

BSAL RESPONSE PLAN

U.S. National Science Foundation Project 2207922:

Socioeconomic and Epidemiological Drivers of Pathogens in Wildlife Trade Networks

<https://www.healthyamphibiantrade.org>

Project Partners:



Introduction

A team of researchers (Table 1) was awarded funding on 1 August 2022 by the National Science Foundation to identify conditions in US pet amphibian trade that reduce the occurrence of pathogens that could be harmful to captive and wild amphibians. This project will lead to recommendations on best practices to reduce business revenue losses and prevent possible spillover of pathogens (harmful microbes) from captive to wild amphibian populations. The project will involve a combination of social and economic surveys of US pet amphibian businesses and consumers, surveillance for harmful microbes in trade, and controlled laboratory experiments that identify best practices to promote healthy trade. The harmful microbes of concern in this project are two species of chytrid fungus (*Batrachochytrium dendrobatidis* [Bd], *B. salamandrivorans* [Bsal]) and ranaviruses (Rv). These pathogens are a threat to commerce and biodiversity of amphibians in the US and elsewhere. Various strains of Bd and Rv have been detected in the US, but Bsal is known to only occur in Europe and Asia. Bsal is endemic (native) to Asia and was recently introduced to Europe, where it is causing mass mortality of several salamander species. Bsal is a skin fungus that can infect frogs but is most lethal to salamanders. Because Bsal has been documented in pet amphibian stores in several European countries, it is believed trade of infected amphibians contributed to its introduction there. Considering that the US has the most salamander biodiversity in the world, there is concern that Bsal could enter the US through trade and find its way (spillover) to wild populations with significant biodiversity loss and environmental effects.

During this project, we are asking businesses to volunteer to collect skin swabs and water samples from their captive stock of amphibians that are housed in terrestrial or aquatic environments. We will also ask participants to complete a survey about various husbandry conditions and biosecurity procedures in their business. The identity of all businesses will remain confidential. There is no cost to businesses for participating in this project other than personnel time to complete the survey and collect samples per study-design protocols.

More specifically, for sample collection, the expectations of businesses are to:

- Watch online training videos and read instructions for properly collecting samples and recording data.
- Collect samples from housed amphibians and provide accurate information about husbandry and biosecurity practices.
- Attempt to meet sample submission deadlines (ideally within 2 weeks of receiving sample kit).
- Withhold tested animals from sale until results are received, or if they are sold, retain buyer contact information.
- Consider further participation if Bsal is detected, by following the decision tree options (described below).

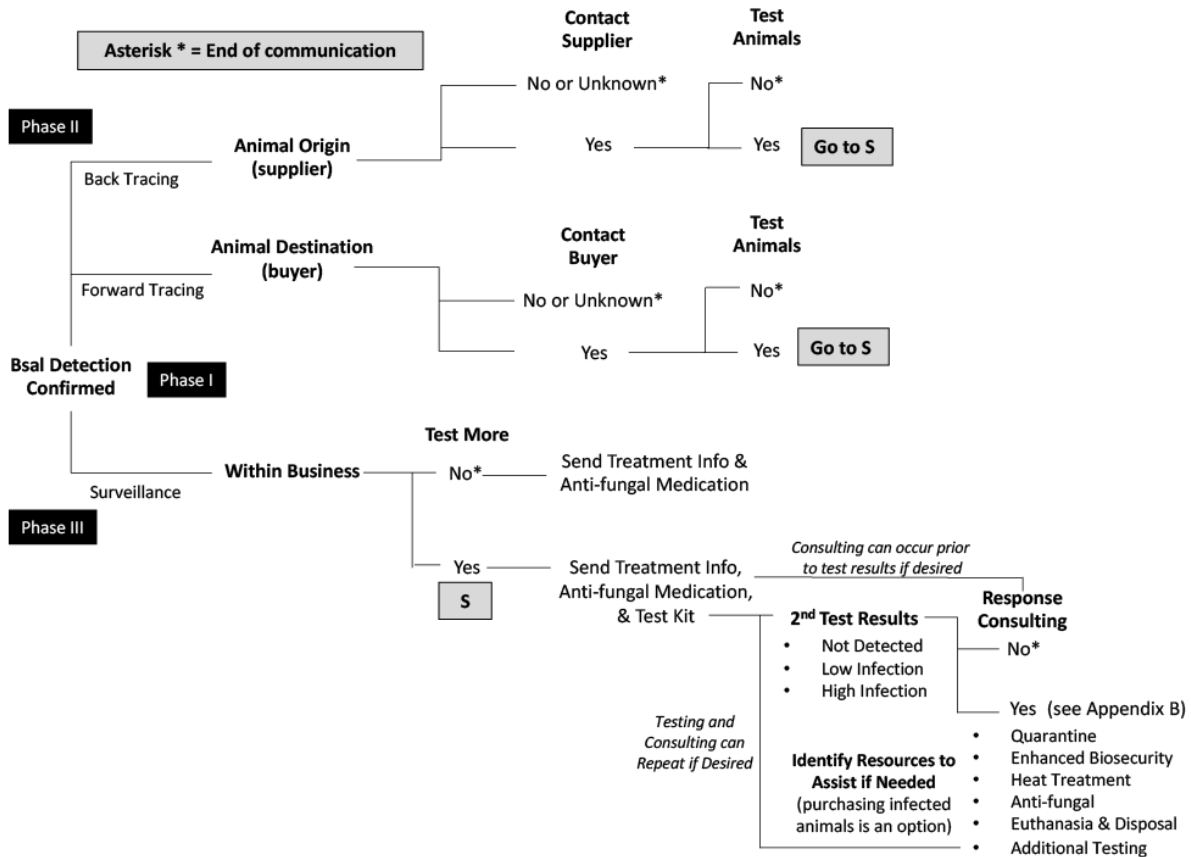
The samples collected by businesses will be sent (pre-paid postage) to and processed either by Washington State University (WSU) or University of Tennessee (UT), and the business will be notified of the test results (Bd, Bsal and Rv) generally within 7 days of receipt. If animals are positive for any of the target pathogens, information will be provided on how to treat the infected amphibians. Because Bsal is not endemic to the US, if there is a positive detection, the research team would like to follow up with additional testing and provide direct guidance to the business to ensure that Bsal is eradicated.

