Detecting Antarctic and New Zealand vertebrates using environmental DNA (eDNA)

A dissertation submitted in partial fulfilment

of the requirements for the Degree of

Master of Antarctic Studies

at the

University of Canterbury

by Lucy Alice Howell

Gateway Antarctica

March 2021

Abstract

Isolated environments can lead to unique biodiversity; however, endemic taxa are often evolutionarily vulnerable to disturbance. While considered very different, both Antarctica and New Zealand face this challenge. To protect these ecosystems, we must first monitor them to assess whether a change falls within the average flux or is the consequence of disturbance. Monitoring programs should utilise various tools to account for biases and ensure that data accurately represents the ecosystem. The development and integration of new technology for monitoring species can improve the resolution of datasets. One method that is increasingly investigated for ecosystem surveillance is Environmental DNA (eDNA). I suggest eDNA is utilised to monitor both Antarctica and New Zealand's endemic fauna. To assess current Antarctic applications, I reviewed the literature on eDNA. While microbiologists have applied eDNA methods extensively, few papers targeted Antarctic vertebrates. I highlight recent developments in population genetics and portable sequencing technologies and discuss how these methods could apply to Antarctic research. As technology and methods develop, so too will the potential for non-invasive monitoring of polar fauna. I identified Orcas (*Orcinus orca*) and Weddell seals (*Leptonychotes weddellii*) as potential target species for future population genetic trials in Antarctica. Thus, my second study investigated Antarctic snow samples as a source of Weddell seal DNA. The snow was collected from a single seal imprint at the Turtle Rock breeding colony in McMurdo Sound. I successfully extracted, amplified and sequenced Weddell seal DNA in seven out of thirty-three snow samples. Though my detection rate was low, to the best of my knowledge, this represents the first time Antarctic vertebrate DNA has been extracted from snow. While all seven sequences fall within the Weddell seal clade on my Neighbour-

Joining tree, the branch lengths suggest a high level of divergence, which could pose challenges for future population genetic applications. I identified that storage time, filter removal and sample size may have impacted our eDNA yield and looked to address this in my final study, primarily through using enclosed Sterivex filter units. I first introduce eDNA surveys in a New Zealand context and highlight the value of eDNA for monitoring a conservation dependent species, the kororā (little blue penguin; *Eudyptula minor*). I extracted DNA from three sample types, feathers, feather soaked water and tank water from the kororā enclosure at the International Antarctic Centre in Christchurch. While amplification from feather samples was unsuccessful, seven out of nine filter water samples were sequenced, and all eight tank samples. As in the Weddell seal sequences, the kororā eDNA, while clustered with *Eudyptula minor*, is highly divergent. Likewise, all but two of the sequences aligned with Eudyptula minor (16srRNS), but in two samples, there were several high matches with non-penguin species. Both my Weddell seal and kororā chapters highlight the challenges of developing an eDNA protocol and the need for extensive optimisation and standardisation to draw confident conclusions. However, both studies are promising for future eDNA studies targeting the two species. And with robust protocols, eDNA could be applied to key management concerns in both areas. For example, monitoring the Ross Sea Marine Protected Area (MPA) in Antarctica and contributing to the surveillance of invasive predator species in New Zealand. Thus informing the conservation of unique taxa on both sides of the Southern Ocean.

Acknowledgements

I acknowledge the mana whenua, Ngāi Tūāhuriri, on whose lands I have spent the past year working towards this degree.

I would like to thank the Christchurch City Council, Gateway Antarctica and New Zealand Antarctic Research Institute for the scholarships and funding that allowed me to spend the past year working towards this dissertation. And to the University of Canterbury for the Aho Hīnātore scholarship that will enable me to continue with this work. As this dissertation is a continuation of my Post Graduate Certificate in Antarctic Science (PCAS), I would also like to thank Antarctica New Zealand, the US and NZ Airforce and the 2019-20 Scott Base team for all the support that got me to my sample site. In particular, I would like to thank our field trainer Steve Greaves and PCAS team Alex Aves, Arek Aspinwall, Hannah Dawson, Chrissy Emeny, Emily McGeorge, Shannon Williams, Bella Zeldis, Michelle LaRue, Rob Lindeman and Adrian McDonald. Thank you also to the International Antarctic Centre for the help with my sampling, especially to penguin keeper Lea who kept me dry in the rain while I filtered samples from the penguin tank.

I am incredibly grateful to my supervisors Sarah Flanagan and Michelle LaRue, for the opportunity, and all their assistance and encouragement this year. I have enjoyed being part of both the Flanagan and LaRue lab groups, I'm glad to have that continue. Thank you also to Tammy Steeves and the ConSERT team for welcoming me into the lab as I move into the PhD. Special thanks to Stephanie Galla, Ilina Cubrinovska and Seoljong Kim for answering all my questions in the lab this year and making me feel welcome. And to Claudia Allen, Molly Magid, Shinae Montie, and Clare Adams for offering their advice when I got stuck.

I would like to say a huge thank you to everyone in the Gateway Antarctica team for being fantastic through all the trials of last year and for keeping us company over zoom in

lockdown. I am grateful for all your friendship this year and glad I don't have to say goodbye just yet. I am also grateful for all the help and advice offered by Ron Doel long after his time with Gateway has finished.

And Finally, to my friends and family, thank you for making me feel completely supported and loved despite the unexpected distance this year. I would especially like to thank Yaren Azman, Babara Brady, Alex Coutts, Shona Elliot-Kerr, Tom McConville, Alana Meehan, Fran Soler and Anna Vassiliadas for the excellent advice and positive reinforcement. And I am endlessly grateful to Mum, Dad, Luke, Molly and Huw who have been in it for the long haul.

Table of Contents

List of Tables

List of Figures

Chapter One

Introduction

The Protocol on Environmental Protection to the Antarctic Treaty (signed in 1991, enforced 1998) establishes the Antarctic continent as a "natural reserve devoted to peace and science' (Article 2) and specifies the value of critical environmental research. However, Antarctica is increasingly influenced by humans through the activities of researchers, tourists, base staff and fisheries staff (Bestly et al. 2020). The extent of cumulative disturbance, both to the ecosystem and wildlife, is often unclear (Pande et al. 2017). The Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) stressed the importance of ecosystem monitoring through the CCAMLR Ecosystem Monitoring Program (CEMP). The two primary objectives of which are 1) to detect significant changes in the marine ecosystem and 2) to distinguish between the natural fluctuation of commercial species and changes in populations due to fishing pressures (Sc-CAMLR 1985; Croxall and Nicol 2004; Kock et al. 2007). It is particularly important that monitoring programs target conservation priority regions such as the Ross Sea Region Marine Protected Area (MPA, Nickols et al. 2019; Gold et al. 2021). Likewise, baseline assessment should be done to the highest possible resolution, requiring multiple, overlapping methods (Bean et al. 2017).

One method for complimentary monitoring is Environmental DNA (eDNA; Bean et al. 2017). Environmental DNA describes genetic material detected in an environmental sample (Ogram et al. 1987). Organisms shed DNA into their environment via a number of natural processes, e.g. through, moulting, defecating or injury (Ficetola et al. 2008; Taberlet et al. 2012; 2018). How DNA then persists in the environment is dependent on a complex interaction of both biotic and abiotic factors (Stewart et al. 2019). This poses a challenge to

eDNA surveys, as the DNA detected is typically of a low quality and quantity (Taberlet et al. 2018). Likewise, most eDNA studies target mitochondrial DNA due to its high copy number, increasing the chance of detection. However, this restricts the detail we can gain from eDNA analyses (Adams et al. 2019; Sigsgaard et al. 2019). Interest in environmental DNA has been increasing over recent years (Beng and Corlett 2020), and so has the available technology (Shokralla et al. 2012; Bohmann et al. 2014; Ducluzeau et al. 2018; Gowers et al. 2019). Recently, eDNA has seen advances into the field of population genetics (Adams et al. 2019; Sigsgaard et al. 2019). Environmental DNA surveys show promise for more sensitive analysis of wildlife including sex determination (Nichols and Spong 2017), estimating population abundance (Tillotson et al. 2018; Spear et al. 2021) and identifying population characteristics (Sigsgaard et al. 2016; Baker et al. 2018).

Despite recent advances in the field, careful consideration is required before implementing an eDNA survey, as there are many decisions that can affect the outcome of a survey (Fig 1.1). Dickie et al. (2018) stressed the need for greater standardisation of eDNA protocols to increase the comparability of datasets and avoid misleading inferences. Such errors are problematic for biodiversity monitoring, where assessing trends across datasets is critical for discriminating between natural environmental variation and a decline in ecosystem health (Pande et al. 2017; Piazza et al. 2020). Both Dickie et al. (2018) and Lear et al. (2018) put forward a number of key considerations that should be assessed prior to designing an eDNA survey. Generally, the early stages of developing a protocol require troubleshooting. Thus the set-up of a DNA protocol is typically the most costly, in both time and money (Smart et al. 2016; Johansson et al. 2020). However, once a robust protocol is in place, eDNA surveys are generally considered more economical than traditional methods (Smart et al. 2016; Lugg et al. 2017; McColl-Gausden et al. 2020). Indeed, the combination of cost-efficiency and ease of sampling has seen eDNA integrated into several government

programs, including the Environmental Protection Agency in New Zealand (Wai Tūwhera o te Taiao, Open Waters Aotearoa programme; www.epa.govt.nz/community-involvement/open-waters-aotearoa/).

Whilst New Zealand may not feel as remote as Antarctica, it's geographical position, and physical isolation has led to distinctive biodiversity. However, oceans are no longer the barrier they once were, and the isolation of New Zealand's taxa has also left them vulnerable to invasive species (Jay et al. 2003). This has resulted in a number of species being classed as conservation dependant (Robertson et al. 2016), thereby requiring permanent management to maintain the population. Conserving endemic species whilst managing introduced species requires constant monitoring of New Zealand's ecosystems. Thus eDNA surveys are equally promising in a New Zealand context. Recent research has seen eDNA discussed for New Zealand's Marine (Jeunen et al. 2020a, 2020b) and terrestrial ecosystems (Holdaway et al. 2017). Considering two very different environment, Antarctica and New Zealand, I will further discuss the potential for eDNA surveys to contribute to conservation management in both ecosystems. My primary aims for this dissertation are to:

1) Identify the state of eDNA in Antarctica and highlight gaps in the current literature and discuss potential avenues for future research on the ice.
2) Begin the development process for an eDNA protocol to detect Antarctic vertebrate DNA (Weddell seal, *Leptonychotes weddelli*) in snow and discuss future applications for snow samples to inform population genetic studies.
3) Further develop my marine mammal protocol by applying it to the captive kororā (*Eudyptula minor*) population at the International Antarctic Centre and discuss environmental DNA surveys in a New Zealand context and the implications they have for a conservation dependent species.

Figure 1.1. A simple eDNA work flow listing some of the considerations required for designing an eDNA survey; highlighting the number of choices that can influence the results of an eDNA study. Abbreviations are PCR (polymerase chain reaction), qPCR (quantitative PCR), HTS (high-throughput sequencing) and OTU's (operational taxonomic units).

1.1 Dissertation structure

This dissertation divides the discussion of eDNA into two distinct themes, eDNA in an Antarctic and a New Zealand context. These themes are covered across my three main chapters:

Chapter Two, *Environmental DNA as a tool for monitoring Antarctic vertebrates*, reviews the literature on Antarctic eDNA studies, outlining a number of environmental samples that have been utilised for non-invasive molecular studies on the ice. I then discuss potential avenues for future research targetting the continent's vertebrate taxa

Chapter Three, *Detection of Weddell seal (Leptonychotes weddellii) eDNA from Antarctic snow samples,* having identified that vertebrate eDNA is understudied on the Antarctic continent, I investigate a novel sample source for Antarctic vertebrates. Specifically, I use snow samples collected from a Weddell seal breeding colony in Antarctica to extract, amplify and sequence Weddell seal eDNA. I then discuss challenges that arose in the study while highlighting future applications for eDNA in Antarctica.

Chapter Four, *Optimising eDNA protocols for little blue penguins Kororā, little blue penguin (Eudyptula minor),* shifts focus from polar research to investigate applications closer to home. While New Zealand may not be as remote as Antarctica, as an island, many of its species have evolved in isolation and are vulnerable in a modern anthropogenic landscape. This chapter first outlines eDNA within a New Zealand context before looking to the kororā. Using samples from International Antarctic Centres' penguin enclosure, I further develop my protocol from Chapter Three and discuss how eDNA can inform the management of a conservation dependant species.

Chapter Five, *Synthesis and conclusions*, summarises the progress made in these two studies and outlines the directions this research can be continued in.

1.2 References

Bean TP, Greenwood N, Beckett R, Biermann L, Bignell JP, Brant JL, Copp GH, Devlin MJ, Dye S, Feist SW. 2017. A review of the tools used for marine monitoring in the UK: combining historic and contemporary methods with modeling and socioeconomics to fulfill legislative needs and scientific ambitions. Frontiers in Marine Science. 4: 263.

Beng KC, Corlett RT. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and conservation. 29(7): 2089-2121.

Bestley S, Ropert-Coudert Y, Bengtson Nash S, Brooks CM, Cotté C, Dewar M, Friedlaender AS, Jackson JA, Labrousse S, Lowther AD 2020. Marine ecosystem assessment for the Southern Ocean: birds and marine mammals in a changing climate. Frontiers in Ecology and Evolution. 8: 338.

Bohmann K, Evans A, Gilbert MTP, Carvalho GR, Creer S, Knapp M, Douglas WY, De Bruyn M. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends in ecology & evolution. 29(6):358-367.

Bylemans J, Furlan EM, Gleeson DM, Hardy CM, Duncan RP. 2018. Does size matter? An experimental evaluation of the relative abundance and decay rates of aquatic environmental DNA. Environmental science & technology. 52(11): 6408-6416.

Commission for the Conservation of Antarctic Living Marine Resources (CCAMLR) 1989. CCAMLR Ecosystem Monitoring Program. [Accessed 2021 Mar 17] https://www.ccamlr.org/en/science/ccamlr-ecosystem-monitoring-program-cemp

Croxall JP, Nicol S. 2004. Management of Southern Ocean fisheries: global forces and future sustainability. Antarctic Science. 16(4): 569.Baker CS, Steel D, Nieukirk S, Klinck H 2018. Environmental DNA (eDNA) from the wake of the whales: droplet digital PCR for detection and species identification. Frontiers in Marine Science. 5: 133.

Dickie IA, Boyer S, Buckley HL, Duncan RP, Gardner PP, Hogg ID, Holdaway RJ, Lear G, Makiola A, Morales SE. 2018. Towards robust and repeatable sampling methods in eDNA-based studies. Molecular Ecology Resources. 18(5): 940-952.

Ducluzeau A-L, Tyson J, Collins R, Snutch T, Hassett B. 2018. Genome sequencing of Sub-Arctic Mesomycetozoean Sphaeroforma sirkka strain B5, performed with the Oxford Nanopore minION and Illumina HiSeq systems. Microbiology Resource Announcements. 7(15):e00848-18

Environmental Protection Agency. Wai Tūwhera o te Taiao – Open Waters Aotearoa [Accessed 2021 Mar 17] https://www.epa.govt.nz/community-involvement/open-waters-aotearoa/

Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. Biology letters. 4(4):423-425.

Gowers G-OF, Vince O, Charles J-H, Klarenberg I, Ellis T, Edwards A. 2019. Entirely off-grid and solar-powered DNA sequencing of microbial communities during an ice cap traverse expedition. Genes. 10(11):902.

Gold Z, Sprague J, Kushner DJ, Marin EZ, Barber PH. 2021. eDNA metabarcoding as a biomonitoring tool for marine protected areas. PLOS ONE. 16(2): e0238557.

Jay M, Morad M, Bell A. 2003. Biosecurity, a policy dilemma for New Zealand. Land Use Policy 20(2): 121-129.

Jeunen G-J, Urban L, Lewis R, Knapp M, Lamare M, Rayment W, Dawson S, Gemmell N. 2020a. Marine environmental DNA (eDNA) for biodiversity assessments: a one-to-one comparison between eDNA and baited remote underwater video (BRUV) surveys. Authorea Preprints.

Jeunen G-J, Lamare MD, Knapp M, Spencer HG, Taylor HR, Stat M, Bunce M, Gemmell NJ. 2020b. Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal. Environmental DNA 2(1): 99-111.

Johansson ML, Lavigne SY, Ramcharan CW, Heath DD, MacIsaac HJ 2020. Detecting a spreading non-indigenous species using multiple methodologies. Lake and Reservoir Management 36(4): 432-443.

Kock KH, Reid K, Croxall J, Nicol S. 2007. Fisheries in the Southern Ocean: an ecosystem approach. Philosophical Transactions of the Royal Society B: Biological Sciences. 362(1488): 2333-2349.

Lear G, Dickie I, Banks J, Boyer S, Buckley HL, Buckley TR, Cruickshank R, Dopheide A, Handley KM, Hermans S. 2018. Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. New Zealand Journal of Ecology. 42(1): 10-50A.

Lugg WH, Griffiths J, van Rooyen AR, Weeks AR, Tingley R. 2018. Optimal survey designs for environmental DNA sampling. Methods in Ecology and Evolution. 9(4): 1049-1059.

McColl-Gausden EF, Weeks AR, Coleman RA, Robinson KL, Song S, Raadik TA, Tingley R. 2020. Multispecies models reveal that eDNA metabarcoding is more sensitive than backpack electrofishing for conducting fish surveys in freshwater streams. Molecular Ecology. Molecular Ecology. Early view.

Nichols RV, Spong G. 2017. An eDNA-based SNP assay for ungulate species and sex identification. Diversity. 9(3): 33.

Nickols KJ, White JW, Malone D, Carr MH, Starr RM, Baskett ML, Hastings A, Botsford LW. 2019. Setting ecological expectations for adaptive management of marine protected areas. Journal of Applied Ecology. 56(10): 2376-2385.

Ogram A, Sayler GS, Barkay T. 1987. The extraction and purification of microbial DNA from sediments. Journal of microbiological methods. 7(2-3): 57-66.

Pande A, Sivakumar K, Sathyakumar S, Kumar RS, Johnson J, Mondol S, Mathur VB. 2017. Monitoring wildlife and their habitats in the Southern Ocean and around Indian research stations in Antarctica. Proceedings of the Indian National Science Academy. 83(2): 483-496.

Piazza P, Gattone SA, Guzzi A, Schiaparelli S. 2020. Towards a robust baseline for long-term monitoring of Antarctic coastal benthos. Hydrobiologia 847(7): 1753-1771.

Protocol on Environmental Protection to the Antarctic Treaty, [Accessed 2021 Mar 17] www.ats.aq/documents/recatt/Att006_e.pdf.

Robertson HA, Dowding JE, Elliott G, Hitchmough R, Miskelly C, O'Donnell CF, Powlesland R, Sagar PM, Scofield RP, Taylor GA 2013. Conservation status of New Zealand birds, 2012, Publishing Team, Department of Conservation.

SC-CAMLR 1985 Report of the 4th meeting of the scientific committee, Hobart, Australia, 2–9 September 1985. In Report of the 4th meeting of the scientific committee, Hobart, Australia. pp. 1–54

Shokralla S, Spall JL, Gibson JF, Hajibabaei M. 2012. Next-generation sequencing technologies for environmental DNA research. Molecular Ecology. 21(8):1794-1805.

Sigsgaard EE, Nielsen IB, Bach SS, Lorenzen ED, Robinson DP, Knudsen SW, Pedersen MW, Al Jaidah M, Orlando L, Willerslev E. 2016. Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. Nature Ecology and Evolution. 1(1):1-5.

Smart AS, Weeks AR, van Rooyen AR, Moore A, McCarthy MA, Tingley R 2016. Assessing the cost-efficiency of environmental DNA sampling. Methods in Ecology and Evolution 7(11): 1291-1298.

Spear MJ, Embke HS, Krysan PJ, Vander Zanden MJ. 2021. Application of eDNA as a tool for assessing fish population abundance. Environmental DNA. 3(1): 83-91.

Stewart KA. 2019. Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. Biodiversity and Conservation 28(5): 983-1001.

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH. 2012. Environmental DNA. Molecular ecology. 21(8):1789-1793.

Taberlet P, Bonin A, Coissac E, Zinger L. 2018. Environmental DNA: For biodiversity research and monitoring. Oxford (UK): Oxford University Press.

Tillotson MD, Kelly RP, Duda JJ, Hoy M, Kralj J, Quinn TP. 2018. Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. Biological Conservation. 220: 1-1

Chapter Two

Literature review: Environmental DNA as a tool for monitoring Antarctic vertebrates

Antarctica is home to numerous species that are vulnerable to environmental change and assessing species responses requires long-term monitoring. However, Antarctica's extreme nature presents limitations to conducting the type of long-term or broad-scale studies necessary for understanding changes in community composition. In this paper, we evaluate the potential for the use of environmental DNA (eDNA) methods in expanding scientific research efforts for biodiversity monitoring and conservation genetics in Antarctica. Through a systematic literature review, we identify that most Antarctic eDNA studies have focused on microbial metabarcoding using samples from soil, sediment, snow, and water. Few eDNA studies in Antarctica have focused on vertebrate biodiversity or population genetics, but we highlight several examples that have effectively and creatively used eDNA to study vertebrates. We highlight the potential for the use of portable sequencing technologies in the future of Antarctic eDNA research. We conclude that eDNA could be a valuable tool for researchers in their efforts to assess, monitor, and conserve biodiversity in the Antarctic.

Keywords: eDNA; environmental sampling; genetic tools; Southern Ocean; Polar; remote sampling; biodiversity; conservation genetics

2.1 Introduction

The extreme nature of the Antarctic environment requires a range of techniques to address the challenges of biodiversity assessment and targeted species detection (Chown et al. 2015; Czechowski et al. 2017; Gutt et al. 2018). Our regional knowledge of biodiversity is directly impacted by the degree of inaccessibility at different locations (Griffiths 2010), and research limitations are not only spatial but temporal, given that Antarctica is largely inaccessible over the dark winter months. A baseline understanding of species' distribution and community composition across the continent and throughout the year is vital for monitoring the effects of increasing human activity and for planning subsequent mitigation strategies (Chown et al. 2012). Assessing this disturbance requires long term data that cover a broad temporal and spatial range, but long-term studies are particularly susceptible to disruptions to planned data collection. Disruptions in fieldwork can result in low sample sizes, which can impede the incorporation of biologically important variables such as age and sex in data analyses (e.g., Nachtsheim et al. 2017). One way to increase scientific flexibility is to use multiple approaches to answer research questions, such as combining traditional visual surveys with molecular tools (Ammon et al. 2018). In this review we will focus on one such tool, environmental DNA, and the opportunities this field offers for current biological survey efforts in the Antarctic region (continental Antarctica, the Southern Ocean, and the Sub-Antarctic Islands).

The term environmental DNA (eDNA) was first used by Ogram et al. (1987) for microbial DNA collected from marine sediments, and generally refers to any genetic material extracted from an environmental sample (Ogram et al. 1987, Taberlet et al. 2012). As an organism interacts with its environment, it sheds genetic material, such as through hair, faeces, or gametes. DNA 'traces' can be detected in a variety of environmental samples (e.g., water, soil, and sediments). Since the early aquatic application of eDNA to detect the

presence of amphibians (Ficetola et al. 2008), the field has expanded, leading to a variety of novel methods, with samples extracted from salt licks (Ishige et al. 2017), honey (Bovo et al. 2018), and leaves (Valentin et al. 2020). While it is possible to detect enough DNA for genome assembly in microbes (Taberlet et al. 2018), most eDNA from macrofauna is no longer protected by intact cells (Levy-Booth et al. 2007; Pietramellara et al. 2009; Taberlet et al. 2018), and such extracellular DNA decays relatively quickly (Collins et al. 2018; Wei et al. 2018). A complex interaction of factors causes rapid decay of DNA (Corinaldesi et al. 2008), such as exposure to UV radiation, lower pH, increased temperature, consumption by microbes, hydrolysis, and enzyme action (Finkel and Kolter 2001; Hebsgaard et al. 2005; Strickler et al. 2015; Barnes and Turner 2016). The short-lived nature of extracellular DNA in the environment may be useful for spatiotemporal inferences of species on detection (Barnes and Turner 2016), however, DNA may also be adsorbed onto organic material and preserved, e.g., Haile et al. (2009) used ancient DNA techniques to detect horse (*Equus* spp.) and mammoth (*Mammuthus* spp.) DNA in Alaskan permafrost sediments.

Within different environmental sources, the method of DNA amplification and sequencing can be approached in one of two ways depending on the research question (e.g., targeting single or multiple species; Taberlet et al. 2018). For single-species questions, such as the detection of an invasive, endangered, or cryptic species (Ficetola et al. 2008; Dejean et al. 2012; Goldberg et al. 2013; Uchii et al. 2016), primers specific to that species' DNA are required (Ficetola et al. 2008). Using these species-specific primers, genotypes from PCR or signals from quantitative PCR (qPCR) act as a fingerprint or 'barcode' to identify target species (Bohmann et al. 2014). Alternatively, a multi-species approach (called metabarcoding) allows for the identification of all taxa present in a sample (Brown et al. 2016; Valentini et al. 2016). Metabarcoding provides a snapshot of biodiversity at a given point in time. Generally, this process requires the use of high-throughput, next generation

sequencing, which allows large amounts of data processing in a single sequencing run (Shokralla et al. 2012; Bohmann et al. 2014). Such a broad, community-level approach is preferable for studies on biodiversity (Bohmann et al. 2014), predator-prey interactions (Deagle et al. 2005), and investigating potential invasions where species-specific information may be unknown, for example in ballast water (Bohmann et al. 2014; Gerhard and Gunsch 2019). Metabarcoding requires universal primers that bind to target DNA regions across taxonomic groups. With the development and commercialisation of high-throughput sequencing technology (Schuster 2008) and datasystems such as the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007), the use of eDNA to monitor ecosystem diversity, particularly the diversity of vertebrate species, has become increasingly feasible (Valentini et al. 2009; Bálint et al. 2018; Cristescu and Hebert 2018). A discussion of the specific methods for metabarcoding is beyond the scope of this review. However, a technical review of high throughput sequencing metabarcoding in Antarctica is provided by Czechowski et al. (2017).

Antarctica is unique in that it is the only continent without an Indigenous human population, and therefore its vertebrates may be susceptible to human disturbance by researchers (Martín et al. 2004; Petel et al. 2007; Coetzee and Chown 2016). Environmental DNA surveys inherently do not require direct contact with target taxa and are therefore considered both non-invasive and 'non-disruptive' to wildlife (Lefort et al. 2019). While many traditional survey methods, such as visual or aerial surveys, do not require invasive samples, they may still lead to a stress response in the target organism. For example, approaching chinstrap penguins (*Pygoscelis antarcticus*) can affect fleeing behaviour (Martín et al. 2004), while prolonged exposure to helicopters inhibits foraging Adélie penguins (*Pygoscelis adeliae*) from returning to their nests (Wilson et al. 1991). An environmental sample however, can be taken without the need to approach, or even sight, the target species.

With this in mind, eDNA surveys present an ideal opportunity to continue to expand our knowledge on Antarctic biodiversity with minimal risk of harm.

In this paper we review the literature on eDNA studies in Antarctica, the Southern Ocean, and the Sub-Antarctic islands. We focus on vertebrate eDNA studies and discuss how methods from Antarctic microbial studies could be translated into vertebrate research. Our review compares current and future applications of eDNA in Antarctica for a number of sample sources, drawing connections to eDNA surveys within the Arctic. The future is bright for the use of eDNA in improving our understanding of Antarctica's unique vertebrate species.

2.2 Literature Search

We reviewed the literature in Scopus, using the term 'Antarctic' in combination with 'environmental DNA' or 'e*DNA'. Alternative abbreviations may be used such as sediment DNA (sedDNA; Ficetola et al. 2018), and further, the term eDNA may also be used to refer to 'extracellular DNA' (Fröls et al. 2012; Ricciardelli et al. 2019). Thus, we also ran multiple searches pairing 'Antarctic' with the terms '*barcoding', "high throughput sequencing", "next generation sequencing" or 'metagenomics'. We then went through the results to determine the source and to identify any papers that discussed environmental vertebrate DNA. We excluded literature reviews, book chapters, notes and errata. The review covers literature from 2000-2020, as of June 29, 2020 (Table 1, supplementary information).

2.3 Summary of Review Results

We found a total of 172 papers for all search terms. Of these papers, only 22 included search terms “environmental DNA” and 7 “eDNA” (Table 1, supplementary information). These results included studies that directly sampled the environment as well as those that analysed data from previously collected metagenomes; we excluded studies that used other sources of genetic material (e.g., seal buccal swabs; Crane et al. 2018). The most common environmental source for DNA was soil (n=47 studies) followed by water, which includes both marine samples (n= 37) and water collected from terrestrial water bodies (n=27). Sediment samples returned 22 studies, and studies involving ice/snow samples returned the fewest, with 14 (Table 1, supplementary information). Samples classed as ‘other’ included faeces, rock and hypoliths, biofilms, microbial mats, and filtered air (Table 1, supplementary information).

Only 4 of the 172 studies in our search recovered vertebrate DNA from the environment (Table 1, supplementary information), which used samples from seawater (Cowart et al. 2018; Mariani et al. 2019), sediment (Ficetola et al. 2018), and faeces (McInnes et al. 2016) as environmental sources (Fig. 1). The vertebrates detected in these four studies ranged from Antarctic specialists including fishes (McInnes et al. 2016; Cowart et al. 2018; Mariani et al. 2019), Weddell seals, and chinstrap penguins (Mariani et al. 2019), to invasive rabbits (Ficetola et al. 2018).

