INVESTIGATION OF eDNA AIR SAMPLING AS A TOOL FOR BAT CONSERVATION AND MANAGEMENT

by

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B.Sc., Simon Fraser University, 2009

A THESIS SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF ENVIRONMENTAL SCIENCES

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June 2025

Thompson Rivers University

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Abstract

Amidst the global biodiversity crisis, efforts to assess population trends for bat species are hampered by a lack of data. Bats are cryptic, nocturnal, and may leave no visible evidence of their roosting. In addition, some species use habitats that are unfeasible or unsafe to enter to conduct survey work. Environmental DNA, or eDNA, survey methods involve sampling environmental media (water, soil, air) and testing for exogenous DNA shed by organisms into their environments to infer species presence. eDNA air sampling is the active collection and filtration of air as a source of eDNA and it has been demonstrated to have utility for surveying tropical bat roosts. This study assesses the readiness of eDNA air sampling, paired with both target-taxa assays and metabarcoding, as a tool for bat conservation and management in a western North American context. eDNA air sampling methods successfully captured bat eDNA in mixed-*Myotis* maternity roosts, an artificial bat cave used as a night roost, and at a known underground mine hibernaculum in winter. This is the first field validation of three new target-taxa eDNA assays developed for western *Myotis* bats. Results of this study are as follows: validation of a genus-wide *Myotis* assay to the level of operational for routine species monitoring; essential validation completed for a species-specific assay for the federally Endangered *Myotis lucifugus*; field validation of a species-specific assay for a morphologically similar co-occurring species, *M. yumanensis*. However, this assay did not successfully detect *M. yumanensis* DNA in any environmental samples so redesign and further laboratory validation is required. Information obtained via eDNA air sampling complemented bat activity patterns recorded using a traditional passive acoustic monitoring approach. With further optimization of eDNA capture from air in low bat abundance and activity sites, eDNA air sampling has the potential to become an efficient, non-invasive, and sensitive way to identify subterranean or inaccessible bat habitats and document species presence in mixed-species roosts.

Keywords: environmental DNA, airborne eDNA, *Chiroptera*, *Myotis*, field validation

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Acknowledgements

I am so grateful for the unwavering support and patience of my family throughout this journey. Braden, thank you for always saying yes, and for being a calming presence and a picker-upper of slack. Madeleine and Nate, I love you to the moon and am so excited to see the impact you will make on this world. Mom, thank you for showing me it is ok to pursue higher learning in motherhood. Dad, thank you for teaching me the value of nature and the importance of balance in all things.

Many thanks are owed to many people who helped make this research project possible. Thank you to Dr. Cori Lausen for first letting me hold a bat! And for all the knowledge you have shared with me ever since. I am incredibly grateful that I have the opportunity to work with and learn from you. Thank you to Dr. Lauch Fraser for taking a chance on a bat project and welcoming me into your lab. Your guidance navigating this process, confidence in my abilities, and thoughtful feedback are greatly appreciated. Thank you to Dr. Naowarat (Ann) Cheeptham for your guidance in all things microscopic, for including me in the amazing teaching ecosystem you have created at TRU, and for the real talk about research and family life. Thank you also to all the members of the Fraser Lab, for your ongoing support, feedback, and good company! I would like to sincerely thank the external examiner Dr. Michael Russello for offering insightful questions and contributing a valuable perspective to the examining committee.

This project would not have come to fruition without the hard work of Dr. Caren Helbing and her lab at the University of Victoria, including Michael Allison, Dr. Louie Lopez, Anna Dema, and Sarah Trilesky. Thank you for answering all my emails and always being available to talk through logistics and results. Thank you to Caren for inspiring my eDNA journey and for all your encouragement. To Dr. Beth Clare, who pioneered eDNA air sampling for terrestrial wildlife, thank you for graciously lending me your air samplers, and for answering my many questions along the way. Thank you also to Dr. Faith Walker and the Bat Ecology and Genetics Lab for your work on the metabarcoding analysis.

Thank you to Skeetchestn Natural Resources for facilitating land access, for sharing your local knowledge, and for all your help in the field. I am grateful to the many mining professionals I met and worked with, especially those at New Gold Inc. and Teck Resources

Ltd., as well as Ron Barkwill, who shared their knowledge of the mining industry in our province. A special thank you to Tyne Roberts for helping the stars align on this research partnership and for supporting this work.

I am very lucky to have an amazing group of women in my corner. You all inspire me everyday, and I cannot thank you enough for being my sounding board and cheering section. I am thankful for the many former colleagues who I have had the opportunity to work with and learn from over my career so far. To Liliana Ortega, my bat sister, thank you for your passion, enthusiasm, good humour, hard work, and encouragement. To Shae, what a happy accident that we embarked on this mission at the same time. I am so appreciative of your support and friendship.

I gratefully received funding for this research from New Gold Inc., Teck Resources Limited, the Natural Sciences and Engineering Research Council of Canada Canadian Graduate Scholarship, Mitacs Accelerate, Economic Trust of the Southern Interior, the Canadian Federation of University Women, the B.C. Technical and Research Committee on Reclamation, the Habitat Conservation Trust Foundation, and in-kind support from Wildlife Conservation Society Canada.

This work was conducted under permits CB24-860599, KA24-861757, and SU24-864296 granted by the British Columbia Ministry of Water, Land, and Resource Stewardship. The work was approved by the Thompson Rivers University Animal Care Committee, protocol no. 103534.

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Chapter 1. Introduction

Our planet is facing a rapid and accelerating loss of global biodiversity, indicating that a mass extinction event is underway (Ceballos et al. 2015). Species extinctions are driven by human activities, particularly land conversion and habitat degradation (Dirzo et al. 2014, Newbold et al. 2015). Biodiversity loss affects the critical processes by which ecological communities capture resources and produce, decompose, and recycle essential nutrients (Cardinale et al. 2012). The global biodiversity crisis impacts human health and well-being via reductions in essential ecosystem goods and services (Cardinale et al. 2012, Johnson et al. 2017, Díaz et al. 2019). Action is needed to stall and reverse biodiversity loss to avoid irreparable changes to ecosystem functioning (Dirzo et al. 2014).

The Importance of Bat Conservation

Bats account for over one fifth of all mammalian biodiversity globally (Burgin et al. 2018, Frick et al. 2020, Mammal Diversity Database 2025). But over one third of all bat species assessed by the International Union for the Conservation of Nature (IUCN) are considered either threatened (15%) or data deficient (18%) (Frick et al. 2020). In North America, many bat populations are declining (ECCC 2018, COSEWIC 2023, Adams et al. 2024), with more than half of all species facing moderate to very high risk of extinction within 15 years (Adams et al. 2024). Key threats considered to have the greatest impact on North American bat populations are climate change and associated drought conditions, invasive species including the fungal pathogen responsible for white-nose syndrome (WNS), agriculture and related land conversion, and energy development, particularly construction of wind turbines (Adams et al. 2024).

In Canada, 14 of 17 species are considered by the IUCN as at-risk (i.e., vulnerable, imperiled, or critically imperiled) (Adams et al. 2024). The highest ranked threat facing bat populations in Canada is WNS (ECCC 2018, Cheng et al. 2021, Adams et al. 2024). This deadly disease has been documented in bats in nine provinces, (White-nose Syndrome Response Team 2024) and now threatens several species with regional or global extinction (Frick et al. 2010, ECCC 2018, Cheng et al. 2021, Adams et al. 2024). The fungal pathogen responsible for WNS, *Pseudogymnoascus destructans* (*Pd*), was detected in British Columbia

(B.C.) in 2022, in guano collected at a bridge roost in the Grand Forks area (Government of B.C. 2023). Though the disease itself has not yet been observed in any B.C. bats, with the presence of the pathogen confirmed, subsequent spread of the disease within B.C. bat populations is likely inevitable. Of Canada's 17 regularly occurring bat species, 15 occur in B.C, and there are 3 additional species that are considered accidental in the province (i.e., no current evidence of breeding populations) (Lausen et al. 2022). Five of B.C.'s bat species are considered Endangered (i.e., facing imminent extirpation or extinction) by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (Government of B.C. 2025). One species is Threatened (i.e., likely to become Endangered), one is Special Concern (i.e., sensitive to human activities or natural events), and one is Data Deficient (i.e., cannot be designated due to lack of information) (Government of B.C. 2025). Protection of bat habitat to support bat populations is challenged by bats cryptic nature, their need for roosts with specific microclimate characteristics which change throughout their annual cycle requiring frequent roost switching, the lack of information on where most bats hibernate in B.C., and the fact that bats often leave no visible evidence of their roosting, so identifying roosts is extremely difficult (Lausen et al. 2022). These factors hamper bat conservation and management efforts in the province.

As bats decline, the ecosystem services they provide will be reduced or lost. Globally, humans derive direct and indirect benefits from ecosystem services provided by bats such as suppression of arthropods including agricultural and forest pests, seed dispersal, pollination, and redistribution of nutrients via guano (Kunz et al. 2011). Insectivorous bats consume large quantities of coleopterans (beetles), dipterans (flies), hemipterans (true bugs), homopterans (cicadas and leaf hoppers), and lepidopterans (moths), including common and costly agricultural pests (Kunz et al. 2011). As an example of the scale of arthropod predation by bats, lactating female *Myotis lucifugus* (Little Brown Myotis) are estimated to consume more than 100% of their body mass per night (Kurta et al. 1989, Kunz et al. 2011). WNS has now killed millions of hibernating bats in North America. When bat insect consumption rates are extrapolated, the loss of one million bats would result in between 660 and 1,320 metric tonnes of insects no longer being consumed each year (Boyles et al. 2011). The loss of bats in North America could result in agricultural losses of more than $3.7 billion per year (Boyles et al. 2011). Concerningly, a recent natural experiment assessing U.S. counties where bat die-

offs from WNS had occurred found that following bat die-offs, farmers increased insecticide use by 31.1% on average, and subsequently human infant mortality from internal causes increased by 7.9% along the same disease spread pathway (Frank 2024).

Tools for Bat Conservation and Management

Global bat conservation efforts are hampered by a lack of data on bat populations compared to other mammals and birds (Frick et al. 2020). Of all bat species, 18% are considered data deficient by the IUCN, compared to 13% of other mammals and just 1% of birds (Frick et al. 2020). Additionally, 57% have unknown population trends, compared to 39% for other mammals and 8% for birds (Frick et al. 2020). Conventional methods to survey bat species have limitations. Bat capture provides valuable information on species, sex, and reproductive status, but requires specialized expertise, equipment, vaccinations, and often permits, and can be labour intensive. Acoustic bat surveys via ultrasonic bat detectors cannot always reliably distinguish between bat species due to overlapping call characteristics. For example, in B.C. an echolocation call with characteristic frequency near 40 kilohertz (kHz) could be one of several bat species, including the Endangered *Myotis lucifugus* (Little Brown Myotis) (Lausen et al. 2022). There is also an acoustic detection bias against species that produce low intensity echolocation calls. For example, bat species that inhabit the interior of tropical rainforest habitats produce faint calls that are not reliably captured on acoustic bat detectors (Frick et al. 2020). In B.C., *Corynorhinus townsendii* (Townsend's Big-eared Bat) produces soft echolocation calls and must echolocate close to a bat detector to be recorded (Lausen et al. 2022). Thus, acoustic surveys may miss or underrepresent certain bats, and cannot always characterize the full diversity of bat assemblages (Frick et al. 2020).

eDNA for the Study of Bats

Environmental DNA (eDNA) presents a novel approach to sampling the environment for exogenous DNA to determine whether a species occupies a certain habitat. eDNA emerged as a method for surveying macroorganisms in 2008, when Ficetola et al. first detected eDNA of American Bullfrog (*Lithobates catesbeianus*) in freshwater samples (Ficetola et al. 2008). Until recently, eDNA sampling efforts have focused on water sampling for detection of aquatic and semi-aquatic species. However, evidence is accumulating that eDNA sampling

also has utility for terrestrial wildlife (Leempoel et al. 2020, Matthias et al. 2021, Mena et al. 2021, Clare et al. 2022, Ryan et al. 2022, Lynggaard et al. 2022, Mas-Carrió et al. 2022).

Genomic methods for the study of bats has expanded in recent years with the advent of next generation sequencing (Garg et al. 2023). Genomic methods have been used to investigate the development of flight and echolocation, ageing, immunity, disease biology, and dietary diversification (Garg et al. 2023). However, adoption of genomic methods for biogeography, biodiversity assessments, and conservation has been slower (Garg et al. 2023). The increasing availability of whole genome assemblies of bat species within the last decade (Garg et al. 2023), has made the development of eDNA tools for detection of bat species possible. Initiatives like the Bat1K Project, the California Conservation Genomics Project, and the iTrackDNA Project are accelerating this progress (Teeling et al. 2018, California Conservation Genomics Project 2023, Capel et al. 2024, Curti et al. 2024, iTrackDNA 2025).

The study of bats using eDNA methods is very new, so applications are just beginning to emerge in the published literature. Bat DNA can be identified from pooled guano samples to determine presence of bat species and describe species assemblages using a *Chiropteran* Order-wide metabarcoding assay (Walker et al. 2016, 2019). eDNA air sampling is the active collection and filtration of environmental samples as a source of eDNA shed by organisms, which can be used to infer presence of taxa of interest. Filtration of air or water can provide sources of eDNA. eDNA from *Eptesicus fuscus* (Big Brown Bat) has been detected via quantitative real-time polymerase chain reaction (qPCR)-based assays using air and water sampled from a captive bat enclosure, from a water sample collected upstream from a mist-netting site, and from a sediment sample collected inside an occupied roost (Serrao et al. 2021). eDNA from *Leptonycteris nivalis* (Mexican Long-nosed Bat) has been detected via both metabarcoding and qPCR on agave plant flowers (*Agave* spp.), which provide a key nectar source during annual migration, and could be used to indicate migration pathways (Walker et al. 2022). In two separate studies, eDNA from arboreal vertebrates including bat taxa were detected inside tree hollows via roller swabs and sediment samples (Newton et al. 2022), as well as from tree bark via roller swabs and around the base of trees in soil samples (Allen et al. 2023). Collection of swabs from artificial bat boxes has very recently been shown to be a viable method for detecting eDNA from *Chalinolobus tuberculatus* (Long-

tailed Bat), in an attempt to determine bat box utilization when bats are not present at the time of survey (Davies et al. 2024).

Both Clare et al. (2022) and Lynggaard et al. (2022) concurrently demonstrated the potential for eDNA air sampling to document biodiversity of terrestrial wildlife communities. eDNA air sampling is hypothesized to have particular utility for the study of bats because they often roost in enclosed or semi-enclosed spaces (e.g., caves, mines, buildings) which may concentrate the eDNA signal in air (Clare et al. 2021, Garrett et al. 2023*a*), and unlike swabbing surfaces or collection of guano or sediment samples, air sampling may not require the surveyor to know exactly where bats are roosting within a particular roosting space. eDNA air sampling could prove particularly useful for the study of bat use of mines and caves, which are often inaccessible or unsafe to enter for surveys (Clare et al. 2022). Air sample collection and processing methods described by Clare et al. (2022) have been refined and applied to tropical bat roosts, first within a field station simulating a roost (Garrett et al. 2023*b*), and then within natural roosts (Garrett et al. 2023*a*). The potential of eDNA methods in general, and air sampling in particular, to become a valuable survey method for bats is considerable, given their nocturnal and cryptic nature, the difficulty in documenting their presence, and the challenges in identifying them to species.

Validating New eDNA Tools

As new eDNA methods are developed for routine monitoring of bats, both the field and laboratory components must be appropriately validated for the environments where they will be applied (Goldberg et al. 2016, Langlois et al. 2021). Extensive laboratory and field testing is required to determine the limits of detection, to estimate detection probabilities via statistical modelling, and to investigate environmental influences on detection (Thalinger et al. 2021). Pilot studies are necessary for any new combined field sampling and laboratory analysis approach, so optimal sampling designs can be determined and appropriate inferences about species presence can be drawn (Goldberg et al. 2016).

Dr. Caren Helbing (University of Victoria) and her team, who specialize in the design and validation of eDNA tools for Canadian wildlife, have developed three new targeted qPCR-based eDNA assays for bats. Each assay was developed following a multistep qPCR-based assay design and validation workflow (Langlois et al. 2021, 2025). Initial development and

laboratory validation was completed by the Helbing lab for three probe-based qPCR eDNA assays: a genus-wide assay for detection of *Myotis* bats known to occur in B.C. (eMyotis1), and species-specific assays for the Endangered *Myotis lucifugus* (Little Brown Myotis, eMYLU4), and *Myotis yumanensis* (Yuma Myotis, eMYYU7), which are morphologically similar and co-inhabit summer roosts in western North America (Lausen et al. 2022).

Confidence in the ability of eDNA air sampling to detect bats if they are present and accurately attribute samples to species or species groups is necessary for eDNA air sampling to be taken up as a useful tool for conservation and management. In addition, information on the origin, state, fate, and transport of bat eDNA within study systems is necessary for advanced field validation that informs our understanding of the target taxa and the environments inhabited by them (Barnes and Turner 2016).

GOAL AND OBJECTIVES

The goal of this study is to assess the readiness of eDNA air sampling as a tool for bat conservation and management in western North America. Each component of the work conducted progresses the overall validation of new targeted probe-based qPCR assays designed for B.C. bats, as well as our understanding of the potential usefulness of eDNA air sampling as a field method for detecting bats. Therefore, the objectives of this study are to:

1. Trial eDNA air sampling collection methods (Garrett et al. 2023*a*) and analysis approaches at known mixed *Myotis lucifugus* and *Myotis yumanensis* maternity roosts in southern B.C. (Chapter 2)
2. Test the feasibility and measure the sensitivity of eDNA air sampling for *Myotis* bats using a genus-wide qPCR-based *Myotis* assay in a simulated underground mine habitat with regular but low levels of bat activity (i.e., few bat passes per night) (Chapter 3)
3. Pilot winter eDNA air sampling at three underground hibernacula to investigate whether eDNA air sampling methods can detect an eDNA signal from *Myotis* bats and understand how eDNA methods compare to a traditional acoustic monitoring approach for assessing bat use of inaccessible underground habitats (Chapter 4).

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Chapter 2. Preliminary field validation of new *Myotis* bat qPCR-based eDNA assays via eDNA air sampling

INTRODUCTION

Recent advancements of genetic tools for species monitoring have opened the door to new and powerful methods for understanding species occurrence on the landscape. One such method is environmental DNA (eDNA) which detects the presence of exogenous DNA that organisms have shed into their habitats in environmental samples. This method allows for the collection of evidence of species presence in certain habitats without relying solely on traditional methods such as capture, visual detections, tracks, camera traps, and acoustic recorders. eDNA may be a useful tool for detecting the presence of rare, cryptic, and elusive species, like bats (Clare et al. 2021). Further, these tools could aid in resolving bat occurrences to species-level identifications, as some species, particularly some bats in the *Myotis* genus, are difficult to distinguish without capture. eDNA detection methods are proving useful in many areas including environmental impact assessments, routine species monitoring and inventory, and biosurveillance (Langlois et al. 2021).

For eDNA tools to be effective at differentiating between bat species, the DNA sequence used as primers and probes must be well conserved within the species of interest but distinct from other sympatric species (Lausen et al. 2008, Langlois et al. 2021, Thalinger et al. 2021). eDNA tools for targeted detection of rare species are primarily based on mitochondrial DNA (mtDNA) sequences because mtDNA is more suitable when analyzing degraded DNA often found in environmental samples (Duarte et al. 2023). mtDNA has many more DNA copies in each cell compared to nuclear DNA, it follows matrilineal inheritance conserving sequences within taxa, and there is greater availability of sequence data in genetic databases (Goldberg et al. 2016, Tsuji et al. 2019, Langlois et al. 2021, Duarte et al. 2023). However, phylogenetics of the *Myotis* bat genus is complex and the genetic trees based on mtDNA versus nuclear DNA for this clade conflict (Platt et al. 2018, Korstian et al. 2022). The conflict may be due to the rapid radiation of New World *Myotis* bats leading to introgressive hybridization, and incomplete lineage sorting (Platt et al. 2018, Korstian et al. 2022). The mtDNA genome of the Endangered *Myotis lucifugus* (Little Brown Myotis) (ECCC 2018) for example, shows more intraspecific variation than the nuclear genome (Lausen et al. 2008).

Therefore, eDNA tools for *Myotis* bats based on mtDNA must account for this intraspecific variation. A lack of carefully and transparently developed eDNA tools for bats may at best slow the adoption of genetic methods among conservation scientists and resource managers and at worst lead to misleading results (Langlois et al. 2021, Curti et al. 2024).

Probe-based eDNA assays used in quantitative real-time polymerase chain reaction (qPCR) are designed to target a highly specific region of DNA that differs between target versus non-target genomes and the amplification process facilitates DNA quantification; qPCR thus has become the most widely used eDNA analysis method for species-specific detection (Tsuji et al. 2019). In addition, qPCR is appealing as a method because it is cost effective, does not require bioinformatics expertise, and can adhere to existing standards to improve transparency and confidence in the resulting data (Helbing and Hobbs 2019, Tsuji et al. 2019, Langlois et al. 2021). However, design of effective targeted eDNA assays is limited by the availability of sequence data for the target species and confounding taxa (Langlois et al. 2021). qPCR-based eDNA assays are highly sensitive for the target sequences they were designed to detect. If the assay-specific region of target species DNA varies from the sequence that the assay is designed to anneal to, the DNA will not be amplified and thus not detected. When sequence data for eDNA assay design is lacking and the degree of intraspecific variation is unknown, as is the case for many bat species, newly developed assays must be rigorously tested against multiple vouchers specimens of both target and confounding taxa ranging across their geographic distribution to ensure assay specificity (Langlois et al. 2021, Thalinger et al. 2021).

eDNA assay design and validation follows six steps to ensure data quality, accuracy, and reliability (Langlois et al. 2021): 1) identification of phylogenies of target taxa as well as confounding taxa, 2) collection and alignment of DNA sequence data, 3) design of the primer and probe, 4) laboratory validation of the primer and probe, 5) laboratory validation of the assay via synthetic DNA, and 6) field validation (Klymus et al. 2020, Langlois et al. 2021, Lesperance et al. 2021, Allison et al. 2023). Dr. Caren Helbing (University of Victoria) and her team, who specialize in the design of eDNA tools for Canadian wildlife, have developed three new targeted qPCR-based eDNA assays based on bat specimens from western Canada (B.C. and Alberta). They have designed a genus-wide assay (eMyotis1) to detect DNA from

all *Myotis* bats, and species-specific assays for *M. lucifugus* (eMYLU4), and *M. yumanensis* (Yuma Myotis, eMYYU7). These two species are morphologically similar and co-inhabit roosts in summer in western North America (Lausen et al. 2022). Dr. Helbing and team completed the first five steps of assay design and validation for these three new qPCR-based eDNA assays (Langlois et al. 2025). This study is the start of the sixth step – field validation of the new assay with environmental samples (Langlois et al. 2021).

Another approach to analysing eDNA samples is using metabarcoding, which depends on universal PCR primers which anneal to highly conserved sequences across taxa (primer binding site), allowing high-throughput sequencing of a region that is highly variable between taxa (Harper et al. 2018). A DNA metabarcoding primer for bat Order *Chiroptera* has been developed based on mtDNA gene *cytochrome c oxidase subunit I* (*COI*) (Walker et al. 2016). Of the 15 species of bats known to occur in B.C., plus three accidental/unconfirmed species, most can be classified to species level with this primer except *M. thysanodes* (Fringed Myotis) versus *M. evotis* (Long-eared Bat), and *M. californicus* versus *M. ciliolabrum*, for which the *COI* sequences do not differentiate the species within each dyad (Bat Ecology and Genetics Lab 2025). A database of taxa that can be detected using the bat metabarcoding *COI* primer is available online: (www5.nau.edu/cefns/forestry/research/bats/public/index/).

Metabarcoding can provide data about multiple bat species in a sample. This is particularly informative in systems where limited or no prior study has occurred (Ruppert et al. 2019, Wood et al. 2019, McColl-Gausden et al. 2023). However, several studies have shown metabarcoding to be less sensitive than qPCR for detection of eDNA from targeted aquatic species in water samples (Harper et al. 2018, Bylemans et al. 2019, Wood et al. 2019, Schenekar et al. 2020, McColl-Gausden et al. 2023). Potential issues with metabarcoding that could lead to lower sensitivity of some species' for targeted species eDNA detection are primer bias, which is when some DNA sequences are amplified more efficiently than other sequences (Clarke et al. 2014, Elbrecht and Leese 2015, Walker et al. 2019, Schenekar et al. 2020), and species masking, which is when DNA from an abundant species dominates the PCR reaction and overpowers the signal from a low abundance species (Adams et al. 2013, Kelly et al. 2014, Evans et al. 2017, Harper et al. 2018). These issues apply most to highly

degraded and low quantity DNA, which is often the case with eDNA studies. A third key issue for detection of species via metabarcoding is that reference databases may be incomplete or inaccurate, leading to non-detections or misassignment of sequences to species (Nilsson et al. 2006, Wangensteen et al. 2018, Schenekar et al. 2020). Bat order *Chiroptera* is diverse and globally distributed, but due to their cryptic nature bats are relatively less studied compared to birds or other mammalian orders (Frick et al. 2020) and reference genomes are lacking (Curti et al. 2024).

Rigorously validated eDNA methods may provide an efficient, non-invasive, and sensitive approach to detect bat species presence. The objective for this study was to trial active eDNA air sample collection methods (Garrett et al. 2023*b*) and analysis approaches at known mixed *M. lucifugus* and *M. yumanensis* maternity colonies in southern B.C. eDNA detection results obtained from two sample analysis approaches, new qPCR-based eDNA assays and a published bat metabarcoding primer, were compared to the traditional bat species detection method of using acoustic bat detectors which record echolocation of bats within detection volume of an ultrasonic microphone. This study aims to validate the three newly designed *Myotis* bat eDNA assays. Here I present the first test of these assays on environmental samples.

METHODS

Study Areas

eDNA air samples were collected at known *Myotis* bat maternity colonies for the purpose of completing preliminary field validation of three newly designed qPCR-based assays to detect B.C. *Myotis* bats: eMyotis1, eMYLU4, and eMYYU7 (Langlois et al. 2025). Three known mixed *M. lucifugus* and *M. yumanensis* maternity colonies were selected in southern B.C. for sampling: Stave Lake Lodge, Tranquille Barn, and Peachland Attic (Figure 2.1). These three sites were selected based on accessibility of the roost for sampling, availability of data on colony composition, and geographic distribution of sites within southern B.C. to capture potential genetic variability. All three colonies are part of local long-term monitoring programs by conservation groups that have determined species presence at each roost site.

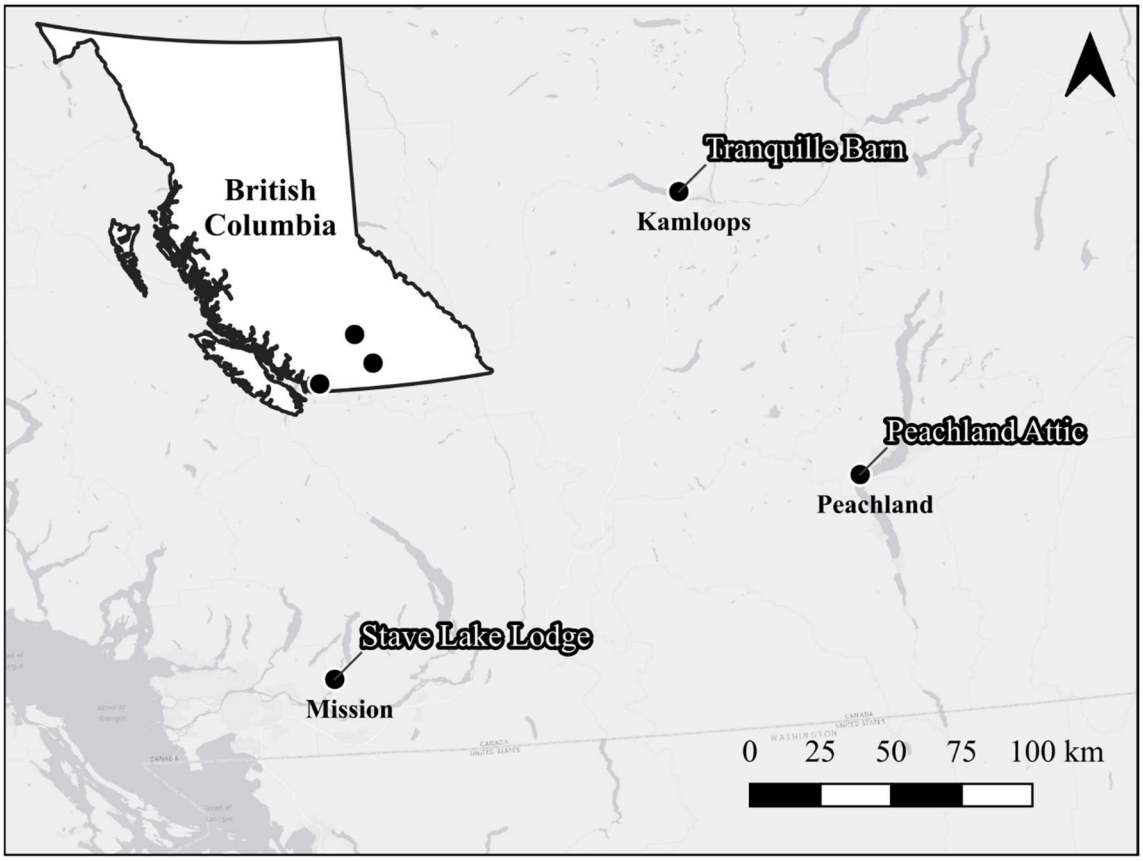

Figure 2.1 Preliminary eDNA air sampling sites at three known mixed *Myotis lucifugus* and *Myotis yumanensis* maternity colonies.

