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Environmental DNA is an Effective Method to Monitor Species in Various Freshwater Habitats

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ENVIRONMENTAL DNA IS AN EFFECTIVE METHOD TO MONITOR AQUATIC SPECIES IN VARIOUS FRESHWATER HABITATS

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ABSTRACT

ENVIRONMENTAL DNA IS AN EFFECTIVE METHOD

TO MONITOR AQUATIC SPECIES IN VARIOUS FRESHWATER HABITATS

by

Kayleigh Mullen

June 2020

This research investigated the use of DNA shed from individuals into the environment (eDNA) to monitor three amphibian species and two trout species associated with habitat intersected by Interstate-90 in Snoqualmie Pass, Washington. This included a large catchment area within creeks and nearby wetlands historically affected by I-90, including sites where significant habitat improvements had been made. Species-specific primers were used to detect three focal amphibians of varying local abundance and two focal trout species. This study showed successful detection of species across both lentic and lotic systems throughout the study area through efficient multiplexing (detection of multiple species in one reaction) via quantitative Polymerase Chain Reaction. Results from this study, overall, showed that eDNA methods can produce results that reliably reflect target species' presence across a large catchment area in an efficient manner.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Freshwater vertebrate species have demonstrated a higher risk of extinction than their terrestrial counterparts and have shown increased rates of decline and extirpation within the past decade (Collen et al. 2013; Wiens 2016). Recent estimates put North American freshwater fish facing an extinction rate over 800 times greater than the background rate (Burkhead 2012). Globally, amphibians are experiencing an extinction rate over 25,000 times that of the background rate (McCallum 2007), with 50% of all amphibians currently at risk of extinction (Gonzalez-del-Pliego et al. 2019). Leading factors contributing to the decline of freshwater species include anthropogenic habitat modifications that lead to the channelization and draining of waterbodies, altered hydrologic regimes, and increased exposure to pollutants (Richter et al. 1997; Aparicio et al. 2000; Vorosmarty et al. 2010).

Traditional monitoring of such freshwater vertebrate species often presents numerous challenges. The elusive nature of amphibians and fish, their diverse morphology and seasonal variations in habitat use add complexity in the use of conventional methods, such as visual encounter surveys, for both the positive detection and identification of species. Additionally, the environment these species inhabit is often times challenging to access and navigate, creating a difficult setting in which to accurately survey. For these reasons, traditional methods of determining the presence

of freshwater species have been deemed time-consuming, ineffective, selective, destructive, and dependent on expertise (Valentini et al. 2015).

The accelerated speed at which freshwater species are declining adds urgency to finding monitoring tools that can quickly and reliably document presence and movement of species throughout freshwater habitats in order to channel limited funding and research into the areas in which it will have the most far-reaching, positive effects. New technologies may be the answer.

Environmental DNA

Environmental DNA (eDNA) is nuclear or mitochondrial DNA that is released from an organism into the environment, commonly through feces, mucous, gametes, shed skin, hair, and the decomposition of carcasses (Pilliod et al. 2013a). The use of eDNA has rapid increased in the past decade as a sampling method for species across multiple taxa, especially in aquatic habitats (Davy et al. 2015). eDNA is being increasingly employed over traditional survey methods for the detection of low-density, cryptic, and rare species (Biggs et al. 2014). We are seeing this shift from traditional methods to eDNA methods for many reasons. eDNA is non-invasive; little to no disturbance on target species or habitat is necessary when taking water samples for filtration, as water can be removed from the edges of ponds and creeks without entry to the waterbody or personal encounters with species. The sterile nature of eDNA equipment lessens the probability that alien pathogens are transferred between sites, critically important in a

time of spreading *Batrachochytrium dendrobatidis* (agent of amphibian chytridiomycosis)*, Myxobolus cerebralis* (agent of whirling disease in fish) and *Piscine novirhabdovirus* (agent of viral hemorrhagic septicemia in fish). eDNA has the flexibility to either target specific species in a waterbody through precise primer design, or through the use of more universal primers, allow the detection of all species within a certain taxonomic group. Without previous knowledge of their occupancy at a site, tools like this are particularly helpful in the early detection of invasive species (Klymus et al. 2017). Such primers also allow for global biodiversity comparisons and assessments with the same technique. Lastly, eDNA techniques have shown a higher probability of detection of rare aquatic species than traditional surveys (Biggs et al. 2015; Spear et al. 2015; Hunter et al. 2015; Pilliod et al. 2013b; Jane et al. 2015; Wineland et al. 2019) and are becoming more cost-effective as primers are developed and shared, and techniques fine-tuned (Dejean et al. 2011).

Although relatively recent in its conception for use in contemporary eukaryotic occupancy, eDNA as a monitoring technique is already the focus of extensive research, with studies into its persistence (Barnes et al. 2014; Collins et al. 2018), transport (Deiner and Altermatt 2014; Pont et al. 2018), degradation (Barnes and Turner 2015; Goldberg et al. 2018) and production rate (Pilliod et al. 2018) recently published.

In the first study to use eDNA for the detection of vertebrates, Ficetola et al. (2008) tested eDNA methods to identify the presence of American bullfrog (*Lithobates catesbeiana*) in both a controlled environment and in natural ponds of known

occupancy and suspected absence. The results not only highlighted eDNA as a reliable method to determine the presence of a freshwater species in wetlands, but also suggested it may be more practical than traditional methods due to its sensitivity in detecting occupancy even at very low densities, which would require huge traditional sampling efforts.

This pattern of outperformance, in both terms of detection and sampling effort, has since been supported by studies involving silver carp (*Hypophthalmichthys molitrix*) (Jerde et al. 2011), bull trout (*Salvelinus confluentus*) (Wilcox et al. 2013), great crested newt (*Triturus* cristatus) (Rees et al. 2014), smooth newt (*Lissotriton vulgaris*) (Smart et al. 2015), eastern hellbender (*Cryptobrancus alleganiensis)* (Olsen et al. 2012), and European weather loach (*Misgurnus fossilis*) (Sigsgaard et al. 2015). Use of eDNA has also been successful in a variety of aquatic habitats including marine systems (Foote et al. 2012; Thomsen et al. 2012b; Kelly et al. 2014), freshwater lakes and ponds (Hunter et al. 2015; Takahara et al. 2012; Moyer et al. 2014), lotic systems (Thomson et al. 2012; Deiner and Altermatt 2014), ground water (Meleg et al. 2013; Niemiller et al. 2018) and snowpack (Kinoshita et al. 2019).

To successfully detect a species, recent studies on various fish, amphibians, and invertebrates have suggested that eDNA must have been recently shed from an individual. Various studies have failed to detect target eDNA in the water column beyond 21 days of removal of the target species in both controlled environments and natural settings (Dejean et al. 2011; Thomsen et al. 2012a; Matsui et al. 2001; Goldberg

et al. 2013). This is due to the rapid degradation of DNA in the freshwater environment. The degradation of eDNA in water is a result of local enzymes, chemicals, UVB radiation, microbial load, or simply the physical mechanics within the waterbody (Barnes and Turner 2015; Lance et al. 2017). The small window of time eDNA is detected after an organism has left the waterbody makes it effective for monitoring *recent* presence, further expanding its usefulness as a non-invasive, real-time monitoring technique.

Results garnered from eDNA methods can provide robust presence/absence data, giving a reliable estimate for species distribution through repeat sampling at sites of interest. Several studies have also shown a positive correlation between the amounts of eDNA amplified from a water sample and the biomass or density of target species in a controlled environment (Takahara et al. 2012; Thomsen et al. 2012a; Doi et al. 2017; Mizumoto et al. 2017). The use of quantitative polymerase chain reaction (qPCR) furthers this use of eDNA, moving it from a technique to gather presence/absence data, to a way of determining relative abundance of different species at a site (Takahara et al. 2012).

The ecology of eDNA, however, is complex and fluid. eDNA itself is polydisperse, made up of differing sized particles, from different source material (Shogren et al. 2017), all of which will react differently to external stimuli. DNA is released into the environment at different rates, dependent on size of an individual (Pilliod et al. 2014), life stage (Maruyama et al. 2014), natural history (Spear et al. 2015), and behavior (Klymus et al. 2014). Similarly, the rate of degradation is dependent on multiple biotic

and abiotic factors. All of these aspects must be taken into account when using eDNA to estimate abundance of a species, and suggest the need for a complex modelling system and fine scale improvements in technique (Yates et al. 2019).

As a case study, Spear et al. (2015) conducted research on how eDNA may improve detectability of eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). The goals of the study were threefold: to determine the presence and relative abundance of eastern hellbenders across their North Carolina range, determine the influence of within-stream distance on quantification of hellbender eDNA, and examine if eDNA can be used to determine reproductive status in hellbender populations.

Firstly, eDNA proved successful in determining presence or absence of hellbenders within the streams tested. This supports the idea that eDNA is a useful tool in determining this rare amphibian's distribution throughout waterways. eDNA sampling showed a 100% success rate in detecting hellbenders at sites where presence was detected using traditional methods in 2012, and a 71% detection rate at sites where historical or recent presence has been noted. eDNA also suggested hellbender occupancy at nine sites with no previous record of their occurrence.

Secondly, this study provided no evidence that eDNA abundance estimates were dependent in any way on the position of the sample collection site in the stream system. Lastly, results suggest that eDNA may, after further research, be used to assess reproductive status in hellbenders when sampled several times over the course of the

year. Every site sampled showed an increase in eDNA at the beginning of their breeding season, although differing in magnitude. The amount of eDNA in samples filtered rose significantly in the run-up to the breeding season, which authors attributed to the fact that males begin fighting over territory, releasing more DNA into the environment. Interestingly, a lone male in captivity also exhibited higher DNA shedding rates in the run-up to breeding season. The increased level of activity and increased number of young individuals in the system during an amphibian's breeding season suggest this to be an ideal time for large-scale surveys on amphibian populations.

The use of eDNA as a technique for monitoring vertebrates continues to be of growing interest within the scientific community, being both vigorously tested and reliably used. Current research continues to show that eDNA is a reliable method for monitoring the recent presence of target species, even at low abundance. This is an important advancement in methods for conservation. Monitoring species is critically important for baseline inventories and documenting species' responses to environmental change and restoration efforts. The latter is particularly important, given the rate and enormity of anthropogenic change on freshwater systems.

Road Ecology

The effects of roads on wildlife have been studied for decades, leading to an abundance of literature examining their impacts. Habitat alteration and destruction (Jaarsma and Willems 2002; Goosem 2007), restricted movement (Richardson et al.

1997), behavior modification (Leblond et al. 2013; Bonnot et al. 2013), and mortality (Coffin 2007) have all been documented, through a wide array of research. Activities in mining, forestry, and recreation have led to roads cutting through the most remote and wild of areas. Roads have continued to create barriers to movement for species, especially those of low mobility, and negatively altered surrounding habitat through destruction, edge effects, fragmentation, and pollution (Goosem 2001; Coffin 2007; van der Ree et al. 2011; Eigenbrod et al. 2009).

Roads often bisect natural waterways through feats of civil engineering. Species show differing levels of aversion to different structures, such as bridges, box culverts and pipe culverts, which can cause restrictions in movements for many animals that use riparian corridors to move between habitats (Kintsch and Cramer 2011). Such structures can also lead to loss of habitat within a stream, even bottomless box culverts alter light and temperature dynamics of the local streambed habitat (Wild et al. 2010) and homogenize the aquatic environment, resulting in a decline in the diversity of aquatic animals (Rahel 2007). Culverts negatively affect dispersal to upstream reaches by creating impassable routes due to increased water velocity (Belford and Gould 1989), perched drop-offs (Warren and Pardew 1998) and aversion behavior (Kemp et al. 2005; Taylor and Goldingay 2010). Culverts also create sediment build-up (Wellman et al. 2000), which destroys habitat for species that seek shelter within the crevices between rocks in the benthic zone. Long- term studies into the changes in the ecology of fish and amphibians within an area that has undergone such development are necessary to

reveal the impacts of civil engineering, such as interstate highways, on freshwater communities.

Amphibians are more likely to be negatively impacted by roads and their by-products than mammals due to their slow movements, permeable skin, and intimate connection to unpolluted wetlands (Hels and Buchwald 2001; Lodé 2000). Many anuran's life histories involve migration between wetland breeding grounds and terrestrial foraging habitat. Their limited mobility increases exposure time on road surfaces when migrating (Trombulak et al. 2000), increasing their risk of predation, vehicle impact, and desiccation. The permeable skin of amphibians makes them exceptionally sensitive to environmental pollutants and chemicals, which increase near and around roads from vehicle lubricants and parts, the application of pesticides, and the use of de-icing compounds for road maintenance (Dale and Freedman 1982). Faced with complex and abundant issues such as disease, invasive species, pollution, predation, habitat destruction, and climate change (Blaustein et al. 2011), amphibians are in need of as much attention as fish when considering freshwater re-connectivity plans.

Freshwater fish, especially diadromous/potamodromous fish, are increasingly impacted by anthropogenic habitat changes. Culverts negatively affect fish passage (Wofford 2005; Norman et al. 2009; Briggs and Galarowicz 2013) through changes in stream hydrology, water velocity (Mahlum et al. 2014), substrate composition, and increased fragmentation of habitats (MacPherson et al. 2012). Additionally, downstream

scouring below culverts can create deep pools, introducing new microhabitats (Wellman et al. 2000) and altering species composition. Alternately, sediment deposition can decrease habitat for both fish and the invertebrates on which they feed (Muck 2010; Marshalonis and Larson 2018). With these changes in habitats, local fish assemblages can be dramatically altered.