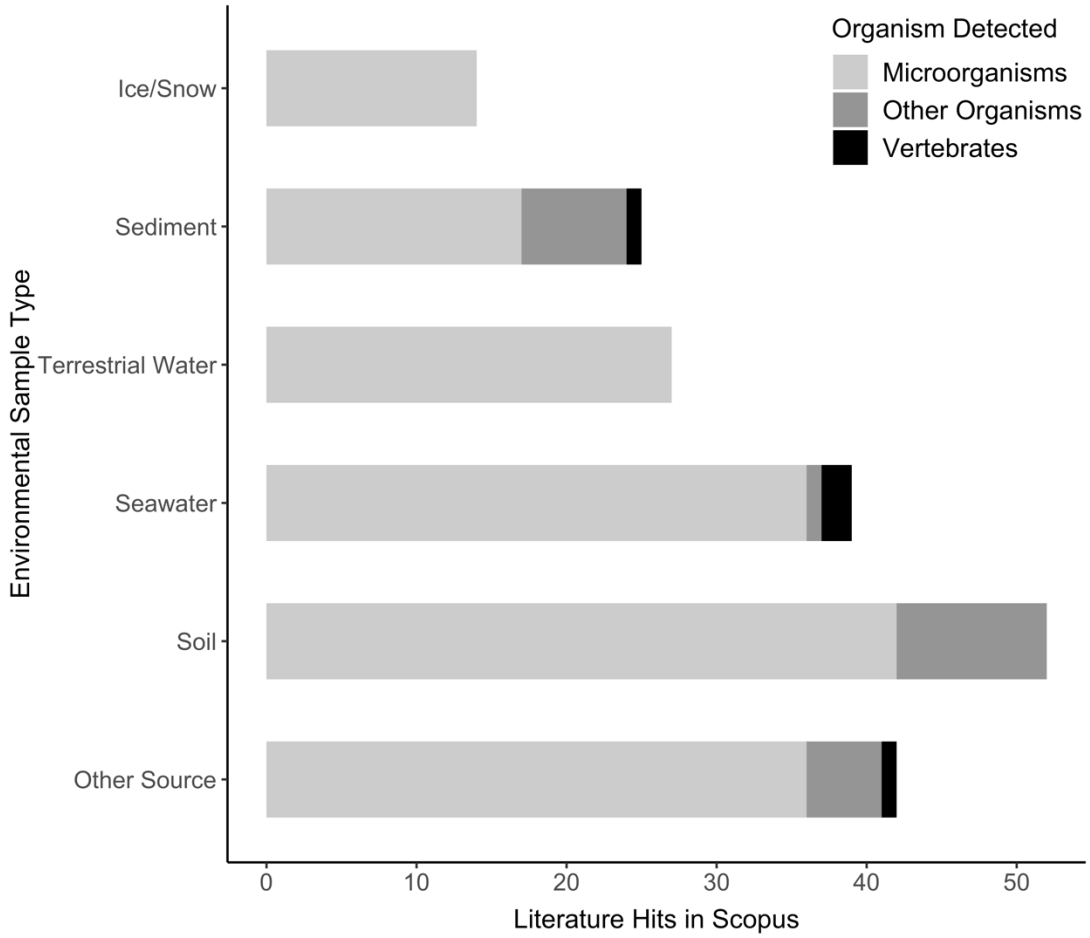

Figure 2.1. Number of papers found by in our systematic review of Antarctic environmental DNA. Categorised by 'Environmental Sample Type' and 'Organism detected', highlighting the discrepancy between microbial and vertebrate eDNA studies in Antarctica. Papers that overlapped between categories and they were counted as a hit for both. Papers in the 'Other Organisms' category included those that targeted one or more of the groups: meiofauna, benthic communities, invertebrates, moss, vascular plants, lichen, as well as macro algae and fungi.

2.4 Polar eDNA Methods

2.4.1 Soil samples

Despite being the most common sample type in our review, none of the 43 soil studies detected vertebrate DNA (Table 1). However, Pansu et al. (2015) made links between the introduction of rabbits to the Sub-Antarctic Kerguelen Islands and soil community compositions, finding a reduction in fungal diversity in locations where rabbits were historically introduced using multi-species barcoding. Furthermore, Pansu et al. (2015) showed that the herbivores' preference for introduced plant species alters the characteristics of soil communities, demonstrating the applications of metagenomic analyses of soil samples for monitoring ecosystem shifts.

2.4.2 Water samples

We found 38 papers that reported extracting DNA from seawater samples. The vast majority report on microbial studies (89 %), although two detected vertebrate eDNA from marine samples (Cowart et al. 2018; Mariani et al. 2019). One of these explored a novel method for collecting DNA using sponges (*Porifera* sp.) as 'natural samplers', capturing the DNA of a number of fishes, as well as both chinstrap penguin and Weddell seal DNA (Mariani et al. 2019). With further validation, sponges could be advantageous for eDNA sampling, filtering up to a thousand-fold more water than traditional filtering methods (Kahn et al. 2015; Mariani et al. 2019). Additionally, their ability to regenerate makes them easy to sample without long-term damage (Mariani et al. 2019).

A survey of invertebrate and vertebrate metazoan diversity in the West Antarctic Peninsula detected the eDNA of fishes using metagenomic analysis of shallow shelf water samples (Cowart et al. 2018). Results were comparable to inventories collated by the Scientific Committee on Antarctic Research-Marine Biodiversity Network (SCAR-MarBIN,

www.scarmarbin.be, www.biodiversity.aq, Griffiths et al. 2011; De Boyer et al, 2014) from peer-reviewed literature, museum specimens and the Census of Antarctic Marine Life (CAML, www.caml.aq, De Broyer and Danis 2011). Cowart et al. (2018) suggest that a combination of the two approaches (eDNA and traditional surveys) will lead to improved detection of faunal assemblages, in turn improving biodiversity assessments and monitoring community change in the Southern Ocean. In-field eDNA detection was supported with laboratory-based assessments of DNA degradation in Antarctic conditions, using Antarctic icefish (*Chionodraco rastrospinosus*) held in tanks (Cowart et al. 2018). Mitochondrial DNA persisted for longer under Antarctic conditions than would be expected in temperate waters, potentially leading to a greater breadth of species detected in future Antarctic eDNA studies.

Fewer studies focused on terrestrial water samples, and all of those were microbial metagenomic studies. This microbial focus is unsurprising; few vertebrates have been associated with Antarctic lakes, with only two instances of fish being sighted in the brackish waters of Beaver Lake (*Trematomus scotti*; Cromer et al. 2005) and Ablation Lake (*Trematomus hemacchii*; Heywood and Light 1975). Nonetheless, eDNA from Antarctic lakes could have potential for genotyping birds that have been associated with a number of lakes on the continent, such as the South Polar skua (*Stercorarius maccormicki*; Laybourn-Parry and Pearce, 2007) and Antarctic petrels (*Thalassoica antarctica*) which have been known to breed hundreds of kilometres inland (Hiller et al. 1988; Brooke et al. 1999).

2.4.3 Sediment samples

Of the papers we reviewed, 22 discussed the use of sediment samples to detect eDNA, with only one detecting vertebrate DNA (Ficetola et al. 2018). One of the benefits of sediment samples is that they provide ideal conditions for preserving DNA. By carbon dating the layers within a sediment core and extracting environmental DNA from those layers, we can collect historical data on species diversity and extinctions. Ficetola et al. (2018) extracted

rabbit and vascular plant DNA from sediment cores collected in the sub-Antarctic Kerguelen Islands to assess the recovery of plant diversity from a herbivore invader. Plant communities were assessed before and after the first detection of rabbit DNA to draw conclusions on the dynamics of the ecosystem and the trajectories of change. Not only were they able to show that changes in plant communities were consistent with the first detection of rabbit DNA, but they accounted for climate change as another potential mechanism driving change. Despite climate change explaining some variation, it was significantly lower than the percentage of variation explained by rabbit abundance. Defining the mechanisms affecting an ecosystem aids in differentiating between a system in 'crisis' versus one that has stabilised to a new equilibrium (Ficetola et al. 2018), a clearer understanding of ecosystem dynamics could impact what is considered the best response in a management situation.

2.4.4 Snow and ice samples

The advantage of snow samples as a source for eDNA is the snow acts as a natural freezer, helping to preserve the DNA within the samples (Hinlo et al. 2017). All of our hits for both snow and ice were microorganism studies; only 5 studies used snow samples and 11 focused on ice samples, with most of these from cryoconite holes (Webster-Brown et al. 2015; Sommers et al. 2018; Lutz et al. 2019; Sommers et al. 2019a; 2019b; Weisleitner et al. 2019). To our knowledge, no studies of Antarctic vertebrate eDNA from snow samples have been published to date. This gap in the literature presents a potential for future eDNA studies on Antarctic wildlife (Box 1); importantly, eDNA could aid in monitoring of ice-dependent species that are likely to be negatively impacted by climate change in the near future (Siniff et al. 2008; Ainley et al. 2010; Jenouvrier et al. 2014; Tranthan et al. 2020).

Future development of eDNA surveys for Antarctic snow samples can look to recent studies in the Northern Hemisphere (Dalén et al. 2007; Franklin et al. 2019; Kinoshita et al. 2019). The varying success of these studies is attributed in part to sample size, ranging from

single footprints (Dalén et al. 2007), five footprints (Kinoshita et al. 2019) to 2 litre samples (Franklin et al. 2019). Detection rates can also be impacted by storage length (Kinoshita et al. 2019), exposure to UV (e.g., targeting shaded versus sunlit sites [Kinoshita et al. 2019]) and laboratory methods (e.g., the use of qPCR over PCR [Franklin et al. 2019]). While the simplified environment of snow samples increases the likelihood of eDNA collection (Franklin et al. 2019), and tracks provide areas where eDNA is potentially condensed (Dalén et al. 2007), it can also increase the risk of contamination. These risks can be minimised through the use of gloves, sterilised equipment, and field blanks (Dalén et al. 2007; Franklin et al. 2019; Kinoshita et al. 2019). Care should be taken to ensure sampling teams have not recently been in contact with target species DNA (Franklin et al. 2019), similarly, it is important laboratories have not been previously contaminated with target species DNA (Dalén et al. 2007; Franklin et al. 2019). An additional benefit of snow samples is the preservation of DNA in compacted layers over time, which can be analysed through the collection of snow-columns (Franklin et al. 2019). By collecting columns from sites where camera traps had recorded lynx (*Lynx lynx*), Franklin et al. (2019) demonstrated the persistence of eDNA in snow and expanded the timeframe for species detection. While there are no terrestrial mammals on continental Antarctica, applying these methodologies to Antarctic snow samples would provide further opportunity to study vertebrates that venture onto the ice (Box 1.)

2.5 Challenges and limitations

A primary problem in working with genetic material is that DNA is vulnerable to degradation, which is exacerbated by exposure to UV, high temperatures, and deviations in pH (Strickler et al. 2015). These factors pose contrasting issues for studying eDNA under Antarctic conditions. For example, Strickler et al. (2015) found that cold, low-UV conditions resulted in less DNA degradation; thus, temperatures in Antarctica would be ideal, but 24 h

sunlight over summer would increase UV exposure. These factors are important to consider in the design of eDNA surveys on the continent. In their review of the applications of DNA, Beng and Corlett (2020) suggested research using eDNA has expanded without a concordant expansion in the understanding of its limitations. In their review covering misuses of DNA, Cristescu and Hebert (2018) suggested the biggest issue with the adoption of widespread eDNA applications was uncertainty introduced by the rates of false positives and false negatives. While false positives can indicate contamination at any stage during collection and analysis, and can be assessed using negative controls, false negatives can be caused by a number of factors, e.g., eDNA dispersal, sampling efforts and insufficient databases, making them difficult to evaluate (Cristescu and Hebert 2018). Misidentification of DNA (Furlan et al. 2020) can lead to incorrect interpretations, with inaccurate data informing management decisions. Drawing robust comparisons between eDNA datasets requires critical study design and methodological consistency (as reviewed in Goldberg, 2016; Dickie et al. 2018; Lear et al 2018; Mathieu et al. 2020). Subjective sampling methods and inadequate methodological information leads to a low level of reproducibility between eDNA studies (Dickie et al. 2018), as does the lack of standardisation between laboratory protocols (Lear et al. 2018; Zinger et al. 2019). Through investigating potential bias, and introducing greater standardisation across eDNA workflows, we will be able to draw sounder conclusions and be better placed to advise on research questions.

2.6. Future directions

2.6.1 Biodiversity and invasive species monitoring

Recently, international programs have prioritized mapping Antarctic diversity (e.g., the Census of Antarctic Marine Life (CAML); Grant and Linse 2009), resulting in the creation of databases such as the Registry of Antarctic Marine Species (RAMS; Griffiths et al. 2011), which is a compilation of known taxa and distribution data in the Antarctic marine environment (De Broyer and Danis 2011). The goal was to assess the current understanding of biodiversity in the region and compile existing gaps that can be identified and targeted, including under-sampled taxonomic groups or specific locations with few existing observations (De Broyer and Danis 2011). RAMS also provides a baseline from which changes in species distributions over time can be identified (Griffiths 2010), and De Broyer and Danis (2011) highlighted the need to incorporate more efficient taxonomic tools to expand the database. One such tool is eDNA; by confirming species presence and mapping distributions we could utilize eDNA to improve the confidence in current databases, identifying the taxa and sites that should be targeted for future biodiversity studies.

The limitations of Antarctic biodiversity assessments in a number of regions arise from their inaccessibility (Griffiths 2010). For example, the Western Weddell and Eastern Ross Seas are particularly poorly sampled due to extensive ice cover, and lack of year-round field stations in the vicinity. Likewise, knowledge of the deep sea is heavily restricted to transit routes (Griffiths 2010). Sampling biases are also temporal, as many winter behaviours and distributions are poorly studied given most research is conducted during the relative safety of austral summer (Griffiths 2010). Environmental DNA offers a tool that addresses some of these existing biases in Antarctic biodiversity databases, enabling us to catalogue taxa in situations where traditional methods are not safe or cost appropriate (e.g., deep sea

underwater surveys; Pawlowski et al. 2011). Furthermore, optimisation of population genetics using eDNA would allow researchers to non-invasively assess the genetic diversity of Antarctic species (Adams et al. 2019). For example, a population genetic eDNA study on orcas (*Orcinus orca*) detected haplotypic variation between orca communities (Baker et al. 2018, see case study in Box 2).

The increasing development and availability of sampling technologies (e.g., remotely operated vehicles (ROVs) and icebreaker vessels) is expanding our capability to monitor Antarctic biodiversity (Griffths 2010). Cowart et al. (2018) found lithodid king crabs (*Neolothodes yaldwyni*) in waters off the West Antarctic Peninsula, which is noteworthy as this area has been suggested as a possible location for such invasions (Thatje et al. 2005; Smith et al. 2012). Along coastal marine systems community eDNA signals remain distinct with minimal signal dispersal (Jeunen et al. 2019; 2020), thus eDNA could play a role in monitoring potential invasion fronts in the Antarctic. Likewise, eDNA sampling can assist biosecurity efforts, through monitoring both present (e.g., ballast water; Rey et al. (2019)) and historic threats (Pansu et al. 2015, Ficetola et al. 2018).

Both Pansu et al. (2015) and Ficetola et al. (2018) used eDNA to show a lag in ecosystem recovery after the eradication of invasive rabbits in the Kerguelen Islands. Understanding how an ecosystem reacted during the initial introduction of a species, and analysing subsequent recovery after eradication, can help inform management on other Sub-Antarctic islands. Environmental DNA surveys are often described as a ‘snapshot’ of a time and location in the current environment, comparatively, ancient DNA allows us to look at historic ‘snapshots’ and construct a much larger picture of an ecosystem through time, informing future conservation efforts.

2.6.2 Community sampling efforts

Community scientists have contributed to successful eDNA surveys in temperate regions, both in freshwater (Biggs et al. 2015) and marine environments (Miralles et al. 2016). Sampling techniques can be easily taught, and the risk of contamination can be minimised by using enclosed filters (Spens et al. 2017). Additionally, standard quality assurance procedures, such as field negatives or controls, are of even greater value when surveyors are inexperienced (Biggs et al. 2015). Though there are limits to non-specialists carrying out eDNA surveys (Biggs et al. 2015), the ability to generate large amounts of data across a greater spatiotemporal range is advantageous for increasing understanding of species distribution and composition. Community-based projects also educate the public about eDNA and raise awareness of species within the target ecosystem, acting as an immersive outreach tool that can enhance science communication (Biggs et al. 2015; Taylor et al. 2019).

Although Antarctica does not have an indigenous human population, communities at research stations and even tourists could contribute to data collection. Over the 2018-2019 summer season the International Association of Antarctic Tour Operators (IAATO) recorded 55,489 visitors (IAATO 2019a), not including scientists, commercial fishers, or base staff. However, few peer-reviewed studies implementing community-engaged projects in Antarctica exist (Casanovas et al. 2015; Mascioni et al. 2019; Taylor et al. 2019; Cusick et al. 2020) and to our knowledge there are no published community science eDNA surveys. Antarctic tourism is predominantly cruise based, so staff can monitor data collection and implement a greater level of standardisation. Further, IAATO is responsive to involvement in research as it corresponds to Resolution 7 (2009) of the Antarctic Treaty system (Taylor et al. 2019). These principles encourage the education of tourists to foster connections with Antarctica, resulting in growing interest in tourist-based data collection and education opportunities e.g., the Polar Citizen Science Collective (http://www.polarcollective.org;

Taylor et al. 2019). Collaborating with tourist companies across the summer season would provide a unique opportunity to build a large-scale baseline of biodiversity across the Antarctic Peninsula, generating a large amount of genetic data with a relatively small individual sampling effort. In addition to tourists, base staff and or researchers heading into the deep-field or overwintering could be provided with eDNA sampling kits, allowing for multiple snapshots of Antarctic biodiversity across a range of temporal and spatial scales.

2.6.3 Miniaturised technology and in situ eDNA analysis

The prospect of highly portable sequencing technology is promising for remote field research. Emerging technology such as the MinION sequencing device (Oxford Nanopore Technologies) could potentially accelerate the process of eDNA surveys, increasing the agility and adaptability of research in the field (Edwards et al. 2016; Johnson et al. 2017; Edwards et al. 2019; Gowers et al. 2019). For example, Truelove et al. (2019) successfully carried out ship based, rapid, in-field analysis of white shark (*Carcharodon carcharias*) DNA, extracted from seawater and sequenced using the MinION device. Results were ground-truthed on-shore with Illumina sequencing and found to be comparable, despite the higher error rate in the MinION device. A decreased turnaround time allows for further, informed sampling a quicker response on time-sensitive issues.

While application of the MinION to vertebrate DNA has been limited (Truelove et al. 2019), the MinION has been applied to microbial studies in both the Arctic (Edwards et al. 2016; Goordial et al. 2017a; Ducluzeau et al. 2018; Edwards et al. 2019; Gowers et al. 2019; Millán-Aguiñaga 2019) and Antarctica (Johnson et al. 2017; Millán-Aguiñaga et al. 2019). In one extreme example, Gowers et al. (2019) packed their entire laboratory onto a sled, travelled 135km from the nearest field station onto the Vatnajökull ice cap in Iceland, and successfully sampled and sequenced eDNA from the ice for metagenomics analysis. These

previous studies in similar environments to Antarctica have highlighted the primary challenge - extreme conditions. In particular, the cold temperatures affect battery life, reagents, and the function of the MinION device and laptops, and contribute to the brittling of resin-based equipment (Gowers et al. 2019). Suggestions for tackling these challenges include the design of an insulating containment system (Johnson et al. 2017) and the use of sheltered field laboratories, utilising either pre-existing structures (Edwards et al. 2016) or tents (Gowers et al. 2019).

2.7 Conclusions

We found 172 papers addressing eDNA studies across 20 years, the vast majority of which focused on microbial diversity. We found eDNA technologies have been used in Antarctica since the beginning of the millennium (Gordon et al. 2000), but few have used eDNA to sequence the DNA of Antarctic vertebrates (McInnes et al. 2016; Cowart et al. 2018; Ficetola et al. 2018; Mariani et al. 2019). This gap highlights an important opportunity for future Antarctic research, particularly for biodiversity analysis, invasive species monitoring, and community science programmes.

As genetic technologies develop, the scope for eDNA surveys will only increase. The MinION sequencer is an example of the opportunity presented by technological advances for in-field, real time analysis of eDNA, increasing the adaptability and the agility of genetic research on the ice (Edwards et al. 2016; Johnson et al. 2017; Edwards et al. 2019; Gowers et al. 2019). There is also the potential to look into differences within species using nuclear eDNA, both for comparisons between individuals (Box 1) or ecotypes in species such as orca (*Orcinus orca*) (Box 2). Though the development of eDNA workflows in fields such population genetics are still in the early days, with much of the research currently in the preliminary phase, Antarctica and the Southern Ocean presents a potential region to explore

these novel applications.

2.8 References

Adams CI, Knapp M, Gemmell NJ, Jeunen G-J, Bunce M, Lamare MD, Taylor HR. 2019. Beyond biodiversity: can environmental DNA (eDNA) cut it as a population genetics tool? Genes. 10(3):192.

Ainley D, Russell J, Jenouvrier S, Woehler E, Lyver POB, Fraser WR, Kooyman GL. 2010. Antarctic penguin response to habitat change as Earth's troposphere reaches 2 C above preindustrial levels. Ecological Monographs. 80(1):49-66.

Ainley DG, Ballard G. 2012. Trophic interactions and population trends of killer whales (*Orcinus orca*) in the Southern Ross Sea. Aquatic Mammals. 38(2):153-160.

Ammon Uv, Wood SA, Laroche O, Zaiko A, Tait L, Lavery S, Inglis GJ, Pochon X. 2018. Combining morpho-taxonomy and metabarcoding enhances the detection of non-indigenous marine pests in biofouling communities. Scientific Reports. 8(1):16290.

Andrews RD, Pitman RL, Ballance LT. 2008. Satellite tracking reveals distinct movement patterns for Type B and Type C killer whales in the southern Ross Sea, Antarctica. Polar Biology. 31(12):1461-1468.

Baker CS, Steel D, Nieukirk S, Klinck H. 2018. Environmental DNA (eDNA) from the wake of the whales: droplet digital PCR for detection and species identification. Frontiers in Marine Science. 5:133.

Barnes MA, Turner CR. 2016. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics. 17(1):1-17.

Beng KC, Corlett RT. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and Conservation. 29(7):2089-2121.

Biggs J, Ewald N, Valentini A, Gaboriaud C, Dejean T, Griffiths RA, Foster J, Wilkinson JW, Arnell A, Brotherton P. 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus). Biological Conservation. 183:19-28.

Bohmann K, Evans A, Gilbert MTP, Carvalho GR, Creer S, Knapp M, Douglas WY, De Bruyn M. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends in ecology & evolution. 29(6):358-367.

Bovo S, Ribani A, Utzeri VJ, Schiavo G, Bertolini F, Fontanesi L. 2018. Shotgun metagenomics of honey DNA: Evaluation of a methodological approach to describe a multi-kingdom honey bee derived environmental DNA signature. PloS one. 13(10):e0205575.

Brown EA, Chain FJ, Zhan A, MacIsaac HJ, Cristescu ME. 2016. Early detection of aquatic invaders using metabarcoding reveals a high number of non-indigenous species in Canadian ports. Diversity and Distributions. 22(10):1045-1059.

Cameron MF, Siniff DB. 2004. Age-specific survival, abundance, and immigration rates of a Weddell seal (*Leptonychotes weddellii*) population in McMurdo Sound, Antarctica. Canadian Journal of Zoology. 82(4):601-615.

Casanovas P, Black M, Fretwell P, Convey P. 2015. Mapping lichen distribution on the Antarctic Peninsula using remote sensing, lichen spectra and photographic documentation by citizen scientists. Polar Research. 34(1):25633.

Chown SL, Lee JE, Hughes KA, Barnes J, Barrett P, Bergstrom DM, Convey P, Cowan DA, Crosbie K, Dyer G. 2012. Challenges to the future conservation of the Antarctic. Science. 337(6091):158-159.

Chown SL, Clarke A, Fraser CI, Cary SC, Moon KL, McGeoch MA. 2015. The changing form of Antarctic biodiversity. Nature. 522(7557):431-438.
Coetzee BW, Chown SL. 2016. A meta-analysis of human disturbance impacts on Antarctic wildlife. Biological reviews of the Cambridge Philosophical Society. 91(3):578-596.
Collins RA, Wangensteen OS, O'Gorman EJ, Mariani S, Sims DW, Genner MJ. 2018. Persistence of environmental DNA in marine systems. Communications Biology. 1(1):1-11.
Corinaldesi C, Beolchini F, Dell'Anno A. 2008. Damage and degradation rates of extracellular DNA in marine sediments: implications for the preservation of gene sequences. Molecular Ecology. 17(17):3939-3951.
Cowart DA, Murphy KR, Cheng CHC. 2018. Metagenomic sequencing of environmental DNA reveals marine faunal assemblages from the West Antarctic Peninsula. Marine Genomics. 37:148-160.
Crane A, Goebel ME, Kraberger S, Stone AC, Varsani A. 2018. Novel anelloviruses identified in buccal swabs of Antarctic fur seals. Virus Genes. 54(5):719-723.
Cristescu ME, Hebert PD. 2018. Uses and misuses of environmental DNA in biodiversity science and conservation. Annual Review of Ecology, Evolution, and Systematics. 49:209-230.
Cromer L, Williams R, Gibson J. 2005. Trematomus scotti in Beaver Lake: the first record of a fish from a non-marine Antarctic habitat. Journal of fish biology. 66(5):1493-1497.
Cusick AM, Gilmore R, Bombosch A, Mascioni M, Almandoz GO, Vernet M. 2020. Polar tourism as an effective research tool. Oceanography. 33(1):50-61.
Czechowski P, Clarke LJ, Breen J, Cooper A, Stevens MI. 2016a. Antarctic eukaryotic soil diversity of the Prince Charles Mountains revealed by high-throughput sequencing. Soil Biology and Biochemistry. 95:112-121.
Dalén L, Götherström A, Meijer T, Shapiro B. 2007. Recovery of DNA from footprints in the snow. The Canadian Field-Naturalist. 121(3):321-324.
De Broyer C, Danis B. 2011. How many species in the Southern Ocean? Towards a dynamic inventory of the Antarctic marine species. Deep sea research Part II: Topical Studies in Oceanography. 58(1-2):5-17.
De Broyer C, Koubbi P, Griffith, HJ, Raymond B, Udekem d'Acoz C, Van de Putte AP, Danis B, David B, Grant S, Gutt J, et al. 2014. Biogeograhical atlas of the Southern Ocean. Cambridge. Scientific Committee on Antarctic Research.
Deagle B, Tollit D, Jarman S, Hindell M, Trites A, Gales N. 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. Molecular Ecology. 14(6):1831-1842.
Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. Journal of Applied Ecology. 49(4):953-959.
Dickie IA, Boyer S, Buckley HL, Duncan RP, Gardner PP, Hogg ID, Holdaway RJ, Lear G, Makiola A, Morales SE. 2018. Towards robust and repeatable sampling methods in eDNA-based studies. Molecular Ecology Resources. 18(5):940-952.
Ducluzeau A-L, Tyson J, Collins R, Snutch T, Hassett B. 2018. Genome sequencing of Sub-Arctic Mesomycetozoean *Sphaeroforma sirkka* strain B5, performed with the Oxford Nanopore minION and Illumina HiSeq systems. Microbiology Resource Announcements. 7(15):e00848-18
Edwards A, Debbonaire AR, Sattler B, Mur LA, Hodson AJ. 2016. Extreme metagenomics using nanopore DNA sequencing: a field report from Svalbard, 78 N. BioRxiv: 073965.

Edwards A, Debbonaire AR, Nicholls SM, Rassner SM, Sattler B, Cook JM, Davy T, Soares A, Mur LA, Hodson AJ. 2019. In-field metagenome and 16S rRNA gene amplicon nanopore sequencing robustly characterize glacier microbiota. BioRxiv: 073965.
Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. Biology letters. 4(4):423-425.
Ficetola GF, Poulenard J, Sabatier P, Messager E, Gielly L, Leloup A, Etienne D, Bakke J, Malet E, Fanget B, Støren E, Reyss JL, Taberlet P, Arnaud F. 2018. DNA from lake sediments reveals long-term ecosystem changes after a biological invasion. Science Advances. 4(5):4292.
Finkel SE, Kolter R. 2001. DNA as a nutrient: Novel role for bacterial competence gene homologs. Journal of Bacteriology. 183(21):6288-6293.
Franklin TW, McKelvey KS, Golding JD, Mason DH, Dysthe JC, Pilgrim KL, Squires JR, Aubry KB, Long RA, Greaves SE. 2019. Using environmental DNA methods to improve winter surveys for rare carnivores: DNA from snow and improved non-invasive techniques. Biological Conservation. 229:50-58.
Fröls S, Dyall-Smith M, Pfeifer F. 2012. Biofilm formation by haloarchaea. Environmental Microbiology. 14(12):3159-3174.
Furlan EM, Davis J, Duncan RP. 2020. Identifying error and accurately interpreting eDNA metabarcoding results: A case study to detect vertebrates at arid zone waterholes. Molecular Ecology Resources. 20(5):1259-1276.
Gelatt TS, Davis CS, Siniff DB, Strobeck C. 2001. Molecular evidence for twinning in Weddell seals (*Leptonychotes weddellii*). Journal of Mammalogy. 82(2):491-499.
Gerhard WA, Gunsch CK. 2019. Metabarcoding and machine learning analysis of environmental DNA in ballast water arriving to hub ports. Environment international. 124:312-319.
Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP. 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science. 32(3):792-800.
Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution. 7(11):1299-1307.
Goordial J, Altshuler I, Hindson K, Chan-Yam K, Marcolefas E, Whyte LG. 2017a. In situ field sequencing and life detection in remote (79°26'N) Canadian high arctic permafrost ice wedge microbial communities. Frontiers in Microbiology. 8:2594.
Gordon DA, Priscu J, Giovannoni S. 2000. Origin and phylogeny of microbes living in permanent Antarctic lake ice. Microbial Ecology. 39(3):197-202.
Gowers G-OF, Vince O, Charles J-H, Klarenberg I, Ellis T, Edwards A. 2019. Entirely off-grid and solar-powered DNA sequencing of microbial communities during an ice cap traverse expedition. Genes. 10(11):902.
Grant RA, Linse K. 2009. Barcoding Antarctic biodiversity: current status and the CAML initiative, a case study of marine invertebrates. Polar Biology. 32(11):1629.
Griffiths HJ. 2010. Antarctic marine biodiversity–what do we know about the distribution of life in the Southern Ocean? PloS one. 5(8):11683
Griffiths HJ, Danis B, Clarke A. 2011. Quantifying Antarctic marine biodiversity: The SCAR-MarBIN data portal. Deep Sea Research Part II: Topical Studies in Oceanography. 58(1-2):18-29.
Gutt J, Isla E, Bertler AN, Bodeker GE, Bracegirdle TJ, Cavanagh RD, Comiso JC, Convey P, Cummings V, De Conto R, De Master D, di Prisco G, d'Ovidio F, Griffiths HJ, Khan AL, López-Martínez J, Murray AE, Nielsen UN, Ott S, Post A, Ropert-Coudert

Y, Saucède T, Scherer R, Schiaparelli S, Schloss IR, Smith CR, Stefels J, Stevens C, Strugnell JM, Trimborn S, Verde C, Verleyen E, Wall DH, Wilson NG, Xavier JC. 2018. Cross-disciplinarity in the advance of Antarctic ecosystem research. Marine Genomics. 37:1-17.

Haile J, Froese DG, MacPhee RD, Roberts RG, Arnold LJ, Reyes AV, Rasmussen M, Nielsen R, Brook BW, Robinson S. 2009. Ancient DNA reveals late survival of mammoth and horse in interior Alaska. Proceedings of the National Academy of Sciences. 106(52):22352-22357.

Harcourt R, Kingston J, Cameron M, Waas J, Hindell M. 2007. Paternity analysis shows experience, not age, enhances mating success in an aquatically mating pinniped, the Weddell seal (*Leptonychotes weddellii*). Behavioral Ecology and Sociobiology. 61(4):643-652.

Hastings KK, Testa JW. 1998. Maternal and birth colony effects on survival of Weddell seal offspring from McMurdo Sound, Antarctica. Journal of Animal Ecology. 67(5):722-740.

Heath C, Xiao PH, Cary SC, Cowan D. 2009. Identification of a novel alkaliphilic esterase active at low temperatures by screening a metagenomic library from Antarctic desert soil. Applied and Environmental Microbiology. 75(13):4657-4659.

Hebsgaard MB, Phillips MJ, Willerslev E. 2005. Geologically ancient DNA: Fact or artefact? Trends in Microbiology. 13(5):212-220.

Hellström A, Wijkmark N, Edbom-Blomstrand C, Hellström P, Näslund J. 2019. Footsteps in the snow-Pilot study for future monitoring of individual lynx (*Lynx lynx*) from eDNA in snow tracks. AquaBiota Report 2019:10.