Stave Lake Lodge is an abandoned two-story building within a park on the shore of Stave Lake hydroelectric reservoir near Mission, B.C. The bat colony here roosts in an interior mechanical room in the building's basement, as well as two external bat boxes and elsewhere in the lodge (e.g., siding) (Figure 2.2A). The colony has been monitored by the B.C. Community Bat Program and Wildlife Conservation Society (WCS) Canada since 2017, with a total of 26 capture events between 2017 to 2023. The average number of bats (a mix of both *M. lucifugus* and *M. yumanensis*) at this roost was estimated to be approximately 150 bats (standard error, SE = 20; Community Bat Programs of B.C., unpublished data) through colony emergence counts, but mark-recapture analyses suggest the number of bats to be closer to 500 (J. Boulanger, Integrated Ecological Solutions, unpublished data). The ratio of each species in the Stave Lake roosts fluctuates as determined by acoustic monitoring, genetic testing of guano pellets, capture and Passive Integrated Transponders (PIT tags) (Community Bat Programs of B.C. & Wildlife Conservation Society Canada, unpublished

data). Some individuals belonging to the colony have been PIT-tagged and PIT-tag readers are located at the access point into the mechanical room as well as at one of the bat boxes to record movement of individuals.

Tranquille Farm is located at the outlet of the Thompson River to Kamloops Lake, near Kamloops B.C. The primary maternity roost at this site is an abandoned two-story horse barn, Tranquille Barn (Figure 2.2B), and bats roost within the walls, hay chutes, and ceiling rafters. The colony has been monitored by the Community Bat Programs of B.C. since 2013. A total of 22 emergence counts have been conducted at Tranquille Barn during the summer maternity period between 2013 and 2023. The average colony size based on these counts is 167 bats (SE = 20; Community Bat Programs of B.C., unpublished data). Based on capture records (2014, 2017, and 2018) this roost is assumed to be occupied primarily by *M. lucifugus* (113 confirmed individuals) with a small number of *M. yumanensis* (6 confirmed), and three that could not be differentiated between these two species. A total of 122 bats were captured (WSC Canada, unpublished data, D. Burles, Wildlife Biologist, unpublished data).

The Peachland Attic colony is located within the attic of the Historic Peachland Primary School, on the shore of Okanagan Lake, B.C (Figure 2.2C). The building has been restored and protected as a bat roost, housing both *M. lucifugus* and *M. yumanensis* (in attic), while functioning as the Peachland Visitors Centre and Art Gallery. The bats here are regularly monitored by the Bat Education & Ecological Protection Society (BEEPS), which conducts regular emergence counts during the maternity season and continuous video monitoring. A total of 56 emergence counts have been completed at this site since 2014, resulting in an average count of 1,194 bats (SE = 47; Community Bat Programs of B.C., unpublished data). Presence of both species within this roost was confirmed via genetic testing of guano in 2016 (two pellets *M. lucifugus*) and 2018 (one pellet *M. lucifugus* and one pellet *M. yumanensis*), as well as via acoustic monitoring (Community Bat Programs of B.C., and WCS Canada, unpublished data).

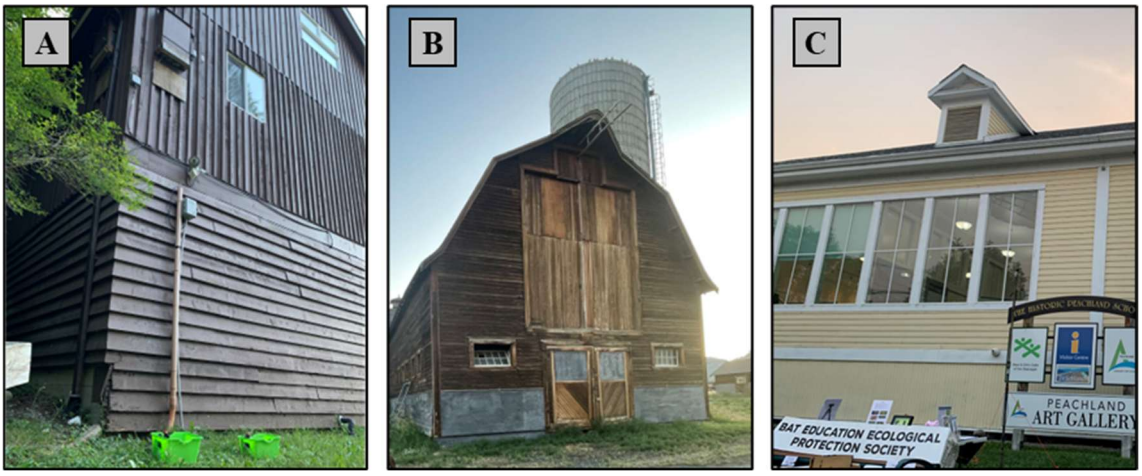

Figure 2.2 Stave Lake Lodge external bat box maternity roost (A), Tranquille Barn maternity roost (B), Peachland Attic maternity roost (C).

eDNA Air Sampling

Three air samples were collected from each site in late July or early August 2023, when roosts were occupied by bats (Table 2.1). An eDNA air sampler prototype design was used consisting of a 3D-printed filter frame and a computer fan, powered by a rechargeable 12 volt (V) battery (Garrett et al. 2023*a*) (Figure 2.3). Prior to deployment, each air sampler was cleaned with a 50% bleach solution followed by a triple rinse with deionized water to prevent contamination of the sampling surface. The filter frame of each air sampler was fitted with an approximately 12 centimeter (cm) by 12 cm piece of filter material (Filtrete 1900 Smart Air Filters "Merv 13" (3M, London, Ontario, Canada)), secured with a ring clamp. The air samplers have a circular sampling surface area with a 9 cm diameter and an estimated air draw (with filter) of 1.8 m/s (Garrett et al. 2023*a*). Each air sampler was placed inside an open plastic tub to keep the air samplers from directly contacting the floor of the roost. The plastic tubs were also cleaned with 50% bleach solution.

Table 2.1 Preliminary eDNA air sampling sites, locations, sampling dates, and times.

Site	Location (lat., long.)	Sampling Date	Local Sunset Time	Sample Deployment Time	Sample Collection Time	Total Sample Run Time
Stave Lake Lodge	49.2434 -122.3652	29-Jul-2023	20:54	20:00	00:15	4:15
Tranquille Barn	50.7225 -120.5148	30-Jul-2023	20:49	19:45	22:45	3:00
Peachland Attic	49.7772 -119.7341	04-Aug-2023	20:35	19:25	22:40	3:15

At Stave Lake Lodge, two air samplers were placed on the ground approximately 3.5 m below an occupied external bat box, and one was deployed on the floor inside the mechanical room which did not contain any bats at the time of sampler deployment but strongly smelled of bats and contained accumulated guano and urine staining. At Tranquille Barn, three air samplers were deployed on the floor in the upper story of the barn. One air sampler was placed adjacent to an accumulation of bat guano and below where two bats could be seen roosting in the ceiling rafters. A second air sampler was placed on the floor near the upper story doors, which have gaps between the wood planks that are used as a primary roost exit based on previous colony counts (L. Ortega, Thompson Region Community Bat Program Coordinator, personal communication). The third air sampler at this site was placed on the floor adjacent to a hay shoot within which bats could be heard at the time of sampler deployment. At the Historic Peachland Primary School, all three air samplers were placed on the floor of the south end of the attic: one near the south attic dormer (a main exit/entry point for bats), and two on either side of the main guano accumulation at the southern end of the attic. Most bats were roosting at the north end of the attic during air sampler deployment, but a group of approximately 250 bats were roosting at the south end of the attic above the air samplers at sample collection. Air samplers were deployed prior to bat emergence at each site, at approximately one hour prior to local sunset time (Table 2.1).

Filters were collected from each air sampler after the peak of bat emergence, once the air samplers had run for approximately three to four hours (Table 2.1). Each filter was placed in a brown paper envelope using forceps sterilized with 50% bleach solution and triple-rinsed with deionized water. All three envelopes were then placed into a plastic zipper top bag with one tablespoon of colour-indicating silica desiccant beads to remove excess moisture that could degrade the samples. All the filters were kept in a cooler with ice for transport from the field and then in a -20°C freezer for temporary storage prior to being shipped to the University of Victoria in a cooler with ice packs for laboratory analysis. A filter was removed from the package of unused filters at each site at the end of each sampling night, to act as a field blank. This field blank was placed inside a paper envelope with forceps and then inside a plastic bag with silica desiccant beads. The field blanks were transported from the field and all zippered bags were stored together.

DNA was extracted and evaluated for integrity and inhibition using IntegritE-DNA test. This test provides a measure of sample viability by assessing the ability of each sample to support amplification of plant chloroplast DNA (Hobbs et al. 2019). Samples that failed integrity testing were cleaned using Zymo OneStep PCR inhibitor removal kit to remove enzyme inhibitors that may block amplification. Once cleaned, samples that initially failed integrity testing were retested using IntegritE-DNA. This step mitigates the potential for false negative results due to poor sample quality (i.e., degraded DNA) or presence of inhibitors (Hobbs et al. 2019). Laboratory analysis of each integrity-confirmed eDNA air sample underwent two processes: 1) qPCR using the three new targeted assays (eMyotis1, eMYLU4, eMYYU7, see Table 2.2), following a well-established eDNA sample processing workflow (e.g., Hobbs et al. 2019, Hocking et al. 2022); 2) the remaining extracted DNA from all samples was pooled within each site and analyzed via bat-specific DNA metabarcoding.

Table 2.2 Newly designed probe-based qPCR eDNA assays for B.C. *Myotis* bats. Assay design and laboratory validation was completed by the Helbing laboratory, University of Victoria (Langlois et al. 2025).

Assay	**Target**	**Gene**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Probe sequence – IDT 5' FAM with ZEN quencher (5'-3')**
eMyotis1	*Myotis* genus	*MT-RNR1*	TTCGCCAGAGTACTACTA	GCTGATTTAGCAAAGATTG	TCGATTATAGAACAGGCTCCTCT
eMYLU4	*Myotis lucifugus*	*MT-ND5*	CCTACTCATCCGATTCTAC	GGTGGTAATTGCTCCTAG	AGAGCAATAAAATAATCCAATCCTTAGCCC
eMYYU7	*Myotis yumanensis*	*MT-ND4*	ATCGCATACTCATCTGTTAG	CCATGAAACTTCAGGGAC	TGAATTAAGACAGCCATAATCACAAGCG

For the qPCR process, each sample was tested with eight qPCR replicates for each target assay, eight negative controls (i.e., no DNA template), and two positive controls (i.e., containing *Myotis*, *M. lucifugus*, or *M. yumanensis* DNA depending on the assay run on a given plate). Target eDNA copy number per sample (including SE) was calculated by the Helbing lab based on standard curves of qPCR cycle threshold (Ct) versus eDNA copy number (Lesperance et al. 2021, Hocking et al. 2022). A sample was considered positive for containing target eDNA via qPCR if a minimum of two of eight PCR replicates amplified (Ct < 50) (Hobbs et al. 2019, Matthias et al. 2021). If only one PCR replicate amplified, the

sample was considered probable for containing target eDNA, with further testing required to confirm the result. If zero PCR replicates amplified, the sample was considered negative for target eDNA.

Once qPCR analysis was completed, the remaining extracted DNA from air samples within a site and location were pooled and sent to a collaborating laboratory at Northern Arizona University (Bat Ecology & Genetics Lab) for analysis via DNA metabarcoding (Walker et al. 2016, 2019).

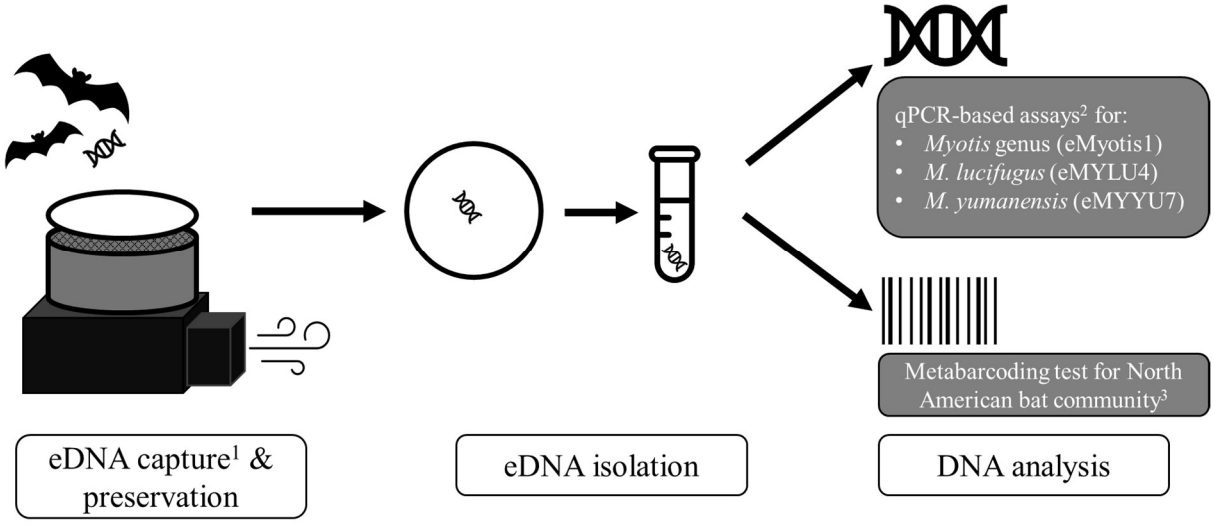

Figure 2.3 eDNA air sampling workflow diagram, including (1) eDNA capture (Clare et al. 2021, 2022, Garrett et al. 2023*a*, *b*) and preservation, eDNA isolation, and DNA analysis via (2) qPCR-based assays (Langlois et al. 2025) and (3) metabarcoding (Walker et al. 2016, 2019). A detailed description of air samplers used is available in Garrett et al. 2023b.

Acoustics

Acoustic monitoring was conducted at roost exits, concurrent to eDNA air sampling, using either an Echo Meter Touch 2 Pro or a Song Meter SM4BAT FS Ultrasonic Recorder with an external U2 ultrasonic microphone (Wildlife Acoustics, Maynard, MA, USA). Bat echolocation calls were recorded in full-spectrum format at each site to verify the presence of each species -- *M. lucifugus* and *M. yumanensis* – during the eDNA sampling period. Each species can be reliably distinguished by species-specific echolocation call traits when recorded in open environments (Lausen et al. 2022), but because acoustic recording was

taking place at roost exits, these were not open environments, making species-level differentiation more difficult. Characteristic frequency is the acoustic frequency at the end of the echolocation call body or the lowest slope portion of the echolocation pulse and it can be used to differentiate some bat species (Lausen et al. 2022). Individual echolocation pulses were binned into two groups based on characteristic frequency: from 35 to 45 kHz likely to be produced by *M. lucifugus* (Lausen et al. 2022), and 45 kHz and greater likely to be produced by *M. yumanensis* (Lausen et al. 2022). A study of 155 genetically confirmed individuals of *M. lucifugus* and *M. yumanensis* across Washington, Oregon, and California found that just 1.3% of *M. yumanensis* had characteristic frequencies less than 45 kHz (Weller et al. 2007). However, 8.6% of *M. lucifugus* had characteristic frequencies greater than 45 kHz (Weller et al. 2007), so this threshold may slightly underestimate *M. lucifugus* acoustic activity.

Acoustic files were first converted from full-spectrum to zero-cross format with no signal enhancement and then non-bat "noise" files were filtered out using Kaleidoscope Pro (Wildlife Acoustics). Filtering based on characteristic frequency was performed in AnalookW (Titley Scientific, Brendale, QLD, Australia). Pulses that did not meet the criteria of either frequency grouping were excluded from analysis as these pulses could be produced by other species. Using the scan function in AnalookW, all echolocation pulses that met the criteria of each frequency filter were counted and summed, giving a relative index of activity for each frequency grouping over the eDNA air sampling period.

RESULTS

eDNA Air Sampling

All three eDNA air sample replicates at all three sites returned strong positive results via qPCR for the genus-wide *Myotis* assay, eMyotis1 (Table 2.3), with eight of eight PCR replicates amplifying in all but two samples which had seven PCR replicates amplify (Stave Lake Lodge samples SLL-01A and SLL02A). All three samples at both the Tranquille Barn and the Peachland Attic sites also returned strong positive results for the *M. lucifugus* assay, eMYLU4 (Table 2.3). However, all three samples collected at Stave Lake Lodge were negative for *M. lucifugus* DNA. The *M. yumanensis* assay, eMYYU7, did not amplify DNA in any sample.

The field blanks collected at both Stave Lake Lodge and Peachland Attic were negative for all three assays; however, the field blank from Tranquille Barn tested positive for eMyotis1 and eMYLU4, suggesting that DNA from the site was transferred to the clean field blank filter during the collection process.

Table 2.3 Preliminary validation eDNA air sample qPCR results from three known *M. lucifugus*/*yumanensis* maternity roosts using a genus-wide assay for all *Myotis* bats (eMyotis1) and a species-specific assay for *Myotis lucifugus* (eMYLU4). All samples were negative (0 of 8 positive PCR replicates) when tested with an assay targeting *Myotis yumanensis* (eMYYU7). The values provided are number of technical qPCR replicates out of 8 that amplified DNA, the estimated eDNA copies per sample, and standard errors.

Site	eDNA Air Samples	eMyotis1			eMYLU4		
		Positive replicates (of 8)	eDNA copies per sample	Standard Error	Positive replicates (of 8)	eDNA copies per sample	Standard Error
Stave Lake Lodge	SLL-01A (beneath bat box)	7	153	75	0	0	0
	SLL-01B (beneath bat box)	8	56	15	0	0	0
	SLL-02A (mechanical room)	7	153	75	0	0	0
Tranquille Barn	TB-01A	8	30,027	1,561	8	1,164	38
	TB-01B	8	6,399	912	8	418	53
	TB-01C	8	16,157	443	8	1,048	80
Peachland Attic	PLAT-01A	8	42,269	1,608	8	932	50
	PLAT-01B	8	46,818	2,209	8	553	26
	PLAT-01C	8	32,630	1,641	8	626	26

Both *M. lucifugus* and *M. yumanensis* DNA sequences were detected in all pooled air samples submitted for DNA metabarcoding analysis (Figure 2.4,Table 2.4). DNA sequences from one additional bat species, *Eptesicus fuscus* (Big Brown Bat), were also detected in the Tranquille Barn pooled air sample with a low number of reads (four, Table 2.4). Five diagnostic acoustic recordings of potential *E. fuscus* (could not be differentiated from *Lasionycteris noctivagans*, Silver-haired Bat) were documented during the sampling period. *E. fuscus* is a common building-roosting species in B.C. (Lausen et al. 2022).

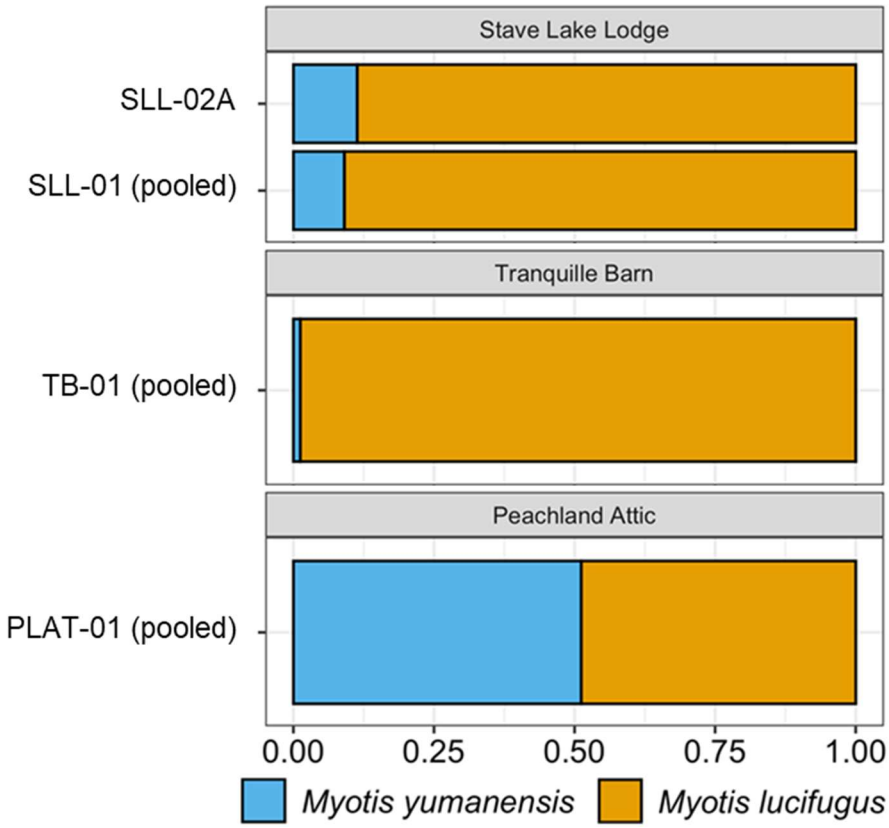

Figure 2.4 Bat species detected in pooled preliminary field validation samples via metabarcoding – sample analyses and figures prepared by Bat Ecology & Genetics Lab at Northern Arizona University. The bar plots represent the proportion of total DNA sequence reads by species, *Myotis lucifugus* and *Myotis yumanensis*, detected in each pooled eDNA air sample. Samples were pooled by site and roosts. *Eptesicus fuscus* was also detected in the TB-01 pooled sampled; however, the detection is not depicted in the figure because the number of DNA sequence reads was very small relative to the other two species detected.

Table 2.4 Bat species eDNA detected via metabarcoding in preliminary field validation eDNA air samples, pooled by site and roost. The values provided are total eDNA sequence read counts from DNA metabarcoding primer for *Chiroptera* based on a short section of mitochondrial DNA gene *cytochrome c oxidase subunit I* (*COI*) **(Walker et al. 2016)**. Laboratory analysis and bioinformatics were completed by the Bat Ecology & Genetics Lab, Northern Arizona University.

Site	Pooled eDNA Air Samples	Species DNA Detected	Number of DNA Sequence Reads	Proportion of Reads in Sample
Stave Lake Lodge	SLL-01A, SLL-01B (beneath bat box)	*M. lucifugus*	26,361	91%
		M. yumanensis	2,619	9%
	SLL-02A (inside mechanical room)	*M. lucifugus*	67,163	89%
		M. yumanensis	8,576	11%
Tranquille Barn	TB-01A, TB-01B, TB-01C	*M. lucifugus*	91,477	99%
		M. yumanensis	1,129	1%
		Eptesicus fuscus	4	<0.01%
Peachland Attic	PLAT-01A, PLAT-01B, PLAT-01C	*M. lucifugus*	54,501	49%
		M. yumanensis	57,099	51%

Acoustics

Echolocation calls of both species were recorded at all three sites in varying proportions during eDNA air sampling (Figure 2.5). These results reflect an index of activity of this species during the air sampling period but do not directly signify species abundance. The proportion of *M. lucifugus* acoustic activity relative to *M. yumanensis* acoustic activity was highest at Tranquille Barn (97:3), followed by Stave Lake Lodge (85:15), and then Peachland Attic (38:62) (Figure 2.5).

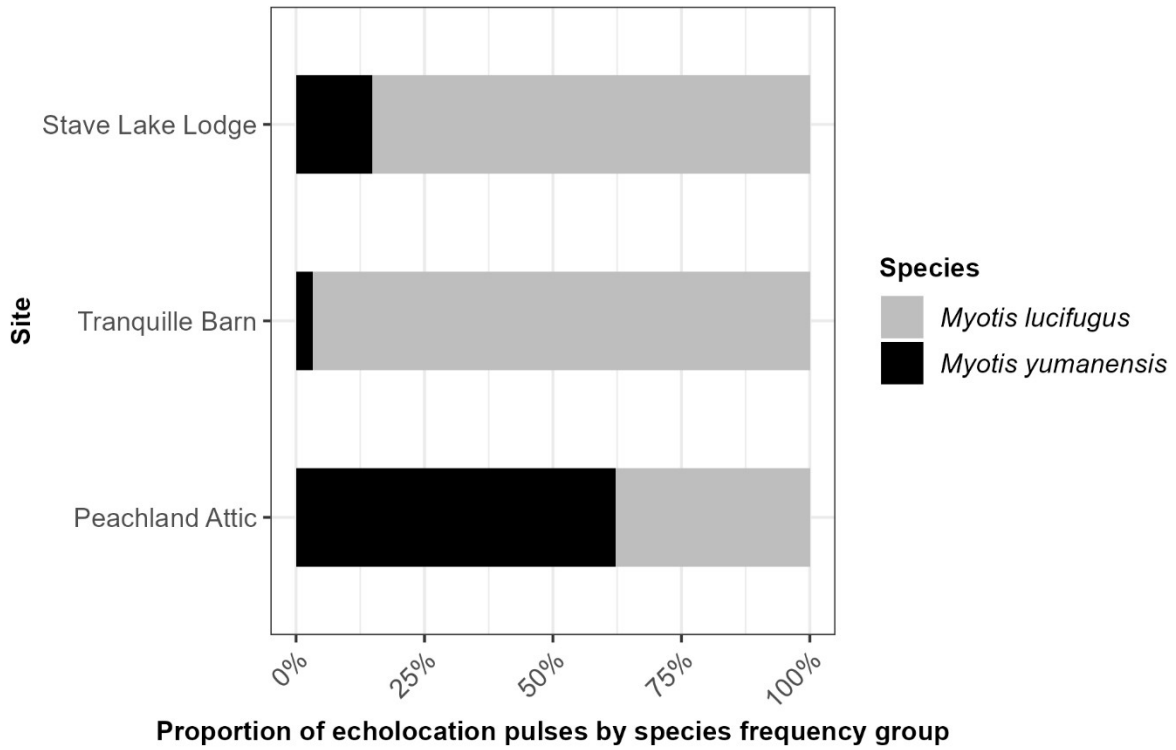

Figure 2.5 Proportion of echolocation pulses of *M. lucifugus* relative to *M. yumanensis* recorded at roost exits concurrent with eDNA air sampling. Calls were classified to frequency group based on characteristic frequency, in AnalookW (Titley Scientific). Pulses with a characteristic frequency greater than 35 and less than 45 kHz were classified as *M. lucifugus*, and those of 45 kHz or greater were classified as *M. yumanensis*.

DISCUSSION

The eDNA air sampling approach described by Garrett et al. (2023*b*) was successful for collecting *Myotis* eDNA from within B.C. maternity roosts. *Myotis spp.* eDNA was detectable via qPCR on all nine air sample filters collected, evidence that the genus-wide *Myotis* qPCR-based eDNA assay, eMyotis1, works to detect *Myotis* DNA in high DNA environments. This method was also sensitive enough to detect *Myotis* eDNA in two samples collected in open air approximately 3.5 m below an occupied bat box. Metabarcoding results for air samples pooled by site and roost confirmed that eDNA air sampling methods captured detectable amounts of *M. lucifugus* and *M. yumanensis* eDNA. All pooled samples had detectable amounts of eDNA for both species and read counts per species ranged from 1,129 (*M. yumanensis* at Tranquille Barn) up to 91,477 (*M. lucifugus* at Tranquille Barn) (Table 2.4).

