Rapid detection of the amphibian chytrid fungus (Batrachochytrium dendrobatidis) using in-situ DNA extraction and a handheld mobile thermocycler

Colleen Kamoroff[1], Caren Goldberg[2], and Robert Grasso[1]

[1]Yosemite National Park
[2]Washington State University

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Abstract

The amphibian chytrid fungus (Bd) has caused extinction of amphibian populations worldwide. Early and accurate Bd detection is essential for management and treatment of susceptible anurans. We analyzed the effectiveness of an in-situ DNA extraction along with handheld mobile quantitative PCR (qPCR) thermocycler to detect Bd on skin frog swabs, and to detect Bd in water samples using environmental DNA (eDNA). We collected duplicate eDNA samples and skin swabs from three Bd positive Rana sierrae populations. We processed one set of samples using a field protocol (a handheld thermocycler), and the other half using a standard lab protocol. We detected Bd DNA in all R. sierrae swabbed across all three sites using both the field and lab protocols. We also detected Bd DNA in eDNA samples collected at two of the three sites using both the field and lab protocols although the field and lab protocol failed to detect Bd eDNA at separate sites. The probability of detecting Bd DNA in the technical replicates was lower for the field protocol compared to samples extracted using the lab protocol, suggesting the field protocol has reduced sensitivity and may not detect low quantities of DNA. Our results suggest the field extraction protocol using a handheld qPCR platform is a promising tool for rapid detection of Bd in susceptible amphibian populations. The field protocol yielded accurate results in less than 60 minutes. However, the applied field protocol may be prone to false negatives when analyzing low-quantity DNA samples (i.e. eDNA).

Introduction:

Chytridiomycosis is a skin disease caused by the invasive amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd; Longcore et al., 1999). Bd is affecting amphibian populations worldwide and is linked to mass extirpation and extinction of over 200 amphibians (Berger et al., 1998; Stuart et al., 2004; Skerratt et al., 2007). In the California Sierra Nevada, historical records show that Bd has been present since the 1970s (Fellers et al., 2001). Bd has been linked to the precipitous declines of two endemic and endangered species of yellow-legged frogs, Sierra Nevada yellow-legged frog (*Rana sierrae*) and southern mountain yellow-legged frog (*R. muscosa*), collectively referred to as the mountain yellow-legged frog complex (Rachowicz et al., 2005; Vredenburg et al., 2009) and referred hereafter as MYLF.

Prior to the arrival of Bd, MYLF populations were already diminished due to the introduction of nonnative trout, which prey on tadpoles and adults (Knapp & Mathews, 2000). Nonnative trout also fragment and isolate MYLF populations by occupying and barring dispersal corridors as well as adjacent water bodies (Bradford & Graber, 1993). Bd has caused declines and localized extirpations of many persisting populations in the few remaining fish-free habitats in the Sierra Nevada (Vredenburg et al., 2010). Given these two prominent causes for decline, MYLF species are listed as endangered by the IUCN (2008; IUCN), the state of California (2003; California Department of Fish and Wildlife), and U.S. Fish and Wildlife Service (2014; U.S Fish and Wildlife Service)

There are few remaining Bd-naïve MYLF populations on protected public lands (e.g., National Parks). The remaining populations are likely susceptible to mass die-off events once Bd is introduced (Rachowicz et al., 2005; Vredenburg et al., 2010; Knapp et al., 2016). Bd positive MYLF populations persisting with the disease are thought to have an adaptive immunity response (Knapp et al., 2016), but both population types (naïve and persisting) require monitoring for adaptive management (e.g., translocation and augmentation), and treatment options such as intervention with antifungal agents at the onset of mass die-off events (Harris et al., 2009).