Bsal Response Phases and Decision Tree

To help participating businesses understand this process and their different decision options, this Bsal Response Plan was developed. Importantly, businesses can remain anonymous to the researchers (if desired) by communicating through the [Pet Advocacy Network \(PAN\)](#), who is a project partner. The specific contact at PAN is Ashley Brinkman (202-452-1525, extension 1010; ashley@petadvocacy.org). Businesses can opt out at any step of the Bsal response process. Below is a decision tree that illustrates the voluntary process of tracing and response if Bsal is detected, with written details that follow.

The Bsal Response Plan is divided into three phases (I = Detection, II = Tracing, III = Response), which are outlined below. **Phase I** explains the process of detection, **Phase II** explains how forward and back tracing of a positive sample can occur, and **Phase III** explains possible Bsal response strategies. Given that every business and possible Bsal-positive case will be unique, the type of response ideally should be tailored for each situation, with guidance provided by the researchers.

Tracing and Response Options if Bsal is Detected during Study



Phase I: Detection

Steps:

1. Business volunteers to participate and agrees to project expectations (see page 2).
2. **PAN** sends sampling kit to business (provided by WSU = business or UT = imports).
3. Business collects samples and sends to WSU or UT (with user-generated PIN).
4. WSU/UT extracts DNA, runs qPCR diagnostic test, and posts results to project website.
5. If the lab results are negative or positive for Bd or Rv, WSU/UT informs **PAN** of results and **PAN** notifies the business with online information about how to treat for Bd and Rv (if positive).
6. If the lab detects a positive sample for Bsal, the testing lab will do the following:
 - Re-run sample within lab, look for contamination with assay for gBlock standard.
 - Rule out a false positive result and **notify PAN**.
 - Send DNA overnight to partner laboratory (UT/WSU), which will re-run the assay and look for contamination with gBlock.
 - Compare results between labs and exclude false positives.
7. If the partnering laboratory confirms positive case, the partnering lab will notify PAN, and tracing is initiated (Continue to Phase II).