Heywood R, Light J. 1975. First direct evidence of life under Antarctic shelf ice. Nature. 254(5501):591-592.

Hiller A, Wand U, Kämpf H, Stackebrandt W. 1988. Occupation of the Antarctic continent by petrels during the past 35 000 years: Inferences from a 14C study of stomach oil deposits. Polar Biology. 9(2):69-77.

Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical Chemistry. 83(22):8604-8610.

Hinlo R, Gleeson D, Lintermans M, Furlan E. 2017. Methods to maximise recovery of environmental DNA from water samples. PloS one. 12(6):e0179251.

IAATO. 2019a. IAATO Overview of Antarctic Tourism: 2018-19 Season and Preliminary Estimates for 2019-20 Season.

Ishige T, Miya M, Ushio M, Sado T, Ushioda M, Maebashi K, Yonechi R, Lagan P, Matsubayashi H. 2017. Tropical-forest mammals as detected by environmental DNA at natural saltlicks in Borneo. Biological conservation. 210:281-285.

Jenouvrier S, Holland M, Stroeve J, Serreze M, Barbraud C, Weimerskirch H, Caswell H. 2014. Projected continent-wide declines of the emperor penguin under climate change. Nature Climate Change. 4(8):715-718.

Jeunen GJ, Knapp M, Spencer HG, Lamare MD, Taylor HR, Stat M, Bunce M, Gemmell NJ. 2019. Environmental DNA (eDNA) metabarcoding reveals strong discrimination among diverse marine habitats connected by water movement. Molecular Ecology Resources. 19(2):426-438.

Jeunen GJ, Lamare MD, Knapp M, Spencer HG, Taylor HR, Stat M, Bunce M, Gemmell NJ 2020. Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal. Environmental DNA. 2(1):99-111.

Johnson SS, Zaikova E, Goerlitz DS, Bai Y, Tighe SW. 2017. Real-time DNA sequencing in the Antarctic Dry Valleys using the Oxford nanopore sequencer. Journal of Biomolecular Techniques. 28(1):2-7.
Kahn AS, Yahel G, Chu JW, Tunnicliffe V, Leys SP. 2015. Benthic grazing and carbon sequestration by deep-water glass sponge reefs. Limnology and Oceanography. 60(1):78-88.
Kinoshita G, Yonezawa S, Murakami S, Isagi Y. 2019. Environmental DNA collected from snow tracks is useful for identification of mammalian species. Zoological science. 36(3):198-207.
LaRue MA, Salas L, Nur N, Ainley DG, Stammerjohn S, Barrington L, Stamatiou K, Pennycook J, Dozier M, Saints J. 2019. Physical and ecological factors explain the distribution of Ross Sea Weddell seals during the breeding season. Marine Ecology Progress Series. 612:193-208.
Laybourn-Parry J, Pearce DA. 2007. The biodiversity and ecology of Antarctic lakes: models for evolution. Philosophical transactions of the Royal Society of London. Series B, Biological sciences. 362(1488):2273-2289.
Lear G, Dickie I, Banks J, Boyer S, Buckley HL, Buckley TR, Cruickshank R, Dopheide A, Handley KM, Hermans S. 2018. Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. New Zealand Journal of Ecology. 42(1):10-50A.
Lefort M-C, Boyer S, Barun A, Khoyi AE, Ridden J, Smith VR, Sprague R, Waterhouse BR, Cruickshank RH. 2019. Blood, sweat and tears: non-invasive vs. non-disruptive DNA sampling for experimental biology. bioRxiv: 385120.
Levy-Booth DJ, Campbell RG, Gulden RH, Hart MM, Powell JR, Klironomos JN, Pauls KP, Swanton CJ, Trevors JT, Dunfield KE. 2007. Cycling of extracellular DNA in the soil environment. Soil Biology and Biochemistry. 39(12):2977-2991.
Lutz S, Ziolkowski LA, Benning LG. 2019. The biodiversity and geochemistry of cryoconite holes in Queen Maud Land, East Antarctica. Microorganisms. 7(6):160.
Mariani S, Baillie C, Colosimo G, Riesgo A. 2019. Sponges as natural environmental DNA samplers. Current Biology. 29(11):401-402.
Martín J, De Neve L, Fargallo JA, Polo V, Soler M. 2004. Factors affecting the escape behaviour of juvenile chinstrap penguins, *Pygoscelis antarctica*, in response to human disturbance. Polar Biology. 27(12):775-781.
Mascioni M, Almandoz GO, Cefarelli AO, Cusick A, Ferrario ME, Vernet M. 2019. Phytoplankton composition and bloom formation in unexplored nearshore waters of the western Antarctic Peninsula. Polar Biology. 42(10):1859-1872.
Mathieu C, Hermans SM, Lear G, Buckley T, Lee KC, Buckley HL. 2020. A systematic review of sources of variability and uncertainty in eDNA data for environmental monitoring. Frontiers in Ecology and Evolution. 8:135.
McInnes JC, Emmerson L, Southwell C, Faux C, Jarman SN. 2016. Simultaneous DNA-based diet analysis of breeding, non-breeding and chick Adélie penguins. Royal Society Open Science. 3(1):150443.
Millán-Aguiñaga N, Soldatou S, Brozio S, Munnoch JT, Howe J, Hoskisson PA, Duncan KR. 2019. Awakening ancient polar actinobacteria: Diversity, evolution and specialized metabolite potential. Microbiology. 165(11):1169-1180.
Miralles L, Dopico E, Devlo-Delva F, Garcia-Vazquez E. 2016. Controlling populations of invasive pygmy mussel (Xenostrobus securis) through citizen science and environmental DNA. Marine Pollution Bulletin. 110(1):127-132.

Nachtsheim DA, Jerosch K, Hagen W, Plötz J, Bornemann H. 2017. Habitat modelling of crabeater seals (*Lobodon carcinophaga*) in the Weddell Sea using the multivariate approach Maxent. Polar Biology. 40(5):961-976.
Ogram A, Sayler GS, Barkay T. 1987. The extraction and purification of microbial DNA from sediments. Journal of microbiological methods. 7(2-3):57-66.
Pansu J, Winkworth RC, Hennion F, Gielly L, Taberlet P, Choler P. 2015. Long-lasting modification of soil fungal diversity associated with the introduction of rabbits to a remote sub-Antarctic archipelago. Biology Letters. 11(9):20150408.
Parsons KM, Durban JW, Burdin AM, Burkanov VN, Pitman RL, Barlow J, Barrett-Lennard LG, LeDuc RG, Robertson KM, Matkin CO. 2013. Geographic patterns of genetic differentiation among killer whales in the northern North Pacific. Journal of Heredity. 104(6):737-754.
Parsons KM, Everett M, Dahlheim M, Park L. 2018. Water, water everywhere: environmental DNA can unlock population structure in elusive marine species. Royal Society open science. 5(8):180537.
Pawlowski J, Fontaine D, da Silva AA, Guiard J. 2011. Novel lineages of Southern Ocean deep-sea foraminifera revealed by environmental DNA sequencing. Deep-Sea Research Part II: Topical Studies in Oceanography. 58(19-20):1996-2003.
Pietramellara G, Ascher J, Borgogni F, Ceccherini M, Guerri G, Nannipieri P. 2009. Extracellular DNA in soil and sediment: fate and ecological relevance. Biology and Fertility of Soils. 45(3):219-235.
Pinfield R, Dillane E, Runge AKW, Evans A, Mirimin L, Niemann J, Reed TE, Reid DG, Rogan E, Samarra FI. 2019. False-negative detections from environmental DNA collected in the presence of large numbers of killer whales (*Orcinus orca*). Environmental DNA. 1(4):316-328.
Polar Citizen Science collective. [accessed 2020 Jul 15]. http://www.polarcollective.org
Ratnasingham S, Hebert PD. 2007. BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). Molecular Ecology notes. 7(3):355-364.
Rey A, Carney KJ, Quinones LE, Pagenkopp Lohan KM, Ruiz GM, Basurko OC, Rodríguez-Ezpeleta N. 2019. Environmental DNA metabarcoding: a promising tool for ballast water monitoring. Environmental Science & Technology. 53(20):11849-11859.
Schuster SC. 2008. Next-generation sequencing transforms today's biology. Nature methods. 5(1):16-18.
Shokralla S, Spall JL, Gibson JF, Hajibabaei M. 2012. Next-generation sequencing technologies for environmental DNA research. Molecular Ecology. 21(8):1794-1805.
Sigsgaard EE, Nielsen IB, Bach SS, Lorenzen ED, Robinson DP, Knudsen SW, Pedersen MW, Al Jaidah M, Orlando L, Willerslev E. 2016. Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. Nature Ecology and Evolution. 1(1):1-5.
Siniff DB, Garrott RA, Rotella JJ, Fraser WR, Ainley DG. 2008. Opinion: Projecting the effects of environmental change on Antarctic seals. Antarctic Science. 20(5):425.
Smith CR, Grange LJ, Honig DL, Naudts L, Huber B, Guidi L, Domack E. 2012. A large population of king crabs in Palmer Deep on the west Antarctic Peninsula shelf and potential invasive impacts. Proceedings of the Royal Society B: Biological Sciences. 279(1730):1017-1026.
Sommers P, Darcy JL, Gendron E, Stanish LF, Bagshaw EA, Porazinska DL, Schmidt SK. 2018. Diversity patterns of microbial eukaryotes mirror those of bacteria in Antarctic cryoconite holes. FEMS Microbiology Ecology. 94(1):fix167.

Sommers P, Porazinska DL, Darcy JL, Zamora F, Fountain AG, Schmidt SK. 2019a. Experimental cryoconite holes as mesocosms for studying community ecology. Polar Biology. 42(11):I973-1984.
Sommers P, Fontenele RS, Kringen T, Kraberger S, Porazinska DL, Darcy JL, Schmidt SK, Varsani A. 2019b. Single-stranded DNA viruses in Antarctic cryoconite holes. Viruses. 11(11):1022.
Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SS, Sigsgaard EE, Hellström M. 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods in Ecology and Evolution. 8(5):635-645.
Stirling I. 1969. Ecology of the Weddell seal in McMurdo Sound, Antarctica. Ecology. 50(4):573-586.
Strickler KM, Fremier AK, Goldberg CS. 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation. 183:85-92.
Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH. 2012. Environmental DNA. Molecular ecology. 21(8):1789-1793.
Taberlet P, Bonin A, Coissac E, Zinger L. 2018. Environmental DNA: For biodiversity research and monitoring. Oxford (UK): Oxford University Press.
Taylor AR, Barðadóttir Þ, Auffret S, Bombosch A, Cusick AL, Falk E, Lynnes A. 2019. Arctic expedition cruise tourism and citizen science: a vision for the future of polar tourism. Journal of Tourism Futures. 6(1):102-111.
Thatje S, Anger K, Calcagno JA, Lovrich GA, Pörtner H-O, Arntz WE. 2005. Challenging the cold: crabs reconquer the Antarctic. Ecology. 86(3):619-625.
Thomsen PF, Møller PR, Sigsgaard EE, Knudsen SW, Jørgensen OA, Willerslev E 2016. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. PloS one 11(11).
Trathan PN, Wienecke B, Barbraud C, Jenouvrier S, Kooyman G, Le Bohec C, Ainley DG, Ancel A, Zitterbart DP, Chown SL, Larue M, Cristofari R, Younger J, Clucas G, Bost C, Brown JA, Gillett HJ, Fretwell, PT. 2020. The emperor penguin - vulnerable to projected rates of warming and sea ice loss. Biological Conservation. 241:108216.
Truelove NK, Andruszkiewicz EA, Block BA. 2019. A rapid environmental DNA method for detecting white sharks in the open ocean. Methods in Ecology and Evolution. 10(8):1128-1135.
Tsuji S, Shibata N, Sawada H, Ushio M. 2020. Quantitative evaluation of intraspecific genetic diversity in a natural fish population using environmental DNA analysis. Molecular Ecology Resources. 20(5):1323-1332.
Uchii K, Doi H, Minamoto T. 2016. A novel environmental DNA approach to quantify the cryptic invasion of non-native genotypes. Molecular Ecology Resources. 16(2):415-422.
Valentin RE, Fonseca DM, Gable S, Kyle KE, Hamilton GC, Nielsen AL, Lockwood JL. 2020. Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect invasive forest insects. Molecular Ecology Resources. 20(3)746-755
Valentini A, Pompanon F, Taberlet P. 2009. DNA barcoding for ecologists. Trends in Ecology and Evolution. 24(2):110-117.
Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular ecology 25(4): 929-942.

Van Polanen Petel T, Giese M, Wotherspoon S, Hindell M. 2007. The behavioural response of lactating Weddell seals (Leptonychotes weddellii) to over-snow vehicles: a case study. Canadian Journal of Zoology. 85(4):488-496.
Von Duyke AL, Kruger E, Wijkmark N, Näslund J, Hellström P, Hellström M. 2019. Evaluation of environmental DNA (eDNA) collected from tracks in the snow as a means to monitor individual polar bears (Ursus maritimus) in the Chukchi and Beaufort Seas. Research Reports, North Slope Borough Department of Wildlife Management, NSB.DWM.PRR.2019-01
Webster-Brown JG, Hawes I, Jungblut AD, Wood SA, Christenson HK. 2015. The effects of entombment on water chemistry and bacterial assemblages in closed cryoconite holes on Antarctic glaciers. FEMS Microbiology Ecology. 91(12):fiv144.
Wei N, Nakajima F, Tobino T. 2018. A microcosm study of surface sediment environmental DNA: decay observation, abundance estimation, and fragment length comparison. Environmental Science and Technology. 52(21):12428-12435.
Weisleitner K, Perras A, Moissl-Eichinger C, Andersen DT, Sattler B. 2019. Source environments of the microbiome in perennially ice-covered lake Untersee, Antarctica. Frontiers in Microbiology. 10:1019.
Wilson RP, Culik B, Danfeld R, Adelung D. 1991. People in Antarctica—how much do Adélie Penguins *Pygoscelis adeliae* care? Polar Biology. 11(6):363-370.
Zinger L, Bonin A, Alsos IG, Bálint M, Bik H, Boyer F, Chariton AA, Creer S, Coissac E, Deagle BE 2019. DNA metabarcoding—Need for robust experimental designs to draw sound ecological conclusions. Wiley Online Library. Molecular Ecology. 28(4):1-6

2.9 Appendix

2.9.1 Box 1: Weddell seals case study

Snow can limit the degradation of eDNA within a sample (Dalén et al. 2007; Kinoshita et al. 2019), and likewise eDNA is not dispersed as it would be in other systems, such as lotic water, making it a promising sample source for studies targeting nuclear eDNA. The use of eDNA from snow samples to make population genetic inferences has not been studied extensively, however, preliminary work in the Arctic has shown promising potential for detecting nuclear eDNA in the footprints of polar bears (*Ursus maritimus;*Von Duyek et al. 2019) and captive lynx (*Lynx lynx*; Hellström et al. 2019). Due to the quality and quantity of DNA required for such studies, accessing freshly deposited DNA left in the environment is best practice. While this may be a challenge in long ranging terrestrial mammals in the Arctic, Antarctica's megafauna are marine organisms, travelling limited distances across the ice and providing ample opportunities to collect fresh samples.

A potential candidate for snow-based eDNA surveys are Weddell seals, which are fast-ice (sea ice literally fastened to the Antarctic coastline) obligates (Stirling 1969), found only sporadically around Antarctica (LaRue et al. 2019). Weddell seals require time out of the water to rest between foraging, and in the austral spring to raise their young (Stirling 1969). The proximity of a Weddell seal population to Scott Base and McMurdo Station, and their limited movement and breeding-season behaviour makes them the ideal target for an annual survey effort (Stirling, 1969). While much is known about this southernmost breeding marine mammal on the planet due to a mark-recapture study that has been ongoing since the mid 1960's (Hastings and Testa 1998; Cameron and Siniff 2004), eDNA surveys could provide additional information. Genetic studies of Weddell seals primarily use flipper skin

punches (Gelatt et al. 2001; Harcourt et al. 2007). These skin samples provide valuable genetic information, but require a high level of training, permitting, and can be hazardous for the researchers involved. Environmental DNA surveys are not a replacement for traditional genetic survey methods, but the ease of collecting non-invasive environmental sampling is beneficial for building more extensive datasets, while limiting disturbance to Antarctic wildlife. One eDNA survey has successfully detected Weddell seals in the West Antarctic Peninsula (Mariani et al. 2019), but their potential for supplementing the traditional genetic sampling methods have not been evaluated. We suggest future species-specific studies investigating the possibility of collecting nuclear eDNA from snow recently vacated by Weddell seals in the breeding colonies of Erebus Bay. As methodologies are developed, they could be applied to other, less accessible pinnipeds, such as the pack-ice inhabiting crabeater seals (*Lobodon carcinophaga*). Success in this field would allow us to draw population-level inferences for Antarctic megafauna from non-invasive eDNA samples (for a review of population genetic inference from eDNA, see Adams et al. (2019)).

2.9.2 Box 2: Orca case study

While eDNA has been primarily utilized to establish the presence or absence of taxa, there is growing interest in population genetics applications (Adams et al. 2019). In marine systems outside of Antarctica, mitochondrial DNA haplotypes have been identified using eDNA for a number of marine vertebrates (Sigsgaard et al. 2016; Baker et al. 2018; Parsons et al. 2018; Tsuji et al. 2020). In one such example, resident and transient communities of Northern hemisphere Orcas (*Orcinus orca*) were distinguished using known mitochondrial haplotypes (Parsons et al. 2013) genotyped from eDNA samples (Baker et al. 2018). Multiple orca ecotypes are known to inhabit the Southern Ocean, with two ecotypes observed in McMurdo Sound (Andrews et al. 2008; Ainley and Ballard 2012). The success of distinguishing orca ecotypes from water samples in the Northern Hemisphere suggests that

eDNA could provide a non-invasive method for differentiating between populations throughout Antarctica.

The specific methodologies used might determine the success of eDNA as a tool for population genetics. In their study on North Pacific orcas in Puget Sound, Baker et al. (2018) successfully identified orca DNA for up to two hours after encountering orcas using droplet digital PCR (Baker et al. 2018), a method based on fractioning samples into droplets which are individually amplified by PCR, aiding with absolute quantification (Hindson et al. 2011). While fragmentation of eDNA limited barcoding, Baker et al. (2018) were able to identify the resident population ecotype in two of their samples, supporting audio and visual data collected during the encounter. In contrast, Pinfield et al (2019) were unable to make population level inferences in their qPCR-based study of Northeast Atlantic orca, finding a high rate of false negatives. Detection may have been limited by weather conditions, animal behaviour and temperature-affected skin sloughing. Colder conditions may lead to a reduction in DNA deposition, as blood flow is restricted to outer skin layers and less skin is shed (Pinfield et al. 2019). Study design is likely also a contributor to the high rate of false negatives, with Pinfield et al. (2019) suggesting sampling behind whales and increasing PCR replicates may lead to more positive results. Future studies on Southern Hemisphere orcas should consider the relationship between detection rates and methodological choices. For eDNA surveys of whales to be viable, more research is needed on optimising workflows, particularly in Antarctica. Nonetheless, population genetic applications of eDNA could be an excellent approach for exploring regional differences across the continent.

Chapter Three

Detection of Weddell seal (*Leptonychotes weddellii*) eDNA from Antarctic snow samples

Accurate, long-term monitoring of community structure and composition is required to quantify the impact of human disturbance and climate change on Antarctica's unique ecosystem. As technology develops, monitoring programmes should be upgraded in order to better reflect the logistical challenges of a complex system. We propose incorporating environmental DNA (eDNA) as an additional sampling tool for Antarctica's vertebrate species and discuss the benefits of non-invasive sampling methods. This project investigates the potential of snow samples as pools of Weddell seal DNA, which we collected from an indent left behind by a seal at the Turtle Rock Breeding colony, McMurdo Sound. We compared three DNA extraction kits, Qiagen DNeasy, Zymo Quick-DNA and Invitrogen PureLink, and used traditional PCR with MarVer3 primers and Sanger sequencing to process our samples. Weddell seal DNA was successfully extracted, amplified and sequenced from seven out of our thirty-three snow samples. Due to limitations in our sample size, we were unable to differentiate between the efficacy of the three extraction kits; however, all three kits contributed to our seven successful sequences. We attribute our low rate of successful amplification to storage length, sample size and filtration methods. A BLASTN search assigned all seven samples to Weddell seal mitochondrial eDNA, though our Neighbour-Joining phylogenetic tree displayed a high level of divergence between our eDNA samples and the 16s rRNA

Weddell seal sequences obtained through GenBank. The variance between our eDNA samples and pre-existing sequences may be due to our short sequence length (~245bp) and a lack of identifiable regions. Despite these challenges, we were able to demonstrate the successful extraction, amplification and sequencing of eDNA from Antarctic snow collected by non-technical volunteers. We discuss the potential for future applications of eDNA within the field of population genetics and highlight the importance of developing robust eDNA protocols.

3.1 Introduction

While Antarctic populations regularly experience fluctuations, long term monitoring is required to differentiate between a normal level of flux and a long term pattern of change (Pande et al. 2017; Piazza et al. 2020). For example, Watters et al. (2020) used long-term observations to suggest that precautionary quotas of krill based on standing biomass failed to reflect predator-prey interactions and could lead to potentially detrimental mismanagement. Differentiating between natural variation and the consequences of disturbance is one of the key objectives of the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) Ecosystem Monitoring Program (CEMP; Croxall and Nicol 2004; Kock et al. 2007). Long term monitoring protocols are critical for establishing baselines in the assessment of climate-mediated ecosystem change and human-induced disturbance (McMahon et al. 2005; Tin et al. 2009; Sydeman et al. 2015; Pande et al. 2017). However, the logistical challenges of the harsh Antarctic environment can bias studies of Antarctic taxa both spatially and temporally, as well as by species, sex and life-history stage (Griffiths 2010; Bestley et al. 2020). For example, mature female seals are more frequently monitored due to extended haul-out periods and because they are typically easier to restrain (Bestley et al. 2020). As technology improves, these logistical challenges are being increasingly met with

remote solutions, e.g. satellite census methods (LaRue et al. 2011, 2019, 2020; Wege et al. 2020). In their review on the current monitoring of Antarctic birds and mammals, Bestley et al. (2020) discuss the value of remote tools in upgrading Antarctic monitoring programmes and highlights the need for creative solutions in a changing environment.

One such tool is environmental DNA (eDNA), this being DNA that is detected in an environmental sample, such as water, snow or soil, that has been shed by organisms through processes such as defecating, moulting or injury (Ogram et al. 1987; Taberlet et al. 2012). The growing interest in the use of eDNA to study cryptic, endangered, and invasive species has been attributed in part to its non-invasive methods (Jiang & Yang, 2017). It is increasingly considered best practice to reduce the need for invasive sampling where possible (Long et al. 2012; Barber-Meyer et al. 2020). While eDNA surveys have primarily been used in freshwater systems (Ficetola et al. 2008; Thomsen et al. 2012; Garlapati et al. 2019), marine applications are increasing (Lafferty et al. 2018; Baker et al. 2018; Garlapati et al. 2019), and there is a growing interest in underutilized sample types such as salt licks (Ishige et al. 2017) and "spray aggregations" from plant surfaces (Valentin et al. 2020). One such sample is snow. The use of snow as a source for eDNA has two primary benefits; DNA degradation is reduced in cold conditions (Smith et al. 2003; Dalén et al. 2007), and the dispersal and dilution of eDNA are limited compared to an aquatic environment (Franklin et al. 2017). While reduced dispersal means that isolating eDNA in randomly collected snow samples is challenging (Hansen and Willerslev 2002), recent studies in the Northern hemisphere have successfully targeted footprints as pools of DNA (Dalén et al. 2007; Franklin et al. 2017; Kinoshita et al. 2019; Barber-Meyer et al. 2020). Snow based eDNA studies require sampling sites with distinct, undisturbed tracks; urban areas with high footfall risk contamination, which can be difficult to exclude and may lead to false positives (Dalén et al. 2007). The benefit of trialling these methods in Antarctica is that the remote environment

and regulations regarding approaching wildlife (Antarctica New Zealand Environmental Code of Conduct; https://adam.antarcticanz.govt.nz/nodes/view/44849) reduce the risk of sampling a pre-contaminated site. Additionally, all Antarctica's ice-obligated mammals are marine and therefore require access to water, limiting travel across the ice and increasing the likelihood of high-quality eDNA deposits.

3.1.1 Weddell seals *(Leptonychotes weddellii)* as a study species

While eDNA presents an opportunity for detecting Antarctica's cryptic species, the development of eDNA methods is challenging when the chance of species detection is low. Weddell seals (*Leptonychotes weddellii*) are arguably the most extensively studied of the pinnipeds (LaRue et al. 2019), making them the ideal study species. The Erebus Bay, Ross Sea population of Weddell seals has been the target of an ongoing mark-recapture study that began in the 1960s (Stirling 1969; Siniff et al. 1977; Cameron and Siniff, 2004; Hadley et al. 2007; Garrott et al. 2012; Rotella et al. 2012, 2016). The extent of research on the Erebus Bay population is largely due to the proximity of the seals to Scott Base, home of New Zealand's national Antarctic programme, and McMurdo Station, a United States Antarctic research station. A number of breeding colonies occur on the local fast ice over the summer months, including a colony directly in front of Scott Base. The logistical benefits and extensive research history of the Erebus Bay Weddell seal colony provide an ideal backdrop for the development of novel genetic methodologies such as eDNA. Additionally, the annual tagging of pups, and consequent access to life-history data on tagged adults (Stirling 1969; Siniff et al. 1977; Cameron and Siniff, 2004; Hadley et al. 2007; Garrott et al. 2012; Rotella et al. 2016), is especially beneficial for exploring novel applications of eDNA surveys to population genetic studies.

Understanding the dynamics of populations is important for ecosystem monitoring (Fenwick, 1973; Zappes et al. 2017). Currently, Weddell seals are considered of least concern by the International Union for Conservation of Nature (IUCN). However, there is uncertainty in the population estimates and population trends, especially for Weddell seal populations outside of McMurdo Sound (LaRue et al. 2011; Hückstädt 2017). Of the Antarctic ice-breeding seals, Weddell seals uniquely meet several parameters linked to the development of population structure; breeding site fidelity, female socialization, monopolization of females by dominant males and regional variation in vocalizations (Stirling and Thomas 2003; Davis et al. 2008). Genetic differentiation is especially evident in the isolated White Island population of Weddell seals, whose offspring are increasingly affected by inbreeding depression (Stirling, 1968; Testa and Scotton 1999; Gelatt et al. 2010). Additionally, Davis et al. (2008) also observed a significant genetic difference between non-enclosed Weddell seal populations over 700km apart, a minimum distance comparable to the geographic variability of vocalizations (Thomas and Stirling 1983; Thomas et al. 1988; Abgrall et al. 2003; Davis et al. 2008). While microsatellites suggest a single Ross Sea population, a high level of haplotypic diversity has been observed between neighbouring colonies (Zappes et al. 2017). Understanding the status of multiple populations is important for monitoring Weddell seal ecology and assessing the impacts of environmental change (Siniff et al. 2008; LaRue et al. 2011). With further optimization, population genetic applications of eDNA surveys (Baker et al. 2018; Adams et al. 2019; Székely et al. 2021) could be utilized to increase sampling efforts for Antarctica's marine mammals and non-invasively supplement traditional sampling methods.

Monitoring Weddell seal populations may prove critical in a changing climate, as Weddell seals are sensitive to fluctuations in sea ice (Learmouth et al. 2006; Younger et al. 2016). For example, population declines correlating with a reduction in sea ice have been

observed in Anvers Island, off the rapidly warming Antarctic Peninsula (Siniff et al. 2008; Younger et al. 2016). Contrarily, while the extent and seasonal breakout of sea ice in the Ross Sea region is not experiencing the same loss of sea ice as the Antarctic Peninsula (Stammerjohn et al. 2012), a reduction in Weddell seal populations was indicated in northern Victoria Land and McMurdo Sound, using a combination of satellite imagery with historic ground counts (Ainley et al. 2015). The toothfish (*Dissostichus mawsoni*) industry has been proposed as a potential source of the population decline, with targeted ecosystem monitoring suggested to assess the disturbance of fisheries in the Ross Sea region (Ainley et al. 2015, 2020). Recent studies have investigated the potential of eDNA surveys as a future fisheries management tool (Lacoursière-Roussel et al. 2015; Thompsen et al. 2016; Baillie et al. 2019; Jerde 2019; Gilbey et al. 2021). Within this framework, Thomsen et al. (2016) highlighted the value of eDNA for monitoring deep water ocean habitats in polar ecosystems. In the only study to detect both Weddell seal and Antarctic toothfish eDNA, Mariani et al. (2019) used sponges as natural filters to metabarcoding local diversity, presenting an affordable tool for biodiversity monitoring. Environmental DNA surveys remain an underutilized tool for Antarctic vertebrates; as evidenced in our literature search (Chapter Two), which returned no examples of eDNA being used to monitor vertebrates in the Ross Sea, despite being the location of one of the world's largest Marine Protected Areas (MPA; CCAMLR, 2016). For a newly implemented MPA, such as the Ross Sea Region MPA, regular monitoring is critical for assessing the efficacy of management efforts (Nickols et al. 2019; Gold et al. 2021). Indeed, on the establishment of the MPA, the 11 objectives designated by CCAMLR included ii) the provision of 'reference areas for monitoring natural variability and long-term change' and iii) the promotion of 'research and other scientific activities (including monitoring) focused on marine living resources' (Conservation Measure 91-05 [2016]). Future implementation of eDNA in Antarctic monitoring programs could contribute to

valuable baseline estimates of biodiversity in the Ross Sea Region MPA. However, before eDNA surveys can be utilized for the Ross Sea Region MPA objectives, eDNA protocols first require optimization and standardization to ensure the production of sound datasets for drawing future comparisons (Dunn et al. 2017; Lear et al. 2018; Zinger et al. 2019).

3.1.2 Aims and objectives of this study

This study aims to develop the methodology for sampling, extracting and amplifying eDNA from non-invasive snow samples, using the Weddell seals of McMurdo sound as a target species. In order to maximize our chances of detecting eDNA, we target a single seal 'snow angel', a depression left behind in the snow by a seal, treating the depression as a potential pool of eDNA. We will use these samples to test each step in the eDNA pipeline, from collection to extraction, amplification and sequencing to analysis. The results of this study should allow us to pursue further applications of eDNA in Antarctica, such as testing the detection of microsatellites for population genetic inferences. We explore the potential of the remote and extreme Antarctic environment as an opportunity to develop and expand upon eDNA applications. Environmental DNA surveys present a promising tool for Antarctic ecologist's biological toolkit. In this chapter, we discuss the potential of the eDNA approach for future Antarctic science, as well as addressing the challenges and necessity of developing robust eDNA protocols.

