

**1. PURPOSE**

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This Standard Operating Procedure (SOP) describes acceptable procedures for fish and aquatic amphibian euthanasia. It ensures that animals are euthanized in the most humane way possible.

**2. RESPONSIBILITY**

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Principal investigator (PI) and their research staff, veterinarian, veterinary care staff.

**3. CONSIDERATIONS**

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The environment should be as quiet and non-stimulatory as possible during euthanasia. Reduce light intensity, use red light illumination as red light does not penetrate water well, or use a dark or opaque container and lid.

Water quality should be similar to that of the environment from which the fish originated, or optimized for that species and situation, for the duration of euthanasia. If the water is of acceptable quality for fish health, the water in which they have been housed or captured should be used. The immersion euthanasia solution should be prepared with water from the housing system and the animals transferred into it or a concentrated form of the anesthetic agent as a solution (containing buffering agent if appropriate) is introduced directly into the container to minimize stressors.

**4. PROCEDURES FOR ZEBRAFISH (*Danio rerio*) AND OTHER SMALL, WARM-WATER LABORATORY FISH**

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**4.1. Zebrafish  $\geq$ 15 days post fertilization:****4.1.1. Rapid cooling (hypothermia):**

- 4.1.1.1. Prepare a tank or insulated cooler containing approximately 5 parts ice to 1 part water to achieve a temperature of 2 to 4 °C.
- 4.1.1.2. Fish should not be in direct contact with the ice in the water. Use a spawning barrier or create a depression in the ice slurry to expose the entire surface of the fish only to the chilled water, not the ice.
- 4.1.1.3. Immerse the fish for at least 10 minutes after opercular movement ceases.
- 4.1.1.4. Where it is difficult to visualize opercular movement, fish should be left in the ice water for at least 20 minutes after cessation of all movement to ensure death by hypoxia.
- 4.1.1.5. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.

**4.1.2. Tricaine methanesulfonate (MS222):**

- 4.1.2.1. MS222 is acidic and causes an aversive reaction in unanesthetized fish. Buffer with sodium bicarbonate to saturation resulting in a solution of pH 7.0 to 7.5.
- 4.1.2.2. Place fish in a solution of MS222 dissolved in water at a concentration of 250-500 mg/L.
- 4.1.2.3. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.
- 4.1.2.4. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.

**4.2. Zebrafish larvae 8-15 days post fertilization (fry):****4.2.1. Rapid cooling (hypothermia):**

- 4.2.1.1. Prepare a tank containing approximately 5 parts crushed ice to 1 part tank water to achieve a temperature of 2 to 4 °C.

- 4.2.1.2. Larvae should not be in direct contact with the ice in the water. Use a spawning barrier or create a depression in the ice slurry to expose the entire surface of the larva only to the chilled water, not the ice.
- 4.2.1.3. Immerse the larvae for at least 20 minutes.
- 4.2.1.4. Follow by an adjunct method of euthanasia:
  - 4.2.1.4.1. Prepare a bleach solution of 1 part sodium hypochlorite 6.15% to 5 parts water.
  - 4.2.1.4.2. Add bleach solution to the culture system water.
  - 4.2.1.4.3. The larvae should remain in this solution at least five minutes prior to disposal to ensure death.
- 4.2.2. Tricaine methanesulfonate (MS222):
  - 4.2.2.1. MS222 is acidic and causes an aversive reaction in unanesthetized fish. Buffer with sodium bicarbonate to saturation resulting in a solution of pH 7.0 to 7.5.
  - 4.2.2.2. Place larvae in a solution of MS222 dissolved in water to a concentration of 250 – 500 mg/L.
  - 4.2.2.3. Larvae should be left in the solution for at least 20 minutes following cessation of opercular movement.
  - 4.2.2.4. Follow by an adjunct method of euthanasia:
    - 4.2.2.4.1. Prepare a bleach solution of 1 part sodium hypochlorite 6.15% to 5 parts water.
    - 4.2.2.4.2. Add bleach solution to the culture system water
    - 4.2.2.4.3. The larvae should remain in this solution at least five minutes prior to disposal to ensure death.
- 4.3. Zebrafish embryos  $\leq$  7 days post fertilization (dpf):
  - 4.3.1. Sodium hypochlorite:
    - 4.3.1.1. Prepare a bleach solution of 1 part sodium hypochlorite 6.15% to 5 parts water.
    - 4.3.1.2. Add bleach solution to the culture system water
    - 4.3.1.3. The embryos should remain in this solution at least five minutes prior to disposal to ensure death.

