study Design:

Eugenol was administered as a static immersion bath at 60 mg/L. Eighty subadult channel catfish (mean length: 27.3 cm, mean weight: 115.7 g; based on

the 40 individually sedated fish) were used and arbitrarily assigned to 8 groups of 10 fish each. Fish in the first 4 groups were individually exposed and fish in the last 4 groups were exposed in groups of 10. The order of treatments was not randomized, and no controls were used. Time to sedation to a handleable condition, time to recovery, general behavior during sedation, and fish length and weight were recorded for all individual fish exposures. For group exposure, time to sedation to a handleable condition and general behavior during sedation were recorded. Water alkalinity, hardness, ammonia, nitrite, and nitrate were measured in the source water. DO concentration and pH were measured in the source water and the exposure containers. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Recovery time for group-treated fish was not recorded. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours.

Results:

Data on time to a handleable condition were summarized but not statistically analyzed. The mean and SD of time to sedation to a handleable condition for subadult channel catfish sedated individually was 1.4 minutes (0.24). The mean time to recovery for individual fish was 4.9 minutes (0.77). The mean time for sedation to a handleable condition for group-exposed fish was 1.1 minutes (0.14).

Water Quality: Hardness, alkalinity, and pH of the source water were 55.2 mg/L $CaCO_3$, 112 mg/L $CaCO_3$, and 8.6, respectively. Mean nitrite, nitrate, and ammonia levels of the source water were 0.1 mg/L, 5.6 mg/L, and 0.0 mg/L, respectively. Mean water temperature was 23.6 °C and mean DO concentration was 8.4 mg/L in the exposure containers.

Behavior: One fish individually exposed displayed slightly agitated behavior during exposure to eugenol; the behavior lasted only a brief time and the fish recovered normally.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports that eugenol administered as an immersion bath at 60 mg eugenol/L is effective for both individual and group sedation of channel catfish (*Ictalurus punctatus*) to a handleable condition. This study shows that small groups of channel catfish (10 fish) can be sedated to a handleable condition together.

17. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% Eugenol) to Sedate Fingerling Common Carp *Cyprinus carpio* to Handleable. (Study No. AQS20E-11-EFF-HQSS-06)

Study Dates: May 5, 2011, to March 22, 2012

Study Location: La Crosse, WI

Study Design:

Eugenol was administered as a static immersion bath at 40 mg/L. Ten fingerling common carp (mean length: 7.1 cm, mean weight 5.1 g) were sedated in individual containers. Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours. Water temperature and DO were measured once in the bulk sedative solution container and in each exposure container before a fish was placed in the solution. Water hardness and alkalinity were measured in source water. pH was measured once in source water and once in the bulk working solution immediately after preparation. One water sample was collected from the eugenol bulk working solution for dose verification.

Results:

Data on time to sedation to a handleable condition and time to recovery were summarized but not statistically analyzed. The mean time for fish exposed to eugenol to become handleable was 1.9 minutes (range 1.5 to 2.4 minutes). The mean time to recovery was 5.9 minutes (range 4.6 to 7.6 minutes).

Water Quality: Water hardness, alkalinity, and pH of the source water were 170 mg/L $CaCO_3$, 127 mg/L $CaCO_3$, and 8.1, respectively. Water pH was 7.7 in the eugenol bulk working solution. Mean water temperature was 18.4 °C and mean DO concentration was 8.6 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 40 mg/L eugenol.

Behavior: No abnormal behaviors were noted during exposure or recovery from eugenol.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports the effectiveness of 40 mg eugenol/L administered as an immersion bath to sedate common carp (*Cyprinus carpio*) to a handleable condition.

18. Clinical Field Study

Title: The Efficacy of AQUI-S20E (10% eugenol) to Sedate Fingerling Fathead Minnows *Pimephales promelas* to handleable. (Study No. AQS20E-11-EFF-HQSS-07)

Study Dates: May 5, 2011, to March 22, 2012

Study Location: La Crosse, WI

Study Design:

Eugenol was administered as a static immersion bath at 40 mg/L. Ten fingerling fathead minnows (mean length: 7.1 cm, mean weight: 3.6 g) were sedated in individual containers. Time to sedation to a handleable condition, time to recovery, general behavior during sedation and recovery, and fish length and weight were recorded. A fish was identified as sedated to a handleable condition when the fish could be easily handled and measured for length and weight with minimal to no movement. A fish was considered recovered from sedation when it regained equilibrium, resumed normal swimming behavior, and could avoid obstacles in its path. Fish were sedated under static conditions, and allowed to recover in fresh, flowing water. Fish were monitored for survival for 24 hours. Water temperature and DO were measured once in the bulk sedative solution container and in each exposure container before a fish was placed in the solution. Water hardness and alkalinity were measured in source water. pH was measured once in source water and once in the bulk working solution immediately after preparation. One water sample was collected from the eugenol bulk working solution for dose verification.

Results:

Data on time to sedation to a handleable condition and time to recovery were summarized but not statistically analyzed. The mean time for fish exposed to eugenol to become handleable was 1.4 minutes (range 1.0 to 2.0 minutes). The mean time to recovery was 6.0 minutes (range 4.4 to 7.7 minutes).

Water Quality: Water hardness, alkalinity, and pH of the source water were 170 mg/L $CaCO_3$, 127 mg/L $CaCO_3$, and 8.1 respectively. Water pH was 7.7 in the eugenol bulk working solutions. Mean water temperature was 18.6 °C and mean DO concentration was 8.7 mg/L in the exposure containers.

Dose Verification: Results showed that the drug administration was appropriate to achieve a concentration of 40 mg eugenol/L.

Behavior: One of 10 (10%) fathead minnows appeared slightly agitated when placed in the eugenol; it lasted only a short time. All fish had a normal recovery following exposure to eugenol.

Adverse Reaction: No adverse reactions were reported in this study.

Conclusion: This study supports the effectiveness of 40 mg eugenol/L administered as an immersion bath to sedate fathead minnows (*Pimephales promelas*) to a handleable condition.

III. TARGET ANIMAL SAFETY

A. Margin of Safety Study

Title: The Safety of AQUI-S®20E (10% Eugenol) as a Sedative to Rainbow Trout *Oncorhynchus mykiss.* (Study No. AQS20E-11-TAS-FISH-02)

Study Dates: May 3, 2012, to June 11, 2013

Study Location: Bozeman, MT

Study Design:

Objective: To identify the maximum safe exposure durations, exposure-duration margins of safety, and safety breakpoint intervals for small rainbow trout exposed to 0 (control), 40, or 60 mg eugenol/L at a water temperature of 15 °C.

Study Animals: 590 fingerling rainbow trout; mean total length and weight of test fish at the start of the study were 5.8 cm and 2.3 g.