Established techniques for Bd detection include swabbing keratinized skin on frogs and mouthparts of tadpoles, followed by analyzing the swabs for Bd zoospores using quantitative polymerase chain reaction (qPCR) techniques (Boyle et al., 2004). More recently, Bd has been detected using environmental DNA (eDNA) techniques (Kirshtein et al., 2007; Walker et al., 2007; Hyman & Collins, 2012; Chestnut et al., 2014). eDNA is a non-invasive alternative survey tool that is not dependent on finding and handling a host organism and can potentially increase detection of aquatic pathogens when few individuals are infected. Species detection using eDNA methods is accomplished by collection and identification of trace DNA particles that are extracted from water samples (Taberlet et al., 2012). A recent study in the Sierra Nevada detected Bd in water samples prior to a MYLF chytridiomycosis die-off event (Kamoroff & Goldberg, 2017).

The National Park Service (NPS) is actively monitoring and managing populations of endangered MYLF in Yosemite National Park (YNP). The NPS and their partners are currently collecting swabs from a known Bd- naïve population to determine if an outbreak is imminent as well as collecting samples from persisting Bd- positive populations to determine Bd- load and epizootic disease dynamics in park populations. Early and accurate detection as well as reliable quantification of Bd is a critical component in managing overall MYLF recovery, a task compounded by the difficulty of reaching occupied sites as MYLF populations inhabit high elevation (>1830 m) alpine lakes often in remote wilderness or wilderness-like settings.

In this study, we analyzed the effectiveness of using an in-situ DNA extraction method combined with a handheld mobile thermocycler for real-time qPCR analysis in the field (Biomeme Inc. Philadelphia, PA). The NPS currently uses lab-based DNA and eDNA extraction/analysis methods for surveillance of Bd across YNP (Yosemite unpublished data). However, the lab-based approaches require hiking samples [?]10 miles out of the field, followed by additional transport time to a temporary storage facility, and further delay during shipping and lab processing which can result in a minimum turnaround time of weeks to months. Our goal was to circumvent this process by rapidly detecting and quantifying Bd DNA using extracted samples collected and analyzed directly in the field. The field-based platform for DNA extraction and mobile real-time qPCR analysis yield results in less than 60 minutes and does not require hiking samples out of the field for lab analysis. We compared the results of the field-based DNA extraction and analysis approach with lab-based extraction and analysis using two sampling strategies, frog skin swabs and eDNA filtered water samples.

Methods:

Study Area:

We collected eDNA samples as well as MYLF skin swabs from three different known Bd-positive populations across YNP (Fig. 1). All three sites were part of YNP's long-term MYLF population and disease monitoring program ranging in elevation from 3069-3202m and a size of 0.3 ha – 4.7 ha.

Sample Collection:

Environmental DNA samples

We collected 500 ml of water every 40 m around the perimeter of each site. Due to varying lake sizes, the amount of water collected varied between sites (Table 1). We split each water sample in half and filtered duplicate 250 ml samples using separate filter cups for comparison testing (Fig. 2). The amount of water collected was 5x the amount of water used for detection of Bd at a site in 2015 (Kamoroff & Goldberg, 2017). At the end of each sample collection, we filtered duplicate 200-250 ml samples of distilled water for a negative

control (Fig. 2). We filtered all water samples using a single-use 47 mm diameter 0.45 µm cellulous nitrate filter membrane (Sterlitech, location) and a polypropylene vacuum flask with a rubber stopper fixed to a hand or motorized pump. To remove the filter membrane, we used single use forceps or forceps soaked in a 50% commercial bleach solution and then rinsed in distilled water. All personnel wore single use disposable latex gloves during sample collection and changed gloves prior to handling filter membranes.

Frog Skin Swabs

We collected Bd skin swabs from adult MYLF at all three populations sites (Table 1). We swabbed 10X on the frog's side, 10X upper arm, 10X lower leg, and between the toes using a cloth-tipped swab (Biomeme Inc. Philadelphia, PA.). We used separate swabs for each side of the frog (i.e. right and left side) for comparison testing (Fig. 2).

All the accessible MYLF populations in YNP were known to be Bd positive. In order to swab potential Bd negative anurans, we opportunistically swabbed four California red-legged frogs (*Rana dryatonii*) recently introduced to Yosemite Valley, YNP, and one American bullfrog (*Lithobates catesbeianus*) in Catalina Island, Catalina National Park. At the time of swabbing, it was unknown whether the *R. draytonii* or the *L. catesbeianus* were Bd positive.

