Determining The Level of Detectability Using Environmental DNA (eDNA) Sampling and Visual Survey Methods to Inventory Amphibians: Improving Amphibian Conservation Efforts on The Bridger-Teton National Forest

Summary of project implementation and findings on the Bridger-Teton National Forest

Prepared by Ashley Egan

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Introduction

Amphibians are highly sensitive and vulnerable to environmental changes, and are proven to be excellent indicators of environmental quality. Their permeable skin makes them susceptible to water and air quality, and disease; and their tendency to occupy small isolated areas greatly increases their susceptibility to habitat loss and degradation. With changing environmental factors, the need for amphibian conservation efforts has become a greater concern.

Current amphibian occupancy techniques on the Bridger-Teton National Forest (BTNF) involves Visual Encounter Surveys (VES) as a means to inventory for sensitive amphibians, whose low abundancy and low detection rates make detecting the presence of amphibians difficult, time consuming, and costly. The BTNF obtained support from the Meg and Bert Raynes Wildlife Fund in 2015 to implement an environmental DNA (eDNA) amphibian sampling effort and evaluate its efficiency and effectiveness.

Project Description

The goals of this project was to evaluate the overall effectiveness of amphibian inventory efforts on the Bridger-Teton National Forest by comparing the results from the eDNA sampling technique to the results from visual inventory techniques. The general efficiency and effectiveness of the eDNA technique and process was assessed based on the techniques ability to successfully identify amphibian presence, and the techniques time and cost effectiveness. The pros and cons from the eDNA sampling technique and visual survey methods were assessed and discussed further in this project completion report.

Aquatic species, such as amphibians, leave DNA in the water from urine, feces, and dead skin cells. By filtering water samples that have been collected at various wetland locations, laboratory technicians can then extract any DNA that is present and use species specific DNA markers to positively detect if a species of interest has been in that water body, a process called environmental DNA or eDNA.

For inventory purposes, successful eDNA analysis could potentially eliminate the need to perform visual survey methods. If successful, the eDNA method would increase certainty of species identification and reduce the likelihood of overlooking species, a common factor with visual surveys. Additionally, successful eDNA analysis would decrease field survey time and to some degree field costs for surveyors and survey equipment.