Creeks divided by roads are also subject to decline in water quality from pollutants. One common example is the use of salts as a de-icing agent on road surfaces, often leading to increased salinization of streams in close vicinity (Cañedo-Argüelles et al. 2013). Various species of fish are extremely intolerant to declines in water quality, be it fluctuations in temperature, sediment alterations or chemistry, resulting in a decrease in population size (Hari et al. 2006; Chapman et al. 2014; Schwindt et al. 2014).

On a broader scale, small streams play an important part in many life stages of fish by providing spawning and rearing grounds. Impassable culverts impede fish migration to such habitats, affecting population numbers across entire stream networks (Favaro et al. 2014). Upstream fish communities and species assemblages can be altered through different swimming and leaping abilities of fish (Nislow et al. 2011). Poorly designed culverts can also prevent upstream migrations of populations in the face of climate change and warming water temperatures at lower elevations.

An abundance of literature supports the need for well-designed culverts. This has helped lead to recent developments implementing passage-friendly culverts, as well as the replacement of older culverts when roads are being re-developed.

Snoqualmie Pass

Snoqualmie Pass is located in an east-west corridor bisecting the Cascade Mountain Range in Central Washington. The Okanogan-Wenatchee National Forest surrounds Snoqualmie Pass, with several more areas of public land further afield: Glacier Peak Wilderness Areas to the north, and Mount Rainier National Park to the south (Fig. 1). Development from King County to the west and Kittitas County to the east creates a wildlands-bottleneck in the area, resulting in Snoqualmie Pass being a critical link for ecological connectivity between the north and south Cascades.

As well as being of vital importance for the movement of wildlife along the Cascades, the pass itself is permanently inhabited by many species. The area is a highly biodiverse ecotone between the dry interior of the eastern Cascades and the wet coastal zones to the west (Hansen et al. 1991).

Snoqualmie Pass has also served as a critical transportation corridor through the Cascades, with the first road being laid in the early 1900s. Since then, its use continually increased, with modifications to the road accommodating the increase in traffic. Today Interstate-90 (I-90) runs through Snoqualmie Pass, linking Seattle and other cities on the coast of the Pacific Northwest to the rest of the country.

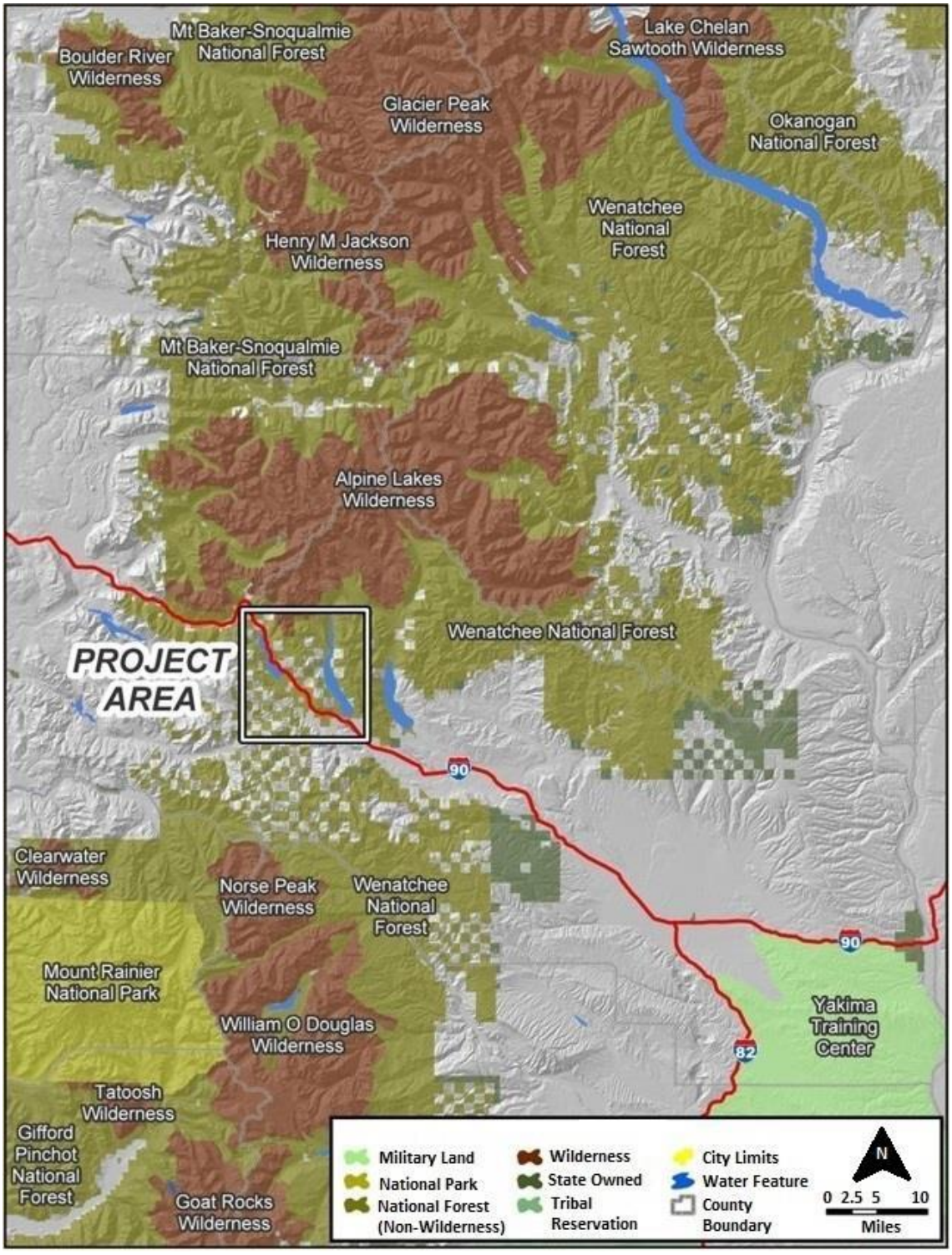

Figure 1: The project area within Washington, highlighting the expanse of public land and wilderness areas both north and south of the project (WSDOT 2006)

In response to an increasing volume of traffic through the corridor, with an expected 41,000 vehicles a day by 2030 (WSDOT 2019), Washington State Department of Transportation (WSDOT) is currently widening a 24-km (15-mile) stretch of I-90 between Hyak and Easton (WSDOT 2016). This stretch crosses 14 tributaries that feed into Keechelus Lake and the Yakima River. Many of these tributaries have been modified through the use of culverts and channelization, with some being filled to such an extent they have lost their hydrological connectivity (WSDOT 2016). Most of the major tributaries in the area have at some point had their channels artificially confined.

In 2008, Washington State Department of Transportation (WSDOT) began a large-scale project on Interstate-90 addressing the anticipated increase in traffic flow through the 24 kilometers of Snoqualmie Pass between Hyak and Easton. The Snoqualmie Pass East Project (SPEP) is focused on increased motorist safety and eased traffic congestion through lane expansion and re-routing the road from the path of avalanches.

To mitigate the negative effects road expansion can have on animal movement, WSDOT included plans to redevelop existing culverts and bridges throughout the project area. These plans include the conversion to widespan bridges, removal of pipe culverts, removal of fish passage barriers, conversion of concrete culverts to bottomless culverts, the addition of wildlife bridges and wetland restoration around the interstate. The variety in crossing structures aims to address the limitations roads impose on the mobility of animals across multiple taxa found in the area, from trout to elk. Upgrading

and retrofitting these structures increases the number of potential crossing sites, not only for charismatic large mammal species, but also for small mammals, amphibians, reptiles, and fish.

The study site spanned several phases of construction during the time of this research. The northwestern-most reaches boast extensive improvements for connectivity. The long-span floodplain bridge over Gold Creek, completed in 2014 (WSDOT), reconnects the stream to its natural floodplain and the expanse of land below the road was widened and naturalized into safe passage for terrestrial animals. Adjacent to Gold Creek, Rocky Run Creek has also undergone improvements under the interstate. A large concrete drop off and narrow pipe culverts were replaced with streambed features, including the addition of deeper pools to improve upstream jumping success. In the mid-sections of the study area, Price Creek and Noble Creek were undergoing heavy development through the widening of culverts and the addition of a wildlife bridge over the interstate. The eastern stretch of the project area was not yet under construction, with relic pipe culverts and poor connectivity between the north and south of the interstate. Having access to both altered and restored creeks allowed us to detect differences in species composition and the return of species upstream of removed barriers.

Focal Species

The region provides important habitat for many Pacific Northwestern amphibian species including the Coastal giant salamande*r (Dicamptodon tenebrous*), western toad *(Anaxyrus boreas)*, Cascades frog (*Rana cascadae*), Pacific treefrog (*Pseudacris regilla*) and coastal tailed frog *(Ascaphus truei)* (WSDOT IES, 2008). In addition, the tributaries to Keechelus Lake are breeding grounds for several fish species of special concern, such as westslope cutthroat trout (O*ncorhynchus clarkii lewisi) and* bull trout (*Salvelinus confluentus)* and the non-native brook trout *(Salvelinus fontinalis*).

This research focused on three of these amphibians (western toad, coastal giant salamander and coastal tailed frog), and two trout species (the native bull trout and the non-native brook trout). The focal amphibian species have been sighted previously within the study site, they have intimate links with the creeks and wetlands in close proximity to I-90, and are all declining throughout the Pacific Northwest (Orchard 1992; Davis and Gregory 2003; Bull and Carter 1996).

Of these five species, particular attention was given to *A. boreas*. This species has the most striking decline in numbers within the study site, a trend mirrored throughout its range (Leonard et al. 1993; Scherer et al. 2005; Deguise and Richardson 2009). This medium-sized toad species exhibits migratory behavior between aquatic and terrestrial habitats, travelling between breeding sites, foraging grounds, and winter hibernacula (Palmeri-Miles 2012). The result of this is an increased sensitivity to development across their range. The usual 300-ft (90-meter) buffer zones put in place around waterbodies to

protect amphibians (Castelle et al. 1992) are often not large enough to accommodate the western toad's movements (Hrubry 2013), with winter hibernacula found over 6000 m from breeding ponds (Bull 2006). Western toads are not thought to use human-altered landscapes for hibernacula (Browne 2010).

Using eDNA to monitor these focal species required sourcing DNA primers that are specific to that species. The chosen sequence must be conserved enough that individuals of the same species carry the sequence, yet polymorphic enough that closely related species can be distinguished (Linacre and Lee 2016). The sequence must be able to amplify from low-quality samples and the same technique must work across all target species. The *cytochrome b* gene, on the mitochondrial genome, exhibits interspecific polymorphisms with few intraspecific polymorphisms (Kocher et al. 1989) and is often used in phylogenetic research. All five of our target species primers were designed to amplify loci from the *cytochrome b* gene. Primers used in this study were designed to target sequences less than 40 base pairs in length to account for the often degraded nature of eDNA.

Aims

Wildlife monitoring is essential for projects such as the Snoqualmie Pass East Project (SPEP) throughout all stages of implementation. Monitoring beforehand obtains baseline data, continued monitoring throughout sheds light into impacts of development, and monitoring post-completion allows researchers to assess the success of methods employed. The extent of connectivity that will be established for habitats each side of this stretch of I-90, for multiple taxa, could set precedent for future mitigation work throughout the United States. For this reason, baseline surveys and future monitoring are of high importance to reveal the effectiveness of work in both the re-colonization of species that were once abundant in the area, and easing the passage of migratory animals through the Cascades.

To understand how species respond to newly created habitats and crossing structures, eDNA methods were used to investigate species distribution and occupancy throughout the SPEP project area. eDNA methods were used to take "snapshots" of target species presence within five major creeks at three points over one year, in one large-scale sweep of 14 creeks, and finally in nine surrounding wetlands for detection of *A. boreas* and potential breeding sites.

This study aimed to test whether eDNA methods can reliably detect aquatic species in both lentic and lotic environments throughout multiple stages of human development and restoration. It served to identify the species in the area, highlighting those whose movements will most likely be affected by development, as well as their spatial and temporal occupancy. Together, this research created baseline data to

compare future monitoring efforts after crossing structures within the project area have been improved. Using eDNA throughout the site and different construction phases also showed the efficiency, ease and cost effectiveness of eDNA during projects, from initial concepts to post-completion monitoring.

CHAPTER II

METHODS

Site Selection

Many of the sampling sites chosen within the project area had either recently undergone restoration work or were due to undergo restoration work, and have historical records of resident amphibians and fish. Gold Creek, Wolfe Creek and Rocky Run Creek, all recently restored, and Price Creek and Noble Creek, which were in in the preliminary stages of redevelopment, were the focal creeks of this study (Fig. 2). Each of these creeks was sampled at multiple points on several occasions over one year.