The eMYLU4 assay successfully detected *M. lucifugus* DNA at two of three sites where they were known to roost, returning strong positive results at both Tranquille Barn and Peachland Attic (eight of eight PCR replicates amplified). These positive results align with the known colony composition and acoustic monitoring results (Table 2.5). However, all Stave Lake Lodge samples were negative for *M. lucifugus* DNA via the eMYLU4 assay. This result contradicts the acoustic monitoring, the capture record, and metabarcoding results (Table 2.5). There was evidence of successful capture of *Myotis* DNA on the filters as indicated by the strong positive detections via the eMyotis1 assay. One explanation for this apparent false negative result is that there could be a difference in assay sensitivity for eMYLU4 compared to eMyotis1. The number of eDNA copies per sample estimated via eMyotis1 was one order of magnitude higher than via eMYLU4 for all samples where *M. lucifugus* was detected. Less *Myotis* DNA overall was captured on the filters at Stave Lake Lodge compared to the other two sites, so it is possible that not enough *Myotis* DNA was captured for eMYLU4 to amplify *M. lucifugus* eDNA at the Stave Lake Lodge site.

Table 2.5 Summary of species historic presence as observed by conservation groups and current detections by site and detection method. An acoustic monitoring detection is a recording of one or more echolocation pulses assigned to frequency group based on characteristic frequency in AnalookW. A qPCR detection is ≥2 PCR replicates amplifying out of 8 versus not detected is 0 PCR replicates amplified. A metabarcoding detection is presence of DNA sequences in the air samples pooled by site that were assigned to each species.

Species	Site	Historic Monitoring	Acoustic Monitoring	eDNA Air Sampling	
				qPCR	Metabarcoding
M. lucifugus	Stave Lake Lodge	Present	Detected	Not detected	Detected
	Tranquille Barn	Present	Detected	Detected	Detected
	Peachland Attic	Present	Detected	Detected	Detected
M. yumanensis	Stave Lake Lodge	Present	Detected	Not detected	Detected
	Tranquille Barn	Present, but in low numbers	Detected	Not detected	Detected
	Peachland Attic	Present	Detected	Not detected	Detected

A second explanation for the false negative result from the eMYLU assay at Stave Lake Lodge is that *M. lucifugus* individuals in this maternity roost may have genetic differences from those at Tranquille Barn and Peachland Attic that were not captured in assay design.

There are six recognized subspecies of *M. lucifugus*, with three occurring in B.C.: *M. lucifugus alascensis* (Miller 1897) found throughout B.C., *M. lucifugus carissima* found in the dry southern interior (Thomas 1904), and *M. lucifugus lucifugus* (Le Conte 1831) found in the far north of B.C. (Lausen et al. 2022). The subspecies distinctions are originally based on pelage colour and forearm length (Miller 1897; Thomas 1904; Le Conte 1831), and no morphological distinction could be found among a purported mixed-subspecies colony despite large mtDNA genetic differences noted, presumably aligning with two different subspecies (Lausen et al. 2008). Because mtDNA underpins eDNA assay design, it is possible that not all subspecies of *M. lucifugus* are recognized by the eMYLU4 assay. This assay is designed based on the *mt-ND5* gene region (Langlois et al. 2025), which is part of the D-loop and shows high intraspecific diversity (Tsuji et al. 2019). The reference genome used for assay design was from a specimen captured in Ottawa, Ontario (J. Zhang, Institute of Ecology, Zhejiang Normal University, personal communication) (Qian and Zhang 2023) and thus possibly belonged to different subspecies than individuals at Stave Lake roosts; the eMYLU4 assay may not account for the intraspecific variation in the mtDNA of *M. lucifugus* and would benefit from further laboratory validation against specimens representing a broader range of geographies to rule out false negatives across subspecies or haplogroups. This result aligns with findings of Serrao et al. (2021) who developed three separate qPCR-based assays for *Eptesicus fuscus* to detect three distinct haplogroups within the species. They suggest that high intraspecific variation must be accounted for in assay design to avoid false negative results (Serrao et al. 2021).

The species-specific qPCR-based assay for *M. yumanensis*, eMYYU7, appears to have returned false negative results at all three sites based on positive acoustic and metabarcoding detections for this species (Table 2.5). These results also highlight the need for field validation of eDNA tools in the geographic regions where assays are intended to be used prior to initiating large-scale field sampling. The eMYYU7 assay was designed based on a reference genome from an individual captured in Brewster County, Texas (D. Ray, Texas Tech University, personal communication) (Platt et al. 2023). The eMYYU7 assay did not amplify eDNA from relevant *M. yumanensis* populations, suggesting a possible genetic difference between the specimens on which the assay was designed and tested in the laboratory, and the individuals in the colonies sampled in the field. There are currently six

putative subspecies recognized for *M. yumanensis*, two of which are found in B.C.: a coastal subspecies called *M. yumanensis saturatus* (Miller 1897) and an interior subspecies called *M. yumanensis sociabilis* (Grinnell 1914). Distinction of subspecies was defined on pelage colour. It is unknown whether these divisions are supported by genetic differences; this work has not yet occurred (Lausen et al. 2022, Curti et al. 2024). Inclusion of data from more reference specimens in assay design will improve the efficacy of the assay across geographies and potential subspecies.

Note that the PIT-tag reader at the Stave Lake Lodge mechanical room roost exit recorded six PIT-tag reads of three *M. lucifugus* individuals and four PIT-tag reads of one *M. yumanensis* during the air sampling period (WCS Canada, unpublished data) suggesting there were undetected day-roosting bats in the mechanical room, or they entered the roost at the beginning of the night. Either way, these PIT-tag reads provide further evidence of presence of both species and false negative results for both species via the qPCR-based assays.

The amount of *Myotis* eDNA captured on the filters at Stave Lake Lodge was one order of magnitude lower than the Tranquille Barn and Peachland Attic samples, as indicated by the estimate of eDNA copies per sample when tested using eMyotis1 (Table 2.3). This result is likely due to the Stave Lake Lodge samples being collected in open air beneath a bat box (SLL-01A and SLL-01B) and in a mechanical room that appeared to be unoccupied by bats during the day immediately prior to air sampling (SLL-02A). In contrast, the Tranquille Barn and Peachland Attic samples were collected from indoor environments where bats day-roosted prior to and during the air sampling period. It is suspected that the eDNA signal in air diffuses more quickly in open air than indoor environments and is weaker if the roost is not occupied directly prior to air sampling, and/or by few individuals. This result aligns with findings from eDNA air samples collected inside and outside animal enclosures in a zoo, where DNA read counts were higher inside an animal's enclosure than outside the enclosure (Clare et al. 2022). These results also suggest that in open air and low DNA environments, air sample collection periods of longer than three to four hours would be advisable. Alternatively, larger air sampling devices that filter greater volumes of air might be considered in some situations where eDNA is expected to be in very low concentration.

Finally, eDNA methods are highly sensitive and are susceptible to contamination if strict quality control procedures are not adhered to (Goldberg et al. 2016, Helbing and Hobbs 2019). The risk of accidental contamination of samples is high in high eDNA environments such as maternity roosts. At these sites, bats, guano, and urine were abundant, and eDNA could easily transfer from the roost itself to field equipment and personnel during air sampler deployment and collection, as indicated by the field blank from Tranquille Barn testing positive for *Myotis* bat and *M. lucifugus* DNA. The eDNA on the field blank was likely picked up by personnel and equipment while inside the roost and transferred onto the filter. This result highlights two potential issues that should be considered for future applications. First, eDNA accidentally transferred to filters from touching surfaces at high eDNA sites like roosts may represent an older eDNA signal (e.g., from guano deposited over time) rather than recent bat presence, which has implications for the interpretation of results. The risk of accidental transfer of eDNA from surfaces onto air sample filters during collection would be greatly reduced in low DNA environments where eDNA methods are more likely to be applied. Second, if surveyors intend to sample multiple roosts, extra caution must be taken to prevent the transfer of eDNA from one roost to the next via equipment and personnel. Generally these protocols align with what is already required for WNS decontamination (B.C. Ministry of Water, Lands and Resource Stewardship and B.C. Ministry of Forests 2017, Canadian Wildlife Health Cooperative 2017). In this case, the field blank was collected at the end of the eDNA sampling period. Perhaps it would be more informative to collect a field blank at each site prior to entering the roost, as a check that equipment was properly decontaminated between sites. Keeping eDNA sampling equipment separated from bat handling equipment will be important.

The samples collected served to validate genetic analysis of filtered air for effective collection of *Myotis* eDNA at occupied B.C. maternity roosts. *Myotis* genus eDNA was highly detectable via the eMyotis1 assay and both *M. lucifugus* and *M. yumanensis* eDNA were identifiable in pooled samples via metabarcoding. In this study, samples were pooled for metabarcoding to maximize detection because other studies have shown that metabarcoding can be less sensitive than qPCR for species detection (Harper et al. 2018, Bylemans et al. 2019, Wood et al. 2019, Schenekar et al. 2020, McColl-Gausden et al. 2023). However, the Stave Lake mechanical room sample (SLL-02A) was not pooled as the other

samples from this roost were collected outside the building, and even though it had one of the lowest estimated *Myotis* genus eDNA copy number via the eMyotis1 assay (153 copies, SE=75) (Table 2.3), both *M. lucifugus* and *M. yumanensis* DNA were detected via metabarcoding (Table 2.4). This result indicates that pooling samples within sites for species detection via metabarcoding was likely not necessary in this study and that the sensitivity of the bat metabarcoding primer was adequate to detect both focal species at all sites. Metabarcoding provided more effective species detection than the targeted qPCR-based assays in this study. How the qPCR-based assays perform in low eDNA environments has yet to be tested (but see Chapter 3).

The results demonstrate that analyzing eDNA samples via metabarcoding can be useful for detection of bat species at roosts, but metabarcoding has several potential issues that have not been fully assessed for the bat metabarcoding primer. Walker et al. (2016) point out that there may be specimen misidentification within the reference database which could lead to mislabelled voucher sequences and thus misidentified genetic samples. Primer bias has not been evaluated for *Myotis* species but has been detected for one species, *Tadarida brasiliensis* (Mexican Free-tailed Bat), via a mock community composition trial (Walker et al. 2019). Both focal species were detected via metabarcoding in all pooled samples in this study so primer bias did not affect the results; however, potential primer bias could impact future efforts to estimate relative abundance of species via eDNA metabarcoding and should be evaluated. Species masking during metabarcoding could result in false negatives in cases where a rare or low abundance species co-inhabits a roost with an abundant species. Again, species masking did not affect the results because DNA from both focal species and from *E. fuscus* were detected, which, based on acoustics, was relatively rare at the site; however, if individuals of another *Myotis* species (e.g., *Myotis volans*, Long-legged Myotis) had been co-roosting in the maternity colonies sampled in low abundance, they were not detected.

eDNA air sampling shows promise as a method for determining species presence in mixed species *Myotis* roosts. Further testing is required to understand the source of the eDNA signal (current versus historic occupancy). Of note, the eDNA sequence read ratios for *M. yumanensis* to *M. lucifugus* via eDNA metabarcoding were similar to the acoustic activity ratios of these two species at all three roosts (compare Figure 2.4 to Figure 2.5). At Stave

Lake Lodge, approximately 15% of acoustic bat echolocation calls were attributed to *M. yumanensis* while 9 to 11% of metabarcoding DNA reads were *M. yumanensis*. At Tranquille barn, 3% of acoustic calls and 1% of DNA reads were *M. yumanensis*, and at Peachland Attic 62% of acoustic calls and 51% of DNA reads were *M. yumanensis*. Further testing is needed to see how this pattern compares to relative abundance estimates obtained via bat capture, and whether it holds across other mixed-*Myotis* roosts. If there is such a correlation, a combined approach of eDNA air sampling and acoustics could be a useful non-invasive technique for estimating ratios of *M. lucifugus* to *M. yumanensis* in mixed-species roosts that occur across western North America.

The results of this study demonstrate that the new eMyotis1 assay works to detect *Myotis* DNA in environmental samples, and this assay is ready for broader application in B.C. A positive result using this assay likely indicates true presence of *Myotis* DNA within the environmental sample, given that quality control procedures are followed. These results do not, however, yet assess the sensitivity of the assay and the probability of failing to detect *Myotis* DNA when it is present. Knowing that mtDNA sequences can vary widely within a species, rigorous testing will be needed that includes all putative subspecies of each *Myotis* species in B.C. Until then, careful interpretation of negative results with this assay are required. The eMYLU assay successfully detected *M. lucifugus* DNA at two of three sites where this species was known to occur. Again, a positive result with this assay seems to indicate true presence of *M. lucifugus* DNA in the environment; however, the sensitivity of this assay is uncertain, and false negatives may reflect genetic differences between target individuals and assays. Further laboratory validation with samples of other *M. lucifugus* voucher specimens is needed.

Finally, the eMYYU assay is not ready for broader field application and requires redesign and further laboratory validation. In the absence of fully validated species-specific qPCR-based eDNA assays, the bat metabarcoding primer currently is the best tool for species detection in high eDNA environments like maternity roosts. Caution should be applied when determining species presence based on one eDNA result as the sole line of evidence for species presence. A second line of evidence, like a secondary genetic analysis (e.g., metabarcoding and qPCR) or acoustics, would reduce uncertainty.

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Chapter 3. Field trial of eDNA air sampling for Myotis bats at an artificial bat cave

INTRODUCTION

Bat populations in North America are declining due to many factors including energy development, resource extraction, climate change, and land conversion (Adams et al. 2024), and the recent arrival in British Columbia (B.C.) of the deadly fungal pathogen responsible for white-nose syndrome (WNS) threatens to exacerbate these impacts locally. In western North America, west of the Rocky Mountains, much is unknown about the ecology of bats through their full annual cycle (Blejwas et al. 2023). The lack of knowledge on fall and winter ecology limits our ability to understand the scale, pathways, and timeframe of disease spread of WNS, or to conduct effective surveillance and mitigation (Blejwas et al. 2021, 2023). Relatively few bat hibernacula have been identified in the west compared to the east, where bats hibernate conspicuously in large aggregations (Weller et al. 2009, 2018). This knowledge gap in overwintering ecology is particularly apparent for western bats in the *Myotis* genus, and several *Myotis* species have suffered high mortality from WNS (Frick et al. 2010, ECCC 2018, Weller et al. 2018).

Where hibernacula for *Myotis* bats have been identified in underground mines and caves in the western United States (U.S.), they primarily hibernate singly or in small groups (i.e., 10 bats or fewer) (Weller et al. 2018). Other types of non-cavernous underground hibernacula, namely rock crevices and Milieu Souterrain Superficiel (MSS), have been identified in the west, and are hypothesized to provide significant overwintering habitat for western *Myotis* bats (Blejwas et al. 2021). For the Endangered *Myotis lucifugus* (Little Brown Myotis) in particular, which is highly susceptible to WNS (Frick et al. 2010, ECCC 2018), no mine or cave hibernacula have been confirmed in B.C. (Lausen et al. 2022), possibly because individuals are dispersed between many confined underground habitats. Among *Myotis* hibernacula sites that have been identified in the west, the shared characteristics are that they are used by low numbers of bats compared to the east, and they are very challenging to locate and access (Blejwas et al. 2023). Innovative research methods are needed to study bats that use inaccessible underground habitats for key life stages like hibernation, and to fill the critical western *Myotis* overwintering ecology knowledge gap (Blejwas et al. 2023).

The recent advent of eDNA air sampling for presence of terrestrial organisms represents a revolutionary approach to evaluating biodiversity (Clare et al. 2022, Lynggaard et al. 2022, Bohmann and Lynggaard 2023). Sampling air for eDNA, as opposed to other media like water or soil, could prove particularly useful for the study of underground habitats, including mines, which are confined spaces that likely minimize dilution of the eDNA signal compared to open air (Clare et al. 2021). Additionally, underground habitats are challenging to study by conventional means like visual surveys because they are dark, often inaccessible, and sometimes dangerous for surveyors to enter (Clare et al. 2021). Recent studies have demonstrated the utility of eDNA air sampling paired with metabarcoding for documenting bat community diversity at known roosts with regular bat activity in a tropical environment (Garrett et al. 2023*a*, *b*). However, for eDNA methods to be useful for assessing *Myotis* bat use of mines in western North America, they must be robust for detection of DNA in conditions of low bat abundance and activity.

A new probe-based quantitative real-time polymerase chain reaction (qPCR) assay designed to detect bats in the *Myotis* genus has recently been developed by Dr. Caren Helbing and her team (University of Victoria). It was designed based on specimens from eight *Myotis* bat species from western Canada (B.C. and Alberta): *Myotis lucifugus*, *M. californicus* (Californian Myotis), *M. ciliolabrum* (Western Small-footed Myotis), *M. evotis* (Long-eared Myotis), *M. thysanodes* (Fringed Myotis), *M. volans* (Long-legged Myotis), *M. yumanensis* (Myotis yumanensis), and *M. septentrionalis* (Northern Myotis). In the laboratory, this new assay demonstrates complete specificity when tested using tissue-derived from target and non-target organisms and highly sensitive amplification of synthetic target DNA sequence (Langlois et al. 2025). However, *in-situ* field tests collecting environmental samples using eDNA capture protocols for the environments of interest are necessary for a thorough validation of any new eDNA sampling approach (Goldberg et al. 2016, Langlois et al. 2021). This field trial step is vital to assess the probability of detecting the target organism using the new approach (Goldberg et al. 2016) and to enable appropriate interpretation of results (Thalinger et al. 2021).

The aim of this field trial was to test the feasibility of eDNA air sampling for *Myotis* bats using a newly designed targeted qPCR assay in a voluminous underground mine habitat

housing few bats. The amount of *Myotis* eDNA in air in this habitat type when bat activity was regular but low (i.e., few bat passes per night) was quantified, and detection probability of our combined eDNA air sampling – targeted qPCR approach was estimated. Goldberg et al. (2016) identify five factors that influence the detection probability of an eDNA sampling method: presence of eDNA in the sampling medium (in this case, air), effectiveness of eDNA capture, effectiveness of eDNA extraction, potential inhibition of qPCR, and assay sensitivity. Key factors that may influence detection probability of eDNA air sampling for *Myotis* bats in an underground mine are considered.

METHODS

Study Area

The New Afton Mine near Kamloops, B.C., provides a unique opportunity to conduct a pilot field trial of eDNA air sampling for *Myotis* bats. New Gold Inc. (New Gold) has undertaken a stewardship project at the New Afton Mine to convert a decommissioned concrete ore bunker into an underground bat habitat feature (the bat cave) (Figure 3.1). This 77 metre (m) long by 4 m wide by 4 m high permanent underground structure is used by bats, as evidenced by recordings of echolocation calls made inside the bat cave; New Gold has conducted monitoring of bat activity at the bat cave using acoustic bat detectors (Anabat Roost Loggers, Titley Scientific, and SM3Bat and SM4Bat, Wildlife Acoustics) periodically since summer 2016, recording a low level of bat activity May through October, as well as some sporadic use in winter (New Gold, unpublished data). Based on the timing of acoustic detections, in the summer period the bat cave is primarily used as a night roost where a small number of bats rest during the night between foraging bouts, presumably at the adjacent slough habitat (New Gold, unpublished data). Limited day-roosting of solitary bats has been documented (New Gold, unpublished data). The conditions at the bat cave approximate what is observed at some underground mine hibernacula in B.C. with *Myotis* bats roosting singly or in small groups and low levels of hibernacula-exiting (i.e., one to few bat flights) on a given night (C. Lausen, Wildlife Conservation Society Canada, unpublished data).

The bat cave has only one entrance / exit point for bats, through a 0.4 m by 1.2 m metal gate at the front of the bat cave (Figure 3.2). The aspect of the front entrance gate is approximately 290 degrees (i.e., facing west-northwest). There is an air vent located at the

back of the bat cave allowing for air movement between the bat cave and the surface; however, this vent is screened preventing bats from using it as an access point. Airflow within the bat cave is minimal (0.1 m/s or less) due to the presence of two large plywood baffle walls located approximately 18 m and 51 m respectively from the entrance. There is also a thick curtain 5 m from the entrance, made of recycled conveyor belt material, that was hung from chains from the ceiling to decrease the portal opening size, but still allow bat passage above the curtain.

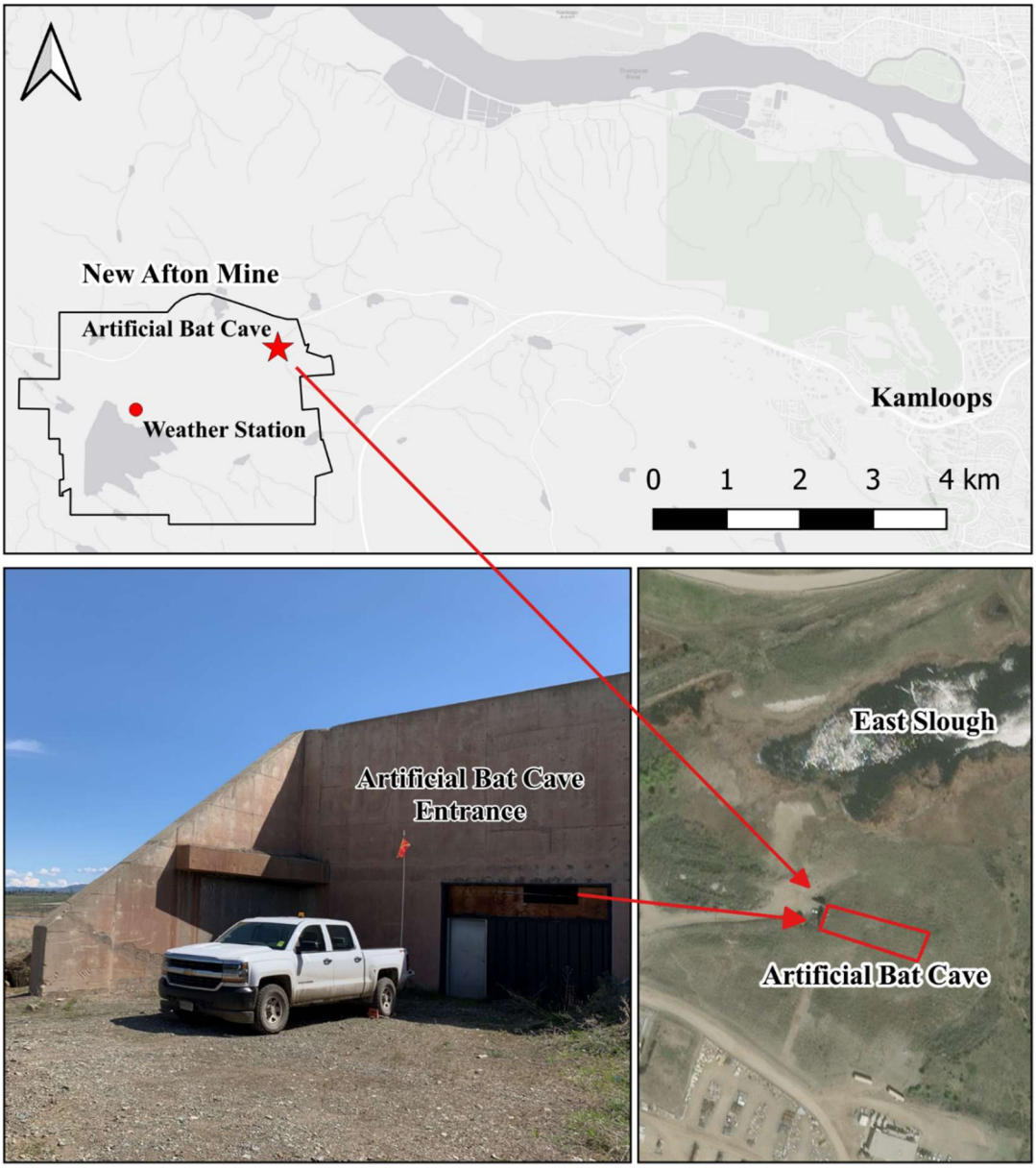

Figure 3.1 New Afton Mine artificial bat cave location, near Kamloops B.C.

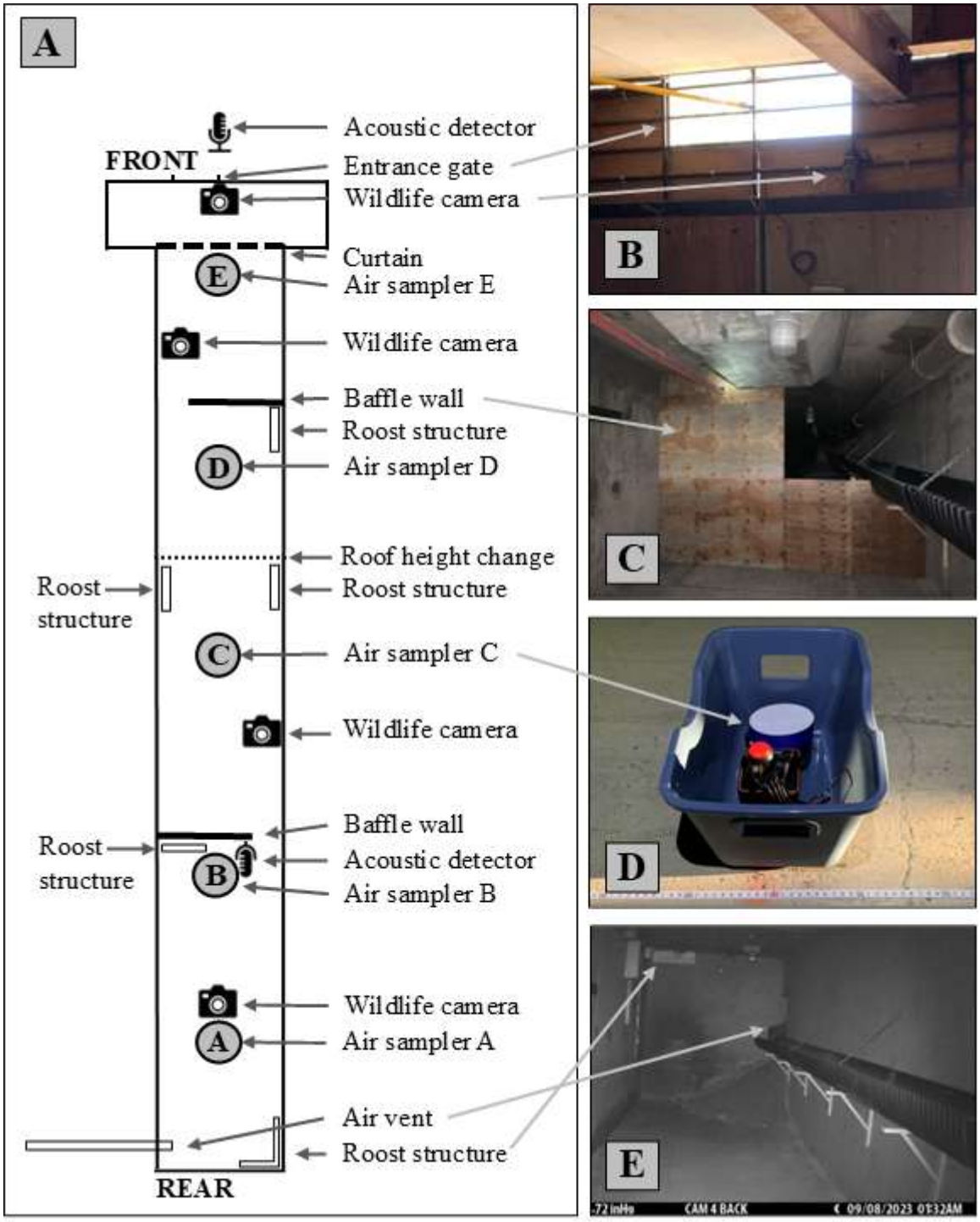

Figure 3.2 Schematic of (a) the New Afton bat cave, with locations of (b) front entrance gate and wildlife cameras, (c) plywood baffle walls, (d) eDNA air samplers, (e) rear air vent, roost structures, and other relevant features. Schematic is not drawn to scale.

eDNA Air Sampling

A pilot field trial of eDNA air sampling for *Myotis* bats was conducted within the bat cave at the New Afton mine. Sampling stations were established at 7.5 m, 22.5 m, 37.5 m, 52.5 m, and 67.5 m from the front entrance (15 m spacing) along the length of the bat cave. An eDNA air sampler design was used as described by Garrett et al (2023*b*). The air samplers have a circular sampling surface area with a 9 cm diameter. Prior to deployment, each air sampler was cleaned with a 50% bleach solution followed by a triple rinse with deionized water to prevent contamination of the sampling surface. Air samplers were deployed on the evening of August 13, 2023, and remained in place until the end of the trial on the evening of August 17, 2023. Each consecutive sampling period was approximately 12 hours minus the time required to collect samples and redeploy new filters, beginning and ending at approximately 07:00 and 19:00 respectively. The primary bat activity period which includes day-roost emergence, foraging, night-roosting, and day-roost return was captured within the nighttime sampling period. Sunrise time shifted from 5:46 to 5:52 and sunset time from 20:25 to 20:17 over the field trial. Sample collection timing was divided between daytime and nighttime due to the battery life being approximately 12 hours.

