



Extraction-free detection of amphibian pathogens from water baths

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ABSTRACT: Detecting and quantifying pathogens with quick, cost-efficient and sensitive methods is needed across disease systems for addressing pertinent epidemiological questions. Typical methods rely on extracting DNA from collected samples. Here we develop and test an extraction-free method from water bath samples that is both sensitive and efficient for 2 major amphibian pathogens — *Batrachochytrium dendrobatidis* and *B. salamandrivorans*. We tested mock samples with known pathogen quantities as well as comparatively assessed detection from skin swabs and water baths from field sampled amphibians. Quantitative PCR (qPCR) directly on lyophilized water baths was able to reliably detect low loads of 10 and 1 zoospores for both pathogens, and detection rates were greater than those of swabs from field samples. Further concentration of samples did not improve detection, and collection container type did not influence pathogen load estimates. This method of lyophilization (i.e. freeze-drying) followed by direct qPCR offers an effective and efficient tool from detecting amphibian pathogens, which is crucial for surveillance efforts and estimating shedding rates for robust epidemiological understanding of transmission dynamics. Furthermore, water bath samples have multiple functions and can be used to evaluate mucosal function against pathogens and characterize mucosal components. The multifunctionality of water bath samples and reduced monetary costs and time expenditures make this method an optimal tool for amphibian disease research and may also prove to be useful in other wildlife disease systems.

KEY WORDS: *Batrachochytrium* · Lyophilization · Pathogen detection · Wildlife disease · Early warning system

1. INTRODUCTION

Quick, cost-efficient and effective methods for detecting pathogens are needed across human, plant and wildlife disease systems. Techniques for detecting organisms, including pathogens, often involve quantitative polymerase chain reaction (qPCR), a highly sensitive technique, on extracted sample DNA. In recent years, qPCR has been the method of choice and has been shown to be a highly sensitive technique for detection of important amphibian pathogens (Boyle et al. 2004, Blooi et al. 2013). These

pathogens, namely *Batrachochytrium dendrobatidis* and *B. salamandrivorans* (*Bd* and *Bsal*), are a cause of global losses in amphibian diversity and shifting ecosystems, making early detection as well as continuous monitoring important endeavors (Weldon et al. 2013, Gray et al. 2015, Waddle et al. 2020). Furthermore, teasing apart transmission dynamics and building robust models from pathogen load data can shape our understanding of infection and disease dynamics (Wilber et al. 2017).

For amphibians, water bath samples have been used for various purposes, including estimation of

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pathogen shedding rates (Hyatt et al. 2007) as well as evaluating mucosal function as an assay for protectiveness of skin secretions and proxy for host susceptibility (Woodhams et al. 2014, Smith et al. 2018). For shedding rates, qPCR is typically performed on DNA extracted from filters of water that previously housed an amphibian host (Reeder et al. 2012, DiRenzo et al. 2014). DNA extraction has been seen as an essential step for performing qPCR as it lyses cells to expose the genomic DNA and purifies the DNA by removing cell debris and other components that can inhibit the qPCR reactions. Extraction methods with the highest efficiency and purity can be expensive and time-consuming while quick methods can often fail to detect low pathogen loads (i.e. have low sensitivity) (Bletz et al. 2015, Brannelly et al. 2020) or inhibit PCR (PrepMan; Boyle et al. 2004).

Mucosal function assays have employed lyophilization of samples to concentrate skin secretions and evaluate their potential to inhibit or kill pathogen cells. Lyophilization is a process that sublimates water from frozen samples, i.e. freeze drying, and requires a standard piece of equipment found in most chemistry or biology departments. This process has the potential to lyse cells or minimize the integrity of cell membrane/walls, and therefore, may make sample DNA available for PCR amplification without formal DNA extraction protocols. Likewise, PCR thermo-cycling can also lyse cells making DNA accessible (Flores et al. 2012, Videvall et al. 2017); therefore, lyophilization coupled with PCR thermo-cycling has the potential to amplify target DNA and allow for detection and quantification of pathogen loads.