Experimental Design:

The study was conducted according to Good Laboratory Practices (GLP). Groups of 15 test fish were exposed to 0, 40 (1X), or 60 (1.5X) mg eugenol/L for 1 of 4 exposure durations. Durations were selected to approximate 50 to 100% survival at each concentration. Each of the 12 combinations of eugenol concentration and exposure duration was replicated 3 times during 3 separate 24-hour periods. An exposure group comprised of an exposure bucket and recovery tank pair; fish were randomly allocated to these exposure groups prior to exposure to eugenol.

A preliminary study was conducted on 30 fish from the reference population to establish an estimated time to sedation to a handleable condition at the 80th percentile (ET80) for each concentration. The ET80 values were then used to determine the exposure durations (T1 to T4) for each eugenol test concentration (Table III.1). The exposure duration for the control groups (0 mg/L) was set to match the 1X exposure durations.

Table III.1. Exposure durations (T1, T2, T3, or T4) for each concentration of eugenol during testing on rainbow trout.

Eugenol concentration	T1 (minutes)	T2 (minutes)	T3 (minutes)	T4 (minutes)
0 mg/L (0X)	3.5	4.5	6.5	8.75
40 mg/L (1X)	3.5	4.5	6.5	8.75
60 mg/L (1.5X)	2.25	2.75	3.5	4.75

Drug Administration: Eugenol was administered at either 40 or 60 mg eugenol/L. The control article was unadulterated hatchery water. All treatment administration was performed in static baths, but recovery was in fresh, flowing water.

Measurements and Observations: From a reference population, 20 healthy fish were collected and examined to establish a baseline for fish health. The examinations included a gross morphologic (20 fish) and microscopic evaluation (10 fish). Source hatchery water was tested for hardness, alkalinity, pH, and water temperature. The pH of the eugenol bulk working solutions was measured. During exposure, fish were monitored for behavior, water temperature and DO concentration in exposure buckets were measured, and water samples were collected for dose verification. In the 24-hour period following exposure, fish were monitored for behavior and mortality. At 24-hour post-exposure, four live fish were collected from each tank for gross morphologic and microscopic evaluation. Any fish that died prior to the end of the 24-hour post-exposure period were also evaluated for gross morphologic and microscopic evaluation. Microscopic evaluation included review of gill, posterior kidney, and liver tissues. These tissues were chosen based on previous information that suggested these were the target tissues.

Statistical Methods: Survival data were analyzed using a generalized linear model with dose as a fixed effect and duration and the dose x duration interaction as covariates. The model assumed a binomial distribution and employed a logit link. For each combination of drug concentration and exposure duration, a 95% confidence interval (CI) for percent survival was estimated by back transforming the least squares estimates from the analysis. Frequency of normal and abnormal behaviors observed during exposure and recovery were tabulated. Prevalence or severity of normal and abnormal lesions observed in sampled test fish was also summarized. The dichotomized versions of pathological (scores of 4 or 5) vs non-pathological findings (scores 0 to 3) were analyzed using a generalized linear model. Statistical significance was indicated when $P < 0.10$.

Results:

Mortality: Table III.2 summarizes the mean survival for each concentration at the exposure durations listed in Table III.1. Estimates in Table III.2 are based on a generalized linear model with dose as a fixed effect and duration and the dose x duration interaction as covariates. The model assumed a binomial distribution and employed logit link. The values presented above are back-transformed from the original analysis.

Maximum exposure durations at 40 and 60 mg eugenol/L, where survival was acceptable (i.e., ≥95%), were approximately 6.5 and 3.5 minutes, respectively.

Safety breakpoint intervals (the exposure duration range during which mean survival of test fish dropped below 95%) for 40 and 60 mg eugenol/L were 6.5 to 8.75 min and 3.5 to 4.75 min, respectively. Margins of safety (the time difference between the maximum safe exposure duration and the time it takes to sedate 80% of fish to a handleable condition) for each concentration tested were 5.5 min and 2.9 min, respectively (Table III.3).

Table III.2. Model-based estimates of mean percent survival and 95% confidence interval (CI) for rainbow trout exposed to 0, 40, or 60 mg eugenol/L.

Eugenol concentration	Duration (minutes)	Mean (95% CI)
0 mg/L	3.5	100.0% (100%, 100%)
0 mg/L	4.5	100.0% (100%, 100%)
0 mg/L	6.5	100.0% (100%, 100%)
0 mg/L	8.75	100.0% (100%, 100%)
40 mg/L	3.5	99.4% (97.0%, 99.9%)
40 mg/L	4.5	98.8% (95.7%, 99.7%)
40 mg/L	6.5	95.1% (90.7%, 97.5%)
40 mg/L	8.75	80.1% (69.3%, 87.7%)
60 mg/L	2.25	98.3% (94.9%, 99.5%)
60 mg/L	2.75	97.4% (93.9%, 98.9%)
60 mg/L	3.5	95.1% (91.4%, 97.2%)
60 mg/L	4.75	86.5% (76.7%, 92.6%)

Table III.3. Margin of safety based on longest exposure time and determined ET80, where the ET80 is 80th percentile time to sedation to a handleable condition for rainbow trout.

Eugenol concentration	Longest safe exposure duration (minutes)	ET80 (minutes)	Safety Margin (minutes)
40 mg/L (1X)	6.5 min	1.02 min	5.48 min
60 mg/L (1.5X)	3.5 min	0.62 min	2.88 min

Fish health and Histopathology: The reference population was considered healthy prior to study conduct. No gross lesions were seen in any fish in any exposure group.

Microscopic findings with marked scores (4/5) in treated fish included gill hypertrophy, posterior kidney degeneration, posterior kidney melanomacrophages, liver degeneration, liver necrosis, and liver vacuolation. These findings were low in frequency, also present in the control fish, or considered clinically insignificant. A significant difference was not detected when comparing lesions or cellular changes in the 0X T4 and the 1X T4 exposure groups or the 0X T4 and 1.5X T4 exposure groups.

Behavior: Behavior was normal in all four 0X exposure durations. Headshaking was observed in >75% of the fish upon immersion in the eugenol solution for both the 1X and 1.5X groups. No headshaking was observed in any of the control fish. Headshaking lasted only a brief period of time (15 to 30 seconds) after initial exposure to the sedative solution, and all recovery behavior was normal.

Dose verification: Results showed that the drug administration was appropriate to achieve a concentration of 40 or 60 mg eugenol/L.

Water Quality: Water hardness, alkalinity, water temperature, and pH of the source water were 214 mg/L $CaCO_3$, 152 mg/L $CaCO_3$, 14.7 °C, and 7.4, respectively. Mean water pH was 7.58 in the eugenol bulk working solutions. Mean water temperature was 15.2 °C and mean DO concentration was 7.7 mg/L in the exposure containers at the start of exposure.