Filters used in this trial were sterile 90 mm diameter mixed cellulose ester reinforced membrane with 0.2 µm pore size (Millipore RW0309000, Oakville, Ontario, Canada). Each air sampler was powered by a portable rechargeable 12-volt battery that was swapped with a fully recharged battery at the start of each consecutive sampling period. Batteries were also cleaned with a 50% bleach solution when they were removed from the site for recharging and before redeployment. A total of 40 eDNA air samples (filters) were collected over the course of the 96-hour field trial (Table 3.1).

Table 3.1 *Myotis* eDNA air sampling field trial sampling scheme including date and time of sample filter deployment, date and time of sample filter collection, sampling duration, timing (night or day), and samples collected (n=40).

Sample Deployment Date and Time	Sample Collection Date / Time	Sampling Duration	Sampling Timing	Samples Collected
13-Aug-2023 19:05	14-Aug-2023 07:40	12:35	Night	1 (A, B, C, D, E)
14-Aug-2023 08:00	14-Aug-2023 18:45	10:45	Day	2 (A, B, C, D, E)
14-Aug-2023 19:15	15-Aug-2023 07:05	11:50	Night	3 (A, B, C, D, E)
15-Aug-2023 07:25	15-Aug-2023 18:45	11:20	Day	4 (A, B, C, D, E)
15-Aug-2023 19:05	16-Aug-2023 07:00	11:55	Night	5 (A, B, C, D, E)
16-Aug-2023 07:25	16-Aug-2023 18:40	11:15	Day	6 (A, B, C, D, E)
16-Aug-2023 19:00	17-Aug-2023 06:55	11:55	Night	7 (A, B, C, D, E)
17-Aug-2023 07:20	17-Aug-2023 18:45	11:25	Day	8 (A, B, C, D, E)

Once removed from the apparatus, each sample filter was folded in half with the sampling surface on the inside and placed in its own labelled brown paper envelope. This step was completed using forceps sterilized with 50% bleach solution and triple-rinsed with deionized water. A field blank was collected after each sample collection which consisted of an unused filter placed inside a paper envelope with forceps. Sample envelopes were then placed into plastic zipper top bags (three envelopes per bag) with one tablespoon of colour-indicating silica desiccant beads. Field blank filters were stored and transported with sample filters. All filters were kept in a cooler with ice for transport from the field and then in a -20°C freezer for temporary storage prior to being shipped to the University of Victoria in a cooler with ice packs for laboratory analysis.

All filters were sent to the Helbing lab at the University of Victoria for analysis via qPCR against the newly designed eMyotis1 assay. Laboratory analysis followed a well established eDNA sample processing workflow (e.g., Hobbs et al. 2019, Hocking et al. 2022). DNA was extracted and evaluated for integrity and inhibition using IntegritE-DNA test. This test provides a measure of sample viability by assessing the ability of each sample to support amplification of plant chloroplast DNA (Hobbs et al. 2019). Two samples required cleaning (5C and 6C). These two samples were cleaned using Zymo OneStep PCR Inhibitor Removal Kit to remove enzyme inhibitors that may block amplification. This step mitigates the

potential for false negative results due to poor sample quality (i.e., degraded DNA) or presence of inhibitors (Hobbs et al. 2019). Once cleaned, both samples were retested and passed integrity testing. Each sample was tested with eight qPCR replicates against the eMyotis1 assay, eight negative controls, and two positive controls (i.e., containing *Myotis* DNA). Due to the low quantities of eDNA captured in the trial, eDNA copy number per sample was estimated (including standard error, SE) by the Helbing lab using a Binomial-Poisson model based on the limit of detection and limit of quantification for the eMyotis1 assay, as well as number of PCR replicates that amplified per sample (Lesperance et al. 2021, Hocking et al. 2022). A sample was considered positive for containing target eDNA via qPCR if a minimum of two of eight PCR replicates amplified (Ct < 50) (Hobbs et al. 2019, Matthias et al. 2021). If only one PCR replicate amplified, the sample was considered probable for containing target eDNA. If zero PCR replicates amplified, the sample was considered negative for target eDNA.

Once qPCR analysis via the eMyotis1 assay was completed, the remaining extracted DNA from all samples with *Myotis* eDNA amplification were pooled and sent to a collaborating laboratory at Northern Arizona University (Bat Ecology & Genetics Lab) for interlaboratory validation via DNA metabarcoding (Walker et al. 2016, 2019). This method used a published DNA metabarcoding primer for bat order *Chiroptera* based on a short section of mitochondrial gene *cytochrome c oxidase subunit 1* (*CO1*) (Walker et al., 2016). Metabarcoding laboratory work and bioinformatics were conducted by the Bat Ecology & Genetics Lab (Northern Arizona University).

Concurrent Activity Monitoring

Bats had unobstructed access to the bat cave through the front entrance gate throughout the field trial and bat activity within and adjacent to the bat cave was monitored via acoustic bat detectors, wildlife cameras, and visual inspection.

Bat echolocation was recorded outside the front entrance of the bat cave using a Song Meter SM3BAT ultrasonic detector (Wildlife Acoustics) with a U2 microphone mounted on a pole approximately 3 m from the entrance gate (**Figure 3.2**). It was set to record in full spectrum starting 30 minutes prior to sunset until 30 minutes after sunrise each day. This detector monitored bat activity in the immediate vicinity of the bat cave entrance and provided full

spectrum recordings to aid in species identification of bats present near the bat cave during the field trial.

A second acoustic detector, a standard sensitivity Anabat RL1 Roost Logger with built-in ultrasonic microphone (Titley Scientific), was deployed inside the bat cave (**Figure 3.2**). The Roost Logger has a directional microphone with a detection distance of 8 to 10 m (C. Corben, Titley Scientific, personal communication). Roost Loggers are designed with shorter range microphone detection distances than the SM3Bat's U2 microphone to optimize recording bat calls inside a confined roost space. The Roost Logger was deployed within the rearmost chamber of the bat cave above sampling station B, facing towards the rear at a height of 2 m. It was programmed to respond to an ultrasonic trigger and record bat echolocation 24 hours a day in zero-cross format. An acoustic detection of a bat on this detector indicated a bat had flown from the front entrance to the rear chamber of the bat cave, over stations E (front of the bat cave), D, C, and B, and within at least 6 to 8 m of station A (rear of the bat cave).

Acoustic recordings were analyzed manually in Anabat Insight (Version 2.1.3, Titley Scientific). A recording with two or more bat echolocation pulses was considered a bat pass. Where possible, bat passes were classified to species based on call characteristics as described by Lausen et al. (2022). Recordings of *Myotis* bats and unidentified calls with minimum frequency greater than 30 kHz were categorized as *Myotis* bat passes for the purpose of comparing acoustic detections to eDNA detections. Recordings of non-*Myotis* species and unidentified calls with minimum frequencies less than 30 kHz were categorized as non-*Myotis* bat passes. Two *Myotis* species (*M. evotis* and *M. thysanodes*) have typical minimum frequencies at or below 30 kHz and therefore poor-quality calls of these two species could inadvertently be included in the non-*Myotis* category.

Four wildlife cameras (Stealth Cam QV20 and Browning Dark Ops Pro X 1080) were deployed at four stations throughout the bat cave (**Figure 3.2**). Camera 1 was mounted inside the bat cave just below the front entrance gate facing toward the rear to capture bats entering and/or exiting the cave. Cameras 2 through 4 were each placed in subsequent chambers created by baffle walls within the bat cave to capture bat movement between each chamber; however, none of these three additional cameras captured any bat flight during the field trial,

only surveyor presence, possibly due to very low light conditions and the small size and quick flight speed of bats. Cameras were programmed to capture 15 second video clips with a 5 second delay between triggers. Video clips were manually reviewed to identify bat passes, number of animals per video, minimum group size, and direction of travel (i.e., in or out of the bat cave).

A visual inspection of the bat cave was completed nine times during the field trial to document presence of roosting bats, once at the start of the trial, and then again following each round of filter collection. If detected, the number, type of bat (i.e., *Myotis* or non-*Myotis*), and location of each bat as well as the date and time of each observation was noted.

Weather data was collected by New Gold at a meteorological station at the New Afton Mine, approximately 2 km southwest of the artificial bat cave. Data was automatically recorded at 15-minute intervals throughout the field trial, and included ambient air temperature, precipitation rate, relative humidity, wind speed, and wind direction. This data was provided by New Gold. Due to the presence of nearby wildfires during the field trial, wildfire smoke conditions were also monitored through the trial period, as recorded at the B.C. Government air monitoring station in downtown Kamloops approximately 12 km east of the bat cave. The weather conditions and wildfire smoke data were used to assess general wind patterns and presence of compounds that could interfere with eDNA sampling within the bat cave (e.g., through transport of eDNA from outside the bat cave to inside or from addition of PCR inhibiting compounds to filters). Additionally, the weather data was used to ensure weather conditions were appropriate for bat activity throughout the trial. In B.C., bat activity is expected when ambient air temperature is greater than 10 °C at emergence time, there is little to no precipitation, and wind speeds are light (Burles et al. 2009, Resources Information Standards Committee 2022).

eDNA Detection Probability

A Bayesian multiscale occupancy model in R package eDNAoccupancy (version 0.2.6) (Dorazio and Erickson 2018) was used to estimate probability of *Myotis* eDNA occupancy (Ψ) within the bat cave as well as the conditional probability of detection of *Myotis* eDNA at both the sample (θ) and replicate (p) level.

Two additional potential covariates were considered that are hypothesized to influence θ, or the conditional probability of capturing *Myotis* eDNA in a sample. These covariates were timing of sampling (night or day) and whether a bat was detected acoustically on the internal bat detector during the sampling period. Nighttime sample collection was hypothesized to improve probability of eDNA capture because bats are more active at night (flying and echolocating) and are therefore more likely to shed eDNA in this time period (Garrett et al. 2023*a*). Similarly, at least one acoustic bat detection during the sampling period was hypothesized to increase the probability of eDNA capture due to bat echolocation being a potential source of eDNA shedding.

The model was fitted using the Markov chain Monte Carlo algorithm in R package eDNAoccupany, with 21,000 iterations and an initial burn-in of 1,000 iterations (Dorazio and Erickson 2018). The posterior median value for each model parameter (Ψ, θ, and p) was computed along with the Bayesian 95% credible interval (CI). Models were checked for convergence and autocorrelation (Dorazio and Erickson 2018, Pope et al. 2020). Model assessment considered widely applicable information criterion (WAIC) (Watanabe 2010, 2013, Dorazio and Erickson 2018).

RESULTS

eDNA Air Sampling

Positive detections of *Myotis* eDNA in air samples via qPCR were returned for three of four nighttime sampling periods and one of four daytime sampling periods (Table 3.2). In total, five air samples were positive for *Myotis* eDNA (i.e., two or more PCR replicates amplified), and an additional three samples were probable for *Myotis* eDNA (i.e., one PCR replicate amplified). Thirty-two samples were negative. The maximum number of samples in any given sampling period that amplified *Myotis* eDNA was three out of five samples on the second and third nights of the field trial. Estimates of the quantity of *Myotis* eDNA captured in each sample were low, ranging from a maximum of 153 eDNA copies per sample (SE = 75) to just 9 (SE = 9) (Table 3.3). The field blank for sampling period 6 (August 16 daytime) was probable for *Myotis* eDNA (1 of 8 PCR replicates amplified). All other field blanks were negative for *Myotis* eDNA.

One bat species, *Myotis volans* (Long-legged Myotis), was detected when all samples that amplified *Myotis* eDNA via qPCR were pooled and analyzed via metabarcoding; however, the total number of sequence reads for *M. volans* was very low, at just eight reads.

Table 3.2 eDNA air sampling qPCR results by station (A through E), date, and sampling period (night vs. day) using a genus-wide assay for *Myotis* bats (eMyotis1). Results are provided in number of PCR replicates that amplified out of eight per sample.

Sampling period		13-Aug Night	14-Aug Day	14-Aug Night	15-Aug Day	15-Aug Night	16-Aug Day	16-Aug Night	17-Aug Day
Sample		1	2	3	4	5	6	7	8
Station	**A** (rear)	0	0	**2**	0	**1**	0	0	0
	B	0	0	0	0	0	0	0	0
	C	**8**	0	**3**	0	0	**2**	0	0
	D	0	0	**1**	0	**2**	0	0	0
	E (front)	0	0	0	0	**1**	0	0	0

Table 3.3 Estimated eDNA copies per sample and standard error for all eDNA air samples that amplified *Myotis* DNA via qPCR using the genus-wide *Myotis* assay (eMyotis1).

Sample	Positive replicates (of 8)	eDNA copies per sample	Standard Error
1C	8	153	75
3A	2	21	15
3C	3	36	15
3D	1	9	9
5A	1	9	9
5D	2	21	15
5E	1	9	9
6C	2	21	15

Concurrent Activity Monitoring

Inside the bat cave, *Myotis* bats were detected acoustically on all four nights of the field trial and during one daytime sampling period (Table 3.4). Bats were also detected on the wildlife camera inside the front entrance of the bat cave while it was operational on both the first and

second nights of the field trial. These wildlife camera detections could not be identified beyond the level of bat; however, only *Myotis* bats were detected acoustically on the internal bat detector, so camera detections were likely to be *Myotis* bats. Wildlife camera detection data was not available for the final two nights of the trial due to equipment failure. Throughout the trial, just one bat, an unidentified *Myotis* bat, was observed day-roosting within the artificial bat cave throughout the third day of the field trial (August 16, 2023; Table 3.4). This bat roosted in a plywood baffle structure mounted on the rear wall of the bat cave, closest to air sampling station A. While it is not possible to definitively confirm that this bat did not fly within the bat cave on this day, no acoustic bat detections were recorded during the daytime while this bat day roosted. Six bat passes were, however, recorded during the daytime on the final day of the trial (August 17, 2023; Table 3.4). No bats were observed roosting within the bat cave on this day, but it is possible that a bat could have day roosted and not been detected visually.

Table 3.4 Number of visual, acoustic, and camera detections of *Myotis* bats inside the bat cave per sampling period.

Sampling period	13-Aug Night	14-Aug Day	14-Aug Night	15-Aug Day	15-Aug Night	16-Aug Day	16-Aug Night	17-Aug Day
Sample	1	2	3	4	5	6	7	8
Visual detections	0	0	0	0	0	**1**	0	0
Acoustic detections	**2**	0	**3**	0	**6**	0	**3**	**6**
Camera detections	**1**	0	**4**	0	NA	NA	NA	NA

Outside the front entrance of the artificial bat cave, *Myotis* bats were detected acoustically on all nights of the field trial with the total number of *Myotis* bat passes ranging from 60 (August 14) to 160 (August 15) (Table 3.5). Non-*Myotis* bats were also detected acoustically on three of four nights of the field trial, but in lower numbers, with the number of non-*Myotis* bat passes ranging from 0 (August 14) to 15 (August 15) (Table 3.5). Overall bat activity, as indicated by total bat passes per night, was highest on August 15 and lowest on August 14. The general pattern of bat activity detected outside the front of the bat cave aligns with what

would be expected for a night roost since hourly bat activity peaked during the middle of the night as opposed to at emergence (Figure 3.3) (Anthony et al. 1981, Barclay 1982).

Table 3.5 Summary of acoustic bat passes each night of the field trial, recorded outside the front entrance of the New Afton artificial bat cave, grouped by species or acoustic group and *Myotis* and other (i.e., likely non-*Myotis*) bat species.

Acoustic group and identification		**13-Aug-2023**	**14-Aug-2023**	**15-Aug-2023**	**16-Aug-2023**
Myotis	Unidentified 40 kHz bat	81	45	120	121
	M. californicus / M. yumanensis	4	14	38	3
	M. evotis	0	0	0	3
	M. lucifugus	0	1	2	2
	Subtotal *Myotis* bat passes	**85**	**60**	**160**	**129**
Non-*Myotis*	*Eptesicus fuscus / Lasionycteris noctivagans*	4	0	7	6
	Lasionycteris noctivagans	0	0	7	1
	Unidentified low frequency bat	0	0	1	0
	Subtotal other bat passes	**4**	**0**	**15**	**7**
	Total all bat passes	**89**	**60**	**175**	**136**

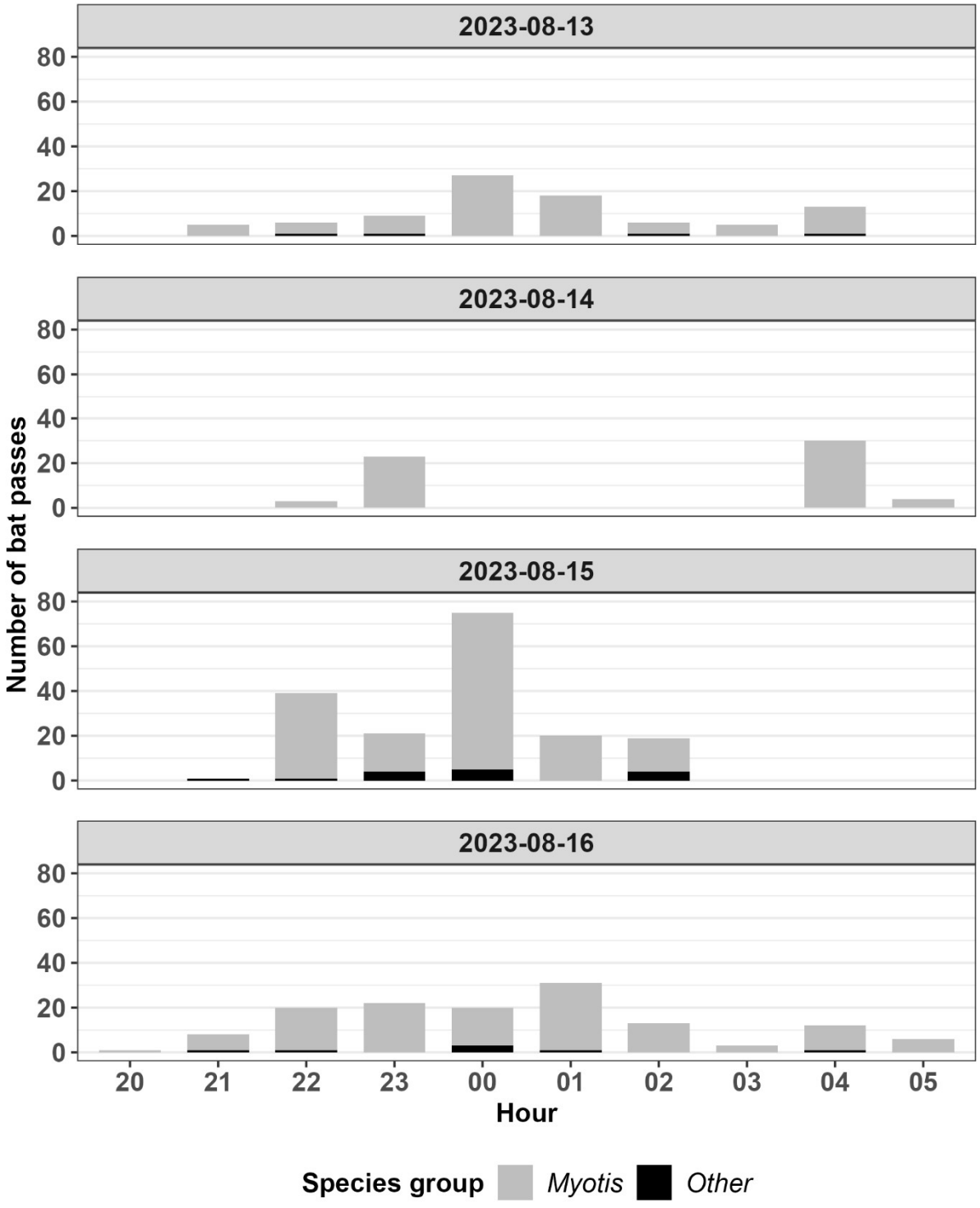

Figure 3.3 Summary of acoustic bat passes each night of the field trial, recorded outside the front entrance of the New Afton artificial bat cave, grouped by *Myotis* bats and other (i.e., likely non-*Myotis*) bat species.

Prevailing wind direction during the field trial was from the southwest and average wind speed was 2.6 meters per second (m/s) (Figure 3.4). The field trial coincided with a heat wave and high-pressure event. Winds were light throughout the field trial until the ridge of high pressure began to breakdown on August 17 with winds gusting to 12.7 m/s on the final afternoon (Figure 3.5). Overnight low temperatures were between 12 and 19 °C and daily maximum temperature ranged from 32 to 36 °C (Figure 3.5). There was no precipitation recorded during the field trial. Wildfire smoke developed beginning on August 14 and accumulated through the remainder of the field trial. Weather conditions were favourable for bat activity on all nights of the trial.

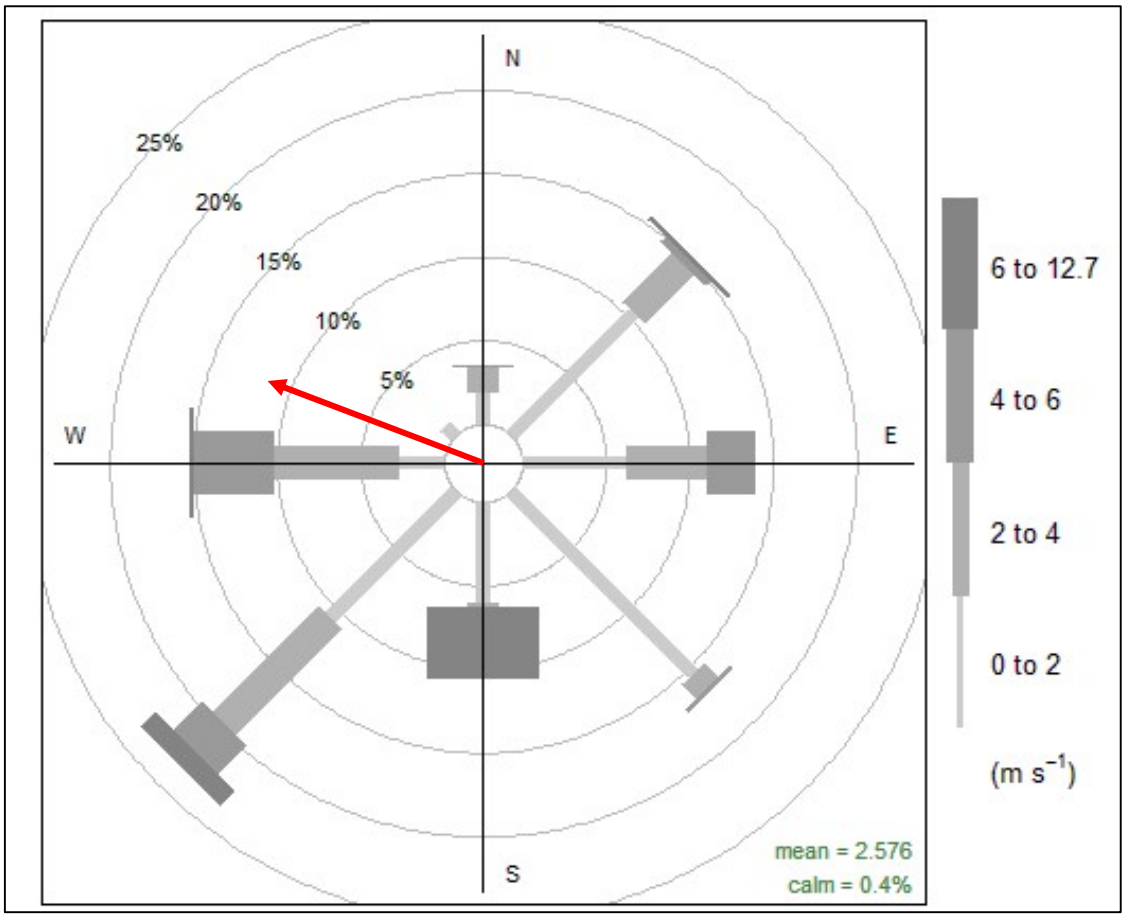

Figure 3.4 Wind rose plot of the frequency of wind direction and wind speed, at the New Afton meteorological station, measured in 15-minute intervals over the course of the field trial, August 13 through 17, 2023. The red arrow represents the approximate aspect of the entrance to the New Afton artificial bat cave (290°).

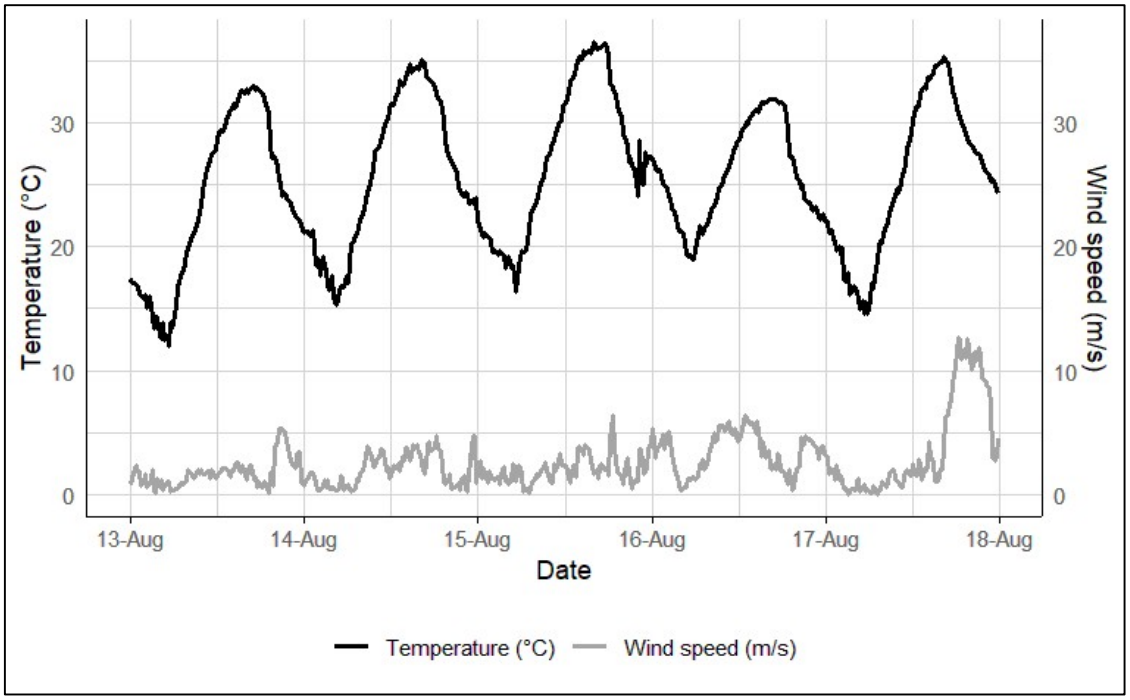

Figure 3.5 Temperature and wind speed measured at the New Afton meteorological station in 15-minute intervals over the course of the field trial, August 13 through 17, 2023.

eDNA Detection Probability

The top multiscale occupancy model of *Myotis* eDNA in the bat cave was the null model which assumes that Ψ, θ, and p are equal for each station, sample, and PCR replicate respectively (Table 3.6). The null model was marginally better than the model that included day versus night or the model that included acoustic detection as covariates of θ. The probability of *Myotis* eDNA occupancy at a given sampling station during the field trial based on the null model was 79% (95% CI: 39 to 99%) (Dorazio and Erickson 2018). The conditional probability of capturing *Myotis* eDNA in a sample was 26% (95% CI: 13 to 45%) and the conditional probability of amplifying *Myotis* eDNA via qPCR in a replicate was 29% (95% CI: 18 to 42%). Comparison of the probability of eDNA capture for samples collected in the daytime versus the nighttime did not reveal a statistically significant difference (Figure 3.6).