Phase II: Tracing

8. **PAN** contacts business with results and inquires if business is willing to allow additional testing [**APPENDIX A: EMAIL TEMPLATE 1**]
 - If yes, send kits for additional testing and antifungal treatment with instructions.
 - If no, provide SOP on how to treat animals and decontaminate aquaria. Also, inquire if the business would like the antifungal treatment sent to them.
9. **PAN** provides recommendations on isolating positive or potentially exposed animals and inquires whether they remain at the facility (*forward tracing*). [**APPENDIX A: EMAIL TEMPLATE 1**]
 - If animals are no longer at the facility, inquire if the buyer can be contacted by PAN or if the business is willing to contact them.
 - Offer to provide free testing or purchase animals from the buyer.
 - If forward tracing occurs, send email to buyer [**APPENDIX A: EMAIL TEMPLATE 2A**].
10. **PAN** inquires if positive animals were recently acquired (*back tracing*). [**APPENDIX A: EMAIL TEMPLATE 1**]
 - If yes, inquire if the supplier can be contacted by PAN or if the business is willing to contact them.
 - Offer free testing to the supplier.
 - If back tracing occurs, send email to seller [**APPENDIX A: EMAIL TEMPLATE 2B**]
11. **PAN** inquires about preferred future communication and anonymity. [**APPENDIX A: EMAIL TEMPLATE 1**]; business' options are as follows:
 - No communication.
 - Communicate with PAN, researchers, or industry partners (Josh's Frogs or RBM).
12. If the business is willing to perform additional testing to understand the distribution and prevalence of Bsal in the facility, Table 2 is followed to identify the minimum to ideal number of samples that should be collected. Continue to Phase III.

Phase III: Response

13. Phase III begins if the business is willing to participate in response, and we have some information on infection prevalence in the facility.
14. **PAN** or **Research Team** provides guidance on response options:
 - Isolation of tanks and area affected – **Appendix B**
 - Enhanced biosecurity (decontamination, glove use) – **Appendix B**
 - Heat treatment (25 C for 10 days) – **Appendix B**
 - Antifungal medication (Bloom et al. 2015) – **Appendix B**
 - Humane euthanasia – **Appendix B**
 - Biohazardous disposal – **Appendix B**
 - Additional testing – Table 2
 - Animals purchased by university, PAN or another partner.
 - No response (only if 2nd testing resulted in no detection)

15. **PAN** or **Research Team** identifies resources to financially help business with response as needed.

Table 1. Researchers (shaded) and pet amphibian industry partners (unshaded) involved in U.S. National Science Foundation Project 2207922.

Name	Organization	Experience	Email
Molly Bletz	University of Massachusetts	Amphibian Disease Specialist; Microbiomes	molly.bletz@gmail.com
Zach Brinks	Josh's Frogs	Frog Breeder/Retailer	Zach@joshsfrogs.com
Jesse Brunner	Washington State University	Amphibian Disease Specialist; Surveillance	jesse.brunner@wsu.edu
Nina Fefferman	University of Tennessee	Disease Modeling Expert	nfefferm@utk.edu
Mark George	Reptiles by Mack	Amphibian Importer, Distributor, and Wholesaler	mark.george@reptilesbymack.com
Matt Gray	University of Tennessee	Project Lead PI: Amphibian Disease Specialist	mgray11@utk.edu
Bob Likens	Pet Advocacy Network	Amphibian Trade Advocacy	bob@petadvocacy.org
Julie Lockwood	Rutgers University	Wildlife Trade Specialist	julie.lockwood@rutgers.edu
John Mack	Reptiles by Mack	Amphibian Importer, Distributor, and Wholesaler	john.mack@reptilesbymack.com
Neil Moherman	Reptiles by Mack	Amphibian Importer, Distributor, and Wholesaler	neil.moherman@reptilesbymack.com
Jonah Plovio-Scott	Washington State University	Amphibian Disease Specialist	jonah.plovio-scott@wsu.edu
Neelam Poudyal	University of Tennessee	Social Science and Economics	npoudyal@utk.edu
Alexa Warwick	Michigan State University	Human Dimensions Specialist-Discussion Facilitator	awarwick@msu.edu
Josh Willard	Josh's Frogs	Frog Breeder/Retailer	josh@joshsfrogs.com

Table 2. Minimum number of amphibian habitats¹ to sample given total number of habitats in your business and the target level of pathogen detection (adapted after Gray et al. 2015, https://link.springer.com/chapter/10.1007/978-3-319-13755-1_8).

Number of habitats in business	Desired level of detection (% habitats with at least one infected individual ³)			
	20 %	10 %	5 %	2 %
25 or less	4	18	20	25
50	5	20	35	50
100	8	23	45	75
250	11	25	50	110
500	13	26	55	130
1,000 or more	15	27	60	145

¹Habitats are defined as an independent living unit that is not connected to another unit. Examples of habitats are tanks, plastic containers, boxes, etc. A habitat is not independent if amphibians can contact each other or the husbandry media is shared, such as a recirculating water system, where water flows among multiple tanks without disinfection of the water between them.

²Target level of detection is the minimum percentage of habitats in your business that you are willing to allow infection. The more infection you are willing to accept, the fewer the tanks you must sample to detect it. If a pathogen is rare, you must sample more tanks to ensure infection does not exist.