Conclusions: The study demonstrates that eugenol is safe for use on rainbow trout when administered in a static bath at a dose of 40 mg eugenol/L for sedation to a handleable condition. There is an adequate margin of safety above 40 mg eugenol/L based on concentration and duration of exposure

B. Margin of Safety Study

Title: The Safety of AQUI-S®20E (10% Eugenol) as a Sedative to Channel Catfish *Ictalurus punctatus.* (Study No. AQS20E-11-TAS-FISH-04)

Study Dates: May 3, 2012, to December 3, 2013

Study Location: Bozeman, MT

Study Design:

Objective: To identify the maximum safe exposure durations, exposure-duration margins of safety, and safety breakpoint intervals for channel catfish exposed to 0 (control), 100, or 150 mg eugenol/L at a water temperature of 26 °C.

Study Animals: 590 fingerling channel catfish; mean total length and weight of test fish at the start of the study were 5.3 cm and 1.5 g.

Experimental Design:

The study was conducted according to GLP. Groups of 15 test fish were exposed to 0, 100 (1X), or 150 (1.5X) mg eugenol/L for 1 of 4 exposure durations. Durations were selected to approximate 50 to 100% survival at each concentration. Each of the 12 combinations of eugenol concentration and exposure duration was replicated 3 times during 3 separate 24-hour periods. An exposure group was comprised of an exposure bucket and recovery tank pair. A completely randomized design was used to allocate 12 exposure regimens among the 12 experimental units, and to determine the order in which the experimental units received fish from the reference population.

A preliminary study was conducted on 30 fish from the reference population to establish an estimated time to sedation to a handleable condition at the 80th percentile (ET80) for each concentration. The ET80 values were then used to determine the exposure durations (T1 to T4) for each eugenol test concentration (Table III.4). The exposure duration for the control groups (0 mg/L) was set to match the 1X exposure durations.

Table III.4. Exposure durations (T1, T2, T3, or T4) for each concentration of eugenol during testing on channel catfish.

Eugenol concentration	T1 (minutes)	T2 (minutes)	T3 (minutes)	T4 (minutes)
0 mg/L (0X)	3.5	4.5	8.0	12.0
100 mg/L (1X)	3.5	4.5	8.0	12.0
150 mg/L (1.5X)	2.5	4.0	6.0	10.0

Drug Administration: Eugenol was administered at either 100 or 150 mg eugenol/L. The control article was unadulterated hatchery water. All treatment administration was performed in static baths, but recovery was in fresh, flowing water.

Measurements and Observations: From a reference population, 20 healthy fish were collected and examined to establish a baseline for fish health. The examinations included a gross morphologic (20 fish) and histological evaluation (10 fish). Source hatchery water was tested for hardness, alkalinity, pH, and water temperature. The pH of the eugenol bulk working solutions was measured. During exposure, fish were monitored for behavior, water temperature and DO concentration in exposure buckets were measured, and water samples were collected for dose verification. In the 24-hour period following exposure, fish were monitored for behavior and mortality. At 24 hours post-exposure, four live fish were collected from each tank for gross morphologic and histological evaluation. Fish that died prior to the end of the 24-hour post-exposure period were also evaluated (up to four per tank) for gross morphologic and histological evaluation. Histological evaluation included review of gill, posterior kidney, and liver tissues. These tissues were chosen based on previous information that suggested these were the target tissues.

Statistical Methods: Survival data were analyzed using a generalized linear model with dose as a fixed effect and duration and the dose x duration interaction as covariates. The model assumed a binomial distribution and employed a logit link. For each combination of drug concentration and exposure duration, a 95% CI for percent survival was estimated by back transforming the least squares estimates from the analysis. Frequency of normal and abnormal behaviors observed during exposure and recovery were tabulated. Prevalence or severity of normal and abnormal lesions observed in sampled test fish were also summarized. The dichotomized versions of pathological (scores of 4 or 5) vs non-pathological findings (scores 0 to 3) were analyzed using a generalized linear model. Statistical significance was indicated when $P < 0.10$.

Results:

Mortality: Table III.5 summarizes the mean percent survival for each concentration at the exposure durations listed in Table III.4. Estimates in Table III.5 are based on a generalized linear model with dose as a fixed effect and duration and the dose x duration interaction as covariates. The model assumed a binomial distribution and employed logit link. The values presented above are back-transformed from the original analysis.

Maximum exposure durations at 100 and 150 mg eugenol/L, where survival was acceptable (i.e., ≥95%), were 4.5 and 2.5 minutes, respectively. Safety breakpoint

intervals (the exposure duration range during which mean percent survival of test fish dropped below 95%) for 100 and 150 mg eugenol/L were 4.5 to 8.0 min and 2.5 to 4.0 min, respectively. Margins of safety (the time difference between the maximum safe exposure duration and the time it takes to sedate 80% of fish to a handleable condition) for each concentration tested were 4.1 min and 2.2 min, respectively (Table III.6).

Table III.5. Model-based estimates of mean percent survival and 95% confidence interval (CI) for channel catfish exposed to 0, 100, or 150 mg eugenol/L.

Eugenol concentration	Duration (minutes)	Estimates of mean percent survival (95% CI)
0 mg/L	3.5	100.0% (100%, 100%)
0 mg/L	4.5	100.0% (100%, 100%)
0 mg/L	8.0	100.0% (100%, 100%)
0 mg/L	12.0	100.0% (100%, 100%)
100 mg/L	3.5	98.0% (93.5%, 99.4%)
100 mg/L	4.5	96.8% (91.3%, 98.9%)
100 mg/L	8.0	85.3% (76.4%, 91.2%)
100 mg/L	12.0	46.6% (32.2%, 61.6%)
150 mg/L	2.5	96.2% (91.0%, 98.5%)
150 mg/L	4.0	92.3% (85.4%, 96.1%)
150 mg/L	6.0	81.4% (72.6%, 87.8%)
150 mg/L	10.0	36.8% (23.6%, 52.2%)

Table III.6. Margin of safety based on longest exposure time and determined ET80, where the ET80 is 80th percentile time to sedation to a handleable condition for channel catfish.

Eugenol concentration	Longest safe exposure duration (minutes)	ET80 (minutes)	Safety Margin (minutes)
100 mg/L (1X)	4.5 min	0.36 min	4.14 min
150 mg/L (1.5X)	2.5 min	0.31 min	2.19 min

Fish health and Histopathology: No gross lesions were seen in any fish in any exposure group. Histologically, there were low levels of gill myxospores, gill monogeneans, and gill *Epitheliocystis* in the reference population. There was lymphocytic gill inflammation that is likely a response to the parasites. Histological results for the reference population were considered normal findings for channel catfish culture.

Histological findings with marked scores (4/5) in treated fish included gill proliferation, gill inflammation, posterior kidney degeneration, posterior kidney proliferation, posterior kidney hypertrophy, and liver nuclear pleomorphism. Marked scores for gill proliferation, gill inflammation, posterior kidney degeneration, and posterior kidney proliferation also occurred in the control groups in similar numbers. Posterior kidney hypertrophy and liver nuclear pleomorphism were likely the result of other disease processes and were not considered test article induced. The only significant difference when comparing lesions or cellular changes between the 0X T4 and the

1X T4 exposure groups or the 0X T4 and 1.5X T4 exposure groups, was gill inflammation between the control and 1X T4 groups (P-value = 0.0174). The gill inflammation was not considered test article induced; more fish had marked scores from the control groups than the treated groups.