Table 3.6 Summary of candidate multiscale occupancy models, assessment of model fit via WAIC scores, and assessment of convergence of the Markov chains via trace and autocorrelation plots in R package eDNAoccupancy **(Dorazio and Erickson 2018)**.

	Candidate models			WAIC	Convergence
	Ψ	**θ**	**p**		
1	~1	~1	~1	27.2	Yes
2	~1	day_night	~1	27.5	Yes
3	~1	acoustic_detection	~1	27.6	Yes
4	~1	day_night + acoustic_detection	~1	27.3	No
5	day_night	~1	~1	Overfit	-
6	acoustic_detection	~1	~1	Overfit	-
7	day_night + acoustic_detection	~1	~1	Overfit	-

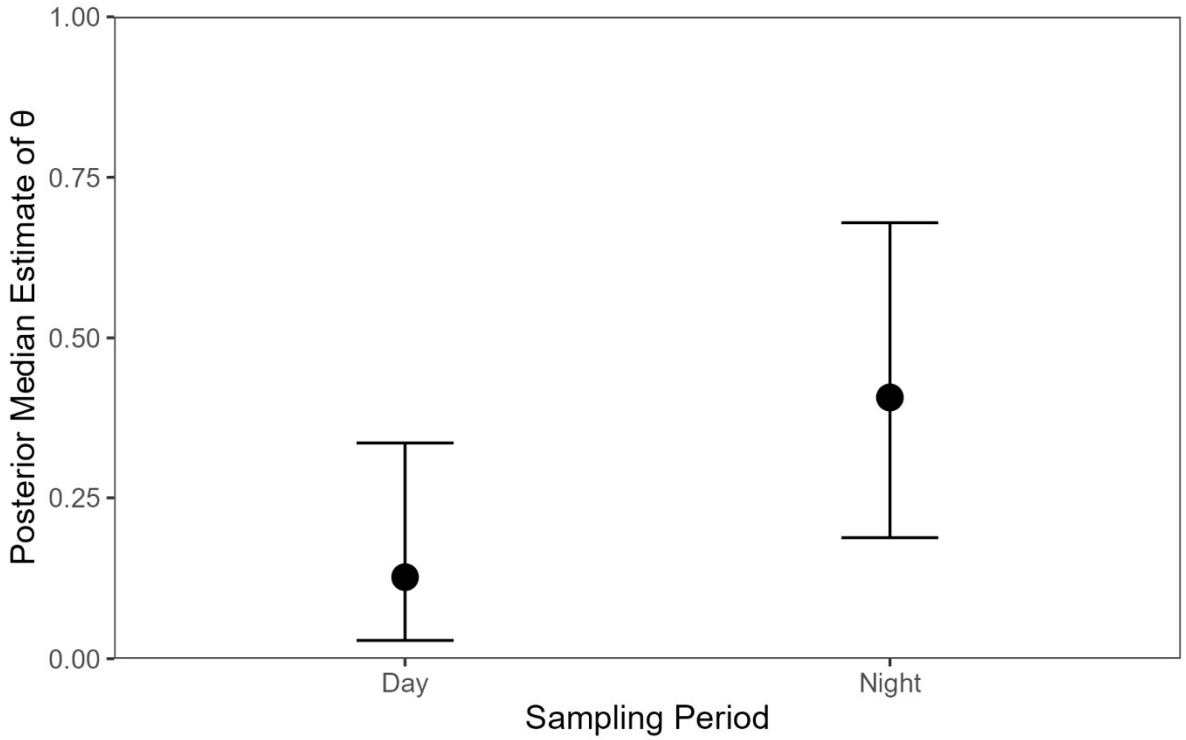

Figure 3.6 Posterior median estimates of θ, or probability of eDNA capture in a sample, for eDNA air samples collected during the daytime versus the nighttime plus Bayesian 95% credible interval.

DISCUSSION

This pilot field trial presents an *in-situ* validation of the eMyotis1 qPCR-based eDNA assay in a low eDNA simulated underground mine environment. Pilot studies are recommended for all new eDNA sampling approaches, to evaluate detection probabilities given the particular sampling and analysis techniques implemented, as well as to identify site characteristics that may influence detection (Goldberg et al. 2016). The aim of the trial was to test eDNA air sampling paired with targeted qPCR for *Myotis* bats as a method for detecting *Myotis* bat use of underground mines. The positive eDNA detections in this study demonstrate that by combining eDNA air sampling with targeted qPCR, bat use of underground mines can be detected, even at low levels of bat activity. While the probability of capturing *Myotis* eDNA in this trial was just 26% (CI: 13% to 45%), *Myotis* eDNA was successfully detected in at least one of five samples on three of four nighttime sampling periods. Improvements to the eDNA capture method are necessary to lower the false negative rate in this environment; nonetheless, this approach holds promise for being highly sensitive and offering valuable insights into species presence in environments that are particularly difficult to study.

The effect of eDNA extraction on detection probability is beyond the scope of this study, but incremental improvements in this factor are expected to have a relatively low overall effect on detection probability compared to the other factors. Each of the four other factors outlined by Goldberg et al. (2016), presence of eDNA in the sampling medium, effectiveness of eDNA capture, potential qPCR inhibition, and assay sensitivity, are addressed here in the context of the pilot field trial, and areas requiring further investigation are identified.

Key to an eDNA pilot study is evaluating whether the sampling strategy targets the appropriate place and time for the ecology of the organism, such that eDNA is present in the environment to collect in a sample (Goldberg et al. 2016). In this case, a confined and semi-controlled artificial bat cave was targeted, simulating an underground mine habitat during a time when regular but low levels of bat flight in and out of the bat cave was expected. Confined roosting spaces with low air flow provide ideal targets for eDNA air sampling for bats, because they concentrate bat activity while minimizing potential dilution effects (Clare et al. 2021, 2022, Garrett et al. 2023*a*). This field trial was conducted in late summer, but the level of bat activity observed approximated what might be expected at an underground mine

hibernacula in B.C. in winter, where *Myotis* bats are anticipated to hibernate singly or in small groups (Weller et al. 2018), and hibernacula-exiting behaviour is regular but in low numbers (i.e., few bat passes in a night) (C. Lausen, WCS Canada, unpublished data). Air was chosen as the sampling medium, as opposed to swabbing surfaces or collecting sediment, to reflect recent *Myotis* bat presence as opposed to past DNA deposition, and due to the impracticality of observing exactly where bats roosted on a given night. During the trial, the probability of presence of *Myotis* bats modelled from the eDNA detection results was 79% (95% CI: 39% to 99%), which aligned with a naïve estimate of *Myotis* bat presence of 75% based on concurrent bat activity monitoring. Bats were detected during the field trial in six of eight sampling periods via acoustics, wildlife cameras, and visual observation. This pilot field trial demonstrates that *Myotis* eDNA is detectable in air within underground mine habitats, even at low levels of bat activity.

Although the *Myotis* eDNA was detectable under the conditions of this trial, the *Myotis* eDNA signal was not dispersed homogenously within the bat cave. In contrast, the eDNA signal in air had no clear spatial pattern, and the false negative rate was high. Out of 30 air samples collected during sampling periods with confirmed *Myotis* bat presence, 22 samples were negative, and just 8 samples amplified *Myotis* eDNA. Additionally, the amount of eDNA captured in samples that amplified eDNA was low. The most *Myotis* eDNA estimated in a single air sample was just 153 copies (SE = 75) in sample 1C. The *Myotis* eDNA signal in air appeared to be short-term, with no amplification in any of the 10 samples collected during the two daytime sampling periods with no concurrent bat activity, despite positive detections on each of the previous nights. This finding provides further evidence supporting the hypothesis that the bat eDNA signal in air is very short-term, aligning with an anecdotal finding by Garrett et al. (2023*a*). What is not known about the sampling environment, was how the bat flew inside the bat cave in relation to the sampling locations. It is possible that filters positive for eDNA were closely approached. While cameras were used, their sensitivity was not enough to be able to record flying bats in the complete darkness. The results indicate that eDNA was present in the artificial bat cave but optimization of eDNA capture is needed to improve the overall performance of this sampling approach in low eDNA environments.

A potential pattern of greater bat detections in eDNA air samples collected at nighttime versus daytime was noted anecdotally by Garrett et al. in their study of tropical bat roosts (2023*a*). A similar non-significant pattern was observed in these field trial results. While not statistically significant, seven of eight samples that amplified *Myotis* eDNA were collected during a nighttime sampling period. The only daytime sample that amplified *Myotis* eDNA (two PCR replicates) was collected on the same day that a single bat was visually observed day-roosting in the bat cave. It is known that bats fly during the day inside enclosed roosts like building attics (as seen in the maternity roosts sampled, see Chapter 2) and thus daytime flight of this bat may have occurred, generating a positive eDNA sample from this day roosting bat. The fact that there was a day-time visual detection at all, together with the low sample sizes for positive detections likely explains the overlapping confidence intervals (Figure 3.6). Further work is needed to confirm whether there is an effect of time of day on the probability of eDNA capture. But for eDNA air sampling at low bat activity sites, like the artificial bat cave, the sample collection period should include the nighttime period when bats are most likely to be active (flying and echolocating). Whether flight of bats is necessary to slough cells into the air for eDNA sampling is not known but hypothesized and requires further study. The short-lived nature of the eDNA signal supports the need for flight, but more study is needed to determine under what conditions bat eDNA may be picked up in a roost if bats are present but not active.

Some aquatic eDNA sampling designs in freshwater environments target pond or wetland outlets to harness the natural transport of eDNA, improving eDNA capture efficiency (e.g., Pope et al. 2020). Similarly, eDNA air sampling schemes for detecting bat use of underground roosts could target constrictions where bats would be forced to fly close to an air sampler. In this study, air samplers were placed down the centre of the bat cave, at 15 m intervals. By placing the air samplers instead in the doorway of each baffle wall for example, eDNA capture efficiency may have been improved as bats passed close to the air samplers.

Transport of *Myotis* eDNA via wind from outside into the bat cave did not appear to influence the detection results. Frequent *Myotis* bat passes (between 60 and 160 per night) were recorded outside the front entrance and over 15% of wind was from the west or northwest, towards the bat cave entrance. Despite these factors, only one PCR replicate from

one nighttime sample collected at Station E, closest to the bat cave entrance, amplified *Myotis* eDNA. The small entrance gate size and presence of a heavy curtain for reducing air flow likely minimized potential transport of *Myotis* eDNA via wind into the bat cave.

Optimizing eDNA capture for low eDNA environments represents the greatest opportunity to advance this method as a conservation tool. These results demonstrate that effectively capturing *Myotis* eDNA when bats are present in low numbers, even within the confines of an underground roost, is challenging. The probability of capturing *Myotis* eDNA in this field trial was just 26% (CI: 13% to 45%). Factors that may be important for improving eDNA capture are filter material, duration of sampling, and the proximity of the air samplers to bats.

In this study, a mixed cellulose ester reinforced membrane filter with 0.2 µm pore size was used (Millipore RW0309000). A filter material that differed from the Filtrete 1900 Smart Air Filters (3 M) used in previous studies (Garrett et al. 2023*b*, *b*), and in Chapter 2 of this thesis, was selected for two reasons. First, the Millipore filters are sterile, so no UV sterilization is required. Availability of ready-to-use filter materials that do not require UV sterilization allows eDNA air sampling to be adopted by a wide variety of end-users (e.g., non-profit conservation groups, environmental practitioners, and land managers) who may not have access to UV sterilization equipment. Second, the smaller pore size was hypothesized to improve eDNA capture by capturing smaller particle sizes. Filtrete 1900 Smart Air Filters are rated MERV 13, meaning they have a capture efficiency of greater than or equal to 90% for particles that are 3.0 to 10.0 µm (US EPA 2019). This efficiency drops to greater than or equal to 50% for particles that are 0.3 to 1.0 µm (US EPA 2019). However, eDNA capture was still low in this study despite the small pore size of the selected filter. This result highlights that other factors besides pore size may be important in filter material selection.

The filters selected for this study were thin and smooth like paper, whereas the Filtrete 1900 Smart Air filters are thicker, rougher texture, and visibly woven. While the low eDNA capture cannot be directly attributed to the filter type selected, a different filter may improve capture by allowing greater air passage and trapping eDNA particles more effectively. Particularly in a dry sampling environment like the bat cave and other underground mines in B.C.'s dry interior, particles may not adhere to a paper-like filter and could be lost prior to DNA extraction. Since the amount of eDNA captured in this environment was low, the ability

of the filter to trap and hold eDNA particles through all stages of sample collection, transport, storage, and initial processing up to DNA extraction is critical. It is possible that some eDNA particles could have been lost when the air sampler stopped drawing air prior to collection, during the collection process as the filter was transferred to the envelope, during transport, or during initial laboratory preparation.

A side-by-side comparison of pre-sterilized filter material options would be beneficial, or a test of whether UV sterilization of Filtrete 1900 Smart Filters is necessary to prevent sample contamination. Further, an investigation of the nature and size of eDNA particles shed by bats (e.g., aerosols from echolocation and/or skin and hair tissue shed during flight) would provide insight into suitable filters as well as other important factors like eDNA deposition rates and anticipated eDNA signal duration.

Degradation of eDNA in the environment can occur from enzymatic activity, mechanical fragmentation, chemical breakdown, or from exposure to radiation (Barnes and Turner 2016). Inside an underground roost habitat in a temperate region, eDNA degradation rates are expected to be low because the filters are protected from high temperatures and UV light (Mena et al. 2021, Garrett et al. 2023*a*). Once captured, eDNA is likely to persist on the filter for an extended period in a dry cool environment. Therefore, eDNA capture efficiency may be improved by longer sampling duration (i.e., several days versus 12 hours as in this study). Walker et al. (2019) were able to amplify bat DNA via metabarcoding from fecal samples stored in a dry cool cave for 30 months. However, eDNA degradation rates will be higher at high humidity sites (Walker et al. 2019). Assessing the temperature, humidity, and UV light conditions at a site prior to eDNA sampling may allow for optimization of sampling that maximizes sampling duration while minimizing the potential for eDNA degradation on the filter.

High assay sensitivity is important for detection of rare or low abundance target organisms, like bats in underground mines in B.C. Assay sensitivity, also referred to as the limit of detection (LOD), is the ability of an assay to detect low quantities of target DNA, and is typically evaluated in a laboratory by performing dilution experiments (Lesperance et al. 2021). The Helbing Laboratory completed this evaluation for the eMyotis1 assay using gBlock™ synthetic DNA and calculated an LOD value for the assay of 0.1 (95% CI: 0.1 to

0.2) copies per reaction, indicating highly sensitive amplification of synthetic target DNA sequence (Langlois et al. 2025). Therefore, assay sensitivity is unlikely to limit the detection probability of this combined eDNA air sampling eMyotis1 qPCR-based eDNA assay approach. In this trial, the probability of amplification of *Myotis* eDNA in a PCR replicate was low, at just 29% (95% CI: 18% to 42%). However, the amount of *Myotis* eDNA captured in this field trial was between 9 (±9) and 153 (±75) copies per sample. The low probability of amplification may have been due to low overall eDNA copy number per sample. Capturing more eDNA copies per air sample would increase PCR amplification and improve detection at the PCR replicate level.

A final factor that may influence detection probability is PCR inhibition (Mauvisseau et al. 2019*b*). Inhibition occurs when a non-target compound interferes with the DNA amplification process causing failure or delay (Goldberg et al. 2016). All air samples but two passed integrity and inhibition testing with Integrit-E DNA, meaning that chloroplast DNA was readily amplified from the air samples. This validation step was developed as a negative control for fresh water eDNA samples (Veldhoen et al. 2016). It tests for the presence of endogenous plant and algae chloroplasts in water samples, as they are ubiquitous in fresh water, to evaluate the presence of amplifiable DNA in field samples and increase confidence in negative results (Veldhoen et al. 2016, Hobbs et al. 2019, Matthias et al. 2021). The transferability of the Integrit-E DNA test as a negative control for air eDNA samples has not been tested; however, all samples but two passed the Integrit-E DNA test. The two samples that did not pass, 5C and 6C, were cleaned to remove enzyme inhibitors and subsequently passed testing. PCR inhibition did not block amplification in this study, as evidenced by the amplification of plant chloroplast DNA in all air samples including in the two samples that required clean-up. However, inhibitor clean-up kits can reduce the yield of target DNA (Goldberg et al. 2016, Mauvisseau et al. 2019*b*), and the degree to which amplification may have been impeded by partial inhibition is unknown. Metal compounds may be present in underground metals mines and therefore testing for inhibition will be an important step in mine site eDNA work to rule out false negative results that may occur.

Wildfire smoke increased throughout the field trial and was high by the end of the trial (air quality health index of 10+, or very high risk). Wildfire smoke can be prevalent in western

North America, particularly in dry interior regions like Kamloops, B.C. Air sampling methods may be susceptible to inhibition from compounds present in wildfire smoke because these compounds could be captured on air filters alongside any target eDNA. Inhibition reduces the repeatability of eDNA sampling by causing false negative results at the PCR level (Mauvisseau et al. 2019*a*). Understanding the degree of inhibition in eDNA detection, particularly for detecting rare or low abundance organisms in mine environments, is important for the accuracy and repeatability of the method (Mauvisseau et al. 2019*b*).

Future Direction

This pilot field trial demonstrates that eDNA air sampling paired with targeted qPCR is sensitive enough to detect bat presence in a simulated underground mine habitat, even at low bat abundance and activity. However, the detection probability was low, suggesting that bat behaviour may heavily influence detection probability, such as proximity of flight to eDNA samplers, or that other alterations may be needed to maximize eDNA capture in low eDNA environments. Determining how to increase the likelihood of detecting a bat in a roost using eDNA air sampling will be important for wider use of this sampling tool for bat conservation and management.

Evidence of the importance of various types of confined underground habitats beyond mines and caves, such as MSS and rock crevices, for overwintering bats in western North American bats is becoming apparent (Lausen and Barclay 2006, Klüg-Baerwald et al. 2017, Neubaum 2018, Weller et al. 2018, Blejwas et al. 2021). Although bats cannot fly in such small volume crevices, sampling of air may still yield eDNA. The detection of not only bat DNA as evidence of occupancy and identification of small cryptic hibernacula, but of the fungus that causes WNS, will be useful for managing this disease in western North America where this disease will be harder to detect. The impetus to identify and characterize winter bat roosts is urgent. Innovative research methods are needed to locate and assess these inaccessible habitats (Blejwas et al. 2023). This study adds to the growing body of evidence that eDNA air sampling is one such method.

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Chapter 4. Detecting winter use of underground roosts by Myotis bats via eDNA air sampling in British Columbia's dry interior region

INTRODUCTION

Amidst the current global biodiversity crisis, efforts to manage wildlife populations are challenged by an increasingly fragmented, altered, and degraded landscape (Dirzo et al. 2014, Newbold et al. 2015, Johnson et al. 2017). Bat populations, like other wildlife, are facing steep global declines due to many factors including habitat loss, human conflict, climate change, and impacts from invasive species (Frick et al. 2020). Underground anthropogenic features, like inactive mines and tunnels, can provide important surrogate habitat for bats (Hayes et al. 2011, Kurta and Smith 2014, Weller et al. 2018, Moran et al. 2023), where natural habitats, like mature forests, caves, and rock features are unavailable or limited due to habitat loss, human encroachment, and disturbance (Ducummon 1999, Grajal-Puche et al. 2024). Mining companies and land managers make decisions about mine closure, including when there is a risk to public safety. Some mine closure decisions can have negative impacts on bats using mines as habitat (Frick et al. 2020). Appropriate mine closure planning requires understanding which bat species are present and the extent of mine habitat use (B.C. Ministry of Environment 2016).

A fulsome understanding of bat presence, abundance, and "timing of use" is needed to effectively inform mine closure decision-making; however, tools to gather this data safely and efficiently are currently lacking. Traditional survey methods recommended for bats at underground mines and tunnels, such as visual surveys, acoustic surveys, and capture, each have limitations (B.C. Ministry of Environment 2016). Internal visual inspections of mines for presence of bats or bat sign are often not possible due to safety considerations of surveyors. Where they are possible, their effectiveness may be limited in B.C. due to small roosting aggregation sizes and limited accumulation of evidence of past use (e.g., bat carcasses, guano, urine staining, and prey remains). Not all hibernacula contain bat guano (B.C. Ministry of Environment 2016). Emergence counts at mine openings are not efficient in winter, when mine-exiting consists of few bats per night (B.C. Ministry of Environment 2016). Counts done in early fall, prior to the onset of the hibernation period, may indicate winter use (B.C. Ministry of Environment 2016). Emergence counts alone do not provide

species identification information, and non-detections of bats during emergence counts cannot be used to infer absence of bats (Sherwin et al. 2009, Moran et al. 2023).

Long-term passive acoustic monitoring is a widely used survey method for determining bat use of inactive underground mines. Acoustic surveys can provide data on presence of bats and patterns of activity; however, information on species identification and intensity of habitat use is limited. If acoustic detectors are deployed within mines or very near to mine entrances such that recordings can be directly attributed to bats using the mine feature, the acoustic call quality often inhibits species identification. If the acoustic detector is set back from the mine opening in a low clutter environment where bats are more likely to make search phase calls, species differentiation is more likely; however, detections are not directly attributable to bat use of the mine.

Detection of bat species eDNA from air within a potential underground habitat feature may add an important and currently missing method to the bat monitoring tool kit. eDNA could present another line of evidence of bat presence in underground habitats that are exceptionally difficult to survey, as well as the ability to resolve species identifications for cryptic and notoriously difficult to distinguish species. Clare et al. (2021) identified caves, hollows, and subterranean systems as likely best-use cases for eDNA air sampling methods because they are spatially confined, potentially limiting dilution of the eDNA signal. Further, Garrett et al. (2023*a*) demonstrated the utility of eDNA air sampling methods to non-invasively detect and monitor biodiversity within tropical bat roosts, including sites that were inaccessible for surveyors. The advancement of eDNA air sampling methods as a tool for bat conservation and monitoring requires additional field pilot studies in new environments. This study expands on the work of previous researchers as a first test of eDNA air sampling in temperate region underground hibernacula.

This pilot study presents winter eDNA air sampling and concurrent long-term acoustic monitoring at three suspected underground hibernacula in B.C.'s dry interior region to investigate whether eDNA air sampling methods as described by Garrett et al. (2023*a*) could detect an eDNA signal from *Myotis* bats. eDNA data was compared to conventional long-term acoustic monitoring data to understand how eDNA methods fit into a larger framework for assessing bat use of inaccessible underground habitats. Recommendations to those

considering adopting these methods in B.C. and beyond are provided and future research directions are identified.

METHODS

Study Area

eDNA air sampling methods were piloted at underground hibernacula sites to assess the readiness and usefulness of this method for investigating winter occupancy of underground habitats by bats. eDNA air sampling was compared to a traditional approach of long-term acoustic monitoring. Selected sites were anthropogenic underground tunnels (mines and a rail tunnel) with known or suspected winter use by *Myotis* bats within the B.C.'s dry interior region near Kamloops, B.C. Selection criteria prioritized sites with one known entrance / exit point for bats, ability to obtain land access permission, surveyor safety, accessibility in winter, and potential for vandalism of field equipment. Three sites were selected for study in the area surrounding Kamloops Lake: Copper Creek Mine, Rail Tunnel, and New Afton Pit Portal (Figure 4.1).

Copper Creek Mine is a historic abandoned Mercury mine located at approximately 490 m above sea level (asl), on the steep south-facing hillside above the northern shoreline of Kamloops Lake. This site is within the Thompson Very Dry Hot (BGxh2) biogeoclimatic zone, characterized by hot to very hot conditions from spring to fall and mild winters with limited snowpack (Ryan et al. 2022). The mine operated from approximately 1890 to 1927 (B.C. Geological Survey 1985). Two adits, horizontal passages leading into a mine for the purposes of access or drainage, were selected for survey that are believed to be unconnected based on historic drawings (B.C. Geological Survey 1985). Copper Creek Mine Adit 1 was the smallest of all underground openings sampled at 1.5 m wide by 0.6 m high. Adit 2 was larger than Adit 1 at 1 m wide by 1.5 m high. The depths of each adit and extent of underground spaces are unknown. For surveyor safety, due to site instability, the underground workings were not entered, and all field work was conducted from the surface at this site.

An abandoned rail tunnel (hereafter called Rail Tunnel) was selected as the second site for pilot sampling, located directly upslope from an active rail line on the north bank of the Thompson River, 3.4 km downstream of the outlet of Kamloops Lake. This site is also within

the BGxh2 at 365 m asl. The tunnel is approximately 380 m long but is collapsed in the middle section preventing passage from one end to the other (Skeetchestn Natural Resources, unpublished data). The openings face east and west respectively and are triangle-shaped at approximately 4 m wide at the base of the opening by 4 m high at the apex. Due to evidence of partial collapse, the tunnel was not entered by surveyors and each opening was sampled and monitored from the surface.

Finally, an inactive portal at the New Afton Mine, at the base of the historic Afton open pit mine was included in the pilot study (hereafter called New Afton Pit Portal). This site is located within the Nicola Very Dry Warm (BGxw1) biogeoclimatic zone which is characterized by mid-elevation grassland (Ryan et al. 2022); however, the landscape surrounding the portal is an active mine site and represents primarily industrial land use. The portal entrance is located at 525 m asl and is approximately 5 m wide by 5 m high. It is a singular blind-ended passage that was previously closed internally with a bulkhead at approximately 150 m deep. Surveyor entry into the portal was permitted so field work occurred here at two sampling locations, one at the portal entrance and at a second internal station approximately 50 m underground.

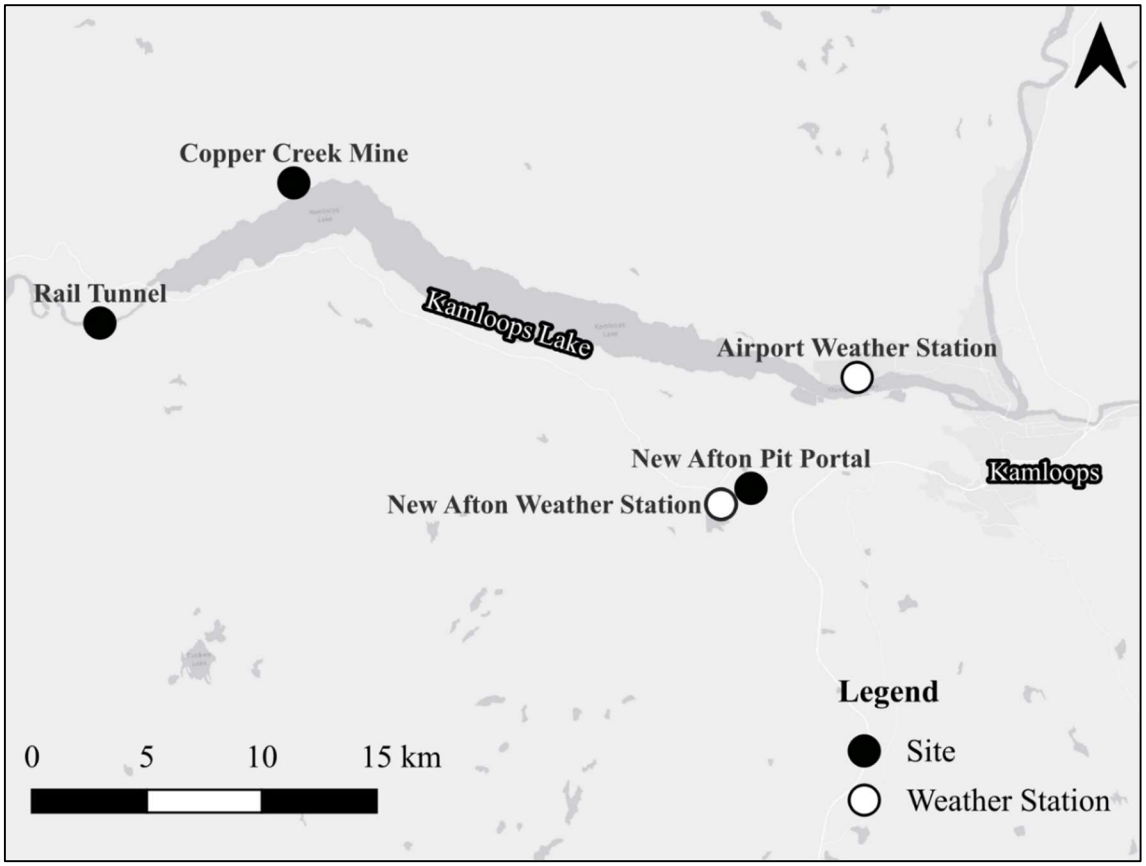

Figure 4.1 Bat hibernacula sites selected for winter eDNA air sampling and long-term passive acoustic monitoring for bats, and weather station locations.

eDNA Air Sampling

eDNA air sampling occurred once at each site in mid-winter (late January to early March 2024). An eDNA air sampler design was used consisting of a computer fan with a 3D printed filter frame, powered by a rechargeable 12V battery with approximately 12 hour battery life, as described by Garrett et al. (2023*a*). Air samplers were placed 1 to 3 m inside tunnel entrances, providing some shelter from direct precipitation and wind, except at the Pit Portal internal sampling location where air samplers were deployed at 50 m inside the tunnel. Three replicate air samples were collected from each sampling location (Table 4.1).