Here we develop and test an extraction-free pathogen detection method from water baths by (1) evaluating efficiency and sensitivity of pathogen detection from mock water baths to which we added known concentrations of *Bd* and *Bsal* zoospores, (2) comparing pathogen quantity estimates from water bath collections in plastic and glass given the surface adherence noted in Hyatt et al. (2007), and (3) assessing *Bd* detection across skin swab and water bath samples from field sampled amphibians given the standard use of swabs in amphibian disease research. It is essential to have a method with high efficiency and sensitivity in order to accurately determine the presence and intensity of the chytrid fungus. Low efficiency and sensitivity could result in false negatives, which can have important consequences for amphibian conservation and disease mitigation decisions and inaccurate modelling of infection and transmission dynamics in natural systems.

2. MATERIALS AND METHODS

2.1. Sample preparation and collection

Mock water bath samples of ~12 ml of artificial pond water (e.g. Provasoli following Wyngaard & Chinnappa 1982; recipe details in Robinson et al. 2020) were prepared and spiked with a dilution series of 1×10^6 to 1×10^0 *Bd* and *Bsal* zoospores to mimic mucosome baths used for mucosal function assays and 1 h shedding rate bath collections. Five replicates of each spike-in dose were prepared ($n = 35$), in addition to 5 negative controls containing only artificial pond water. *Bd* (JEL 423) and *Bsal* (AMFP13/1) were maintained in 1 % tryptone broth. *Bd* zoospores were harvested from tryptone agar plates that were seeded with 1 ml of 3–5 d old liquid cultures, and *Bsal* zoospores were harvested from synchronized liquid cultures (Robinson et al. 2020). Artificial pond water was used for harvesting both pathogens. Harvested zoospores were subsequently counted on a hemocytometer with 4 replicate counts by 3 individuals. Dilutions of 10^6 – 10^0 zoospores were prepared in artificial pond water so 1 ml of prepared zoospore solutions could be added to the mock water baths to obtain the target spike in amounts. Samples were then frozen in 15 ml conical tubes at -80°C , lyophilized, and rehydrated in 1 ml sterile Milli-Q water. For lyophilization, 15 ml tubes (HDPE, CellTreat # 229411) were uncapped, covered with parafilm, and made porous by pricking 3–5 holes in the parafilm to allow sublimation while also protecting the sample contents. The lyophilizing procedure was carried out on a LabConco FreezeZone 2.5 l Benchtop System; the temperature was -85°C and the pressure was 0.006 mBar.

Additionally, 6 field water bath samples (collection methods followed field procedure outlined below) from swab-positive leopard frogs were processed to demonstrate if detection occurs from ‘complex’ samples containing amphibian secretions. Initial rehydration was performed in a ‘concentrated’ form where a smaller volume of 100 μl was used to see if detection increased or was hindered by concentration. (See ‘Supplementary methods’ and ‘Supplementary results’ in the Supplement for more details; www.int-res.com/articles/suppl/d146p081_supp.pdf). Samples were subsequently diluted 1:10 in MilliQ water to equate to adding 1 ml to the lyophilized sample, which is standard for mucosome function testing from water bath samples.

An additional trial was performed to determine if completion of 1 h water baths in plastic (e.g. Whirl-Paks) versus glass jars affected qPCR detection esti-

mates. To test this, *Bsal* zoospores were quantified using 4 replicate counts on a hemocytometer, and 1000 *Bsal* zoospores were spiked into 12 ml of artificial pond water in both Whirl-Pak bags and Pyrex glass containers. Seven replicates of *Bsal* spike-in samples and 3 controls containing only 12 ml of artificial pond water were prepared in each container type (total N = 20) and incubated for 1 h. After 1 h, mock baths were transferred to 15 ml plastic conical tubes, frozen, lyophilized, and rehydrated in 1 ml of sterile Milli-Q water.

Lastly, we compiled data from 313 field-collected 1 h skin washes and skin swabs to assess whether skin wash and swab values mirror each other. Post-metamorphic leopard frogs (*Lithobates [Rana] pipiens*, n = 105; *L. sphenoccephalus*, n = 167; *L. blairi*, n = 41) were sampled in rural ponds and surrounding habitats at 5 locations including Vermont, Pennsylvania, Tennessee, Louisiana, and New Mexico, USA, between February and November of 2017. Sampled leopard frogs ranged from 0.8–83.0 g in mass (measured using a Pesola scale). Individuals were first swabbed following Hyatt et al. (2007) with rayon-tipped swabs (Medical Wire, MW113). Fresh, clean pairs of gloves were used for each individual to avoid cross contamination. Swabs were stored in 1.5 ml tubes. Subsequently, 1 h water baths were collected by bathing the frog in 15 ml of artificial pond water in sterile Whirl-Pak bags. Three water bath controls were taken during field sampling where the sampling procedure was completed without an amphibian being placed in the bag. After the bath, washes were transferred to 15 or 50 ml conical tubes for storage. Both swabs and washes were stored on ice until access to a –20 or –80°C freezer was possible.