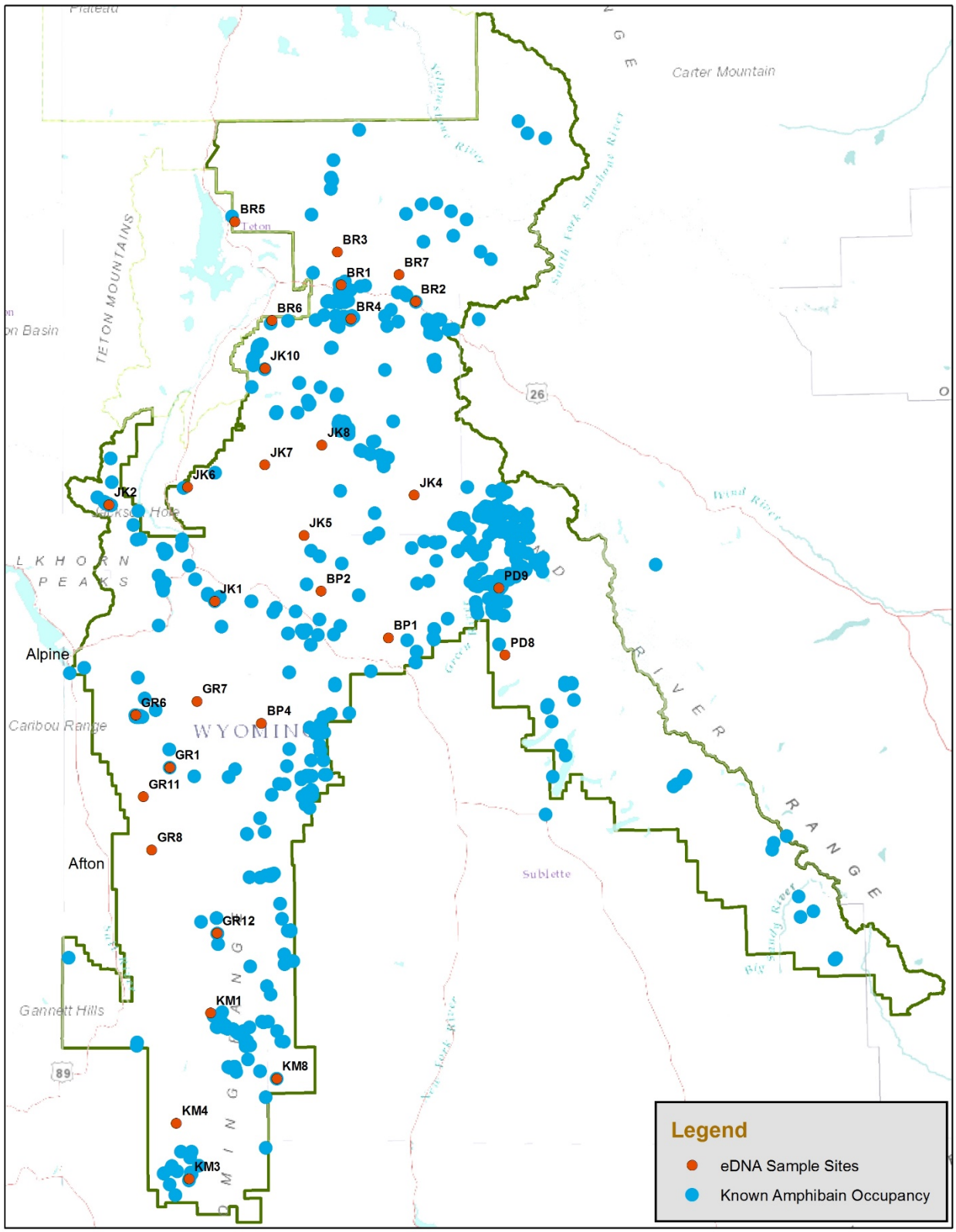
Project Methods

eDNA sampling was implemented on the BTNF from July to October of 2015. A total of 30 individual wetland sites were surveyed using the eDNA method. In order to increase the accuracy and probability of amphibian DNA detection, 3 water samples were collected at each of the 30 wetland sites. Unfortunately one sample was lost in the field, therefore a total of 89 water samples were collected for eDNA analysis on the Bridger-Teton National Forest.

Fewer eDNA water samples were collected than originally planned in the project proposal. This was foreseeable given this was the first year implementing such a large-scale project across the Forest and there were a lot of logistics, planning, and modified priories involved. For example, some sites selected for sampling where not completed due to limited accessibility, some sites no longer had water present once they were visited, and some sites were just not feasible given their location and the time that was allotted for the project work.

Of the 30 sample site locations, 20 sites had unknown amphibian occupancy, and 10 sample sites were collected in locations where amphibian occupancy was known (Map1). Occupancy was known in areas because visual surveys had been implemented on the Forest prior to the 2015 eDNA project. Using areal imagery and National Wetland Inventory (NWI) datasets, all eDNA sample sites were located where amphibian habitat was likely present. Additionally, the water samples themselves were collected in areas where optimal amphibian habitat characteristics where present. For example, water samples were collected on the edge of a site where cover was available, and slow moving, shallow water was present. The intention was to increase amphibian detectability to the extent possible, since the objective was to compare the ability for eDNA and Visual Encounter Survey techniques to detect amphibians. The objective was not to simply assess what sites have amphibians and what sites do not. Visual methods were conducted in the same fashion—technicians were trained to conduct surveys within optimal amphibian habitat within a site.

Following water sample collection, samples were filtered using the "eDNA Sample Collection and Filtration Protocol" (Egan 2016 unpublished) within 24 hours from the time the sample was collected. Samples where then stored in a cool, dark location until the sample collection and filtration process was complete for all 89 water samples. Amphibian eDNA samples were then sent to Wyoming Natural Diversity Database (WYNDD) Cooperative Laboratory for DNA extraction and analysis, during the winter of 2016.



Map 1: eDNA Sample Locations and Known Amphibian Occupancy on the Bridger-Teton National Forest.

Results

2015 eDNA analysis results from WYNDD were received in May of 2017. Laboratory technicians tested for the presence of Boreal chorus frogs (*Pseudacris maculata*), Barred tiger salamanders (*Ambystoma mavortium*), Boreal toads (*Anaxyrus boreas*), Northern leopard frogs (*Lithobates pipiens*l), Wood frogs (*Lithobates sylvaticus*), Columbia spotted frogs (*Rana luteiventris*), and Chytrid fungus (*Batrachochytrium dendrobatidis*). Wood frogs and Leopard frogs are not known to occur in the project area. However, given those species are already integrated into the laboratories current molecular tests, they were included in the analysis. Chytrid fungus was included in the analysis given the ability to detect the presence of the fungus and the important role the fungus has on amphibian mortality.