This study represents the first test of these air samplers in Canadian winter conditions and so the sampler deployment strategy necessarily was adapted to account for in-the-field learnings as the pilot study progressed. At the Rail Tunnel site, the first site sampled, 9 cm diameter mixed cellulose ester reinforced membrane filters with 0.2 µm pore size (Millipore) were

used (Figure 4.2, panels A and B, and as trialed at the New Afton artificial bat cave, see Chapter 3); however, one of the three air sample filters from Rail Tunnel East blew off the air sampler prior to collection, spoiling the sample. At the subsequent sites, 12 cm by 12 cm pieces of Filtrete 1900 Smart Air Filters "Merv 13" furnace filter material (3M) that could be secured to the air samplers with ring clamps, as described by Garrett et al. (2023), were used for Copper Creek Mine and New Afton Mine (Table 4.1). Copper Creek Mine presented two additional challenges. The mine tunnels were very unstable, and the mine entrances were small and in the case of Adit 2 sloped downwards, making air sampler deployment very challenging. Evidence of *Neotoma cinerea* (Bushy-tailed Woodrat) activity at both adits was observed (i.e., scat), which may interfere with the air sampling equipment. To combat these two problems, air samplers at this site were fastened inside wire mesh cages and deployed using a 3 m extendable pole with a hook, requiring the use of filters that could be secured to the air sampler with a ring clamp (as described above and in Garrett et al. 2023). This deployment strategy allowed the surveyors to remain outside the mine tunnel, kept the filters securely fastened to the air samplers during the deployment, and prevented interference with the equipment by non-target wildlife (Figure 4.2, panels C and D). Wire mesh cages were not required at either the Rail Tunnel or New Afton Mine **(**Figure 4.2, panels E and F). Air samplers at these two sites were deployed inside open-topped plastic tubs to prevent direct contact of the air sampler, battery, and wires with the ground.

Prior to deployment, each air sampler was cleaned with a 50% bleach solution followed by a triple rinse with deionized water to prevent contamination of the sampling surface. Air samplers were deployed in the afternoon and left to run overnight with the vacuum pump actively sampling for approximately 12 hours, through the bat emergence period after sunset. Air samplers and filters were collected the following morning. Sample envelopes were placed into plastic zipper top bags (three envelopes per bag) with one tablespoon of colour-indicating silica desiccant beads. One field blank was collected per site after sample retrieval, for a total of three field blanks. Field blank filters were stored and transported with sample filters. All filters were kept in a cooler with ice for transport from the field and then in a -20°C freezer for temporary storage prior to being shipped to the University of Victoria in a cooler with ice packs for laboratory analysis.

All filters were sent to the Helbing lab at the University of Victoria for analysis via qPCR against the newly designed eMyotis1 assay. Laboratory analysis followed a well established eDNA sample processing workflow (e.g., Hobbs et al. 2019, Hocking et al. 2022). DNA was extracted and evaluated for integrity and inhibition using IntegritE-DNA test. Each sample was tested with eight qPCR replicates against the eMyotis1 assay, eight negative controls (i.e., no DNA template), and two positive controls (i.e., containing *Myotis* DNA). eDNA copy number per sample (including standard error, SE) was estimated by the Helbing lab using a Binomial-Poisson model based on the limit of detection and limit of quantification for the eMyotis1 assay, as well as number of PCR replicates that amplified per sample (Lesperance et al. 2021, Hocking et al. 2022).

Samples that were positive via the eMyotis1 qPCR-based assay were pooled by site and sent to Northern Arizona University (Bat Ecology & Genetics Lab) for analysis via DNA metabarcoding based on a short section of *cytochrome oxidase subunit 1* (*COI*) (Walker et al. 2016, 2019). Of the 15 species of bats known to occur in B.C., plus three accidental/unconfirmed species, most can be classified to species level with this primer except *M. thysanodes* (Fringed Myotis) versus *M. evotis* (Long-eared Bat), and *M. californicus* versus *M. ciliolabrum*, for which the *COI* sequences do not differentiate the species within each metabarcoding dyad (Bat Ecology and Genetics Lab 2025).

Though not the focus of this study, non-bat DNA incidentally recovered during the metabarcoding was also reported and corroborated against detections of non-bat species throughout the field work. Non-bat values were cross-referenced against the National Center for Biotechnology Information's Genbank database using BLAST and then classified using Lowest Common Ancestor analysis in MEGAN (v6). Metabarcoding laboratory analysis and bioinformatics were completed by the Bat Ecology & Genetics Lab, Northern Arizona University.

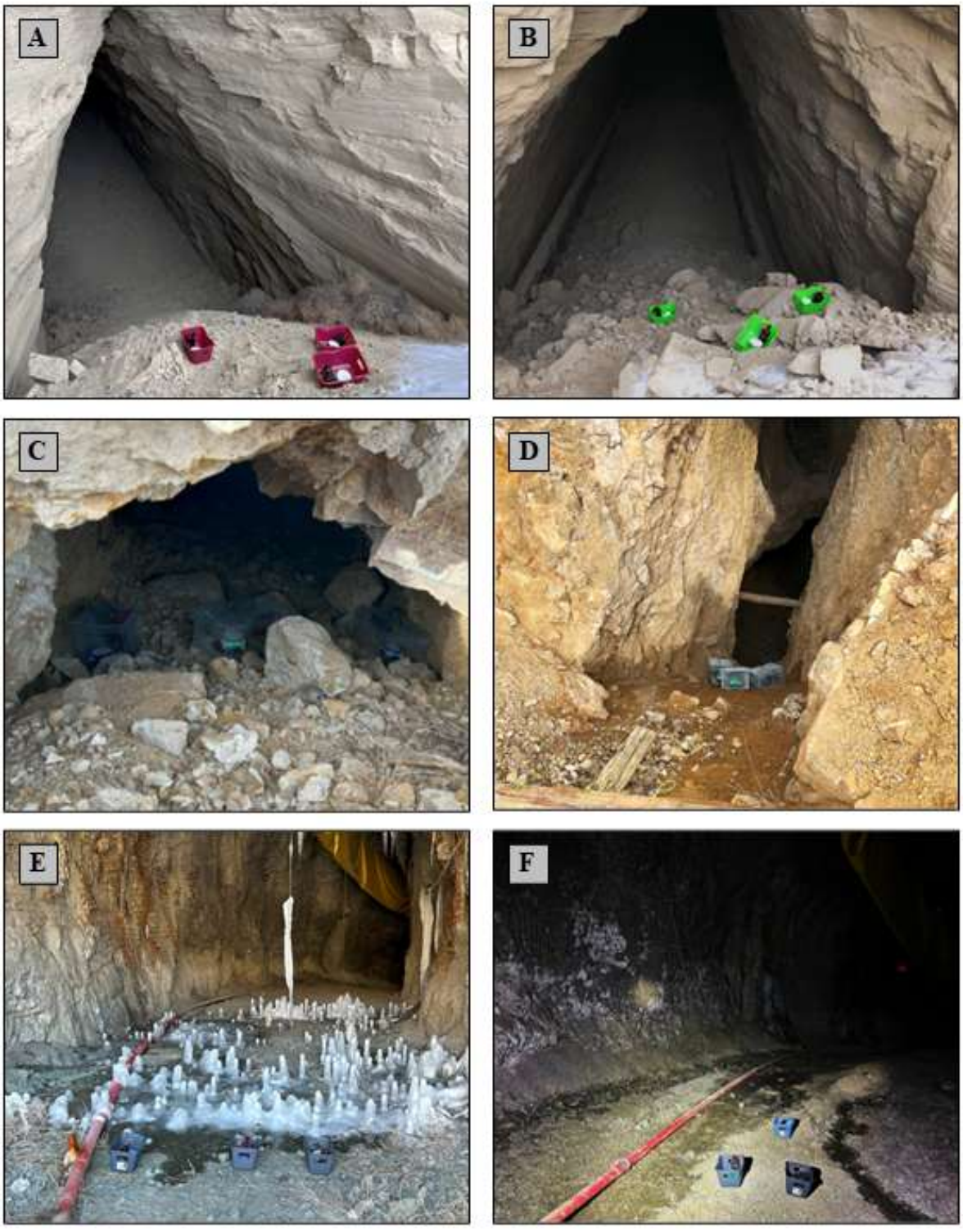

Figure 4.2 eDNA air sampler deployments at Rail Tunnel West (A) and East (B), Copper Creek Mine Adit 1 (C) and Adit 2 (D), and New Afton Pit Portal entrance (E) and internal (F) sampling locations.

Table 4.1 Bat eDNA air sampling scheme including site, sampling location, date and time of air sampler deployment, collection, and sampling duration, details of the deployment, and number of samples.

Site	Location	Deployment date / time	Local sunset time	Collection date / time	Deployment duration	Wire mesh cage	Filter type	# of samples
Rail Tunnel	West	20-Jan-2024 15:05	16:32	21-Jan-2024 9:55	18:50	No	Millipore	3
	East	20-Jan-2024 15:35	16:32	21-Jan-2024 10:35	19:00	No	Millipore	2
Copper Creek Mine	Adit 1	29-Feb-2024 12:15	17:42	01-Mar-2024 9:40	21:25	Yes	Filtrete	3
	Adit 2	29-Feb-2024 13:10	17:42	01-Mar-2024 10:15	21:05	Yes	Filtrete	3
New Afton Mine	Internal	06-Mar-2024 15:00	17:52	07-Mar-2024 9:55	18:55	No	Filtrete	3
	Entrance	06-Mar-2024 15:05	17:52	07-Mar-2024 10:00	18:55	No	Filtrete	3

Acoustic Monitoring

Passive ultrasonic acoustic bat detectors were deployed through the winter at each of the five underground tunnels where eDNA air sampling occurred (Table 4.2). A Roost Logger Anabat RL1s (Titley Scientific) was deployed within 1 m of each opening at the Rail Tunnel (West and East) and Copper Creek Mine sites (Adit 1 and 2) on t-post at a height of 1 m, with microphones directed towards the tunnel openings. A Roost Logger was also deployed inside the New Afton pit portal at the internal sampling location. This detector was mounted on the wall of the tunnel at a height of 1.5 m with the microphone pointed across the tunnel. The Roost Loggers were deployed with internal lithium batteries to withstand cold winter temperatures. A Song Meter SM4Bat FS Ultrasonic Recorder with external U2 microphone (Wildlife Acoustics) was also deployed approximately 10 m outside the New Afton Mine Pit Portal entrance to capture free-flying bat calls and aid in species identification. The SM4Bat operated on a solar panel and external 12V battery and the microphone was boosted to a height of 3 m on a pole. These long-term passive monitoring units were deployed in fall 2023 (late October or early November) and recorded bat echolocation each night from 30 minutes prior to sunset to 30 minutes after sunrise until the units were retrieved in spring 2024. An

additional SM4Bat FS Ultrasonic Recorder with external U2 microphone was deployed during the night of eDNA sample collection at both Rail Tunnel East and Copper Creek Mine Adit 2 to capture free-flying bat calls and aid in species identification. This unit was 10 m from each tunnel opening with the microphone boosted on a 3 m extendable pole.

Table 4.2 Summary of acoustic detector deployments at each underground opening.

Site	Sampling Location	Acoustic Detector Deployment Date	End date	Total # recording nights	Detector type
Rail Tunnel	West	09-Nov-2023	03-May-2024	175	Roost logger
	East	09-Nov-2023	03-May-2024	175	Roost logger
	East*	20-Jan-2024	21-Jan-2024	1	SM4Bat
Copper Creek Mine	Adit 1	02-Nov-2023	29-Apr-2024	179	Roost logger
	Adit 2	02-Nov-2023	29-Apr-2024	179	Roost logger
	Adit 2*	29-Feb-2024	01-Mar-2024	1	SM4Bat
New Afton Mine	Internal	18-Oct-2023	03-Jul-2024	259	Roost logger
	Entrance	18-Oct-2023	21-Jun-2024	247	SM4Bat

Acoustic recordings were analyzed manually in Anabat Insight (Version 2.1.3, Titley Scientific). A recording with two or more bat echolocation pulses was considered a bat pass. Where possible, bat passes were classified to species based on call characteristics as described in by Lausen et al. (2022). However, in many cases, due to poor quality recordings, generic acoustic groupings were used, each containing three or more species. Based on minimum call frequencies, two basic groups were used: *Myotis* and non-*Myotis* (Table 4.3). Recordings of *Myotis* bats and unidentified calls with minimum frequency greater than 30 kHz were categorized as *Myotis* bat passes for the purpose of comparing acoustic detections to eDNA detections of *Myotis* bats. The most common clustering of *Myotis* recordings is 40 kHz; species with minimum call frequencies approximating 35 to 42 kHz include *M. lucifugus* (Little Brown Myotis, MYLU), *M. ciliolabrum* (Western Small-footed Myotis, MYCI), and *M. volans* (Long-legged Myotis, MYVO) (Lausen et al. 2022). Two lower frequency (30 kHz or less) *Myotis* species *M. evotis* (Long-eared Myotis, MYEV) and *M. thysanodes* (Fringed Myotis, MYTH) can be differentiated from the other *Myotis* species in many cases to the species level, although the later can be acoustically confused with

Corynorhinus townsendii (Townsend's Big-eared Bat, COTO). Poor quality recordings of these two low frequency *Myotis* species could inadvertently be include in the non-*Myotis* category (see below). Two high frequency *Myotis* bats were differentiated in their own grouping, MYCAMYYU, because *Myotis californicus* (California Myotis, MYCA) and *M. yumanensis* (Yuma Myotis, MYYU) are the only two species of bats in B.C. that produce calls with minimum frequencies above 45 kHz (typically categorized as 50 kHz *Myotis* bats).

Recordings of non-*Myotis* species and bat passes that could not be identified to a species or acoustic group that had minimum frequencies less than 30 kHz were categorized as non-*Myotis* bat passes. The low frequency bat species that could cluster into this generic non-*Myotis* category include *Eptesicus fuscus* (Big Brown Bat, EPFU) and *Lasionycteris noctivagans* (Silver-haired Bat, LANO) that are often identified as a low frequency non-*Myotis* acoustic dyad when there are no species-specific diagnostic call features; these two species are difficult to differentiate acoustically (Betts 1998). The non-*Myotis* generic low-frequency category also can include poor quality recordings of these species: *Antrozous pallidus* (Pallid Bat), though this species is not confirmed in the study area (Lausen et al. 2022); and *Lasiurus cinereus* (Hoary Bat), though this species is known to migrate out of the province for winter, and does not use underground features as roosting habitat (Lausen et al. 2022). There is one additional species of bat known from the study area that produces such low frequencies that it is unlikely to be confused with other bat species: *Euderma maculatum* (Spotted Bat, EUMA) was classified to species-level when recorded.

Table 4.3 Summary of acoustic groupings, acoustic classifications, and species or potential species that comprise each classification.

Primary acoustic grouping	Acoustic classification	Species or potential species
Myotis	MYCA	*Myotis californicus*
	MYCI	*Myotis ciliolabrum*
	MYEV	*Myotis evotis*
	MYLU	*Myotis lucifugus*
	MYTH	*Myotis thysanodes*
	MYVO	*Myotis volans*
	MYYU	*Myotis yumanensis*
	MYCAMYYU	MYCA or MYYU
	40 kHz *Myotis*	MYCI or MYLU or MYVO
	Unidentified *Myotis*	MYCA or MYCI or MYEV or MYLU or MYTH or MYVO or MYYU
Non-*Myotis*	ANPA	*Antrozous pallidus*
	COTO	*Corynorhinus townsendii*
	EPFU	*Eptesicus fuscus*
	EUMA	*Euderma maculatum*
	LANO	*Lasionycteris noctivagans*
	EPFULANO	EPFU or LANO
	Low frequency bat	ANPA or COTO or EPFU or LANO or possibly MYEV or MYTH
	Unidentified bat	Bat that could not be distinguished to species.

RESULTS

eDNA Air Sampling

One positive detection of *Myotis* eDNA in an air sample was obtained via the eMyotis1 qPCR-based assay at Copper Creek Mine Adit 1 (Sample 1-A,Table 4.4). Five of eight PCR replicates amplified *Myotis* DNA in this sample. One probable detection of *Myotis* DNA was obtained at Copper Creek Mine Adit 2 (Sample 2-B,Table 4.4), meaning one of eight PCR replicates amplified in this sample. Remaining extract from the two samples from Copper Creek Mine that amplified *Myotis* DNA via qPCR were pooled and analyzed via metabarcoding. DNA from two bat species and one species dyad were detected in the pooled sample: one non-*Myotis* species, *Corynorhinus townsendii*, and two *Myotis* DNA sources,

Myotis lucifugus, and the *M. ciliolabrum / M. californicus* genetic dyad (Figure 4.3, Table 4.5).

No winter hibernacula samples collected at either Rail Tunnel or New Afton Mine amplified *Myotis* DNA via qPCR. All three samples collected at Rail Tunnel West failed integrity and inhibition using IntegritE-DNA test and were deemed poor quality. One of the three samples collected at Rail Tunnel East blew off the air sampler prior to filter collection and was discarded. Two air samples collected at Rail Tunnel East were also pooled and sent for metabarcoding analysis and no bat DNA was detected. DNA classified as *Columba* spp. was detected, however, in the pooled Rail Tunnel East eDNA air samples (33,433 reads). Both Rail Tunnel entrances were used as roost sites for *Columba livia* (Rock Pigeon). This species flushed from each entrance on every visit to the site through the winter monitoring period. Air samples from Rail Tunnel West and New Afton Mine were not submitted for metabarcoding analysis.

Incidental non-bat DNA metabarcoding detections from Copper Creek Mine aligned with non-genomic detections throughout the field work, and species that could be reasonably expected at the site. In the pooled eDNA air samples, DNA from seven non-bat animals was detected, from three bird and four mammal sources (Table 4.5). DNA classified as *Columba* spp. was detected (108,528 reads, Table 4.5) and *Columba livia* was also observed roosting at Copper Creek Mine within Adit 2. An accumulation of bird feces was also observed within Adit 2 and two individuals flushed from the mine opening immediately prior to the eDNA air sample collection on March 1, 2024. *Bonasa umbellus* (Ruffed Grouse) DNA was also detected (1,901 reads, Table 4.5). This species was not observed visually near the mine entrances; however, it was detected in the area auditorily (i.e., drumming) during a fall site reconnaissance visit (October 19, 2023). *Accipter* spp. DNA was detected (154 reads, Table 4.5) and an unidentified *Accipter* spp., *A. cooperii* (Cooper's Hawk) or *A. striatus* (Sharp-shinned Hawk), flushed from Adit 2 as the surveyors approached the opening to deploy the air samplers on February 29, 2024. This animal grazed its body against the ceiling of the mine tunnel as it exited, shedding several feathers at the eDNA air sampling location as it flew away.

Two of the four non-bat mammal taxa detected at Copper Creek Mine, *Bos* spp. (domestic cattle) and *Neotoma cinerea* (Bushy-tailed Woodrat), are both supported by visual detections during the field work. The area surrounding the mine is used for cattle grazing and cattle were seen upslope of the mine during the site reconnaissance (October 19, 2023). Cattle scat was abundant along the path used to access the mine. *N. cinerea* scat was observed at the entrance to both Adit 1 and Adit 2. While no evidence corroborating presence of *Peromyscus maniculatus* (Deer Mouse) or *Vulpes* spp. (fox species) was observed, presence of both *P. maniculatus* and *Vulpes vulpes* (Red Fox) at the mine site is plausible.

Table 4.4 Winter underground hibernacula air sampling qPCR results for Copper Creek Mine using a genus-wide assay for all *Myotis* bats (eMyotis1). The values provided are number of qPCR replicates out of 8 that amplified DNA, the estimated eDNA copies per sample, and standard errors.

Site	Sampling location	Samples	qPCR via eMyotis1		
			Positive replicates (of 8)	eDNA copies per sample	Standard Error
Copper Creek Mine	Adit 1	1-A	5	72	36
		1-B	0	0	0
		1-C	0	0	0
	Adit 2	2-A	0	0	0
		2-B	1	9	9
		2-C	0	0	0

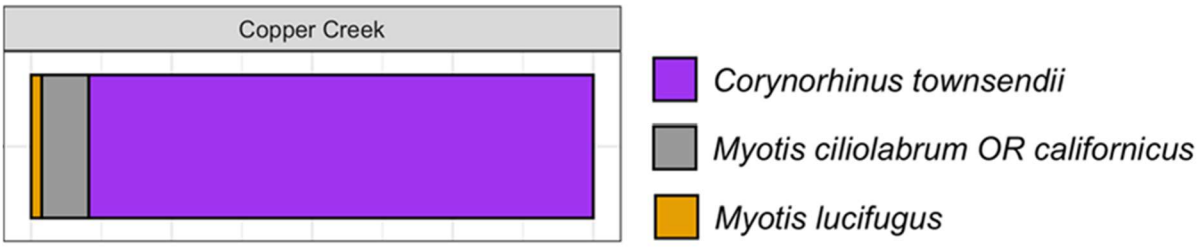

Figure 4.3 Bat species detected via metabarcoding in pooled winter underground hibernacula eDNA air samples collected at two adits at Copper Creek Mine. Sample analyses and figure prepared by Bat Ecology & Genetics Lab at Northern Arizona University. The bar plot represents the proportion of total DNA sequence reads by species. Note, *M. ciliolabrum* and *M. californicus* cannot be distinguished to the species-level based on the *COI* sequence used for metabarcoding.

Table 4.5 Bat and non-bat DNA detected via metabarcoding in pooled winter air samples collected at Copper Creek Mine. Laboratory analysis and bioinformatics were completed by the Bat Ecology & Genetics Lab, Northern Arizona University.

DNA detected by taxa			Number of DNA sequence reads
Detection type	**Scientific name**	**Common name**	
Bat detections	*Corynorhinus townsendii*	Townsend's Big-eared Bat	45,684
	M. ciliolabrum / *californicus*	Western Small-footed Myotis / California Myotis	4,257
	M. lucifugus	Little Brown Myotis	951
Non-bat detections	*Accipiter spp.*	Unidentified *Accipiter* hawk	154
	Bonasa umbellus	Ruffed Grouse	1,901
	Columba spp.	Unidentified pigeon	108,528
	Bos spp.	Domestic cattle	4,050
	Neotoma cinerea	Bushy-tailed Woodrat	29,854
	Peromyscus maniculatus	Deer Mouse	19,657
	Vulpes spp.	Unidentified fox	784

Acoustic Monitoring

The Copper Creek Mine site had regular winter bat activity, particularly at Adit 2 which had more nights with acoustic detections of bat echolocation than Adit 1 (Table 4.6). *Myotis* bats were detected acoustically at both adits in all months of the monitoring period (November 15, 2023, to April 29, 2024). Most acoustic detections were attributed to the *Myotis* bat group (Table 4.7). At both Adit 1 and Adit 2, the number of nights with acoustic detections of *Myotis* bats decreased from November (18.8% and 56.3%) to the lowest levels in January (3.2% and 29.0%r respectively), then increased again from February through April (33.3% and 86.7%, Table 4.6). The only acoustic bat detection on the additional full spectrum detector deployed at Adit 2 on the night of eDNA sampling (February 29, 2024) was one bat pass classified to the *Eptesicus fuscus* / *Lasionycteris noctivagans* acoustic dyad.

The Rail Tunnel site had the most acoustic bat activity of the three sites monitored, and of the two monitoring locations at this site, Rail Tunnel East had more acoustic activity than Rail Tunnel West (Table 4.8 and Table 4.9). *Myotis* bats were detected acoustically at Rail Tunnel East on every night monitored except for six nights: January 11 through 14, February 10 and

February 27, 2024 (Figure 4.6). At Rail Tunnel West, *Myotis* bats were detected acoustically on approximately 75% of nights monitored in November. *Myotis* bats were detected acoustically on approximately 15 to 20% fewer nights in December through February, with the lowest number of nights in February (55.2%). Nights with *Myotis* acoustic detections increased again to 75% of nights in March, and 100% of nights in April (Table 4.8). Most bats detected acoustically at both Rail Tunnel East and West were classified to the *Myotis* bat group (Table 4.9). An additional 17 bat passes were recorded at Rail Tunnel East on the full spectrum bat detector deployed during the night of eDNA air sampling on January 20, 2024. All 17 bat passes were classified to the *M. californicus* / *M. yumanensis* acoustic dyad.

The New Afton Mine site had the highest proportion of non-*Myotis* bat activity of all three sites monitored. Almost 40% of all bat passes recorded at New Afton Mine (internal and external detections combined) were classified as other bats (i.e., likely non-*Myotis*) (Table 4.11). Overall bat activity recorded on the internal bat detector was low compared to the external detector, with just 42 bat passes detected during the monitoring period inside the Pit Portal (Table 4.11). *Myotis* acoustic activity was lowest both inside and outside the portal in January, with *Myotis* acoustic detections on only approximately 3% of nights (Table 4.10). At the Afton Pit external detector, *Myotis* acoustic activity increased substantially in April to approximately 77% of nights (Table 4.10); however, interestingly, at the Pit Portal internal detector there were no acoustic detections of any bats after the night of March 20, 2024, potentially indicating a seasonal movement of bats out of this site.

Table 4.6 Summary of *Myotis* bat acoustic activity detected at Copper Creek Mine by detector location and month, including number of nights with *Myotis* acoustic detections, percentage of detector nights with *Myotis* acoustic detections, and total, mean (±SE), and maximum *Myotis* bat acoustic detections.

Acoustic detector location	Month	Detector nights	*Myotis* bat acoustic detections				
			Nights with activity	% detector nights with activity	Total detections	Mean detections per night ± SE	Max. detections per night
Adit 1	Nov	16	3	18.8	3	<1	1
	Dec	31	3	9.7	3	<1	1
	Jan	31	1	3.2	2	<1	2
	Feb	29	1	3.4	1	<1	1
	Mar	31	8	25.8	10	<1	2
	Apr	29	10	34.5	21	<1	5
Adit 2	Nov	16	9	56.3	32	2.0 ± 0.7	12
	Dec	31	15	48.4	31	1.0 ± 0.2	4
	Jan	31	9	29.0	24	<1	5
	Feb	29	11	37.9	39	1.3 ± 0.9	26
	Mar	31	16	51.6	537	17.3 ± 10.6	322
	Apr	29	26	89.7	289	9.6 ± 2.9	82

Table 4.7 Summary of all bat acoustic detections at Copper Creek Mine by detector location, species group (*Myotis* bat or other), acoustic classification, and month. * Indicates one EPFULANO bat pass recorded on the additional full spectrum bat detector deployed on the night of eDNA air sampling (February 29, 2024). Acoustic classifications are defined in Table 4.3.

Detector location	Species group	Acoustic classification	Nov	Dec	Jan	Feb	Mar	Apr	Total
Adit 1	*Myotis* bats	40 kHz *Myotis*	0	0	1	0	1	2	4
		MYCAMYYU	0	0	0	0	2	2	4
		Unidentified *Myotis*	3	3	1	1	7	17	32
		Sub-total *Myotis*	3	3	2	1	10	21	**40**
	Other bats	-	0	0	0	0	0	0	**0**
		Total	**3**	**3**	**2**	**1**	**10**	**21**	**40**
Adit 2	*Myotis* bats	40 kHz *Myotis*	2	1	3	0	11	19	36
		MYCAMYYU	10	3	1	12	347	148	521
		MYEV	0	0	0	1	0	0	1
		Unidentified *Myotis*	20	27	20	26	179	122	394
		Sub-total *Myotis*	32	31	24	39	537	289	**952**
	Other bats	COTO	0	0	0	0	0	4	4
		EPFULANO	0	0	0	1*	0	0	1
		Low frequency bat	3	5	0	0	1	2	11
		Unidentified bat	4	3	0	0	2	66	75
		Sub-total Other	7	8	0	1	3	72	**91**
		Total	**39**	**39**	**24**	**40**	**540**	**361**	**1,043**

Table 4.8 Summary of *Myotis* bat acoustic activity detected at the Rail Tunnel site by detector location and month, including number of nights with *Myotis* acoustic detections, percentage of detector nights with *Myotis* acoustic detections, and total, mean (±SE), and maximum *Myotis* bat acoustic detections.