3.2 Methods

3.2.1 Sampling collection

We sampled from the Weddell seal breeding colony at Turtle Rock, Ross Dependency, Antarctica (S77°44'42.3 E166°46'15.9) between 3:03 pm and 3:13 pm on the 30th November 2019. At the time of sampling, there was complete cloud coverage (8 oktas), a wind speed of 0-10 kn, a temperature of 0—5°C. Prior to sample collection, there had been light to moderate snowfall. In order to maximize the likelihood of detecting eDNA, necessary for our methods development, we targeted a single seal 'snow angel', a depression in the snow that had recently been vacated. The sample site was chosen based on the safety of access and in keeping with our ACA permit (PCAS 2019-2020). Samples were taken from an area approximately 2 m by 0.5 m to a depth of 3 cm using sterilized plastic scoops and 500 mL Nalgene bottles; our permit allowed for 20 bottles to be filled. To minimize contamination, samplers wore gloves and stood downwind of the sample site. Samples were initially stored in an icebox packed with snow and then transferred to the -20°C freezer facility at Scott Base for two weeks before being transported to New Zealand on ice and stored at -4°C. Due to delays in processing, samples were not defrosted until the 23rd January 2020. None of the samplers or equipment used at any stage had been in contact with Weddell seal DNA.

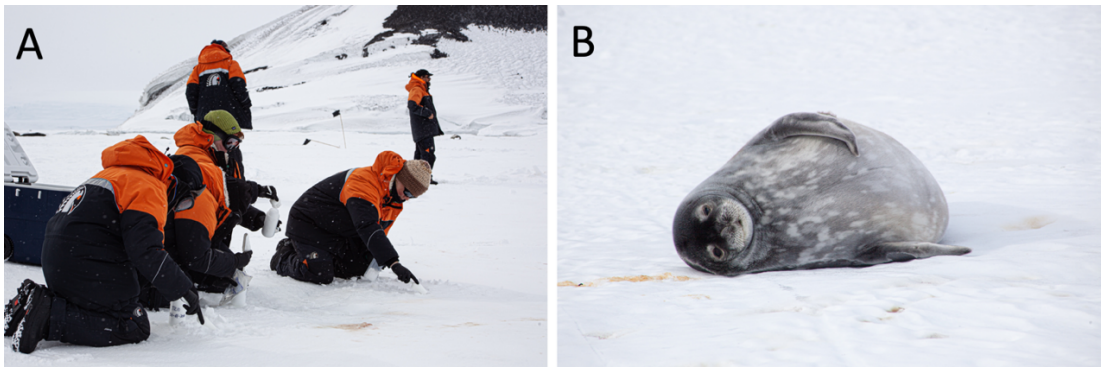

Figure 3.1 A) Sampling site at Turtle rock, Antarctica. Showing volunteers collecting snow samples. Flattened discoloured snow is a site recently vacated by a Weddell seal B)Weddell seal pup and example of seal 'snow angel', flattened discoloured snow representing a potential pool for eDNA. Photography by Bella Zeldis. Taken under Antarctic Conservation Act (ACA) permits (PCAS 2019)

3.2.2 Filtration and extraction

Snow samples were thawed at room temperature immediately prior to processing. Melted samples were shaken and then filtered using glass fibre filters (diameter 47 mm and pore size 1.5 µm) in 250 mL Nalgene funnels (Carim et al. 2016). For overnight thawing or filtering, snow samples were placed in the fridge at 3°C. Any liquid remaining after 12h was pulled through using a syringe fixed to the base of the funnel. After trialling two single snow samples and noting that 500 mL snow samples were reduced to approximately 200 mL liquid, we combined subsequent snow samples so that two bottles were used for each filter. We filtered MilliQ water as our negative control (C). We then quartered filters, flame-sterilizing scissors between each filter, storing the first quarter (S), and extracting eDNA from the remaining three using one of three extraction kits: Qiagen DNeasy Blood and Tissue kit (Q), Zymo Quick-DNA miniprep kit (Z) and Invitrogen Purelink Genomic DNA mini kit (V). For biosecurity purposes, filtered water was sterilized for 24 h using bleach to a ratio of 0.3:1 bleach to water before being diluted to 1 L and drained in the sink.

DNA extractions were carried out according to each kit's respective protocol; any changes to the protocol are summarised in Table 3.1. In cases where there were excessive hair and debris still present in the solution, 10 µL of Proteinase K was added and the solution left to incubate for an extra 24 h. The lysis solution was vortexed then filters were removed with flame-sterilized forceps after the binding step. Any hairs present on the filters were removed and stored at -4 °C. Post-extraction, the yield of DNA was quantified by processing 1.5 µL of each solution using a NanoDrop ND-1000 Spectrophotometer (3.8.1, Thermo Scientific) set to nucleic acids, and 2 µL of each sample using a Qubit Fluorometer (Invitrogen) set to High Sensitivity (HS) with HS reagents. Finally, we ran an Agarose Gel Electrophoresis in a Sodium Borate solution using Invitrogen Sybr-Safe stain. We mixed 2.5 µL of the sample (or control) with 2.5 µL dye and compared these to an Easy Ladder. The gel

ran at 90-100 V for 45 minutes. We photographed the gel using a Syngene G:Box (Synoptics 2.0MP camera) with the GeneSys software (Version 1.0.9.0, Database version 1.47).

Table 3.1 Summary of changes made to the protocols for each of the three extraction kits used in this study, Qiagen, Invitrogen and Zymo. Including the expanded incubation time and reduced elution volume utilized. Primary changes are elution volume in microlitres (µL), incubation time in hours (h), vertexing and spinning in seconds (s), and the use of a shaking heat block set to 750 revolutions per minute (rpm).

Extraction kit	Elution volume (µL)	Incubation time (h)	Changes to protocol
Qiagen *DNeasy Blood and Tissue kit* ***(Q)***	100	48	To ensure the filter was completely covered, we doubled Buffer ATL (260 µL) and Proteinase K (40 µL). Prior to lysis vortex 5s, spin 5s. Incubated on a shaking heat block set to 5 minute intervals at 750rpm.
Invitrogen *Purelink Genomic DNA mini kit* ***(V)***	100	48	To ensure the filter was covered, we doubled the genomic buffer (260 µL) and Proteinase K (40 µL). Prior to lysis vortex 5s, spin 5s. Incubated on a shaking heat block set to 5 minute intervals at 750rpm.
Zymo Quick *DNA miniprep Kit* ***(Z)***	50	48	To ensure the filter was completely covered, we doubled MilliQ H_2O (190 µL) solid tissue buffer (190 µL) and Proteinase K (20 µL). Prior to lysis vortex 5s, spin 5s. Incubated on a shaking heat block set to 5 minute intervals at 750rpm.

3.2.3 PCR conditions and development

We used the Biotaq PCR kit (Bioline) for all our PCR reactions. 5 µL of DNA was used for each reaction. For each 20 µL reaction, the master mix concentrations were; 2 µL buffer, 2 µL dNTP, 8 µL MilliQ H_2O, 0.6 µL $MgCl_2$, 0.4 µL TAQ and 2 µL primers (combined forward and reverse). Each reaction included a MilliQ H_2O negative control and both a λ DNA and pipefish (*Stigmatopora nigra*) DNA positive control, while the negative control used the same primers as the samples being run (Table. 3.2) the positive control used λ specific primers. Thermal cycling conditions followed the protocols by Valsecchi et al. (2020) for MarVer3 (initial denaturation step at 94°C or 4 minutes, then 38 cycles of 30 s at 95°C, 30 s at annealing temperatures 54/55/56/57°C for 8/10/10/10 cycles respectively,

followed by 40 s at 72°C and a final extension of 5 minutes at 72°C) and Taberlet et al. (2018) for Tele02 (initial denaturation step at 95°C for 30 s, then 40 cycles of 30 s at 95°C, 60 s at 54°C and 30s at 72°C, with a final extension of 5 minutes 72°C). However, due to limited samples, after testing Tele02 on samples that had sucessfully amplified with MarVer3 primers with no result, consequent trials were done in Chapter Four. Post PCR, all samples were checked for successful amplification by running a 1.4 % Agarose Gel Electrophoresis in a Sodium Borate solution with Sybr-Safe stain (Invitrogen) at 90V for 45 min. All samples that displayed bands on the gel were then cleaned in preparation for sequencing using the DNA Clean & Concentrator-5 (Zymo Research) following the manufacturer's instructions, with a Binding buffer:sample ratio of 5:1 at 100 µL buffer to 20 µL sample and an elution volume of 25 µL. The DNA in our cleaned samples was quantified using the Qubit fluorometer before being submitted for Sanger sequencing to the Canterbury Sequencing and Genotyping Facility (University of Canterbury, Te Whare Wānanga o Waitaha).

Table 3.2 Published primers tested in this study using the Biotaq PCR kit (bioline), showing both the forward (F) and reverse (R) sequence length in base pairs (bp), the amplicon length (the product of amplification) in BP and the source literature.

Name	Sequence (5'-3')	Length (BP)	Region	Annealing temp (°C)	Amplicon size (BP)	Reference
MarVer3	F: AGACGAGAAGACCCTRTG R: GGATTGCGCTGTTATCCC	18	16S	54-57	232-274	*Valsecchi et al. 2020*
Tele02	F: AAACTCGTGCCAGCCACC R: GGGTATCTAATCCCAGTTTG	F: 18 R: 20	12S	54	129-209	*Taberlet et al. 2012*

3.2.4 Phylogenetic analysis

The eDNA sequences were queried against the National Centre for Biotechnology Information (NCBI) nucleotide database using the Basic Local Alignment Tool (BLASTN 2.11.0+, Altschul et al. 1990) Megablast algorithm (Zhang et al. 2000; Morgulis et al. 2008) to confirm taxonomy. We downloaded the FASTA files for the 16s rRNA gene of taxa that returned with a percentage identity above 90 % (percentage identity being the percentage match of bases between our query and the database sequence when aligned), and an E-value greater than e-70 (E-value, or Expect value describing the number of hits between our query and the database sequence alignment that are expected by chance, a low value reflects biological similarity). Additional seal sequences were obtained through NCBI GenBank (Clark et al. 2016; www.ncbi.nlm.nih.gov/genbank/), including the outgroup, the Antarctic fur seal (*Arctocephalus forsteri,* Assession number: BK010918.1). All eDNA and GenBank sequences were uploaded into Geneious Prime v.2021.0.3 (Biomatters Ltd., New Zealand; Kearse et al. 2012; https://www.geneious.com). To form the tree, our eDNA samples and downloaded sequences were aligned using the Geneious alignment algorithm (Biomatters Ltd) set to default settings with 70 % similarity. Alignments were visually checked before a neighbour-joining tree was generated, assuming a Hasegawa–Kishono–Yano (HKY; Hasegawa et al. 1985) model of substitution. The Antarctic Fur seal (*Arctocephalus forsteri,* Assession number: BK010918.1) was selected as an outgroup, and a Resample tree selected, with bootstrapping replicates set to 1000. The phylogenetic tree was then uploaded to the Interactive Tree of Life (iTOL, v.4; Letunic and Bork 2019) for editing.

3.3 Results

We successfully extracted, amplified and sequenced eDNA from seven out of thirty-three samples. Our success rate was low, despite Qubit analysis indicating eDNA in the majority of our extractions (Table 3.3). The initial concentration of our successfully amplified sequences ranged from 1.43 Ng/µl to 22.6 Ng/µl, though there were extractions of a higher concentration that were not amplified with the MarVer3 primers (TR01+01Z, 28.5 Ng/µl; TR03+04Z, 37.7 Ng/µl). Successful PCR reactions and sequencing spanned all three extraction kits; however, our sample size is too small to conclusively preference one over another. None of the samples tested with the Tele02 primers showed sign of amplification, even in cases where samples that had been amplified previously using MarVer3 primers. The PCR product sequences ranged from 243 to 251 bp. All successful amplifications displayed a band in the 250 bp region. Assessment with the BLASTN tool indicated the sequences were Weddell seal DNA. BLASTN queries for all seven sequences returned the closest alignment to *Leptonychotes weddellii* full mitochondrial genomes *(*Accession number: MT755639.1 and AM181025.1) and had E-values below 4e-72 and query cover of above 85 %. Likewise. All samples have a percentage identity above 90%, except for TR13+14Q which was 89.22%. TR13+14Q also scored the lowest in E-value (4e-72) and in Maximum and Total score, both 283 (Table 3.4).

All of our eDNA snow samples fall clearly within the Weddell seal clade in our Neighbour-Joining tree, with an associated bootstrap value of 99.6 (Fig. 3.3). With the exception of TR15Z (99.6) and TR10+11Q (94.9), there is a lot of uncertainty within the eDNA cluster. TR03+04Q, TR10+11V, TR18+17Z, TR08Z and TR13+14Q are all associated with a bootstrap value of 19.9, and all bootstrap values within the cluster are below 64.1. While the bootstrap cut-offs used in phylogenies vary, a common cut-off is 70% (Hahn 2007) which would see these branches collapsed (Fig. 3.3). While our eDNA samples sit within the

Weddell sea clade, they show a much longer branch length than those associated with our downloaded sequences. In addition to showing the weakest alignment with the matched Weddel seal sequences in our BLASTN query, TR13+14Q has the greatest distance from our outgroup (*Arctocephalus gazella)* at 0.26 substitutions per sequence site, compared to 0.18 for TR15Z, the shortest branch within the cluster. Comparatively, distances between the outgroup and the three GenBank Weddell seal samples sit at 0.11-0.12 substitutions per site.

Table 3.3. Qubit fluorometer results in nanograms per microlitre (Ng/µl) for Weddell seal eDNA from snow samples. Including Qubit results post PCR for samples that were successfully amplified. Results have been adjusted to account for differences in elution volume. Grey boxes indicate successfully amplified PCR products.

Sample	**Invitrogen (Ng/µl)**		**Qiagen (Ng/µl)**		**Zymo (Ng/µl)**	
	Pre-PCR	***Post PCR***	***Pre-PCR***	***Post PCR***	***Pre-PCR***	***Post PCR***
TR01+02	11.8	-	6.02	-	28.5	-
TR03+04	6.72	-	***6.10***	***11.3***	37.7	-
TR05+06	1.83	-	2.54	-	2.43	-
TR07+09	4.16	-	3.72	-	27.3	-
TR10+11	***5.70***	***11.2***	***5.70***	***18.6***	11.7	-
TR12+16	0.43	-	*0.61*	-	1.67	-
TR13+14	2.42	-	***1.43***	***8.11***	<0.01	-
TR17+18	6.22	-	5.34	-	***18.01***	***20.5***
TR19+20	4.92	-	6.28	-	9.64	-
TR15	0.96	-	2.42	-	***2.50***	***17.4***
TR08	1.31	-	1.62	-	***2.86***	***22.6***

Table 3.4. Results of a BLASTN query showing identities of the closest alignments of the eDNA samples that were successfully amplified and sequenced. The letter associated with each sample name indicates the extraction kit used, either Qiagen DNeasy (Q), Zymo Quick-DNA (Z) or Invitrogen Purelink (V). Table shows percentage identification, Expect value (E-value), Query cover (%), the highest alignment score (Max score), the sum of alignment scores (Total score) and Query length in base pairs (Bp).

Sample	Species Match	Accession number	Percentage ID (%)	E-Value	Query Cover (%)	Max score	Total score	Query length (BP)
TR03+04-Q	*Leptonychotes weddellii*	MT755639.1/ AM181025.1	97.25	3e-97	88	366	366	243
Tr08-Z	*Leptonychotes weddellii*	MT755639.1/ AM181025.1	94.47	4e-86	87	329	329	245
TR10+11Q	*Leptonychotes weddellii*	MT755639.1/ AM181025.1	92.52	1e-76	85	298	298	249
TR10+11V	*Leptonychotes weddellii*	MT755639.1/ AM181025.1	95.89	1e-92	86	351	351	251
TR13+14Q	*Leptonychotes weddellii*	MT755639.1/ AM181025.1	89.22	4e-72	92	283	283	249
Tr15-Z	*Leptonychotes weddellii*	MT755639.1/ AM181025.1	95.13	9e-93	91	351	351	243
TR17+18-Z	*Leptonychotes weddellii*	MT755639.1/ AM181025.1	94.88	1e-86	86	331	331	247

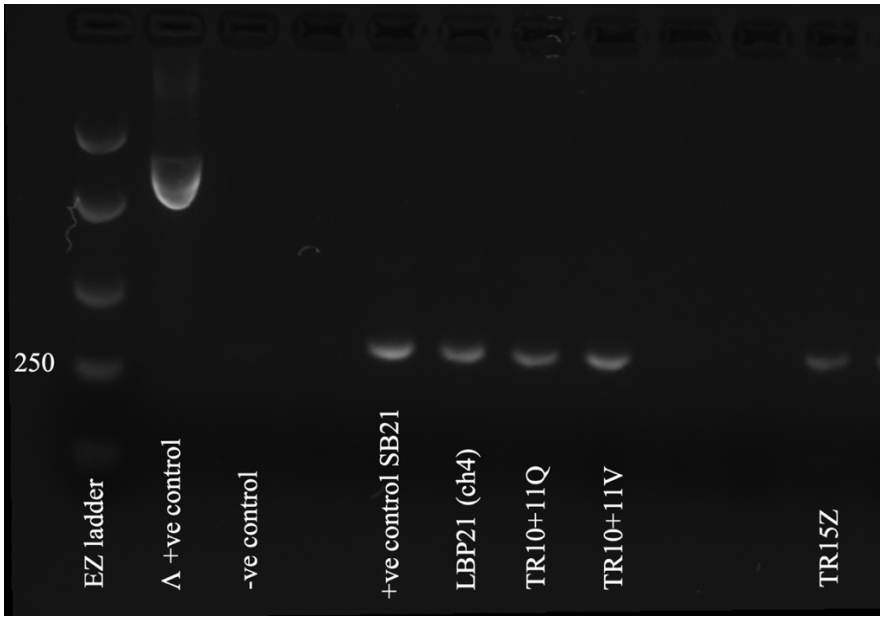

Figure 3.2. Representative gel electrophoresis image showing an example of all three extraction kits successfully amplifying eDNA from snow: TR10+11Q (Qiagen), TR10+11V (Invitrogen) and TR15Z (Zymo). Sequences were amplified by PCR using MarVer3 primers. All bands appeared in the 250 bp region as expected. Positive controls are lambda DNA (λ +ve control), and pipefish DNA (+ve control SB21) and negative control (-ve control) is MilliQ water. LBP21 is a *Eudyptula minor* eDNA sample (Chapter Four).

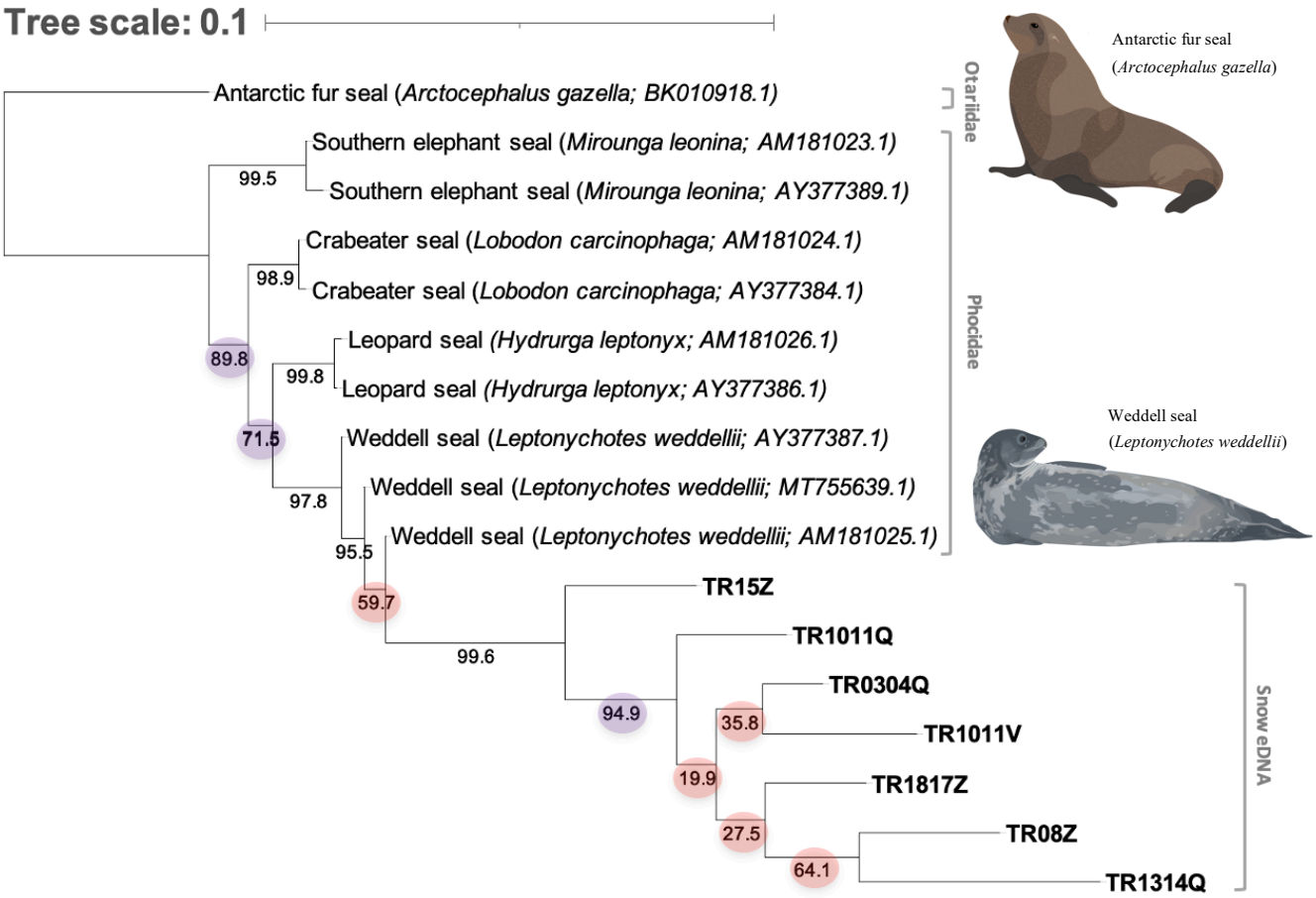

Figure 3.3 Neighbour-joining Phylogenetic tree comparing the 16s fragments for our snow eDNA sequences extractions to the 16s sequence for a number of pinnipeds, showing our eDNA samples sit within the Weddell sea clade. Arctocephalus gazella (Otariidae) is included as an outgroup. Bootstrap values are based on 1000 resamplings. Red circles represent bootstrap values <70, while purple is 70<n<95. Branch length scale=0.1 substitutions per sequence site.

3.4 Discussion

To the best of our knowledge, this pilot study represents the first extraction of Antarctic vertebrate DNA from the snow. While our success in extracting and amplifying eDNA from snow samples was minimal, with seven sequences resulting from thirty-three samples, all seven of those sequences returned matches to Weddell seals through our BLASTN query (Table 3.4). Likewise, our Neighbour-Joining tree places all of our sequences within the Weddell seal clade (Fig. 3.2). However, the branch lengths for our eDNA cluster suggest our samples display a larger divergence than we expected. This is likely due to sequence degradation and low coverage as a result of fragmentation (Sproul and Maddison 2017; Jensen et al. 2020). To consider future applications in population genetics, these issues would need to be explored and, where possible, resolved to improve sequence resolution (Adams et al. 2019; Sigsgaard et al. 2019; Jensen et al. 2020).

3.4.1. Challenges and limitations

The characteristics and ecology of eDNA have likely slowed progress in the field (Taberlet et al. 2018). Critically, the degradation and low yield of eDNA pose a challenge for analysis. While our amplified sequences were identified as Weddell seal DNA, the branch lengths associated with our samples (fig 3.2) pose an issue for future applications. The long branches suggest a relatively high level of divergence between our samples and the GenBank Weddell seal sequences (fig 3.2). Given we collected snow from a single seal imprint, this divergence is likely due to low sequence quality and poor alignment (Wu et al. 2012). This is concerning for future applications of eDNA to population genetics. Long branches separating similar samples could lead to populations being incorrectly interpreted as highly divergent (Schwartz and Muller 2010; Molina-Venegas and Rodríguez 2017). As a result of this, phylogenetic histories could be misconstrued (Richie et al. 2021). Though we are confident in

the identity of our samples, inaccurate inferences of divergence may lead to erroneous species delineation in studies investigating multiple taxa (Reid and Carstens 2012). Recognizing branch lengths is critical for assessing ecological communities, especially those where the distribution of closely related and distantly related species is uneven (Weiblen et al. 2006).

Potential biases in estimates of genetic diversity may arise from PCR and sequencing errors (Smith et al. 2014; Adams et al. 2019; Sigsgaard et al. 2019). Amplifying and sequencing degraded samples can lead to false alleles or a loss of allelic variation, which can in turn bias estimates of genetic diversity (Smith et al. 2014; Adams et al. 2019). Such genotyping errors have been shown to correlate with the amount of DNA in a sample and thus are of particular concern in non-invasive genetic samples (McKelvey et al. 2010). Random errors during amplification or sequencing can lead to a sequence incorrectly being defined as a new individual, and therefore, genotyping errors have been flagged as a concern for genetic population estimates (Taberlet et al. 1999; Morin et al. 2001; McKinley et al. 2010). For example, Creel et al. (2003) sampled wolf (*Canis lupus*) faeces in the United States and found that the incorrect identification of sequences as individuals can lead to a 5.5-fold increase in population estimates. Given the risk of dilute, degraded samples leading to inaccurate conclusions, it is important we consider, quantify and reduce error rates when optimizing new protocols (Taberlet et al. 1999; Morin et al. 2001; McKinley et al. 2010; Adams et al. 2019; Sigsgaard et al. 2019), for example, increasing sampling efforts, using positive and negative controls, implementing strict contamination procedures (such as those in a dedicated clean laboratory) and increased replication (Taberlet et al. 1999; Morin et al. 2001; Creel et al. 2003; Broquet et al. 2006; McKinley et al. 2010; Ficetola et al. 2015; Goldberg et al. 2016; Adams et al. 2019; Sigsgaard et al. 2019). We suggest evaluating existing protocols for the analysis of highly degraded samples within the fields of ancient DNA and forensic analysis, which could be adapted for the analysis of eDNA (Handt et al.

1994; Gilbert et al. 2005; Fondevilla et al. 2008; Alaeddini et al. 2010; Lan and Lindqvist 2018; Adams et al. 2019). Additionally, eDNA studies primarily target mitochondrial DNA due to its high copy number within environmental samples. However, the evolutionary rate for mitochondrial DNA is far greater than nuclear DNA, and the inheritance of mitochondrial DNA down the maternal line can exclude critical non-maternal genetic variation, potentially impacting phylogenies and lending to biases in branch length (Rubinoff et al. 2006; Grealey et al. 2017; Adams et al. 2019). This can lead to variations in the observed divergence between nuclear eDNA derived phylogenies compared to phylogenies derived from mitochondrial DNA (Rubinoff et al. 2006; Carranza et al. 2016; Pratlong et al. 2016; Adams et al. 2019; Sigsgaard et al. 2019). This highlights the value in developing protocols for the extraction of nuclear eDNA from samples such as snow (Hellström et al. 2019; Von Duyek et al. 2019; Barber-Meyer et al. 2020).

It is possible that the branch lengths of our sequences are indicative of bioinformatic decisions rather than the sequences themselves (Hahn 2007). In a phylogenetic analysis, model choice can impact the inferred divergence of aligned sequences (Phillips 2009). While Neighbour-Joining trees have seen multiple applications in eDNA studies to construct quick trees (Agersnap et al. 2017; Kundu et al. 2019; Suter et al. 2020), generally, Maximum Likelihood analysis is considered more accurate, despite not always being favoured due to a higher computational cost (Hahn 2007; Stark et al. 2010.). In their study aiming to identify a cryptic fox species from tracks in the snow, Dalén et al. (2007) first generated a Neighbour-Joining starter tree to estimate the substitution parameters for further Maximum Likelihood based analysis. For future optimization of this protocol, it would be beneficial to investigate more sophisticated phylogenetic methods, such as Maximum Likelihood and Bayesian algorithms, especially for complex analyses such as population inferences.

The divergence seen in our sequences may be a cause of degraded samples. Once shed into the environment, DNA undergoes degradation through a number of biotic and abiotic processes (Barnes and Turner 2016; Taberlet et al. 2018; Stewart et al. 2019). While cold temperatures have been shown to reduce eDNA degradation, the twenty-four hour polar sun may seasonally increase the rate of eDNA degradation in Antarctica (Strickler et al. 2015; Cowart et al. 2018). Kinoshita et al. (2019) identified both UV and freeze-thaw cycles as potential sources of degradation in their snow-sourced eDNA samples. Additionally, Kinoshita et al. (2019) observed the failure of samples after a five day storage period (freezing <0°C) far shorter preservation than our two months. We recommend limiting storage length to minimize the risk of DNA degradation. However, this recommendation may be challenging for Antarctic eDNA applications as samples collected in proximity to Scott Base need to be held until they can be transported back to New Zealand, and delays on the ice can be frequent. This could potentially be addressed by transferring laboratory steps into the field. For example, samples could be filtered using enclosed filter units such as Sterivex units (Merck Millipore; Sigsgaard et al. 2017; Kinoshita et al. 2019; Tingley et al. 2019; Takahashi et al. 2020; Székely et al. 2021). Preservation could be further optimized with the use of ethanol or Longmire's buffer (Spens et al. 2017). Additionally, enclosed filter units would reduce contamination risk, filtering time and necessary storage capacity (Tingley et al. 2019; Takahashi et al. 2020). DNA degradation during storage could be further mitigated through the development of on-ice sequencing protocols, using portable sequencing technology such as the MinIon Nanopore sequencer (Oxford Nanopore Technologies; Johnson et al. 2017; Millán-Aguiñaga et al. 2019).

The nature of Antarctic fieldwork placed restrictions on our sample design. Samples were collected under a permit that allowed for twenty 500 mL bottles of snow, which equated to approximately half the volume of water. The general consensus for aquatic eDNA samples

suggests that more filtrate leads to a greater chance of eDNA capture (Dalén et al. 2007; Mächler et al. 2016; McColl-Gausden 2020). The same appears to be true in the minimal literature that exists on snow. Dalén et al. 2007 had limited success in detecting eDNA from a single footprint in the snow, while Franklin et al. (2017) and Kinoshita et al. (2019) had greater success with samples of two litres and five footprints, respectively. Barber-Meyer et al. (2020) were able to make species-level inferences from ten snow tracks but proposed trials of twenty to fifty tracks to assess the minimum volume required for nuclear eDNA extraction. However, while it is likely the volume of our filtered snow impacted our success rate, we did successfully extract, amplify and sequence eDNA from two samples that used only a single bottle of snow, whereas a number of our extractions from combined bottles did not successfully amplify. Importantly, our limited sample size also meant we were unable to collect field blanks (Franklin et al. 2017, Kinoshita et al. 2019). We cannot confidently preference one extraction kit, however, we had successful sequences derived from all three kits. Further analysis is necessary to draw comparisons, however, it should be noted that all prior eDNA extractions from snow used the Qiagen DNeasy Blood and Tissue Kit (Dalén et al. 2007, Franklin et al. 2019, Hellström et al. 2019; Kinoshita et al. 2019; Von Duyek et al. 2019; Barber-Meyer et al. 2020). The subjectivity of eDNA survey design, and poor reporting of critical methodology, can inhibit reliable comparisons between datasets (Lear et al. 2018; Dickie et al. 2018). Thus standardization should be considered when developing a eDNA protocol (Dickie et al. 2018).