To prevent the spread of disease, we disinfected all field gear (e.g., boots, nets, etc.,) in a 0.016% solution of quaternary ammonia between sample sites (Johnson et al., 2003). We did not enter the water with gear prior to eDNA sample collection.

Lab Protocol for DNA Analysis

We stored the filters and swabs in 95% ethanol at room temperature away from any light source (Minamoto et al. 2016) and extracted the samples at Washington State University lab within 6 mo. following collection. For eDNA filters, we cut each filter in half, used half the filter for DNA extraction, and stored the other half in 95% ethanol as a reserved. We used a QIAshredder/Qiagen DNeasy Blood and Tissue DNA extraction protocol (Goldberg et al., 2011) in a limited-access room using best practices for eDNA (Goldberg et al., 2016). For frog swabs, we extracted DNA from the entire swab using Qiagen DNeasy Blood and Tissue DNA extraction protocol without QIAshredder in a tissue lab. We analyzed the samples using previously published Bd qPCR assay (Boyle et al., 2004), with the substitution of Environmental Master Mix (ThermoFisher, Waltham, MA), 3 µl of DNA extract in triplicate reactions, and running for 50 cycles, on a BioRad quantitative PCR (qPCR) machine (BioRad Laboratories, Hercules, CA). To quantify initial DNA copy number of Bd in the eDNA and swab samples, we created a standard curve by using a four-point serial dilution (10-10,000 copies) of a synthesized gene (gBlocks; Integrated DNA) in duplicate on each plate. We can detect quantities of DNA outside the range set by the standard curve, but exact quantities cannot be determined if they are outside the set range. All wells included an exogenous positive control to ensure no qPCR inhibition had occurred (IPC; ThermoFisher). We created and analyzed negative extraction and qPCR controls with every extraction batch and plate.

Filed Protocol for DNA Analysis

We used reagents, assay, handheld qPCR and protocols from Biomeme Inc. (Biomeme Inc, Philadelphia PA). We extracted all samples in the field within 10 min of collection using M1 Sample Prep Kit and protocol for eDNA or DNA respectively and we extracted DNA from the entire swab or filter. We analyzed the samples using Go-Strips, custom shelf-stable assay developed from previously published Bd qPCR assay (Boyle et al., 2004). The Go-Strips contain primer, probe, master mix, and internal positive control and only require the addition of extracted DNA. The exception to this was at site 1, where we added pre-mixed primer and probe stored on ice to the Go-Strips that contained master mix and internal positive control. We ran our samples on a two3 mobile real-time PCR machine (˜ 2 lbs.). To quantify initial DNA copy number of Bd in the samples, we created a standard curve by using a three-point serial dilution (100-10,000 copies) of a synthesized gene (gBlocks; Integrated DNA) prior to running reactions. We used 20-40 µl of DNA extract in each reaction (20 µl at site 1, and 40 µl at site 2 and 3). The two3 contains three wells and can run two channels. We ran

each sample in triplicate (three technical replicates using all available wells) with an internal positive control (duplex reaction using both channels). As a result, we could not run a standard curves or field negative simultaneously (as described in Sepulveda et al. 2018). To run all skin swab reactions, we used a cycle of 15 min at 95°C followed by 45 cycles at 94°C for 60 s and 60°C for 60 s. To run all eDNA reactions, we used cycles of 15 min at 95°C followed by 50 cycles at 94°C for 60 s and 60°C for 60 s. If inhibition occurred, we diluted samples 1:1 using molecular grade water and re-ran. We diluted inhibited samples in the field using a 20µl pipette. We analyzed the eDNA negative control samples collected on site as the negative control for field PCR.

Data analysis:

We considered Bd to have been detected in a sample if [?]1 PCR technical replicated tested positive for frog skin swabs. For eDNA samples, we considered Bd to have been detected at the site if [?]1 technical replicate was positive in [?]1 eDNA sample collected at the site. We considered a technical replicate to be positive if an exponential increase occurred at any point during the qPCR cycles (as described in Goldberg et al., 2013, see also Ellison et al., 2006).

