***Batrachochytrium salamandrivorans* (Bsal)**

**Rapid Response Template**

**Last revised June 8, 2018**

**PLEASE NOTE:** Within this document are **explanatory notes and questions to stimulate discussion** to help clarify the intent of the information provided for end users and to facilitate their customization of the template. These are **placed throughout the text in *blue italics*** thus distinguishing these notes from other guidance provided for the purpose of responding to a Bsal detection or outbreak.

**Purpose:** This document and recommendations that follow serve as a **template to be customized** by any agency or institution with management jurisdiction over wild or captive amphibians, respectively, when actions in response to a disease may be warranted. *This purpose statement may be further customized as needed for individual entities.*

Herein is provided an outline and guidance for **local, rapid response** actions that could be triggered upon initial or subsequent detections of Bsal, in either wild or captive amphibian populations. *The scenarios are based on what an entity using this plan might do after receiving information regarding Bsal detection status from a diagnostic laboratory with expertise in Bsal diagnostics. In other words, all recommended actions occur after the laboratory has made its determinations based on the Case Definition of Bsal (White et al. 2016).* Also provided are considerations for *in situ* containment (i.e., in the existing location of the population) as well as establishment of *ex situ* populations (i.e., outside of the natural location, such as in captive assurance colony). Rapid containment and response measures may prevent broad impacts. *The USGS Amphibian Research and Monitoring Initiative (ARMI) is also working to assist entities in making decisions regarding wildlife disease management, including the customization of this template. Contact ARMI Decision Support Lead, Dr. Evan Grant (*[*ehgrant@usgs.gov*](mailto:ehgrant@usgs.gov)*) for assistance.*

This template was produced by the **Bsal Response Working Group** as part of their work with the **Bsal Task Force’s Technical Advisory Committee** (see [www.salamanderfungus.org](http://www.salamanderfungus.org) for additional information), and is considered a living document that will be updated as more information becomes available.

At the time of this version, Bsal is not known to occur in North America and suggested responses are consistent with the high-alert condition of Bsal being yet undetected or rare in North America. This document is intended to be incorporated into a National Bsal Strategic Framework, where larger surveillance and monitoring strategies, research needs, development of a Bsal reporting database, policy needs, and related prevention strategies, along with public outreach and communication, are addressed.

***Batrachochytrium salamandrivorans* (Bsal)**

**Rapid Response Template**

**Preliminary definitions and resources**

*This section sets forth how terms are defined within the rest of the document. These definitions will also occur in the larger Action Plan. However, entities customizing this template should add other definitions as they deem appropriate.*

**Definitions:**

1. *Bsal-susceptible host species* – We use this phrase broadly to indicate both species for which Bsal can be fatal and species that can be infected by Bsal but not develop disease symptoms, hence may serve as reservoirs and carry Bsal. At the time of this version, experimental and field evidence suggests that anurans may carry it (Stegen et al. 2017; Yuan et al. 2018). Thus, we assume that all amphibian species *may* be susceptible to Bsal or be carriers of Bsal unless it is demonstrated that a species cannot be infected. *Bsal has been termed the “salamander fungus” because it was described from infected fire salamanders in Europe and has been shown to either infect or to be carried by several other salamander species (Martel et al. 2014). New evidence suggests that some anurans can also be infected and carry the pathogen, potentially without developing clinical signs of infection (Stegen et al. 2017; Yuan et al. 2018). This template and this definition will be updated when new evidence of species-specific susceptibility becomes available.*
2. *Wild host population* – Free-ranging population of Bsal-susceptible species.
   1. Naïve (no prior Bsal detections known at a given site)
   2. Exposed (prior Bsal detections documented at a given site)
   3. Unknown (no or insufficient Bsal surveillance has been performed to know the status)
3. *Captive host population –* Any population that is not free-ranging, including outdoor enclosed spaces or fenced runs where contact with wild amphibians or disease vectors may be possible (e.g., zoo, aquarium, research facility, university).
   1. Naïve (no prior Bsal detections known from the captive location)
   2. Exposed (prior Bsal detections documented from the captive location)
   3. Unknown (no or insufficient Bsal surveillance has been performed to know the status)
4. *Mortality event*, wild – Death of one or more free-ranging amphibians in the environment, whether or not the Bsal pathogen has been detected.
5. *Mortality event*, captive – Death of one or more amphibians in a captive environment, whether or not the Bsal pathogen has been detected.
6. *Eradication* – The assumed elimination of Bsal from individual amphibians *held in captivity* based on four (4) consecutive, negative PCR tests, each one week apart, per individual, as described in Blooi et al. (2014).
7. *Participating Laboratory*. The particular laboratory that has been engaged during a testing or response effort; see also Resources below regarding the Diagnostic Laboratory Network.
8. *Reporting Individual(s)*. The individual(s) who submitted the sample(s) (e.g., swabs, carcasses, live animals) to a laboratory for diagnostics. This is the person(s) the laboratory is to contact to provide results. In some cases, this may be a scientific researcher. *At the time of this version, the Bsal Task Force is working to develop a statement and working with key scientific journal editors to ensure that sharing of scientific findings with management agencies in order to facilitate early detection and rapid response actions will not diminish the value or integrity of the scientific findings or the person(s) involved.*
9. *Core Response Team* (CRT). The group of authorized professionals, and other parties involved in the initial discovery, that evaluates the situation and makes recommendations for next steps. The CRT may include other trusted parties, as appropriate, where information can be securely shared, and will not compromise scientific integrity (see suggested composition in Resources)**.** *We reference the use of such a team as part of the recommended actions in the response scenarios described in this template. We suggest that certain members of this team be identified in advance, to facilitate a rapid response. Below, we offer additional suggestions regarding role and composition. However, the use or role of the team is ultimately at the discretion of the entity customizing this template.*

**Resources**

1. **Diagnostic Laboratory Network.** A consortium of participating laboratories equipped to handle Bsal testing requests, and to employ specific protocols (as recommended by the Bsal Task Force’s Diagnostics team) for Quality Assurance and Quality Control (QA/QC). Assists with coordination of sample handling. The list of known labs capable of Bsal testing is provided on the Bsal Task Force Website: [www.salamanderfungus.org/resources/labs](http://www.salamanderfungus.org/resources/labs). *Entities customizing this template may benefit from contacting their nearest laboratory(-ies) to understand their sample submission protocols, fees for services (as applicable), and any other requirements to collaborate in the event of a disease outbreak (whether Bsal or other pathogen).*
2. **Core Response Team (CRT).**  *As noted above, we reference the use of such a team as part of the recommended actions in the response scenarios described in this template. Here, we offer suggestions on the charge and composition of the team. However, the use or role of the team is ultimately at the discretion of the entity customizing this template.*
   1. **Purpose**. The CRT is an advisory group who discusses the specific scenario and helps to make initial decisions regarding response actions and related communications. Any member of the CRT is expected to keep the shared information **confidential** until the **management agency or entity with jurisdiction** (i.e., the authority to make decisions about the species or the lands affected)indicates how, where, when information may be shared.