Behavior: Behavior was normal in all fish.

Dose verification: Results showed that the drug administration was appropriate to achieve a concentration of 100 or 150 mg eugenol/L.

Water Quality: Water hardness, alkalinity, water temperature, and pH of the source water were 242 mg/L $CaCO_3$, 146 mg/L $CaCO_3$, 27.5 °C, and 8.6, respectively. Mean water pH was 8.1 in the eugenol bulk working solutions. Mean water temperature was 26.2 °C and mean DO concentration was 6.2 mg/L in the exposure containers at the start of exposure.

Conclusions: The study demonstrates that eugenol is safe for use on channel catfish when administered in a static bath at a dose of 100 mg eugenol/L for sedation to a handleable condition. There is an adequate margin of safety above 100 mg eugenol/L based on concentration and duration of exposure.

C. Margin of Safety Study

Title: The Safety of AQUI-S®20E (10% Eugenol) as a Sedative to Yellow Perch *Perca flavescens.* (Study No. AQS20E-11-TAS-FISH-03)

Study Dates: May 3, 2012, to December 12, 2013

Study Location: Bozeman, MT

Study Design:

Objective: To identify the maximum safe exposure durations, exposure-duration margins of safety, and safety breakpoint intervals for small yellow perch exposed to 0 (control), 80, or 120 mg eugenol/L at a water temperature of 19 °C.

Study Animals: 590 fingerling yellow perch; mean total length and weight of test fish at the start of the study were 6.1 cm and 2.7 g.

Experimental Design:

The study was conducted according to GLP. Groups of 15 test fish were exposed to 0, 80 (1X), or 120 (1.5X) mg eugenol/L for 1 of 4 exposure durations. Durations were selected to approximate 50 to 100% survival at each concentration. Each of the 12 combinations of eugenol concentration and exposure duration was replicated 3 times during 3 separate 24-hour periods. An exposure group was comprised of an exposure bucket and recovery tank pair. A completely randomized design was used to allocate 12 exposure regimens among the 12 experimental units, and to determine the order in which the experimental units received fish from the reference population.

A preliminary study was conducted on 30 fish from the reference population to establish an estimated time to sedation to a handleable condition at the 80th percentile (ET80) for each concentration. The ET80 values were then used to determine the exposure durations (T1 to T4) for each eugenol test concentration (Table III.7). The exposure duration for the control groups (0 mg/L) was set to match the 1X exposure durations.

Table III.7. Exposure durations (T1, T2, T3, or T4) for each concentration of eugenol during testing on yellow perch.

Eugenol concentration	T1 (minutes)	T2 (minutes)	T3 (minutes)	T4 (minutes)
0 mg/L (0X)	5.0	7.0	9.0	10.5
80 mg/L (1X)	5.0	7.0	9.0	10.5
120 mg/L (1.5X)	4.0	5.0	6.0	6.75

Drug Administration: Eugenol was administered at either 80 or 120 mg eugenol/L. The control article was unadulterated hatchery water. All treatment administration was performed in static baths, but recovery was in fresh, flowing water.

Measurements and Observations: From a reference population, 20 healthy fish were collected and examined to establish a baseline for fish health. The examinations included a gross morphologic evaluation. Source hatchery water was tested for hardness, alkalinity, pH, and water temperature. The pH of the eugenol bulk working solutions was measured. During exposure, fish were monitored for behavior, water temperature and DO concentration in exposure buckets were measured, and water samples were collected for dose verification. In the 24-hour period following exposure, fish were monitored for behavior and mortality.

Statistical Methods: Survival data were analyzed using a generalized linear model with dose as a fixed effect and duration and the dose x duration interaction as covariates. The model assumed a binomial distribution and employed a logit link. For each combination of drug concentration and exposure duration, a 95% CI for percent survival was estimated by back transforming the least squares estimates from the analysis. Frequency of normal and abnormal behaviors observed during exposure and recovery were tabulated.

Results:

Mortality: Table III.8 summarizes the mean percent survival for each concentration at the exposure durations listed in Table III.7. Estimates in Table III.8 are based on a generalized linear model with dose as a fixed effect and duration and the dose x duration interaction as covariates. The model assumed a binomial distribution and employed logit link. The values presented above are back-transformed from the original analysis. (Note: *mean percent survival estimates are obtained from 14 fish).

Maximum exposure durations at 80 and 120 mg eugenol/L, where survival was acceptable (i.e., ≥95%), were 10.5 minutes and 6.0 minutes, respectively. The safety breakpoint interval (the exposure duration range during which mean percent survival of test fish dropped below 95%) for 80 mg eugenol/L was beyond 10.5 min. The safety breakpoint interval for 120 mg eugenol/L was 6.0 to 6.75 min. Margins of safety (the time difference between the maximum safe exposure duration and the

time it takes to sedate 80% of fish to a handleable condition) for each concentration tested were 9.6 min and 5.2 min, respectively (Table III.9).

Table III.8. Model-based estimates of mean percent survival and 95% confidence interval (CI) for yellow perch exposed to 0, 80, or 120 mg eugenol/L.

Eugenol concentration	Duration (minutes)	Estimates of mean percent survival (95% CI)
0 mg/L	5.0	100.0% (100%, 100%)
0 mg/L	7.0	100.0% (100%, 100%)
0 mg/L	9.0	100.0% (100%, 100%)
0 mg/L	10.5	100.0% (100%, 100%)
80 mg/L	5.0	100.0%* (100%, 100%)
80 mg/L	7.0	100.0% (100%, 100%)
80 mg/L	9.0	100.0% (100%, 100%)
80 mg/L	10.5	95.7% (90.0%, 98.2%)
120 mg/L	4.0	100.0% (100%, 100%)
120 mg/L	5.0	100.0% (100%, 100%)
120 mg/L	6.0	100.0% (100%, 100%)
120 mg/L	6.75	93.0%* (86.6%, 96.5%)

*mean percent survival estimates are obtained from 14 fish.

Table III.9. Margin of safety based on longest exposure time and determined ET80, where the ET80 is 80th percentile time to sedation to a handleable condition for yellow perch.

Eugenol concentration	Longest safe exposure duration (minutes)	ET80 (minutes)	Safety Margin (minutes)
80 mg/L (1X)	10.5 min	0.9 min	9.6 min
120 mg/L (1.5X)	6.0 min	0.78 min	5.2 min

Fish health: The reference population was considered healthy prior to study conduct. No gross lesions were seen in any fish in any exposure group.

Behavior: Behavior was normal in all four 0X exposure durations. Headshaking was observed in ≥75% of the fish upon immersion in the eugenol solution for both the 1X and 1.5X groups. No headshaking was observed in any of the control fish. Headshaking lasted only a brief period of time (15 to 30 seconds) after initial exposure to the sedative solution, and all recovery behavior was normal.