2.2. Molecular methods

For all water bath samples, *Bd/Bsal* qPCR duplex reactions that amplify the internal transcribed spacer (ITS) region were run directly on the rehydrated samples following Blooi et al. (2013). For swab samples, DNA was first extracted using a Qiagen Blood and Tissue Kit following the manufacturer's protocol for animal tissue with one modification of a double elution step to increase total DNA yield (final vol. = 200 µl). For all qPCR reactions (bath and swab extracts) 5 µl of DNA was added to the reactions. For mock water bath samples, AzuraQuant Probe Fast qPCR Mix (2X), and all qPCR reactions were performed in duplicate (or triplicate in some cases) on a BioRAD CFX machine. gBLOCK standards were

used to estimate ITS copies of both *Bd* and *Bsal* in samples (Standish et al. 2018). Samples were considered positive when at least 2 qPCR replicates had a positive amplification signal with typical amplification curves. Estimated copies from the qPCR were adjusted to represent total sample volume; that is, we divided by 5 to get a per µl estimate and then multiplied the estimate by 200 to account for total extraction volume from swab samples or by 1000 to account for total rehydration volume of 1 ml for water bath samples.

For the field samples, the same duplex reaction protocol was followed (Blooi et al. 2013), except bovine serum albumin (final concentration 400 ng µl⁻¹, 1 µl in 25 µl reaction; Garland et al. 2010) was added to each sample to minimize potential inhibition. SensiFAST Probe Lo-ROX qPCR Mix (2X) was used for these samples. An internal positive control (IPC) (Hyatt et al. 2007) was also included in each sample to allow for evaluation of inhibition within qPCR reactions. Ten water bath samples were re-run at a 1:10 dilution due to IPC-detected inhibition. Standards consisted of a 7-fold dilution series of plasmid-based *Bd* and *Bsal* sequences ($4.2 \times 10^6 - 4.2$ plasmid copies µl⁻¹; Pisces Molecular). These 313 field samples are part of another ongoing study, and each swab extract and field water bath was tested in singlicate to maximize cost efficiency (Kriger et al. 2006). Estimated loads from the qPCR were adjusted to represent total extraction and rehydration volume for swabs and baths, respectively, as described above. All swab extraction controls, field water bath controls, and qPCR negative controls were negative for *Bd* and *Bsal*.

2.3. Data analysis

All statistical analyses and plot generation were completed in R v4.0.2 (R Core Team 2020). The proportion of samples with positive detections was calculated across the spiked water bath samples for *Bd* and *Bsal* as well as the field samples in the standard and concentrated preparations. Fisher's exact tests and generalized linear models (GLM) with a binomial distribution were used to analyze detection (i.e. presence/absence). Estimated ITS copies were compared using linear models (LM) and Kruskal-Wallis (KW) tests depending on data distributions. Additionally, Kendall-tau correlations were calculated across the dilution series of both pathogens as well as for the standard and concentrated spike-in samples. Correlations were also calculated between swab and water

bath estimates from field-collected samples. Detection rates of swabs and water baths were calculated from the field-collected samples, and Bayes theorem was used to calculate conditional probabilities.

3. RESULTS

3.1. Direct qPCR from water baths exhibits high sensitivity

Detection from direct lyophilized mock water baths occurred in 5/5 samples for both *Bd* and *Bsal* for the 10^6 – 10^2 spike-in doses. Detection was 4/5 and 3/5 at the 10 zoospore spike in and 4/5 and 1/5 at the 1 zoospore spike in for *Bd* and *Bsal*, respectively (Fig. 1). All negative control bath samples were negative for *Bd/Bsal* in qPCR. Detection did not differ from 100% across doses for *Bd* and only dropped significantly for *Bsal* at the 100 spike-in dose (Fisher's exact: $p = 0.048$). Detection did not significantly differ between *Bd* and *Bsal* at spike-in doses of 10^6 – 10^1 (Fisher's exact: $p > 0.05$), but detection was significantly lower at the 1 zoospore spike-in quantity for *Bsal* compared to *Bd* (Fisher's exact: $p = 0.033$). Furthermore, there was a strong correlation between the number of zoospores spiked into the samples and the number of ITS copies detected via qPCR for both *Bd* and *Bsal* (*Bd*—LM: $\log_{10}$ zoospores added, $F_{(33,1)} = 343.0$, $R^2 =$