**Composition**:  ***The composition of this team may change depending on the specific circumstance. A brief explanation on the suggested composition: a)*** *Individual who discovered the mortality event or was involved in research that led to a Bsal-positive detection may have ability to assist in response-related actions or follow-up work at the site; b) Agency with management jurisdiction, or land manager will be able to confirm actions that can or cannot be taken; c) The state or provincial/territorial fish and wildlife agency is the primary management authority for amphibians & can assist with appropriate species management actions on non-federal lands; d) Amphibian experts can advise on most current science. Specifically,* ***the Bsal Technical Advisory Committee was formed to include appropriate expertise in the event of a Bsal outbreak,*** *and is at your disposal for confidential advisory assistance.*

* + 1. Reporting Individual(s)
    2. Agency or entity with jurisdiction over the affected species or lands
    3. Land or Facility manager(s)/owner(s) where samples were collected, if different from the entity in (2)(b)(ii)
    4. State agency personnel in charge of amphibians
       1. NOTE: The Association of Fish and Wildlife Agencies’ Amphibian & Reptile Program Manager, Priya Nanjappa ([pnanjappa@fishwildlife.org](mailto:pnanjappa@fishwildlife.org)), can assist in determining the appropriate state contacts.
    5. Key amphibian expert scientists who can provide recommendations, **in a confidential consulting capacity**, for short and long-term responses based on best available science:
       1. NOTE: Please consider contacting the Bsal Technical Advisory Committee leadership ([response@salamanderfungus.org](mailto:response@salamanderfungus.org)); one or more members will be available to assist in a confidential advisory capacity.

1. **Points of Contact (PoCs):** *Entities customizing this template should populate with preferred PoCs.*
   1. Provide a list of key contacts in a given state, federal agency, or management unit (e.g. unit director or manager; staff veterinarian, lead herpetologist or wildlife biologist) to inform when there is a positive/after CRT and Reporting Individual.
   2. Include Permit coordination contacts (state, federal, ESA, etc.)
2. **Wildlife Health Expert Networks.** Qualified wildlife experts to assist in treatment of captive or privately-owned animals, issuing health certifications or other documentation to verify animal health, emergency responses, etc., may be found via:
   1. The Diagnostic Laboratory Network established via the Bsal Task Force’s Diagnostics Working Group (see [www.salamanderfungus](http://www.salamanderfungus).org).
   2. Veterinary experts:
      1. [Association of Reptile and Amphibian Veterinarians](http://arav.org/%20) (ARAV);
      2. [Board-certified zoological medicine veterinarians](http://www.aczm.org/content.aspx?page_id=22&club_id=366916&module_id=48992); or
      3. The [American Association of Wildlife Veterinarians](http://www.aawv.net/) (AAWV)
   3. Wildlife Epidemiologists or Wildlife Disease Ecologists
3. **Facilities.** A list of available captive housing or breeding facilities, with contacts (e.g., Amphibian Ark (AArk), Association of Zoos and Aquariums (AZA)-accredited zoos, other local facilities)
   1. Treatment. *Entities customizing this template should identify secure, emergency facilities in their network to temporarily house moribund (dying, unable to right themselves) or sick but potentially treatable animals, and infected animals without disease symptoms that may be treatable to avoid their becoming vectors of pathogen transmission*.
   2. Rescue colonies. *Entities customizing this template should identify facilities to house rescued animals or those collected for the purpose of captive breeding and reintroduction.*
   3. Museums or other storage facilities. *Entities customizing this template should identify facilities for vouchered animals, or archived tissue samples, swabs or extracted DNA.*

***Questions:*** *What AArk or AZA facilities are local? Are you familiar with the appropriate contacts there? What local museums are able to accession animals? Can they also accession tissues, swabs, DNA?*

1. **Protocols.** *Along with those below, also consider other protocols that may be useful, e.g., data submission or management protocols.* Recommended guidance can be found at the Bsal Task Force website, [www.salamanderfungus.org](http://www.salamanderfungus.org), via the Diagnostics or Research pages, but see also Pessier & Mendelson (2017), including:
   1. Biosecurity protocols for field, lab, use of live cultures, etc.
   2. Swabbing and storage (and transportation) protocols
   3. **See also Appendix I**, where pertinent portions of the guidance manual have been included and adapted for quick reference.
2. **Bsal Reporting Database: amphibiandisease.org** This web portal is available for reporting the results of Bsal surveillance, including both Bsal detections and no-detections. Also, planned projects can be reported here to improve the efficiency of taxonomic- or geographic-specific surveillance efforts, and to aid communication among managers and scientists.

**RESPONSES**

**NOTE:** The scenarios below pertain to mortality or PCR detection events (and subsequent confirmation of causative agent), however, any suspicious-appearing amphibians should be investigated. Examples of suspicious-appearing amphibians would be sick or lethargic individuals, those with black circular or oblong lesions, or inability to right themselves. In this heightened state of awareness, all such amphibians should be reported. The Partners in Amphibian and Reptile Conservation (PARC) Disease Task Team has established an alert system to help connect people to the appropriate experts and authorities quickly; please see [the PARC Disease Task Team website](http://parcplace.org/resources/parc-disease-task-team/) for information and for how to send a report to [herp\_disease\_alert@parcplace.org](mailto:herp_disease_alert@parcplace.org).

**Scenario 1: Mortality event, cause unknown; Wild**

Mortality events may be due to any number of causative agents. The actions below include collection of samples to confirm a diagnosis and activities to be considered while results are pending. These should be implemented at the discretion of the jurisdictional management unit depending on the level of response they are able to take to help minimize potential impacts. Contact [your local amphibian expert or member of a Veterinary Expert Network] to assist. *Entities customizing this document should identify appropriate amphibian experts local to your jurisdiction.*

**When uncertain how to proceed or whom to contact**, the Partners in Amphibian and Reptile Conservation (PARC) Disease Task Team has established an alert system to help connect people to the appropriate experts and authorities; send a report to [herp\_disease\_alert@parcplace.org](mailto:herp_disease_alert@parcplace.org). *Reports should include your name and email address, what you saw, where you saw it, what types of animals were involved such as species and life stage (e.g., larvae, adults), whether the disease event is ongoing, and any photographs (if available) or other relevant information.*

***Actions recommended (one or more, items 2-6 in no priority order and as feasible):***

**Notification to agency with management jurisdiction.** To facilitate a Bsal early detection and rapid response, contact the management agency with jurisdiction where the mortality event occurred (which may be your own agency) to ensure they are aware of the testing event and impending results. ***Important --*** *Given the heightened state of alert for Bsal and the critical nature of early detection and rapid response, when customizing this template, please consider including this recommended action of contacting the management agency with jurisdiction where the mortality event occurred, even if this may be your own agency, to be sure they are aware that a mortality event and testing is underway, while results are pending.*