Dose verification: Results showed that the drug administration was appropriate to achieve a concentration of 80 or 120 mg eugenol/L.

Water Quality: Water hardness, alkalinity, water temperature, and pH of the source water were 216 mg/L $CaCO_3$, 169 mg/L $CaCO_3$, 19.8 °C, and 7.8, respectively. Mean water pH was 8.2 in the eugenol bulk working solutions. Mean water temperature was 18.8 °C and mean DO concentration was 7.0 mg/L in the exposure containers at the start of exposure.

Conclusions: The study demonstrates that eugenol is safe for use on yellow perch when administered in a static bath at a dose of 80 mg eugenol/L for sedation to a handleable condition. There is an adequate margin of safety above 80 mg eugenol/L based on concentration and duration of exposure. Because the margin of safety above 80 mg eugenol/L is demonstrated with 120 mg eugenol/L, a dose of 100 mg eugenol/L is also considered safe.

IV. HUMAN FOOD SAFETY

A. Microbial Food Safety

This drug is not an antimicrobial. Therefore, the Agency determined that a microbial food safety assessment is not required.

B. Toxicology

1. Summary of Toxicology Studies

FDA conducted a toxicological evaluation of eugenol based on information from publicly available scientific literature. Four key references used in this evaluation are summarized below:

a. Carcinogenesis Studies of Eugenol (CAS No. 97-53-0) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies), 1983, National Toxicology Program (NTP) No. 223, National Institute of Health, Public Health Service, U.S. Department of Health and Human Services.

Rats:

In the 14-day study, F344/N rats (5/sex/group) were fed with 6,000, 12,500, 25,000, 50,000, or 100,000 ppm of eugenol in the diet. No control group was used. Body weight (bw) and survival were evaluated. One high dose male and all high dose females died during the study. A dose-associated decrease in mean body weight gain was observed in both male and female rats at or above 25,000 ppm (equivalent to 3,542 mg/kg bw/day).

In the 13-week study, F344/N rats (10/sex/group) were given 0, 800, 1,500, 3,000, 6,000, or 12,500 ppm (equivalent to 0, 45, 85, 170, 340, or 708 mg/kg bw/day, respectively) of eugenol in the diet for 13 weeks. Observations for clinical signs or mortality were made twice daily, and animals were weighed weekly. At the end of the 13-week study, surviving animals were killed, necropsies were performed on all animals, and tissues from the controls and the highest dose group were taken for histopathologic analysis. Weight gain was reduced about 10% in the highest dose males and about 6% in the highest dose females relative to controls. No histopathological effects were observed. A No-Observed-Effect Level (NOEL)/No-Observed-Adverse-Effect Level (NOAEL) of 340 mg/kg bw/day was determined based on the weight gain decreases at the highest dose.

In the carcinogenicity study, eugenol was given to female F344/N rats (40/sex for the control group, 50/sex for dosed groups) at 0, 0.6, or 1.25% in the diet

(equivalent to 0, 240, or 500 mg/kg bw/day, respectively) and male F344/N rats at 0, 0.3, or 0.6% in the diet (equivalent to 0, 120, or 240 mg/kg bw/day, respectively) for 103 weeks. A dose-related effect on weight gain, especially in females, and small decreases in food consumption were noted. Increases in tumor incidences were noted in low dose males (alveolar/bronchiolar adenomas), low dose females (C-cell adenomas of the thyroid gland), and high dose females (endometrial stromal polyps of the uterus). None of these neoplasms could be attributed to eugenol treatment. It was concluded that eugenol was not carcinogenic in rats under the conditions of the study.

Mice:

In the 14-day study, B6C3F_1 mice (5/sex/group) were given 6,000, 12,500, 25,000, 50,000, or 100,000 ppm of eugenol in the diet for 14 days. No control group was used. Body weight and survival were evaluated. Deaths occurred in 3 of 5 males that received 50,000 ppm (equivalent to 18,750 mg/kg bw/day) of eugenol and in all males and females that received 100,000 ppm (equivalent to 37,500 mg/kg bw/day) of eugenol. A dose-associated decrease in mean body weight gain was observed in males at 12,500 ppm (equivalent to 4,688 mg/kg bw/day) and above, and in females at 25,000 ppm (equivalent to 9,375 mg/kg bw/day) and above.

In the 13-week study, B6C3F_1 mice (10/sex/group) were fed 0, 400, 800, 1,500, 3,000, or 6,000 ppm (equal to 0, 100, 200, 375, 750, or 1,500 mg/kg bw/day, respectively) of eugenol in the diet. Observations for clinical signs or mortality were made twice daily, and animals were weighed weekly. At the end of the 13-week study, surviving animals were killed, necropsies were performed on all animals, and tissues from the controls and the highest dose group were taken for histopathologic analysis. There was no mortality, and no effect on body weights or compound-related gross or microscopic pathology.

For the carcinogenicity study, eugenol was given in the diet of male and female B6C3F_1 mice (50/sex/group) at diet concentrations of 0, 0.3, or 0.6% (equivalent to 0, 590, or 1,138 mg/kg bw/day, respectively) for 103 weeks. A small dose-related decrease in weight gain was found for both males and females throughout the study. No compound-related clinical signs were reported. The incidence of hepatocellular tumors (carcinoma and adenoma combined) was 14/50, 39/45, and 19/49 in the control, low dose, and high dose males, respectively. The corresponding incidence in females was 2/50, 7/49, and 9/49. Males at the low dose, but not the high dose, showed a statistically significant increase in hepatocellular tumors. It was concluded that eugenol was not carcinogenic in mice under the conditions of the study.

Overall, there is no clear evidence indicating that eugenol is carcinogenic based on the results of the above carcinogenicity studies in rats and mice.

b. Toxicity of the Mucigogue, Eugenol, Administered by Stomach Tube to Dogs. Lauber, FU, and Hollander. F. 1950, Gastroenterology. 15, 481-486

A single dose of eugenol was intragastrically administered to a total of 5 female dogs in 20 experiments. In these experiments, 4 dogs were treated at 500 mg/kg bw in 6 experiments, 4 dogs at 250 mg/kg bw in 7 experiments, 3 dogs at 200 mg/kg bw in 6 experiments, and 1 dog at 100 mg/kg bw in 1 experiment. Vomiting, ataxia, and some pathological findings (marked venous congestion of the liver and kidneys and small sites of hemorrhage in the stomach and duodenum) were noted at 250 and 500 mg/kg bw. Mortality was reported at 500 mg/kg bw.

Eugenol was administered at 200 mg/kg bw per dose to three dogs for 10 days, with 2- to 3-day intervals between doses. No clinical signs of toxicity, gross or microscopic changes were observed.

