



COLLECTION, PRESERVATION, PACKAGING & SHIPPING OF Amphibians

FOR DIAGNOSTIC EXAMINATIONS

I. PURPOSE: This Standard Operating Procedure (SOP) provides guidelines for selecting, collecting, preserving, packaging and shipping amphibians related to ongoing mortality to optimize diagnostic value.

II. SCOPE: This SOP applies to normal-appearing live, sick and dead amphibians up to mudpuppy size.

III. EQUIPMENT & SUPPLIES

A. Hard-sided plastic cooler

B. Ice packs (not wet ice)

C. Plastic bags (heavy mil), zip-top bags, and/or plastic food-container boxes of varying sizes. Screw cap plastic jars (for fixative samples)

D. NWHC Mortality Reporting and Diagnostic Services Request Worksheet: www.usgs.gov/nwhc/submit

E. Labels for containers

F. Dissecting scissors or scalpels

G. 75% ethanol (or greater concentration) – Best is “denatured ethyl alcohol” available with painting supplies or from laboratory suppliers. Isopropyl alcohol will work in a pinch, but is not ideal. –Molecular grade is even better. Or 10% formalin

H. Packing foam, crumpled newspaper, bubble-wrap, etc.

I. Tape: nylon-reinforced tape or wide clear wrapping tape

J. Sharpie pens (fine point and regular) and pencils

IV. BACKGROUND

Amphibians and aquatic animals decompose rapidly after death, which mean amphibian carcasses nearly always will have large numbers of decomposition bacteria and fungi throughout their bodies. This rapid decomposition (autolysis) makes determining cause of death difficult. Carrying supplies in work vehicle and using collection and submission techniques outlined in this protocol will help maximize utility of specimens for diagnostic purposes.

Many amphibian die-offs are fleeting. This means the casualties must be collected as soon as they are found. Returning to the casualty site the next day to collect sick amphibians and carcasses is not optimal because of the highly efficient activity of scavengers during the night and rapid autolysis of carcasses. If any other sick/dead amphibians, mollusks, fish, reptiles, birds or mammals are present at the site, record an estimate of species and numbers. Collect other vertebrate species if they are observed sick or dead. Contact the NWHC to discuss whether to attempt to collect and submit any other species (NWHC generally does not accept fish or invertebrates).

V. COLLECTION & PACKAGING METHODS

Each individual should be individually bagged. Individual bags can be placed into a larger bag for each collection site by date. Label them accordingly.

Amphibian specimen can be collected and submitted live to NWHC for diagnostics. This is usually the best method to preserve diagnostic suitability, but the collection and packaging should be done by personnel with general knowledge of herptiles and experience handling live amphibians. Depending on your agency/institution, you may need Animal Care and Use Committee approval or special permits for collection of live animals and should always work in consultation with your agencies wildlife health or veterinary staff.

****please pre-clear a shipment with live amphibians with a NWHC epidemiologist BEFORE sending**** Detailed protocols are in section C, *but you are responsible for proper approvals.*

A. Collection of live & Sick or apparently healthy amphibians that can be collected and euthanized

Tadpoles, larvae, neonates, and adults: If possible collect, euthanize, and chill the carcasses (do not freeze) and ship within 24 hours. If prompt shipping isn't feasible, then follow (B) for dead amphibians. Please note that depending on your agency/institution, you may need Animal Care and Use Committee approval for euthanasia and should always work in consultation with your agencies wildlife health or veterinary staff. Below are recommended humane methods, *but you are responsible for proper approvals*.

To Euthanize

- 1) If you have MS222 (Tricaine methanesulfonate) available, immerse the amphibian into MS222 at 250-300mg/L concentration. Make sure the MS22 is neutral buffered to pH 7-7.5. This should be done in individual plastic bags and then leave the carcass in the MS222 and chill them. Do not freeze.
 - a) An alternative to MS-222 is to use a 20% benzocaine gel such as Orajel. Apply a strip along the ventral skin of the animal and package as above. American Veterinary Medical Association suggests ~2-3 cm strip for African clawed frogs. Use ~5-10cm strip for a large adult mudpuppy to ensure rapid anesthetic action and death. Use only products that are "gels." Liquid formulations often have high concentrations of alcohol, which is an irritant.
- 2) Keep carcasses in individual plastic bags with a small volume of whatever euthanasia agent you use, keep on blue ice packs, and ship to the NWHC as soon as possible.
- 3) See packaging "Freshly dead specimen" under section D. for more details of how to package