0.91, $p < 0.001$; *Bsal*—LM: $\log_{10}$ zoospores added, $F_{(31,1)} = 160.2$, $R^2 = 0.83$, $p < 0.001$; Fig. 1). Concentrated water bath samples (i.e. those rehydrated in 100 μ l) minimized pathogen detection compared to samples with the 1 ml rehydration volume and resulted in spurious, atypical amplification curves (see the Supplement).

3.2. Collection container does not affect pathogen detection

Bsal was detected in all spike-in samples from both container types ($n = 7$ each), and control samples were all negative for *Bsal* ($n = 3$ each). Estimated *Bsal* ITS copies from mock water bath samples did not differ between Whirl-Pak and glass collection jars (Whirl-pak = $1.55 \times 10^4 \pm 2.15 \times 10^3$ [mean $\pm$ SE], glass = $1.89 \times 10^4 \pm 1.76 \times 10^3$; GLM: $F_{1,28} = 1.28$, $p = 0.270$).

3.3. Water bath samples exhibit higher detection rate than swabs

Quantitative PCR was performed on extracted swab DNA and lyophilized water baths collected from 313 field samples from *Lithobates pipiens*, *L. sphenoccephalus* and *L. blairi*. All field collected control samples ($n = 3$), swab extraction controls (1/ex-

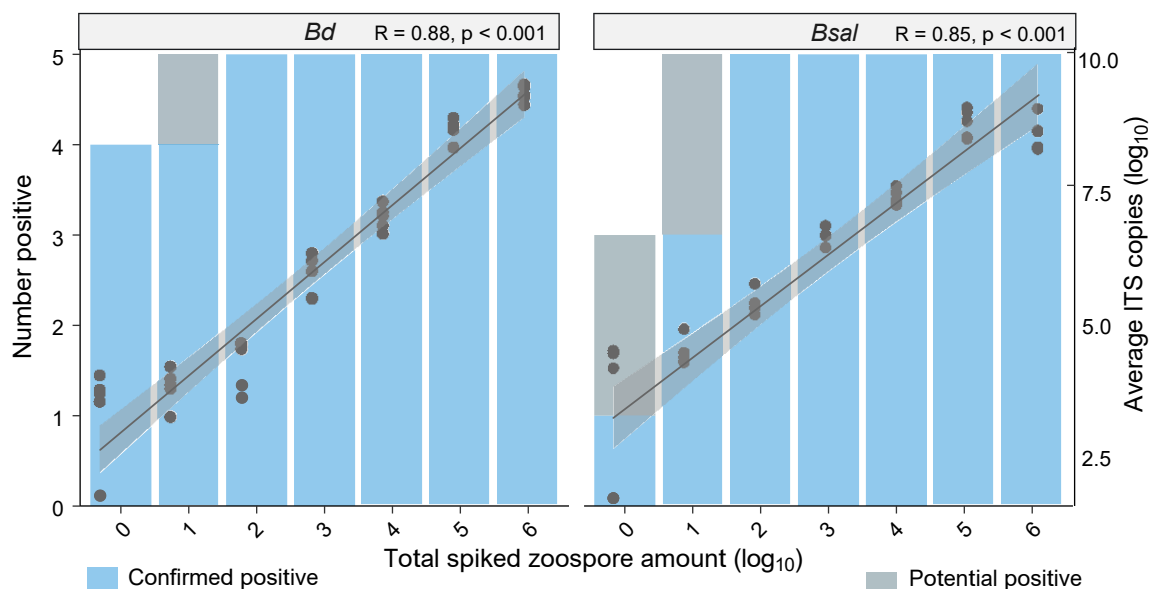


Fig. 1. Detection of *Bd* and *Bsal* from direct quantitative PCR (qPCR) testing from lyophilized water baths spiked with known zoospore quantities. Left axis: count of positive samples (i.e. detection). Blue fraction of bar: samples with clear duplicate or triplicate qPCR amplifications (i.e. confirmed positives); gray fraction: samples with singlicate qPCR amplification (1/2 or 1/3, i.e. unconfirmed positives). Right axis: average *Bd/Bsal* internal transcribed spacer (ITS) copies estimates corresponding to the linear fit lines