A NOEL/NOAEL of 200 mg/kg bw was established based on liver toxicity, vomiting, and punctate hemorrhages observed at higher dose levels in these two studies.

c. Developmental Toxicity of Eugenol when Administered *via* Gavage in CD Sprague-Dawley Rats (NTP No. TRP92043, 1993, National Institute of Health, Public Health Service, U.S. Department of Health and Human Services).

Eugenol was administered by daily gavage to mated female CD Sprague-Dawley rats (16/group) at 0, 100, 400, 600, 800, or 1,000 mg/kg bw/day from gestation days 6 through 15. Clinical observations and body weights were recorded. The number of implantation sites, the number of early and late resorptions, the number of dead fetuses, the number of live fetuses, live litter weight, and gravid uterine weight were unaffected by treatment. An examination of skeletal and soft tissue alterations was not performed. Maternal body weight gain was decreased at 800 mg/kg bw/day and above. No other effects were observed. A maternal NOEL/NOAEL was established at 600 mg/kg bw/day.

d. The Role of Eugenol in the Reduction of Teratogenic Effects of Retinoic Acid on Skeletal Morphology of Mice Embryo. Amini, A, Cheraghi, E, Safaee, M.R., and Hill, M. 2003, Cell Journal, Volume 4, 195-200.

Eugenol was administered by daily gavage to mated female mice (numbers per group unidentified) at 0 and 100 mg/kg bw/day from gestation days 5 through 15. Five females from the eugenol treated group and six females from the control group produced litters. The number of early and late resorptions, the number of live fetuses, and live litter weight were unaffected by treatment. An examination of skeletal and soft tissue alterations was performed on half of the fetuses. No effects were reported at all doses tested.

Additional groups of pregnant female mice were also fed with retinoic acid, or retinoic acid plus eugenol in this study. The results of this study suggested that eugenol may decrease the teratogenic effects of retinoic acid.

2. Microbiological effects (i.e., effects on human intestinal flora)

a. Determination of whether there is a concern for microbiological effects

Step 1: Are eugenol residues microbiologically active against representative human intestinal bacteria?

Yes. Eugenol is not considered an antimicrobial drug, but it is known to have limited activity against various types of microorganisms, including bacteria.

Step 2: Do eugenol residues enter the human colon?

Yes. Published pharmacokinetic data for eugenol indicate that the compound is readily absorbed following oral dosing in humans - less than 5% of ingested eugenol is expected to enter the human colon. Thus, it is expected that a small proportion of eugenol residues enter the human colon.

Step 3: Do eugenol residues entering the human colon remain microbiologically active?

No. Based on the following considerations: a) the eugenol activity against bacteria is relatively weak, b) under the conditions of use, the total amount of eugenol entering the human colon is relatively small, c) colon contents will interfere and diminish the activity. Therefore, the concentration of eugenol residues in the colon is low and is not expected to be microbiologically active.

Conclusion: The Agency determined that there is no concern for the effects of eugenol residues on human intestinal flora.

C. Establishment of the ARfD

1. Considerations for the Acute Reference Dose (ARfD) Approach

Fisheries professionals routinely use sedatives in field-based fishery management activities, such as collection of samples or morphometric data. The investigation into the likelihood of human consumption of fish containing residues of sedatives from field-based fishery management involves several steps:

a) Characterizing fishery management scenarios: The U.S. Geological Survey and U.S. Fish and Wildlife Service analyzed various fishery management activities across U.S. freshwater environments. Data were collected on fish capture rates and tagged fish recapture rates for various activities and locations.

b) Identifying the worst-case scenario: Their analysis identified that the salmonids returning to the Pacific Northwest could represent the worst-case

scenario. This is likely due to the high frequency and volume of these fish being captured and tagged.

c) Quantitative probabilistic analysis: FDA conducted a quantitative analysis to model the probability of human consumption based on this worst-case scenario. Key parameters applied in the model included conservative estimations of fish capture rate, tagged fish recapture rate, and recapture rate on the first day of release. Fish capture rate was set at 20%, meaning 20% of the targeted fish population is captured during fishery management activities. The tagged fish recaptured rate was set at 20%, indicating that 20% of tagged fish are recaptured. Various rates of fish recaptured on the first day of release were considered (0.8%, 5%, 10%, or 20%).

The analysis suggests that the probability of a human consuming sedated fish during a one-year period would be low. Even with the conservative rates applied in the model, such events were found to be rare and infrequent.

The key references used in this evaluation are summarized below:

- Hayes, D.B., C.P. Ferreri, and W.W. Taylor. 2012. Active fish capture methods. Pages 267-304. A.V. Zale, D.L. Parrish, and T.M. Sutton, editors. Fisheries Techniques, 3rd edition. American Fisheries Society, Bethesda, Maryland.

- Hubert, W.A., K.L. Pope, and J.M. Dettmers. 2012. Passive capture techniques. Pages 223-265. A V. Zale, D.L. Parrish, and T.M. Sutton, editors. Fisheries Techniques, 3rd edition. American Fisheries Society, Bethesda, Maryland.

- Bumgarner, J.D. and J.T. Dedloff. 2009. Lyons Ferry Complex Hatchery Evaluation: Summer Steelhead Annual Report 2006 and 2007 Run Year. Washington Department of Fish and Wildlife.

- Keefer, M.L., C.A. Peery, W.R. Daigle, M.A. Jepson, S.R. Lee, C.T. Boggs, K.R. Tolotti, and B.J. Burke. 2005. Escapement, harvest, and unknown loss of radio-tagged adult salmonids in the Columbia River – Snake River Hydrosystem. Canadian Journal of Fisheries and Aquatic Sciences 62: 930-949.

- Sanquer, A, G. Wackowiez, and B. Havrileck, 2006. Qualitative assessment of human exposure to consumption of injection site residues. Journal of Veterinary Pharmacology Therapy. 29, 345-353.

Because human exposure to eugenol residues due to consumption of fish treated with eugenol is considered an infrequent event under the worst-case scenario of field applications for fishery management activities, an ARfD approach for acute exposure is appropriate for toxicological evaluation.

2. Determination of Toxicological NOEL/NOAEL for Acute Exposure

   The NOEL/NOAEL of 200 mg/kg bw from the acute toxicity study in dogs is selected for the determination of the ARfD for total residue of eugenol for acute exposure.

3. Acute Reference Dose (ARfD)

   The ARfD is calculated using the following formula based on the NOEL/NOAEL of 200 mg/kg bw from the acute oral toxicity study in dogs and a safety factor of 100.

$$\text{ARfD} = \frac{\text{NOEL/NOAEL}}{\text{Safety Factor}} = \frac{200\ \text{mg/kg bw}}{100} = 2\ \text{mg/kg bw}$$

   The ARfD for eugenol is 2 mg/kg bw.