Overall, eDNA detection rates for all species was low on the BTNF (Table1). Wood frogs and Leopard frogs where not detected, as to be expected, given the BTNF is outside their home range. Chorus frogs and Spotted frogs were found to be positive in single replicates. Although, when those samples where reanalyzed, none of the positives were confirmed. Therefore based on the laboratories criteria, those samples were classified as negative. There were no positive detections for Boreal toads for any of the samples. Tiger salamanders and Chytrid fungus (Bd) are the only species found present in any of the samples based on the laboratories criteria. Tiger salamanders where detected at 4 of the 30 locations on the BTNF, within 7 of the 89 individual samples. Chytrid fungus was detected at 3 of the 30 locations on the BTNF, within 3 individual samples.

Of the 4 locations in which Tiger salamanders where detected with eDNA sampling, none of the locations had detections with the visual method. Conversely, the 1 location that did have a visual salamander detection in 2015, was not picked up with the eDNA method (Table 1). While no other sites resulted in positive eDNA amphibian detections, the visual method detected Chorus frogs at 4 sites, Columbia spotted frogs at 3 sites, and Boreal toad and Tiger salamander was observed at 1 site. In summary (excluding Bd since that is not detectable via visual methods), 9 (7.5%) amphibian detections where made using the visual survey method, compared to 4 (3.3%) amphibian detections using the eDNA sampling method, and no detections overlapped between sampling method. Additionally, visual surveys detected 4 individual amphibian species—all species known to occur in the project area, while eDNA only detected 1 species, aside from Bd. The results suggest that the visual method was more effective at detecting amphibian presence than the eDNA method. While the visual method missed species that the eDNA method positively detected, the visual method detected more species, at more locations across the BTNF, than eDNA. Although it is not entirely consistent, the results suggest that the visual method had a higher rate of detectability than eDNA sampling on the BTNF in 2015.

Given the rates of detection were low compared to the laboratories research and others using identical protocols and analysis, WYNDD re-extracted DNA from a subset of samples and reanalyzed them to rule out laboratory error. The second analyses contained samples from sites with both known and unknown amphibian presence. According to the laboratory summary, the results mirrored those from the first analyses, indicating DNA concentrations where low and that negative detections where in fact the case.

In order to further assess how successful eDNA and visual methods are, they were not only compared against each other, as the results above indicate, but also against what was already known about the site from previous surveys. The type of species detected during the 2015 project was compared against the species that were previously known to occupy that site.

Of the 30 eDNA sample sites, 10 sites were located in areas where amphibian species were present prior to project implementation. The eDNA method detected at least one species previously known to occur at 3 of the 10 occupied sites, while visual methods detected at least one previously known species at 5 of the 10 occupied sites. However, both the eDNA and visual survey methods missed species at all of the 10 sites where they were previously known to occur. Although, the visual method missed less species, generally. In summary, where species were already known to occur, the visual survey method was more successful than the eDNA method at detecting those species again during the 2015 project implementation.

Of the 20 sites where amphibian occupancy was unknown, the eDNA method positively detected an amphibian species at 1 site, while visual survey methods detected an amphibian species at 3 sites. 16 of the 20 sites had no detections regardless of method, and 2 of those 16 where consistent with previous surveys in that no detections have ever been made. This suggested that the visual survey method was more successful at detecting amphibians at new sites. However, whether or not amphibian presence was previously known or unknown, amphibian detections were low, regardless of method.