***Questions:*** *Do you know the appropriate contacts for disease response in the agencies with management jurisdiction in your state? (If not, the PARC Disease Task Team may be able to assist; send a message to* [herp\_disease\_alert@parcplace.org](mailto:herp_disease_alert@parcplace.org) *requesting information on the appropriate contacts.)* ***For management agencies:*** *Are there other partners that you need to engage and if so, should it be at this stage or after results are received?*

1. Tissue collection for diagnostics.
   1. Collect any live but apparently moribund (dying, unable to right themselves) or lethargic animals, using humane euthanasia procedures, as applicable (**see** **Appendix I, Section A**); submission to Participating Laboratory. Swabs alone are insufficient to confirm a Bsal diagnosis.
   2. Carcass collection, fresh-dead (see **Appendix I, Section A**), for diagnostic necropsy and submission to Participating Laboratory.
   3. Sampling of other live amphibians (e.g., swabbing skin for use in a PCR assay), if area is high risk and if feasible (**Appendix I, Section B**).
2. Biosecurity protocols, as established (**Appendix I, Section A**(**3**)), implemented for all field gear especially as part of implementing #2 above, and also upon leaving die-off site.

***Questions:*** *Have you considered establishing an approved set of biosecurity protocols for sampling or surveillance in a disease-affected site?*

1. Heightened alert considerations.
   1. Increased surveillance
   2. Local personnel notification. *It may be helpful to form and consult the CRT (see Resources above) or to assess notifications at this stage, and could be handled on a “need to know” basis.*
2. Containment considerations. The following are options that might help prevent spread of pathogens.
   1. Restricted public access to the exposed area(s).
   2. Signage at or around the exposed area(s).
   3. Local personnel notification and access restrictions to the exposed area(s). *Again here, it may be helpful to consult the CRT or to assess notifications at this stage, and could be handled on a “need to know” basis.*
3. **See below for “Definitive detection, Wild” for additional response**

**Scenario 2: Mortality event, cause unknown; Captive**

Mortality events may be due to any number of causative agents. The actions below include collection of samples to confirm a diagnosis and activities to be considered while results are pending. These should be implemented at the discretion of the captive management facility depending on how conservative or comprehensive of a response they are able to take to help minimize impacts. Contact *[your local amphibian expert or member of a Veterinary Expert Network]* to assist.

**When uncertain how to proceed or whom to contact**, the Partners in Amphibian and Reptile Conservation (PARC) Disease Task Team has established an alert system to help connect people to the appropriate experts and authorities; send a report to [herp\_disease\_alert@parcplace.org](mailto:herp_disease_alert@parcplace.org). *Reports should include your name and email address, what you saw, where you saw it, what types of animals were involved such as species and life stage (e.g., larvae, adults), whether the disease event is ongoing, and any photographs (if available) or other relevant information.*

***Actions recommended (one or more, items 2–6 in no priority order and as feasible):***

1. **Notification of state or provincial/territorial fish & wildlife agency.** To maintain transparency and open communications regarding Bsal and to facilitate early detection and rapid response, we recommend contacting the state or provincial/territorial fish & wildlife agency where the mortality event occurred to ensure they are aware of the testing event and impending results. ***Important --*** *Given the heightened state of alert for Bsal and the critical nature of early detection and rapid response, when customizing this template, please consider including this recommended action of contacting the state or provincial/territorial fish & wildlife agency where the mortality event occurred, to be sure they are aware of the mortality event and that testing is underway, while results are pending. This allows them to consider additional surveillance or management actions to further protect wild populations.*

***Questions:*** *Do you know the appropriate contacts for disease response in the agencies with management jurisdiction in your state? (If not, the PARC Disease Task Team may be able to assist; send a message to* [herp\_disease\_alert@parcplace.org](mailto:herp_disease_alert@parcplace.org) *requesting information on the appropriate contacts.)* ***For management agencies or industries:*** *Are there other partners that you need to engage and if so, should it be at this stage or after results are received?*

1. Tissue collection for diagnostics.
   1. Collect tissue and/or moribund (dying, unable to right themselves), abnormally behaving, or co-located live animals, as feasible and using humane euthanasia procedures, as applicable (**Appendix I, Section A**); submission to the facility’s pathologist, where applicable, or, after confirming closest lab that is able to handle the specific case, to a Participating Laboratory (see also the Diagnostic pages of [www.salamanderfungus.org](http://www.salamanderfungus.org)).
   2. Carcass collection, fresh-dead animals for diagnostic necropsy; submission to Participating Laboratory (**Appendix I, Section A**).
   3. Consider collecting swabs from living animals without symptoms contained in the same enclosures or nearby.
2. Biosecurity protocols, as established in Pessier & Mendelson (2017), implemented for:
   1. Disinfection of captive caging/housing facilities and materials prior to reuse for treated or new animals.
   2. Treatment and disinfection of water prior to disposal.
   3. Treatment of plant or soil substrate materials prior to disposal.

***Questions:*** *Have you established/considered establishing an approved set of biosecurity protocols for disease-affected populations/housing materials in captivity?*

1. Containment considerations. For exposed, captive animals that remain living, we suggest the following:
   1. Individual quarantine for all potentially exposed animals until causative agent is determined.
      1. Consult with your local amphibian or veterinary expert and consider prophylactic treatments, and post-treatment testing and monitoring, as per guidance in Blooi et al. (2015a, b).
   2. Halt transport/commerce of exposed, co-located, co-shipped, or all amphibians until health conditions and pathogen eradication can be verified.
   3. Retrieve chain-of-contact/custody information (i.e., individuals or entities throughout the history of possession of the animals or lot in which the affected amphibians originated).
      1. Inform and recommend to all potential points of transmission to follow quarantine, testing, and treatment recommendations.
   4. Ensure biosecurity standards have been met (see #3) prior to resumption of any transport or commerce of animals or caging materials, in accordance with existing federal, state, or local laws.
2. **See below for “Definitive detection, Captive” for additional response.**

**Scenario 3: Detection of Bsal Presence, by Polymerase Chain Reaction (PCR) (Wild or Captive)**

**This scenario is defined as:** Detection of *B. salamandrivorans* DNA, as determined by a Participating Laboratory, based on swab or tissue samples of individual amphibians or from the environment (e.g., environmental DNA sampling), using PCR testing. *Ideally, the Participating Laboratory will have also verified the result by a second Participating Laboratory.*

This scenario indicates potential presence of Bsal, but is NOT considered a “definitive detection” of Bsal until additional evidence of Bsal has also been determined. However, in the heightened state of alert, the guidance below is to facilitate early detection, rapid response efforts, while confirmation of Bsal presence is pending.

*A detection of Bsal presence via PCR could occur a) in an instance where no clinical sign or histopathologic evidence, nor evidence of a current mortality event, exists that is indicative of an active Bsal outbreak, or b) as an outcome of Scenarios 1 or 2 above, or c) may arise independently via surveillance or research of wild or captive populations*.