traction set) and qPCR negative controls (1/plate) tested negative for *Bd/Bsal* by qPCR. *Bd*-ITS copy estimates from swab extracts and water baths were significantly correlated (Kendall: $R = 0.36$, $p < 0.001$; KW: $\chi^2 = 222.4$, $p < 0.001$, Fig. 2). Swab extracts and water baths had matched results in 69% of the samples. A total of 134 samples were negative for *Bd* in both swabs and water baths, 81 samples were positive for both swabs and water baths (i.e. matched detection), 63 were positive only for water baths, and 35 were positive only for swab extracts. If any detection is considered true (therefore combining positive matches, water bath only, and swab only, $n = 179$), the detection rate from water baths was 80% (20% failure rate) and 65% for swabs (35% failure rate). Furthermore, for all species, water baths showed higher detection rates than swabs (Table S1 in the Supplement). Using Bayes theorem to calculate conditional probabilities, we found that there was a 60% chance of detecting *Bd* from a swab if *Bd* was detected in the water bath, and there was a 71% chance of detecting *Bd* from the water bath if *Bd* was detected on the swab. Of the samples where both were positive ($n = 81$), 53 had higher ITS copy estimates from water baths and 29 had higher estimates

from skin swabs. Furthermore, the average number of estimated ITS copies from skin washes was significantly lower for samples where only the skin wash sample detected *Bd* compared to samples where both skin wash and swab detected *Bd* (wash only: $1.04 \times 10^6 \pm 1.02 \times 10^6$ (SE); matched: $6.24 \times 10^5 \pm 3.23 \times 10^5$ (SE); KW: $\chi^2 = 10.032$, $p = 0.002$).

4. DISCUSSION

Water bath samples from amphibians are used for a variety of purposes including mucosal function assays (Woodhams et al. 2014, Smith et al. 2018), pathogen detection (Shin et al. 2014), and pathogen shedding rate (Reeder et al. 2012, DiRenzo et al. 2014). The purpose of this study was to develop and evaluate a protocol for pathogen detection and quantification from water bath samples without inclusion of a DNA extraction step. We found that qPCR on the directly lyophilized water bath samples rehydrated in 1 ml of ultrapure water can not only detect 2 major amphibian pathogens, *Bd* and *Bsal*, but can do so with high sensitivity (i.e. detect low pathogen loads). Furthermore, we found that the type of water bath collection container (plastic vs glass) had no effect on estimated pathogen loads and that ‘concentrating’ samples through rehydration in less than 1 ml resulted in qPCR inhibition, which, at times, led to a failure to detect the pathogens altogether. Finally, we found that overall water bath and swab extracts correlated in their estimated *Bd* quantity, and Bayes theorem suggests our protocol from water bath samples may be moderately better at detecting *Bd* compared to swabs. Collectively these results show that our protocol for water bath samples is effective and highly sensitive and may outperform standard swabbing for pathogen detection. Furthermore, this means pathogen detection, estimate of shedding rates, and mucosal function can be determined from a single sample, reducing cost and combined time during field and wet lab procedures (Table 1).

Quantitative PCR on extracted DNA is a standard and widely used method for detection of specific pathogens in and on host organisms. In amphibian systems DNA extraction from skin swabs and filters from water baths followed by qPCR have been used for pathogen detection and shedding rate calculations from host amphibians (Reeder et al. 2012, DiRenzo et al. 2014). Herein, we show pathogens can be efficiently detected by our simple method of lyophilizing (freeze-drying) collected water baths and performing qPCR directly on the rehydrated