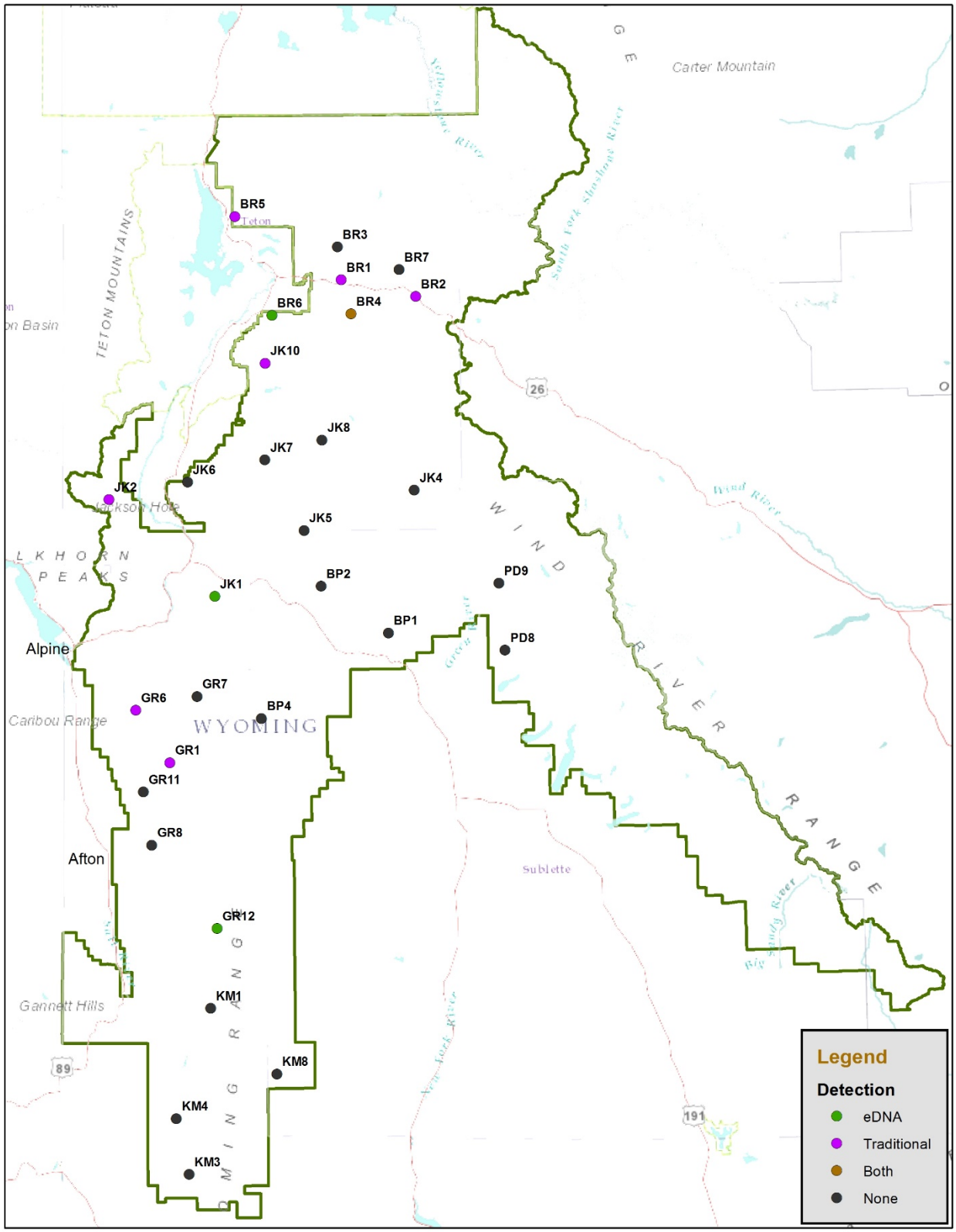
Amphibian detections from both the eDNA and visual survey methods were made across all districts on the BTNF except the Big Piney and Pinedale Ranger Districts. Visual surveys were implemented by a variety of surveyors depending on location, possibly contributing to inconsistent results due to varying identification skills and knack for surveying amphibians. Other data collected included air and water temperature, pH, vegetation characteristics, fish occupancy, and waterbody and substrate type. All detections made from visual methods overlapped with a wide range of the site characteristics. However, all eDNA detections had a common characteristic in that that the sample was collected within some form of pond complex where enclosed, slow moving water was the objective eDNA sample location. At the time, this was thought to increase the probability of detection because the site would "hold" more DNA than a flowing water source.

Summary

While amphibian detectability as a result of both the visual and eDNA survey methods were generally low, the visual survey method was more successful at detecting amphibian species on the Bridger-Teton National Forest. The eDNA method did result in positive detections, thereby proving to be successful, and the visual survey method missed species that eDNA positively detected. However, the visual survey method detected more amphibian species, and it detected amphibian species at more locations across the Forest. This was the case when comparing both methods against each other from the 2015 effort, as well as when comparing the two methods independently against survey results from previous data collected.