For each of these five creeks, visual encounter surveys (VES) were conducted during the summer, with at least 10 cumulative hours on each creek. Presence and number of each amphibian study species were noted to establish a solid baseline of amphibian species in the study area. To compare data derived from traditional methods with eDNA results, these creeks were also sampled for eDNA both 30 m north and 30 m south of I-90 crossing structures in winter and the following summer (2016). During one sampling event (Fall, 2015) each of these focal creeks was sampled at an additional point upstream, away from I-90 disturbance.

To test the use of eDNA as a “rapid-fire” sampling method over a large area, multiple creeks between Hyak and Easton were sampled at one point, in summer 2016. In addition to the five focal creeks, we also sampled Coal Creek, Mill Creek, Cold Creek, Resort Creek, Meadow Creek, Mosquito Creek, Swamp Creek, Toll Creek and Cedar

Creek (Fig. 2). This led to 117 field samples (including 39 blank controls) within the creeks. Full sampling methodology is explained in greater detail below.

To further understand the population of *A. boreas* within Snoqualmie Pass, eDNA methods were used to identify *A. boreas* breeding grounds. Three breeding sites are known in the study area: Swamp Lake, Mardee Lake and Keechelus Dam Ponds. After surveying these sites in 2015, the study area was searched for sites of similar environmental characteristics for further potential breeding sites. These characteristics include large shallow areas, gently sloping banks, aquatic vegetation, woodland surrounds, and still or slow moving water. Six sites were identified: Gold Creek Wetlands, Gold Creek Mitigation Ponds, Price-Noble wetlands, Toll Creek ponds, Cedar Creek wetlands and Crystal Springs (Fig. 3).

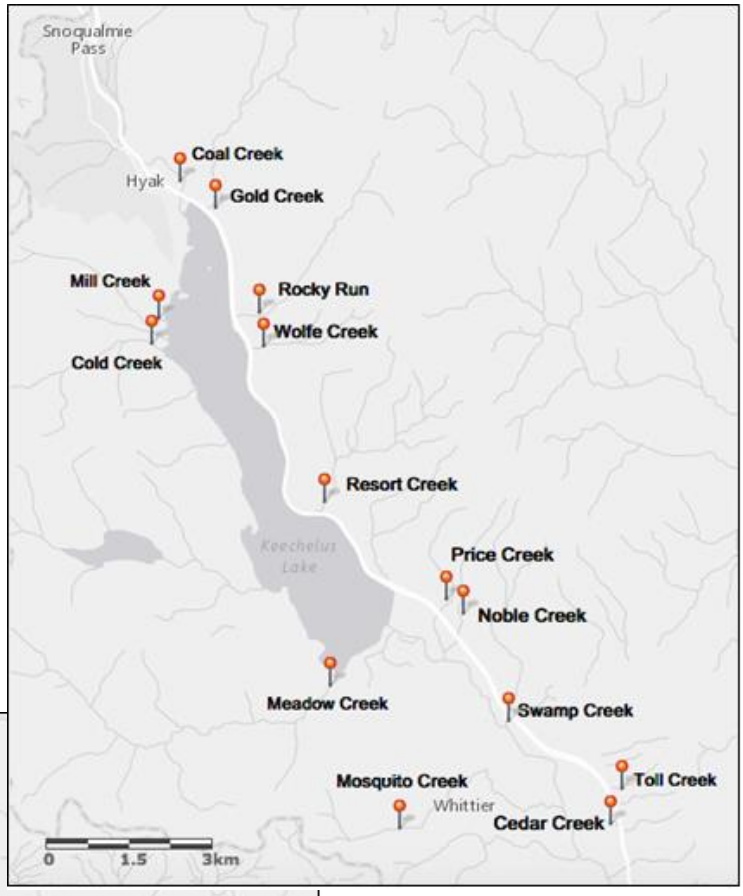

Figure 2: The location of creeks sampled for amphibians and fish (ESRI, 2015)

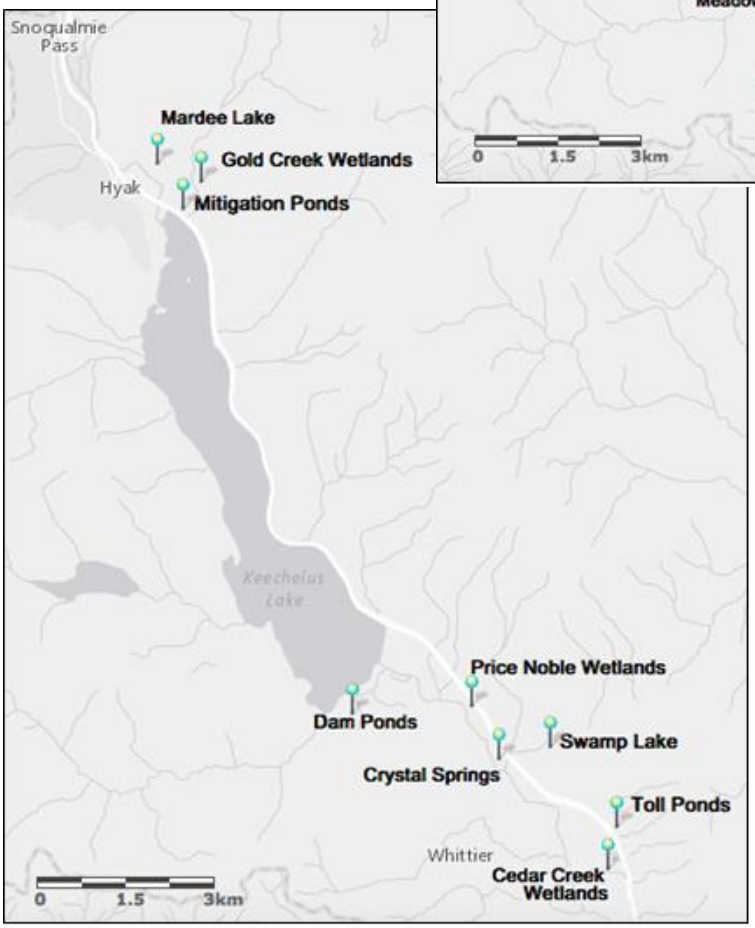

Figure 3: The location of wetlands sampled for breeding amphibians (ESRI, 2015)

Creek Sample Collection and Processing

Water samples for eDNA extraction were collected at 10 creeks that flow into Keechelus Lake and are intersected by I-90 and four creeks to the west of Keechelus Lake that are not interrupted by I-90 (Fig. 2). At each site three filters were used. One filter served as a control for contamination: the two bottles used for creek sample collection were rinsed with 500ml distilled water each, this 1 L was then filtered through the control filter to ensure no DNA was previously in sample bottles. These two bottles were then used to each collect separate 1 L creek samples. Each of these 1 L samples was made up of two ~500 ml submersion events. A 1 L water sample was taken from the middle thalweg, water which is flowing over the deepest part of the channel, and the other 1 L water sample taken from the slower moving edges or side pools. When taking a water sample, disturbance of sediment was kept minimal. All samples were taken from the water surface against the direction of flow.

When sampling a creek at several points along its length, downstream sites were sampled first to decrease contamination risk by the unidirectional flow of water. Equipment was kept downstream of the sample site and away from the water. Boots and equipment were sterilized before reaching the bank. For each site, I recorded GPS location, time, date, weather, and temperature of both water and air.

Five creeks were sampled at three points along their length, three times over the course of one year. Gold Creek, Rocky Run Creek, Wolfe Creek, Price Creek and Noble

Creek were sampled in fall (09/15/2015 – 09/22/16), winter (01/08/16 - 01/15/16) 2016, and summer (06/07/19 - 06/13/16).

To test eDNA methods as a rapid-fire sampling technique over multiple tributaries, nine additional creeks were sampled at only one point once in the summer of 2016 (7 samples collected 06/07/16 and 7 samples collected 06/13/16).

Each sample point was based on a 1 L sample pumped through a 250-ml 0.45µm cellulose nitrate membrane analytical test filter funnel (Thermo Scientific). eDNA from filters was analyzed for five focal species: *Anaxyrus boreas, Ascaphus truei, Dicamptodon tenebrous, Salvelinus confluentus,* and *Salvelinus fontinalis.*

Wetland Sample Collection and Processing

Nine wetlands were sampled for eDNA during *A. boreas* breeding season (April 2016): Mardee Lake, Gold Creek Wetlands, Gold Creek Mitigation Ponds, Keechelus Dam Ponds, Price/ Noble Wetlands, Swamp Lake, Crystal Springs, Toll Ponds and Cedar Creek Wetlands. Wetlands were each sampled on two occasions, within the anticipated *A. boreas* breeding timeframe. This resulted in 54 field samples (including 18 blank controls) from breeding sites.

For wetland sampling to determine *A. boreas* breeding sites, a similar eDNA collection methodology was used: two samples of filtered water, taken twice throughout the breeding season at these nine sites. The first round of wetland sampling

took place between April 12th and April 16th. The second round of sampling took place between April 19th and April 23rd.

Within lentic systems, eDNA has a patchy distribution of heterogeneous concentration compared to lotic system, due to limited water mixing and the uneven distribution of organisms due to preferences in microhabitats (Takahara et al. 2012; Goldberg et al. 2018). For this reason, each 1 L sample was comprised of four different submersion events at four different points of each water body (merged sampling) to increase the chance of detection.

Visual encounter surveys at each of the wetland sites were carried out to determine breeding. Positive breeding was noted if adults in amplexus, tadpoles or eggs were seen.

DNA Extraction and Detection

All extractions were performed in a lab in which no invasive genetic sampling took place, separate from the room where water samples were filtered and from where qPCR was carried out. Filters from both wetland and creek samples were processed in the same way.

Filters were halved and one half removed from buffer, stored individually in a tube and air dried overnight. DNA was extracted from each half-filter using Qiagen Blood and Tissue DNeasy Kit and QIAShredder columns (Qiagen; www.qiagen.com,

protocol followed from Goldberg et al. 2011). Once dry, 180 µl tissue lysis buffer (ATL) and 20 µl proteinase K was added, vortexed and incubated at 55°C overnight. Samples were spun at 11,000 RPM through Qiashredder spin columns and buffer AL added to the resulting supernatant. After incubating for 10 minutes at 70°C, 200 µl ethanol was added and the mixture added to DNeasy spin columns. Samples were centrifuged through three separate ethanol washes. DNA was then eluted from the spin column using 100 µl Tris-Cl (buffer AE). DNA extractions were stored at -15°C until qPCR analysis.

DNA was detected by quantitative polymerase chain reaction (qPCR). All qPCR reactions were run on an IQ5 Real-Time Thermal Cycler (BioRad). Conditions of qPCR per 20 µl well were as follows: 10 µl PrimeTime Master Mix (IDT), 1 µl custom assays per species containing primers and probe (IDT), 1 µl Hex-labelled IAC, 1 µl IAC sample, 5 µl eDNA sample, and brought to a standard 20 µl with PCR grade water. Conditions for qPCR were a 3-minute hotstart at 95°C followed by 50 cycles with annealing temperatures ranging from 50-55°C dependent on primer melting temperature (Tm).

An exogenous internal positive control (IPC) was included in every plate well, in the form of a yeast species not found in the environment in our study area. The addition of this IPC and its subsequent amplification show the PCR reaction to be successful and rules out reaction malfunctions that may otherwise be interpreted as a false negative. A negative control was also included on each plate, to ensure the master mix of reagents

had no DNA contamination. All samples were run in duplicate or, on the few occasions duplicate samples had opposing amplification results, triplicate.

Each of the five species-specific primer set was tested against positive samples of target species DNA extracted from live or frozen individuals at Central Washington University. Each assay worked and was paired by Tm and fluorescent dye to allow multiplexing, the testing of samples for multiple target species during the same PCR run (Appendix 1).

CHAPTER III

RESULTS

eDNA was consistently detectable throughout samples collected (examples in Fig. 4) and both positive and negative controls indicated only rare instances of inhibition or contamination (once each). Every positive detection, regardless of species, had very high cycle thresholds (after 30), meaning each sample had very low concentrations of eDNA.