³These sample sizes assume that all individuals in a habitat have an equal likelihood of infection and that diagnostic assays have minimal error in pathogen detection. More elaborate sample size calculation methods exist and can be generated by contacting the research team (<https://www.healthyamphibiantrade.org>).

Appendix A: Email templates for NSF Project.

EMAIL TEMPLATE #1: First email from PAN to Business with positive Bsal sample

SUBJECT LINE = RESPONSE NEEDED: Bsal Detection in Samples

Dear Amphibian Healthy Trade Study Participant:

Pet Advocacy Network (PAN) was notified by the researchers of this project that at least one of the samples you submitted to them has evidence of an amphibian being infected with the pathogen, *Batrachochytrium salamandrivorans* (Bsal). The testing results for your samples can be accessed via this website (add hyperlink) using the unique PIN and password for your business (sent with the supplies).

Bsal is a fungus that can kill amphibians, is native to Asia, and hasn't been detected yet in the United States. Because of this pathogen's possible threat to captive amphibians and native amphibians in the wild, we would like to help you with eradicating Bsal from your facility. We also would like to help with tracing the origin of the positive animal (called *back tracing*) and finding the animals' current location(s) if they were sold (called *forward tracing*). You can participate in all, a portion, or none of the response steps. Also, communications can remain anonymous through PAN, or with your permission, we can share the identity of your business with the researchers (who will keep your business confidential) for faster and more directed guidance on response steps.

The response is divided into a series of steps and decisions, which are outlined in this plan ([LINK TO PLAN ONLINE](#)). In order to initiate or decline assistance with response, we ask that you fill out this brief survey as soon as possible (<2 days from receiving this email):

LINK with Questions: See below

If you would prefer to talk confidentially with me, my phone number is: 202-452-1525 (ext. 1010). You can also respond to this email. Even if you decide not to participate, we ask that you please indicate "no" in the survey or in an email to me.

To expedite effective response, we are proactively sending swabs for collecting and testing samples from additional animals (free of charge). In the package, we also will send a medication with instructions for treating Bsal-infected amphibians. We also are willing to purchase infected animals to limit financial burden and risk to other amphibians in your business.

We hope that you will consider testing additional animals, treating infected ones or selling them to us, and allowing our team to give guidance during response. Thank you for your willingness to participate in this project. I look forward to hearing from you by email, phone or the survey.

Best regards,
Ashley Brinkman, Director of Government Affairs, Pet Advocacy Network

EMAIL #1 Questionnaire:

NOTE: Insert decision tree at the top of the survey.

- 1) What is your business' unique 4-digit PIN?
- 2) Please indicate your preference for future communication and anonymity.
 - I prefer to remain anonymous and communicate with PAN.
 - I prefer that my business' identify is shared with researchers and I would like to communicate directly with them.
- 3) Please indicate if you are willing to collect additional samples from your facility and send to the researchers for testing.
 - Yes
 - No
- 4) Please indicate if you want to receive guidance on eradicating Bsal from your facility, including information of biosecurity practices and animal treatment.
 - Yes
 - No
- 5) Please indicate if all the animals associated with the positive samples, remain at your business.
 - Yes
 - No
- 6) If you responded "no" to question 5, do you know the identity of the buyer(s)?
 - Yes
 - No
- 7) If you responded "yes" to question 6, are you willing to contact the buyer(s) or give permission to Pet Advocacy Network to contact them for additional testing.
 - Yes
 - No
- 8) Do you know the identity of the business that you acquired (purchased) the positive animal?
 - Yes
 - No
 - Animal was captive bred
 - Animals was wild caught
- 9) If you responded "yes" to question 8, are you willing to contact the seller(s) or give permission to Pet Advocacy Network to contact them for additional testing?
 - Yes
 - No
- 10) If you have questions or concerns, please indicate below otherwise leave blank.

EMAIL TEMPLATE #2A: Forward Tracing – contacting the consumer.

SUBJECT LINE = RESPONSE NEEDED: Bsal Detection in Collection

Dear Buyer:

{Business' Name} is involved in a research project organized by five universities and the [Pet Advocacy Network](#) that is looking at the commonness of various microorganisms in US pet amphibian trade (please see project website: <https://www.healthyamphibiantrade.org/>). As part of that project, we tested some of the amphibians in our business and discovered (after sale) that one or more of the amphibians you purchased from us was infected with *Batrachochytrium salamandrivorans* (also called Bsal). Bsal is a fungal pathogen that has no health risk to humans or warm-blooded pets but could make other amphibians in your collection sick. In addition, the origin of this pathogen is Asia and could negatively affect wild amphibians if spillover were to occur. The researchers involved in this project would like to test up to 30 amphibians in your collection free of charge. If you are willing, they will send supplies and instructions to you. If your animals are positive for Bsal, they can provide treatment options or purchase potentially infected stock. Additionally, we are (or {Business' Name}) willing to provide a full refund for the purchase or equivalent in store credit. To claim your refund, please respond to this email. To give permission for free health assessments of your amphibians by the researchers, please fill out the following survey as soon as possible (within two days of receipt).