D. Safe Concentrations for Total Residues in Edible Tissues

The calculation of the tissue safe concentrations is based on the "General Principles for Evaluating the Human Food Safety of New Animal Drugs Used in Food-Producing Animals" (FDA/CVM, Guidance for Industry #3, revised May 2022). The daily consumption value of the edible tissue of fish (muscle with skin in natural proportions) is approximated as 300 g. The safe concentration of total residues of eugenol (ppm) in the edible tissue of fish (muscle with skin in natural proportions) is calculated using the following formula:

$$\text{Safe Concentration} = \frac{\text{ARfD x Human Body Weight}}{\text{Food Consumption Value}}$$

$$= \frac{2\ \text{mg/kg bw x 60 kg}}{300\ \text{g}} = 400\ \mu\text{g/g} = 400\ \text{ppm}$$

Therefore, the safe concentration for total eugenol residues is 400 ppm in fish muscle with skin in natural proportions.

E. Residue Chemistry

1. Summary of Residue Chemistry Studies

   a. Study to Determine Optimal Exposure Parameters

      Title: Determining Exposure Parameters that Maximize Eugenol Residues in the Fillet Tissue and Determining the Sample Times that Will Adequately Characterize the Depletion of Eugenol Residues from the Fillet Tissue of Rainbow Trout Exposed to AQUI-S® 20E. (Study No. AEH-11-EUGNL-01)

      Study Dates: March 9, 2012, to May 17, 2012

      Study Location: La Crosse, WI

Study Design:

Objective: To determine the appropriate dosing parameters for the total residue and metabolism study, e.g., the dosing parameters that result in the maximum tissue residues.

Experimental Design: Rainbow trout (*Oncorhynchus mykiss*), weighing 167 to 404 g (mean 254 g), were used. The study was conducted in two parts. First, the exposure conditions that result in maximum eugenol concentrations in fillet tissue were determined by a series of absorption experiments. Second, the exposure conditions that resulted in the maximum eugenol tissue concentrations were used in a depletion experiment. The fish were exposed to a specific concentration of eugenol in a tank that contained 17°C water.

Table IV.1. Concentration of eugenol in exposure tank to determine absorption, number of exposed fish for each concentration, and minutes of exposure of rainbow trout to AQUI-S® E10%.

Eugenol (mg/L)	Number of exposed fish	Duration of Exposure (minutes)
5	40	5, 10, 15, 30, 60, 240, 720, 1440
10	35	5, 10, 15, 30, 60, 120, 240
25	25	15, 30, 60, 120, 240
50	25	15, 30, 45, 60, 90
100	25	15, 30, 45, 60, 90

After each exposure period, five fish were randomly sampled. Fillet tissue (muscle with adhering skin) was collected and then analyzed for eugenol using a High Performance Liquid Chromatography (HPLC) method.

Results: The following table describes the maximum tissue eugenol concentration found at each exposure concentration tested and the duration of exposure at which it occurred.

Table IV.2. Duration of exposure of rainbow trout to AQUI-S® E10% that resulted in the maximum eugenol concentration in fillet tissue.

Eugenol (mg/L)	Exposure Duration (minutes)	Mean eugenol (ppm) ± standard deviation	Minimum-Maximum Eugenol (ppm)
5	720	38.8 ± 9	27.4-51.1
10	240	62.0 ± 10	50.4-77.3
25	240	26.7 ± 4.7	19.3-32.4
50	90	26.8 ± 7.2	17.1-37.3
100	90	34.8 ± 4.3	29.4-40.1

The fish exposed to 10 mg/L eugenol had the highest eugenol residue concentrations in their fillet. The highest eugenol residue (77.3 ppm) was

found in the fillet of fish exposed for 240 minutes. However, there was no significant difference between the mean eugenol concentrations in the fillet of fish exposed for 30, 60, 120, and 240 minutes. The 60-minute exposure was selected for the depletion experiment because that time period was likely to be the longest exposure time when sedating finfish with eugenol for field-based fishery management activities. The following table describes the eugenol concentrations in fillet tissue of rainbow trout exposed to 10 mg eugenol/L for exposure periods from 5 to 240 minutes.

Table IV.3. Eugenol concentrations in fillets of rainbow trout exposed to 10 mg/L eugenol.

Exposure Duration (minutes)	Mean (ppm) ± standard deviation	Minimum-Maximum Eugenol (ppm)
5	15.4[a] ± 3.4	11.5-20.5
10	23.8[b] ± 5.0	16.6-28.6
15	28.6[b] ± 4.3	21.7-32.2
30	50.0[c] ± 4.0	45.9-55.4
60	58.2[c] ± 10	49.8-74.8
120	53.6[c] ± 9.2	41.3-62.8
240	62.0[c] ± 10	50.4-77.3

Means with the same superscript are not significantly different (p>0.05).

To determine depletion of eugenol, 35 fish were exposed to AQUI-S® 20E using the concentration and duration that resulted in the maximum tissue residues (10 mg/L eugenol for 60 minutes). After exposure, all of the fish were transferred to fresh, flowing water. Groups of 5 fish were randomly sampled from the holding tank at 30, 60, 120, 240, 360, and 720 minutes, and 4 fish were sampled at 1440 minutes after exposure.

Table IV.4. Eugenol concentration (ppm) in fillet tissue of rainbow trout exposed to 10 mg/L eugenol for 60 minutes.

Time after Exposure (minutes)	Mean (ppm) ± standard deviation	Minimum-Maximum Eugenol (ppm)
30	19.7 ± 5	13.5-27.3
60	10.5 ± 4	6.3-16.7
120	4.09 ± 2.3	2.16-8.13
240	0.51 ± 0.55	0.15-1.46
360	0.44 ± 0.29	0.13-0.86
720	0.06 ± 0.032	0.03-0.11
1440	0.01 ± 0.015	0.00-0.03

b. Total Residue and Metabolism Study

Title: Eugenol Total Residue Depletion from the Skin-on Fillet Tissue of Rainbow Trout Exposed to ^{14}C Labeled AQUI-S® 20E (Study No. AEH-11-EUGNL-02)

Study Dates: October 16, 2012, to November 26, 2012

Study Location: La Crosse, WI

Study Design:

Objective: To determine the concentrations of total residues in skin-on fillet of rainbow trout exposed to ^{14}C labeled AQUI-S® E10% to a nominal concentration of 10 mg ^{14}C-eugenol/L for 60 minutes in 17°C water.

Experimental Design: Rainbow trout (*Oncorhynchus mykiss*), weighing 358 grams (range 255 to 491 g), were used. The test fish were transferred from a holding tank to an exposure tank where they were exposed to a nominal concentration of 10 mg ^{14}C-eugenol/L for 60 minutes in 17°C water. Six test fish were sampled immediately after exposure to eugenol (0-minute time point). The remaining fish were transferred back to the holding tank. Groups of 6 test fish were sampled at 30, 60, and 120 minutes after exposure. The fish were euthanized, and skin-on fillet was removed from each fish. Total residues were measured by liquid scintillation counting techniques and were characterized by liquid chromatography. Tissue samples also were analyzed for eugenol using an HPLC procedure.