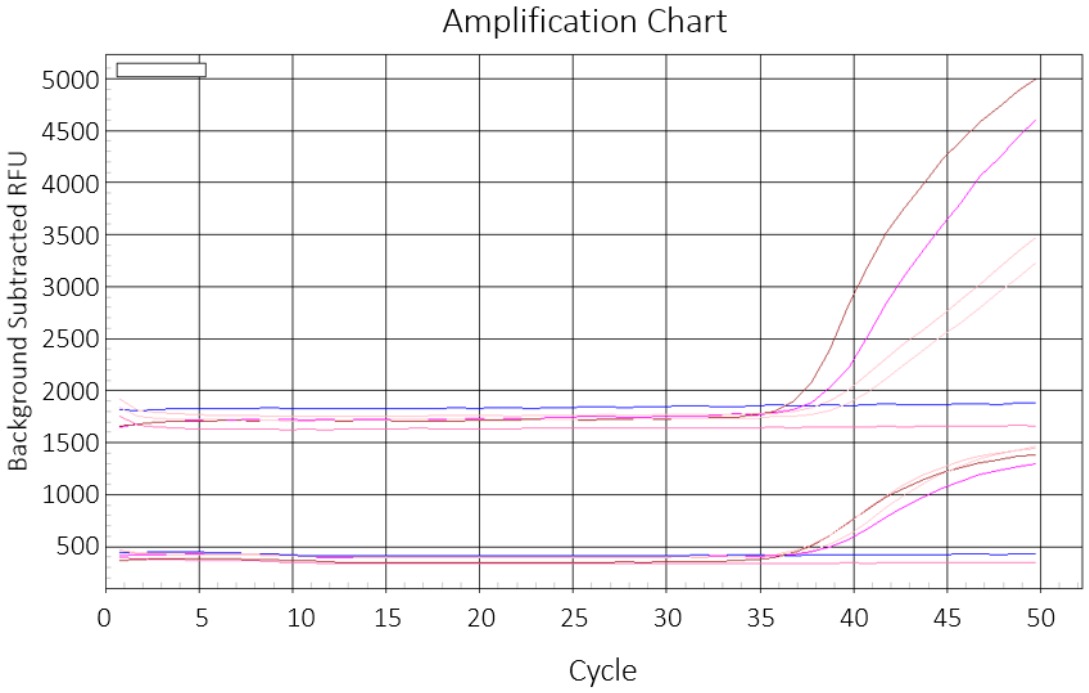

Figure 4: Visual of qPCR results, with two examples of positive results each alongside their negative controls. Upper lines (baseline between 1600-1800), from two Dicamptodon tenebrosus *samples (red, purple and light pink) run in duplicate; lower lines (baseline between 300-500), from two* Ascaphus truei *samples (red, purple and light pink), run in duplicate.*

Detection in Proximity to I-90

Amplification of eDNA shed from the three focal amphibian species and two focal trout species in this study was successful throughout. During the fall 2015 sampling in the five focal creeks *A. truei* and *D. tenebrosus* were detected in three and four of the five creeks respectively, whereas *A. boreas* was detected in only two (Table. 1). eDNA from the *Salvelinus* species was detected in each of the five creeks, with both *S. fontinalis* and *S. confluentus* detected in Gold Creek, but each being detected in different creeks farther east (Table. 1).

Table 1: Detection results by qPCR for focal species at three points in each of the five major creeks: 30m south of I-90 crossing structure, 30m north of I-90 crossing structure and an upstream 'undisturbed site', fall 2015. '+' indicates a positive detection via eDNA and '-' indicates species not detected. 'I' shows an occasion where qPCR was inhibited (one occasion).

	Gold Creek			Rocky Run Creek			Wolfe Creek			Price Creek			Noble Creek		
	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper
ANBO	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
DITE	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
ASTI	-	-	-	-	-	+	-	+	+	-	-	-	+	+	+
S.FON	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-
S.CON	+	+	+	-	+	-	-	+	-	-	I	-	-	-	-

During the Fall 2015 sampling, extra sites were sampled upstream and away from the disturbance of I-90. Positive detection from around I-90 (from either the 30 m north sample or the 30 m south sample) were combined as a single I-90 site and compared to the upstream site (Table. 2).

Table 2: Pooled samples both north and south of I-90 as a single "I-90" site compared to sites further upstream away from I-90. Detection results by qPCR for focal species near I-90 (within 35m) or not near I90 (>100m). '+' indicates a positive detection via eDNA and '-' indicates species not detected.

	Gold Creek		Rocky Run Creek		Wolfe Creek		Price Creek		Noble Creek	
	I90	Upper	I90	Upper	I90	Upper	I90	Upper	I90	Upper
ANBO	-	-	-	-	-	-	+	-	+	-
DITE	-	-	+	+	+	+	+	+	+	-
ASTI	-	-	-	+	+	+	-	-	+	+
S.FON	+	+	-	-	-	-	+	-	+	-
S.CON	+	+	+	-	+	-	-	-	-	-

Salvelinus confluentus eDNA was detected near the I-90 crossing in all three restored creeks: Gold Creek, Rocky Run Creek and Wolfe Creek. *Salvelinus fontinalis* eDNA was detected in Gold Creek and the yet to be restored Price Creek and Noble Creek, reflecting their hardier nature. None of the focal amphibian species' eDNA was detected within Gold Creek. This holds true throughout all Gold Creek samples throughout the project.

Visual Encounter Surveys and eDNA Detection

To compare eDNA methods and results to traditional amphibian survey results (visual encounter) the following tables summarize VES results through several different parameters. First, positive visual detection of amphibians in the 21 days leading up to the eDNA sample being taken (Table. 3) to compare visual encounter results and eDNA results within the time frame of suggested eDNA degradation. Second, positive detection of focal amphibian species within the first six cumulative hours of VES on each focal creek to compare time efficiency of each method (Table. 4). Finally, positive detection of any of the three focal amphibians at any point during the survey season by VES (Table. 5).

Dicamptodon tenebrosus was the most frequent amphibian species encountered during the 2015 visual survey season, followed by *A. truei*, then *A. boreas*, a result replicated by eDNA detection results.

Table 3. Visual encounter survey for amphibians within 21 days prior to eDNA water sample taken, 2015. '+' indicates a positive detection via VES and '-' indicates species not detected.

	Gold Creek			Rocky Run Creek			Wolfe Creek			Price Creek			Noble Creek		
	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper
ANBO	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DITE	-	-	-	+	+	-	+	+	+	+	+	+	-	-	+
ASTI	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-

Table 4. Amphibian species seen within six field hours of surveying each site, May- September 2015. '+' indicates a positive detection via VES and '-' *indicates species not detected*.

	Gold Creek			Rocky Run Creek			Wolfe Creek			Price Creek			Noble Creek		
	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper
ANBO	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DITE	-	-	-	-	+	-	+	+	+	+	+	+	+	-	+
ASTI	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-

Table 5. Positive visual detection of amphibian species at the five major creeks during the entirety of the 2015 survey season (May-September). '+' indicates a positive detection via VES and '-' indicates species not detected.

	Gold Creek			Rocky Run Creek			Wolfe Creek			Price Creek			Noble Creek		
	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper	South	North	Upper
ANBO	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
DITE	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+
ASTI	-	-	-	-	-	+	+	+	-	+	-	+	+	+	-

When comparing positive detection of each of the focal amphibian species by eDNA and VES, treating creeks as one waterbody (combining different sampling sites), the results are remarkably similar (Table. 6).

Table 6: Comparison of detection by visual encounter surveys and eDNA methods, combining results from all three survey sections of each creek for 2015 field season. '+' indicates a positive detection via VES/ eDNA and '-' indicates species not detected.

	Gold Creek		Rocky Run Creek		Wolfe Creek		Price Creek		Noble creek	
	VES	EDNA	VES	EDNA	VES	EDNA	VES	EDNA	VES	EDNA
ANBO	-	-	-	-	-	-	+	+	+	+
DITE	-	-	+	+	+	+	+	+	+	+
ASTI	-	-	+	+	+	+	+	-	+	+

Seasonal Comparisons

Anaxyrus boreas was not detected within any creeks sampled in the winter. *Dicamptodon tenebrosus* was detected only marginally less frequently in the winter (Table 7). One negative control did show contamination for *D. tenebrosus* (Rocky Run Creek, North). *Ascaphus truei* was detected at one only sample point in Resort Creek on one occasion. Both *Salvelinus* species were detected in Gold Creek during winter.

Table 7: Focal species detection via eDNA methods in six creeks during winter, January 2016. '+' indicates a positive detection via eDNA and '-' indicates species not detected. 'A' represents a sample in which the blank control tested positive for the species, indicating contamination, and so results cannot be reliably translated.

	Gold Creek		Rocky Run Creek		Wolfe Creek		Price Creek		Noble Creek		Resort Creek	
	North	South	North	South	North	South	North	South	North	South	North	South
ANBO	-	-	-	-	-	-	-	-	-	-	-	-
ASTI	-	-	-	-	-	-	-	-	-	-	-	+
DITE	-	-	A	+	+	+	-	+	-	+	+	+
S. FON	+	+	-	-	-	-	-	-	-	-	-	-
S. CON	+	+	-	-	-	-	-	-	-	-	-	-

Dicamptodon tenebrosus was consistently detected in each creek at least once, at one sampling point, in each season sampled. A*scaphus truei* was found within the three of the five creeks sampled over several seasons: Rocky Run Creek, Wolfe Creek and Noble Creek. I found no positive detections for *A. boreas* or *A. truei* in winter sampling. Both *S. fontinalis* and *S. confluentus* were detected throughout the year in the Gold Creek samples. *Salvelinus confluentus* was detected in both recently restored creeks, Rocky Run Creek and Wolfe Creek (Table. 8).

Table 8: Seasonal difference in eDNA detection of focal amphibian species in restored (Gold, Rocky Run, and Wolfe) and unrestored (Price and Noble) creeks. '+' indicates a positive detection via eDNA and '-' indicates species not detected.

	A. boreas			*A. truei*			*D. tenebrosus*		
	fall	winter	summer	fall	winter	summer	fall	winter	summer
Gold	-	-	-	-	-	-	-	-	-
Rocky Run	-	-	-	+	-	+	+	+	+
Wolfe	-	-	-	+	-	+	+	+	+
Price	+	-	-	-	-	-	+	+	+
Noble	+	-	-	+	-	+	+	+	+

Table 9: Detection of focal trout species by season in five major creeks. '+' indicates a positive detection via eDNA and '-' indicates species not detected.

	S. fontinalis			*S. confluentus*		
	Fall	Winter	Summer	fall	winter	summer
Gold	+	+	+	+	+	+
Rocky Run	-	-	-	+	-	-
Wolfe	-	-	-	+	-	-
Price	+	-	-	-	-	-
Noble	+	-	+	-	-	-

Rapid-fire Sampling

The rapid-fire sampling results showed *D. tenebrosus* and *A. truei* to be positively detected via eDNA in nine of the 14 creeks sampled, with some overlapping occupancy. *Anaxyrus boreas* was detected in 2 of the fourteen creeks. These results come from sampling the creek at one point, with a two-bottle methodology (Table. 10).

Table 10: Focal amphibian species detected via eDNA throughout the study area via rapid sampling, summer 2016. '+' indicates a positive detection via eDNA and '-' indicates species not detected.

Creek	*A. boreas*	*D. tenebrosus*	*A. truei*
Coal Creek	+	+	+
Gold Creek	-	-	-
Rocky Run Creek	-	+	+
Wolfe Creek	-	+	+
Resort Creek	-	-	-
Price Creek	-	+	-
Noble Creek	-	+	+
Swamp Creek	-	-	-
Toll Creek	-	-	+
Cedar Creek	-	-	+
Mosquito Creek	-	+	-
Meadow Creek	+	+	+
Cold Creek	-	+	+
Mill Creek	-	+	+

Salvelinus fontinalis was detected in Gold Creek, Swamp Creek and Noble Creek, with *S. confluentus* only detected in Gold Creek during the summer eDNA sampling round (Table. 11).

Table 11: Focal trout species presence, summer 2016. '+' indicates a positive detection via eDNA and '-' indicates species not detected.

Creek	*S. fontinalis*	*S. confluentus*
Coal Creek	-	-
Gold Creek	+	+
Rocky Run Creek	-	-
Wolfe Creek	-	-
Resort Creek	-	-
Price Creek	-	-
Noble Creek	+	-
Swamp Creek	+	-
Toll Creek	-	-
Cedar Creek	-	-
Mosquito Creek	-	-
Meadow Creek	-	-
Cold Creek	-	-
Mill Creek	-	-

Anaxyrus boreas Breeding-site Surveys

Visual confirmation of *A. boreas* breeding (eggs or tadpoles) occurred at three of the wetland sites: Mardee Lake, Swamp Lake, and a new site, East of Dam Ponds (Table 12). During the first round of sampling, eDNA methods showed positive detection of *A. boreas* in both Mardee Lake and Swamp Lake, two known *A. boreas* breeding sites. During the second round of sampling, Mardee Lake and Swamp Lake again came back positive; additionally, the ponds east of the Keechelus dam had a positive eDNA detection, as did Crystal Springs (Table. 12).

Table 12. Detection results of A. boreas *from both visual surveys and two rounds of eDNA sampling. 1st round between 12th and 16th April, second round between 19th and 23rd April. '+' indicates a positive detection via eDNA and '-' indicates species not detected.*

Site	Visual Detection	eDNA detection 1st sample round	eDNA detection 2nd sample round
Mardee Lake	+	+	+
Gold Creek Wetlands	-	-	-
Gold Creek Mitigation Ponds	-	-	-
East of Dam Ponds	+	-	+
Price Noble Wetlands	-	-	-
Swamp Lake	+	+	+
Crystal Springs	-	-	+
Toll Pond	-	-	-
Lower Cedar Creek Wetlands	-	-	-

CHAPTER IV

DISCUSSION

Environmental DNA as a Monitoring Tool

This research investigated the use of eDNA to monitor multiple freshwater species throughout a large catchment area within creeks affected by human alteration, as well as surrounding wetlands. Species-specific primers successfully detected all three focal amphibian species and both focal trout species. Successful multiplexing between species-specific primers during qPCR highlighted how eDNA can be used to monitor species over extended periods of time and may even outperform traditional methods of monitoring. Results from this study, overall, show eDNA methods to produce results that reliably reflect target species presence in both lentic and lotic systems. eDNA is a credible and sensitive monitoring tool.

The process of obtaining reliable results from eDNA methods is both intricate and rigorous and it is necessary to have checks throughout. A primary concern for many professionals when deciding to use eDNA as a sampling method is its reliability in giving valid results and the occurrence of false positives and false negatives (Type 1 and Type 2 errors, respectively). This study took thorough measures at multiple stages of collection, extraction, and detection to control for both false positives and false negatives.