To model Bd DNA detection probability, we used a multi-scale occupancy models in in R (version 3.6.0; R Project for Statistical Computing, Vienna, Austria) and package eDNAoccupancy (as described in Sepulveda et al., 2018; see also Dorazio & Erickson, 2017). We compared a null model to models fitted with covariates that affected the occurrence of Bd DNA in the sample ($\vartheta\cdot$ sample type [swab vs. eDNA]), and covariates that affected the detection of Bd DNA in the technical replicate or subsample (p; analysis approach [field vs. lab]). We assessed the models using posterior-predictive loss criterion (PPLC) and widely acceptable information criteria (WAIC). We calculated detection probability and their standard errors using a Markov chain containing 11,000 iterations (1,000 burn-in).

Results:

We detected Bd DNA on all Sierra Nevada yellow-legged frogs (n=6) swabbed for comparison testing across all three sites using the field and lab protocols (Table 1 & Fig. 3). Of the anurans with unknown Bd levels, we detected Bd DNA on swabs from 75% of the *R. draytonii*(n=4) as well as the *L. catesbeianu* s (n=1) using the field protocol. We did not detect Bd on one *R. draytonii* swab. Lab analysis of the *R. draytonii and L. catesbeianus* swabs verified the negative and positive results.

We detected Bd DNA in eDNA samples collected at two out of the three sites using both the lab and the field protocols. We did not detect Bd DNA for Site 3 using the field protocol and we did not detect Bd DNA for Site 2 using the lab protocol (Table 1 & Fig. 3).

We were unable to quantify DNA from both the eDNA and swab samples using the field protocol because all DNA levels were below the standard curve (<100 copies). All positive Bd eDNA and swab samples extracted and analyzed using the lab protocol were >100 copies and within the limits of quantification (Table 1).

We detected Bd in more technical replicates using the lab protocol compared to the field protocol (Fig. 3 and Fig. 4). The best fit detection probability model with the lowest PPLC and WAIC included sample type (swab vs eDNA) and analysis approach (field vs. lab) as covariates for the sample (ϑ) and sub-sample (p) respectively. The mean derived estimated of ϑ was higher for swab samples compared eDNA samples, as expected, and the mean derived estimated of $p$was higher for lab protocol compared to the field protocol (Fig. 4). The mean ϑ ($\pm$ 95% credible limits) was 0.47 (0.42-0.59) for the eDNA samples and 0.80 (0.70-0.94) for skin swabs. The mean p ($\pm$ 95% credible limits) was 0.65 (0.49-0.75) for the field protocol and 0.97 (0.90-0.99) for the lab protocol. Approximately 1.5 technical replicates would have to be analyzed using the field protocol in order to have the same mean detection probability of one technical replicate using the lab protocol. Reduced detection probability of the field-based approach is compounded when collected as eDNA samples, where 1.7 technical replicates would need to be analyzed in order to have the same mean detection probability of one technical replicate from a frog swab.

While using the field protocol, one skin swab and one eDNA sample experienced inhibition at Site 3. To

resolve, we removed the inhibition in the skin swab and eDNA sample through two serial dilutions (1:1) with molecular grade water (final dilution: 10 µl DNA and 30 µl H_20). We detected Bd DNA in the skin swab but failed to detect Bd DNA in the eDNA sample. However, we did not detect Bd DNA in any of the uninhibited eDNA samples collected at Site 3 using field analysis techniques. There was no issue with inhibition for all samples extracted and analyzed using the lab protocol.

All the negative control samples, extraction negatives, and qPCR negatives tested negative for both the lab and field protocols.