***Actions recommended (one or more as feasible):***

1. Initial diagnostic results communicated by Participating Laboratory to:
   1. Reporting Individual(s), who in turn informs:
      1. Detection site landowner/manager
      2. Wildlife agency or entity with management authority
2. Agency or entity with management authority forms and convenes the Core Response Team (CRT). *Some entities customizing this template may consider developing an* [*Incident Command System*](https://en.wikipedia.org/wiki/Incident_Command_System) *to help coordinate across other agencies or stakeholders.* 
   1. Consider also engaging the Bsal Task Force Technical Advisory Committee leadership ([response@salamanderfungus.org](mailto:response@salamanderfungus.org)), who are available to assist by advising on resources and responses, and will keep the information confidential. *Through the Task Force’s working groups, additional assistance can be provided on next steps following a PCR detection.*
   2. Consider developing a communications plan that facilitates internal agency and Core Response Team communications to external stakeholders and the public (including signage for affected sites, intended visitor behavior modifications). *These are potential, suggested components of a communications plan; customized actions may differ.* ***Questions:*** *Is there any cultural or archaeological significance of the site? Is it a popular visitor site that may require a visitor management plan, or additional staffing to advise the public and help avoid disturbance or public contact with affected areas?*
3. Further investigation. Additional diagnostic testing should be conducted as feasible (e.g., sequencing and phylogenetic analyses, isolation by fungal culture, necropsy and histopathologic examination of associated dead animals or tissues where applicable) by a Participating Laboratory for a definitive diagnosis (White et al. 2016).
4. Management Actions, **Wild populations**
   1. Biosecurity protocols, as established (**Appendix I, Section A(3)**), implemented for all field gear used at the Bsal-positive site.
   2. Increased surveillance at Bsal-positive site.
      1. If available, test any archived amphibian tissues from the site of detection for Bsal.
      2. Evaluate known amphibian species composition at the site, with special consideration for presence of federally-listed, state-listed, and at-risk salamander species.
         1. If listed and/or at-risk species are present, evaluate need and opportunity available for taking healthy individuals from the wild and placing them in captivity for establishment of a breeding (captive assurance) colony.
      3. Conduct additional sampling of amphibians and water at the site of detection.
      4. Evaluate movements of other animals in or out of the site
   3. Heightened awareness by managers at the Bsal-positive site.
      1. Collect any morbid or dead amphibians at that site and submit to Participating Laboratory for testing.
      2. Review any existing data from vicinity of site for evidence of population or mortality trends.
      3. Initiate population monitoring of affected amphibian species to determine if stable or declining.
   4. Containment Considerations. Consider options that might help prevent the spread of Bsal:
      1. Restricted public access to the exposed area(s).
      2. Signage at or around the exposed area(s).
      3. Local personnel notification and access restrictions to the exposed area(s).
      4. Direct actions, when evaluating risk and with an abundance of caution. ***Questions****: Is drying or treating the site an option? Is the harm of taking an extreme action greater than doing nothing?*

*Treatment of animals and sites, and reporting to the Bsal database is covered in Scenario 4, Definitive Detection, Wild.*

1. Management Actions, **Captive populations**
   1. Containment
      1. Ensure no shared water sources or water flowing out of the animals’ caging/housing.
      2. Individual quarantine. Isolate affected animals, including any that were housed with affected individuals.
         1. Perform additional diagnostics on co-located individuals.
         2. Eradicate Bsal sources.
            1. For live, captive animals whose samples return a positive Bsal result, eradication may be attempted:

For failsafe eradication, we recommend humane culling or euthanasia, and either:

Preservation of infected individuals, per **Appendix 1(A),** for further histological analysis (consult with your CRT and your Participating Laboratory to confirm necessity).

Disposal of infected individuals using strict biosecurity protocols. See Section 8.6 in Pessier & Mendelson, 2017, or humane methods in accordance with the *AVMA Guidelines for the Euthanasia of Animals: 2013 edition* (<https://www.avma.org/KB/Policies/Documents/euthanasia.pdf>).

If there are reasons to maintain the animals, eradication of Bsal may be possible and has been demonstrated in published literature (Blooi et al. 2015a; Blooi et al. 2015b). *There may be reasons to maintain and treat animals, e.g., with threatened or endangered species. However, there may also be reasons to maintain infected animals, e.g., for additional diagnostics or research. Consult with the CRT and your Participating Laboratory to determine options.*

As such, we suggest the following:

Treat per guidance in Blooi et al. (2015a, b). *As new treatments and research are being investigated, we will update this template.* ***Please note:*** *the methods tested to date only are confirmed in Fire Salamanders (Salamandra salamandra); keep in mind that species differences may come into play with respect to treatment validity and effectiveness. This is why multiple swabs for PCR testing over time are necessary to confirm eradication.*

Swab treated animals post-treatment (see Appendix I, Section B) and submit repeat samples to a Participating Laboratory to confirm Bsal eradication.

Repeat treatment regime(s) and post-treatment swabbing until confirmation of Bsal eradication.

* 1. Disinfection, per Pessier & Mendelson (2017):
     1. All caging/housing materials and equipment prior to reuse.
     2. All water prior to disposal.
     3. All plants, soils, or other organic materials prior to disposal.
  2. Captive population monitoring. Evaluate the exposure to other co-located amphibians, including:
     1. Determine other places it could be in the facility, and disinfect these areas.
     2. Assess other potential sources of spread or origin of the pathogen, including through shared water sources or uses and movements, and quarantine or disinfect these sources.
     3. Assessment across the collection to determine whether it is clinically stable or if there is a trend of increasing morbidity and mortality.
  3. Reporting, and additional testing, throughout the chain of custody (i.e., individuals or entities throughout the history of possession of the animals or lot in which the affected amphibians originated).

At minimum, swab amphibians for PCR analysis throughout the chain of custody.

Consider additional monitoring, as in 5(c) above.

*Treatment of animals and sites, and reporting to the Bsal database is covered in Scenario 5, Definitive Detection, Captive.*

1. Document Bsal treatment. Prior to resumption of transport or sale (in accordance with existing federal, state, or local laws), consider obtaining a health certificate or other documentation from a member of one of the Veterinary Expert Networks verifying Bsal treatment and eradication for *each individual animal* that tested positive for Bsal and was treated and for which Bsal was shown to be eradicated. *Entities customizing this template should keep in mind that each state may or may not have specific laws regarding “official” health certifications or alternative options; it is important to consult your state fish and wildlife agency and state department of agriculture regarding either the recommendation being an official or unofficial form of documentation.*
2. Additional management guidance via CRT
3. Messaging considerations.
   * 1. CRT will advise on and assist in development of preliminary detection messaging for the *Reporting Individual(s)* or the agency/entity with management jurisdiction over the site of detection to disseminate.
4. Movement restrictions, voluntary or mandatory, implemented by landowner/manager, captive population owner, or agency with jurisdiction over the captive animals, to reduce further transmission (e.g., prohibitions on collecting wild salamanders from the wild site; temporary moratorium on movement or sale of salamanders from the captive facility until further information is known). *Entities customizing this template may consider including additional guidance for tracking animals that were documented to be infected and then treated, including reporting or other requirements upon relocation to new jurisdictions.*
5. Subsequent communications:
   1. If the Bsal Technical Advisory Committee has not been engaged in prior steps, consider contacting them regarding the findings and actions ([response@salamanderfungus.org](mailto:response@salamanderfungus.org)).
   2. Internal communications as required by *Reporting Individual’s* agency/organization.
   3. Internal communications within the agency or entity with management jurisdiction of the detection site as management decisions are made, on a need-to-know basis.
   4. Local stakeholder and chain-of-contact/custody outreach.
   5. No further communications until detection status is definitive. *Limiting communications to a “need to know” group of people may help until confirmations of Bsal (or other pathogen) detection is received, to avoid unnecessary attention or public reaction.*