A false positive in eDNA research is the incorrect positive detection of a species. False positives of target species in a sample can result from contamination, ancient DNA, and the transport of eDNA from a different area. To address the issue of contamination during this research, blank controls were used at every stage: during

collection, during extraction and during qPCR to detect contamination from equipment. Full and proper sterilization of all equipment between sites is vital to prevent cross contamination, which can lead to a false positive detection. The use of blank controls at multiple stages throughout the process allows the point of contamination of a sample to be highlighted and corrected for, increasing our confidence in the end results.

eDNA degrades rapidly within freshwater systems (Dejean et al. 2011), reducing the probability of positively detecting species from populations that are long extirpated. However, eDNA that becomes embedded within stream sediment can persist for a longer amount of time (Turner et al 2015). Sampling this sedimentary eDNA could lead to an inaccurate representation of the real-time, current distribution of focal species. During this study, samples were carefully collected to minimize disturbance of the stream bed to avoid positive detection of an individual that has long since inhabited the waterbody.

Despite meticulous collection and extraction protocols, some external factors are harder to control. eDNA could move long distances by water (Deiner and Altermatt 2014; Jane et al. 2015), which would inaccurately reflect species composition within a set study area. Research into the introduction of eDNA into an area by vectors and fomites, such as predator feces, has shown this to be a credible concern (Merkes at al. 2014; Creer et al. 2016). This highlights the importance of multiple sampling days throughout the study and the benefit of pairing eDNA with another method, such as traditional visual encounter surveys or historical records.

False negatives can also occur within eDNA samples, wherein a target species is present in the research area, but eDNA methods do not detect it – that is, the results inaccurately reflect the absence of target species in the area. A common cause of detection failure is poor primer design.

Primers are a vital part of any PCR assay. Poorly designed primers result in reduced precision and sensitivity and could ultimately lead to the failure of target amplification (Kelly et al. 2019). Within this study all primers used were successfully tested against positive controls through a dilution series before they were run on field samples. This was particularly important for the two very closely-related trout species. Additionally, each qPCR plate included a known positive sample to ensure conditions within the qPCR cycler were ideal for each primer.

Another complication in eDNA sampling that can result in a false negative is the over-dispersion of eDNA within the water column. eDNA may be present within the study area but may be collected in amounts insufficiently low for qPCR to amplify them within the set cycle number (Ellison et al. 2006). To combat this, full 1-L water samples were collected and filtered from each of two points in the waterbody: the thalweg, and within the slower side channels (resulting in 2 filters for each sampling point). This increased the likelihood of detection of target species that occupy different aquatic zones. It also helped tackle the issue that eDNA is not evenly dispersed throughout the water column (Furlan et al. 2015).

PCR is a sensitive process and multiple factors can prevent the amplification of nucleic acids. The extraction and purification of eDNA involves methods to remove

inhibitors from samples, and the use of Internal Amplification Controls (IAC) within each plate well highlights inhibition during PCR. During this research, primary steps were taken to reduce inhibition in samples, including washing DNA with buffers when bound to the silica gel column during DNA extraction. Samples were in duplicate to strengthen the validity of results, and an IAC was added to every sample run to ensure that inhibition was not mistaken for a false negative. I saw only one sample with inhibition in this study. Possible explanations for this are the increased leaf matter in creeks with the onset of fall or high levels of humic acid from decaying matter or tannins from the surrounding pines.

A recent study tackling the complex topic of inhibition in eDNA samples concluded that a multi-filter protocol to eliminate filter clogging, increasing the amount of water filtered, and using CTAB as a storage buffer (Hunter et al. 2019) could reduce inhibition in eDNA samples. My study did not use such methods as it was anticipated that only small amounts of eDNA would be extracted from the sample and inhibitor removal can reduce the amount of eDNA available for qPCR. Indeed, all samples in this study had high cycle thresholds, indicating consistently low eDNA concentrations. In the future, a similar study could implement the use of a post-extraction inhibitor removal step, which can remove humic substances interfering with PCR of the sample (Turner et al. 2015; Robson et al. 2016).

Through the various checks and balances throughout the process of using eDNA methods for monitoring species within this study, I am confident in its use as a credible monitoring tool.

Comparison of eDNA Methods to Visual Encounter Surveys

My study provided the opportunity to compare eDNA methods to more traditional monitoring methods such as visual encounter surveys (VES) and electrofishing. The total time for eDNA results to become available, from water collection to finished qPCR run, was an estimated six work-hours. Comparing eDNA detection rates (Table. 1) to six cumulative hours of visual surveys (Table. 4) suggests the less often a species is seen, the more valuable eDNA methods are as a survey tool. Results gathered from eDNA methods matched well, but not perfectly, the results gathered from the first cumulative six hours of amphibian VES on the same creeks. When comparing the eDNA results to those of VES, eDNA methods seemed to outperform visual surveys for each *A. boreas, D. tenebrosus,* and *A. truei* on most occasions, in terms of work hours before positive detection.

However, there were some discrepancies with the eDNA results. Several of which came from within Price Creek. Here, *A. truei* was seen through traditional survey methods at two sites (south and upper), but eDNA results showed no positive detection. *Salvelinus fontinalis* are also known to have a year-round resident population in Price Creek, yet eDNA did not detect eDNA from this species on most sampling occasions, except in Fall 2015.

Also, eDNA sampling at Resort Creek gave negative results for both *A. truei* and *D. tenebrosus* when previous traditional survey results showed they occupy this creek. An explanation for this may be the sample was taken too close to the mouth of the

creek, where mixing with Keechelus Lake water (in which eDNA from either species is very low or nonexistent) might prevent detection.

Among the creeks *A. boreas* was positively detected at two sites via eDNA during fall sampling: Price Creek and Noble Creek, which correlated with results from VES that season, and two during summer sampling: Coal Creek and Meadow Creek. The positive eDNA detection at Meadow Creek may warrant further breeding-site surveys throughout wetlands in the vicinity of these creeks. Mardee Lake, a known breeding site for *A. boreas*, flows into Coal Creek, which may explain the positive detection.

On several occasions, *A. boreas* were seen within the large box culvert of Price Creek under I-90. This may indicate the creek is a passage of importance for this species when migrating north and south of I-90. With the terrestrial ability of the amphibians within this study, within-stream structures may hinder, but not prevent, movement.

My research showed eDNA to be incredibly effective at detecting *D. tenebrosus. Dicamptodon tenebrosus* was detected via eDNA at nine of the 14 creeks sampled (Table 10). Traditional visual surveys on aquatic *D. tenebrosus* are particularly time-intensive, difficult to conduct, and cause habitat disturbance as these salamanders are nocturnal and inhabit the benthic zone, often well hidden under rocks and other stream features.

Over the course of the study, *A. truei* was positively detected within nine creeks via eDNA and five creeks via VES. However, positive visual detections at two sites, both at points along Price Creek, had no positive eDNA detection in either fall or summer. This poses an interesting question as to why eDNA detection of *A. truei* was successful in Noble Creek and not Price Creek when VES encountered *A. truei* within both. Price Creek

was also the only creek that produced a sample that was inhibited within this study. Visually, Price Creek and Noble Creek have very similar physical features in terms of gradient, canopy cover and dominant substrate (Gustafson, 2018); however, water chemistry differences could be at play.

Season Affects eDNA Detection

To most effectively use eDNA as a method to detect the presence of a species, both fiscally and logistically, a basic understanding of the ecology of the species is essential. For example, prior knowledge of seasonal activity, migration patterns, and habitat use can govern sampling times and locations. This knowledge also helps validate results obtained from eDNA studies. During this study, five creeks were sampled for eDNA from five target species over three seasons: Gold Creek, Rocky Run Creek, Wolfe Creek, Price Creek, and Noble Creek. Species were not detected in samples taken in months their ecology would suggest they not be present. For example, *A. boreas* was not detected within creeks during the winter months, as would be expected (Palmeri-Miles 2012).

Extensive surveys in the area have shown the majority of *S. confluentus* individuals in the study area to be lacustrine-adfluvial, spending much of the year in Keechelus Lake, migrating up tributaries in late summer to spawn in the cool headwaters (Dr Paul James, pers. comm.). The fall detection of *S. confluentus* in the recently restored Rocky Run Creek and Wolfe Creek may show that the restoration measures are working, almost instantly. *Salvelinus confluentus* were not consistently

present in Rocky Run Creek or Wolfe Creek in pre-restoration surveys around I-90, although one survey in 2013 positively detected one individual in Rocky Run Creek (Dr. Paul James, pers. comm.). The detection of this species in these creeks north of I-90 may indicate that site mitigation through retrofitting crossing structures has enhanced habitat availability for trout in the area. The potential increase in available spawning habitat to *S. confluentus* within headwater streams is of increased importance to this particular species' population. Listed as threatened under the Endangered Species Act (USFWS, 1998) this population is considered warranted for multiple recovery plans put forward by the U.S. Fish and Wildlife Service (2015) the Bureau of Reclamation (2015) and the local Yakima Bull Trout Action Plan (2012). Finally, surveys for *S. confluentus* using traditional methods, such as electroshocking, did not result in positive detection of bull trout in either of these two recently restored creeks during the same time period (Dr Paul James, pers. comm.), which demonstrates the effectiveness of eDNA to detect species with low abundance.

Salvelinus fontinalis had positive detection by eDNA methods throughout the year in Gold Creek, in fall and summer in Noble Creek, and just once in the fall sampling in Price Creek. The more widespread and frequent detection of *S. fontinalis* over *S. confluentus* is troubling but expected. A more generalist species, brook trout can tolerate broader environmental conditions than *S. confluentus*, which attributes to their invasive nature throughout the West. The more frequent positive detection of both trout species via eDNA in the fall months is consistent with their seasonal upstream

migrations (Wissmar and Craig 1997) and the consequent increased eDNA in these lotic systems.

However, it is known that *S. fontinalis* are year-round residents in Price Creek, a result not reflected by eDNA results. Taken over three seasons, only the fall samples (2015) resulted in a positive detection (both north and south of I-90). Price Creek was the only creek which had inhibition present an issue in a sample during this research. It is possible something within the creek, be it water chemistry, higher levels of humic acid or more complex issues, is causing eDNA to degrade quicker in this creek than others, resulting in a higher number of false negatives. Further research is necessary into this matter.

The three focal amphibian species in this research have very different life histories. Both *A. truei* and *D. tenebrosus* have aquatic stages that remain in the stream year-round. If conditions are favorable, *D. tenebrosus* often remain year-round within the creeks they inhabit. Sexually mature peadomorphs may choose to remain in the creeks instead of metamorphosing into the terrestrial form. This lengthy aquatic stage means it is possible to have positive detection of this species throughout the year and the eDNA results show consistent positive detection throughout three seasons within creeks (Table 8).

Ascaphus truei was detected via eDNA methods in Rocky Run Creek, Wolfe Creek, and Noble Creek in both fall and summer sampling. *Ascaphus truei* are also year-round residents, with tadpoles having an extended larval stage, taking around four years to metamorphose (Daugherty and Sheldon, 1982). Our results, however, do not detect

A. truei in every season sampled, with a negative result in winter for three creeks in which they were positively detected in both fall and summer. Once *A. truei* were thought to hibernate during winter (Metter 1964); although now it is thought that although they do not hibernate, they do exhibit a dramatic decrease in activity over the colder winter months. These results were reflected in a more recent study comparing eDNA detection rates by season for *A. truei* (Smith, 2017).

In a study by Goldberg and colleagues in 2011, eDNA was used to detect presence of species closely related to our target resident amphibians, the Rocky Mountain tailed frog (*Ascaphus montanus*) and Idaho giant salamander (*Dicamptodon aterrimus*). Results suggested detection via eDNA was more difficult during the spring months than the fall months, likely due to the decreased activity and metabolism seen in the species in the colder months of the year. These lower detection rates in winter again bring to light the importance of knowing the ecology of the species within a detection study and further emphasize the importance of multiple sampling sessions for increased reliability in results garnered by eDNA methods.

The effectiveness of eDNA methods detecting creek-dwelling amphibians such as *D. tenebrous* and *A. truei* are clear. The negative results for both species at Gold and Swamp Creeks are expected as multiple visual surveys have failed to produce positive results. *D. tenebrous* has not been seen at Cedar Creek or Toll Creek, which often runs dry for multiple months a year. An anomaly did arise, however, in the negative detection within Resort Creek, where *D. tenebrosus* are known to reside.

As expected, *A. boreas* was not detected in any of the creeks sampled in winter, due to their retreat to terrestrial hibernacula. This is a good sign that *A. boreas* in the area are staying true to their annual life histories despite the alterations and continued disruption in their range. This adds confidence to the short-term nature of eDNA in lotic systems, showing its use as a method with fine temporal accuracy.

There was positive detection of *A. boreas* in fall in both Price Creek and Noble Creek. The fall months are when toads of this species migrate from their breeding grounds to hibernacula. Detection at both of these geographically close creeks suggests this is a migratory route for this species and highlights the importance for restoration at Price Creek and Noble Creek, which was indeed completed after data collection for this study had taken place.