Discussion:

We detected Bd DNA in all positive frog skin swabs collected across multiple sampling locations and amphibian species using rapid in-the-field methods. Mobile handheld real-time PCR thermocyclers are promising tools for rapidly detecting Bd in susceptible amphibian populations. Previously, to detect Bd in individuals, amphibian skin swabs had to be stored to prevent DNA degradation, transferred out of field, and processed in a lab. The difficulty of collecting and processing swabs in a lab is compounded by species that inhabit remote wilderness or otherwise difficult to access locations. The rapid, in-situ method we applied yielded accurate results for all amphibians swabbed in less than 60 minutes and did not require the transfer of samples out of the field for lab analysis. Rapid detection of Bd is critical to predict and/or minimize epizootic outbreaks (e.g., mass die-off events) and initiating intervention / treatment options such as salvaging animals for captive rearing efforts or on site anti-fungal treatments (Harris et al., 2009). The ability to detect Bd presence in a population in less than 60 min will significantly aid to the recovery of MYLF and other Bd susceptible amphibians where high loads are expected if Bd is present.

We also detected Bd DNA in eDNA samples at the same number of sites using the rapid in-the-field method compared to traditional lab methods. We did not detect Bd DNA for Site 3 using field analysis and we did not detect Bd DNA for Site 2 using lab analysis (Table 1); swabs from both sites tested positive as swabs samples have a higher probability of detecting Bd DNA than eDNA samples (Fig. 4). The false negative result in both the lab and field methods suggest that a more sensitive eDNA surveillance strategy should be used (i.e. increase number and quantity of water samples collected and/or increase number of technical replicates), as generally recommended for eDNA surveys (Goldberg et al., 2016).

We detected Bd DNA in more technical replicates for the samples processed in the lab compared to samples processed in the field, suggesting that our field-based methods may not be as sensitive. Approximately 1.5 technical replicates would have to be analyzed using the field protocol in order to have the same mean detection probability of 1 technical replicate using traditional lab protocol. Our findings are consistent with Sepulveda et al. (2018) who found lower detection of northern pike in eDNA samples and subsamples processed with field protocol compared to traditional lab techniques. As a result, the field extraction approach failed to detect DNA in areas collected with low densities of northern pike (Sepulveda et al., 2018). Additionally, we used a liberal positive criterion where a sample was considered positive if [?] 1 technical replicate detected Bd DNA. A more conservative approach where a sample is considered positive if [?]2 or 3 technical replicates detects the target DNA, as is typically applied to eDNA analysis, would likely increase false negative rates of the field-based protocol. eDNA is typically used as a surveillance tool for rare or elusive species (Rees et al., 2014) or for early detection/ monitoring for invasive species (Jerde et al., 2011; Hunter et al., 2015; Kamoroff et al., 2019); being able to detect trace amount of DNA in a water sample is critical for successful use of eDNA techniques. Prior to the use of rapid field techniques, further assessment should be made to ensure eDNA samples (or other low-quality DNA samples) can be detected at low quantities, and to assess false positive rate at the technical replicate level.

All quantities of DNA detected using rapid in-the-field techniques were below the standard curve, further evidence in the field methods lack sensitivity. Binary detection (i.e. presence/absence) of Bd DNA is an important metric for understanding disease dynamics and host risk. However, DNA quantification of both eDNA and swab samples is critical to the ecological interpretation of the results. Vredenburg et al. (2010) found Bd prevalence increased rapidly and infection intensity increased exponentially with declines of MYLF

evident after average infection intensity of ~10,000 zoospores swab^{-1}. Determining when Bd levels and infection intensities rapid/ exponential grown before lethal threshold levels is critical for management to implement conservation strategies. Such determination can only be accomplished through accurate quantification of Bd load on skin swabs and potentially eDNA samples.