## **5. PROCEDURES FOR OTHER FISH SPECIES**

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- 5.1. Tricaine methanesulfonate (MS222):
  - 5.1.1. MS222 is acidic and causes an aversive reaction in unanesthetized fish. Buffer with sodium bicarbonate to saturation resulting in a solution of pH 7.0 to 7.5.
  - 5.1.2. Place fish in a solution of MS222 dissolved in water at a concentration of 250-500 mg/L.
  - 5.1.3. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.
  - 5.1.4. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.
- 5.2. Eugenol, isoeugenol, clove oil:
  - 5.2.1. Use products with standardized, known concentrations of essential oils to ensure accurate dosing.
  - 5.2.2. Prepare a stock solution by mixing 1-3 ml eugenol, isoeugenol, or clove oil in 10 ml of ethanol.
  - 5.2.3. Mix 10 ml of this solution to 1L of water.
  - 5.2.4. Immerse the fish for at least 10 minutes after opercular movement ceases.
  - 5.2.5. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.

- 5.3. Benzocaine hydrochloride:
  - 5.3.1. Buffer benzocaine hydrochloride solutions to a pH 7.0 to 7.5 to avoid tissue irritation.
  - 5.3.2. Immerse fish into a bath of benzocaine hydrochloride solution of >250 mg/L.
  - 5.3.3. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.
  - 5.3.4. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.
- 5.4. 2-phenoxyethanol:
  - 5.4.1. Place fish into a bath of 2-phenoxyethanol solution at a concentration of 0.5 to 0.6 mL/L or 0.3 to 0.5 mg/L.
  - 5.4.2. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.
  - 5.4.3. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.
- 5.5. Sodium pentobarbital injection:
  - 5.5.1. Inject sodium pentobarbital intravenously at a dose of 60 to 100 mg/kg body weight.
  - 5.5.2. Verify the animal is dead by monitoring for opercular movement and lack of response to sharp tail pressure. Time to effect may vary, with death occurring in up to 30 minutes.
  - 5.5.3. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.
- 5.6. Liquid anesthetics (isoflurane, sevoflurane):
  - 5.6.1. Prepare a tank or container for euthanasia.
  - 5.6.2. Add liquid anesthetic to the water at a dose of 5 to 20 ml/L using a syringe and needle to facilitate dispersal into the water.
  - 5.6.3. Immerse the fish for at least 10 minutes after opercular movement ceases.
  - 5.6.4. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.
- 5.7. Physical Methods:
  - 5.7.1. Anesthesia or sedation must be applied prior to the use of physical techniques unless approved by the Facility Animal Care Committee (FACC).
  - 5.7.2. Decapitation:
    - 5.7.2.1. Use sharp equipment of the appropriate size for the species to be euthanized to ensure that the head is quickly separated from the body rapidly and completely.
    - 5.7.2.2. Follow decapitation with pithing or freezing.
  - 5.7.3. Pithing:
    - 5.7.3.1. Insert a rigid metal rod into the foramen magnum which is identified by the slight midline skin depression posterior to the eyes when the neck is flexed. Ensure that both the brain and the proximal end of the spinal cord are destroyed.
  - 5.7.4. Freezing:
    - 5.7.4.1. May be used as the second step of a 2-step procedure when fish have been rendered unconscious.

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## 6. PROCEDURES FOR AQUATIC AMPHIBIANS

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- 6.1. Sodium pentobarbital and sodium phenytoin injection:
  - 6.1.1. Inject sodium pentobarbital 1100 mg/kg and 141 mg/kg sodium phenytoin intracoelomically.
  - 6.1.2. Place animal in water. Time to effect may vary, with death occurring in up to 1 hour.

- 6.1.3. Verify the animal is dead before disposing of the carcass by monitoring for respiratory movement, heart contractions, and lack of response to stimuli.
- 6.1.4. Follow by an adjunctive method of euthanasia such as decapitation or pithing to complete euthanasia. Note that frogs require double-pithing (brain and spinal cord).
- 6.2. Tricaine methanesulfonate (MS222):
  - 6.2.1. MS222 is acidic and causes an aversive reaction in unanesthetized amphibians. Buffer with sodium bicarbonate to saturation resulting in a solution of pH 7.0 to 7.5.
  - 6.2.2. Place animals in a solution of MS222 dissolved in water at a concentration of 3 to 5 g/L. Time to effect may vary, with death occurring in up to 1 hour.
  - 6.2.3. Verify the animal is dead before disposing of the carcass by monitoring for respiratory movement, heart contractions, and lack of response to stimuli.
  - 6.2.4. Follow by an adjunctive method of euthanasia such as decapitation or pithing to complete euthanasia. Note that frogs require double-pithing (brain and spinal cord).
- 6.3. Benzocaine hydrochloride gel (cutaneous application):
  - 6.3.1. Apply a 2cm × 1mm strip of 20% benzocaine gel directly from the tube on the ventral abdomen. No special preparation of the skin is required.
  - 6.3.2. Place animal in wet container without water. Euthanasia may take up to 3-5 hours
  - 6.3.3. Verify the animal is dead before disposing of the carcass by monitoring absence of respiratory movement, heart contractions, and lack of response to stimuli.
  - 6.3.4. Follow by an adjunctive method of euthanasia such as decapitation or pithing to complete euthanasia. Note that frogs require double-pithing (brain and spinal cord).
- 6.4. Physical Methods:
  - 6.4.1. Anesthesia or sedation must be applied prior to the use of physical techniques unless approved by the Facility Animal Care Committee (FACC).
  - 6.4.2. Decapitation:
    - 6.4.2.1. Use sharp equipment of the appropriate size for the species to be euthanized to ensure that the head is quickly separated from the body rapidly and completely.
    - 6.4.2.2. Follow decapitation with pithing or freezing.
  - 6.4.3. Pithing:
    - 6.4.3.1. Insert a rigid metal rod into the foramen magnum which is identified by the slight midline skin depression posterior to the eyes when the neck is flexed. Ensure that both the brain and the proximal end of the spinal cord are destroyed.
  - 6.4.4. Freezing:
    - 6.4.4.2. May be used as the second step of a 2-step procedure when fish have been rendered unconscious.