Rapid-fire Sampling

The widespread rapid-fire sampling of 14 creeks in the area in 2016 further showed the efficiency of eDNA as a large-scale sampling method. Importantly, it shows how efficient eDNA can be, these results (five species across 14 creeks) were gathered in only three qPCR runs. Each creek was sampled at only one point, the furthest downstream as was safe and unaffected by lake input. Even with this minimal sampling effort, eDNA revealed the presence of different species widespread throughout the study area.

Salvelinus fontinalis was detected in three of 14 creeks sampled: Gold Creek, Noble Creek, and Swamp Creek through rapid-fire eDNA methods (Table 11). They were

also positively detected through traditional surveying methods in a similar time period in Rocky Run Creek (October 2015) and Wolfe Creek (June 2015), but each of these records was of only one individual (Dr. Paul James, pers. comm.), which suggests low abundance, perhaps too low to be detected by eDNA methods.

During this rapid-fire sampling round, *S. confluentus* was detected in only Gold Creek, despite having positive eDNA detection in Rocky Run Creek and Wolfe Creek just months before. Repeated sampling and further research would be needed to conclude whether these results represent a difference in sampling effort or season. eDNA did not detect *S. confluentus* in Coal Creek, despite visual confirmation in this creek several years in a row (August 2014, June 2015, November 2015, July 2016). These results highlight the need for repeat sampling in waterbodies with low abundance of target species.

A 2017 eDNA study into the presence of *S. confluentus* in the Upper Yakima Basin (Parrish 2017) sampled several creeks included in my research. Samples from Coal Creek, Cold Creek, Meadow Creek, Resort Creek, Rocky Run Creek, and Wolfe Creek all returned negative eDNA results for *S. confluentus*. With positive detection of the species through VES in Coal Creek and via eDNA in Rocky Run Creek and Wolfe Creek just the year before, these results again may highlight the importance of multiple sampling with creeks of interest, both spatially and temporally.

Results gathered from eDNA methods were strengthened by complementing results in samples gathered in both fall 2015 and summer 2016, with the same detection

result (positive/ negative) for both *D. tenebrosus* and *A. truei* in Gold Creek, Rocky Run Creek, Wolfe Creek, Price Creek, and Noble Creek.

Despite positive detection in Price Creek and Noble Creek in fall, *A. boreas* showed positive eDNA detection in only Coal Creek and Meadow Creek in the single sampling round of summer 2016. This difference in location across seasons could be attributed to *A. boreas* migratory behaviors and could suggest wetlands that may have congregations of toads in the breeding season near Coal and Meadow Creeks. Indeed, Mardee Lake, a positive *A. boreas* breeding site flows out into Coal Creek. This may strengthen the hypothesis that an unknown *A. boreas* breeding site is linked to Meadow Creek.

eDNA techniques proved very efficient for detection of amphibians in the study area. Even with the multiple use of blank controls, eDNA detected *A. truei* in four creeks that visual surveys did not in the previous survey season- Coal Creek, Toll Creek, Meadow Creek and Mill Creek. Despite a negative eDNA result, *A. truei* tadpoles and adults have been seen in Price Creek, with more sightings in the upper reaches than around I-90. Mosquito Creek also gave negative eDNA results, although *A. truei* have been seen in low numbers, which may give rise to very low concentrations of eDNA in the water system. *Anaxyrus boreas* was detected in Coal Creek and Meadow Creek through eDNA detection but not visual surveys. Throughout the study *D. tenebrosus* was not visually detected in any creek with a negative eDNA result via the rapid-fire sampling. The higher abundance of *D. tenebrosus* in the study area may have led to this increased accuracy in eDNA detection.

The rapid-fire sampling shows the highly efficient manner in which eDNA can be used to get quick and reliable results of species within an area. Multiple sampling over time will increase reliability. In terms of work-hours spent monitoring large-scale catchment areas, eDNA methods outweigh traditional methods, especially when monitoring species from multiple taxa.

Anaxyrus boreas Breeding-site Detection

Previous knowledge of the ecology of the target species is important and increases detectability chances (Block et al. 2001) by ensuring samples are taken at the correct time of year and in locations with a likelihood of occupancy. eDNA sampling for *A. boreas* detection in lentic waterbodies took place in April, as several years of monitoring data in the area suggest that *A. boreas* do not leave hibernacula until mid-March. Amplexus has been seen in April and toadlets are often seen in the area in June.

eDNA results showed positive detection of *A. boreas* in Mardee Lake, Swamp Lake and Keechelus Dam Ponds. VES reflected these positive detections and also confirmed breeding activity in each of these sites. Crystal Springs was the only wetland that had a positive detection through eDNA for *A. boreas* during the breeding season, with no toads or signs of breeding activity seen during visual encounter surveys. The positive eDNA detection was only during the second round of sampling. This wetland is downstream from Swamp Lake, and we can speculate, but not confirm, the possibility that *A. boreas* eDNA was transported along Swamp Creek to the Crystal Springs wetland.

Sampling lentic waterbodies has a different set of challenges than lotic waterbodies. eDNA is found in the water at incredibly low concentrations (Goldberg et al. 2016) and is not distributed within the water evenly (Takahara, 2012), so it is important to sample at multiple points throughout a waterbody. Due to the higher level of suspended solids and organic debris commonly seen in smaller lentic waterbodies, filters often clogged after only a portion of the sample had been filtered, making filtration of the samples laborious and time-consuming.

This could have been tackled by a multi-level system of filtering using decreasingly smaller pores, however it is still unknown how much eDNA is attached to such organic matter and how it could affect detection rates when at already low concentrations. During this study, I did not use pre-filtration methods, but instead accepted longer filtering times, to maintain the maximum yield of eDNA.

The results suggest that eDNA methods are as reliable and perhaps more time effective than traditional VES in detecting *A. boreas* during breeding season. Sampling several times over the suspected breeding season could help narrow down a more precise time when adults start arriving at the breeding sites. This would help in prioritizing *A. boreas* conservation when making land management decisions in the surrounding area.

Conclusions

Using eDNA methods I was able to survey creeks throughout the Snoqualmie Pass East Project Area, comparing creeks with recent restoration work in the form of improved crossing structures, to creeks without. Within recently-restored Gold Creek, both *S. fontinalis* and *S. confluentus* were positively detected across samples 30 m south and 30 m north of I-90, as well as at the upstream sites away from I-90.

Five creeks were sampled over three seasons: Gold Creek, Rocky Run Creek, Wolfe Creek, Price Creek and Noble Creek. This allowed for a more in-depth look at three creeks that had been recently restored (Gold Creek, Rocky Run Creek, Wolfe Creek) compared to creeks being prepared for restoration (Price Creek, Noble Creek).

To the east of Gold Creek, *S. fontinalis* was detected in Price Creek and Noble Creek and *S. confluentus* was detected in Rocky Run Creek and Wolfe Creek (Table 1). The positive eDNA detection of *S. confluentus* in Rocky Run Creek and Wolfe Creek in the fall sampling round is an exciting result. eDNA results from Gold Creek, corroborate with extensive monitoring throughout the area during this study (Bunce, 2016). Positive eDNA detection in Rocky Run and Wolfe were not backed up by visual detection over the course of this study, although *S. confluentus* was detected north of I-90 in Rocky Run Creek in 2013.

Interestingly, neither trout species was detected at sites sampled farther upstream in any of these four creeks (Rocky Run, Wolfe, Price, Noble). This suggests that either the habitat is unsuitable, the grade is too steep, or there are further crossings north of I-90 that are impassable for these fish.

Although Gold Creek tested positive for both trout species along its reaches, only *Pseudacris regilla* (Pacific chorus frog,a widespread species) was seen in this creek. At no point during this study did Gold Creek test positive for any of the focal amphibian species. This may suggest Gold Creek is be poor habitat for aquatic amphibians, or it may be that the higher trout numbers in this creek exert greater predation pressure on amphibians such as *D. tenebrosus* and *A. truei* that breed within the creek system. *Pseudacris regilla,* on the other hand, breed in shallow wetlands adjacent to the creek, perhaps free from the predation pressure from trout.

Dicamptodon tenebrosus was detected via eDNA at all three sites (north and south of I-90 and an upstream, undisturbed site) in each Rocky Run Creek, Wolfe Creek, Price Creek and Noble Creek. This is fitting, as with their terrestrial life stage *D. tenebrosus* may have a greater potential for migrating upstream, because the terrestrial adults have been observed to move up to 50 m from the water (Fessler 2012), thus providing them the ability to circumvent within-stream barriers.

Ascaphus truei seek out cool, fast-moving streams, which are often characteristic of higher headwaters. This could result in more positive detections of *A. truei* in the upper sites, rather than the lower downstream sites, typically characterized by a lower slope gradient, and so slower waters. This matches 2016 VES data, where *A. truei* tadpoles were found in upper Price Creek site. *A. truei* was not detected via eDNA methods in Price Creek, but was detected at all three sample sites in the nearby Noble Creek. *A. truei* was detected both north of I-90 and the upstream undisturbed site of Wolfe Creek and solely in the upper sample in Rocky Run Creek. Further investigation

would be necessary to explore the variables that affect the distribution of *A. truei* around Snoqualmie Pass and I-90.

A less studied creek within the area is Meadow Creek. Meadow Creek runs into Keechelus Lake from the west, and eDNA results from this study showed positive detection of all three focal amphibian species. Protecting a creek with such diversity in species will create a refuge from which amphibians can disperse once habitat restoration is complete.

A creek within the study area that drastically highlights the importance of well-designed culverts in habitat connectivity is Cedar Creek. During one visual encounter survey in 2016 over 50 *A. truei* were found south of I-90 (downstream) and 0 were seen north of I-90.The large-drop pipe culvert to the creek below measured almost 2 meters in height and the congregation of *Rana cascadae* at its base suggested it was impassable.

Collecting information on the spatial distribution of focal species is the first step in any conservation action planning. Whether the species in question is invasive or endangered, their occupancy across the landscape is the primary piece of information on which to build an effective plan. eDNA methods consistently showed to be a beneficial method of monitoring species' recent occupancy throughout this study. eDNA proved reliable in restored habitats, altered habitats, undisturbed habitats, as well as in both lentic and lotic systems. It proved effective for closely-related trout species as well as amphibians, both aquatic and terrestrial, that rely on aquatic sites during certain life stages.

Although traditional methods provide more data about the complex ecology of a population, such as demography and fitness, they lack the increased sensitivity eDNA provides when monitoring for rare and cryptic species. For baseline inventory monitoring, eDNA showed in this study to be an optimal method for surveying freshwater species.

During this research, only half of each filter was used in processing the samples, the other half stored for use in the future. This paves the way for further research to be done within these creeks without further fieldwork, using different primers on the already collected and filtered eDNA. This research used a targeted approach. Future eDNA research in the area could use a non-targeted, metabarcoding approach making use of universal primers. Next-generation sequencing methods are becoming more refined and more accessible, which will allow full sweeps of species inventories of waterbodies to be completed in a time and cost-effective manner. Such methods may also allow the detection of invasive species far earlier than traditional methods (Goldberg et al. 2013).

Using eDNA methods to monitor freshwater species also reduces personnel effort while identifying all species present. This is especially advantageous when species within the study are morphologically similar, such as the *Salvelinus* species in this study. The speed at which eDNA can identify occupied sites for multiple species at once promises to aid in up-to-date species atlases, critical in monitoring invasive species spread and at-risk populations.

There is evidence that eDNA can be used to reliably estimate the biomass, and therefore abundance, of a species. Some studies have shown a positive correlation between concentration of eDNA in a sample and relative biomass of target species (Takahara et al. 2012; Salter et al. 2019) although not always significantly (Matsuhashi et al. 2016). The potential for eDNA to answer questions beyond just presence/ absence is certainly there but relies on the development of complex modelling systems taking in multiple factors: biomass of species, species-specific shedding rates, time of year and seasonal activity, and stream dynamics to name a few.

eDNA found in the sediment of waterbodies exhibits a much lower decay rate than aqueous eDNA, allowing retrospective genetic monitoring. Sedimentary eDNA analysis will further increase the use of eDNA, allowing researchers to monitor species on a different timescale and reveal shifts in species composition over time (Sakata et al. 2020).

Multiple checks throughout sample processing increases the level of confidence in results gathered via eDNA methods. Moreover, the strong overlap between field sampling and the eDNA results demonstrate the effectiveness of eDNA to identify sites occupied by these aquatic species. To further strengthen our knowledge in our focal species distribution and movement throughout the SPEP area, I suggest repeat sampling over several years.

Management Implications

Several of the focal species within this study are of special importance in Washington State. *Salvelinus confluentus* is federally classified as threatened and, alongside *A. boreas,* is identified by Washington State Department of Fish and Wildlife (WDFW) as a Species of Greatest Conservation Need. Results garnered from this study help delineate the species range and habitat preferences, as well as provides a methodology for using eDNA to conduct large-scale, time-efficient monitoring throughout the state for recognized important aquatic species.

The Snoqualmie Pass East Project tackles a number of stressors on wetland habitat listed in the 2015 State Wildlife Acton Plan, such as roads and development, alteration of hydrology and habitat degradation. The positive detection of *S. confluentus* in creeks where crossing structures have been improved (Gold Creek, Resort Creek and Rocky Run Creek) highlights the success of the restoration work carried out within the SPEP area so far. As such, methods used for restoration can be implemented in other areas around the state.