Acoustic detector location	Month	Detector nights	*Myotis* bat acoustic detections				
			Nights with activity	% of detector nights with activity	Total detections	Mean detections per night ± SE	Max. detections per night
Rail Tunnel East	Nov	16	16	100.0	406	25.4 ± 5.3	81
	Dec	31	31	100.0	687	22.2 ± 3.1	72
	Jan	31	27	87.1	506	16.3 ± 3.5	80
	Feb	29	27	93.1	503	17.3 ± 2.5	46
	Mar	31	31	100.0	789	25.5 ± 4.8	107
	Apr	30	30	100.0	1,390	46.3 ± 4.8	133
Rail Tunnel West	Nov	16	12	75.0	53	3.3 ± 1.4	23
	Dec	31	18	58.1	236	7.6 ± 3.0	85
	Jan	31	19	61.3	162	5.2 ± 2.1	60
	Feb	29	16	55.2	72	2.5 ± 0.8	18
	Mar	31	23	74.2	231	7.5 ± 2.0	43
	Apr	30	30	100.0	610	20.3 ± 2.4	54

Table 4.9 Summary of all bat acoustic detections at Rail Tunnel by detector location, species group (*Myotis* bat or other), acoustic classification, and month. * Indicates the total number of bat passes includes 17 bat passes recorded on the additional full spectrum bat detector deployed on the night of eDNA air sampling (January 20, 2024). Acoustic classifications are defined in Table 4.3.

Detector location	Species group	Acoustic classification	Nov	Dec	Jan	Feb	Mar	Apr	Total
Rail Tunnel East	*Myotis* bats	40 kHz *Myotis*	1	5	2	2	24	18	52
		MYCAMYYU	225	333	257	213	338	441	1,807
		MYEV	0	3	0	0	0	1	4
		Unidentified *Myotis*	180	346	267*	288	427	928	2,433
		MYYU	0	0	0	0	0	2	2
		Sub-total	406	687	523	503	789	1,390	**4,298**
	Other bats	Unidentified bat	0	1	0	0	0	5	6
		EPFU	3	0	0	1	0	0	4
		Low frequency bat	0	1	0	0	1	3	5
		Sub-total	3	2	0	1	1	1,398	**15**
								Total	**4,313**
Rail Tunnel West	*Myotis* bats	40 kHz *Myotis*	0	0	2	0	0	12	14
		MYCAMYYU	3	58	31	19	42	190	343
		Unidentified *Myotis*	50	178	129	53	189	408	1,007
		Sub-total	53	236	162	72	231	610	**1,364**
	Other bats	Unidentified bat	0	1	1	1	2	0	5
		Low frequency bat	0	0	0	0	0	1	1
		Sub-total	0	1	1	1	2	1	**6**
								Total	**1,370**

Table 4.10 Summary of *Myotis* bat acoustic activity detected at New Afton Mine by detector location and month, including number of nights with *Myotis* acoustic detections, percentage of detector nights with *Myotis* acoustic detections, and total, mean (±SE), and maximum *Myotis* acoustic detections.

Acoustic detector location	Month	Detector nights	*Myotis* bat acoustic detections				
			Nights with detections	% of detector nights with activity	Total detections	Mean detections per night ± SE	Max. detections per night
Afton Pit (external)	Nov	16	11	68.8	21	1.3 ± 0.4	6
	Dec	31	9	29.0	21	<1	6
	Jan	31	1	3.2	1	<1	1
	Feb	29	5	17.2	8	<1	3
	Mar	31	14	45.2	32	1.0 ± 0.1	6
	Apr	30	23	76.7	448	14.9 ± 4.2	95
Pit Portal (internal)	Nov	16	5	31.3	10	<1	5
	Dec	31	6	19.4	18	<1	7
	Jan	31	1	3.2	1	<1	1
	Feb	29	1	3.4	1	<1	1
	Mar	31	3	9.7	8	<1	4
	Apr	30	0	0.0	0	0	0

Table 4.11 Summary of all bat acoustic detections at New Afton Mine by detector location, species group (*Myotis* bat or other), acoustic classification, and month. Acoustic classifications are defined in Table 4.3.

Detector location	Species group	Acoustic classification	Nov	Dec	Jan	Feb	Mar	Apr	Total
Afton Pit (external)	*Myotis* bats	40 kHz *Myotis*	14	12	1	1	23	104	155
		MYCAMYYU	0	0	0	0	0	15	14
		MYLU	0	0	0	0	0	4	4
		Unidentified *Myotis*	7	9	0	7	9	325	357
		Sub-total	21	21	1	8	32	448	**531**
	Other bats	COTO	0	0	0	2	7	10	19
		EPFU	0	0	0	0	4	2	6
		EPFULANO	17	40	8	48	68	129	310
		EUMA	0	0	0	0	0	1	1
		LANO	2	1	0	0	3	0	6
		Low frequency bat	2	2	0	3	2	1	10
		Sub-total	21	43	8	53	84	143	**352**
								Total	**883**
Pit Portal (internal)	*Myotis* bats	40 kHz *Myotis*	0	0	0	0	2	0	2
		MYCAMYYU	0	1	0	0	0	0	1
		Unidentified *Myotis*	10	17	1	1	6	0	35
		Sub-total	10	18	1	1	8	0	**38**
	Other bats	Unidentified bat	0	3	0	0	1	0	4
		Sub-total	0	3	0	0	1	0	**4**
								Total	**42**

Integrated Results

Bats were detected both acoustically and via eDNA air sampling at Copper Creek Mine (Table 4.12). Bat eDNA was detected from samples at both adits via the eMyotis1 qPCR-based eDNA and in the pooled sample via metabarcoding. On the night of eDNA sampling, February 29, 2024, concurrent acoustic monitoring at Adit 1 did not detect any acoustic bat activity (Figure 4.4). There was, however, one acoustic detection of a *Myotis* bat (*M. californicus* / *M. yumanensis* dyad) on the subsequent evening, March 1, 2024 (Figure 4.4). The most recent acoustic bat activity at Adit 1 prior to the night of eDNA sampling, was

recorded 11 nights prior to the eDNA sampling event, on the night of February 18, 2024, when one *Myotis* bat pass recorded. At Adit 2, concurrent acoustic monitoring on the night of eDNA air sampling detected 1 bat pass which was identified as *M. evotis* (Figure 4.5). One bat pass was also recorded on the additional full spectrum detector deployed approximately 10 m back from the Adit 2 entrance during the night of eDNA sampling; This bat pass was identified to the *Eptesicus fuscus* / *Lasionycteris noctivagans* acoustic dyad.

At the Rail Tunnel site no bat eDNA was detected via the eMyotis1 qPCR assay or metabarcoding (Rail Tunnel East only) in air samples collected on the night of January 20, 2024, despite nine concurrent *Myotis* acoustic detections at Rail Tunnel East (Figure 4.6) and three concurrent *Myotis* acoustic detections at Rail Tunnel West (Figure 4.7). Of the nine acoustic bat detections at Rail Tunnel East, six were classified to the *M. californicus* / *M. yumanensis* acoustic dyad, and three bat passes were classified to the general *Myotis* species group. The three concurrent *Myotis* bat passes at Rail Tunnel West were all classified to the general *Myotis* group. A total of 17 bat passes were recorded on the additional full spectrum bat detector deployed at Rail Tunnel East on the night of eDNA sampling. All bat passes recorded on this detector were *Myotis* bats that were classified to the *M. californicus* / *M. yumanensis* acoustic dyad.

There were no acoustic detections of any bats on either acoustic detector at New Afton Pit Portal on the night of eDNA air sample collection, March 6, 2024 (Table 4.12, Figure 4.8, and Figure 4.9). The last night with acoustic bat activity prior to eDNA air sampling was four nights prior, on March 2, 2024, when two *Myotis* bat passes were detected at the Afton Pit external detector (Figure 4.8). These two bat passes were classified to the 40 kHz *Myotis* bat group. At the New Afton Pit Portal internal detector, the most recent acoustic bat detection was 15 nights prior to the eDNA air sampling event, on February 21, 2024 (one *Myotis* pass, Figure 4.9).

Table 4.12 Synthesis of eDNA air sampling results and acoustic detection results, by site and sampling location, for the night of eDNA air sampling at each site respectively. Acoustic classifications are defined in Table 4.3.

Site and sampling location	eDNA results			Concurrent acoustic detections	Nights since last acoustic detection
	Sampling Night	qPCR eMyotis1	Metabarcoding		
Copper Creek Mine Adit 1	29-Feb-2024	Positive (1 sample)	COTO MYCI / MYCA dyad MYLU	None	11 nights (1 Unidentified *Myotis* pass)
Copper Creek Mine Adit 2	29-Feb-2024	Probable (1 sample)		1 MYEV pass (at mine opening) 1 EPFULANO pass (at 10 m setback)	0 nights
Rail Tunnel West	20-Jan-2024	Negative	Did not analyze	3 *Myotis* passes	0 nights
Rail Tunnel East	20-Jan-2024	Negative	No bats detected	6 MYCAMYYU passes and 3 *Myotis* passes (at tunnel opening) 17 MYCAMYU passes (at 10 m setback)	0 nights
New Afton Pit Portal Internal	06-Mar-2024	Negative	Did not analyze	None	4 nights (2 passes of 40 kHz *Myotis*)
New Afton Pit Portal External	06-Mar-2024	Negative	Did not analyze	None	15 nights (1 Unidentified *Myotis* pass)

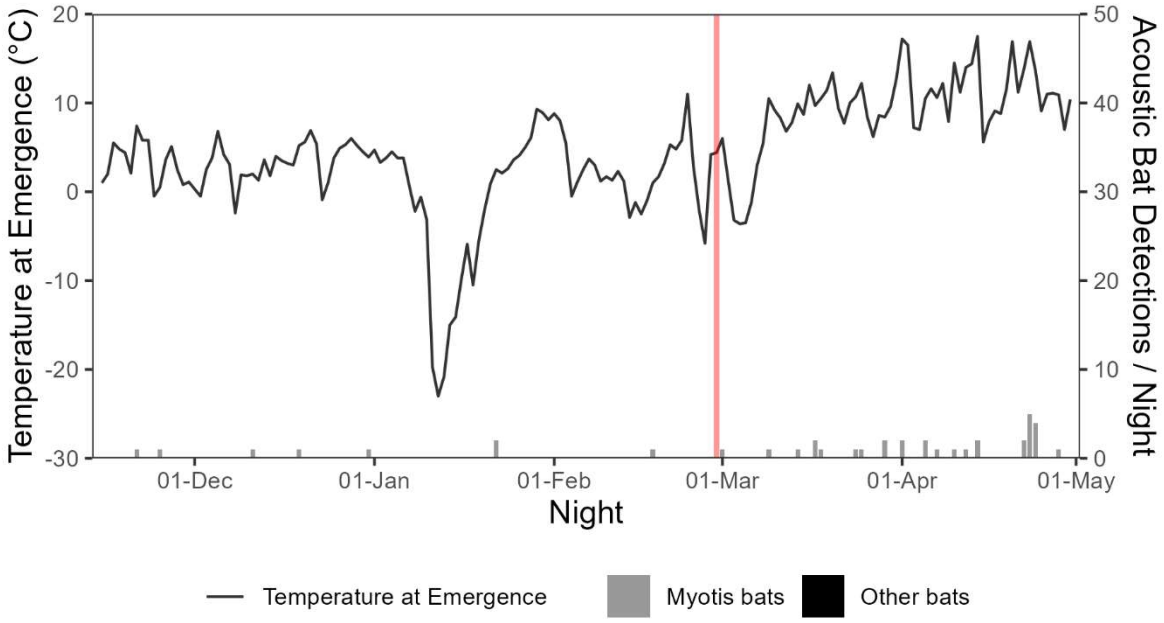

Figure 4.4 All bat detections (*Myotis* bats and other species) at the Copper Creek Adit 1 Roost Logger throughout the passive acoustic monitoring period with temperature at emergence measured at the Kamloops Airport weather station. The red line indicates the night of eDNA air sampling, February 29, 2024.

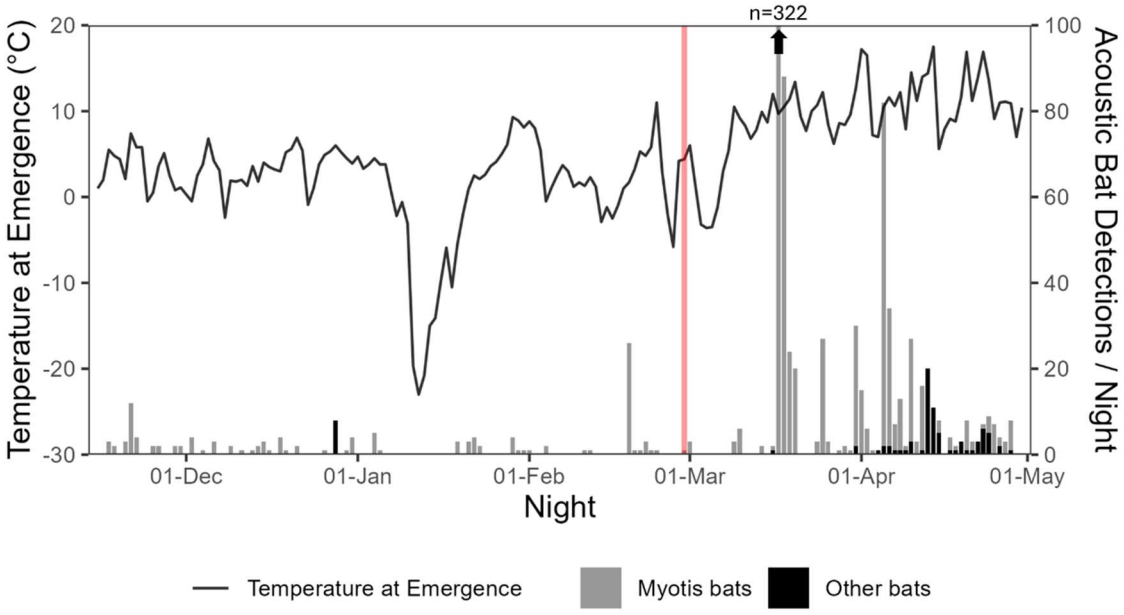

Figure 4.5 All bat detections (*Myotis* bats and other species) at the Copper Creek Adit 2 Roost Logger throughout the passive acoustic monitoring period with temperature at emergence measured at the Kamloops Airport weather station. The total number of acoustic bat detections on the night of March 17, 2024 (n=322) is not shown on the figure. The red line indicates the night of eDNA air sampling, February 29, 2024.

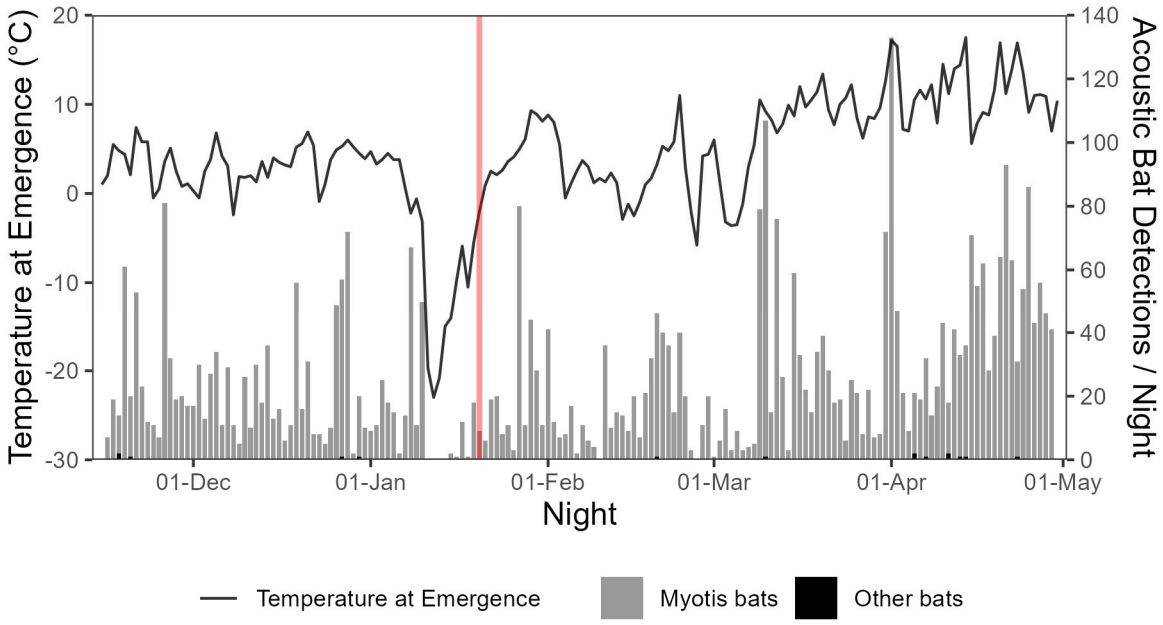

Figure 4.6 All bat detections (*Myotis* bats and other species) at the Rail Tunnel East Roost Logger with temperature at emergence measured at the Kamloops Airport weather station. The red line indicates the night of eDNA air sampling, January 20, 2024.

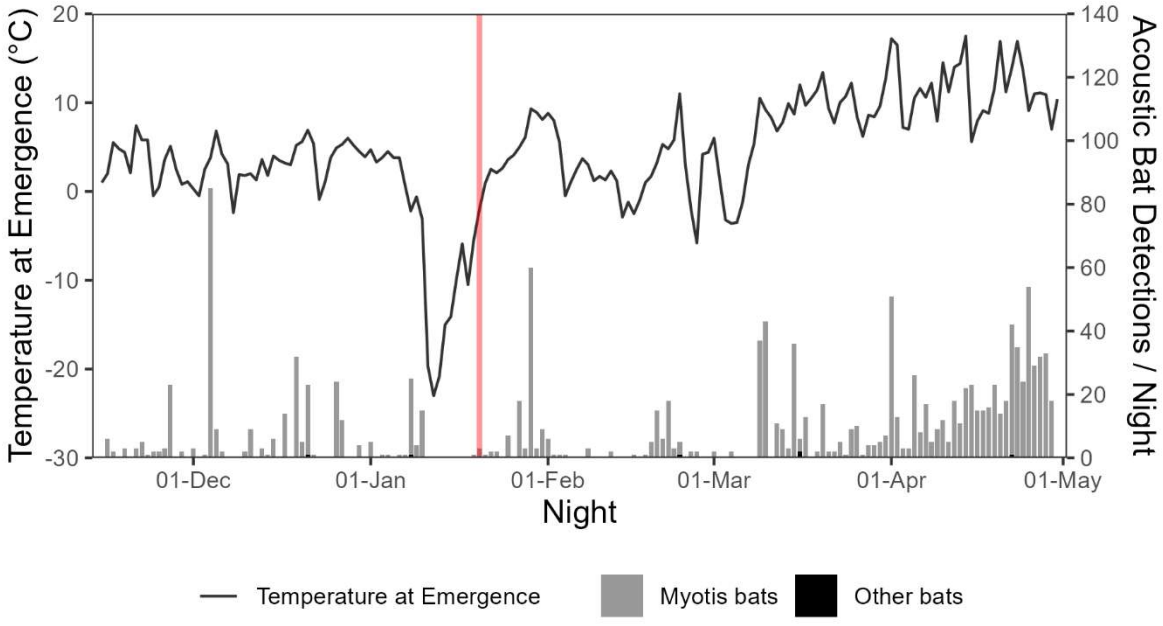

Figure 4.7 All bat detections (*Myotis* bats and other species) at the Rail Tunnel West Roost Logger with temperature at emergence measured at the Kamloops Airport weather station. The red line indicates the night of eDNA air sampling, January 20, 2024.

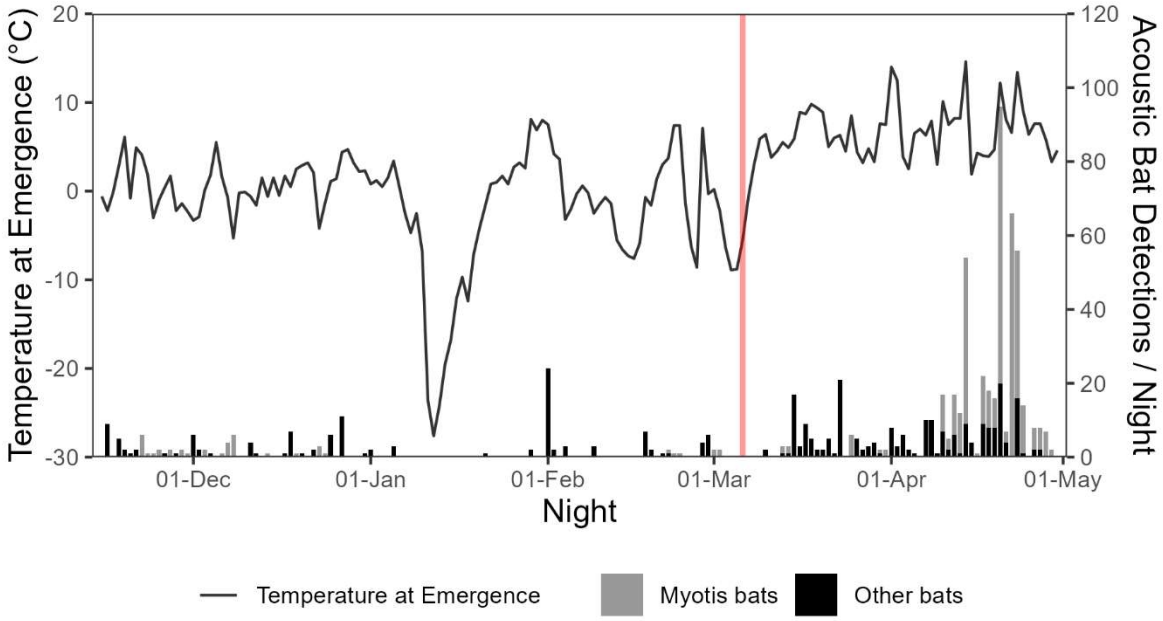

Figure 4.8 All bat detections (*Myotis* bats and other species) at the New Afton Pit external SM4 bat detector throughout the passive acoustic monitoring period with temperature at emergence measured at the New Afton Mine weather station. The red line indicates the night of eDNA air sampling March 6, 2024.

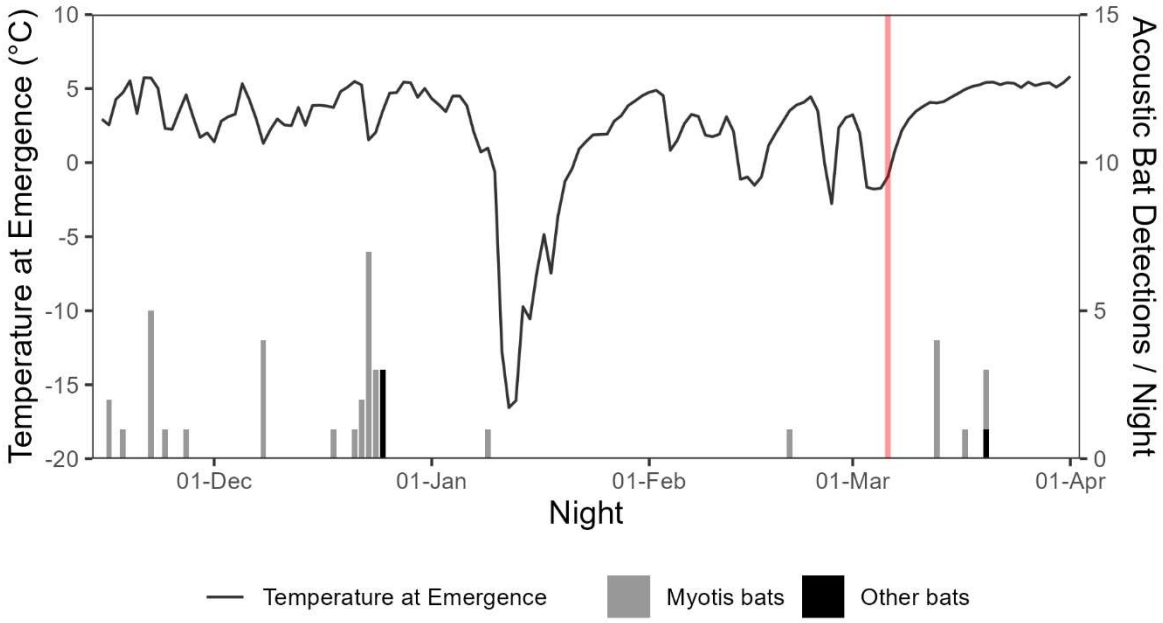

Figure 4.9 All bat detections (*Myotis* bats and other species) at the Afton Pit Portal internal Roost Logger bat detector throughout the passive acoustic monitoring period with temperature at emergence, measured at the internal sampling station on a Hobo logger. The red line indicates the night of eDNA air sampling March 6, 2024.

DISCUSSION

Bat eDNA was successfully detected in air samples via qPCR and metabarcoding at an abandoned underground mine near Kamloops, B.C. The positive winter eDNA air sampling detections at Copper Creek Mine demonstrate the utility of eDNA air sampling to provide a valuable source of information for habitat assessment and management efforts at sites and in seasons that are extremely challenging to survey. Copper Creek Mine was not safe for surveyors to enter to complete a visual inspection for bats or bat sign. The findings of this study also highlight the limitations of eDNA air sampling as a sole survey method for winter bat presence. eDNA air sampling at two hibernacula sites produced negative results via a *Myotis* genus qPCR-based assay despite acoustic detections of *Myotis* bats during the winter (Table 4.12). At all three sites, acoustic detection data demonstrated a pattern of regular bat activity throughout the winter that supports their use as hibernacula. At Copper Creek Mine, the eDNA results added further species resolution that was not achieved with acoustics alone.

Over the course of the winter acoustic monitoring period at Copper Creek Mine, two species and two acoustic dyads were identified from diagnostic call characteristics: *Corynorhinus townsendii*, *M. evotis*, the *M. californicus* / *M. yumanensis* dyad, and the *Eptesicus fuscus* / *Lasionycteris noctivagans* dyad (Figure 4.10). Presence of 40 kHz *Myotis* was confirmed via acoustics, with calls detected that could belong to any of *M. ciliolabrum, M. volans*, and the federally Endangered *M. lucifugus*; however, presence of any one of these three species could not be confirmed based on acoustics data. All remaining passes were classified in generic acoustic groups due to lack of diagnostic call features or poor call quality. The eDNA air sampling results from the night of February 29, 2024, detected the presence of *Myotis* DNA via qPCR in two samples. These two samples were then pooled and analyzed via metabarcoding, which detected DNA from *Corynorhinus townsendii*, the *M. ciliolabrum* / *M. californicus* genetic dyad, and *M. lucifugus* (Figure 4.10). Taken together, the acoustic and eDNA results support confirmed winter presence of *Corynorhinus townsendii*, likely presence of *M. californicus* and/or *M. ciliolabrum*, and *M. lucifugus*, and/or *M. evotis*, and possible presence of *M. yumanensis*, and *M. volans*, and *Eptesicus fuscus* and/or *Lasionycteris noctivagans* at Copper Creek Mine (Figure 4.10). Diagnostic *Corynorhinus townsendii* acoustic calls were not recorded until April, making confirmation of presence of this species in winter from acoustics uncertain; however, the genetic identification of this

species in winter confirms its use of Copper Mine as a hibernaculum. This species does not typically produce loud echolocation calls and therefore may be underrepresented in acoustic surveys (Lausen et al. 2022). Additionally, the *Eptesicus fuscus / Lasionycteris noctivagans* acoustic dyad was detected on the additional full spectrum acoustic detector set 10 m back from the mine entrance. This acoustic detector has a detection radius of approximately 50 m so this bat pass cannot be directly attributed to use of the mine.