Bd detection for conservation and management projects needs to be reliable as well as able to meet budget and time constraints. Typical costs for lab extraction and analysis of swabs and eDNA samples are ~$10-35 and ~$50-150 respectively depending on type of lab, extraction method, and number of samples processed. Typical qPCR machines used in lab analysis have a 96 well capacity and can multiplex up to five targets per well resulting in a high-volume throughput per run. The M1 sample prep kit for field-based DNA and eDNA extraction cost was $15 per sample and the custom Go-Strips were $10 per well (Biomeme inc, Philadelphia PA.). Total cost of analysis using the Biomeme field methods is $45 for both eDNA and DNA extractions run in triplicate wells. The two3 mobile real-time PCR machine has a three well capacity and can multiplex two targets per well (the target species and an internal positive control). The limited wells inherent to a small handheld qPCR machine will take much longer to run a large number of samples compared to a lab-based machine. As a result, for projects requiring high numbers of samples, lab-based extraction and analysis may be more cost and time efficient.

Conclusion:

We demonstrated that rapid and accurate detection of Bd in frog skin swabs can be accomplished using field extraction and analysis techniques with a mobile real-time PCR device. The methods applied worked for presence/ absence detection data of high-quality (swab) samples. The field-based DNA extraction and qPCR analysis is a promising management tool to aid in the recovery of declining amphibian species. However, there are tradeoff between using the rapid in-the-field methods as traditional lab methods are more sensitive to low quantities of DNA (Sepulveda et al., 2018). Currently, our methods could not be used for accurate DNA quantification and should be used cautiously when detecting low quality DNA samples (i.e. eDNA) especially when the target specimen is present a low densities. Additionally, scope of operation should be considered as it may be more cost and time efficient to run high volumes of samples using lab-based approaches.

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Author Contributions:

C.K. led the writing of manuscript, collected DNA and eDNA aquatic samples, and analyzed samples in the field. C.G. helped develop the field-based protocol, oversaw the processing and analysis of DNA and eDNA samples at WSU eDNA lab, and contributed to manuscript writing. R. G. contributed to writing of the manuscript, overseeing field work, as well as obtaining funding and resources for project. All authors contributed critically to the drafts and gave final approval for publication.

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Yosemite National Park (2019) Unpublished data

Table 1. Sample collection meta-data and results across all 3 sites and sample types: control sample (Blank), environmental DNA sample (eDNA), and frog swab (Swab). "# of Samples Collected" refers to number of frogs swabbed, eDNA samples collected, and eDNA blanks collected for PCR analysis of *Batrachochytrium dendrobatidis (Bd)*DNA. *Bd* DNA detection and quantification results for field and lab methods are indicated by "Field:" or "Lab:" respectively. Mean*Bd* DNA quantification is the average number of *Bd* DNA copies found across all samples and technical replicates with standard deviation (SD) across all samples. – indicates not applicable for respected cell type. DNA below the level of the standard curve is marked by "<100".

Site	Sample Type	Total filtered	# Samples Collected	Field: DNA detected	Lab: DNA detected	
Site 1	eDNA	2380 ml	4	Yes	Yes	
Site 2	eDNA	3150 ml	3	Yes	No	
Site 3	eDNA	1500 ml	3	No	Yes	
Site 1	Swab	-	2	Yes	Yes	
Site 2	Swab	-	1	Yes	Yes	
Site 3	Swab	-	3	Yes	Yes	
Site 1	Blank	250	1	No	No	
Site 2	Blank	200	1	No	No	
Site 3	Blank	250	1	No	No	

* Note that lab quantities listed have been scaled up to represent total quantities in the sample because only a fraction of the DNA extracted from each sample is quantified during PCR analysis.