Sincerely,

Best regards,

Ashley Brinkman, Director of Government Affairs, Pet Advocacy Network
(or business with Bsal case)

EMAIL TEMPLATE #2B: Back tracing – contacting the seller

SUBJECT LINE = RESPONSE NEEDED: Bsal Detection in Collection

Dear Seller:

{Business' Name} is involved in a research project organized by five universities and the [Pet Advocacy Network](#) that is looking at the commonness of various microorganisms in US pet amphibian trade (please see project website: <https://www.healthyamphibiantrade.org/>). As part of that project, we tested some of the amphibians in their business and discovered that one or more of the amphibians we purchased from you was infected with *Batrachochytrium salamandrivorans* (also called Bsal). Bsal is a fungal pathogen that has no health risk to humans but could make other amphibians in your collection sick. In addition, the origin of this pathogen is Asia and could negatively affect wild amphibians if spillover were to occur. The researchers involved in this project would like to test up to 30 amphibians in your collection

free of charge. If you are willing, we will send supplies and instructions to you. If your animals are positive for Bsal, we can provide treatment options or purchase potentially affected animals. To give permission for free health assessments of your amphibians by the researchers, please fill out the following survey as soon as possible (within two days of receipt).

Sincerely,

Ashley Brinkman

Director of Government Affairs, Pet Advocacy Network
(or business with Bsal case)

EMAIL #2 Questionnaire:

- 1) Please indicate your name or business' name, address, email and phone number below.
- 2) Are you willing to give permission to Pet Advocacy Network or the researchers to contact you regarding testing amphibians (free of charge) in your collection for Bsal?
 - Yes
 - No
- 3) Are you interested in receiving treatment information for Bsal infections?
 - Yes
 - No

Appendix B: Standard Operating Procedures (SOPs) for possible Bsal Response Strategies and Animal Disposal Practices

DISCLAIMER:

These SOPs are not intended to replace veterinary guidance. The authors of these guidelines cannot legally provide individualized medical advice or prescribe medications. Businesses are highly encouraged to establish a relationship with a local herptile veterinarian if not already established. The Association of Reptile and Amphibian Veterinarians has a helpful tool to find herptile veterinarians by location: <https://arav.site-ym.com/search/custom.asp?id=3661>. Many of these SOPs are most useful if implemented in collaboration with a veterinary team. Veterinary teams offer many benefits including translating these SOPs into more detailed dosing regimens, contextualizing Bsal and other diagnostic results within your collection, prescribing and dispensing medications if warranted, and identifying and addressing any other health concerns that might be occurring.

Quarantining and Isolating Animals

Purpose:

Quarantine is intended for newly arrived animals to ensure they are healthy before they are allowed to mix with and potentially infect resident animals.

Isolation is intended for animals that have become sick, tested positive for infections, or were co-housed or exposed to sick or infected animals. The goal is to contain (potentially) affected animals to prevent further transmission while responding to the infections.

Housing and Infrastructure:

- Ideally there will be a physically separate **room** for quarantine and isolation with access limited only to those trained in biosecurity protocols and working with infected animals. At a minimum, a distinct, physically separated area of an animal room could be reserved for quarantine and isolation.
 - Both the walls and floor of the room or area should be easily disinfectable (i.e., have non-porous surfaces, minimal cracking, chemically-resistant paints).
 - Ventilation is important, especially when chemical disinfectants are used regularly, but ventilation of the quarantine room should ideally be separated from that of the non-quarantine room(s).
- Separate **housing containers** (i.e., tanks, tubs, containers), **equipment** (e.g., nets, forceps, funnels & tubing, etc.), **water** and **food** should be used in quarantine and isolation spaces. That is, there should be no mixing of anything that might incidentally carry or contain pathogens (= “fomites” in the literature).

- **Waste** of all kinds—water, bedding, trashing—should be kept physically separate from that of the rest of the facility and must be appropriately treated before being disposed (see Disposal SOP later in the Plan).
- To prevent the movement of pathogens out (or into) the quarantine room or area, there should be separate **footwear** (e.g., sandals or boots) and coats or **outerwear** at the entrance that are donned and used only in the quarantine room or area. At a minimum, the entry should include a disinfectant **footbath** that is regularly filled and cleaned.