The results gathered in this eDNA study can be used in wider programs, such as The Aquatic eDNAtlas Project (Young et al. 2018) and The Rangewide Bull Trout eDNA Project (Young et al. 2017). Further uses of eDNA could include the detection of fungi and parasites that cause diseases, such as chytrid and whirling disease, which will help map their occurrence and direct conservation actions to prevent their spread. eDNA continues to revolutionize the way wildlife managers and researchers survey and monitor for multiple taxa throughout various environments.

LITERATURE CITED

Aparicio E, Vargas MJ, Olmo JM and Sostoa A. 2000. Decline of native freshwater fishes in a Mediterranean watershed on the Iberian Peninsula: A quantitative assessment. Environmental Biology of Fishes. 59(11): 11-19

Arnell A, Williams P, Dunn F. 2015. Using eDNA to develop a national citizen science – based monitoring program for the Great Crested Newt (*Tristus cristatus*). Biological Conservation. 183: 19-28

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL and Lodge DM. 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environmental Science Technology. 48(3): 1819-1827

Barnes MA, Turner CR. 2015. The ecology of environmental DNA and implications for conservation genetics. Conservation Biology. 17(1): 1-17

Beebee T. 2013. Effects of road mortality and mitigation measures on amphibian populations. Conservation Biology. 27(4): 657-668

Berger L, Spear R, Hyatt A. 1999. Chytrid fungi and amphibian declines: overview, implications and Future Directions. Campbell A (ed.) Declines and Disappearances of Australian Frogs. Environment Australia. Canberra.

Biggs J, Ewald N, Valentini A, Gaboriaud C, Griffiths RA, Foster J, Wilkinson J, Arnell A, Williams P, Dunn F. 2014. Analytical and methodological development for improved surveillance of the Great Crested Newt. Freshwater Habitats Trust. Oxford

Biggs J, von Fumetti S, Kelly-Quinn M. 2016. The importance of small waterbodies for biodiversity and ecosystem services: implications for policy makers. Hydrobiologia. 793: 3-39

Blaustein AR, Han BA, Relyea RA, Johnson PT, Buck JC, Gervasi SS, Kats LB. 2011. The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Annals of the New York Academy of Sciences. 1223: 108–119

Block WM, Franklin AB, Ward JP, Ganey JL, White GC. 2001. Design and implementation of monitoring studies to evaluate the success of ecological restoration on wildlife. Restoration Ecology. 9(3): 293-303

Briggs AS, Galarwoicz TL. 2013. Fish passage through culverts in central Michigan warmwater streams. North American Journal of Fisheries Management. 33(3): 652-664

Browne C. 2010. Hibernation sites of western toads (*Anaxyrus boreas*): Characterization and management implications. Herpetological Conservation and Biology. 5(1): 49-63

Bull EL, Carter BE. 1996. Tailed frogs: distribution, ecology, and association with timber harvest in northeastern Oregon. Res. Pap. PNW-RP-497. Portland, OR: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station

Bull EL. 2006. Sexual differences in the ecology and habitat selection of Western Toads (*Bufo boreas*) in northeastern Oregon. Herpetological Conservation and Biology. 1(1): 27-38

Bunce A. 2016. Seasonal microhabitat use of young-of-the-year bull trout, *Salvelinus confluentus*, All Master's Theses, Central Washington University. 507. http://digitalcommons.cwu.edu/etd/507

Burkhead NM. 2012. Extinction rates in freshwater fishes 1900-2010. Bioscience. 62(9): 798-808

Castelle AJ, Conolly C, Emers M, Metz ED, Meyer S, Witter M, Mauermann S , Erickson T, Cooke S. 1992. Wetland Buffers: Use and Effectiveness. Adolfson Associates, Inc., Shorelands and Coastal Zone Management Program, Washington Department of Ecology, Olympia, Pub. No. 92-10

Chapmam JM, Proulx CL, Veilleux AN, Levert C, Bliss S, André M, Lapointe NWR, Cooke SJ. 2014. Clear as mud: A meta-analysis on the effects of sedimentation on freshwater fish and the effectiveness of sediment-control measures. Water Research. 56: 190-202

Coffin AW. 2007. From roadkill to road ecology: A review of the ecological effects of roads. Journal of Transport Geography. 15: 396-406

Collen B, Whitton F, Dyer E, Baillie JEM, Cumberlidge N, Darwall WRT, Pollock C, Richman NI, Soulsbury A, Böhm M. 2013. Global patterns of freshwater species diversity, threat and endemism. Global Ecology and Biogeography. 23(1): 40-51

Creer S, Deiner K, Frey S, Porazinska D, Taberlet, Thomas WK, Potter C, Bik HM. 2016. The ecologists field guide to sequence-based identification of biodiversity. Methods in Ecology and Evolution. 7: 1008-1018

Dale JM, Freedman B. 1982. Lead and zinc contamination of roadside soil and vegetation in Halifax, Nova Scotia. Proceedings of tile Nova-Scotian Institute of Science. 32: 327-336

Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious diseases. 5: 735-748

Davis TM, Gregory PT. 2003. Local decline and extinction of the western toad *Bufo boreas*, on Southern Vancouver Island, British Columbia, Canada. Herpetological Review. 34(4): 350-352

Davy CM, Kidd AG, Wilson CC. 2015. Development and validation of environemntal DNA (eDNA) markers for detection of freshwater turtles. PLoS ONE. 10(7):e0130965

Deguise I, Richardson JS. 2009. Prevalence of the chytrid fungus (*Batrochochytrium dendrobatidis*) in western toads in northwestern British Columbia, Canada. Northwestern Naturalist. 90: 35-38

Deiner K, Altermatt F. 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS ONE. 9(2): e88786

Dejean T, Valenti A, Duparc A, Pellier-Cuit S, Pompanon F, Taberlet P, Miaud C. 2011. Persistence of environmental DNA in freshwater ecosystems. PLoS ONE. 6(8): e23398

Downing JA, Prairie YT, Cole JJ, Duarte CM, Tranvik LJ, Streigl, McDowell WH, Kortelainen P, Caraco NF, Melack JM, Middleburg JJ. 2006. The gloabal abundance and size distribution of lakes, ponds and impoundments. Limnology and Oceanography. 51:2388-2397

Eigenbrod F, Hecnar SJ, Fahrig L. 2009. Quantifying the road-effect zone: threshold effects of a motorway on anuran populations in Ontario, Canada. Ecology and Society. 14(1): 24

Ellison SLR, English CA, Burns ML, Keer JT. 2006. Routes to improving the reliability of low level DNA analysis using real-time PCR. BMC Biotechnology. 6(3): 33

ESRI. 2015. ArcGIS Online. Redlands, CA: Environmental Systems Research Institute. https://www.esri.com/en-us/arcgis/products/arcgis-online/overview, viewed June 2015

Favaro C, Moore JW, Reynolds JD, Beakes MP. 2014. Potential loss and rehabilitation of stream longitudinal connectivity: fish populations in urban streams with culverts. Canadian Journal of Fish and Aquatic Science. 71: 1-12

Fessler B. 2012. Spatial ecology, behavior, and habitat use of terrestrial coastal giant salamanders (*Dicamptodon tenebrosus*) in the central Washington cascades. All Master's Thesis, Central Washington University.

Ficolet GF, Maud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. Biological Letters. 4: 423-425

Foote AD, Thomsen PF, Sveegaard S, Wahlberg M, Kielgast J, Kyhn LA, Salling AB, Galatius A, Orlando L, Thomas M, Gilbert P. 2012. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PLoS ONE 7(8): e41781

Furlan EM, Hardy CM, Duncan RP. 2015. A framework for estimating the sensitivity of eDNA surveys. Molecular Ecology Resources. 16: 641-654

Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP. 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*), Freshwater Science. 32: 792−800

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomson PF, Murphy MA, Spear SF, McKee A, Oyler Mckance SJ, Cornman RS, Laramie MB, Mahon AR, Lance RF, Pilliod DS, Stickler KM, Waits LP, Fremier AK, Takahara T, Herder JE and Taberlet P. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution. 7(11): 1299-1307

Goldberg CS, Strickler KM, Fremier AK. 2018. Degradation and dispersal limit environmental DNA detection of rare amphibians in wetlands: increasing efficacy of sampling designs. The Science of the Total Environment. 15(633): 695-703

Gonzalez-del-Pliego P, Frecklton RP, Edwards DP, Scheffers BR, Pryon RA, Jetz W. 2019. Phylogenetic and trait based prediction of extinction risk for data-deficient amphibians. Current Biology. 29(9): 1557-1563

Goosem M. 2001. Effects of tropical roads on small mammals: inhibition of crossing movements. Wildlife Research. 28(4) 351-364

Gustafson A. 2018. Occupancy of stream- associated amphibians within the Interstate 90 Snoqualmie Pass corridor. All Master's Theses, Central Washington University. 884. https://digitalcommons.cwu.edu/etd/884

Hansen AJ, Spies TA, Swanson FJ, Ohmann JL. 1991. Conserving biodiversity in managed forests. Bioscience. 41:6 (382-392)

Hari RE, Livingstone DM, Siber R, Burkehardt- Holm P, Guttinger H. 2006. Consequences of climatic change for water temperature and brown trout populations in Alpine rivers and streams. Global Change Biology. 12(1): 10-26

Harper LR. Buxton AS, Rees HC, Bruce K, Brys R, Halfmareten D, Read DS, Watson HV,Sayer CD, Jones EP, Priestley V, Mächler E., Múrria C, Garcés-Pastor S, Medupin C, Burgess K, Benson G, Boonham N, Griffiths RA, Handley LL, Hänfling B. 2019. Prospects and challenges of environmental DNA monitoring in freshwater ponds. Hydrobiologica. 82(1): 25-41

Hels T. and Buchwald E. 2001. The effect of road kills on amphibian populations. Biological Conservation. 99: 331-340

Hruby T. 2013. Update on Wetland Buffers: The State of the Science, Final Report, October 2013. Washington State Department of Ecology. Publication 13-06-11

Hunter ME, Oyler-McCance SJ, Dorazio RM, Fike JA, Smith BJ, Hunter CT, Reed RE, Hart KM. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. PLoS ONE. 10(4): e0121655

Hunter ME, Ferrante JA Migs-Friend G, lmer A. 2019. Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. Scientific Reports. 9: 5259

Jane SF, Wilcox TM, Mckelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR. 2015. Distance, flow, and PCR inhibition: eDNA dynamics in two headwater streams. Molecular Ecology Resources. 15(1): 216-227

Jerde CL, Mahon AR, Chadderton WL, Lodge DM. 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA. Conservation Letters. 4(2): 150–157

Kelly RP, Port JA, Yamahara KM, Crowder LB. 2014. Using environmental DNA to census marine fishes in a large mesocosm. PLoS ONE. 9(1): e86175

Kelly RP. Shelton AO, Gallego R. 2019. Undertstanding PCR processes to draw meaningful conclusions from environmental DNA studies. Scientific Reports. 9: 12133

Kemp PS, Gessel MH, Williams JG. 2005. Seaward migrating subyearling Chinook salmon avoid overhead cover. Journal of Fish Biology. 67(5): 1381- 1391

Kinoshita G, Yonezawa S, Murakami S, Isagi Y. 2019. Environmental DNA collected from snow tracks is useful for identification of mammalian species. Zoological Science. 36(3): 198-207

Kintsch J and Cramer PC. 2011. Permeability of existing structures for terrestrial wildlife: a passage assessment system. WSDOT Research Report No WA-RD 777.1. Washington State Department of Transportation, Olympia, WA

Klymus KE, Richter CA, Chapman DC, Paukert C. 2014. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix.* Biological Conservation. 183: 77-84

Klymus KE, Marshall NT, Stepien CA. 2017. Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. PLoS ONE. 12(5): e0177643

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablance FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolutions in animals: amplification and sequencing with conserved primers. PNAS. 86(18): 6196-6200

Lance RF, Klymus KE, Richter CA, Guan X, Farrington HL, Carr MR, Thompson N, Chapman DC, Baerwaldt. 2017. Experimental Observations on the decay of environmental DNA from bighead and silver carps. Management of Biological Invasions. 8: 343-359

Leonard WP, Brown HA, Jones LC, McAllister KR, Storm RM. 1993. Amphibians of Washington and Oregon. Seattle Audubon Society. Seattle, Washington

Linacre A, Lee JC. 2016. Species Determination: The role and use of the *cytochrome b* gene. Methods in Molecular Biology. 1420: 287-296

Lodé T. 2000. Effect of a motorway on mortality and isolation of wildlife populations. Ambio. 29: 163-166

Long RA, Begley JS, MacKay P. 2012. Baseline Wildlife Monitoring at I-90 Snoqualmie Pass East, Prior to the Installation of Wildlife Crossing Structures. Final report to the Washington State Department of Transportation, Yakima, Washington. Western Transportation Institute, Montana state University, Bozeman, Montana. 270pp

MacPherson L, Sullivan MG, Foote AL, Stevens CE. 2012. Effects of culverts on stream fish assemblages in the Alberta Foothills. North American Journal of Fisheries Management. 32(3): 480-49