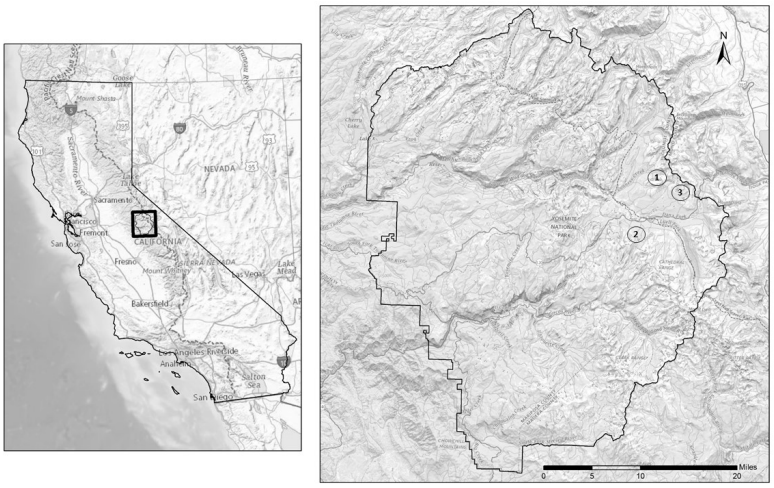

Figure 1. Left) Yosemite National Park insert within the State of California. Right) Sites visited across Yosemite National Park. Site 1, Site 2, Site 3 are mapped at 1, 2, and 3 respectively. Sites are general location as to protect sensitive Sierra Nevada yellow-legged frog populations.

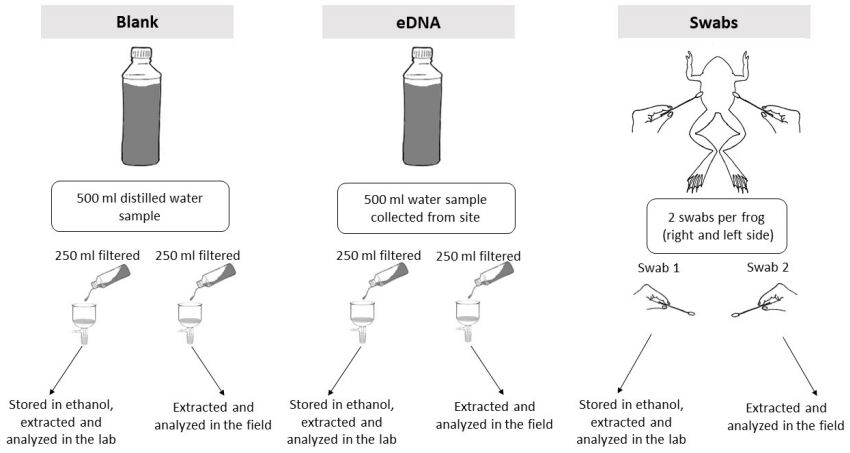

Figure 2. Experimental design for lab and field comparison testing of environmental DNA (eDNA), frog swab, and blank samples collected at three sites across Yosemite National Park and analyzed for the presence of *Batrachochytrium dendrobatidis* (Bd) DNA.

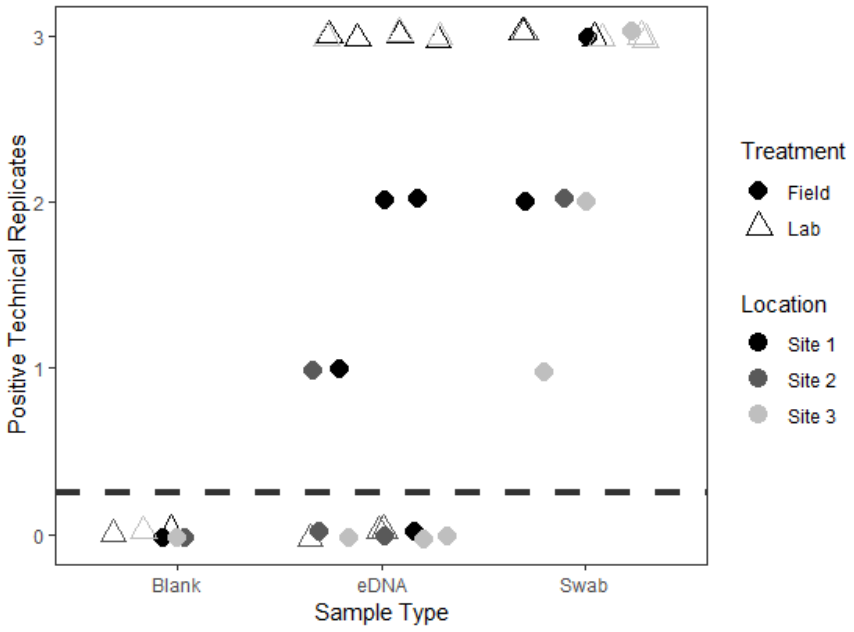

Figure 3. Number of technical replicates that detected*Batrachochytrium dendrobatidis* (Bd) DNA for each sample across all three sites and sample types: control sample (Blank), environmental DNA sample (eDNA), and frog swab (Swab). We detected Bd DNA in at [?]1 technical replicate for icons above dashed line. We did not detect Bd DNA in any technical replicate for icons below the dashed line.