Order of operations:

Quarantine and isolation areas or rooms should be entered last, after working with the rest of the animals. Ideally, personnel that enter the quarantine will not re-enter other animal areas until after changing clothes or otherwise ensuring no pathogens can re-enter with them.

Enhanced Biosecurity

1) Use of Gloves

Purpose:

Using disposable gloves can prevent the movement of pathogens between groups of animals, ensuring those involved in husbandry do not become inadvertent “vectors” of infection. There is plenty of evidence that contaminated gloves can result in transmission of amphibian pathogens from an infected to uninfected individual (see Gray et al. 2018).

Procedure:

- Disposable gloves should be worn while handling, feeding, or otherwise caring for animals and changed between containers.
 - Gloves can contain powders or other unknown chemicals on them that may be harmful to animals, so it is recommended that gloves first be rinsed with fresh water before handling animals (see Greer et al. 2009).
 - Since it can be difficult to get gloves on wet or sweaty hands, one can “double glove,” putting on one set of gloves on top of another, and simply change the outer layer.
 - Ideally, gloves should be changed between housing containers (independent habitats) unless there is strong confidence that animals are pathogen-free (Gray et al. 2018).

Gray, M. J., J. A. Spatz, E. D. Carter, C. M. Yarber, R. P. Wilkes, and D. L. Miller. 2018. Poor biosecurity could lead to disease outbreaks in animal populations. [PLoS ONE 13\(3\): e0193243](https://doi.org/10.1371/journal.pone.0193243).

Greer, A. L., D. M. Schock, J. L. Brunner, R. A. Johnson, A. M. Picco, S. D. Cashins, R. A. Alford, L. F. Skerratt, and J. P. Collins. 2009. Guidelines for the safe use of disposable gloves with

amphibian larvae in light of pathogens and possible toxic effects. *Herpetological Review* 40:145-147.

2) Use of Disinfectants at Approved Concentrations

Purpose:

Disinfectants are used to inactivate pathogens and may be used prophylactically (=preemptively) or in response to known contamination. Note that disinfected does not imply sterility, which is a much higher standard, nor does it remove all traces of pathogens (e.g., pathogen DNA may still be present). There are many types and brands of disinfectants that can be organized by their active ingredient. They may have different efficacies against various pathogens, different working concentrations and required contact times, different longevities (and thus frequency of being remixed), and different costs and benefits (see Table 3)..

Table 3. Common disinfectants used for inactivating *Batrachochytrium dendrobatidis* (Bd), *B. salamandrivorans* (Bsal), and ranavirus and approximate cost (in 2023 USD).

Product	Chemical ³	Minimum Application Concentration ^{1,2}			Cost (\$)
		Bd	Bsal	Rv	
Liquid Bleach	Sodium hypochlorite (6%)	4% (5 oz)	20% (25 oz)	4% (5 oz)	\$3/gal
Granulated Pool Shock	Calcium hypochlorite (70%)	0.5% (0.65 oz)	2.5% (3.25 oz)	0.5% (0.65 oz)	\$9/lb
Ethanol	EtOH (100%)	70% (90 oz)	70% (90 oz)	70% (90 oz)	\$100/gal
Virkon®	Potassium peroxymonosulfide (20%)	1% (1.3 oz)	1% (1.3 oz)	1% (1.3 oz)	\$7/lb
Novalsan®	Chlorhexidine diacetate (2%)	1% (1.3 oz)	1% (1.3 oz)	1% (1.3 oz)	\$100/gal

¹Ounces are the amount of product that needs to be added to 1 gallon of water. Disinfectants will remain stable for 7 days if not exposed to sunlight; effectiveness will decrease thereafter. Disinfectants should remain on surfaces for at least 5 minutes, and surfaces should be free of organic matter or debris.

²Based on research published in Johnson et al. (2003), Bryan et al. (2009), Gold et al. (2013), and Van Rooij et al. (2017).

³These chemicals can be toxic to amphibians, thus surfaces should be rinsed thoroughly with water after use.

Note on containment and labeling:

- It is best practice to place disinfectants in a secondary container (e.g., a bin or bucket) in case of spills. Be sure to physically separate caustic chemicals from other chemicals or supplies.
- Be sure containers are clearly labeled with the contents and the date of purchase and expiration date. Containers with diluted chemicals should be similarly labeled with the contents and date when mixed up.

Disinfecting surfaces:

- Use a spray bottle or apply to a clean rag or paper towel to apply enough of the appropriately diluted disinfectant to the surface that it is wet and the entire surface is covered.
- Let sit for the required contact time (see Table 3). A timer is helpful.
- Wipe up with a clean rag or paper towel.
- If necessary (e.g., if the disinfectant is corrosive such as bleach or the surface will contact animals) rinse several times with clean water to remove any residue.