**Scenario 4: Definitive detection, Wild**

**This scenario is defined as:** Evidence of *both* 1) the presence of Bsal, as determined by the Participating Laboratory through either PCR-testing, or through isolation of a Bsal fungal culture as identified with genetic sequencing; *and* 2) signs of infection, as determined by the Participating Laboratory based on either clinical signs of disease in individual animals, or by histopathological characterization consistent with Bsal infection. *Evidence of presence without evidence of infection is not enough to determine definitive detection of Bsal (see Iwanowicz et al. 2017). Laboratory determinations are based on the Case Definition for Bsal chytridiomycosis (White et al. 2016), accepted by the Diagnostic Working Group of the Bsal Task Force.*

***Actions recommended (one or more, as feasible):***

1. Results communicated by Participating Laboratory to:
   1. Reporting Individual(s), who in turn informs:
2. Detection site landowner/manager
3. Wildlife management agency with jurisdiction over species and/or land
4. Agency or entity with management authority forms and convenes the Core Response Team (CRT)
   1. Consider also engaging the Bsal Task Force Technical Advisory Committee leadership ([response@salamanderfungus.org](mailto:response@salamanderfungus.org)), who are available to assist by advising on resources and responses, and will keep the information confidential.
5. Subsequent Communications (in order of priority)
6. Internal communication as required by the Reporting Individual’s agency/organization.
7. If the Bsal Task Force Technical Advisory Committee leadership has not yet been informed, notify them of the findings ([response@salamanderfungus.org](mailto:response@salamanderfungus.org)).
8. Formal stakeholder notifications (e.g., partner institutions or agencies).
9. Public announcement/press release as appropriate.
10. Local stakeholder outreach (e.g., public groups who use the affected sites and could be asked to either disinfect gear and to report observations of dead amphibians).
11. Scientific publication outlet.
12. Bsal reporting database. *Laboratory findings can now be entered into the Bsal reporting database at amphibiandisease.org*
13. Emergency Meeting convened among parties identified in 2a, and possibly 3a–b, above to discuss:
    1. Risk/threat assessment. *Some considerations for potential risk assessment include species movements, people’s activities, water movements, etc., and risk level to co-occurring species.*
    2. Management actions and considerations:
       1. Containment of mortality/detection site:
          1. Landowner/manager restrictions on public access to site, except for approved personnel.
          2. Strict use of approved biosecurity protocols (Appendix I, Section A(3)) for all personnel, their gear, vehicles, etc. when exiting site.
             1. Establish dedicated equipment/gear including nets, footwear, etc. for the site.
          3. Deployment of fencing or other containment measures to reduce or prevent spread by other wildlife.
          4. Demarcation of the affected area(s) to minimize or prevent trespass by personnel or public.
       2. Establishment of *ex situ* colony(-ies):
          1. Engage additional partners (Amphibian Ark, AZA, American Association of Zoo Veterinarians, etc.) to assist.
          2. Initiate rescue/captive assurance populations:
             1. Based on conservation status (e.g., federally or state-listed).
             2. Based on proportion of local population affected and proportion of total population represented locally.
             3. As an attempt to salvage/save affected, but treatable, individuals.
       3. Priority surveillance:
          1. Detection site
             1. Sampling of other amphibian species at the detection site, particularly any within those families shown to be susceptible in Martel et al. 2014 and Stegen et al. 2017 (or more recent publication, if available).
             2. Additional sampling of exposed amphibian species or substrates.
          2. Non-independent sites (e.g., potential transmission pathways of water bodies connected to the detection site by permanent or ephemeral water flow or watershed considerations, and adjacent terrestrial areas).
          3. Adjacent waters or lands within natural movement distances of the affected species.
          4. Nearby sites that may serve as refugia for translocating uninfected salamanders.
       4. Movement restrictions and prohibitions on collections of wild salamanders from affected site.
       5. Other interventions as feasible, e.g., antifungal treatments for surviving animals, as described by Blooi and colleagues (2015b), or possibly habitat treatments or disinfection. *As new information becomes available on pending research and mitigation strategies, we will update this template.* *Preliminary data show some habitat treatments may be effective in eradicating the related pathogen,* Batrachochytrium dendrobatidis *(Bd; Bosch et al. 2015). In the early stages of Bsal detection and rapid responses, these may be the best opportunities to address site-level habitat treatments as part of containment and eradication. The Bsal Task Force’s Response and Management working groups are examining policies for rapid responses to pathogens such as Bsal.*
          1. *Culling/Euthanasia*
          2. *Chemical treatments of animals or site*
          3. *Draining or drying*
          4. *Site closures (including physical barriers)*
          5. *Signage or additional staffing to address desired visitor behavior modifications*

**Questions:** *Whom might you contact for each of the above possible actions? Is there an “expert team” you could develop and have on call for the different actions above? The Bsal Task Force can assist in identifying a few national contacts, and perhaps also some local contacts, as a start.*

*What local, state, or federal resources are there to accomplish the actions above (e.g., labs, chemical application or water draining equipment)?*

*What local, state, and federal laws may apply for environmental compliance? Do agency or local law enforcement contacts need to be informed or engaged?*

**Scenario 5: Definitive detection, Captive**

**This scenario is defined as**: Evidence of *both* 1) the presence of Bsal, as determined by the Participating Laboratory through either PCR-testing, or through isolation of a Bsal fungal culture as identified with genetic sequencing; *and* 2) signs of infection, as determined by the Participating Laboratory based on either clinical signs of disease in individual animals, or by histopathological characterization consistent with Bsal infection. *Evidence of presence without evidence of infection is not enough to determine definitive detection of Bsal (see Iwanowicz et al. 2017). Laboratory determinations are based on the Case Definition for Bsal chytridiomycosis (White et al. 2016), accepted by the Diagnostic Working Group of the Bsal Task Force.*