The variety of steps that can be taken to minimize DNA loss highlights the importance of communicating methodological challenges as well as successes; continued optimization and standardization of eDNA protocols across the field can lead to faster resolutions (Goldberg et al. 2016; Spens et al. 2016; Lear et al. 2018; Tsuji et al. 2019; McColl-Gausden et al. 2020). Our methodological choices impact the outcome of our eDNA studies and investing time in

the development of sound protocols is critical for robust conclusions (Goldberg et al. 2016; Lear et al. 2018; Wilcox et al. 2018).

3.4.2 Population genetics

Environmental DNA applications have expanded considerably over the past decade (Cristescu and Herbert 2018; Beng and Corlett 2020). While the method is typically used to detect the presence of cryptic, rare, or invasive taxa (Ficetola et al. 2008; Goldberg et al. 2011; Díaz-Fergeson & Moyer, 2014; Bass et al. 2015; Uchii et al. 2015), the field is currently growing in the direction of population genetics (Adams et al. 2019; Sigsgaard et al. 2019). Mitochondrial haplotypes have been applied to a number of species, from bivalves to cetaceans (Sigsgaard et al. 2016; Baker et al. 2018; Parsons et al. 2018; Marshall and Stepien 2019; Stepien et al. 2019; Pinfield et al. 2019; Tsuji et al. 2020), while targeting multi-allelic microsatellites can help differentiate between individuals (Adams et al. 2019, Monge et al. 2018; Sisgaard et al. 2020). Recent advances have seen single-nucleotide polymorphisms (SNPs) utilized to distinguish between genetic signatures (Aylward et al. 2018; Marshall and Stapien 2019) and investigate the potential for sex determination from eDNA (Nichols and Spong, 2017). Environmental DNA surveys primarily rely on mitochondrial DNA due to its abundance in the cell and, consequently, in eDNA samples (Goldberg et al. 2016; Tsuji et al. 2019). However, improvements in the sensitivity of assays in targeting nuclear eDNA could allow us to make more detailed population-level inferences about taxa of conservation and management interest (Hellström et al. 2019; Pierszalowski et al. 2013; Von Duyek et al. 2019).

The use of eDNA to make population genetic inferences, while promising, is not as robust as traditional sampling methods and continues to face a number of challenges (see reviews by Adams et al. 2019; Sigsgaard et al. 2019). However, incorporating non-invasive

sampling can support increased data collection without needing to weigh up animal welfare concerns (Bearzi 2000; Romero and Reed 2005; Coetzee and Chown 2015; Steinmetz et al. 2021). Additionally, eDNA does not require the sighting, or capture, of the target taxa which may prove critical in studies of endangered, cryptic or dangerous species (Mondol et al. 2009; Janečka et al. 2011; Sharma et al. 2011; Hellström et al. 2019; Von Duyek et al. 2019). Indeed, these advantages have been utilized for preliminary studies on Polar bears *(Ursus maritimus;* Von Duyek et al. 2019), lynx (*lynx lynx;* Hellström et al. 2019*)* and wolves (*Canis lupus;* Barber-Meyer et al. 2020). While success in this area is limited, early work suggests it is possible to extract nuclear eDNA from snow samples (Hellström et al. 2019; Von Duyek et al. 2019). Further optimization and validation of nuclear eDNA protocols are required before the tool can be utilized for population genetic inferences (Hellström et al. 2019; Von Duyek et al. 2019; Barber-Meyer et al. 2020). The benefit of trialling population genetic studies with Weddell seals is in their behaviour; Weddell seals travel on their stomachs and remain close to ice holes, increasing the chances of collecting a high-quality eDNA sample. In their study addressing population genetics in wolves, Barber-Meyer et al. (2020) yielded the most eDNA from samples containing urine and suggested blood stains as another potential source of nuclear eDNA. The snow at Weddell seal breeding colonies is often stained (Fig 3.1), and it may be useful for targeting potential pools of high-quality nuclear eDNA. Despite this, our study returned highly degraded sequences, requiring further optimization of our protocol and investigation into degradation factors such as UV (Strickler et al. 2015; Kinoshita et al. 2019).

3.4.3 Monitoring and management applications

Antarctica's top predators are all dependent on the marine ecosystem, and consequently, shifts in their reproductive success can be indicative of a decline in ecosystem health (Pande et al. 2017; Hazen et al. 2019; Bestley et al. 2020). However, long-term data is required in order to differentiate between natural fluctuations in populations and a pattern of decline (Magurran et al. 2010; Pande et al. 2017; Bestley et al. 2020 Piazza et al. 2020). In a recent review on birds and mammals for the first Marine Ecosystem Assessment for the Southern Ocean (MEASO), Bestley et al. (2020) attributed an increasing understanding of the marine ecosystem in part to an increase in novel and more economical survey methods and technologies. However, current data on Antarctic vertebrates remains bias by accessibility, life-history stage, sex and seasonality (Griffiths et al. 2011; Bestley et al. 2020). For example, despite being highlighted as a potential indicator species for krill fisheries, the crabeater seal (*Lobodon carcinophagus*) is not considered a useful indicator by the CCAMLR Ecosystem Monitoring Program (CEMP) as they are pack-ice obligates and therefore difficult to target for repeated monitoring (https://www.ccamlr.org/en/science/ccamlr-ecosystem-monitoring-program-cemp; Wege et al. 2020). Genetic studies of crabeater seals have typically been carried out using samples from historic sealing, skin biopsy darts or direct handling (Lehman et al. 2004; Davis et al. 2008; Curtis et al. 2007, 2009, 2011; Lehnert et al. 2017). In particular, invasive chemical restraint can come with a number of risks to pinnipeds, including mortalities (Baylis et al. 2015). Additionally, direct handling requires accessing a seal before it can retreat into the water. While sampling snow for eDNA analysis of pack-ice seals would still require the logistical challenges of boat access, genetic sampling efforts could be increased by targeting recently vacated sites, allowing for the sampling of seals disturbed prior to capture.

The Ross Sea region is important for both pinniped and cetacean populations and thus has been declared an Important Marine Mammal Area (IMMA) by the IUCN Marine Mammal Protected Areas Task Force (http://www.marinemammalhabitat.org/; Lauriano et al. 2020). Comparable to other Southern Ocean regions, the trophic interactions in the Ross Sea are distinctly complex due to the shelf's unique geographical features and the resulting prominence of notothenioid fishes prominent as both predator and prey within the food web (Smith et al. 2017; Bestley et al. 2020; McCormack et al. 2021). In 2016 the Ross Sea Region Marine Protected Area was established by CCAMLR to protect an area deemed one of the worlds least anthropogenically altered marine systems (Halpern et al. 2008; Ballard et al. 2012). In order to achieve the best possible estimation of the current state of the system, MPA monitoring should utilize a variety of survey tools (Bean et al. 2017; Freiwald et al. 2018; Gold et al. 2021). Environmental DNA has been suggested as a novel monitoring tool for both established and proposed MPAs (Aizu et al. 2017; Cottier-Cook et al. 2019; Turon et al. 2020; Gold et al. 2021). The use of eDNA sampling alongside traditional methods such as scuba surveys (Lindfield 2014; Gold et al. 2021) provides a complementary approach for the construction of baseline estimates of biodiversity that can be used to overlay gaps and address biases in traditional monitoring data (Bani et al. 2020; Gold et al. 2021). For example, complex seabed structure provides a challenge for traditional trawl surveys, whereas eDNA surveys are not impacted by obstacles in the environment (Thompsen et al. 2012, 2016; Lacoursière-Roussel et al. 2018; Closek et al. 2019). Additionally, traditional methods often rely on the taxonomic expertise of those undertaking sampling; the molecular identification of eDNA is comparatively less subjective, and samples can be collected with minimal training (Thompson et al. 2012; Hansen et al. 2018), as was the case in our pilot study with all our snow samples collected by non-technical volunteers. The simplicity of eDNA

sampling potentially allows for the outsourcing of data collection across a greater spatial and temporal field (Chapter 2.6.2).

A draft Research and Monitoring Plan for the Ross Sea Region MPA highlights the necessity for the inclusion of monitoring directly outside of the MPA, including studies from fishery vessels (CCAMLR, 2017). The inclusion of eDNA in fisheries monitoring could assist with non-lethal stock assessments (Hansen et al. 2018; Jerde 2019). As with novel population genetic applications of eDNA (3.4.2), there has been progress in the use of eDNA for estimates of species abundance (Pilliod et al. 2013; Doi et al. 2015; Knudsen et al. 2019; Lacoursière-Roussel et al. 2015, 2016; Stoeckle et al. 2017; Tillotson et al. 2018; Lawson Handley et al. 2019; Spear et al. 2020; Yates et al. 2020). Spikes in eDNA concentration have been linked to spawning events (Bylemans et al. 2016; Tillotson et al. 2018; Antognazza et al. 2019; Bayer et al. 2019; Hayer et al. 2020; Tsuji et al. 2020) and migrations (Uchii et al. 2017; Wu et al. 2019; Thalinger et al. 2019; Halvorsen et al. 2020). While these studies indicate a correlation between eDNA concentration and species biomass, the complex interaction of biotic and abiotic factors, e.g. organismal eDNA production, degradation and dispersal, impact the consistency and reliability of these estimates, and the methods require further optimization (Hinlo et al. 2018; Jerde 2019). Both Klymus et al. (2015) and Iversen et al. (2015) stress the need for cautious conclusions as this field develops. Current quantitative estimates are limited by the reliance on a number of assumptions. The inclusion of data on species-specific eDNA persistence, such as in notothenioid icefish in the West Antarctic Peninsula (Cowart et al. 2018), will strengthen future estimates, as will the integration of models that account for the ecology and transport of eDNA (Fukaya et al. 2020). Progress in eDNA abundance estimates would assist Antarctic biodiversity assessments and fisheries monitoring (Jerde 2019) and could contribute to our understanding of the state of the Ross

Sea toothfish population, a prey species of Weddell seals (Ainley et al. 2009, 2020) and Type-C orcas (*Orcinus orca*; Pitman and Ensor 2003; Lauriano et al. 2020).

3.5 Conclusions

We successfully detected Weddell seal DNA in seven of our thirty-three snow samples collected by volunteers. While our eDNA yield was low, this pilot study is the first example of vertebrate DNA being extracted from Antarctic snow samples. We suggest a number of ways to further optimize eDNA protocols in future applications of these methods, particularly in the storage and filtration of samples. The extreme nature of Antarctica makes it an ideal environment to investigate potential novel applications of eDNA surveys in fields such as population genetics. While we identified population genetics as a promising direction for future Antarctic eDNA surveys, we caution that sequence quality and fragmentation could lead to misinterpretations of population divergence and potential errors in species assignment (Adams et al. 2019; Sigsgaard et al. 2019). Environmental DNA is a rapidly developing field and is continually improving as more research is done on its methodologies and applications (Lear et al. 2018; Adams et al. 2019; Sigsgaard et al. 2019; Zinger et al. 2019). While eDNA is not suitable as a replacement for traditional methods, it presents an opportunity to complement current species' monitoring (Oleksiak et al. 2019; Steinmetz et al. 2021). The ability to non-invasively sample Antarctic vertebrate species, such as Weddell seals, is a promising addition to current biological surveys in Antarctica. Environmental DNA surveys represent an easily accessible, non-invasive diagnostic tool that shows promise for informing current and future research on the continent through the contribution to long term monitoring efforts.

3.6 References

Abgrall P, Terhune JM, Burton HR. 2003. Variation of Weddell seal (*Leptonychotes weddellii*) underwater vocalizations over mesogeographic ranges. Aquatic Mammals. 29(2): 268-277.

Adams CI, Knapp M, Gemmell NJ, Jeunen G-J, Bunce M, Lamare MD, Taylor HR. 2019. Beyond biodiversity: can environmental DNA (eDNA) cut it as a population genetics tool? Genes. 10(3): 192.

Agersnap S, Larsen WB, Knudsen SW, Strand D, Thomsen PF, Hesselsøe M, Mortensen PB, Vrålstad T, Møller PR. 2017. Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. PLoS One. 12(6): e0179261.

Ainley DG, Siniff DB. 2009. The importance of Antarctic toothfish as prey of Weddell seals in the Ross Sea. Antarctic Science. 21(4): 317.

Ainley DG, Larue MA, Stirling I, Stammerjohn S, Siniff DB. 2015. An apparent population decrease, or change in distribution, of Weddell seals along the Victoria Land coast. Marine Mammal Science. 31(4): 1338-1361.

Ainley DG, Cziko PA, Nur N, Rotella JJ, Eastman JT, Larue M, Stirling I, Abrams PA. 2020. Further evidence that Antarctic toothfish are important to Weddell seals. Antarctic Science. 33(1): 17-29

Aizu M, Seino S, Sado T, Miya M. 2017. Environmental DNA metabarcoding with MiFish primer reveals marine fish fauna of Tsushima Island, Nagasaki for establishing a marine protected area. Symposium Proceedings: The JSFS 85th Anniversary-Commemorative International Symposium “Fisheries Science for Future Generations”.

Alaeddini R, Walsh SJ, Abbas A. 2010. Forensic implications of genetic analyses from degraded DNA—a review. Forensic science international: genetics. 4(3): 148-157.

Antognazza CM, Britton JR, Potter C, Franklin E, Hardouin EA, Gutmann Roberts C, Aprahamian M, Andreou D. 2019. Environmental DNA as a non-invasive sampling tool to detect the spawning distribution of European anadromous shads (*Alosa spp.*). Aquatic Conservation: Marine and Freshwater Ecosystems. 29(1): 148-152.

Aylward ML, Sullivan AP, Perry GH, Johnson SE, Louis Jr EE. 2018. An environmental DNA sampling method for aye-ayes from their feeding traces. Ecology and evolution. 8(18): 9229-9240.

Baillie SM, McGowan C, May-McNally S, Leggatt R, Sutherland BJ, Robinson S. 2019. Environmental DNA and its applications to Fisheries and Oceans Canada: National needs and priorities. Canadian Technical Report of Fisheries and Aquatic Science. 3329

Baker CS, Steel D, Nieukirk S, Klinck H 2018. Environmental DNA (eDNA) from the wake of the whales: droplet digital PCR for detection and species identification. Frontiers in Marine Science. 5: 133.

Ballard G, Jongsomjit D, Veloz SD, Ainley DG. 2012. Coexistence of mesopredators in an intact polar ocean ecosystem: the basis for defining a Ross Sea marine protected area. Biological Conservation. 156: 72-82.

Bani A, De Brauwer M, Creer S, Dumbrell AJ, Limmon G, Jompa J, von der Heyden S, Beger M. 2020. Informing marine spatial planning decisions with environmental DNA. Advances in Ecological Research. 62: 375-407.

Barber-Meyer SM, Dysthe JC, Pilgrim KL. 2020. Testing environmental DNA from wolf snow tracks for species, sex, and individual identification. Canadian Wildlife Biology and Management. 9 (1): 12-20.

Barnes MA, Turner CR. 2016. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics. 17(1): 1-17.
Bass D, Stentiford GD, Littlewood D, Hartikainen H. 2015. Diverse applications of environmental DNA methods in parasitology. Trends in parasitology. 31(10): 499-513.
Bayer SR, Countway PD, Wahle RA. 2019. Developing an eDNA toolkit to quantify broadcast spawning events of the sea scallop *Placopecten magellanicus*: moving beyond fertilization assays. Marine Ecology Progress Series. 621: 127-141.
Baylis AM, Page B, Staniland I, Arnould JP, McKenzie J. 2015. Taking the sting out of darting: risks, restraint drugs and procedures for the chemical restraint of Southern Hemisphere otariids. Marine Mammal Science. 31(1): 322-344.
Bean TP, Greenwood N, Beckett R, Biermann L, Bignell JP, Brant JL, Copp GH, Devlin MJ, Dye S, Feist SW. 2017. A review of the tools used for marine monitoring in the UK: combining historic and contemporary methods with modeling and socioeconomics to fulfill legislative needs and scientific ambitions. Frontiers in Marine Science. 4: 263.
Bearzi G. 2000. First report of a common dolphin (*Delphinus delphis*) death following penetration of a biopsy dart. Journal of Cetacean Research and Management. 2(3): 217-222.
Beng KC, Corlett RT. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and conservation. 29(7): 2089-2121.
Bestley S, Ropert-Coudert Y, Bengtson Nash S, Brooks CM, Cotté C, Dewar M, Friedlaender AS, Jackson JA, Labrousse S, Lowther AD 2020. Marine ecosystem assessment for the Southern Ocean: birds and marine mammals in a changing climate. Frontiers in Ecology and Evolution. 8: 338.
Bylemans J, Furlan EM, Hardy CM, McGuffie P, Lintermans M, Gleeson DM. 2017. An environmental DNA-based method for monitoring spawning activity: a case study, using the endangered Macquarie perch (*Macquaria australasica*). Methods in Ecology and Evolution. 8(5): 646-655.
Cameron MF, Siniff DB. 2004. Age-specific survival, abundance, and immigration rates of a Weddell seal (*Leptonychotes weddellii*) population in McMurdo Sound, Antarctica. Canadian Journal of Zoology. 82(4): 601-615.
Carim KJ, McKelvey KS, Young MK, Wilcox TM, Schwartz MK. 2016. A protocol for collecting environmental DNA samples from streams. Gen. Tech. Rep. RMRS-GTR-355. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station. 18: 355.
CCAMLR CM 91-05.(2016). Ross sea region marine protected area.
Chapple DG, Ritchie PA, Daugherty CH. 2009. Origin, diversification, and systematics of the New Zealand skink fauna (Reptilia: *Scincidae*). Molecular Phylogenetics and Evolution. 52(2): 470-487.
Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW 2016. GenBank. Nucleic acids research. 44(D1): D67-D72.
Closek CJ, Santora JA, Starks HA, Schroeder ID, Andruszkiewicz EA, Sakuma KM, Bograd SJ, Hazen EL, Field JC, Boehm AB. 2019. Marine vertebrate biodiversity and distribution within the central California Current using environmental DNA (eDNA) metabarcoding and ecosystem surveys. Frontiers in Marine Science. 6: 732.
Coetzee BW, Chown SL. 2016. A meta-analysis of human disturbance impacts on Antarctic wildlife. Biological Reviews. 91(3): 578-596.

Commission for the Conservation of Antarctic Living Marine Resources (CCAMLR) 1989. CCAMLR Ecosystem Monitoring Program. [Accessed 2021 Mar 17] https://www.ccamlr.org/en/science/ccamlr-ecosystem-monitoring-program-cemp
Cottier-Cook EJ, Minchin JD, Giesler R, Graham J, Mogg AO, Sayer MD, Matejusova I. 2019. Biosecurity implications of the highly invasive carpet sea-squirt *Didemnum vexillum* Kott, 2002 for a protected area of global significance. Management of biological invasions. 10(2): 311-323.
Cowart DA, Murphy KR, Cheng C-HC. 2018. Metagenomic sequencing of environmental DNA reveals marine faunal assemblages from the West Antarctic Peninsula. Marine Genomics. 37: 148-160.
Cristescu ME, Hebert PD. 2018. Uses and misuses of environmental DNA in biodiversity science and conservation. Annual Review of Ecology, Evolution, and Systematics. 49: 209-230.
Croxall JP, Nicol S. 2004. Management of Southern Ocean fisheries: global forces and future sustainability. Antarctic Science. 16(4): 569.
Curtis C, Stewart BS, Karl SA. 2007. Sexing pinnipeds with ZFX and ZFY loci. Journal of Heredity. 98(3): 280-285.
Curtis C, Stewart BS, Karl SA. 2009. Pleistocene population expansions of Antarctic seals. Molecular Ecology. 18(10): 2112-2121.
Curtis C, Stewart BS, Karl SA. 2011. Genetically effective population sizes of Antarctic seals estimated from nuclear genes. Conservation Genetics. 12(6): 1435-1446.
Dalén L, Götherström A, Meijer T, Shapiro B. 2007. Recovery of DNA from footprints in the snow. Canadian Field-Naturalist. 121(3): 321-324.
Davis CS, Stirling I, Strobeck C, Coltman DW. 2008. Population structure of ice-breeding seals. Molecular Ecology. 17(13): 3078-3094.
Díaz-Ferguson EE, Moyer GR. 2014. History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments. Revista de biologia tropical. 62(4): 1273-1284.
Dickie IA, Boyer S, Buckley HL, Duncan RP, Gardner PP, Hogg ID, Holdaway RJ, Lear G, Makiola A, Morales SE. 2018. Towards robust and repeatable sampling methods in eDNA-based studies. Molecular Ecology Resources. 18(5): 940-952.
Doi H, Uchii K, Takahara T, Matsuhashi S, Yamanaka H, Minamoto T. 2015. Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. PloS one. 10(3): e0122763.
Dunn A, Vacchi M, Watters G. 2017. The Ross Sea region Marine Protected Area research and monitoring plan. CCAMLR document SC-CAMLR-XXXVI/20, Hobart, Australia.
Fenwick G. 1973. Breeding biology and population dynamics of the Weddell seal, *Leptonychotes weddelli*: a review. New Zealand Natural Sciences Mauri Ora. 1: 29-36
Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. Biology letters. 4(4): 423-425.
Fondevila M, Phillips C, Naverán N, Cerezo M, Rodríguez A, Calvo R, Fernández L, Carracedo Á, Lareu M. 2008. Challenging DNA: assessment of a range of genotyping approaches for highly degraded forensic samples. Forensic Science International: Genetics Supplement Series. 1(1): 26-28.
Franklin TW, McKelvey KS, Golding JD, Mason DH, Dysthe JC, Pilgrim KL, Squires JR, Aubry KB, Long RA, Greaves SE. 2019. Using environmental DNA methods to improve winter surveys for rare carnivores: DNA from snow and improved noninvasive techniques. Biological Conservation. 229: 50-58.

Freiwald J, Meyer R, Caselle JE, Blanchette CA, Hovel K, Neilson D, Dugan J, Altstatt J, Nielsen K, Bursek J. 2018. Citizen science monitoring of marine protected areas: case studies and recommendations for integration into monitoring programs. Marine Ecology. 39: e12470.

Fukaya K, Murakami H, Yoon S, Minami K, Osada Y, Yamamoto S, Masuda R, Kasai A, Miyashita K, Minamoto T. 2020. Estimating fish population abundance by integrating quantitative data on environmental DNA and hydrodynamic modelling. Molecular ecology. Early view.

Garlapati D, Charankumar B, Ramu K, Madeswaran P, Murthy MR. 2019. A review on the applications and recent advances in environmental DNA (eDNA) metagenomics. Reviews in Environmental Science and Bio/Technology. 18(3): 389-411.

Garrott RA, Rotella JJ, Siniff DB, Parkinson CL, Stauffer GE. 2012. Environmental variation and cohort effects in an Antarctic predator. Oikos. 121(7): 1027-1040.

Gelatt TS, Davis CS, Stirling I, Siniff DB, Strobeck C, Delisle I. 2010. History and fate of a small isolated population of Weddell seals at White Island, Antarctica. Conservation Genetics. 11(3): 721-735.

Gilbert MTP, Bandelt H-J, Hofreiter M, Barnes I. 2005. Assessing ancient DNA studies. Trends in ecology & evolution. 20(10): 541-544.

Gilbey J, Carvalho G, Castilho R, Coscia I, Coulson MW, Dahle G, Derycke S, Francisco SM, Helyar SJ, Johansen T. 2021. Life in a drop: Sampling environmental DNA for marine fishery management and ecosystem monitoring. Marine Policy. 124: 104331.

Gold Z, Sprague J, Kushner DJ, Marin EZ, Barber PH. 2021. eDNA metabarcoding as a biomonitoring tool for marine protected areas. PLOS ONE. 16(2): e0238557.

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in ecology and evolution. 7(11): 1299-1307.

Griffiths HJ. 2010. Antarctic marine biodiversity–what do we know about the distribution of life in the Southern Ocean? PLOS ONE. 5(8): e11683.

Griffiths HJ, Danis B, Clarke A. 2011. Quantifying Antarctic marine biodiversity: The SCAR-MarBIN data portal. Deep Sea Research Part II: Topical Studies in Oceanography. 58(1-2): 18-29.

Hadley GL, Rotella JJ, Garrott RA. 2007. Evaluation of reproductive costs for Weddell seals in Erebus Bay, Antarctica. Journal of Animal Ecology. 76(3): 448-458.

Hahn MW. 2007. Bias in phylogenetic tree reconciliation methods: implications for vertebrate genome evolution. Genome biology. 8(7): 1-9.

Halpern BS, Walbridge S, Selkoe KA, Kappel CV, Micheli F, D'Agrosa C, Bruno JF, Casey KS, Ebert C, Fox HE. 2008. A global map of human impact on marine ecosystems. science. 319(5865): 948-952.

Halvorsen S, Korslund L, Gustavsen PØ, Slettan A. 2020. Environmental DNA analysis indicates that migration barriers are decreasing the occurrence of European eel (*Anguilla anguilla*) in distance from the sea. Global Ecology and Conservation. 24: e01245.

Handt O, Höss M, Krings M, Pääbo S. 1994. Ancient DNA: methodological challenges. Experientia. 50(6): 524-529.

Hasegawa M, Kishino H, Yano TA. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of molecular evolution. 22(2): 160-174.

Hayer CA, Bayless MF, George A, Thompson N, Richter CA, Chapman DC. 2020. Use of Environmental DNA to Detect Grass Carp Spawning Events. Fishes 5(3): 27.

Hazen EL, Abrahms B, Brodie S, Carroll G, Jacox MG, Savoca MS, Scales KL, Sydeman WJ, Bograd SJ. 2019. Marine top predators as climate and ecosystem sentinels. Frontiers in Ecology and the Environment. 17(10): 565-574.
Hellström M, Wijkmark N, Edbom-Blomstrand C, Hellström P, Näslund J. 2019. Footsteps in the snow–Pilot study for future monitoring of individual lynx (*Lynx lynx)* from eDNA in snow tracks. AquaBiota Report 10.
Hinlo R, Lintermans M, Gleeson D, Broadhurst B, Furlan E. 2018. Performance of eDNA assays to detect and quantify an elusive benthic fish in upland streams. Biological Invasions. 20(11): 3079-3093.
Hückstädt L 2015. Leptonychotes weddellii (Weddell Seal). The IUCN red list of threatened species 2015: eT11696A45226713. [Accessed 15 March] 2017. https://dx.doi.org/102305/IUCNUK2015-4RLTST11696A45226713en
Ishige T, Miya M, Ushio M, Sado T, Ushioda M, Maebashi K, Yonechi R, Lagan P, Matsubayashi H. 2017. Tropical-forest mammals as detected by environmental DNA at natural saltlicks in Borneo. Biological conservation. 210: 281-285.
Iversen LL, Kielgast J, Sand-Jensen K. 2015. Monitoring of animal abundance by environmental DNA—An increasingly obscure perspective: A reply to Klymus et al., 2015. Biological Conservation. 100(192): 479-480.
Janečka JE, Munkhtsog B, Jackson RM, Naranbaatar G, Mallon DP, Murphy WJ. 2011. Comparison of noninvasive genetic and camera-trapping techniques for surveying snow leopards. Journal of Mammalogy. 92(4): 771-783.
Jensen MR, Sigsgaard EE, Liu S, Manica A, Bach SS, Hansen MM, Møller PR, Thomsen PF. 2020. Genome-scale target capture of mitochondrial and nuclear environmental DNA from water samples. Molecular Ecology Resources. 21(3): 690-702
Jerde CL. 2019. Can we manage fisheries with the inherent uncertainty from eDNA? Journal of fish biology. 98(2): 341-353
Jiang L, Yang Y 2017. Visualization of international environmental DNA research. Current Science. 1659-1664.
Johnson SS, Zaikova E, Goerlitz DS, Bai Y, Tighe SW 2017. Real-time DNA sequencing in the Antarctic dry valleys using the Oxford Nanopore sequencer. Journal of Biomolecular Techniques: JBT. 28(1): 2.
Kinoshita G, Yonezawa S, Murakami S., Isagi Y. 2019. Environmental DNA collected from snow tracks is useful for identification of mammalian species. Zoological science. 36(3): 198-207.
Klymus KE, Richter CA, Chapman DC, Paukert CP. 2015. A reply to Iversen et al.'s comment “Monitoring of animal abundance by environmental DNA-An increasingly obscure perspective”. Biological Conservation. 192: 481-482.
Knudsen SW, Ebert RB, Hesselsøe M, Kuntke F, Hassingboe J, Mortensen PB, Thomsen PF, Sigsgaard EE, Hansen BK, Nielsen EE. 2019. Species-specific detection and quantification of environmental DNA from marine fishes in the Baltic Sea. Journal of experimental marine biology and ecology. 510: 31-45.
Kock KH, Reid K, Croxall J, Nicol S. 2007. Fisheries in the Southern Ocean: an ecosystem approach. Philosophical Transactions of the Royal Society B: Biological Sciences. 362(1488): 2333-2349.
Kundu S, Rath S, Laishram K, Pakrashi A, Das U, Tyagi K, Kumar V, Chandra K. 2019. DNA barcoding identified selected ornamental fishes in Murti river of East India. Mitochondrial Dna Part B. 4(1): 594-598.
Lacoursière-Roussel A, Côté G, Leclerc V, Bernatchez L. 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. Journal of Applied Ecology. 53(4): 1148-1157.

Lafferty KD, Benesh KC, Mahon AR, Jerde CL, Lowe CG. 2018. Detecting southern California's white sharks with environmental DNA. Frontiers in Marine Science. 5: 355.
Lan T, Lindqvist C. 2018. Technical advances and challenges in genome-scale analysis of ancient DNA. Paleogenomics, Springer. Pp. 3-29.
LaRue MA, Rotella JJ, Garrott RA, Siniff DB, Ainley DG, Stauffer GE, Porter CC, Morin PJ. 2011. Satellite imagery can be used to detect variation in abundance of Weddell seals (*Leptonychotes weddellii*) in Erebus Bay, Antarctica. Polar Biology. 34(11): 1727.
LaRue MA, Ainley DG, Pennycook J, Stamatiou K, Salas L, Nur N, Stammerjohn S, Barrington L. 2020. Engaging 'the crowd'in remote sensing to learn about habitat affinity of the Weddell seal in Antarctica. Remote Sensing in Ecology and Conservation. 6(1): 70-78.
LaRue MA, Salas L, Nur N, Ainley DG, Stammerjohn S, Barrington L, Stamatiou K, Pennycook J, Dozier M, Saints J. 2019. Physical and ecological factors explain the distribution of Ross Sea Weddell seals during the breeding season. Marine Ecology Progress Series. 612: 193-208.
Lauriano G, Pirotta E, Joyce T, Pitman RL, Borrell A, Panigada S. 2020. Movements, diving behaviour and diet of type-C killer whales (*Orcinus orca*) in the Ross Sea, Antarctica. Aquatic Conservation: Marine and Freshwater Ecosystems. 30(12): 2428-2440.
Lawson Handley L, Read DS, Winfield IJ, Kimbell H, Johnson H, Li J, Hahn C, Blackman R, Wilcox R, Donnelly R. 2019. Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. Environmental DNA. 1(1): 26-39.
Lear G, Dickie I, Banks J, Boyer S, Buckley HL, Buckley TR, Cruickshank R, Dopheide A, Handley KM, Hermans S. 2018. Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. New Zealand Journal of Ecology. 42(1): 10-50A.
Learmonth JA, MacLeod CD, Santos MB, Pierce GJ, Crick H, Robinson R. 2006. Potential effects of climate change on marine mammals. Oceanography and Marine Biology. 44: 431.
Lehman N, Decker DJ, Stewart BS. 2004. Divergent patterns of variation in major histocompatibility complex class II alleles among Antarctic phocid pinnipeds. Journal of Mammalogy. 85(6): 1215-1224.
Lehnert K, Weirup L, Harding K, Härkönen T, Karlsson O, Teilmann J. 2017. Antarctic seals: molecular biomarkers as indicators for pollutant exposure, health effects and diet. Science of the Total Environment. 599: 1693-1704.
Letunic I, Bork P. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic acids research. 47(W1): W256-W259.
Lindfield SJ, Harvey ES, McIlwain JL, Halford AR. 2014. Silent fish surveys: bubble-free diving highlights inaccuracies associated with SCUBA-based surveys in heavily fished areas. Methods in Ecology and Evolution. 5(10): 1061-1069.
Long RA, MacKay P, Ray J, Zielinski W. 2012. Noninvasive survey methods for carnivores. Washington (DC): Island Press.
Mächler E, Deiner K, Spahn F, Altermatt F. 2016. Fishing in the water: effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. Environmental science & technology. 50(1): 305-312.
Magurran AE, Baillie SR, Buckland ST, Dick JM, Elston DA, Scott EM, Smith RI, Somerfield PJ, Watt AD. 2010. Long-term datasets in biodiversity research and monitoring: assessing change in ecological communities through time. Trends in ecology & evolution. 25(10): 574-582.