District	eDNA Station	Drainage	Chorus Frog		Tiger Salamander		Wood Forg		Columbia Spotted Frog		Northern Leaopard Frog		Borea Toad		Chytrid Fingus	Known Occupancy
			Traditional	eDNA	Traditional	eDNA	Traditional	eDNA	Traditional	eDNA	Traditional	eDNA	Traditional	eDNA	eDNA	
Jackson	JK1	Willow Creek	-	-	-	YES	-	-	-	-	-	-	-	-	-	Yes
	JK2	Mosquito Creek	YES	-	-	-	-	-	-	-	-	-	-	-	-	Yes
	JK4	Grose Ventre Ponds	-	-	-	-	-	-	-	-	-	-	-	-	-	
	JK5	Strawberry Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	JK6	Cache Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	JK7	Flat Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	JK8	Crystal Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	JK10	S Fork Ditch Creek	YES	-	-	-	-	-	-	-	-	-	-	-	YES	Yes
Blackrock	BR1	Buffalo Fork	-	-	-	-	-	-	-	-	-	-	YES	-	-	Yes
	BR2	Blackrock Meadows	-	-	-	-	-	-	YES	-	-	-	-	-	-	Yes
	BR3	Lava Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	BR4	Lilly Lake Ponds	-	-	-	YES	-	-	YES	-	-	-	-	-	-	Yes
	BR5	Bailey Creek	YES	-	-	-	-	-	-	-	-	-	-	-	-	Yes
	BR6	Dry Lake Wetlands	-	-	-	YES	-	-	-	-	-	-	-	-	-	
	BR7	South Buffalo Fork	-	-	-	-	-	-	-	-	-	-	-	-	-	
Greys River	GR1	Pearson Creek	-	-	-	-	-	-	YES	-	-	-	-	-	-	Yes
	GR6	Murphy Lakes	YES	-	YES	-	-	-	-	-	-	-	-	-	-	Yes
	GR7	Blind Bull Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	GR8	Willow Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	GR11	Strawberry Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	
	GR12	Greys River/Box Canyon	-	-	-	YES	-	-	-	-	-	-	-	-	YES	Yes
Kemmerer	KM1	LaBarge Meadows	-	-	-	-	-	-	-	-	-	-	-	-	-	No
	KM3	Hams Fork	-	-	-	-	-	-	-	-	-	-	-	-	-	Yes
	KM4	West Fork Hams Fork	-	-	-	-	-	-	-	-	-	-	-	-	YES	
	KM8	Big fall Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	Yes
Pinedale	PD8	Lower Boulder Basin	-	-	-	-	-	-	-	-	-	-	-	-	-	
	PD9	Gypsum Creek	-	-	-	-	-	-	-	-	-	-	-	-	-	Yes
Big Piney	BP1	Slide Lake	-	-	-	-	-	-	-	-	-	-	-	-	-	
	BP2	Shoal Creek Trib	-	-	-	-	-	-	-	-	-	-	-	-	-	
	BP4	Hoback River	-	-	-	-	-	-	-	-	-	-	-	-	-	No

Table 1. eDNA Project Site Summary on the Bridger-Teton National Forest



Map 2: eDNA Sample Locations and Survey Detection Results by Location, on the Bridger-Teton National Forest

Discussion

Results were reviewed in order to 1) determine the ability for eDNA sampling to positively identify amphibian species at 30 wetland locations across the Bridger-Teton National Forest and 2) compare the eDNA sampling technique to visual survey methods, and assess the strengths and weaknesses of each respective technique.

eDNA Effectiveness

As a result from this effort, amphibian detectability using eDNA sampling was low. This was particularly interesting where the eDNA sample was collected in a site that also had a visual amphibian detection. However, there are many attributes that could affect the ability for the laboratory analysis to detect amphibian DNA in a water sample. Some having to do with the site itself, the method, and simply DNA presence or absence. Below we discuss the potential causes:

(1) Laboratory factors can reduce the amount of DNA detected and an error in the DNA extraction process is possible, but the re-extraction and testing as described in the results section indicates this is not likely the case for this project. Additionally, the positive controls showed a very low failure rate, indicating the labs analysis design was functional.

(2) Species presence could result in low DNA concentration within the water, and therefore the sample. If amphibian distribution in the project area is low, there is a large amount of unoccupied wetland habitat, and/or amphibian densities at occupied sites are low, then eDNA concentrations in the water is likely low. While this may not explain the case for the eDNA samples that were collected near an amphibian, there are other factors that may influence the detectability of DNA. We know that there are many areas throughout the BTNF where amphibian occupancy is low, distribution is patchy, and amphibians are not typically observed in high densities. This suggests that environmental factors specific to the BTNF likely influenced the effectiveness of eDNA sampling to some degree.

(3) Water quality is a large factor in eDNA detection. For example if a survey site is particularly saline, acidic, warm and/or exposed to excessive ultraviolet radiation, any eDNA in the water will tend to degrade far faster and will likely be more difficult to detect (Jane et al. 2015; Strickler et al. 2015). While we have the ability to minimize some factors factors, they are likely inevitable and could have impacted amphibian eDNA sampling in 2015.