***Actions recommended (one or more, as feasible):***

1. Results communicated by Participating Laboratory to:
   1. Reporting Individual(s), who in turn informs:
      1. Captive animal owner/captive facility manager or veterinarian
      2. State or provincial/territorial agency(-ies) with jurisdiction over captive animal health and movement (e.g., wildlife management agency, or state/provincial/territorial department of agriculture)
2. Agency or entity with management authority forms and convenes the Core Response Team (CRT)
   1. Consider also engaging the Bsal Task Force Technical Advisory Committee leadership ([response@salamanderfungus.org](mailto:response@salamanderfungus.org)) is available to assist by advising on resources and responses, and will keep the information confidential.
3. Subsequent Communications (in order of priority)
   1. Internal Reporting agency/organization (if applicable)
   2. Pet store, or importer, or zoological institution where animals were acquired
4. Chain-of-contact/custody stakeholders (i.e., individuals or entities throughout the history of possession of the affected amphibians, and other associated individuals or entities).
   1. Formal stakeholder notifications (per CRT guidance)
      1. State veterinary health official
      2. AZA Taxonomic Advisory Group or Species Survival Plan contacts
   2. Scientific publication outlet.
   3. Bsal reporting database.
   4. Public announcement/press release as appropriate (and in collaboration with captive animal/facility owner).
5. Emergency Meeting convened among parties identified in 2 and possibly 3(a–c) above to discuss:
   1. Risk/threat assessment.
   2. Management actions.
      1. Containment.
         1. Ensure no running water out of the housing of the animals
         2. Eradicate Bsal sources.
            1. For live, captive animals whose samples return a positive Bsal result, eradication may be attempted:

For failsafe eradication, we recommend humane culling or euthanasia and disposal of infected individuals using strict biosecurity protocols. See Section 8.6 in Pessier & Mendelson, 2017, or humane methods in accordance with the *AVMA Guidelines for the Euthanasia of Animals: 2013 edition* (<https://www.avma.org/KB/Policies/Documents/euthanasia.pdf>).

If there are reasons to maintain the animals, eradication of Bsal may be possible and has been demonstrated in published literature (Blooi et al. 2015a; Blooi et al. 2015b). *There may be reasons to maintain and treat animals, e.g., with threatened or endangered species. However, there may also be reasons to maintain infected animals, e.g., for additional diagnostics or research. Consult with the CRT and your Participating Laboratory to determine options.* As such, we suggest the following:

Treat per guidance in Blooi et al. (2015a, b).  *As new treatments and research are being investigated, we will update this template.* ***Please note:*** *the methods tested to date only are confirmed in Fire Salamanders (*Salamandra salamandra*); keep in mind that species differences may come into play with respect to treatment validity and effectiveness. This is why multiple swabs over time are necessary to confirm eradication.*

Swab treated animals post-treatment (see Appendix I, Section B) and submit samples to a Participating Laboratory to confirm Bsal eradication.

Repeat treatment regime(s) and post-treatment swabbing until confirmation of Bsal eradication.

* + 1. Quarantine. Isolate any potentially affected individual animals, including any that were housed nearby or co-located with affected individuals.
       1. Perform additional diagnostics on quarantined, co-located individuals.
       2. Employ strict use of biosecurity protocols (see Pessier & Mendelson, 2017), for all people/personnel handling the affected species, particularly prior to exiting quarantine area.
    2. Disinfection, per Pessier & Mendelson (2017):
       1. All caging/housing materials and equipment prior to reuse.
       2. All water prior to disposal.
       3. All plants, soils, or other organic materials prior to disposal.
    3. Captive population monitoring. Evaluate the exposure to other co-located amphibians, including:
       1. Determine other places it could be in the facility, and disinfect these areas.
       2. Assess other potential sources of spread or origin of the pathogen, including through shared water sources or uses and movements, and quarantine or disinfect these sources.
       3. Assessment across the collection to determine whether it is clinically stable or if there is a trend of increasing morbidity and mortality.
       4. Evaluate other sources of infection, including new acquisitions.
    4. Reporting, and additional testing, throughout the chain of custody (i.e., individuals or entities throughout the history of possession of the animals or lot in which the affected amphibians originated).
       1. At minimum, swab amphibians for PCR analysis in either direction throughout the chain of custody.
       2. Consider additional monitoring, as in 4(b)(iv) above.

*Laboratory findings can now be entered into the Bsal reporting database: amphibiandisease.org*

* + 1. Voluntary surveillance of affected populations.
       1. Additional sampling of affected species and captive environment (plant and other substrates).
       2. Sampling of all other amphibian species in the facility.
       3. Sampling of stock of original importer or zoological collection
          1. Exposed animals
          2. Other co-located animals
       4. Sampling throughout the chain-of-contact/custody of exposed individual animals.
    2. Voluntary movement restrictions/prohibitions of movement or sale of affected species.
       1. Place a temporary moratorium of sale or movement of all salamanders from same zoological collection, captive breeder, pet supplier, or importer. *The entity customizing this document can determine whether to qualify this action as “mandatory” or “required” or other descriptor. When there is a definitive detection of Bsal, we suggest the strongest possible measures to reduce risk of spread and facilitate containment.*
       2. Document Bsal treatment. If animals are treated prior to resumption of transport or sale (in accordance with existing federal, state, or local laws), consider obtaining a health certificate or other documentation from a member of one of the Veterinary Expert Networks verifying Bsal treatment and eradication for *each individual animal* that tested positive for Bsal and was treated and for which Bsal was shown to be eradicated. *Entities customizing this template should keep in mind that each state may or may not have specific laws regarding “official” health certifications or alternative options; it is important to consult your state fish and wildlife agency and state department of agriculture regarding either the recommendation being an official or unofficial form of documentation.*

**APPENDIX I.**

**Protocols and procedures for sampling from mortality events,**

**and for sampling from living animals, for diagnostic testing**

**Text adapted, with permission, from:  
Pessier, A.P. and J.R. Mendelson (eds.). 2017. A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs, Ver. 2.0. IUCN/SSC Conservation Breeding Specialist Group: Apple Valley, MN. [Available** [here](http://www.amphibianark.org/pdf/Disease-Manual-2017.pdf)**.]**

1. **TISSUE COLLECTION DURING MORTALITY EVENTS.** Mortality events where multiple animals are found dying or dead are observed in amphibian survival assurance colonies as well as wild amphibian populations. Although well‐known infectious diseases of amphibians (e.g., chytridiomycosis or *Ranavirus* infection) may be strongly suspected, it is important to keep an open mind and always consider other potential causes. Many different disease conditions can initially look very similar and require laboratory investigation to achieve a definitive diagnosis.

It is always advisable to contact the lab where you intend to send samples and discuss with them their preference on how to prepare and ship the animals. *If possible, well in advance of a mortality event, consider contacting your nearest diagnostic laboratory to find out their preferences for preparing and shipping animals in various scenarios of a mortality event.*

The initial goal of investigating mortality events is to collect and preserve representative samples that can be used for the different types of laboratory techniques that may be needed.

Complex protocols can be designed for sample collection during mortality events—especially if veterinary guidance is available—however, a simple and basic approach is also sufficient for most situations.