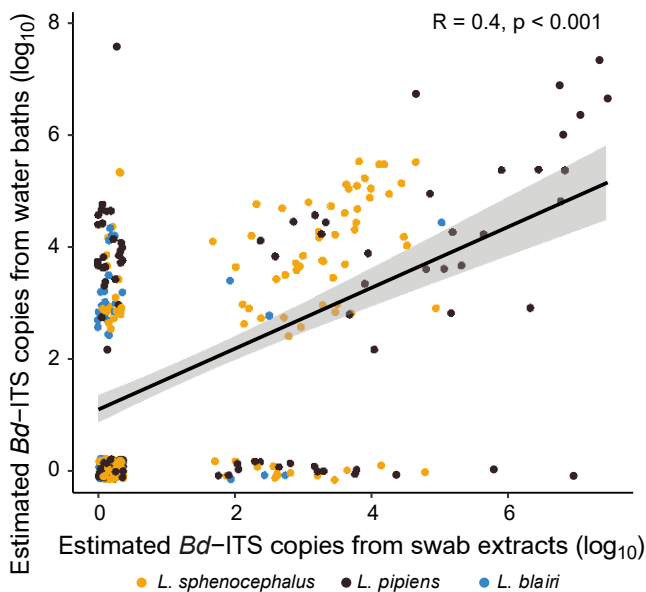


Fig. 2. Relationship between *Bd* internal transcribed spacer (ITS) copies estimated from swab extracts and 1 h water bath from field sampled frogs. Points representing zeros for either axis have been jittered with ggplot::geom_jitter to allow visualization of overlapping points. Non-zero values are displayed un-jittered. Points are colored by host species: blue: *Lithobates blairi*; black: *L. pipiens*; yellow: *L. sphenoccephalus*. Kendall-tau correlation results denoted in the upper right

Table 1. Comparison of water bath and swab sampling broken down by field sampling, sampling processing, and detection rate. Cost estimates, hands-on time, and number of samples are provided. Elements of sampling and processing that are equal between methods are excluded (e.g. capture time, quantitative PCR, freezers)

Field sampling	Water bath	Swab
Sample time	60–75 min (60 min bath of individuals + transfer time) for 30 individuals	30–40 min for 30 individuals
Supplies	Collection bags, 15 ml tubes, artificial pond water, ice cooler	Collection bags, swabs, 1.5 ml tubes, ice cooler (optional)
Sample processing time	15 min of hands-on time (total time to obtain final DNA ‘extract’ = 48 h)	2–2.5 h hands-on time (total time to obtain final DNA ‘extract’ = 3 h)
Sample number	60 samples at a time (assuming 4 nozzle benchtop lyophilizer)	24 samples at a time (assuming 24 rotor centrifuge and single tube extractions)
Freezer space	Space for 15 ml tubes	Space for 1.5 ml tubes
Cost	\$0 (USD) per sample	\$2.30–3.04 (USD) per sample
Equipment	Lyophilizer, Vortex Cost: \$ 18 000 + \$ 350 = \$ 18 350 (USD)	Centrifuge, Heat block, Vortex Cost: \$ 1800 + \$ 800 + \$ 350 = \$ 2950 (USD)
Detection rate	80%	65%

- Collection & Processing Protocol**
detection of pathogens from water baths
- (1) Bath amphibian in 12–15 ml of sterile artificial pond water within a Whirl-pak bag for 1 hr.
 - (2) Transfer bath solution to a 15 or 50 ml conical tube and freeze at -80°C .
 - (3) Once frozen, prepare samples by partially unscrewing the caps to allow sublimation.
 - (4) Place on lyophilizer and lyophilize until dry.
 - (5) Resuspend dried sample in 1 ml of ultrapure (e.g. MilliQ) water, vortex ~10 sec, and centrifuge for 10 sec.
***DO NOT use a rehydration solution with added salts**
Optional. Transfer the 1ml to a 1.5 ml tube for ease of storage.
 - (6) Run a standard qPCR reaction with 5 μl of sample of the resuspension liquid.