Mariani S, Baillie C, Colosimo G, Riesgo A. 2019. Sponges as natural environmental DNA samplers. Current Biology. 29(11): R401-R402.
Marine Mammal Protected Areas Task Force. 2016. Important marine mammal areas [Accessed 2021 Mar 17] http://www.marinemammalhabitat.org/
Marshall NT, Stepien CA. 2019. Invasion genetics from eDNA and thousands of larvae: A targeted metabarcoding assay that distinguishes species and population variation of zebra and quagga mussels. Ecology and evolution. 9(6): 3515-3538.
McColl-Gausden EF, Weeks AR, Tingley R. 2020. A field ecologist's guide to environmental DNA sampling in freshwater environments. Australian Zoologist 40(4): 641.
McCormack SA, Melbourne-Thomas J, Trebilco R, Blanchard JL, Raymond B, Constable A. 2021. Decades of dietary data demonstrate regional food web structures in the Southern Ocean. Ecology and evolution. 11(1): 227-241.
McMahon CR, Burton HR. 2005. Climate change and seal survival: evidence for environmentally mediated changes in elephant seal, *Mirounga leonina*, pup survival. Proceedings of the Royal Society B: Biological Sciences. 272(1566): 923-928.
Millán-Aguiñaga N, Soldatou S, Brozio S, Munnoch JT, Howe J, Hoskisson PA, Duncan KR. 2019. Awakening ancient polar Actinobacteria: diversity, evolution and specialized metabolite potential. Microbiology. 165(11): 1169-1180.
Molina-Venegas R, Rodríguez MÁ. 2017. Revisiting phylogenetic signal; strong or negligible impacts of polytomies and branch length information? BMC evolutionary biology. 17(1): 1-10.
Mondol S, Karanth KU, Kumar NS, Gopalaswamy AM, Andheria A, Ramakrishnan U. 2009. Evaluation of non-invasive genetic sampling methods for estimating tiger population size. Biological Conservation. 142(10): 2350-2360.
Monge O, Dumas D, Baus I. 2020. Environmental DNA from avian residual saliva in fruits and its potential uses in population genetics. Conservation Genetics Resources. 12(1): 131-139.
Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. 2008. Database indexing for production MegaBLAST searches. Bioinformatics. 24(16): 1757-1764.
Nichols RV, Spong G. 2017. An eDNA-based SNP assay for ungulate species and sex identification. Diversity. 9(3): 33.
Nickols KJ, White JW, Malone D, Carr MH, Starr RM, Baskett ML, Hastings A, Botsford LW. 2019. Setting ecological expectations for adaptive management of marine protected areas. Journal of Applied Ecology. 56(10): 2376-2385.
Ogram A, Sayler GS, Barkay T. 1987. The extraction and purification of microbial DNA from sediments. Journal of microbiological methods. 7(2-3): 57-66.
Pande A, Sivakumar K, Sathyakumar S, Kumar RS, Johnson J, Mondol S, Mathur VB. 2017. Monitoring wildlife and their habitats in the Southern Ocean and around Indian research stations in Antarctica. Proceedings of the Indian National Science Academy. 83(2): 483-496.
Parsons KM, Everett M, Dahlheim M, Park L. 2018. Water, water everywhere: environmental DNA can unlock population structure in elusive marine species. Royal Society open science. 5(8): 180537.
Piazza P, Gattone SA, Guzzi A, Schiaparelli S. 2020. Towards a robust baseline for long-term monitoring of Antarctic coastal benthos. Hydrobiologia 847(7): 1753-1771.
Pierszalowski S, Ferrari M, Glockner-Ferrari D, Mizroch S, Clapham P, Dickerson B. 2013. Investigating the feasibility of using DNA from sloughed skin for individual identification and kinship analysis in humpback whales (*Megaptera novaeangliae*). Marine mammal science. 29(3): 533-541.

Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences. 70(8): 1123-1130.
Pinfield R, Dillane E, Runge AKW, Evans A, Mirimin L, Niemann J, Reed TE, Reid DG, Rogan E, Samarra FI. 2019. False-negative detections from environmental DNA collected in the presence of large numbers of killer whales (*Orcinus orca*). Environmental DNA. 1(4): 316-328.
Pitman RL, Ensor P. 2003. Three forms of killer whales (*Orcinus orca*) in Antarctic waters. Journal of Cetacean Research and Management. 5(2): 131-140.
Reid NM, Carstens BC. 2012. Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. BMC evolutionary biology. 12(1): 1-11.
Ritchie AM, Hua X, Cardillo M, Yaxley KJ, Dinnage R, Bromham L. 2021. Phylogenetic diversity metrics from molecular phylogenies: modelling expected degree of error under realistic rate variation. Diversity and Distributions. 27(1): 164-178.
Roh C, Villatte F, Kim B-G, Schmid RD. 2006. Comparative study of methods for extraction and purification of environmental DNA from soil and sludge samples. Applied biochemistry and biotechnology. 134(2): 97-112.
Romero LM, Reed JM. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 140(1): 73-79.
Rotella JJ, Paterson JT, Garrott RA. 2016. Birth dates vary with fixed and dynamic maternal features, offspring sex, and extreme climatic events in a high-latitude marine mammal. Ecology and Evolution. 6(7): 1930-1941.
Rotella JJ, Link WA, Chambert T, Stauffer GE, Garrott RA. 2012. Evaluating the demographic buffering hypothesis with vital rates estimated for Weddell seals from 30 years of mark–recapture data. Journal of Animal Ecology. 81(1): 162-173.
Schwartz RS, Mueller RL. 2010. Branch length estimation and divergence dating: estimates of error in Bayesian and maximum likelihood frameworks. BMC evolutionary biology. 10(1): 1-21.
Schweiss KE, Lehman RN, Drymon JM, Phillips NM. 2020. Development of highly sensitive environmental DNA methods for the detection of Bull Sharks, *Carcharhinus leucas* (Müller and Henle, 1839), using Droplet Digital™ PCR. Environmental DNA. 2(1): 3-12.
Sharma R, Stuckas H, Bhaskar R, Khan I, Goyal SP, Tiedemann R. 2011. Genetically distinct population of Bengal tiger (*Panthera tigris tigris*) in Terai Arc Landscape (TAL) of India. Mammalian Biology. 76(4): 484-490.
Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology. 7(1): 539.
Sigsgaard EE, Nielsen IB, Bach SS, Lorenzen ED, Robinson DP, Knudsen SW, Pedersen MW, Al Jaidah M, Orlando L, Willerslev E. 2016. Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. Nature ecology & evolution. 1(1): 1-5.
Siniff D, DeMaster D, Hofman R, Eberhardt L. 1977. An analysis of the dynamics of a Weddell seal population. Ecological Monographs. 47(3): 319-335.
Smith Jr WO, Ainley DG, Cattaneo-Vietti R. 2007. Trophic interactions within the Ross Sea continental shelf ecosystem. Philosophical Transactions of the Royal Society B: Biological Sciences. 362(1477): 95-111.

Spear MJ, Embke HS, Krysan PJ, Vander Zanden MJ. 2021. Application of eDNA as a tool for assessing fish population abundance. Environmental DNA. 3(1): 83-91.
Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SS, Sigsgaard EE, Hellström M. 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods in Ecology and Evolution. 8(5): 635-645.
Sproul JS, Maddison DR. 2017. Sequencing historical specimens: successful preparation of small specimens with low amounts of degraded DNA. Molecular Ecology Resources. 17(6): 1183-1201.
Stammerjohn S, Massom R, Rind D, Martinson D. 2012. Regions of rapid sea ice change: An inter-hemispheric seasonal comparison. Geophysical Research Letters. 39(6).
Stark M, Berger SA, Stamatakis A, von Mering C. 2010. MLTreeMap-accurate Maximum Likelihood placement of environmental DNA sequences into taxonomic and functional reference phylogenies. BMC genomics. 11(1): 1-11.
Steinmetz K, Murphy S, Cadhla OÓ, Barnett J, Mirimin L. 2021. Enabling pinniped conservation by means of non-invasive genetic population analysis. Conservation Genetics Resources: 1-12.
Stepien CA, Snyder MR, Elz AE. 2019. Invasion genetics of the silver carp *Hypophthalmichthys molitrix* across North America: Differentiation of fronts, introgression, and eDNA metabarcode detection. PLOS ONE. 14(3): e0203012.
Stewart KA. 2019. Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. Biodiversity and Conservation 28(5): 983-1001.
Stirling I. 1969a. The seals at White Island: a hypothesis on their origin. Antarctic, 4: 310-313. 1967. Population studies on the Weddell seal. Tuatara. 13: 133-141.
Stirling I. 1969b. Ecology of the Weddell seal in McMurdo Sound, Antarctica. Ecology. 50(4): 573-586.
Stoeckle MY, Soboleva L, Charlop-Powers Z. 2017. Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. PLOS ONE. 12(4): e0175186.
Strickler KM, Fremier AK, Goldberg CS. 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation. 183: 85-92.
Suter L, Polanowski AM, Clarke LJ, Kitchener JA, Deagle BE. 2020. Capturing open ocean biodiversity: Comparing environmental DNA metabarcoding to the continuous plankton recorder. Molecular Ecology. Early view.
Sydeman WJ, Poloczanska E, Reed TE, Thompson SA. 2015. Climate change and marine vertebrates. Science. 350(6262): 772-777.
Székely D, Corfixen NL, Mørch LL, Knudsen SW, McCarthy ML, Teilmann J, Heide-Jørgensen MP, Olsen MT. 2021. Environmental DNA captures the genetic diversity of bowhead whales (*Balaena mysticetus*) in West Greenland. Environmental DNA. 3(1): 248-260
Taberlet P, Bonin A, Zinger L, Coissac E. 2018. Environmental DNA: For biodiversity research and monitoring. United Kingdom (UK): Oxford University Press.
Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E. 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular ecology. 21(8): 2045-2050.
Takahashi S, Sakata MK, Minamoto T, Masuda R. 2020. Comparing the efficiency of open and enclosed filtration systems in environmental DNA quantification for fish and jellyfish. PLOS ONE. 15(4): e0231718.

Testa JW, Siniff DB. 1987. Population dynamics of Weddell seals (*Leptonychotes weddelli*) in McMurdo sound, Antarctica. Ecological Monographs. 57(2): 149-165.
Testa JW, Scotton BD. 1999. Dynamics of an isolated population of Weddell seals (*Leptonychotes weddellii*) at White Island, Antarctica. Journal of Mammalogy. 80(1): 82-90.
Thomas J, Puddicombe R, George M, Lewis D. 1988. Variations in underwater vocalizations of Weddell seals (*Leptonychotes weddelli*) at the Vestfold Hills as a measure of breeding population discreteness. Biology of the Vestfold Hills, Antarctica, Springer. Pp. 279-284.
Thomas JA, Stirling I. 1983. Geographic variation in the underwater vocalizations of Weddell seals (*Leptonychotes weddelli*) from Palmer Peninsula and McMurdo Sound, Antarctica. Canadian Journal of Zoology. 61(10): 2203-2212.
Thomsen PF, Møller PR, Sigsgaard EE, Knudsen SW, Jørgensen OA, Willerslev E. 2016. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. PLOS ONE. 11(11): e0165252.
Thomsen PF, Kielgast J, Iversen LL, Wiuf C, Rasmussen M, Gilbert MTP, Orlando L, Willerslev E. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular ecology. 21(11): 2565-2573.
Tillotson MD, Kelly RP, Duda JJ, Hoy M, Kralj J, Quinn TP. 2018. Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. Biological Conservation. 220: 1-11.
Tin T, Fleming ZL, Hughes KA, Ainley D, Convey P, Moreno C, Pfeiffer S, Scott J, Snape I. 2009. Impacts of local human activities on the Antarctic environment. Antarctic Science. 21(1): 3-33.
Tingley R, Greenlees M, Oertel S, van Rooyen A, Weeks A. 2019. Environmental DNA sampling as a surveillance tool for cane toad *Rhinella marina* introductions on offshore islands. Biological invasions. 21(1): 1-6.
Tsuji S, Shibata N. 2021. Identifying spawning events in fish by observing a spike in environmental DNA concentration after spawning. Environmental DNA. 3(1): 190-199
Tsuji S, Shibata N, Sawada H, Ushio M. 2020. Quantitative evaluation of intraspecific genetic diversity in a natural fish population using environmental DNA analysis. Molecular Ecology Resources. 20(5): 1323-1332.
Turon M, Angulo-Preckler C, Antich A, Præbel K, Wangensteen OS. 2020. More than expected from old sponge samples: a natural sampler DNA metabarcoding assessment of marine fish diversity in Nha Trang Bay (Vietnam). Frontiers in Marine Science. 7: 605148
Uchii K, Doi H, Minamoto T. 2016. A novel environmental DNA approach to quantify the cryptic invasion of non-native genotypes. Molecular Ecology Resources. 16(2): 415-422.
Valentin RE, Fonseca DM, Gable S, Kyle KE, Hamilton GC, Nielsen AL, Lockwood JL. 2020. Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect invasive forest insects. Molecular ecology resources. 20(3): 746-755.
Von Duyke AL, Kruger E, Wijkmark N, Näslund J, Hellström P, Hellström M. 2019. Evaluation of environmental DNA (eDNA) collected from tracks in the snow as a means to monitor individual polar bears (*Ursus maritimus*) in the Chukchi and Beaufort Seas. North Slope preliminary research report. NSB.DWM.PRR.2019-01
Watters GM, Hinke JT, Reiss CS. 2020. Long-term observations from Antarctica demonstrate that mismatched scales of fisheries management and predator-prey

interaction lead to erroneous conclusions about precaution. Scientific reports. 10(1): 1-9.
Wege M, Salas L, LaRue M. 2020. Citizen science and habitat modelling facilitates conservation planning for crabeater seals in the Weddell Sea. Diversity and Distributions. 26(10): 1291-1304.
Weiblen GD, Webb CO, Novotny V, Basset Y, Miller SE. 2006. Phylogenetic dispersion of host use in a tropical insect herbivore community. Ecology. 87(sp7): S62-S75.
Wilcox T, Carim K, Young M, McKelvey K, Franklin T, Schwartz M. 2018. Comment: The importance of sound methodology in environmental DNA sampling. North American Journal of Fisheries Management. 38(3): 592-596.
Wu Q, Kawano K, Ishikawa T, Sakata MK, Nakao R, Hiraiwa MK, Tsuji S, Yamanaka H, Minamoto T. 2019. Habitat selection and migration of the common shrimp, *Palaemon paucidens* in Lake Biwa, Japan—An eDNA-based study. Environmental DNA. 1(1): 54-63.
Yates MC, Glaser D, Post JR, Cristescu ME, Fraser DJ, Derry AM. 2020. The relationship between eDNA particle concentration and organism abundance in nature is strengthened by allometric scaling. Molecular Ecology. Early view.
Young MJ, Dutoit L, Robertson F, van Heezik Y, Seddon PJ, Robertson BC. 2020. Species in the faeces: DNA metabarcoding as a method to determine the diet of the endangered yellow-eyed penguin. Wildlife Research. 47(6): 509-522.
Younger JL, Van Den Hoff J, Wienecke B, Hindell M, Miller KJ. 2016. Contrasting responses to a climate regime change by sympatric, ice-dependent predators. BMC evolutionary biology. 16(1): 1-11.
Zappes IA, Fabiani A, Sbordoni V, Rakaj A, Palozzi R, Allegrucci G. 2017. New data on Weddell seal (*Leptonychotes weddellii*) colonies: A genetic analysis of a top predator from the Ross Sea, Antarctica. PLOS ONE. 12(8): e0182922.
Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. Journal of Computational biology. 7(1-2): 203-214.
Zinger L, Bonin A, Alsos IG, Bálint M, Bik H, Boyer F, Chariton AA, Creer S, Coissac E, Deagle BE. 2019. DNA metabarcoding—Need for robust experimental designs to draw sound ecological conclusions. Molecular Ecology. 28(8): 1857-1862.

Chapter Four

Optimizing eDNA protocols for Kororā, little blue penguin (*Eudyptula minor*)

New Zealand's long history of isolation has led to its unique biodiversity; however, this has been threatened by the introduction of predatory mammals and consequently has led to the decline in endemic fauna. Whilst it is critical to protect New Zealand's threatened, and endangered taxa, many species considered of least concern are also conservation dependant. One of these species is the kororā (or little penguin; *Eudyptula minor*). Breeding kororā have also been proposed as an indicator species for the health of the local marine ecosystem; therefore, monitoring their populations has multiple benefits. However, to fully assess how an organism functions in its environment, it's important to utilise multiple monitoring methods to account for various methodological biases and build a more comprehensive understanding of the situation. In this study, we look to environmental DNA (eDNA) as a non-invasive monitoring tool and discuss its current and future applications within a New Zealand context. This study utilises shed feathers and tank water from the rescue population of kororā at the International Antarctic Centre in Christchurch. In addition to direct feather extractions, we trialled filtering self-made feather-water and penguin tank water using enclosed Sterivex filter units and MarVer3 universal marine vertebrate primers to extract, amplify and sequence kororā DNA. While our direct feather amplifications were unsuccessful, we were able to retrieve sequences from both the feather water and tank water samples. A

BLASTN query and the construction of a Neighbour-Joining tree identified our samples as kororā. However, our branch lengths indicated a high level of divergence and the BLASTN metrics showed similar alignments with non-target species, likely due to a low quantity and quality of DNA and potential contamination. The protocol, therefore, requires further optimisation to ensure high confidence in future conclusions. Despite these challenges, we suggest that eDNA surveys could be applied to monitoring wild populations of kororā in the future, highlighting potential applications that could inform future management of the species.

4.1 Introduction

Anthropogenic modification of the marine environment is a global issue and requires long term monitoring to identify trends in biodiversity and ecosystem health (Luypaert et al. 2020). However, extensive monitoring programmes can be logistically challenging and difficult to implement, limiting our understanding of change (Chilvers 2017). Likewise, the ocean is a vast, complex environment and it is not possible to survey the entirety of its taxa, particularly given the number of cryptic organisms the marine system encompasses. Though all monitoring tools have biases, such as taxa size, niche, behaviour, etc., by combining methodologies, we can build a more comprehensive estimate of an ecosystem (Costello et al. 2017). It is important to develop, trial and standardise emerging technologies to confidently refine our understanding of biodiversity and trophic interactions (Dickie et al. 2018; Lear et al. 2018; Jeunen et al. 2019). One such emerging technology is Environmental DNA (eDNA), that being DNA shed by an organism into the environment and subsequently extracted from a sample such as water, air or soil (Taberlet et al. 2012; 2018). There has been growing interest in eDNA due to its ability to non-invasively sample taxa across a broad spatial scale without

the need to sight, track, or trap an individual. This technology's application is especially beneficial for cryptic, endangered and invasive species (Taberlet et al. 2012; 2018; Beng and Corlett 2020). Once an eDNA assay is established, eDNA has the benefit of a rapid turnaround at a relatively low cost (Evans et al. 2017; Lugg et al. 2018; Beng and Corlett 2020). Environmental DNA can apply to questions ranging from a multi-species approach (metabarcoding) to species-specific detection. In recent years the field has seen the development of eDNA applications beyond species detection to questions of population genetics and abundance estimates (Adams et al. 2019; Sigsgaard et al. 2019). While eDNA is a promising monitoring tool, the currently limited databased can lead to poor taxonomic resolution (Kress et al. 2015; Zaiko et al. 2018; Furlan et al. 2020). Additionally, there is a risk of false positives and false negative invalidating conclusions. In particular, contamination at any stage in the process can introduce false positives and must be accounted for. Further research should prioritise the development, refinement and standardisation of workflows to minimise these risks (Cristescu and Herbert 2018; Lear et al. 2018; Dickie et al. 2018; Taberlet et al. 2018; Furlan et al. 2020).

New Zealand presents a unique landscape for investigating the potential applications of eDNA methods both within the marine (Jeunen et al. 2020a, 2020b) and terrestrial environment (Holdaway et al. 2017). New Zealand's fauna's evolution, in a landscape uniquely free of mammals except for three bat species (pekapeka; orders: *Chalinolobus* and *Mystacina*), has left many of the endemic species evolutionarily vulnerable to pests (Jay et al. 2003). Due to established invasive species, New Zealand has an extensive history of national monitoring programmes and research through institutions such as the National Institute of Water and Atmospheric Research (NIWA) and the Department of Conservation (DOC; Parkes et al. 2003; Innes et al. 2009; King and Forsyth 2021). Recent advances in eDNA applications and technology have led to the proposal of eDNA as a potential monitoring tool

within these frameworks (Holdaway et al. 2017; Wallis 2019). For example, the Environmental Protection Agency (EPA) has developed a community-driven initiative, Wai Tūwhera o te Taiao, Open Waters Aotearoa programme (www.epa.govt.nz/community-involvement/open-waters-aotearoa/), utilising eDNA samples collected by the public to map New Zealand's biodiversity. As eDNA surveys are increasingly used to survey New Zealand's taxa, we must consider the accessibility and ownership of the data generated. While eDNA programmes are reliant on extensive, open, reference databases, researchers must consider the question of data management. Currently, the field of eDNA lacks the enforcement of a clear ethical framework for data sovereignty. It is critical researchers engage and collaborate with indigenous stakeholders, iwi and hapū, and consider culturally significant taxa, such as mahinga kai (food species) and taonga (treasured species), that may be detected and sequenced in the process of an eDNA survey (Holdaway et al. 2017; Collier-Robinson et al. 2019). In regards to eDNA, Handsley-Davis et al. (2020) discuss the risks of inadvertently revealing information that can be damaging or distressing to first nations people, especially in the case of ancient eDNA (aeDNA). While the paper is written in the context of Australia's traditional owners, these considerations also apply in New Zealand.

4.1.1 Biosecurity

Protecting New Zealand's biodiversity calls for strict biosecurity measures, which is increasingly challenging in a globalised world (Jay et al. 2003). Biosecurity management requires a framework that detects and prevents the entry of potential threats (Jay et al. 2003; Collins et al. 2013; Zaiko et al. 2018). Additionally, that framework should include surveillance systems for the early detection of non-indigenous species and programmes for monitoring and managing established pests (Jay et al. 2003; Zaiko et al. 2018). Positive biosecurity outcomes are best achieved by amalgamating complementary methods, i.e.

utilising methods that address different biases or limitations (Von Ammon et al. 2018; Banks et al. 2020). The integration of eDNA surveys in biosecurity monitoring in New Zealand could be beneficial across invasion timelines (Zaiko et al. 2018). The utilisation of eDNA at the New Zealand border could assist in biosecurity prevention measures. For example, identifying live imports (Collins et al. 2013), screening baggage and footwear in airports (McNeill et al. 2011; Bulman et al. 2018) and assessing marine vectors such as bilge and ballast water (Zaiko et al. 2015, 2020; Ponchon et al. 2017).

Should a species make it past the New Zealand border, early detection is critical (Riccardi et al. 2017; Von Ammon et al. 2018). Similarly, once a species becomes established, understanding distribution is important for control and management (Zaiko et al. 2020; Wood et al. 2019, 2020; Von Ammon et al. 2019a, 2019b; Banks et al. 2020). Banks et al. (2020) compared eDNA methods to hand net counts for a freshwater pest, the mosquito fish (*Gambusia affinis)* and suggested eDNA sampling may resolve access limitations at private or inaccessible sites. In the marine environment, researchers have suggested eRNA may have benefits over eDNA for inferring living invasive organisms in a locality such as invasive Mediterranean fanworms (*Sabella spallanzanii*; Von Ammon et al. 2019; Wood et al. 2020). Continued research on the factors that impact eDNA and eRNA persistence is critical as we consider applications to invasive species monitoring and environmental assessments (Zaiko et al. 2018; Von Ammon et al. 2019).

4.1.2 Environmental monitoring

Environmental DNA can assist in our understanding of anthropogenic disturbance across a landscape. For example, Laroche et al. (2016, 2017, 2018) assessed the impacts of offshore oil extraction in the South Taranaki Bight by metabarcoding local benthic communities, suggesting the formation of site-specific assemblages. Likewise, eDNA surveys apply to other New Zealand industries' biomonitoring, such as agriculture (Wakelin et al. 2016) and aquaculture (Ponchon et al. 2015; Pearman et al. 2020). Environmental DNA extracted from sediment samples can be used to infer a relationship between community composition and New Zealand salmon farms (Ponchon et al. 2015; Pearman et al. 2020). Thus eDNA surveys could inform management programmes and provide critical information to develop thresholds and mitigation strategies. In addition to clear sources of disturbance, eDNA surveys can target vulnerable ecosystems such as wetlands (Woods et al. 2021). Importantly, by adapting the design of an eDNA survey, we can investigate various research questions. Hogg et al. (2019) reviewed potential eDNA and DNA barcoding surveys' potential applications to the restoration of New Zealand lakes, e.g. assessing water quality, monitoring invasive species, identifying cryptic taxa and estimating community assemblages. Routine monitoring using eDNA will continue to improve as reference databases grow (Hogg et al. 2019). While the use of eDNA for population estimates may not currently be applicable in the fisheries industry, a workshop held by the Ministry for Primary Industries (MPI) and Fisheries New Zealand (2020) proposed the collection and storage of eDNA samples in preparation for future advances (Mace et al. 2020). While future developments of genetic methods and technology are promising, current e-DNA surveys already offer a cost-effective environmental assessment tool (Ramón-Laca et al. 2014).

4.1.3 Trophic interactions

Environmental DNA applies to questions on the connectedness of taxa across New Zealand's ecosystem. By metabarcoding faecal samples, we can make inferences about trophic dynamics. Metabarcoding of Bryde's whales (*Balaenoptera edeni brydei*) faeces suggest prey preference influences the whales' persistence in the Hauraki Gulf (Carroll et al. 2019). Thus eDNA can contribute to management by inferring bottom-up trophic pressures (Carroll et al. 2019). Likewise, dietary analysis can be used to evaluate the competition between New Zealand fauna and industry. Environmental DNA has been used to demonstrate a 10 % overlap between local fisheries catch and the diet of the kekeno (New Zealand fur seal, *Arctocephalus forsteri;* Emami-Khoyi et al. 2016*)*. Additionally, ancient eDNA (aeDNA) from coprolites (fossilised faeces) can infer foraging behaviour and niche partitioning in an extinct species such as the moa (Order: Dinornithiformes; Wood et al. 2008, 2012, 2013, 2020; Boast et al. 2018). Similarly, we can establish the diet of a critically endangered species, the kākāpō (*Strigops habroptilus),* prior to current range restrictions (Boast et al. 2018). By investigating the role an extinct or invasive species played in dispersal, aeDNA can infer the legacy of anthropogenic disturbance, which can inform modern conservation (Boast et al. 2018; Wood et al. 2020).

Environmental DNA from faeces can be especially valuable in the management of endangered species. Traditional morphological analysis of faeces is bias against soft-bodied organisms; however, studies applying eDNA to dietary analysis have highlighted it's potential to address this concern (Boyer et al. 2011; Carroll et al. 2019). One such example is from endangered carnivorous land snails (*Powelliphanta augusta*), where faecal DNA analysis confirmed an earthworm diet (Boyer et al. 2011; 2013). Importantly, Boyer et al. (2013) established that *P.augusta* diet is not specific to a single earthworm species. Understanding diet specificity is key to conservation efforts and is necessary for informing restoration and

translocation programmes (Boyer et al. 2013; Waterhouse et al. 2014). Another important consideration in conservation research and management is ethical best practice (Costello et al. 2016; Zemanova et al. 2020). Environmental DNA surveys are typically both non-invasive and non-disruptive, which can be critical for monitoring endangered species such as the hoiho (yellow-eyed penguin, *Megadyptes antipodes*; Young et al. 2020). While eDNA lacks the resolution of traditional methods such as stomach flushing, metabarcoding of faecal samples can support complimentary, ethical, long-term monitoring of an endangered species (Young et al. 2020).

4.1.4 Detecting threatened species

Monitoring threatened species distribution can be challenging due to low detection probability (Robinson et al. 2018; Folt et al. 2019). However, eDNA surveys do not require the sighting, or capture, of target taxa, thus addressing some of the limitations and biases that arise in traditional monitoring (Thomsen and Willerslev et al. 2015; McColl-Gausden et al. 2020; Neice and McRae 2021). While there has been minimal application of eDNA to mapping New Zealand's threatened species (Doyle et al. 2015, Jeunen et al. 2020), the methods have seen success elsewhere. For example, eDNA extracted from Brisbane waterways in Australia, coupled with historical observations, suggest regions inhabited by threatened platypus have declined (Lugg et al. 2018). Likewise, comparisons with traditional Fyke net surveys for platypus in Victoria found eDNA not only to be more cost-efficient, but the surveys had a comparatively reduced sampling risk (McColl-Gausden et al. 2020). Additionally, Victorian platypus surveys utilised community scientists, increasing both public engagement in conservation and sampling efforts (Melbourne Waterways, 2019; McColl-Gausden et al. 2020).

While New Zealand has no freshwater aquatic mammals, eDNA methods apply to other native species. Researchers in the United States investigated eDNA surveys as a tool for assessing the distribution of threatened black rails (*Laterallus jamaicensis*), which are easily concealed in their wetland habitats (Neice and McRae 2021). Validated with traditional callback surveys, black rail eDNA surveys highlighted the value of small pools for accumulating DNA (Neice and McRae 2021). These methods could apply to many of New Zealand's threatened wetland and riverine species, and indeed eDNA has already shown promise for monitoring whio (blue duck, *Hymenolaimus malacorhynchos*; Doyle 2015). Environmental DNA surveys can also apply to species not typically associated with water. In northern Australia, Day et al. (2019) detected threatened Gouldian finches in the waterholes they use to drink. Importantly, eDNA detection indicated finch presence within seventy-two hours but stressed the need for further investigation into eDNA persistence in waterholes. Thus, Day et al. (2019) highlight captive populations' value for initial controlled trials and optimisation before applying to wild populations. One species in New Zealand that meets these criteria is the kororā (little blue penguin, *Eudyptula minor*), with a captive population at the International Antarctic Centre in Christchurch. While assessed as a species of least concern by the IUCN (BirdLife International, 2020), the kororā is largely considered conservation dependent, particularly in the face of ongoing threats from invasive predators and urbanisation (Challies et al. 2004, 2015; Allen et al. 2011; Robertson et al. 2016; Wilson and Mattern 2018; Cargill et al. 2020).