(4) The water filtration process could also impact DNA detection rates. If too little water is filtered, there may not be enough DNA adhering to the filter, especially as DNA breaks down into smaller particles (Turner et al. 2014). The goal for this project was to filter the recommended rate of 1,000 ml of water per sample. However, with increased sediment, plant, or soil particles in the water sample, the filtering process became difficult and often time the filter clogged prior to reaching the recommended amount. While this was avoided to the extent practical, murky water with natural sediments is prevalent throughout the project area and the recommended filtered amount was often not achieved for many

samples collected. This can be mitigated through a pre-filtration process where larger particles are filtered through a cheese cloth prior to the final filtration. This mitigation was not utilized at the time and in fact, the problem we found on the BTNF is that most of the clogged filters were from large quantities of small, fine sediments, and pre-filtering may not be sufficient. Another solution could be using multiple filters per water sample. In other words use as many filters as it takes until a sufficient amount of water is filtered. The caveat is the increase in cost due to additional filters and laboratory DNA extractions and analysis.

(5) How the sample is stored between the time of sample collection and the time the DNA extraction is complete can impact detection rates. To avoid DNA degradation to the extent possible, it is recommended that water samples are kept cool in the field (i.e. avoid sitting in the sun, hot vehicles, etc.), water should be filtered within 24 hours of collection, filters should be immediately transferred to 95-100% ethanol and maintained at room temperature short-term (days – 1-2 weeks), and then stored at or below -20° C long-term. The potential for DNA to degrade could have occurred to any of the samples collected on the BNTF. The samples where not necessarily kept at an ideal temperature during their transfer from the field site to their filtration location, unfortunately this simply wasn't considered. This could easily be mitigated by placing water samples in a cooler with ice packs. Additionally, the length of time the filters where stored at room temperature (short-term) before they were sent to the laboratory for analysis, likely exceeded 2 weeks, particularly for samples collected during the start of project implementation. This storage recommendation was not known at the time.

With modifications and improvements to the sampling method, eDNA remains a valuable tool for detecting the presence of amphibian. While detection rates where low during 2015 project implementation, eDNA was successful to some degree, and even more importantly is the information we gained from the project. The importance of maximizing the amount of water filtered and the prevention of DNA degradation are the two major factors that likely effected the projects effectiveness, and these are just a few of our lessons learned. Tiger salamander and Chytrid fungus were the only DNA detected in this effort. However, can this be contributed to the fact these species are more concentrated and move less across wetlands than the more terrestrial species such as a Chorus frog, Boreal toad, and Spotted frog? Something to consider.

The are many benefits from incorporating eDNA sampling into future inventory efforts on the BTNF. From the lessons learned, and with increasing functionality of eDNA sampling, the method has proven the potential to be fast, reliable, and affordable. As a result form the project, the BTNF was able to recognize the benefits from eDNA sampling as an inventory technique. The BTNF can now asses how to utilize the eDNA sampling method in addition to visual methods and help increase the effectiveness of eDNA sampling. The Forest can work with partners on improving landscape-level conservation efforts and monitoring by sharing the lesson learned, and recommendations for improving eDNA sampling.

<u>Technique Comparison</u>

The visual method used to survey for amphibian species on the BTNF has proven to be more successful than the eDNA method, and while there remains room for improvement, the visual method also lacked in accuracy, efficiency, and cost. We found that while the eDNA sampling technique was lacking in amphibian detectability, the method has many benefits.

(1) There is less error in species identification with the eDNA sampling method. During visual surveys, technicians are trained to identify species accurately. However, there is more likely to be an error with visual/aural detections when compare to positive detections from DNA analysis.

(2) The visual survey method is far less efficient than the eDNA sampling method. This is due to the amount of time spent training technicians, and the large amount of time it takes to survey one waterbody or wetland. With eDNA, multiple waterbodies can be surveyed in the time it takes to survey a single waterbody with a Visual Encounter Survey.

(3) Visual survey methods can be costly when considering the salaries of field technicians and the equipment expenses required to conduct the surveys. The caveat is that eDNA sampling can be costly as well. eDNA sampling equipment can be a significant expense, and laboratory analysis to extract and analyze DNA can be expensive depending on the sample size. With that said, there are many laboratories that are willing to provide researches with eDNA sample kits and work with land managers on funding limitations.

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