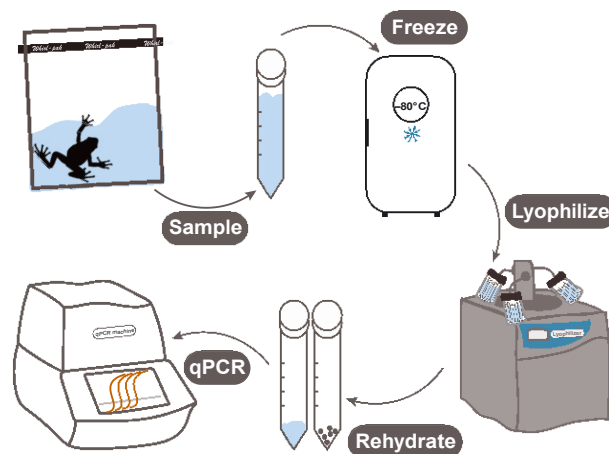


Fig. 3. Step by step protocol for pathogen detection from water bath method

products, bypassing the DNA extraction step (Fig. 3). Both *Batrachochytrium* pathogens were detectable with high sensitivity. The detection limit mirrored that of optimal DNA extraction methods (see swab extraction comparisons in Bletz et al. 2015 and Brannelly et al. 2020) with detection only being significantly reduced for the 1 zoospore level. This reduction in detection was observed for *Bsal* only and not *Bd*. This difference may be associated with ITS copy number differences between the pathogens, which has been reported for different strains of *Bd* (Longo et al. 2013, Rebollar et al. 2017). If *Bsal* has lower copies per genome, capturing and successfully amplifying DNA with low zoospores amounts could be below the threshold of detection. Alternatively, the existence of encysted spores with more robust

cell walls for *Bsal* (but not *Bd*) may also contribute to this, given it likely makes it more difficult to access the DNA in these more robust spores (Stegen et al. 2017).

Bypassing the extraction step can reduce both supply costs and hands-on lab time (Table 1). While lyophilizers might not be present in every molecular lab, it is a typical piece of equipment within biology or chemistry departments at universities with multifunctionality across fields (e.g. Rockinger et al. 2021). Multiple studies have proposed cost-saving measures at the extraction and qPCR step through extraction pooling (Hyatt et al. 2007, Sabino-Pinto et al. 2019a,b), and singlicate qPCR (Kriger et al. 2006). For efficient DNA extraction kits that produce high-quality, pure DNA, costs are approximately \$2.30–\$3.04 USD per

sample (catalog prices for IBI and Qiagen), and hands-on lab time to complete a standard spin column extraction is approximately 3 h (Sabino-Pinto et al. 2019a). The method tested here, therefore, is one of the most economical and person-time efficient approaches available (Table 1). With that said, it is important to consider that for certain field sites which involve traversing rough terrain on foot, carrying water and ice packs may be challenging. Furthermore, if pathogen surveillance is the only purpose of sampling, it would be likely more time efficient in the field to swab amphibians; however, it will be important to factor in lab time and detection probability when making method decisions.

Plastic vials and bags are commonly used for amphibian samples; previous studies have suggested that zoospores and cellular material can stick or adhere to collection container surfaces (Hyatt et al. 2007). However, we found that HDPE plastic and glass vials yielded matching results. The short duration of bath time (1 h) may reduce potential adherence. This result is useful in the context of field research where bringing large numbers of glass jars could be cumbersome.

It is important to note that qPCR amplification and detection was not improved by rehydrating samples in smaller volumes (i.e. concentrating the sample by 10×). While concentrating DNA in highly pure samples can be seen as a way to increase the likelihood of detection, in this case the opposite occurred; the reaction was hindered. In fact, concentrating the samples reduced detection and estimated quantities, and caused atypical exponential curves. While no IPC controls were run to confirm inhibition, this failure of the qPCR reactions is likely due to increased salt concentrations (as demonstrated by nanodrop 260:230 reading less than 1.8; range of tested samples: 0.06–0.3), which interfere with the Taq polymerase or other components of the qPCR reaction (Schrader et al. 2012). Water baths of amphibians are necessarily completed in a water solution with added ions (e.g. Provasoli, reconstituted reverse osmosis water) to minimize interfering with the osmotic balance across sensitive amphibian skin. During lyophilizing, this salt and other sample contents are dried and therefore concentrated when rehydrated. Minimizing the rehydration volume to less than 1 ml or rehydrating in a solution containing ions, therefore, can push salt concentrations to levels not suitable for qPCR. For baths of smaller individuals or larval stages, a reduced bath volume could also help reduce the amount of salt being rehydrated; here we used up to 15 ml of bath water with no apparent

qPCR inhibition when rehydrated in 1 ml Milli-Q water. Importantly, amphibians have different shapes, sizes, and secretions; therefore, soaking and rehydration volumes may need to be validated and adjusted depending on the study system. Further testing to evaluate the effects of environmental conditions (e.g. temperatures and other field conditions) that may affect DNA degradation and PCR inhibition may also be important.