Disinfecting equipment:

- Fill a tub with appropriately diluted disinfectant and soak equipment for required contact time, then rinse thoroughly and dry.
 - Note that some chemical disinfectants are inactivated by organic matter and should be disposed of after disinfecting many dirty items.
- Alternatively, spray the equipment with appropriately diluted disinfectant being sure to cover all surfaces thoroughly, letting sit for the required contact time before rinsing thoroughly.

Disinfecting water or spills:

- Wastewater should be collected (e.g., in a bucket) and then the appropriate disinfectant (e.g., bleach) can be added to the water to achieve the required dilution and mix thoroughly to ensure a homogenous solution. Let sit for the required contact time before disposing water as per usual.
 - Note that because some chemical disinfectants are inactivated by organic matter, etc., it may be appropriate to aim for a higher concentration than that recommended (e.g., 10% bleach instead of 5% bleach). Granulated chlorine (used for shocking pools) can be an effective way to disinfect large volumes of water.
- Small spills can be sprayed with disinfectant, wiped up with a disposable rag or paper towel, sprayed again and let sit for the required contact time before being wiped up again and the area rinsed.
- Large spills may require adding a higher concentration of the disinfectant so that the overall concentration is at least that recommended for disinfection before being

mopped up. The area should then be sprayed again with disinfectant, let set for the required contact time, and then wiped up and rinsed.

Heat Treatment SOP

Blooi et al. 2015 [[10.1038/srep08037](https://doi.org/10.1038/srep08037)]; **Main Conclusion** - Exposing *B. salamandrivorans* infected salamanders to 25°C for 10 days resulted in complete clearance of infection and clinically cured all experimentally infected animals.

Protocol:

1. Individually house amphibians in adequately sized terraria/aquaria for the species. Simple setups that allow for easy cleaning are ideal. Consider a moist paper towel or sphagnum moss substrate with a cover object.
2. Begin with frogs or salamanders housed at the typical temperature and incrementally raise temperature 1 degree per day until 25 C / 77 C.
3. Maintain individuals at 25C/77F for a period of 10 days.
4. Monitor animals daily to assess health and wellbeing.
5. Change substrate every 2 or 3 days to remove any shed pathogen from the environment.
6. After 10 days, incrementally return animals to their typical temperature.
7. Bsal retesting should occur to verify that the individual(s) have cleared the infection (see Table 2).

Antifungal Treatment SOP

Blooi et al. 2015 [[10.1038/srep11788](https://doi.org/10.1038/srep11788)]; **Main Conclusion** - topical treatment of *Bsal* infected animals with a combination of polymyxin E (2000 IU/ml) and voriconazole (12.5 µg/ml) at an ambient temperature of 20 °C resulted in clearance of *Bsal* infections.

Protocol:

1. Individually house amphibians in adequately sized terraria/aquaria for the species. Simple setups that allow for easy cleaning are ideal. Consider a moist paper towel or sphagnum moss substrate with a cover object.
2. House animals at least 20 C / 70 F. Incrementally raise/decrease temperature 1 degree per day if temp is outside of their typical temperature.
3. Change substrate every 2 or 3 days to remove any shed pathogen from the environment.
4. Implement treatment twice a day for 10 days.
 - First, administer Polymyxin E bath (2000 IU/ml) via submersion for 10 minutes
 - Second, administer voriconazole (12.5 µg/ml) through spraying the animals and tissue in their housing container.
5. After 10 days of treatment, return animals to typical housing conditions.

6. Basal retesting should occur to verify that the individual(s) have cleared the infection (see Table 2).

Other Resource for Bd Treatment:

<http://www.amphibianark.org/wp-content/uploads/2018/07/Amphibian-Disease-Manual-Chapter-8-Disease-Treatment.pdf>

Amphibian Euthanasia Guidelines

(follow 2020 AVMA Guidelines for the Humane Euthanasia of Animals)