4.1.5 Kororā (*Eudyptula minor*) as a study species

Found across the coastlines of New Zealand and Southern Australia, the kororā, also known as the little penguin, and the fairy penguin, is the smallest of the penguins (Reilly 1994). The kororā has caused some contention regarding taxonomic delineation (Kinsky and Falla 1976; Banks et al. 2002; 2010; Clark et al. 2012). Historically the kororā was divided into six subspecies, based on morphological characteristics and regional specificity (Kinsky and Falla 1976; Talor 2000a, 2000b), whereas they are currently considered a single species (Wilson and Mattern 2018; BirdLife International, 2020). However, these two conflicting taxonomic delineations coexisted, with *Eudyptula minor* considered a single species as early as 1990 (Marchant and Higgins 1990). Current taxonomic understanding suggests *Eudyptula minor* should be recognised as two clades, a New Zealand and an Australian clade, though the kororā of Otago fall within the Australian clade (Banks et al. 2002; 2010; Peuker et al. 2009; Clark et al. 2012, Waugh et al. 2016). Grosser et al. (2015) targeted the mitochondrial control region, mitochondrial cytochrome c oxidase subunit 1 (COI). They found a greater divergence between Australian/Otago and New Zealand *Eudyptula minor* than between Magellanic penguins (*Spheniscus magellanicus*) and African penguins (*Spheniscus demersus*). In addition to mitochondrial evidence (Banks et al. 2002; Grosser and Waters 2015), Observed variations in morphology, vocalisations, and behaviour further support the existence of two clades (Banks et al. 2002; Grosser et al. 2015, 2016, 2017).

The white-flippered penguin, a morphological variant of *Eudyptula minor* unique to the Canterbury region in New Zealand (Challies & Burleigh 2004), has also caused debate. Baker et al. (2006) reported a genetic split between white-flippered penguins (previously referred to as *Eudyptola albosignata* or *Eudyptola minor albosignata*) and other *Eudyptula minor* morphotypes. However, Banks et al. (2002) and Peuker et al. (2009) found little mitochondrial evidence supporting phylogenetic separation. Despite this, recent genomes

published by Pan et al. (2019) refer to the Australian little penguin (*Eudyptula novaehollandiae*) as a separate species, while dividing the New Zealand kororā into two subspecies; the New Zealand kororā (*Eudyptula minor minor*) and the white-flippered penguin (*Eudyptula minor albosignata*). Similarly, in a recent summary of the conservation status of New Zealand Birds, the Department of Conservation (DOC; Robertson et al. 2016) not only refers to the white-flippered penguin as a subspecies but includes three other subspecies; the northern blue penguin (*Eudyptula minor iredalei*), the southern blue penguin *(Eudyptula minor minor*) and the Australian little blue (*Eudyptula minor novaehollandiae*). In the report, *Eudyptula minor novaehollandiae* was classed as recovering. The remaining three subspecies were categorised as at risk, with a partial decline and, in the case of the white-flippered penguin, conservation dependent. Notably, the white-flippered penguin was upgraded from threatened due to successful conservation management (Robertson et al. 2016). Wilson and Mattern (2018) stressed the importance of clarifying the taxonomy of the kororā as it could have important implications for conservation management. For example, should the white-flippered penguin be reassessed as a subspecies, its management priority would likely increase.

Kororā have been proposed as a potential indicator species for the marine ecosystem (Dann and Chambers 2013; Chilvers 2017). An indicator species being an organism whose population reflects the state of the environment or other communities' health within an ecosystem (Niem and McDonald 2004; Chilvers 2017). Despite their generalist diet, kororā are range-restricted during breeding season due to the need to frequent their nest. Thus, local impacts are likely to affect the species, and changes in kororā survival and reproductive output could indicate the health of local coastlines (Dann and Chambers 2013; Chilvers 2017). The Banks Peninsula kororā population has previously undergone an extensive census (Challis et al. 2004), and recent efforts have seen the implementation of community science

to reassess the population (https://bluecradle.org/penguin-project/). Understanding this population dynamics could build a baseline for comparisons across temporal and spatial scales (Agnew et al. 2014). Notably, while *Eudyptula minor* has been extensively studied, most of this research has centred on the Phillip Island population in Australia (Wilson and Mattern 2018). It is important to investigate New Zealand populations of kororā as not only are colonies typically much smaller than in Phillip Island, but the plasticity of kororā ecology means that colonies vary in several factors critical for conservation management (Wilson and Mattern 2018). Kororā require both a healthy marine environment and a protected terrestrial habitat; therefore, understanding the population could inform management across the marine and terrestrial spheres. Wilson and Mattern (2018) identified critical research priorities for kororā, including taxonomic clarification, population monitoring, marine ecology, breeding biology and threats (e.g. predation). Environmental DNA presents one tool in which we can begin to address key concerns for kororā. For example, eDNA surveys could help map kororā habitat use and distribution, both regionally and across colonies. Thus we could investigate different disturbance scenarios faced by colonies, such as the predator-free Motunau Island (Beach et al. 1997), Banks Peninsula, with its extensive mammal trapping programme and marine reserve (Robertson et al. 2016) and the heavily urbanised St Kilda breakwater in Melbourne (Giling et al. 2008). Importantly, the captive population of kororā at the International Antarctic Centre in Christchurch means that eDNA methods can be developed and tested in a controlled setting before surveying the wild kororā of Banks Peninsula. The development of an eDNA protocol for kororā could, with further optimisation and standardisation, be applied to conservation programmes for New Zealand's endemic species.

4.1.6 Aims and objectives of this study

This study aims to optimise eDNA methods for monitoring a species of conservation concern, the kororā. Methods for the sampling, extraction, amplification and sequencing of kororā DNA will be tested using water samples from the captive kororā population at the International Antarctic Centre in Christchurch. We discuss the challenges of developing an eDNA survey and highlight the importance of optimising sampling and laboratory protocols before implementing an eDNA survey. Establishing and standardising the laboratory component of an eDNA survey or programme is often the most costly component in both time and money and can mean the difference between robust conclusions and a high level of uncertainty (Dickie et al. 2018; Zinger et al. 2019). In designing an eDNA protocol for the extraction of kororā DNA, this study looks towards establishing methods that could be applied to current monitoring efforts for the wild kororā population on the Banks Peninsula. We discuss key considerations in the New Zealand context and look to future management applications of eDNA for conserving New Zealand's unique taxa.

4.2 Methods

4.2.1 Sampling and filtration

We tested three sample types for penguin eDNA extraction, all of which were provided by the International Antarctic Centre in Christchurch. First, directly from kororā feathers, which had been stored at room temperature for an unknown period. Feathers were primarily down, though we were supplied with some moult feather samples that still appeared to have skin cells attached. For our second sample type, we used feathers soaked in water. Having observed faecal matter on the feathers, we filled sterile 50 mL tubes with feathers and MilliQ H2O and left them to soak for 2-7 days at 4 °C. Finally, our third sample type was

water directly from the penguin tank. Water was collected immediately before scheduled cleaning of the International Antarctic centre tank, which houses a number of Adult kororā, including the white-flippered morphotype. The penguin tank was exposed, and at the time of collection (approximately midday), it was overcast and raining. During this period, there were no penguins in the water and sampling was done from a single point of the tank, away from the colony. Using sterile 60 mL syringes, 300 mL of tank water was filtered through 0.22 µL (polyethersulfone membrane, gamma-irradiated) Sterivex filters (Merck, Millipore; Spens et al. 2017). After filtration, empty syringes were used to push air through the filter and remove any remaining liquid. Gloves were worn throughout the entire process. Before DNA extraction, filters were stored for a maximum of three days at 4°C.

4.2.2 Extractions

Our first sample type were kororā feather root tip extractions. These extractions were done using a modified version of the Invitrogen PureLink Genomic DNA Mini Kit protocol for mammalian tissue and mouse/rat tails. Root tips were cut directly into 180 µL Genomic digestion buffer. Initially, we used three root tips (LBP03-LBP06). After little success, we increased this to five tips. Root tips were cut, crushed and pressed so that they would sink in the digestion buffer. Feather root tips were left in the digestion buffer at room temperature overnight. Pro-K was then added, as directed in the protocol, and the mixture was vortexed for 5s and spun for 5s. The lysate was moved to the shaking heat block (set to 56°C and 750 rpm for 5 minute intervals). Samples were left for a further 48h, and an additional 20 µL Pro-k was added 2h before the end of the incubation period. Before proceeding with the protocol, the lysate was vortexed (5s) and spun (5s). The final elution volume was 50 µL, which was left to incubate at room temperature for 7 minutes prior to centrifugation.

Our second and third sample types, feathers soaked in water and penguin tank water respectively, were extracted using the same protocol. Both extractions were done directly from the enclosed Sterivex units using a modified spin-column protocol for the Qiagen DNeasy Blood and Tissue Kit (Tingley et al. 2019). One end of the Sterivex unit was sealed with parafilm, and 540 µL of ATL buffer and 40 µL Pro-k was then pipetted directly into the filter unit. The leur lock end was then sealed, and the entire unit was placed in a rotating hybridisation oven set to 56°C for 3h. Both ends of the Sterivex unit were then unsealed, and a sterile syringe was used to push the lysate into a microcentrifuge tube. 500 µL of buffer AL was added to the lysate, which was then vortexed before adding 500 µL of ethanol. The mixture volume required adding to the DNeasy Mini spin columns and centrifuging in two parts. The extraction then returned to the standard Qiagen DNeasy Blood and Tissue protocol. The only additional change was an elution volume of 50 µL, which was incubated for 7 minutes before centrifugation.

4.2.3 PCR and sequencing

Successful DNA extractions were amplified using PCR and combined forward and reverse MarVer3 primers (Valsecchi et al. 2020). MarVer 3 primers were forward: AGACGAGAAGACCCTRTG and reverse: GGATTGCGCTGTTATCCC. We used the Biotaq PCR kit (Bioline) for our reaction mix. For each PCR run, we included two positive controls (λ DNA and pipefish, *Stigmatopora nigra*) and negative control (MilliQ water). Each PCR mix used 2 µL of primers. In most cases this was the MarVer3 mix, however, the λ DNA positive control used a λ DNA specific primer. In the case of two amplifications LBP27A and LBP27B, we trialled 5 µL and 7 µL of the sample respectively, but due to similar gel electrophoresis runs (Fig. 4.1), we opted to use 5 µL for the remaining samples. Reactions were then made up to 20 µL using a master mix of 8 µL MilliQ H2O, 2 µL buffer,

2 µL dNTP0.6 µL MgCl2 and 0.4 µL TAQ per reaction. The thermocycling conditions were: an initial denaturation step for 4 minutes at 94°C followed by 38 cycles at 95°C for 30 seconds then 30 seconds at 8/10/10/10 cycles for the annealing temperatures 54°C/55°C/56°C/57°C. This was followed by 40 seconds at 72°C with a final extension at 72°C for 5 minutes (Valsecchi et al. 2020). 2.5 µL of PCR products were combined with 2.5 µL dye and electrophoresed on a 1.4 % Agarose Gel for 45 minutes at 90V. The gel was submerged in a 1x Sodium Borate buffer, and we used Invitrogen Sybr-Safe stain. An EZ ladder was also run for comparison. Successfully amplified sequences were cleaned with the DNA Clean & Concentrator-5 (Zymo Research) according to the supplied protocol. The final product was checked using High Sensitivity reagents and the Qubit fluorometer and was then given to the Canterbury Sequencing and Genotyping facility for Sanger sequencing. In addition we trialled Tele02 primers (Table 3.2) using two gradient PCRs with annealing temperatures from 44.9°C to 55.1°C and from 39.9°C to 60.3°C. The thermocycling conditions were 30 s at 95°C, 40 cycles at 95°C for 30 s, then 60 s at the annealing temperature and 30 s at 72°C, the final extension was for 5 minutes at 72°C. When this returned no results, all subsequent PCR's were run using the MarVer3 primers.

4.2.4 Phylogenetic analysis

The resulting eDNA sequences were assessed using the Megablast algorithm (Zhang et al. 2000; Morgulis et al. 2008) in the Basic Local Alignment Tool (BLASTN 2.11.0+, Altschul et al. 1990) and run against the National Centre for Biotechnology Information (NCBI) nucleotide database. We assessed sequences with a low Expect value (E-value) and a percentage identity greater than 90 %. For sequences identified as high similarity, we downloaded the GenBank 16srRNA sequences. We also downloaded the sequence for our outgroup, the emperor penguin (*Aptenodytes forsteri*, NC027938.1), in FASTA format from

the GenBank database (www.ncbi.nlm.nih.gov/genbank/), as well as sequences from the *Eudyptes* genus that scored highly (for our BLASTN search. Sequences were then assessed in Geneious Prime (v.2021.0.3; Biomatters Ltd., New Zealand; Kearse et al. 2012; https://www.geneious.com). We aligned and visually checked our sequences with the Geneious alignment algorithm (Biomatters Ltd) under default settings with a similarity of 70 % selected. One of our sequences (IAC06) did not align and was excluded from our tree. We constructed a Neighbour-Joining tree using the Hasegawa–Kishono–Yano (HKY; Hasegawa et al. 1985) model of substitution with bootstrapping replicated set to 1000. The Neighbour-Joining was rooted to the emperor penguin outgroup. The resulting tree was then edited in the Interactive Tree of Life programme (iTOL, v.4; Letunic and Bork 2019).

4.3 Results

We had no success extracting and amplifying DNA directly from the kororā feather root tips. However, we successfully extracted and amplified eDNA from seven of our nine feather-water samples and all of our tank water samples, despite initial Qubit results for our tank samples all below 0.335 Ng/ µL (Table 4.1). Comparably our feather samples range from undetectable to 1.7 Ng/ µL and none were successfully amplified, while the feather water samples ranged from 1.5-6.7 Ng/ µL (Table 4.1). Gel electrophoresis runs all showed bands in the 250 bp range as expected with the MarVer3 primers. However, the gel runs for the penguin tank samples showed a faint band in the negative control, though further analysis with the Qubit Fluorometer returned a value of <0.1 ng/µL. The successfully amplified sequences queried in BLASTN ranged from 239-263 base pairs (bp). In two cases (IAC06 and IAC08), no similar sequences when queried in BLASTN. However, all other samples returned similar alignments to three *Eudyptula minor* sequences (accession numbers: AF362763.1, DQ137164.1, DQ137163.1 [*albosignata*]) using the BLASTN metrics for similar alignment, sorted by the Expect value (E-value; Table 4.2). However, a number of our

E-values were low (Table 4.2), with one falling below e-3 for the feather water trials (LBP33, 1e-16) and three for the tank water trials (IAC02, 7e-30; IAC03, 4e-22; IAC04, 2e-15). An additional two of the feather water samples fall below e-5 (LBP24, 7e-34; LBP35, 1e-36) as do two tank samples (IA05, 6e-40; IAC07, 6e-35). While four of our tank samples had a query cover off less than 50 % (IAC03, 44 %; IAC04, 40 %; IAC05, 46 %; IAC07, 46 %). For sample LBP29 sixty-three sequences aligned with a percentage identity >90 %, with only the top four sequences from *Eudyptula minor,* most of these sequences were not from New Zealand species or species know to be sequenced within the laboratory except for the kākāpō (*Strigops habroptilus*). However, the query cover for most of these alignments was below 50 %, except for *Eudyptula minor* (80-88 %), the kākāpō (query cover 85 %, E-value 4e-37, percentage identity 92.37, max score 167 and total score 281) and the white-bellied heron (*Ardea insignis*, query cover 87 %, E-value 5e-36, percentage identity 90.91, max score 163 and total score 262) a species not found in New Zealand. Similarly, the top 100 BLASTN results for IAC01 had a percentage identity greater than 90 %, including high similarity to the northern rockhopper penguin (*Eudyptes moseleyi*; query cover 73 %, E-value 6e-65, percentage identity 91.98, max score 259 and total score 259) as well as the royal penguin (*Eudyptes schlegeli),* macaroni penguin (*Eudyptes chrysolophus)* and snares penguin (*Eudyptes robustus*) all returning a query cover 73 %, E-value 4-62, percentage identity 90.91, max score 250 and total score 250. However, our Neighbour-Joining tree shows all eDNA sequences clustered with the GenBank *Eudyptula minor* sequences (Fig 4.3), except for IAC6, which did not successfully align. The bootstrap value associated with this cluster is 99.9 showing high confidence in their position. However, the associated branch lengths are long. This is particularly true for the samples taken from the International Antarctic Centre penguin tank which range from 0.45-0.63 substitutions per site, showing a high level of

divergence. Comparatively, the branch lengths for the GenBank *Eudyptula minor* sequences show a distance of 0.09 from the *Aptenodytes fosteri* outgroup.

Table 4.1. Qubit Fluorometer results (Ng/ µL) for little penguin (*Eudyptula minor)* trials. Indicating the source (Feather, Feather Water and Tank Water), whether DNA was successfully amplified (Y=yes, N=no) and the pre- and post-PCR DNA yield after amplification with MarVer3 primer in nanograms per microlite (Ng/ µL).

Sample	***Source***	***DNA Amplified***	***Ng/ µL Pre-PCR***	***Ng/ µLPost-PCR***
LBP03	Feathers	N	<0.01	-
LBP04	Feathers	N	<0.01	-
LBP05	Feathers	N	<0.01	-
LBP06	Feathers	N	1.7	-
LBP07	Feathers	N	0.27	-
LBP08	Feathers	N	0.44	-
LBP09	Feathers	N	0.40	-
LBP10	Feathers	N	<0.01	-
LBP11	Feathers	N	0.06	-
LBP12	Feathers	N	0.19	-
LBP13	Feathers	N	0.20	-
LBP14	Feathers	N	0.27	-
LBP15	Feathers	N	0.12	-
LBP16	Feathers	N	0.21	-
LBP17	Feathers	N	0.26	-

LBP18	Feathers	N	<0.01	-
LBP19	Feathers	N	<0.01	-
LBP20	Feathers	N	0.42	-
LBP22	Feathers	N	0.18	-
LBP23	Feathers	N	0.89	-
LBP24	Feathers	N	0.94	-
LBP21	Feather water	Y	0.15	22.3
LBP25	Feather water	Y	1.99	24.0
LBP27	Feather water	Y	6.70	11.4
LBP28	Feather water	Y	1.43	23.3
LBP29	Feather water	Y	0.83	27.4
LBP32	Feather water	N	3.01	-
LBP33	Feather water	Y	2.01	4.8
LBP34	Feather water	N	2.20	-
LBP35	Feather water	Y	0.62	24.3
IAC01	Tank water	Y	0.335	19.1
IAC02	Tank water	Y	0.220	17.8
IAC03	Tank water	Y	0.265	10.4
IAC04	Tank water	Y	0.272	9.9
IAC05	Tank water	Y	0.297	22.8
IAC06	Tank water	Y	0.323	11.7

IAC07	Tank water	Y	0.294	11.6
IAC08	Tank water	Y	0.335	6.6

Table 4.2. BLASTN query results indicating the species and accession numbers for the sequences most closely aligned to our eDNA sequences. LBP samples are all from feather water extractions, while IAC samples were from tank water at the International Antarctic Centre. The table shows the Query length of our eDNA sequence in base pairs (bp) as well as metrics for similarity; Percentage identification, Expect value (E-value), Query cover (%) as well as the highest alignment score (Max score)and the sum of these scores (Total score).

Sample	Species Match	Accession Number	Percentage ID (%)	E-value	Query cover (%)	Max Score	Total Score	Length (BP)
LBP21	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	90.76	7e-34	78	156	273	239
LBP25	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	96.02	3e-97	88	366	366	253
LBP27A	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	90.09	2e-73	88	287	287	250
LBP27B	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	91.44	9e-78	86	302	302	252
LBP28	*Eudyptula minor/*	AF362763.1 DQ137164.1 DQ137163.1	88.64	1e-66	86	265	265	251

	E.m. (albosignata)							
LBP29	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	93.28	4e-46	88	196	312	247
LBP33	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	86.52	1e-16	61	99	188	255
LBP35	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	91.60	1e-36	88	165	288	247
IAC01	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	97.33	4e-82	73	316	316	253
IAC02	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	88.24	7e-30	77	143	266	260
IAC03	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	84.62	4e-22	44	143	264	263
IAC04	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	83.33	2e-15	40	95.3	95.3	249
IAC05	*Eudyptula minor/*	AF362763.1 DQ137164.1	93.28	6e-40	46	176	176	254

	E.m. (albosignata)	DQ137163.1						
IAC06	*No sig. similarity found*	-	-	-	-	-	-	261
IAC07	*Eudyptula minor/ E.m. (albosignata)*	AF362763.1 DQ137164.1 DQ137163.1	90.76	6e-35	46	159	159	255
IAC08	*No sig. similarity found*	-	-	-	-	-	-	259

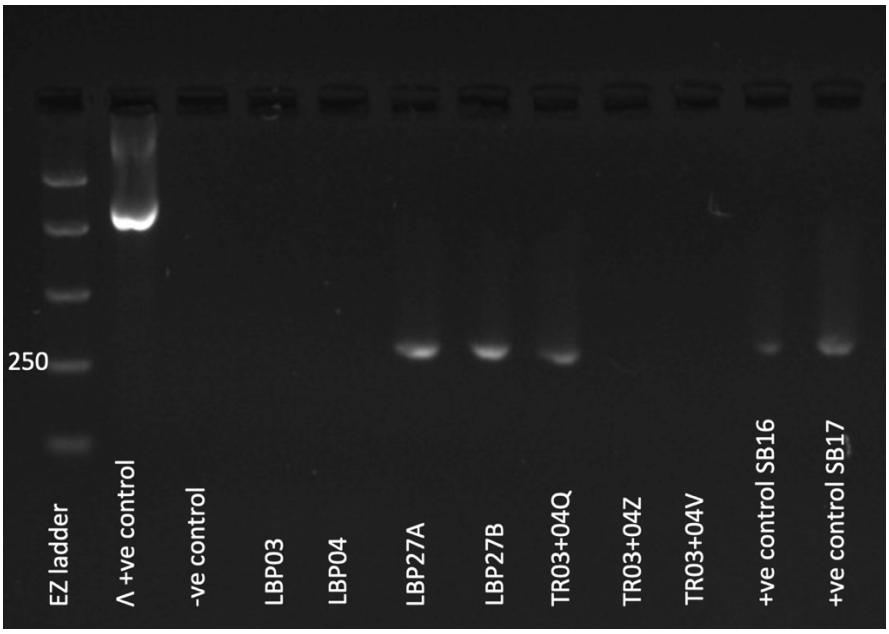

Figure 4.1. A 1.4% Agarose Gel electrophoresis image with all successful bands showing in the 250 bp region. The run included three positive controls, a lambda DNA control (λ +ve control), and two pipefish DNA controls (+ve control SB16 and SB17), in addition to a negative control (-ve control). All samples were amplified using MarVer3 primers. LBP03 and LBP04 are direct *Eudyptula minor* feather extractions. The LBP27 was amplified with different volumes of sample, 5 µL (A) and 7 µL (B). TR03+04 (Q, V, Z) are all from snow samples (Chapter Three)

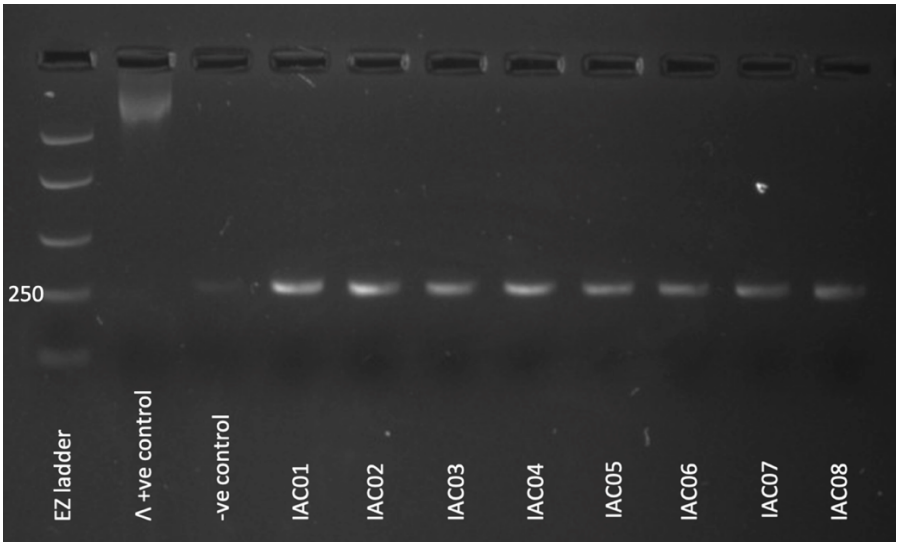

Figure 4.2. Gel electrophoresis image showing the eDNA samples from the Eudyptula minor tank at the International Antarctic Centre in Christchurch. All bars are in the 250bp region. There is some contamination in the negative control (-ve control). The lambda positive control (λ +ve control) is not expected to show bands in the same region.

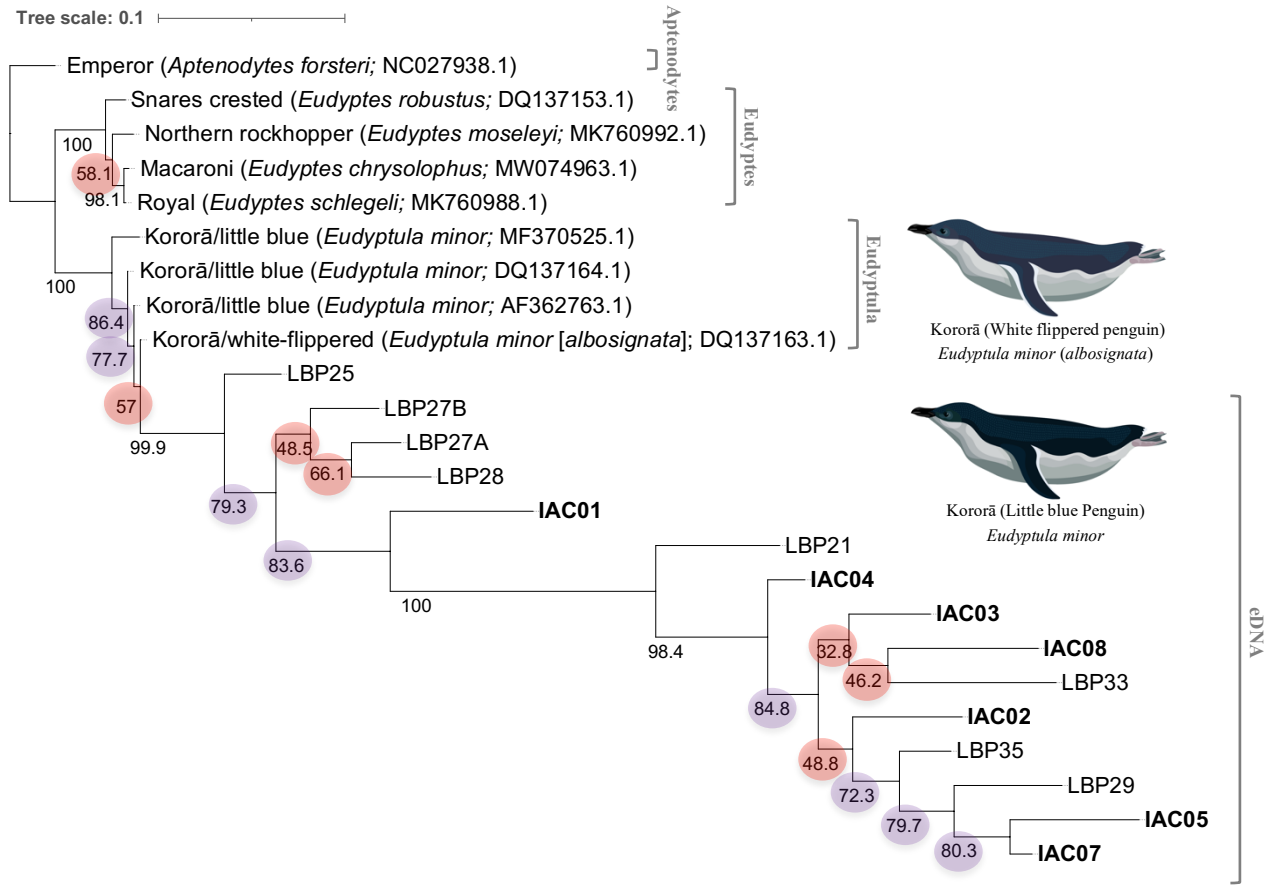

Figure 4.3. Neighbour-Joining comparing feather water and tank water eDNA samples to penguin (order *Sphenisciformes*) 16s rRNA sequences retrieved from Genbank. Using the Emperor penguin (Aptenodytes forsteri) as our outgroup. The neighbour-joining tree was constructed using the Hasegawa–Kishono–Yano (HKY) distance model with bootstrap values of 1000 replicated. Red circles indicate values <70, purple 70<n<95. Samples taken from the International Antarctic centre tank are shown in bold.

4.4 Discussion

This study investigated the potential application of non-invasive samples for monitoring kororā, specifically using feather and water samples. While we had no success extracting and amplifying DNA from the feather root tips, we were able to retrieve DNA sequences from both self-made feather water and the penguin tank at the International Antarctic Centre in Christchurch, highlighting the value of eDNA surveys. All but one (IAC06) of these sequences aligned with GenBank *Eudyptula minor* sequences through a BLASTN query (Table 4.2). Likewise, all but IAC06 were clustered with *Eudyptula minor* on our Neighbour-

Joining tree, and the cluster was associated with a high bootstrap value (Fig 4.1). However, long branch lengths in our phylogenetic tree suggest a higher degree of divergence than we would expect within the species, likely due to the low quality of the sequence and degradation (Wu et al. 2012). The tank water at the International Antarctic Centre is treated with Ozone which may be contributing to DNA degradation (Cataldo 2006). Similarly, the BLASTN results for two of our samples (IAC06 and IAC08) failed to align with sequences in the database. Additionally, two samples (LBP29 and IAC01) flagged a high number of close alignments with other species, the majority of which are not found in New Zealand. As we move forward with these methods we will need to investigate these issues as they could lead to erroneous conclusions. Further optimisation is therefore required to improve the sensitivity of our protocol design before this study can be expanded to monitor wild kororā populations.

We were unable to extract and amplify DNA directly from the little penguin feathers, despite using a protocol that had previously been successful with feathers from other species. While we used the Invitrogen PureLink extraction kit, Clark et al. (2013) successfully extracted DNA from penguin feathers used a modified version of the Qiagen DNeasy kit. Comparatively, Banks et al. (2002) used a high salt protocol (White et al. 1990). Therefore, future analysis could explore alternative kits for extractions from feathers. However, both Banks et al. (2002) and Clarke et al. (2012) used feathers plucked directly from penguins while our feather samples were of unknown age and had been stored at room temperature; therefore, DNA degradation is likely to be higher. Peucker et al. (2009) used recently moulted feathers in their extractions, which could be a non-invasive option for further feather analysis. Despite DNA degradation posing a challenge in our study, DNA has been successfully extracted from the subfossil feathers of moa using the Qiagen Dneasy kit (Rawlence et al. 2009). We suggest looking to aDNA protocols when extracting potentially degraded DNA, including those designed for museum specimens (Horváth et al. 2004;

Speller et al. 2011). While we increased our incubation step considerably from the manufacturer's protocol to improve cell digestion, other steps could be taken to potentially increase the success of extractions, for example, by heating the buffer to 70 °C immediately before the lysis step (Peters et al. 2020), alternatively using buffers with a higher concentration of dithiothreitol (DTT) or detergents (Campos and Gilbert 2019). Given the primary purpose of this study was to develop eDNA methods, and we had success extracting DNA from feather water, we did not pursue this avenue further and instead focused on feather water and tank samples.