Results:

Table IV.5. Concentrations of eugenol-equivalent ^{14}C-residues and percentage of total ^{14}C-residues in fillet from rainbow trout exposed to 10 mg/L ^{14}C-eugenol for 60 minutes in 17°C water.

Time after Exposure (minutes)	Number of Fish Collected	Mean Eugenol concentration (ppm equivalents) ± standard deviation	%Total ^{14}C-eugenol
0	6	38.77 ± 7.9	98.6
30	6	12.42 ± 1.7	97.6
60	6	7.41 ± 2.0	94.3
120	6	3.95 ± 0.81	92.4
240	5	1.77 ± 0.47	85.7

Table IV.6. Concentrations of eugenol in fillet from rainbow trout exposed to 10 mg/L ^{14}C-eugenol for 60 minutes in 17°C water.

Time after Exposure (minutes)	Number of Fish Collected	Mean (ppm) ± standard deviation
0	6	44.47 ± 8.8
30	6	12.80 ± 2.0
60	6	6.16 ± 2.5
120	6	2.42 ± 0.8
240	5	0.47 ± 0.19

Total residues were highest at zero withdrawal, depleting quickly over time. The majority (mean 90%) of total residues are composed of parent eugenol. Therefore, the marker residue is eugenol.

c. Comparative Metabolism Study

Ninety percent of the extractable residue in muscle with skin was eugenol and no other significant metabolites were found. Therefore, the requirement for a comparative metabolism study was waived.

d. Study to Establish Withdrawal Period

Title: Marker Residue Depletion from the Skin-on Fillet Tissue of Rainbow Trout Exposed to AQUI-S 20E (Study No. AEH-13-EUGNL-05)

Study Dates: October 15, 2014, to December 9, 2014

In-Life and Analytical Study Location: La Crosse, WI

Experimental Design: 101 rainbow trout (*Oncorhynchus mykiss*), weighing 316 g to 672 g, were used. The test fish were transferred from a holding tank supplied with flowing well water to an exposure tank where they were exposed to a nominal concentration of 10 mg eugenol/L for 60 minutes in 9°C water. Sixteen test fish were sampled immediately after exposure to eugenol (0-minute time point). The remaining fish were transferred to a depuration tank supplied with flowing well water in an environmentally controlled chamber holding the water at 9°C. Groups of 16 test fish were sampled at 15, 30, 90, and 150 minutes after exposure. The fish were slaughtered by electric shock and skin-on fillet was removed from each fish. Tissue samples were analyzed for eugenol using an HPLC-UV procedure.

Results:

Table IV.7. Mean eugenol concentrations in fillet tissue from rainbow trout sampled after treatment with AQUI-S® E10% at 10 mg/L eugenol for 60 minutes at 9°C in a freshwater exposure bath.

Withdrawal Time (minutes)	Mean Eugenol in Muscle and Skin (ppm ± standard deviation)
0	48 ± 6.5
15	29 ± 3.8
30	19 ± 3.4
90	4.5 ± 1.1
150	1.9 ± 0.65

Eugenol concentrations in fish tissue were highest at zero withdrawal (mean 48 ppm), depleting quickly to a mean of 1.9 ppm at 150 minutes withdrawal.

2. Target Tissue and Marker Residue

   The target tissue is muscle with skin in natural proportions because that is the edible tissue for all fish (except those where the skin is inedible, i.e., catfish and eel). The marker residue is eugenol because that is the major compound found in the edible tissues after exposure to eugenol.

3. Tolerance

   A tolerance is not assigned for eugenol used for field use because the total residue concentrations are substantially below the safe concentration for acute exposure to eugenol (400 ppm).

4. Withdrawal Period

   A withdrawal period of 0 days is assigned for field use. Results from Study No. AEH-11-EUGNL-02 show that eugenol-equivalent ^{14}C-residues (total residues) are substantially below the safe concentration of 400 ppm in muscle. Results from Study No. AEH-13-EUGNL-05 confirm that mean eugenol concentrations in rainbow trout fillet at zero-day withdrawal, calculated from the percentage of total ^{14}C-residues found as ^{14}C-eugenol in fillet tissue (Study No. AEH-11-EUGNL-02), are significantly below the safe concentration calculated for muscle (48 ppm *vs.* 400 ppm).

F. Analytical Method for Residues

1. Description of Analytical Method

   A validated HPLC method for measuring eugenol in fish edible tissue was provided.

2. Availability of the Method

   The validated analytical method for analysis of residues of eugenol is on file at the Center for Veterinary Medicine CPK1, 5001 Campus Drive, College Park, MD 20740.

   To obtain a copy of the analytical method, please submit a Freedom of Information request to:
   https://www.accessdata.fda.gov/scripts/foi/FOIRequest/requestinfo.cfm.

V. USER SAFETY

The product labeling contains the following information regarding safety to humans handling, administering, or exposed to AQUI-S® E10%:

Not for use in humans. Keep out of reach of children. Harmful if swallowed. May cause skin and eye irritation and skin sensitization. Eugenol may cause an allergic skin reaction in some people. Personal protective equipment (e.g., gloves, lab coats, eyewear) should be worn when handling the product.

Wash hands and face with soap and water after handling product or treating fish. If skin or hair contact occurs, flush affected area with running water. If splashed in eyes, flush eyes copiously with water.

In case of ingestion, contact Poison Help Hotline at 1-800-222-1222, or a medical doctor immediately.

Additional information is listed in the Safety Data Sheet (SDS). To obtain a copy of the SDS, contact Merck Animal Health at 1-800-521-5767.

VI. AGENCY CONCLUSIONS

The data submitted in support of this NADA satisfy the requirements of section 512 of the Federal Food, Drug, and Cosmetic Act (FD&C Act) and 21 CFR part 514. The data demonstrate that AQUI-S® E10%, when used according to the label, is safe and effective for the conditions of use in the General Information Section above. Additionally, data demonstrate that residues in food products derived from species treated with AQUI-S® E10% will not represent a public health concern when the product is used according to the label.

A. Marketing Status

This product can be marketed over the counter (OTC) because the approved labeling contains adequate directions for use by laypersons and the conditions of use prescribed on the label are reasonably certain to be followed in practice.

B. Exclusivity

AQUI-S® E10%, as approved in our approval letter, qualifies for SEVEN years of exclusive marketing rights beginning as of the date of our approval letter. This drug qualifies for exclusive marketing rights under section 573(c) of the FD&C Act because it is a designated new animal drug under section 573(a) of the FD&C Act. Except as provided in section 573(c)(2) of the FD&C Act, we may not approve or conditionally approve another application submitted for such new animal drug with the same intended use as AQUI-S® E10%. Because this is the first time we are approving this active moiety in a new animal drug application submitted under section 512(b)(1) of the FD&C Act, this drug also qualifies for five years of exclusivity under section 512(c)(2)(F)(i) of the FD&C Act. The exclusive marketing rights and exclusivity for this drug run concurrently.

C. Patent Information

For current information on patents, see the Green Book Reports in the Animal Drugs @ FDA database.