* If wildlife health expert guidance is not available or if animals are small:
  + Perform the carcass-fixation necropsy method (see Chapter 9 in Pessier & Mendelson, 2017) on one‐half to two‐thirds of the dead animals.
  + For the remaining animals, freeze the carcasses whole as soon as possible and label with the species name, individual identification number and date.
    - For freezing of entire carcasses or individual tissue samples, ultracold temperatures (–70°C or below) or in liquid nitrogen are preferable. However, regular household freezer temperatures (–20°C) are sufficient for short‐term storage.
    - As a last resort, if a freezer or liquid nitrogen is unavailable, fixation of carcasses or tissue samples in 70% ethanol (instead of formalin) may still allow application of some molecular diagnostic techniques.
* If wildlife health expert guidance or an individual experienced with amphibian anatomy is available, perform the dissection necropsy method (see Pessier & Mendelson, 2017) on the dead animals.
* In addition to saving samples from all major organs in fixative solution for histopathology, freeze additional samples of individual organs.
  + Suggested samples for freezing include skin, liver, kidney, lung, intestine, brain and any tissue thought to be abnormal during dissection (e.g., enlarged or discolored organs or organ nodules). In addition, stomach contents, coelomic fat bodies and skeletal muscle can also be saved, especially if exposure to a toxic substance is a possibility.
  + Organ samples are saved in sterile Whirl‐Pak® style bags (Nasco, USA, www.enasco.com) or cryovials such as Nunc CryoTubesTM or Vangard CryosTM (Sumitomo Bakelite Co., Ltd. Japan, [www.sumibe.co.jp/english/](http://www.sumibe.co.jp/english/)).
  + Containers should be labeled with the species name, individual animal ID number, specimen type, date, and county and state where collected.
* If moribund (dying) animals are found, consideration should be given to humanely euthanize some of these individuals for necropsy and sample collection (see Section 8.6 in Pessier & Mendelson, 2017). This provides very fresh samples that are ideal for most laboratory methods used for disease investigation.
  1. **BASIC TISSUE SAMPLE COLLECTION PROTOCOL FOR AMPHIBIAN MORTALITY EVENTS (Wildlife Health Expert Not Available, or Field Situation with Limited Equipment)**
* For half of the dead animals, make an incision into the coelomic cavity and expose the internal organs.
  + For very small animals or if a knife is not available, just fix the carcasses intact.
  + Place the opened carcass into a fixative solution such as 10% neutral buffered formalin (preferred) or 70% ethanol. The ideal ratio is 1 part animal carcass to 9 parts fixative solution.
* For the other half of the dead animals, freeze the carcasses whole or keep them cool (such as in a portable ice‐chest) until they can be transported to a location where freezing is possible.
  + It is always better to save both fixed (formalin or ethanol) and frozen samples. If this is not possible, preference should be given to saving tissues fixed in formalin or ethanol.
  + Saving only frozen samples should be a last resort (but is better than no samples at all).
    - If freezing of samples is not possible, fixation in ethanol may allow for both histopathology as well as some molecular diagnostic tests (e.g., PCR)
  1. **SHIPMENT OF SAMPLES.** For shipment of tissues that have been preserved in a fixative solution. Once carcasses or tissues have been in formalin or other fixative solution for a minimum of 48 hours, they are removed from fixative, wrapped in paper towels or gauze moistened with fixative, packed into sealed plastic bags and shipped to a pathologist. This minimizes the potential for leakage during shipment and reduces package weight (and shipment costs).
* Materials should be shipped in a manner that follows International Air Transport Association (IATA) regulations for Dangerous/Hazardous Materials (see also <https://www.gpo.gov/fdsys/pkg/FR-2011-07-20/pdf/2011-17687.pdf>). Some general guidelines include:
  + Samples should be enclosed in a primary receptacle that is leak‐proof.
  + The primary receptacle is then placed within a leak‐proof secondary receptacle.
  + An absorbent material (e.g., paper towels) should be placed between the primary and secondary receptacles. The volume of material should be sufficient to absorb all of the fluid within the primary receptacle.
* Major shipping companies have guidelines available to help with proper shipping of biological samples. More information available here: <http://images.fedex.com/downloads/shared/packagingtips/pointers>
  1. **Disinfection and Biosecurity in the Field.** Concerns about the possibility of moving amphibian pathogens to new locations as the result of field research conducted on wild amphibians have led to a number of protocols for reduction of this risk (e.g., <http://northeastparc.org/disinfection-protocol/>). There are variations and sometimes contradictions between the different protocols, however, the basic principles of biosecurity for biologists working on wild amphibian populations are similar. Peer‐reviewed publications including the addition of risk calculators to assist the biologist in making good biosecurity decisions have recently become available (St‐Hilaire et al. 2009; Phillott et al. 2010). A summary of recommended field practices includes:
* **Definition of the field site.** The first precaution against the possible spread of disease among amphibian populations is careful definition of the field site or sites. Researchers should use natural and man‐made boundaries to help define the sites. Whenever possible, plans should be made ahead of time to work in only one site per outing, or have different groups working at each individual site to avoid cross‐contamination (and transmission of disease) between sites.
* **On‐site hygiene and biosecurity of equipment.** The use of disposable equipment discarded after use at a single site or on a single individual amphibian reduces the risk of spreading disease. All reusable equipment, including footwear, should be disinfected between sites, or dedicated to a single site (e.g., a single pair of rubber boots is purchased for each field site and used ONLY at that site). Consult the table in Section 5.10 of Pessier & Mendelson (2017) for details on the use of specific disinfectants including recommended concentrations and contact times.
  + Footwear and other reusable equipment should be made of materials that are easy to clean and disinfect (e.g., rubber boots are better than leather hiking boots).
  + Thorough cleaning of equipment is essential for removal of dirt and organic material prior to disinfection in the field. As noted in other sections, organic material inactivates many disinfectants. Scrub brushes and other implements to remove dirt should be part of the field equipment. If disinfectant solutions become contaminated with organic material or dirt they should be changed.
  + The quaternary ammonium compounds (see Section 5.2 in Pessier & Mendelson, 2017) have been recommended for field situations because they are concentrated and easy to transport into field situations (Johnson et al. 2003; Webb et al. 2007).
  + If disinfection is undertaken in the field, consideration should be given to the toxicity of chemicals to the environment. The quaternary ammonium compounds and Virkon® (see Section 5.2 of Pessier & Mendelson, 2017) are more environmentally friendly options compared to chlorine bleach (Johnson et al. 2003; Webb et al. 2007; Schmidt et al. 2009). If ranaviruses are a special concern Virkon® may have some advantages over the quaternary ammonium compounds (Bryan et al. 2009). Powdered bleach is another easily portable suggestion.
  + Vehicles are less likely to be a vector for the transmission of disease than footwear and field equipment, but still should be disinfected, especially if used to cross or enter a known contaminated site. The wheels and tires should be cleaned of all dirt and organic material and disinfected prior to leaving the site, using the same disinfectant that was used on footwear. Always remember to disinfect footwear before getting into a vehicle to prevent pathogens from transferring to the floor or pedals.
* **Handling and collection of samples from *live* amphibians:** When handling live amphibians in the field, even within the same site, precautions should be taken to minimize the risk of transmitting pathogens between individual animals.
  + Non‐powdered disposable gloves are the best choice when handling amphibians. Powdered gloves should be rinsed free of powder. A new pair of gloves should be used for each animal. If gloves are unavailable, it is slightly preferable to use bare hands, and wash hands between handling different animals (Mendez et al. 2008).
  + The greatest risk for spreading disease when handling amphibians occurs when animals are placed together in the same container or when containers are reused without being disinfected. Do not re‐use collecting bags and utilize a new one for each animal.
  + Always handle animals as little as possible. Procedures that are quick, even if potentially painful, may cause less stress than longer procedures.
  + Animals should only be released at the site of capture and any sick or dead amphibians found should be humanely euthanized (if applicable) and preserved in 10% buffered formalin solution and submitted for disease diagnosis (see Chapter 9, Necropsy, in Pessier & Mendelson, 2017).
  + Instruments used for sample collection should be disinfected between use on different animals. For surgical instruments (e.g., scissors) and weighing equipment 70% ethanol is rapidly acting against the amphibian chytrid fungus (Johnson et al. 2003).
  + Although mentioned in some amphibian handling protocols the use of iodine-based compounds for sanitizing the skin prior to procedures such as toe‐clipping or microchip implantation is not recommended because of toxicity concerns. Potential substitutes include 0.75% chlorhexidine or 2mg/L benzalkonium chloride (Wright, 2001).