B. Dead Amphibians

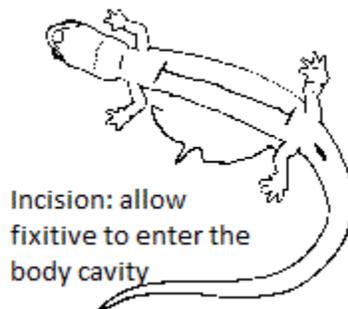
Examine the carcasses carefully in the field to determine the quality of postmortem condition for testing.

Freshly dead specimens. Freshly dead specimens show no signs of scavenging, no decomposition, no flies laying eggs, no odor, no bloating, eyes that are not sunken, and skin that appears normal with minimal physical damage, skin sloughing or slime/watermold. If there are any fresh carcasses, collect, chill, and ship immediately (details in section D). If they cannot be shipped immediately, follow guidance of "Not freshly dead specimens" below.

Not freshly dead specimens. Carcasses that are not clearly very freshly dead (this will be most cases), but not yet scavenged or decomposing should be preserved with 1/2 of the individuals frozen and 1/2 fixed in preservative.

- 1) **Fixing****. About half the dead amphibians should be immediately placed into 10% buffered neutral formalin or 70-75% ethanol for histologic examinations. When possible, the freshest carcasses (those with the least amount of decomposition) should be selected for fixation. Prior to immersing the carcass in the fixative, slit open the body cavity along the ventral midline to assure the fixative gets inside the body cavity to reach internal organs. Clean and disinfect scissors or scalpel between individuals by wiping clean and immersing in alcohol for 1 minute, then air drying. Allow specimens to soak in fixative 3-4 days before shipping. For the first 3-4 days of fixation, the volume of fixative to volume of carcasses should be 10:1. Add more fixative if volume drops owing to evaporation. After 3-4 days of fixation, the carcasses may be transferred to a minimal amount of fresh fixative that prevents drying of the specimen and shipped. Allowing the carcasses to fully fix, allows us to avoid cumbersome chemical declarations for shipping.

**Note for large amphibians (greater than 3cm wide)- to efficiently fix be sure to make the incision so that the body cavity is open and the liquid can reach the internal organs. Place the carcass in a gallon zip-top bag and then add the fixative to fully submerge the carcass. Seal the bag, removing most of the air (do this and store in a well-ventilated area), and place on a tray or in a bucket in case of accidental spills. After 3-4 days, carefully pour off liquid, reseal, and the carcass is ready to ship. A small amount of the fixative should remain to keep the specimen from drying out, but there should not be so much that the liquid sloshed in the bottom of the bag.



- 2) **Freezing**. About half the carcasses should be promptly frozen. Preferred freezing temperature is -40 degrees C, but any freezing temperature is preferable to a chilled carcass. **Do NOT freeze amphibians in water**. Frozen carcasses can be used for virus cultures, toxicological examinations, and molecular (DNA) tests. If freezing in a commercial freezer with a freeze-thaw cycle, surround with ice packs to keep frozen during the thaw cycle.

Decomposed carcasses. Clearly decomposed carcasses may have some diagnostic usefulness for molecular testing and toxicological analyses. If fresher carcasses are not available, very decomposed carcasses with fluffy

growths of fungus on the skin; maggots in the mouth, vent and body cavity; or those that consist of just skin and bones, should be frozen and saved,. *Do not send these unless directed with prior NWHC consultation.*