<https://www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf>

Euthanasia should be performed by individuals with appropriate training. Tricaine methanesulfonate (MS 222) can be added to non-circulating water at a concentration of 5 – 10 grams per liter of water (or 0.7 – 1.4 oz per gal) to euthanize amphibians. MS-222 is acidic and should be buffered to pH = 7 (neutral) using sodium bicarbonate (baking soda) to ensure exposure is not painful to the amphibian. MS-222 has been linked to prostrate cancer, so extreme care should be taken when handling it. Similarly, a water bath with benzocaine hydrochloride can be used to euthanize amphibians at a concentration of ≥ 250 milligrams per liter of water (or 0.03 oz per gal). For both euthanasia agents, the amphibian needs to be at least partially submersed in the solution so it can absorb the chemical through its skin. We recommend immersion of at least one hour. Ensure that the amphibian can't crawl out of the solution. Alternatively, over-the-counter 7-20% benzocaine gel (used in humans for toothaches) can also be applied topically to the ventrum (belly) of an amphibian for humane euthanasia. Additional euthanasia methods are available through a veterinarian. A second form of euthanasia should be used after the amphibian becomes unconscious (i.e., no eyelid movement when eyeball touched, no movement when turned on the back or when toe/tail pinched very hard), such as pithing or decapitation. Loss of heartbeat for at least 30 seconds should be confirmed if possible.

Biohazardous Disposal Options of Dead Infected Animals:

Purpose:

Animal carcasses can harbor many viable pathogens and so should be disposed of to ensure no further transmission or contamination is possible. Infected animals may also be of great interest and utility to researchers (e.g., for pathology, pathogen isolation, DNA analyses). We recommend that a subsample of diseased animals (up to 20) is sent to the researchers (Table 1), while the remaining animals can be decontaminated then disposed.

1) Animals for Researchers

If animal carcasses are destined for researchers, ideally the researchers should be contacted to provide preferred options for storing or preserving animals. In general, we recommend that half of the animals are kept cold (but not frozen) and the other half are frozen. Another option is to preserve the animals in 70% ethanol. Details are provided below.

- Amphibians are kept cold in the refrigerator, but not frozen, for histological examination or pathogen isolation. Carcasses should be placed in individual bags (e.g., Ziploc bags) that are then placed in a secondary bag or container to prevent leaks. Animals should be kept separate of food or supplies, ideally in a separate refrigerator.
- Preserved frozen, for pathogen isolation generally placed in individual bags that are then placed in a secondary bag or container to prevent accidental leaks.
 - Labels written on pieces of paper are preferable to using pen or marker on the bags, which may come off with chemicals or in ultracold freezers.
 - A typical frost-free freezer will occasionally warm up to defrost before cooling down again. This freezing and thawing tends to degrade samples, so if non-frost-free freezer is available, this would be preferable. If not, the samples can be surrounded by ice blocks or the equivalent.
- Ethanol preserved, usually with $\geq 70\%$ ethanol (as opposed to “rubbing alcohol”, isopropanol).
 - Glass (Mason) jars are best as ethanol will leak through or degrade many plastic containers. Glass jars should be wrapped in bubble wrap or a similar material to prevent jostling.
 - In general, there should be ≥ 10 times as much ethanol as volume of animals.
 - Larger animals can be cut open to allow the ethanol to penetrate internal tissues for better preservation.

2) Animals for Immediate disposal

There are several options for disposal depending on what options are locally available:

- Small and soft-bodied animals can be placed in a high concentration of commercial bleach (e.g. 50% bleach) for at least 6 hours to inactivate most pathogens. Use of disposable gloves and splash goggles are recommended when handling bleach.
- Animals can be autoclaved, if available, or baked at 250°F (120°C) for 30-60 minutes before disposal.
- Decontaminated animals should be placed in double-layered, opaque trash bag, and closed using a square-knot. The outside of the bag should be disinfected prior to depositing in regular trash.
- Burial or disposal outside should be avoided as the carcasses may be dug up and can contain large concentrations of viable pathogens.
- A final option is to contact a biohazardous waste company, such as Stericycle, and arrange for pick up. Stericycle will have biohazardous bags for waste. Amphibians can be put in a regular, double layered trash bag and frozen prior to pick up.

General notes:

- Gloves should be worn when handling animals and changed before touching any other surfaces or materials.
- The outside of the secondary bag or container should be sprayed with an appropriate disinfectant to inactivate any pathogens that might have contaminated the outside of the bag or container.

Proper Disposal of Potentially Contaminated Aquarium Contents

Purpose:

Materials in aquaria or terraria that have housed (potentially) infected animals can harbor pathogens and thus serve as a source of infection to other animals. Proper disposal ensures the risk of further transmission is minimal.

Procedure:

- Substrates, plants, wood, and other structure should be soaked in an appropriate disinfectant for the required contact time before being disposed of in the trash.
 - Note that organic material can inactivate certain disinfectants (e.g., the active ingredient in bleach) and so should be mixed at a higher concentration when disinfecting aquarium contents.
- Autoclaving aquaria and their contents is also a possibility.