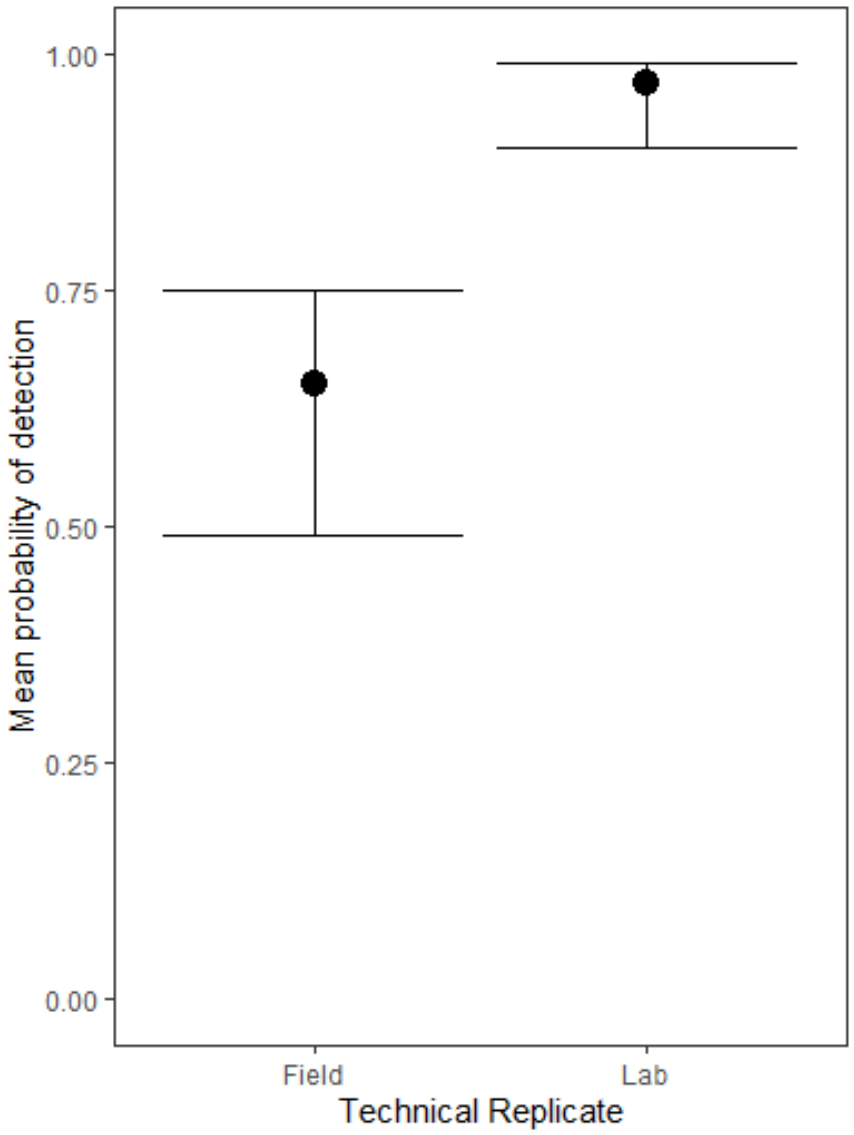

Figure 4. Left) mean conditional detection probability (±95% C.I.) of *Batrachochytrium dendrobatidis* (Bd) DNA occurrence in eDNA or swab samples. Right) mean conditional detection probability (±95% C.I.) of Bd detection in technical replicate (sub-sample) using the field protocol (Field) and lab protocol (Lab).