



STANDARD OPERATING PROCEDURE 402 FISH AND AQUATIC AMPHIBIAN EUTHANASIA

1. PURPOSE

This Standard Operating Procedure (SOP) describes acceptable Fish and aquatic amphibian euthanasia procedures. It ensures that animals are euthanized in the most humane way possible. It is according to the AVMA 2021 Guidelines for Euthanasia

2. RESPONSIBILITY

Principal investigator (PI) and their research staff.

3. GENERAL CONSIDERATIONS

- 3.1. All animal euthanasia must be performed by appropriately trained personnel approved by the Animal Use Protocol.
- 3.2. All euthanasia procedures must be continuously monitored by the person(s) performing the procedure until confirmation of euthanasia is complete.
- 3.3. Animals must not be left unattended until the procedure is complete.
- 3.4. 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.
- 3.5. Water quality should be similar to the environment from which the animals 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 they have been housed or captured should be used.
- 3.6. 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

4.1 Zebrafish ≥ 15 days post fertilization:

4.1.1. **Rapid cooling (hypothermia):**

- 4.1.1.1. Prepare a tank or insulated cooler containing approximately five 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 the 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. Followed 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 250-500 mg/L concentration.

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. Followed 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 five 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. To ensure death, the larvae should remain in this solution for at least five minutes before disposal.

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. To ensure death, the larvae should remain in this solution for at least five minutes before disposal.

4.3. Zebrafish embryos ≤ 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. To ensure death, the embryos should remain in this solution for 5 minutes before disposal.

5. PROCEDURES FOR OTHER FISH SPECIES

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 250-500 mg/L concentration.
- 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 with 1L of water.
- 5.2.4. Immerse the Fish for at least 10 minutes after opercular movement ceases.
- 5.2.5. Follow an adjunctive method of euthanasia, such as decapitation, pithing, or freezing, to complete euthanasia.

5.3 Sodium pentobarbital injection:

- 5.3.1. Inject sodium pentobarbital intravenously at a dose of 60 to 100 mg/kg body weight under MS22 or eugenol anesthesia.
- 5.3.2. Verify that the animal is dead by monitoring for opercular movement and lack of response to sharp tail pressure.
- 5.3.3. Time to effect may vary, with death occurring in up to 30 minutes.
- 5.3.4. Follow by an adjunctive method of euthanasia such as decapitation, pithing, or freezing to complete euthanasia.

5.4 Physical Methods:

- 5.4.1. Anesthesia or sedation must be applied before physical techniques unless approved by the BGU Animal Care Committee.
- 5.4.2. Decapitation:
 - 5.4.2.1. Use sharp equipment appropriate for the species to be euthanized to ensure that the head is quickly separated from the body rapidly and completely.
 - 5.4.2.2. Follow decapitation with pithing or freezing.
- 5.4.3. Pithing:
 - 5.4.3.1. Insert a rigid metal rod into the foramen magnum, identified by the slight midline skin depression posterior to the eyes when the neck is flexed. Ensure that the brain and the proximal end of the spinal cord are destroyed.

5.4.4 Freezing:

- 5.7.4.1. May be used as the second step of a 2-step procedure when Fish has been rendered unconscious.

6. PROCEDURES FOR AQUATIC AMPHIBIANS

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 the animal in the water. Time to effect may vary, with death occurring in up to 1 hour.
- 6.1.3 Verify that the animal is dead before disposing of the carcass by monitoring respiratory movement, heart contractions, and lack of response to stimuli.
- 6.1.4 Follow an adjunctive method of euthanasia, such as decapitation or pithing, to complete euthanasia.

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 3 to 5 g/L. Time to effect may vary, with death occurring in up to 1 hour.
- 6.2.3 Verify that the animal is dead before disposing of the carcass by monitoring respiratory movement, heart contractions, and lack of response to stimuli.
- 6.2.4 Follow an adjunctive method of euthanasia, such as decapitation or pithing, to complete euthanasia.

6.3 Physical Methods:

- 6.3.1 Anesthesia or sedation must be applied before physical techniques unless BGU Animal Care Committee approves.
- 6.3.2 Decapitation:
 - 6.3.2.1. Use sharp equipment appropriate for the species to be euthanized to ensure that the head is quickly separated from the body rapidly and completely.
 - 6.3.2.2. Follow decapitation with pithing or freezing.
- 6.3.3 Pithing:
 - 6.3.3.1. Insert a rigid metal rod into the foramen magnum, identified by the slight midline skin depression posterior to the eyes when the neck is flexed. Ensure that the brain and the proximal end of the spinal cord are destroyed.
- 6.3.4 Freezing:
 - 6.3.4.1. May be used as the second step of a 2-step procedure when Fish has been rendered unconscious.

7. SAFETY PRACTICES

7.1 Tricaine methanesulfonate (MS222):

- 7.1.1 Wear protective clothing, gloves, and eye protection when handling the MS222 powder.
- 7.1.2 Wear gloves to handle animals exposed to MS222

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.
Making eugenol solutions:

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 the sink or drain

SOP 402 FISH AND AQUATIC AMPHIBIAN EUTHANASIA

Written on (dd-mm-yyyy): 01.11.2022

Revised on (dd-mm-yyyy): 13.03.2023

Approved by the BGU Animal Policy and Welfare Oversight Committee