Furthermore, our tested field samples did not experience significant qPCR inhibition (successful amplification of the internal standard) in either water bath or swab samples. There were 10 water bath samples that did show inhibition; when rerun at a 1:10 dilution, inhibition was ameliorated, and samples were negative for *Bd*. We cannot exclude the possibility that this dilution influenced the ability to detect *Bd* by pushing it below the detection limit. Inhibition in water baths could have potentially occurred due to increased environmental debris or feces, indicating the importance of reducing these in the bath before freezing. Importantly, the majority of field samples (96.8%) amplified with no inhibition, suggesting that frog secretions are unlikely to affect detection. Continued inclusion of IPCs should be considered standard even though these data suggest inhibition is low.

As compared to skin swabs, water bath sample collection may serve as a more robust technique for detecting amphibian pathogens, *Bd* and *Bsal*. Swabbing amphibians is a standard method widely used in amphibian disease research (Hyatt et al. 2007) for pathogen detection and quantification. While, overall, swabs and water bath results correlate, mirroring results of some studies (Reeder et al. 2012, compared to tissue in Hall et al. 2020), detection probabilities from water baths were greater than swabs. This improvement in detection of *Bd* in water baths should also be considered conservative, as some zoospores that may have been added to the water bath samples were first swabbed from the skin. This finding is similar to that of Shin et al. (2014), who found that swabs can also miss lower-level infections compared to water baths. Indeed, we found that the average estimated quantity from skin washes was significantly lower for samples where only the skin wash sample detected *Bd* compared to samples where both sample types showed detection, suggesting the washes can detect lower infection loads. Our results add to their study, showing that baths of only 1 h (vs. 12, 24 h in Shin et al. 2014) are effective and more robust than swabs for detection. Water baths had a 15% lower failure rate than swabs and showed no evi-

dence of contamination from field collection or sample processing. Water baths also remove some common biases associated with swabbing, such as surface area swabbed, swabbing pressure, number of swabbing motions, and other factors (Simpkins et al. 2014). Water baths also remove the harmful effects of swabbing delicate larvae, which can sometimes lead to death when swabbing oral disks of tadpoles and the thin skin of larval salamanders (authors' pers. obs.). We also note the importance of using artificial pond water, rather than pure water, for bathing larval amphibians sensitive to osmotic stress. Detection from water baths may be greater than from swabs given that shedding seems to be uneven through time, making the prolonged sampling duration of a water bath more likely to pick up pathogen cells (Shin et al. 2014). Contact with water may also promote zoospore discharge. Further testing with shorter bath durations may reveal optimal field handling times for pathogen surveillance. Furthermore, water bath samples also can provide additional data, e.g. shedding rates and mucosal function, which can synergize to address questions in the disease ecology of amphibian systems. Estimating shedding rates can add valuable information about individual amphibian contributions to transmission and infection dynamics (Wilber et al. 2017), and mucosal function assays can provide crucial information on host susceptibility (Woodhams et al. 2014). Perhaps water baths of other potential pathogen reservoir hosts, such as crayfish (McMahon et al. 2013), will prove useful when swabbing is not possible.

The multifunctionality of water bath samples, better detection rates, reduced monetary costs, and hands-on time expenditures make this method a useful tool for amphibian disease research systems, and it may also prove to be useful in other wildlife disease systems.

Acknowledgements. We thank Michel Ohmer, Laura Brannelly, Nina McDonnell, Mason Ryan, Ian Latella, and Jamie Voyles for assistance with field sample collection, and Dan Wetzel, Caitlin Nordheim, and Joana Sabino Pinto for assistance with laboratory sample processing. We thank Cori Richards-Zawacki for coordination of the SERDP project team. This investigation was funding by SERDP Contract RC-2638 to D.C.W., NSF EEID grant 1814520 to D.C.W., and David H. Smith Postdoc Fellowship to M.C.B.

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Editorial responsibility: Catherine Collins,
Jouy-en-Josas, France
Reviewed by: L. Campbell and 2 anonymous referees

Submitted: March 19, 2021
Accepted: July 13, 2021
Proofs received from author(s): September 20, 2021