C. Packaging and Sending Live and Sick Amphibians

******Pre-clear a shipment with live amphibians with the NWHC BEFORE sending******

1. Eggs. Place eggs in heavy mil plastic bag or plastic container. Equal volumes of air and water should be present in the bag or container to assure adequate oxygen exchange. Do NOT fill bags or containers completely with water. If possible, place plastic bags in a solid container for support and to avoid crushing specimens or puncture of the bag.
2. Tadpoles, Larvae & Neotenes. Same as for eggs. For small amphibians (<2 grams each), multiple live animals may be placed in one container, but avoid mixing species. For larger aquatic larvae and neotenes, one animal per bag or container is recommended. It is important to assure enough air is present in each container; containers that have a large surface area of water to air are preferred; hence, flat food storage-type plastic boxes with lids (available at nearly any grocery store) are preferred to tall narrow plastic bottles.
3. Adult amphibians (terrestrial amphibians). Plastic boxes or bottles with wide lids may be used for mailing. Sick amphibians should be mailed in separate containers. Two or more live adult amphibians of the same species may be placed in one container, but avoid crowding. Note: if an infectious disease is the cause of the casualties, the disease may be transmitted between amphibians in the container if more than one animal is placed in each container. Wet unbleached (brown) paper towels or wet local vegetation should be added to the container to prevent dehydration of the animal; do not use sponges, because many contain chemicals that are toxic to amphibians. Three or more small holes should be made in the lid of each container. Plastic bags are not recommended for terrestrial amphibians. If plastic bags are the only alternative, be sure to allow plenty of air in the bag to avoid suffocation during shipping.

D. Packaging specimens

Each individual should be individually bagged and labeled. Individual specimen bags can then be placed into a larger bag for each collection site by date.

- 1) **Chilled (Found freshly dead or euthanized and never frozen).** Place in cooler with plenty of ice packs *and* wrap the specimen bags in paper towel or something else to insulate slightly and keep from freezing against ice packs.
- 2) **Fixed (preserved).** Once specimens have fixed in a large volume of formalin or ethanol for 3-4 days, the preserved samples should be mailed in a minimal amount of preservative that prevents drying. Do not mail large volumes of liquid fixative. It is prohibited by law. Wrap fixed carcasses in paper towel or other absorbent material that is moistened with the fixative inside the primary bag. Double bag the specimen. Include a few ice packs
- 3) **Frozen.** Frozen specimens should be mailed with plenty of ice packs. If possible, ship frozen carcasses in separate coolers than fixed or chilled carcasses. If frozen and fresh dead (or fixed specimens) must be mailed in the same cooler, separate the shipping container into two compartments with styrofoam panels or other insulating material and place most of the ice packs at one end of the container next to the frozen samples.

E. Labels and data

Place labels with each carcass. They can be placed inside the primary plastic bag (within their own plastic bag) or taped to that bag. Paper labels written **in pencil** are preferred, especially if there is ethanol in any containers. Most ink will dissolve in ethanol or become streaked during freezing and thawing. Each label should have the following information:

- species
- date collected
- location (state/county/town)
- found dead or euthanized and method
- preservation method (chilled, frozen, fixative solution)

Also fill out a NWHC diagnostic request form (attached) with the contents of the cooler, submitter contact, and any other observations about the mortality event

E. Mailing.

1. **Shipping container.** Use a hard-sided plastic cooler and follow labeling instructions on the general shipping protocol. www.usgs.gov/nwhc/submit

2. **Ice.** Ice packs (“blue ice”) minimize leaking during shipment and keep the fedex or UPS staff happy.

4. **Packing the shipping container.** Prepare specimen, as described above. Place unfrozen specimen into plastic containers or add other bracing material (crumpled newspaper or packing peanuts) to keep ice packs from crushing the specimen. Include a copy of the diagnostic specimen submission form (placed inside a plastic bag so it does not get wet) taped to inside of lid or on top of all packing material in the cooler.

5. **Shipping.** Overnight courier service should be used (Fedex or UPS is best). Select the ~10am delivery time. Ship Monday-Wednesday, unless there is a freshly dead specimen, then shipping Thursday is acceptable. Do not ship for weekend or holiday delivery. Securely tape the cooler or box and mail to:

USGS National Wildlife Health Center

Necropsy Loading Dock

6006 Schroeder Road

Madison WI 53711

6. **Notice of Shipping.** You must notify NWHC prior to shipping by email (nwhc-epi@usgs.gov) that includes the overnight tracking number and an electronic copy or scan of the specimen submission form. Alternatively call an NWHC epidemiologist to provide the expected delivery date, tracking # and what is coming (608-270-2480). Use these contacts for shipping notification and to ask any questions about observations of sick or dead wildlife, as well as for help with packaging and shipping.