1. **SAMPLE COLLECTION FOR Bsal PCR.** As of this version, some of the sample collection options for Bsal have not yet been documented; this information is provided based on techniques used for *Batrachochytium dendrobatidis* (Bd)and will be updated as new information becomes available. Based on what is known for Bd, the PCR procedure can be performed using a variety of different sampling methods including skin swabs, water bath, and tissue samples (e.g., toe clip; Hyatt et al. 2007).

* Skin swabs. The skin swab procedure is simple, minimally invasive and samples multiple areas of the skin that may be infected with Bsal (increasing the likelihood that infected areas will be sampled). Skin swabs generally are the preferred sampling method for Bsal PCR.
* Water bath. Samples using the water bath procedure require immediate centrifugation or micropore filtration *and are not practical in many settings*.
* Tissue samples. Toe clipping is an invasive procedure with associated ethical concerns and has the disadvantage of sampling only a small portion of potentially infected skin.
  1. **Materials Needed.** The materials listed below are general guidelines needed to perform the skin swab procedure for Bsal PCR using realtime or quantitative PCR (qPCR) methods. There may be differences depending on the preferences of the laboratory processing the samples and the environmental conditions under which the swabs are obtained.
* Powder‐free latex or nitrile disposable gloves.
* Sterile applicators (“swabs”); see “Swab Selection” in Pessier & Mendelson (2017).
* 1.5 ml microcentrifuge tubes/cryovials.

Storage of dry swabs at controlled room temperature/refrigeration or freezing is preferred, but 70% ethanol is an alternative especially if samples will be exposed to variable climate conditions, especially heat. Individual laboratories may have preferences about sample storage conditions; be sure to check in advance with the Participating Laboratory to which samples will be sent. For additional information see the section on “Storage of Skin Swab Samples” below.

* 1. **Swabbing Procedure 101.** Several videos demonstrating swabbing and associated biosecurity and prevention of contamination have been developed.
     1. Swabbing technique for qPCR: <http://amphibiaweb.org/chytrid/index.html>
     2. Swabbing using wooden‐stemmed swabs suitable for conventional PCR (see “How to Swab a Frog for Chytrid”): <http://www.amphibianark.org/frog_gallery.html>
     3. General swabbing and associated biosecurity procedures: <https://www.youtube.com/watch?v=a5CtPrGOK8c>
  2. **Avoiding Cross‐contamination of Samples.** The PCR assays are very sensitive tests and can detect very small amounts of Bsal DNA. This is good for detecting animals that have very low‐level infections with Bsal, but increases the risk that samples from a non‐Bsal infected animal can have false-positive results if they become contaminated with even small amounts of Bsal DNA from an infected animal. Therefore, it is very important to take precautions to avoid sample cross‐contamination which include:
* A new pair of disposable latex or nitrile gloves should be used for each animal handled for testing (Mendez et al. 2008).
* Avoid contact of swabs (especially swab tips) with surfaces or substrates other than the skin of the animal to be tested.
* If instruments are used to cut the tip of the swab into cryovials, a freshly disinfected instrument must be used for each sample.
  + To disinfect instruments for this purpose, dip in 70% ethanol followed by flaming under an alcohol lamp.
  + Avoid using bleach solutions for disinfection because this can degrade Bsal DNA in swab samples (resulting in false‐negative tests; Cashins et al. 2008).
  1. **Avoiding PCR Inhibitors in Samples.** Foreign material such as dirt or plant matter can contain materials that inhibit the PCR reaction. This can result in a false‐negative test result (animal is infected with Bsal, but it is not detected by the PCR test).
* Prior to skin swabbing efforts should be made to manually remove heavy skin contamination. Animals may be gently rinsed with clean water prior to sampling, but vigorous washing should be avoided because of the potential to also rinse off Bsal infected skin cells or organisms.
* If rinsing with water is used for cleaning, the water should not originate from the animal’s enclosure or environment.
* Laboratories that perform PCR for Bsal should always use exogenous internal positive controls to detect PCR inhibitors (Hyatt et al. 2007).
  1. **Storage of Skin Swab Samples.** Storage of swabs after sample collection is an important consideration. Swabs can be stored air-dried or in 70% ethanol. Be sure to check in advance with the Participating Laboratory to which samples will be sent; individual laboratories may have preferences about sample storage conditions.

For air-dried swabs, the major concern is high temperature extremes:

* The DNA on air‐dried skin swabs is remarkably stable, and experimentally, swabs have been stored for up to 18 months at room temperature (23°C) without a reduction in the sensitivity of the assay (Hyatt et al. 2007).
* In contrast, exposure of swabs to very high temperatures (> 38°C) for 7 days can result in decreased recovery of pathogen DNA that could result in false‐negative results for animals with low‐level Bsal infections (Van Sluys et al. 2008).

Therefore, it is recommended that air‐dried skin swab samples be stored at as low a temperature as possible (Skerratt et al. 2008).

* At 25°C (refrigerator) or lower.
* Samples should be frozen (–20°C or below) if sample analysis is not performed within six months of sample collection.
* See alternatives to low temperature storage (i.e., where refrigeration may not be possible) in Pessier & Mendelson (2017).
  1. **Shipment of Swabs to the Laboratory.**
* Ideally ship swabs by overnight or 2‐day courier service (e.g., Federal Express; UPS).
* Consider using cold packs to guard against high temperature extremes.
* Samples that have been previously frozen should be sent on dry ice to prevent freeze‐thaw cycles.

**APPENDIX II.**

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