## **7. SAFETY PRACTICES**

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- 7.1. Tricaine methanesulfonate (MS222):
  - 7.1.1. Wear protective clothing, gloves and eye protection when handling the MS222 powder.
  - 7.1.2. When possible, work in a chemical fume hood or Class II Type B Biological Safety Cabinet, or wear a respirator when handling the MS222 powder.
  - 7.1.3. Wear gloves to handle animals exposed to MS222
  - 7.1.4. Making MS222 solutions:
    - 7.1.4.1. Contact Environmental Health and Safety Department for safe handling, use, and storage procedures.
  - 7.1.5. Disposal of MS222 waste:

7.1.5.1. Contact the Waste Management department for disposal procedures.

7.2. Eugenol:

7.2.1. Wear protective clothing, gloves, and eye protection when handling eugenol.

7.2.2. Wear gloves to handle animals exposed to eugenol.

7.2.3. Making eugenol solutions:

7.2.3.1. Contact Environmental Health and Safety Department for safe handling, use, and storage procedures.

7.2.4. Disposal of eugenol waste:

7.2.4.1. Contact the Waste Management department for disposal procedures.

7.2.5. Thoroughly wash hands after handling or administering eugenol.

7.3. Sodium hypochlorite (bleach):

7.3.1. Wear protective clothing, gloves, and eye protection when handling bleach.

7.3.2. Wear gloves to handle animals exposed to bleach.

7.3.3. Making bleach solutions:

7.3.3.1. Work in a well-ventilated area.

7.3.3.1. Work areas should be protected from spills by placing an absorbent pad with absorbent material facing up.

7.3.4. Disposal of bleach waste:

7.3.4.1. Discard directly into sink or drain.

## 8. REFERENCES

- 8.1. Wilson JM, Bunte RM, Carty AJ. Evaluation of Rapid Cooling and Tricaine Methanesulfonate (MS222) as Methods of Euthanasia in Zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* : JAALAS 2009;48(6):785-789.
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- 8.3. American Veterinary Medical Association. [AVMA Guidelines for the Euthanasia of Animals](#): 2020 Edition.
- 8.4. Canadian Council on Animal Care. [CCAC guidelines on euthanasia of animals used in science](#) (2010).
- 8.5. Canadian Council on Animal Care. [CCAC guidelines on the care and use of fish in research](#) (2005).
- 8.6. Canadian Council on Animal Care. [CCAC guidelines: Zebrafish and other small, warm-water laboratory fish](#) (2020).
- 8.7. Fernandes IM, Bastos YF, Barreto DS, Lourenço LS, Penha JM. The efficacy of clove oil as an anaesthetic and in euthanasia procedure for small-sized tropical fishes. *Braz J Biol.* 2017 Jul-Sept;77(3):444-450. [doi: 10.1590/1519-6984.15015](#).

## SOP REVISION HISTORY

DATE	NEW VERSION
2024.01.12	2. RESPONSIBILITY Principal investigator (PI) and their research staff, <b>veterinarian</b> , veterinary care staff.
2024.01.12	6.1.4. Follow by an adjunctive method of euthanasia such as decapitation or pithing to complete euthanasia. <b>Note that frogs require double-pithing (brain and spinal cord).</b>
2024.01.12	6.2.4. Follow by an adjunctive method of euthanasia such as decapitation or pithing to complete euthanasia. <b>Note that frogs require double-pithing (brain and spinal cord).</b>
2024.01.12	6.3.4. Follow by an adjunctive method of euthanasia such as decapitation or pithing to complete euthanasia. <b>Note that frogs require double-pithing (brain and spinal cord).</b>
2024.01.12	<b>7.1.2. When possible, work in a chemical fume hood or Class II Type B Biological Safety Cabinet, or wear a respirator when handling the MS222 powder.</b>