Mahlum S, Cote D, Wiersma YF, Kehler D, Clarke KD. 2014. Evaluating the barrier assessment technique derived from FishXing Software and the upstream movement of brook trout through rod culverts. Transactions of the American Fisheries Society. 143: 39-48

Marshalonis D and Larson C. 2018. Flow pulses and fine sediments degrade stream macroinvertebrate communities in King County, Washington, USA. Ecological Indicators. 93: 365-378

Maruyama A, Nakamura K, Yamanaka H, Kondoh M, Minamoto T. 2014. The release rate of environmental DNA from juvenile and adult fish. PLoS ONE. 9(12): e114639

Matsui M, Honjo M, Kawabata Z. 2001. Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. Aquatic Microbial Ecology. 26: 95–102

Matheson CD, Guerney C, Esau N, Letho. 2010. Assessing PCR inhibition from humic substances. The Open Enzyme Inhibition Journal. 2: 38-45

McCallum, M. 2007. Amphibian decline or extinction? Current decline dwarf background extinction rate. Journal of Herpetology. 41(3): 483-491

McKee AM, Spear SF, Pierson TW. 2015. The effect of dilution and the use of post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA sample., Biological Conservation, 183: 70-76

Meleg IN, Zakšek V, Fišer C, Kelemen BS, Moldovan OT. 2013. Can environment predict cryptic diversity? The case of *Niphargus* inhabiting western Carpathian groundwater. PLoS ONE. 8(10): e76760

Merkes CM, McCalla SG, Jensen NR, Gaikowski MP, Amberg JJ. 2014. Persistence of DNA in carcasses, clime and avian feces may affect interpretation of environmental DNA data. PLoS ONE. 9(11): e113346

Metter D. 1964. A morphological and ecological comparison of two populations of the tailed frog, *Ascaphus truei stejneger*. Copeia. 181-195

Mizumoto H, Urabe H, Kanbe T, Fukushima M, Araki H. 2018. Establishing an environmental DNA method to detect and estimate the biomass of Sakhalin taimen, a critically endangered Asian salmonid. Limnology. 19(2): 219-227

Moyer GR, Díaz-Ferguson E, Hill JE, Shea C. 2014. Assessing environmental DNA detection in controlled lentic systems. PLoS ONE. 9(7): e103767

Muck J.2010. USFWS. Biological Effects of sediment on bull trout and their habitat-guidance for evaluating effects. Lacey, WA

Nislow KH, Hudy M, Letcher BH, Smith EP. 2011. Variation in local abundance and species richness of stream fishes in relation to dispersal barriers: implications for management and conservation. Fresh Water Biology. 56: 2135-2144

Norman JR, Hagler MM, Freeman MC, Freeman BJ. 2009. Application of multistate model to estimate culvert effects on movement on small fishes. Transactions of The American Fisheries Society. 18(4):826- 838

Orchard SA .1992. Amphibian population declines in British Columbia. Declines in Canadian amphibian populations: designing a national monitoring strategy. CA Bishop and KE Petit, eds., Canadian Wildlife Service, 10-13

Palmeri-Miles A. 2012. Seasonal Movement Patterns and Overwintering of Western Toads (*Anaxyrus boreas*) Near Snoqualmie Pass, WA. All Master's Thesis, Central Washington University.

Parrish C. 2017. Upper Yakima Basin Bull Trout eDNA. Technical Report. Mid-Columbia Fisheries Enhancement Group, WA

Pilliod DS, Goldberg CS, Laramie MB, Waits LP. 2013a. Application of environmental DNA for inventory and monitoring of aquatic species: U.S. Geological Survey Fact Sheet 2012-3146

Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2013b, Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Science. 20: 1123-1130

Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2014. Factors influencing detection of eDNA from a stream dwelling amphibian. Molecular Ecology Resources. 14(1):109-116

Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2018. Factors influencing detection of eDNA from a stream dwelling amphibian. Molecular Ecology Resources. 14(1):109-116

Rahel FJ. 2007. Biogeographic barriers, connectivity and homogenization of freshwater faunas: it's a small world after all. Freshwater Biology. 52: 696–710

Rees HC, Bishop K, Middleditch DJ, Patmore JRM, Maddison BC, Gough KC. 2014. The application of eDNA for monitoring of the Great Crested Newt in the UK. Ecology and Evolution. 4(21): 4023–4032

Richter DB, Braun DP, Mendelson MA, Master LL. 1997. Threats to imperiled freshwater fauna. Conservation Biology. 11(5): 1081-1093

Rieman E, Peterson JT, Myers DL. 2006. Have brook trout (*Salvelinus fontinalis*) displaced bull trout (*Salvelinus confluentus*) along longitudinal gradients in central Idaho streams? Canadian Journal of Fisheries and Aquatics Sciences. 63(1): 63–78

Robson HLA, Noble TH, Saunders RF, Robson SKA, Burrows DW, Jerry DR. 2016. Fine tuning or the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystem. Molecular Ecology Resources. 16(4): 922–932

Robinson AT, Paroz YM, Clement MJ, Franklin TW, Dysthe JC, Young MK, McKelvey KS, Carim KJ. 2019. Environmental DNA sampling of small bodied minnows: performance relative to location, species and traditional sampling, North American Journal of Fisheries Management. 39: 1073–1085

Sagar J. 2004. Movement and Demography of Larval Coastal Giant Salamanders (*Dicamptodon tenebrosus*) in Streams with Culverts in the Oregon Coast Range. Master's Thesis, Oregon State University.

Sakata MK, Yamamoto S, Gotoh RO, Miya M, Yamanaka H, Minamoto T. 2020. Sedimentary eDNA provides different information on timescale and fish species composition compared with aqueous eDNA. Environmental DNA. https://doi.org/10.1002/edn3.75

Salter I, Joenson M, Kristiansen R, Steingrund P, Vestergaard. 2019. Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. Communications Biology. 2: 461

Scherer RD, Muths E, Noon BR, Corn PS. 2005. An Evaluation of Weather and Disease as Causes of Decline in Two Populations of Boreal Toads. 2005. USGS Staff -- Published Research. Paper 109.

Schwindt AR, Winkleman DL, Keteles K, Murphy M and Vajda AM. 2014. An environmental oestrogen disrupts fish population dynamics through direct and transgenerational effects in survival and fecundity. Journal of Applied Ecology. 51(3): 582-591

Shogren AJ, Tank JL, Andruszkiewicz, Olds B, Mahon AR, Jerde CL, Bolster D. 2017. Controls on eDNA movement in streams: transport, retention and resuspension. Scientific Reports 7. 5065

Sigsgaard EE, Carl H, Moller PR, Thomsen PF. 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biological Conservation. 183: 46–52

Smart AS, Tingley R, Weeks AR, Van Rooyen AR, McCarthy MA. 2015. Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader, Ecological Applications. 25(7): 1944-1952

Smith L, 2017, Seasonal comparison of environmental DNA and traditional sampling techniques for coastal tailed frogs (*Ascaphus trueii*) in Northern California. Thesis and Projects, Humboldt. digitalcommons.humboldt.edu/etd/42

Spear SF, Groves JD, Williams LA, Waits LP. 2015. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobanchus alleaniensis*) monitoring programme. Biological Conservation. 183: 38-45

Species at Risk Committee. 2014. Species status report for Western Toad (*Anaxyrus boreas)* in the Northwest Territories. Species at Risk Committee, Yellowknife, NT.

Stoeckle BC, Beggel S, Cerwenka AF, Motivans E, Eist J. 2017. A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. PLoS ONE. 12(12): e0189119

Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z. 2012. Estimation of fish biomass using environmental DNA. PLoS ONE. 7(4): e35868

Taylor DD, Goldingay RL. 2010. Roads and wildlife: impacts, mitigation and implications for wildlife management in Australia. Wildlife Research. 37(4): 320- 331

Terrell VCK, Engbrecht NJ, Pessier AP, Lannoo MJ. 2014. Drought reduces chytrid fungus (*Batrochochytrium dendrobatidis*) infection intensity and mortality but not prevalence in adult crawfish frogs. Journal of Wildlife Diseases. 50(1): 56-62

Thomsen PF, Kielgast J, Iversen LL, Wiuf C, Rasmussen M, Gilbert TP, Orlando L, Willerslev E. 2012a. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology. 21(11): 2565-2573

Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E. 2012b. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS ONE. 7(8): e41732

Trombulak SC, Frissel CA. 2000. Review of ecological effects on roads. Conservation Biology. 14(1): 18-30

Turner CR, Uy KL, Everhart RC. 2015. Fish environmental DNA is more concentrated in aquatic sediments than in the water, Biological Conservation. 183: 93 - 102

U.S. Fish and Wildlife Service. 2015. Recovery plan for the coterminous United State population of bull trout (*Salvelinus confluentus*). Portland, Oregon. xii + 179 pages.

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F, Gaboriaud C, Jean P, Poulet N, Roset N, Copp GH, Geniez P, Pont D, Argillier C, Baudoin JM, Peroux T, Crivelli AJ, Olivier A, Acqueberge M, Le Brun M, Møller PR, Willerslev E, Dejean T. 2015. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology. 25(4): 929-942

Van der Ree R, Jaeger JAG, Van der Grift EA, Clevenger AP. 2011 Effects of roads and traffic on wildlife populations and landscape function: road ecology is moving towards larger scales. Ecology and Society. 16(1): 48

Vorosmarty CJ, McIntyre PB, Gessner MO, Dudgeon D, Prusevich A, Green P, Glidden S, Bunn SE, Liermann CR, Davies PM. 2010. Global threats to human water security and river biodiversity. Nature. 467: 555-561

Voyles J, Rosenblum EB, Berger L. 2011. Interactions between *Batrochochytrium dendrobaditis* and its amphibian hosts: a review of pathogenesis and immunity, microbes and infection. Microbes and Infection. 13(1): 25- 32

Walker SF, Salas MB, Jenkins D, Garner TWJ, Cunningham AA, Hyatt AD, Bosch J, Fisher MC. 2007. Environmental detection of *Batrochochytrium dendrobatidis* in a temperate climate. Diseases of Aquatic Organisms. 77(2): 105-112

Warren ML, Pardew MG. 1998. Road crossings as barriers to small stream fish movement. Transactions of the American Fisheries Society. 127: 637-644

Washington Department of Transportation (WSDOT). 2006. Recommended Package: Interstate 90 Snoqualmie Pass East Mitigation Development Team. Washington State Department of Transportation, Yakima, WA.

Washington Department of Transportation (WSDOT), 2008. Environmental Impact Statement- I-90 Snoqualmie Pass East, Federal Highway Administration. Washington State Department of Transportation, Yakima, WA

Washington Department of Fish and Wildlife. 2015. Washington's State Wildlife Action Plan: 2015 Update. Washington Department of Fish and Wildlife, Olympia, Washington, USA.

Weins JJ. 2016. Climate related local extinctions are already widespread among plant and animal species. PLoS Biol. 14(12): e2001104

Wellman JC, Combs DL, Cook SB. 2000. Long term impacts of bridge and culvert constructon on replacement on fish communities and sediment characteristic of streams. Journal of Freshwater Ecology. 15: 317-328

Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, et al. 2013. Robust detection of rare species using environmental DNA: The importance of primer specificity. PLoS ONE. 8(3): e59520

Wild, TC, Bernet, JF, Westling EL, and Lerner, DN. 2010, Deculverting: Reviewing the evidence on the 'daylighting' and restoration of culverted rivers. Water and Environment Journal. 23(3): 412-421

Wind EL, Dupuis LA. 2002. COSEWIC status report on the western toad *Bufo boreas* in Canada. Committee on the Status of Endangered Wildlife in Canada. Ottawa

Wineland SM, Arrick RF, Welch SM, Pauley TK, Mosher JJ, Apodaca JJ, Olszack M, Holmes JN, Waldron JL. 2019. Environmental DNA improves Eastern Hellbender (*Cryptobranchus allganiensis* alleganiensis) detection over conventional sampling methods. Environmental DNA. 1(1): 86-96

Wissmar RC, Craig S. 1997. Bull trout spawning activity, Gold Creek, Washington. Fisheries Research Institute, University of Washington

Wofford JEB, Gresswell RE, and Banks, MA. 2005. Influence of barriers to movement on within-watershed genetic variation of coastal cutthroat trout. Ecological Applications. 15(2): 628–37

Yates MC, Fraser DJ, Derry AM. 2019. Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. Environmental DNA. 1(1): 5-13

Young MK, Isaak DJ, McKelvey KS, Schwartz MK, Carim KJ, Wilcox TM, Taylor M, Franklin TW, Chandler GL, Greaves SE, Parkers-Payne SL, Sharon L, Horan DL, Wollrab SP. 2017. Species occurrence data from the Range-Wide Bull Trout eDNA Project. Fort Collins, CO: Forest Service Research Data Archive. Updated 08 November 2019. https://doi.org/10.2737/RDS-2017-0038

Young MK, Isaak DJ, Schwartz MK, McKelvey KS, Nagel DE, Franklin TW, Greaves SE, Dysthe JC, Pilgrim KL, Chandler GL, Wollrab SP, Carim KJ, Wilcox TM, Parkers-Payne SL, Horan DL. 2018. Species Occurrence data from eDNAtlas database. Fort Collins, CO: Forest Service Research Data Archive. Updated 08 November 2019 https://doi.org/10.2737/RDS-2018-0010