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ENVIRONMENTAL DNA (EDNA) SAMPLING FOR AMPHIBIAN PATHOGENS

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Introduction to Environmental DNA Sampling:

The isolation and identification of DNA from environmental samples—environmental DNA (eDNA)—is a valuable tool for characterizing biological communities. The detection of eDNA is an effective way to survey for a wide variety of taxa, ranging in size from viruses to large megafauna. Some biologists make a distinction between surveys targeting microbial organisms, where an environmental sample contains the entire living organism, and those targeting DNA that is shed into the environment by larger organisms, with the latter being classified as true "environmental DNA". We acknowledge the differences between these classifications, but view them as opposite ends of a continuum of environmental DNA studies. Somewhere along this continuum lies environmental sampling for amphibian pathogens. While many amphibian studies use molecular techniques for the detection of pathogens, most of the techniques are optimized for DNA extraction from swabs or tissue samples collected directly from the host. Here, we describe the detection of amphibian pathogens directly from the environment, focusing on two of the most frequently studied pathogens: amphibian chytrid fungi (*Batrachochytrium* spp.) and ranaviruses (*Ranavirus* spp.).

Environmental DNA Sampling for Chytrid Fungi:

A variety of protocols for the collection, isolation, and detection of *Batrachochytrium dendrobatidis* (*Bd*) DNA from environmental samples have been tested and published. To the best of our knowledge, no such studies have been published for *Batrachochytrium salamandrivorans* (*Bs; Bsal*). We suspect that many of the protocols described below will apply to *Bsal*, but additional research is needed. What follows is a description of the current state of the field and a brief review of techniques and strategies used for detecting amphibian pathogens using eDNA sampling. Please consult individual references for additional detail.

Before scientists attempted to detect *Bd* fungi in eDNA samples, they developed PCR and quantitative PCR (qPCR) protocols for detecting *Bd* in skin swabs and tissue samples. Annis *et al.* (2004) described the first PCR assay for the detection of *Bd.* Martel *et al.* (2013) described the first PCR assay for the detection of *Bsal*. Boyle *et al.* (2004) described a quantitative PCR (qPCR) assay for the detection of *Bd*, and Hyatt *et al.* (2007) further elaborated on its appropriate implementation. As the name suggests, qPCR has an advantage over traditional PCR in that

concentrations of target DNA are more easily quantified. Additionally, qPCR assays have the potential to be more sensitive than traditional PCR, especially with the inclusion of species-specific (e.g., TaqMan®) probes. Kirshtein *et al*. (2007) and Walker *et al.* (2007) were the first to use a qPCR assay for the amplification of *Bd* DNA from environmental samples, each showing that *Bd* could successfully be amplified from water samples. Negative or inconclusive results were found for sediment, however. Kirshtein *et al.* (2007) compared a reformulation of the qPCR assay of Boyle *et al.* (2004) using SYBR Green reagents against their new TaqMan® assay. They found that the two performed similarly on mock eDNA samples created in the laboratory, but that the Boyle *et al.* (2004) SYBR assay detected *Bd* in more field samples. The latter also showed more non-specific amplification late in the qPCR. Later studies have modified and implemented the assays developed by Boyle *et al.* (2004; i.e., Walker *et al.* 2007; Hyman and Collins 2012; Kolby *et al.* 2015b; Kolby *et al.* 2015c) and Kirshtein *et al.* (2007; i.e., Schmidt *et al.* 2013; Chestnut *et al.* 2014; Kolby *et al.* 2015a).

Several collection protocols have been used in studies attempting to detect *Bd* from environmental samples. The most common method used is the isolation of *Bd* eDNA from water, including rainwater (Kolby *et al.* 2015a). After water is collected, it is filtered through a 0.2 – 0.45 μm capsule or membrane filter with a syringe or hand-, electric-, or battery-powered vacuum pump (Kirshtein *et al.* 2007; Hyman and Collins 2012; Chestnut *et al.* 2014; Kolby *et al.* 2015a,b,c). Notably, Kirshtein *et al.* (2007) unsuccessfully attempted to collect *Bd* eDNA from aquatic sediment samples, but Kolby *et al.* (2015c) swabbed leaves where amphibians had been sitting and detected *Bd* eDNA. Furthermore, Freeman *et al*. (2009) isolated and identified eDNA from free-living and non-pathogenic species of chytrid fungi. Their techniques could potentially be useful in extracting *Bd* eDNA from soil in amphibian habitats, but consideration should be given to the ecological differences between these fungi and pathogenic chytrid fungi.

Methods for preservation and storage of *Bd* eDNA samples include short term storage on ice or dry ice (Kirshtein *et al.* 2007; Hyman and Collins 2012; Chestnut *et al.* 2014), long term storage at freezing or refrigerated temperatures (Freeman et al. 2009; Hyman and Collins 2012; Chestnut *et al.* 2014; Kolby *et al.* 2015a,), addition of cell lysis buffer and/or phosphate buffered saline (Hyman and Collins 2012; Chestnut *et al.* 2014; Kolby *et al.* 2015a, 2015c), storage in ethanol, and desiccation (Kolby *et al.* 2015b).