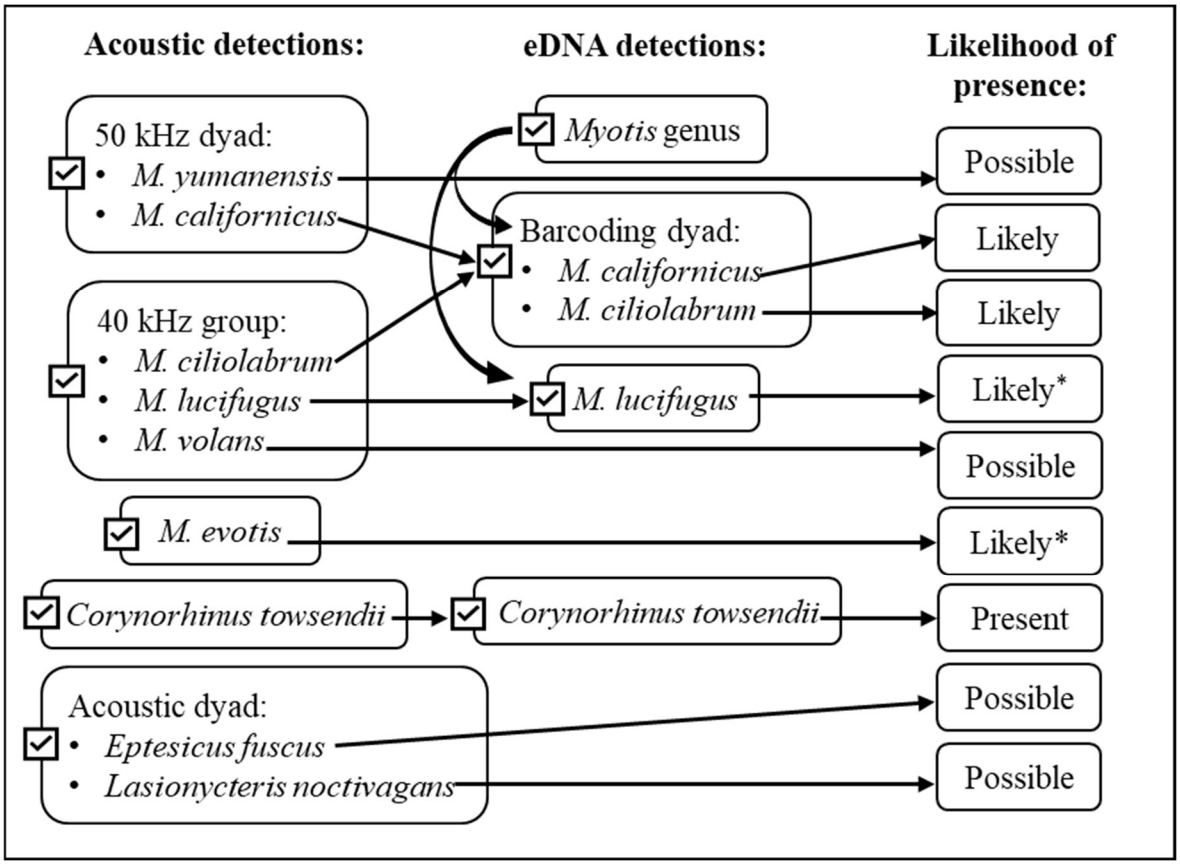

Figure 4.10 Acoustic and eDNA bat detections at Copper Creek Mine, and an assessment of the likelihood of winter presence of each species based on the combined results: possible, likely, or confirmed present. **M. lucifugus* was classified as likely due to uncertainty resolving some populations from *M. evotis* using mitochondrial DNA (D. Paetkau, Wildlife Genetics International, unpublished data). *M. evotis* was classified as likely due to recording of diagnostic call pattern (Lausen et al. 2022).

Further, eDNA was detected in the pooled air samples from Copper Creek Mine via metabarcoding that matched to a federally Endangered species, *Myotis lucifugus*. Mine closure decisions necessarily must consider factors such as presence of at-risk species. Winter

hibernacula sites for *M. lucifugus* have been defined as Critical Habitat for this species under the federal Recovery Strategy (ECCC 2018). However, very little information exists on the winter ecology *of M. lucifugus* in B.C. and there are no documented hibernation records of *M. lucifugus* in the province (Lausen et al. 2022). In high clutter environments, like roost entrances, *M. lucifugus* cannot be reliably distinguished from two other 40 kHz *Myotis* species with overlapping call characteristics (Lausen et al. 2022). While one might assume that the positive genetic identification of this species at Copper Creek (Fig. 4.10) should provide confirmation of its presence, its presence is being listed only as likely because of potential uncertainty surrounding the ability to resolve some populations of *M. lucifugus* from *M. evotis* using mitochondrial DNA (D. Paetkau, Wildlife Genetics International, unpublished data). The only bat pass acoustically recorded on the Roost Logger located 1 m from the mine entrance on the night of eDNA sampling at Copper Creek Mine was of *M. evotis*. Follow-up confirmatory sampling is thus warranted, and if *M. lucifugus* is confirmed during winter, Copper Creek Mine would be one of few confirmed hibernacula for *M. lucifugus* in B.C. and would warrant an update for critical habitat as defined in the species' federal recovery strategy (ECCC 2018). However, this mine is being assessed for closure (B. Quist, B.C. Ministry of Mining and Critical Minerals, personal communication). The caution being applied to the interpretation of *M. lucifugus* genetic results also highlights the limitations that currently exist for bat genomic tools (E. Clare, York University, personal communication): many have yet to be developed, validated and/or adapted to capture intraspecies variation, especially in wide-ranging species (e.g., Lausen et al. 2008). Because of the difficulty in identifying some bat species acoustically and the current paucity of genetic tools for accurately differentiating bat species, it is critical that additional resources are invested in genomic tools that consider genetic variation across species' ranges. Accurate species-specific genetic tools are essential for suitable mine closure planning, to inform decisions regarding the necessity for, and the appropriate design of, bat-friendly gates.

This pilot project has highlighted that eDNA collection methods may not always detect bat eDNA at entrances to occupied underground hibernacula in winter. The methods used for eDNA air sample collection should be considered carefully. Bat eDNA was not detected at two underground hibernacula in this pilot study, despite bats being recorded acoustically during the winter at both sites. These non-detection results highlight some potential obstacles

to capturing eDNA from air at underground hibernacula. First, the Millipore filter used at the Rail Tunnel site was not appropriate for winter mine sampling because it could not be securely fastened to the air sampler. Second, the sizes of the tunnel entrances and depth of the sites varied so the proximity between the air samplers and flying and roosting bats likely varied at each site. Copper Creek Mine had the smallest tunnel entrances so mine-exiting bats would have had to pass closer to the air samplers than at the other two sites (i.e., within about 1.5 m versus 4 to 5 m at the other sites). Copper Creek is likely also the shallowest site so air samplers may have been deployed closer to roosting bats. Further investigation is needed to determine the distance a bat must be from an air sampler, flying and roosting, to reliably capture eDNA. Thirdly, the timing of eDNA air sampling compared to bat activity appears to be important, particularly at large and deep sites where air samplers might be deployed far from roosting bats. eDNA air sampling at New Afton Mine Pit portal coincided with a period of no acoustic bat activity, and no *Myotis* eDNA was captured on either the external or internal air sample filters. It is suspected that at underground roosts in winter, the primary sources of bat eDNA are likely to be sloughing of cells during flight and production of aerosols via echolocation, so timing eDNA sampling to coincide with winter flight is likely important. To optimize eDNA capture in low eDNA environments, like underground hibernacula in western North America, further investigation to understand the source and signal duration of bat eDNA in air is needed.

In addition to challenges with eDNA capture at underground roosts, false negative results from eDNA could result from the inhibition of amplification of eDNA in the samples. PCR inhibiting substances can reduce or block amplification of target eDNA (Wilson 1997, Mauvisseau et al. 2019*a*). All three samples collected at Rail Tunnel West failed integrity and inhibition testing via IntegritE-DNA and were deemed poor quality. These samples could have been degraded if bleach residue remained on the air samplers following decontamination, or if PCR inhibiting substances were collected concurrently on the filter during sampling (e.g., phenolic compounds, humic acids, and heavy metals) (Wilson 1997, Schrader et al. 2012). While the Rail Tunnel site had no obvious sources of PCR inhibiting compounds, inactive mine sites generally may contain substances like heavy metals that could inhibit PCR, warranting further consideration in study design.

Based on the study's findings, it is recommended that for detection of bat species via eDNA air sampling at underground hibernacula, collection of samples should coincide with periods of highest potential for winter bat flight. In the pilot study of hibernacula in the Kamloops region, the highest levels of acoustic bat activity recorded at hibernacula entrances occurred in November or March (Figure 4.11). This finding aligns with a study of hibernacula exiting behaviour by *M. ciliolabrum* and *Corynorhinus townsendii* at nine caves in Idaho, U.S., which also recorded the highest acoustic bat activity at cave entrances in March, followed by November (Whiting et al. 2021). For underground hibernacula sites in the B.C. interior, optimal eDNA air sampling timing would be November and March. However, no bats were detected acoustically on the internal detector at the New Afton Pit Portal after March 20, highlighting the importance of timing eDNA sampling schedules to consider possible seasonal movement away from hibernacula in early spring. In this study, acoustic bat activity at hibernacula entrances was lowest in January and February at all sites except Rail Tunnel West which had the lowest detections in February and December (Figure 4.11). The mid-winter period has been shown in other studies to represent the period of lowest winter bat activity (Johnson et al. 2016, Whiting et al. 2021, Jackson et al. 2022), and therefore likely also represents the lowest probability of winter eDNA capture at hibernacula entrances.

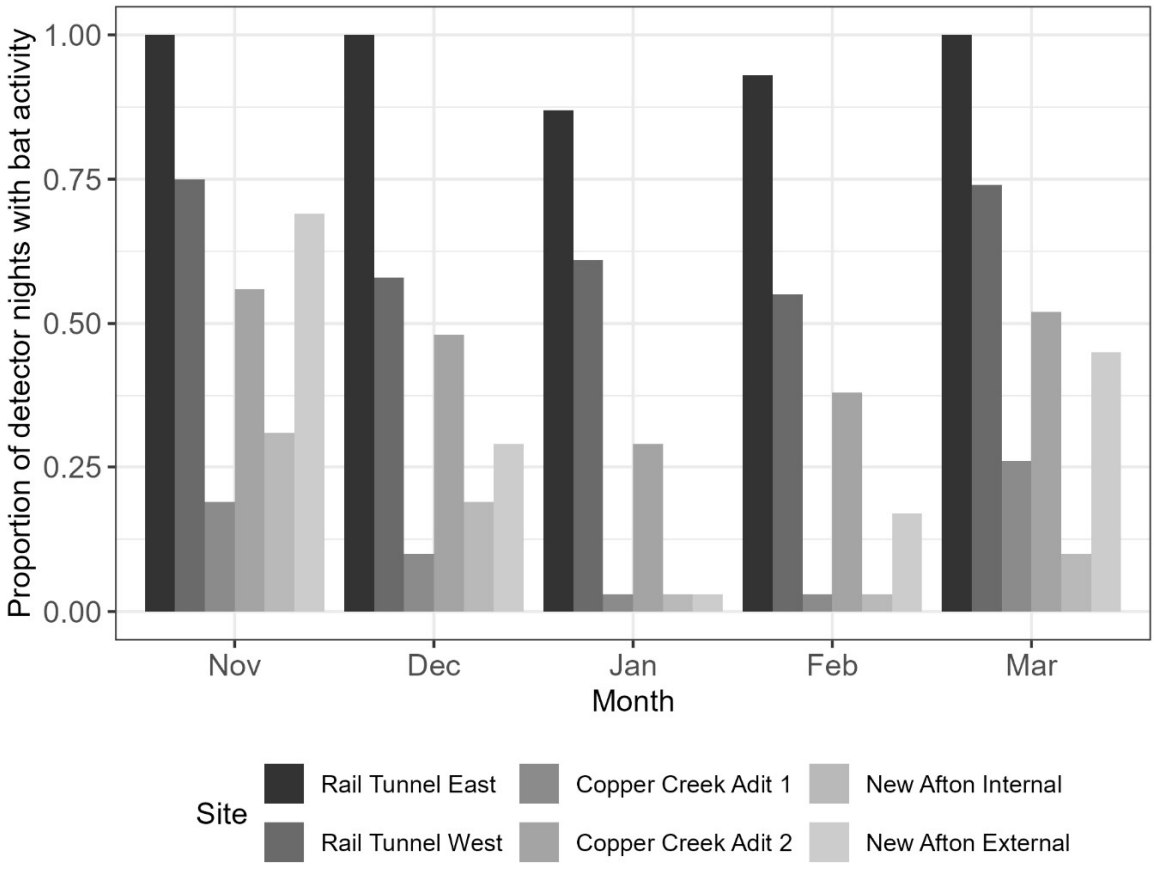

Figure 4.11 Proportion of acoustic detector nights with recorded bat activity, by month and site.

Further investigation of the frequency and drivers of winter bat flight may allow for optimization of winter eDNA air sample collection by focusing sampling efforts on conditions with the highest likelihood of winter activity. The significance of regular winter bat flight and hibernacula-exiting for western temperate region bats is not well understood, as compared to eastern caverniculous species that hibernate in large aggregations (Klüg-Baerwald et al. 2016). Generally, winter bat flight has been associated with warmer temperatures and lower wind speeds (Lausen and Barclay 2006, Johnson et al. 2016, Klüg-Baerwald et al. 2016, 2024, Whiting et al. 2021, Jackson et al. 2022, Andersen et al. 2024). But winter bat flight has been documented in western North America at temperatures below 0°C (Lausen and Barclay 2006, Schwab and Mabee 2014, Johnson et al. 2016, Klüg-Baerwald et al. 2016, De Freitas 2023). At the three sites included in this study, all but four winter acoustic bat detections recorded between November 15 and March 31 (n = 4,722)

occurred at temperatures at emergence greater than -6°C. All four detections at colder temperatures were recorded at Rail Tunnel East, two on January 15 (-14.1°C), one on January 16 (-9.8°C), and one on January 18 (-10.5°C). Targeting eDNA air sampling on nights with temperature at emergence greater than -6°C and low wind speeds may increase probability of winter bat flight and thus eDNA capture. Implementing multiple sampling events per season per site may mitigate both low eDNA capture in air samples and uncertainty in bat activity patterns.

Conclusions and Future Directions

There are few tools available to land managers and conservation scientists to support assessment of bat presence at underground habitats like inactive mines. Bat capture via trapping or mist-netting at mine entrances can provide valuable information on species, sex, and reproductive status, but bat handling requires expertise and permitting and may be prohibitively labour intensive in winter when bat activity is minimal (Sherwin et al. 2009, B.C. Ministry of Environment 2016). Radiotelemetry has limited value for confirming winter hibernation at mines because the signal from a bat inside a mine can be blocked by the surrounding rock (B.C. Ministry of Environment 2016). Long-term passive acoustic monitoring at mine entrances provides important activity pattern information, but species resolution can be limited, and as a sole line of evidence of presence, can leave considerable uncertainty. Detection of bat eDNA from air samples at underground habitats adds an additional line of evidence supporting bat presence and seasonal use, as well as potentially providing greater species resolution.

eDNA metabarcoding has been touted as a potentially revolutionary tool for wildlife monitoring in a mine reclamation context (Fernandes et al. 2018). The results of this pilot study demonstrate the utility of eDNA air sampling for bats to inform mine closure planning. Underground tunnels are useful sampling sites for bats because they concentrate bat activity on the landscape and therefore likely also concentrate bat eDNA within a relatively confined air space (Clare et al. 2022), making detection of these cryptic animals possible. Underground tunnels may similarly concentrate the DNA of other wildlife that could use these spaces (Armstrong et al. 2022). As suggested by others, eDNA metabarcoding is particularly useful for providing a snapshot of biodiversity at sites, like inactive mines, where

little is known (Bohmann et al. 2014, Fernandes et al. 2018, Bohmann and Lynggaard 2023, Littlefair et al. 2023). This study's non-bat eDNA metabarcoding results were compelling (e.g., detection of *Accipter* eDNA), highlighting the utility and sensitivity of eDNA air sampling at underground habitat features for wildlife beyond bats.

The general *Myotis* qPCR-based eDNA assay successfully detected *Myotis* eDNA. Future development of species-specific qPCR-based assays for target bat species may allow for even greater species resolution, by clarifying barcoding dyads (e.g., *M. californicus* versus *M. ciliolabrum*) and testing explicitly for species of regulatory or management interest (e.g., *M. lucifugus*). Further experimentation of eDNA air sampling under various environmental conditions will uncover the scenarios where it provides the greatest benefit. As the methodology is developed and optimized, eDNA air sampling methods should be integrated into a monitoring framework to complement traditional monitoring approaches.

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Chapter 5. Conclusion

The use of environmental DNA (eDNA) methods for species detection has become increasingly prevalent in the published literature since its first use in 2008 (Ficetola et al. 2008, Sahu et al. 2023). While there has been excitement about the potential for eDNA to revolutionize biodiversity monitoring (Deiner et al. 2017, Fernandes et al. 2018, Bohmann and Lynggaard 2023, Littlefair et al. 2023, Sahu et al. 2023), there have also been many efforts to standardize approaches, to establish minimum criteria, and to identify limitations and sources of uncertainty (Goldberg et al. 2016, Beng and Corlett 2020, Mathieu et al. 2020, Langlois et al. 2021, Thalinger et al. 2021, Kelly et al. 2024). Ready or not, eDNA tools are here, and their many benefits, including non-invasiveness, sensitivity, and relative ease of sample collection, make them an appealing choice for environmental practitioners. As eDNA tools get taken up in new applications for wildlife conservation and management, it is imperative that they are rigorously validated so that results can be interpreted appropriately by land managers and decision makers.

In this study, the readiness of eDNA air sampling as a tool for bat conservation and management was assessed by undertaking three separate field trials (Chapters 2, 3, and 4). Each of the three trials advanced our understanding of the strengths and limitations of eDNA air sampling for detecting bats in a western North American context. This project also advances the overall validation of new qPCR-based eDNA assays for western bats, designed and developed by Dr. Caren Helbing and her team at the University of Victoria. Multi-disciplinary collaborations like this one, between experts in biochemistry, genetics, ecology, and industry, as end-users of eDNA tools, are critical for the development of tools and methods that are rigorous, defensible, and effective.

Findings and Significance

In this project, eDNA air sampling collection methods described by Garrett et al. (2023*a*) were trialed in three different habitats representing different aspects of the ecology of *Myotis* bats in western North America. First, *Myotis* eDNA was successfully captured in all air samples collected in summer at mixed-*Myotis* maternity roosts located in buildings and bat boxes. Next, *Myotis* eDNA was captured in 8 of 40 air samples collected at an artificial bat cave feature that is primarily used as a night roost in late summer by low levels of bats. A

new filter type (Millipore) was used for this trial that does not require pre-sterilization and was easier to deploy and collect, because no ring clamp was needed. However, the probability of eDNA capture was estimated to be just 26% (CI: 13% to 45%) in this field trial, indicating further optimization of eDNA capture for low eDNA environments would improve the usefulness of this method. Finally, eDNA air sampling methods were piloted at three underground hibernacula sites in winter to extend the field validation into a real-world scenario and determine whether eDNA air sampling can help to identify bat use of underground mines. Bat eDNA was successfully captured at one of three hibernacula sampled in mid-winter. The air sample collection method was adapted to the specific field challenges encountered at each site, requiring abandonment of the Millipore filter and deployment of the air samplers inside protective wire mesh cages with 3 m extendable poles. Altogether, the results from this project add to the findings of Garrett et al. (Garrett et al. 2023*a*, *b*) demonstrating the utility of eDNA air sampling for bats at a variety of roost types and in different seasons.

This study represents the first field tests of three new qPCR-based eDNA assays for B.C. bats: one genus-wide assay for all *Myotis* species (eMyotis1), and two species-specific assays for *M. lucifugus* (eMYLU4) and *M. yumanensis* (eMYYU7). The eMyotis1 assay detected *Myotis* eDNA in all air samples collected at positive control sites at known mixed-*Myotis* maternity roosts in coastal and interior B.C. The eMyotis1 assay also detected *Myotis* eDNA at the artificial bat cave and the Copper Creek Mine hibernaculum, which both represent lower bat abundance and activity sites. These detections demonstrate the sensitivity of this assay in a variety of environments. Finally, a first effort to estimate the detection probability of eDNA air sampling paired with the eMyotis1 assay for detecting *Myotis* bats in an underground roost was completed. This milestone brings the eMyotis1 assay into the initial stages of Level 5 validation, indicating it is operational for routine monitoring (Thalinger et al. 2021) (Table 5.1). However, further testing of ecological and physical factors that influence *Myotis* eDNA in the environment is needed for advanced validation (Thalinger et al. 2021).

Based on the results outlined in Chapter 2, the eMYLU4 assay has been validated to Level 3 (Essential) because it did not detect *M. lucifugus* eDNA at one of the three positive control

sites, the Stave Lake Lodge maternity roost, despite confirmed capture of *Myotis* eDNA on the filters and known presence of the species at the site. Results obtained from this assay can be used to infer presence of *M. lucifugus*, but non-detection results cannot be used to draw conclusions regarding species absence (Thalinger et al. 2021). The eMYYU7 assay has been validated to Level 1 (Incomplete), because it has not yet successfully been used to detect *M. yumanensis* eDNA from environmental samples (Thalinger et al. 2021). Further laboratory validation work and redesign efforts for both species-specific assays are already underway.

Table 5.1 Assessment of readiness of three new qPCR-based bat eDNA assays for routine species monitoring, based on 5-level validation scale **(as per Thalinger et al. 2021)**.

eDNA assay	Current validation level	Key validation accomplishments	Interpretation of results
eMYYU7 (*M. yumanensis*)	Level 1 Incomplete	Assay designed and tested on target tissue.	Cannot tell if target is present or absent.
eMYLU4 (*M. lucifugus*)	Level 3 Essential	Level 1 + assay optimized, tested on closely related non-target species, tested on environmental samples, positive detections obtained, and sample processing steps reported.	Can be used to infer target presence, but non-detection results cannot be used to infer absence.
eMyotis1 (*Myotis genus*)	Level 5 Operational	Level 3 + LOD established, extensive testing on co-occurring non-target species.	Can be used to infer target presence, non-detection results can be used to infer absence including probability of false negative results.

A metabarcoding approach to laboratory analysis proved successful for detecting bat eDNA in all three trials conducted. The metabarcoding results were compelling for determining species composition in roosts in both summer (Chapter 2) and winter (Chapter 4). At all three maternity roost sites sampled, eDNA from both *M. lucifugu*s and *M. yumanensis* was detected via metabarcoding, even when the relative abundance of *M. yumanensis* was thought to be very low, as at Tranquille Barn. Metabarcoding may be less sensitive than qPCR for detection of rare and low abundance species (Harper et al. 2018, Bylemans et al. 2019, Wood et al. 2019, Schenekar et al. 2020, McColl-Gausden et al. 2023). In this project, eDNA air samples were pooled for metabarcoding analysis to increase the likelihood of detection. Pooling of air samples was likely not necessary at the summer maternity roost sites where

eDNA was abundant and readily detected via metabarcoding. Comparatively, at the artificial bat cave field trial, all eight samples that amplified *Myotis* eDNA via the eMyotis1 qPCR-based assay were pooled and submitted for metabarcoding, but only eight sequence reads of *Myotis* eDNA were detected. Bat eDNA was, however, readily detected via metabarcoding in two pooled winter eDNA air samples collected at the abandoned Copper Creek Mine hibernaculum.

Bat eDNA was readily captured at high eDNA maternity roost sites; however, to improve the likelihood of eDNA capture at low eDNA sites, like underground hibernacula, use of particle-trapping filter materials, like the Filtrete material used by Garrett et al. (2023*a*) is recommended. Samplers should be deployed to coincide with periods of highest anticipated bat activity. In the winter pilot study, the optimal timing for sampling was November and early to mid-March at temperatures at emergence greater than -6 °C. Additionally, pooling of several filters, longer sampling durations (i.e., longer than one sampling night), and multiple sampling events per season are recommended to mitigate both low eDNA capture in air samples and uncertainty in bat activity patterns at low bat abundance and activity sites.

Finally, data obtained via eDNA air sampling was complemented by acoustic data in all three trials. Bat activity patterns obtained from acoustic monitoring cannot be replaced by eDNA sampling, which provides only a snapshot of presence/non-detect information. eDNA data is useful as an additional line of evidence for species presence, as seen with the detection of *Corynorhinus townsendii* eDNA in winter samples collected at the Copper Creek Mine hibernaculum. This species is known to inhabit inactive underground mines, however, it is often underrepresented in acoustic data sets because of its soft echolocation calls (Lausen et al. 2022). In this trial, no diagnostic acoustic recordings of this species were made until spring. If the only data available had been the acoustic results, considerable uncertainty regarding presence of *Corynorhinus townsen*dii in winter would have remained. eDNA from the federally Endangered species, *Myotis lucifugus*, was also detected, and this species cannot always be distinguished from two other *Myotis* species based on acoustics alone. These results highlight the utility of eDNA data as a complement to passive acoustic monitoring, and other methods depending on the feasibility and safety of site access, for understanding bat presence and seasonal use at underground habitats.

Future Directions

As the fungal pathogen responsible for the deadly disease white-nose syndrome (WNS) inevitably begins to spread in B.C., eDNA air sampling holds promise for identifying hibernacula (this study), tracking the spread of the disease (through eDNA detection of the fungus *Pseudogymnoascus destructans*), and monitoring population responses to both the disease among B.C. bats and any mitigation measures that are implemented. eDNA air sampling can provide valuable information about bat presence at inaccessible or unsafe roosting spaces, like underground mines, caves, and non-cavernous subterranean features (e.g., Milieu Souterrain Superficiel) (Blejwas et al. 2021, 2023, Clare et al. 2021, Garrett et al. 2023*a*). Innovative approaches, such as eDNA air sampling, are needed to study and monitor bats inhabiting inaccessible habitats (Blejwas et al. 2023).

In western North America, a distinct challenge exists for researchers working to monitor and address the impacts of WNS on the Endangered *Myotis lucifugus*. In the west, overwintering habitats for this species are largely unknown and therefore winter season population surveillance is not feasible (Blejwas et al. 2023). In the summer, *M. lucifugus* co-inhabits maternity roosts with a morphologically similar species, *M. yumanensis*, so summer roost count efforts for population monitoring are complicated by the presence of two species that are difficult to distinguish (Weller et al. 2007, Blejwas et al. 2023). WNS has had differential effects on species and therefore, being able to identify changes in roost composition overtime is critical for understanding the species-specific effects of this disease (Langwig et al. 2012, 2015, Frank et al. 2014, Blejwas et al. 2023). Currently, determining the relative abundance of *M. lucifugus* to *M. yumanensis* in a maternity roost requires capture so that individuals can be distinguished based on morphological characteristics (Weller et al. 2007). Acoustics can be used to identify presences of both species at roosts because *M. lucifugus* typically uses a characteristic frequency below 45 kHz, while *M. yumanensis* makes calls above 45 kHz (Weller et al. 2007, Lausen et al. 2022). However, an approach for extrapolating estimates of relative abundance from acoustic data at maternity roosts has yet to be developed. In this study, when the proportion of metabarcoding read counts for each species at each maternity roost was compared to the relative acoustic activity for each species, the relative proportions aligned across all three sites (Chapter 2). Further investigation is needed to determine how these two potential non-invasive indices of relative abundance compare to estimates obtained

from bat capture, and whether the pattern holds for other mixed-*Myotis* roosts. If validated, a mixed-*Myotis* roost monitoring approach that combines acoustics and eDNA air sampling (as described in Chapter 2), could be an efficient, simple, and accessible approach to population surveillance. Importantly, this approach would not require high levels of involvement by taxonomic and wildlife handling experts, so surveillance efforts could be readily scaled up to harness non-expert conservation and citizen science groups.

But adoption of eDNA methods does not remove the need for taxonomic expertise (Van Leeuwen and Michaux 2023). In fact, this study highlights the significant upfront effort required from taxonomic experts to appropriately design and validate eDNA tools. eDNA tools must necessarily be developed as a collaboration between experts in biochemistry, genetics, and ecology, with practicality for the end-user kept at the forefront. It is the role of taxonomic experts to identify confounding species, subspecies, and haplogroups that could lead to false positive or negative results, to develop appropriate field sampling strategies for target taxa, and to recognize factors that influence the origin, state, fate, and transport of target eDNA in study systems (Barnes and Turner 2016, Goldberg et al. 2016, Langlois et al. 2021). Critical review of eDNA-derived data is also needed as confidence in new tools is established. The development of eDNA tools for biodiversity assessment and species detection in terrestrial environments is still in its infancy. In a time of unprecedented human-induced biodiversity loss, when such tools are critically needed, continued efforts to undertake rigorous and transparent methodological validation will propel this field forward.

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