All studies seeking to detect *Bd* in environmental samples have used modified protocols from commercial DNA extraction kits (e.g., Qiagen DNeasy; Goldberg *et al.* 2011). These kits are advantageous in that many reagents and supplies (e.g., spin-columns) are individually wrapped and sterilized, reducing the risk of contamination. However, it is worth noting that alternative protocols have been used in other eDNA systems and have distinct advantages worth exploring (e.g., Renshaw *et al.* 2015).

Environmental DNA Sampling for Ranaviruses:

Compared to *Bd*, relatively few studies have attempted to detect ranavirus DNA from environmental samples. Miller *et al.* (2015) described traditional diagnostic tests for detecting ranavirus in amphibians. Brunner and Collins (2009), Robert *et al.* (2011), and many others (see references cited in Brunner *et al*. 2015) showed that amphibians can be infected by ranaviruses shed into the water by other infected individuals, suggesting that the detection of ranaviruses by environmental DNA methods might be possible. Nazir *et al.* (2012) and others (e.g., Munro *et al.* 2016) also demonstrated that ranaviruses can persist in the environment.

Picco *et al.* (2007) described the first qPCR assay designed for the detection of ranaviruses in amphibians using a TaqMan® assay. Johnson and Brunner (2014) used the same qPCR assay on pseudo-environmental samples (i.e., water samples with spiked ranavirus) to detect ranavirus. They collected 10 mL of water from experimental microcosms, filtered the sample through Millex-GV PVDF 0.22 μm vacuum filters, stored filters in phosphate-buffered saline without magnesium or calcium, and extracted DNA following Kirshtein *et al.* (2007) and Hyman and Collins (2012). Ranavirus eDNA was detected in samples using the qPCR protocol of Picco *et al.* (2007).

Monsen-Collar *et al*. (2013) collected asymptomatic *Bufo fowleri* tadpoles from a population with a documented ranavirus outbreak, allowed those tadpoles to swim in small volumes (100 mL) of sterile water for 30 seconds, and attempted to detect ranavirus eDNA from those water samples. Water samples were frozen and DNA extracted using a Qiagen QIAamp DNA Mini Kit. The authors used PCR and qPCR with primers designed by Mao *et al.* (1997); ranavirus was not detected in PCR assays but was detected in some qPCR assays, perhaps due to low virus levels in the water.

Kolby *et al.* (2015b) used a modified version of the Picco *et al.* (2007) qPCR assay to detect ranavirus DNA in water collected from captive amphibian enclosures and natural water bodies. They detected ranavirus in some samples from captive amphibian enclosures, but not from natural water bodies.

Hall *et al.* (2016) were successful and detecting and quantifying ranavirus DNA in water samples collected at sites in the wild with ongoing ranavirus outbreaks. They collected 250 mL water samples and filtered them through 0.2 um cellulose nitrate filter paper. Filters were stored in 100% ethanol, extracted using the modified Qiagen DNeasy protocol of Goldberg *et al.* (2011), and amplified using the qPCR protocol of Picco *et al.* (2007). They found a strong correlation between ranavirus DNA copies detected in water and the outbreak status in *Rana sylvatica* populations.

Quality Control and Statistical Analysis

Like all detection methods, the interpretation of results from eDNA techniques must account for false negatives and false positives. The following section describes general practices for preventing and recognizing these potentially confounding factors and, more broadly, for placing eDNA results in a statistical framework.

Other compounds are sometimes co-extracted from eDNA samples, and some of these compounds can inhibit PCR or qPCR. Because this inhibition can cause false negatives, it is important to attempt to remove inhibitors in the lab and account for inhibition in statistical analyses. A useful method for detecting inhibition is the inclusion of an 'internal positive control' in an assay. In short, this is the addition of extraneous DNA and appropriate primers (and probe in qPCR) to amplify the DNA. If this DNA does not amplify (or if the amplification is delayed in qPCR), inhibition can be inferred.

Additional measures can be taken to prevent or reduce PCR and qPCR inhibition, such as extraction of DNA using kits designed to remove inhibitors, additional clean-up steps to remove inhibitors, dilution of samples, and the addition of bovine serum albumin (BSA) to PCR reactions. Garland *et al.* (2010) described a method for reducing inhibition in the qPCR amplification of *Bd* DNA, although it was tested on DNA derived from swabs, not environmental

samples. McKee *et al.* (2015) tested and reported upon the effectiveness of several methods for removing inhibitors from metazoan eDNA samples.

Throughout the process of collecting, processing, and analyzing eDNA samples, care must also be taken to reduce the risk of false positives. Because these processes involve the replication of target DNA from very low concentrations, they are especially sensitive to contamination. Thus, the use of typical measures for sterilizing and decontaminating equipment (e.g., wearing disposable gloves and bleaching equipment) is of the utmost importance. Most researchers do this work in a separate laboratory or laboratory space dedicated to low-concentration DNA work, where no post-PCR samples or reagents are allowed. Other practices designed to reduce the risk of contamination (e.g., the use of filter tips for pipetting) are also critical. To guard against unexpected contamination, it is necessary to include negative controls throughout the experiment, ranging from 'blank' samples collected in the field to extraction and PCR controls.

Appropriate statistical analysis of these data is dependent upon the goals and methods used in each experiment. For some examples of how detection probability and occupancy have been modeled in sampling for *Bd* eDNA, see Schmidt *et al.* (2013) and Chestnut *et al.* (2014).

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