The use of enclosed Sterivex units for filtering our samples improved the first stage of the eDNA process. Using a syringe was faster than gravity filtration and didn't require leaving eDNA samples at room temperature for extended periods. Likewise, filter units negated the need to transport large amounts of liquid into the laboratory and allowed for filtering directly from the penguin tank. The process of filtering was simple and could easily be outsourced to community scientists with minimal training. Sterivex filters have previously yielded greater amounts of eDNA compared to other filter types, including glass fibre filters (Spens et al. 2017). However, Li et al. (2018) found Sterivex units did not perform as well as Mixed Cellulose Ester (MCE) membrane filters, though the difference was minimal, and Sterivex units were still recommended for filtering water samples in the field. Takahashi et al. (2020) also compared Sterivex units to Glass Fibre filters and found a greater sensitivity in the Sterivex units. However, whilst Sterivex units outperformed Glass Fibre filters when the concentration of eDNA in the environment was low, the opposite was true when concentration was high. It is important to consider such variations in future studies, especially when looking to draw comparisons between datasets.

In this study, we utilised traditional PCR and while traditional PCR is used for single-species detection in eDNA studies (Ficetola et al. 2008; Jerde et al. 2011; Goldberg et al.

2011; Dejean et al. 2012), as the field has progressed, quantitative PCR (qPCR) has become a more common method (Mcoll-Gausden et al. 2019). The benefit of qPCR is that it allows for a quantification of copy number and an improved understanding of the amount of eDNA in the environment (Cristescu and Herbert 2018; Beng and Corlett 2020). Recent advances in technology have seen the application of methods such as droplet digital PCR (ddPCR), the development of this technology has been critical for the novel application of eDNA surveys to fields such as population genetics (Doi et al. 2015; Baker et al. 2018). Regardless of the method used, the amplification of DNA can lead to the introduction of biases in eDNA studies. These concerns have been highlighted and reviewed extensively by Lear et al. (2018). In particular, contamination can introduce false positives and lead to erroneous conclusions. While the uncertainty we saw in our BLASTN results may be down to multiple sources of DNA in the penguin tank, contamination may also have been introduced during our laboratory work. This is of particular concern for our tank samples as the gel indicated there had been a low level of contamination in the negative control (Fig. 4.2). In their extensive review on eDNA methods Lear et al. (2018) recommends the use of a laminar flow cabinet with UV light for a PCR setup, the use of both positive and negative controls and an awareness of the potential biases and error in amplification methods. For eDNA analysis, replicate PCRs are recommended to account for amplification error. Moving forward in our protocol design and considering applying our methods to wild populations, we need to further investigate minimising and accounting for potential contamination in the laboratory.

When applying eDNA surveys in a New Zealand context, it is critical to consider the management of the genetic data generated. The kororā is a taonga species and therefore require a consultation with the relevant Iwi, Rūnanga, and Hapū. However, researchers must take care not to project a one-sided discussion and continue communications and collaboration beyond these early stages (Collier-Robinson et al. 2019). Research frameworks

should be co-developed regarding genetic data (Collier-Robinson et al. 2019; Holdaway et al. 2017; Handsley-Davis et al. 2020). Likewise, Māori voices, priorities and concerns should be centred in New Zealand's genetic research, and eDNA is no exception. In their publication of the genomes of five taonga penguin species, including the kororā, Pan et al. (2019) stressed that though the data is publicly available, it is critical for researchers seeking to utilise taonga genomes engage with Iwi, Hapū and Rūnanga and show respect to the cultural significance of genomic data. Alternatively, genomic data has been stored on password protected databases, ensuring communication (Galla et al. 2019, 2020). If implemented collaboratively, with protections in place for genetic data, applying eDNA in the New Zealand context could help improve the conservation outcomes for taonga species such as the kororā, while minimising the impact on a culturally significant species.

Non-invasive molecular tools, such as eDNA, should be investigated for monitoring New Zealand's endemic fauna as stress responses in wildlife are often difficult to assess (Bejder et al. 2009; Romero et al. 2015; Tablado and Jenni 2017). Reported responses of kororā to human interactions vary from no response to having an impact on breeding (Klomp et al. 1991; Dann 1992; Carroll et al. 2016). Captive penguins have been reported to increase aggression and avoidance in response to visitors at zoos (Sherwen et al. 2015) while increasing visitor distance reduced stress behaviours (Chiew et al. 2019). Understanding stress in response to tourism is important, as kororā are a species that attracts tourists across both Australia and New Zealand (Dann and Chambers 2013; Carroll et al. 2016). While the long-term effects of research disturbance on reproductive success are unclear (Vertigan and McMahon 2012), kororā experience a greater increase in the hormone corticosterone than other penguin species in response to stressors, such as handling (Carroll et al. 2016; Cockrem et al. 2017). Carroll et al. (2016) found that prior disturbance led to an increase in both hormone response and aggressive behaviour during consequent handing. However, Cockrem

et al. (2017) found little difference in corticosterone levels across two successive years of sampling. Despite this uncertainty, it is considered best practice to avoid the disturbance of species where possible (Costello et al. 2016; Zemanova et al. 2020; Steinmetz et al. 2021). While handling by researchers may be stressful for an individual, the information gained through the research can be invaluable; however, non-invasive methods can be implemented alongside traditional surveys (Young et al. 2019; Steinmetz et al. 2021). Carroll et al. (2016) highlighted the value of investigating less invasive methods for monitoring kororā. We suggest eDNA should be considered for non-invasive genetic sampling. As a growing field, eDNA surveys could be used to inform conservation concerns for kororā and other endemic species.

4.4.1 Understanding trophic interactions

DNA metabarcoding of faecal DNA can be used to make inferences about trophic interactions. For example, a recent metabarcoding study used faeces from latrine sites to assess the died of an endangered New Zealand penguin, the hoiho (or yellow-eyed penguin; Young et al. 2019). Similar methods have already been utilised to monitor the Australian population of little penguins on Phillip Island (Cavallo et al. 2018, 2020). In the case of soft-bodied prey, eDNA outperforms traditional dietary analyses, which are bias against gelatinous organisms such as salps, a component of the Phillip island little penguin diet (Cavallo et al. 2018). Thus, eDNA not only provides comparable identification data to traditional monitoring methods, but eDNA surveys can provide additional information on trophic interactions. While eDNA surveys can address biases in traditional monitoring methods such as stomach flushing, they are not without biases themselves. Critically, eDNA surveys do not provide data on prey quality metrics such as size, biomass or proportion, and are proposed as a complementary tool, not a replacement for conventional dietary analysis (Young et al. 2019). Likewise, eDNA cannot differentiate between direct predation and

secondary prey consumption, e.g. DNA may be detected from taxa previously consumed by prey species prior to predation (Cavallo et al. 2018), though the likelihood of the DNA of gelatinous organisms persisting through multiple digestions is low (Cavallo et al. 2018) and secondary prey is still a key component of the food web. Long term monitoring of faecal DNA can infer both short-term (e.g. weekly; Cavallo et al. 2020) and long-term (e.g. seasonal; Cavallo et al. 2018) shifts in diet and be used to assess community dynamics, particularly in the face of disturbance. Further, faecal eDNA could be utilised to inform ecosystem-based management through comparisons with concurrent datasets such as local fisheries catch or annual kororā reproductive success. For example, Cavallo et al. (2018) combined faecal DNA metabarcoding with data on breeding stage to assess changes in foraging patterns. In addition to breeding stage, Cavallo et al. (2020) combined faecal DNA data with data from Automated Penguin Monitoring Systems (APMS; Chiaradia and Kerry 1999). These monitoring systems generate data on penguin weights and foraging lengths by scanning passive identification transponder (PIT) tags as the penguins pass over weighbridges between foraging trips. Cavallo et al. (2020) discussed the benefits of combined monitoring tools for more sensitive monitoring of prey availability in the local ecosystem. This could be particularly beneficial in prey species that quickly outgrow kororā predation, as it could be used to monitor juvenile cohorts (Cavallo et al. 2020). Long term monitoring of diet is important for increasing the sensitivity of ecosystem monitoring, and faecal DNA metabarcoding could increase the adaptability of kororā conservation by providing an early warning for potential disturbances in the food chain (Cavallo et al. 2018, 2020). It is critical that monitoring of kororā diet is implemented regionally as kororā prey species can vary considerably between colonies (Fraser and Lalas 2004; Flemming et al. 2013; Flemming and van Heezik 2014; Wilson and Mattern 2018). Thus, molecular tools

could inform regionally specific conservation management for the Banks Peninsula kororā and contribute to our understanding of the health of the local marine ecosystem.

4.4.2 Distribution mapping

Environmental DNA can be used to look at species across an ecosystem. A recent study by Jeunen et al. (2020) compared eDNA extractions to Baited Remote Underwater Video (BRUV) surveys at Rakiura (Stewart Island, New Zealand). While the study indicated several potential biases, Jeunen et al. (2020) highlight the breadth of taxa that can be detected using eDNA, including penguins. Few papers have looked at the detection of penguins in the marine environment. Mariani et al. (2019) detected chinstrap penguins in sponges (*Porifera*) collected from the Antarctic Peninsula. While previous studies have extracted penguin DNA in controlled systems such as aquariums (Valsecchi et al. 2020) and zoos (Ushio et al. 2018). Environmental DNA surveys could potentially be implemented across New Zealand to detect the foraging grounds of kororā. We recommend such surveys also target kororā predators and prey DNA to get a more complete picture of species interactions. While there is value in monitoring kororā as a conservation dependent species, they have also been suggested as potential indicators of disturbance in the marine environment (Dann and Chambers 2013; Chilvers 2017). Through metabarcoding such indicator species as proxies, we can inform assessments of marine health (Barnes and Turner 2016; Baillie et al. 2019; Closek et al. 2019; DiBattista et al. 2020). In order to implement an ecosystem-wide survey, further work would be required on DNA persistence and dispersal, though current research indicates a distinction between the eDNA detected at different marine sites and different depths (Jeunen et al. 2018, 2019, 2020). Additionally, the early success of community science initiatives such as the Open Waters Aotearoa, Wai tūwhera o te Taiao programme (https://www.epa.govt.nz/commVunity-involvement/open-waters-aotearoa/) indicates a

potential for public engagement. Involving the public in eDNA surveys for kororā could increase engagement with a conservation dependant taonga species. New Zealand's coastal species are often impacted by both marine and terrestrial disturbances, which can pose a challenge to conservation efforts (Hocken et al. 2000; Croxall et al. 2012; Wilson and Mattern 2018; Whitehead et al. 2019). Integrating eDNA surveys with the fisheries may present an opportunity to better assess overlap with species such as kororā and the impact of primary industry on marine health (Lacoursière-Roussel et al. 2015; Thompsen et al. 2016; Hansen et al. 2018; Baillie et al. 2019; Jerde 2019; Gilbey et al. 2021). Whilst still an application requiring further investigation, recent studies suggest eDNA may assist with future estimates of fish abundance, thus better-informing catch quotas (Thompsen et al. 2016; Hansen et al. 2018; Salter et al. 2019; Muri et al. 2020). However, we must first address the challenges posed by the ecology of eDNA (Elbracht and Leese 2015; Fonesca 2018; Bylemans et al. 2018). Monitoring disturbances in the marine environment is important, not just for kororā, but for a number of New Zealand's important marine species, including the five other penguin species that breed around New Zealand's waters; the endangered hoiho (yellow-eyed penguin) and near-threatened tawaki (Fiordland crested penguin, *Eudyptes pachyrhynchus*) which both breed on the mainland, as well as the endangered erect-crested penguin (*Eudyptes sclateri*), the vulnerable snares penguin (IUCN Red List, BirdLife 2020) and the eastern rockhopper penguin (*Eudyptes filholi*).

4.4.3 Invasive species management

While this study utilised eDNA to detect kororā in water samples, the protocol could be adapted for the surveillance of invasive species in their terrestrial habitat. In a survey of kororā mortalities in Otago, Hocken (2000) found that 25 % of the fatalities were predation events. In the cases where the cause of predation was identifiable, kororā were killed primarily by dogs (14 %), Mustelids (9.4 %) and sharks (8 %). Of the Mustelids, stoats

(*Mustela erminea*), weasels (*Mustela nivalis*) and ferrets (*Mustela furo*) all pose a threat to New Zealand's endemic fauna (Hocken 2000; Challis 2015; Wilson and Mattern 2018). A study by Hocken (2000) suggested ferrets were the most significant of the mustelid predators. Similarly, the predation of white-flippered penguins in Harris Bay on the Banks peninsula was attributed primarily to ferrets by Challies (2015). However, while ferrets pose a considerable threat to the kororā of Banks Peninsula, their distribution is patchy (Parkes, 2009; Curnow et al. 2017), and their numbers declined in the early 2000s as the result of rabbit haemorrhagic disease (Challies 2015). By contrast, stoats are distributed across the Peninsula (Parkes, 2009; Curnow et al. 2017). Studies on the predation of kororā correlate trapping data with data on fatalities attributed to Mustaleids; thus, the species responsible for the fatality cannot always be determined (Challis 215, Wilson and Mattern 2018). One potential tool for addressing this uncertainty is eDNA. Predator species can be identified by extracting DNA from residual saliva in bite wounds (Mumma et al. 2014; van Bleijswijk et al. 2014; Peelle 2016; Drymon et al. 2019; Piaggio et al. 2020). These methods are also applicable to egg predation (Steffens et al. 2012; Hopken et al. 2016). However, in a study comparing camera traps to saliva DNA from the predated eggs of black-fronted terns (*chlidonias albostriatus*) in the Marlborough region of New Zealand, Steffens et al. (2012) cautioned that detectability may vary among predators. Such methods require thorough optimisation and validation before they are implemented in management programmes. Molecular methods have also been proposed for assessing prey species in the stomach contents of trapped stoats, with an assay designed to identify kiwi DNA (*Apteryx spp*.; Harmoinen 2019). Such studies could contribute to the research priorities identified by Mattern and Waters (2018), specifically identifying the cause of mortalities in monitored colonies, such as the white-flippered penguins of Banks Peninsula. Additionally, eDNA surveys could contribute to identifying the regional impacts of predators on kororā.

Mapping the distribution of invasive predators informs subsequent management and control; thus, monitoring is an essential aspect of New Zealand's Predator Free 2050 initiative (Tompkins, 2018). In addition to an extensive trapping programme (Pickerell et al. 2014), the Department of Conservation utilises predator detection methods, e.g. tracking tunnels and camera traps (Pickerell et al. 2014), to assess the success of trapping in an area or for the surveillance of predator-free sites (Parlato and Armstrong 2018). Environmental DNA surveys could contribute to a clearer picture of trapping success or failure. In Europe, Ushio et al. (2017) utilised water samples to detect terrestrial mammals, including Mustelids. Similar applications could improve our understanding of Mustelid distribution in regions such as the Banks Peninsula, contributing to our understanding of the threat faced by kororā.

4.5 Conclusions

We successfully extracted little penguin DNA in a controlled setting from both our feather water and our tank water samples. While we were unsuccessful with direct feather extractions, this challenge emphasises the value of using environmental samples. Short, degraded sequences leading to poor alignments and a high level of inferred divergence between samples is a concern for species delineation in future applications. However, we suggest that with further optimisation, our protocol will apply to wild populations of kororā. We recommend considering the use of enclosed filter units such as Sterivex for simplifying filtration steps and reducing the contamination risk during the sampling stage. With further optimisation, eDNA surveys could contribute to the monitoring and management of a conservation dependant taonga species, the kororā. Importantly, eDNA surveys are non-invasive. Thus, eDNA methods present a promising tool for monitoring New Zealand's unique taxa while minimising the risks of causing harm, inducing stress and impacting

behaviour. It is critical that eDNA studies are carried out in consideration of their relevance to Māori whakapapa and are built on open communication with respect for data sovereignty. Increasingly, researchers in New Zealand are utilising eDNA for monitoring New Zealand's unique fauna. With continued optimisation and the development of novel applications, eDNA represents a promising survey method for future research in Aotearoa.

4.6 References

Adams CI, Knapp M, Gemmell NJ, Jeunen G-J, Bunce M, Lamare MD, Taylor HR. 2019. Beyond biodiversity: can environmental DNA (eDNA) cut it as a population genetics tool? Genes .10(3): 192.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology. 215(3): 403-410.

Baillie SM, McGowan C, May-McNally S, Leggatt R, Sutherland BJ, Robinson S. 2019. Environmental DNA and its applications to Fisheries and Oceans Canada: National needs and priorities, Canadian Technical Report of Fisheries and Aquatic Science. 3329

Baker AJ, Pereira SL, Haddrath OP, Edge KA. 2006. Multiple gene evidence for expansion of extant penguins out of Antarctica due to global cooling. Proceedings of the Royal Society B: Biological Sciences. 273(1582): 11-17.

Baker CS, Steel D, Nieukirk S, Klinck H. 2018. Environmental DNA (eDNA) from the wake of the whales: droplet digital PCR for detection and species identification. Frontiers in Marine Science. 5:133.

Banks JC, Mitchell AD, Waas JR, Paterson AM. 2002. An unexpected pattern of molecular divergence within the blue penguin (*Eudyptula minor*) complex. Notornis. 49(1): 29-38.

Banks JC, Kelly LT, Falleiros R, Rojahn J, Gabrielsson R, Clapcott J. 2021. Detecting the pest fish, Gambusia affinis from environmental DNA in New Zealand: a comparison of methods. New Zealand Journal of Zoology. 1-15.

Barnes MA, Turner CR. 2016. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics. 17(1): 1-17.

Beach G, Wilson K-J, Bannock CA. 1997. A survey of the birds, lizards and mammals of Motunau Island, Canterbury, New Zealand, with emphasis on the effects of vegetation change on the breeding success of burrowing seabirds. Lincoln University Wildlife Management Report series 14.

Bejder L, Samuels A, Whitehead H, Finn H, Allen S. 2009. Impact assessment research: use and misuse of habituation, sensitisation and tolerance in describing wildlife responses to anthropogenic stimuli. Marine Ecology Progress Series. 395: 177-185.

Beng KC, Corlett RT. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and conservation. 29(7): 2089-2121.

BirdLife International. 2020. Eudyptula minor. The IUCN Red List of Threatened Species 2020: e.T22697805A184753545. https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697805A184753545.en. Downloaded on 17 March 2021.
Blue Cradle. (2020). Penguin project. [Accessed 2021 Mar 17] https://bluecradle.org/penguin-project/
Boast AP, Weyrich LS, Wood JR, Metcalf JL, Knight R, Cooper A. 2018. Coprolites reveal ecological interactions lost with the extinction of New Zealand birds. Proceedings of the National Academy of Sciences 115(7): 1546-1551.
Boyer S, Yeates GW, Wratten SD, Holyoake A, Cruickshank RH. 2011. Molecular and morphological analyses of faeces to investigate the diet of earthworm predators: example of a carnivorous land snail endemic to New Zealand. Pedobiologia .54: S153-S158.
Boyer S, Wratten SD, Holyoake A, Abdelkrim J, Cruickshank RH. 2013. Using next-generation sequencing to analyse the diet of a highly endangered land snail (Powelliphanta augusta) feeding on endemic earthworms. PLoS One 8(9): e75962.
Bulman SR, McDougal RL, Hill K, Lear G. 2018. Opportunities and limitations for DNA metabarcoding in Australasian plant-pathogen biosecurity. Australasian Plant Pathology. 47(5): 467-474.
Bylemans J, Furlan EM, Gleeson DM, Hardy CM, Duncan RP. 2018. Does size matter? An experimental evaluation of the relative abundance and decay rates of aquatic environmental DNA. Environmental science & technology. 52(11): 6408-6416.
Campos PF, Gilbert MTP. 2019. DNA Extraction from Keratin and Chitin. Ancient DNA, Springer. Pp. 57-63.
Cargill CP, Judkins AG, Weir JS. 2020. Distribution of little penguins (*Eudyptula minor*) along the greater Kaikōura coastline, New Zealand. New Zealand Journal of Marine and Freshwater Research: 1-16.
Carroll E, Gallego R, Sewell M, Zeldis J, Ranjard L, Ross H, Tooman L, O'Rorke R, Newcomb R, Constantine R. 2019. Multi-locus DNA metabarcoding of zooplankton communities and scat reveal trophic interactions of a generalist predator. Scientific reports 9(1): 1-14.
Carroll G, Turner E, Dann P, Harcourt R. 2016. Prior exposure to capture heightens the corticosterone and behavioural responses of little penguins (*Eudyptula minor*) to acute stress. Conservation Physiology 4(1).
Cataldo F 2006. DNA degradation with ozone. International journal of biological macromolecules 38(3-5): 248-254.
Cavallo C, Chiaradia A, Deagle BE, Hays GC, Jarman S, McInnes JC, Ropert-Coudert Y, Sánchez S, Reina RD. 2020. Quantifying prey availability using the foraging plasticity of a marine predator, the little penguin. Functional Ecology 34(8): 1626-1639.
Challies CN. 2015. Predation of white-flippered penguins (*Eudyptula minor albosignata*) by ferrets (Mustela furo) in Harris Bay, Banks Peninsula, New Zealand. Notornis 62: 202-208.
Challies CN, Burleigh RR. 2004. Abundance and breeding distribution of the white-flippered penguin (*Eudyptula minor albosignata*) on Banks Peninsula, New Zealand. Notornis 51(1): 1-6.
Chiaradia AF, Kerry KR. 1999. Daily nest attendance and breeding performance in the little penguin *Eudyptula minor* at Phillip Island, Australia. Marine Ornithology. 27: 13-20.
Chiew SJ, Butler KL, Sherwen SL, Coleman GJ, Fanson KV, Hemsworth PH. 2019. Effects of regulating visitor viewing proximity and the intensity of visitor behaviour on little penguin (*Eudyptula minor)* behaviour and welfare. Animals. 9(6): 285.

Chilvers BL. 2017. Comparison of New Zealand's little blue penguins, *Eudyptula minor*, diving behaviour. Polar Biology 40(10): 1965-1974.
Clark JA, Banks JC, Waas JR. 2013. Economical genotyping of little blue penguin (*Eudyptula minor)* clades from feather-based DNA. New Zealand Journal of Ecology: 146-150.
Closek CJ, Santora JA, Starks HA, Schroeder ID, Andruszkiewicz EA, Sakuma KM, Bograd SJ, Hazen EL, Field JC, Boehm AB. 2019. Marine vertebrate biodiversity and distribution within the central California Current using environmental DNA (eDNA) metabarcoding and ecosystem surveys. Frontiers in Marine Science. 6: 732.
Cockrem JF, Candy EJ, Barrett DP, Agnew P, Potter MA. 2017. Individual variation and repeatability of corticosterone responses of little penguins (*Eudyptula minor*) sampled in two successive years at Oamaru, New Zealand. General and Comparative Endocrinology 244: 86-92.
Collier-Robinson L, Rayne A, Rupene M, Thoms C, Steeves T. 2019. Embedding indigenous principles in genomic research of culturally significant species: a conservation genomics case study. New Zealand Ecology 43(3): 2289
Collins RA, Armstrong KF, Holyoake AJ, Keeling S. 2013. Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. Biological Invasions. 15(6): 1209-1215.
Costello MJ, Basher Z, McLeod L, Asaad I, Claus S, Vandepitte L, Yasuhara M, Gislason H, Edwards M, Appeltans W.. 2017. Methods for the study of marine biodiversity. The GEO handbook on biodiversity observation networks, Springer, Cham. Pp. 129-163.
Cristescu ME, Hebert PD.. 2018. Uses and misuses of environmental DNA in biodiversity science and conservation. Annual Review of Ecology, Evolution, and Systematics. 49: 209-230.
Croxall JP, Butchart SHM, Lascelles BEN, Stattersfield AJ, Sullivan BEN, Symes A, Taylor P.. 2012. Seabird conservation status, threats and priority actions: a global assessment. Bird Conservation International. 22(1): 1-34.
Curnow M, Kerr GN. 2017. Predator free Banks Peninsula: scoping analysis, Lincoln University. LEaP Research Report 44.
Dann P. 1991. Distribution, population trends and factors influencing the population size of little penguins *Eudyptula minor* on Phillip Island, Victoria. Emu. 91(5): 263-272.
Dann P, Chambers L. 2013. Ecological effects of climate change on little penguins *Eudyptula minor* and the potential economic impact on tourism. Climate Research. 58(1): 67-79.
Day K, Campbell H, Fisher A, Gibb K, Hill B, Rose A, Jarman SN. 2019. Development and validation of an environmental DNA test for the endangered Gouldian finch. Endangered Species Research 40: 171-182.
Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus. Journal of applied ecology. 49(4): 953-959.
Di Muri C, Lawson Handley L, Bean CW, Li J, Peirson G, Sellers GS, Walsh K, Watson HV, Winfield IJ, Hänfling B. 2020. Read counts from environmental DNA (eDNA) metabarcoding reflect fish abundance and biomass in drained ponds. Metabarcoding and Metagenomics. 4: 97-112.
DiBattista JD, Reimer JD, Stat M, Masucci GD, Biondi P, De Brauwer M, Wilkinson SP, Chariton AA, Bunce M. 2020. Environmental DNA can act as a biodiversity barometer of anthropogenic pressures in coastal ecosystems. Scientific Reports. 10(1): 8365.

Dickie IA, Boyer S, Buckley HL, Duncan RP, Gardner PP, Hogg ID, Holdaway RJ, Lear G, Makiola A, Morales SE. 2018. Towards robust and repeatable sampling methods in eDNA-based studies. Molecular Ecology Resources. 18(5): 940-952.
Doi H, Uchii K, Takahara T, Matsuhashi S, Yamanaka H, Minamoto T. 2015. Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. PloS one. 10(3): e0122763.
Doyle EJ. 2015. Assessing the use of molecular techniques to determine terrestrial invertebrate diversity in New Zealand mangroves and detection of blue ducks in New Zealand streams. [MAsters thesis] University of Waikato.
Drymon JM, Cooper PT, Powers SP, Miller MM, Magnuson S, Krell E, Bird C. 2019. Genetic Identification of Species Responsible for Depredation in Commercial and Recreational Fisheries. North American Journal of Fisheries Management 39(3): 524-534.
Environmental Protection Agency. Wai Tūwhera o te Taiao – Open Waters Aotearoa [Accessed 2021 Mar 17] https://www.epa.govt.nz/community-involvement/open-waters-aotearoa/
Elbrecht V, Leese F. 2015. Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass—sequence relationships with an innovative metabarcoding protocol. PloS one 10(7): e0130324.
Emami-Khoyi A, Hartley DA, Paterson AM, Boren LJ, Cruickshank RH, Ross JG, Murphy EC, Else T-A. 2016. Identifying prey items from New Zealand fur seal (Arctocephalus forsteri) faeces using massive parallel sequencing. Conservation Genetics Resources 8(3): 343-352.
Evans NT, Shirey PD, Wieringa JG, Mahon AR, Lamberti GA. 2017. Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. Fisheries 42(2): 90-99.
Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. Biology letters 4(4): 423-425.
Flemming SA, van Heezik Y. 2014. Stable isotope analysis as a tool to monitor dietary trends in little penguins *Eudyptula minor*. Austral Ecology 39(6): 656-667.
Flemming SA, Lalas C, van Heezik Y. 2013. Little penguin (*Eudyptula minor*) diet at three breeding colonies in New Zealand. New Zealand Journal of Ecology: 199-205.
Folt B, McGowan C, Steen DA, Piccolomini S, Hoffman M, Godwin J, Guyer C. 2020. Modeling strategies and evaluating success during repatriations of elusive and endangered species. Animal Conservation 23(3): 273-285.
Fonseca VG. 2018. Pitfalls in relative abundance estimation using eDNA metabarcoding. Molecular Ecology Resources. 18(5): 923-926
Fraser MM, Lalas C. 2004. Seasonal variation in the diet of blue penguins (*Eudyptula minor)* at Oamaru, New Zealand. Notornis. 51(1): 7-15.
Furlan EM, Davis J, Duncan RP. 2020. Identifying error and accurately interpreting environmental DNA metabarcoding results: A case study to detect vertebrates at arid zone waterholes. Molecular ecology resources. 20(5): 1259-1276.
Galla SJ, Forsdick NJ, Brown L, Hoeppner MP, Knapp M, Maloney RF, Moraga R, Santure AW, Steeves TE 2019. Reference Genomes from Distantly Related Species Can Be Used for Discovery of Single Nucleotide Polymorphisms to Inform Conservation Management. Genes 10(1): 9.
Galla SJ, Moraga R, Brown L, Cleland S, Hoeppner MP, Maloney RF, Richardson A, Slater L, Santure AW, Steeves TE 2020. A comparison of pedigree, genetic and genomic estimates of relatedness for informing pairing decisions in two critically endangered

birds: Implications for conservation breeding programmes worldwide. Evolutionary Applications 13(5): 991-1008.
Gilbey J, Carvalho G, Castilho R, Coscia I, Coulson MW, Dahle G, Derycke S, Francisco SM, Helyar SJ, Johansen T. 2021. Life in a drop: Sampling environmental DNA for marine fishery management and ecosystem monitoring. Marine Policy. 124: 104331.
Giling D, Reina RD, Hogg Z.. 2008. Anthropogenic influence on an urban colony of the little penguin *Eudyptula minor.* Marine and Freshwater Research .59(7): 647-651.
Goldberg CS, Pilliod DS, Arkle RS, Waits LP. 2011. Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. PloS one. 6(7): e22746.
Grosser S, Waters JM. 2015. Development and characterisation of 20 novel microsatellite markers for the little blue penguin (*Eudyptula minor*) using next-generation sequencing. Conservation Genetics Resources. 7(1): 143-145.
Grosser S, Scofield RP, Waters JM. 2017. Multivariate skeletal analyses support a taxonomic distinction between New Zealand and Australian *Eudyptula* penguins (*Sphenisciformes: Spheniscidae*). Emu - Austral Ornithology 117(3): 276-283.
Grosser S, Rawlence NJ, Anderson CNK, Smith IWG, Scofield RP, Waters JM. 2016. Invader or resident? Ancient-DNA reveals rapid species turnover in New Zealand little penguins. Proceedings of the Royal Society B: Biological Sciences. 283(1824): 20152879.
Handsley-Davis M, Kowal E, Russell L, Weyrich LS. 2021. Researchers using environmental DNA must engage ethically with Indigenous communities. Nature Ecology & Evolution. 5(2): 146-148.
Hansen BK, Bekkevold D, Clausen LW, Nielsen EE. 2018. The sceptical optimist: challenges and perspectives for the application of environmental DNA in marine fisheries. Fish and Fisheries. 19(5): 751-768.
Harmoinen K. 2019. Kiwi and introduced mammalian predators: A case study on the use of molecular techniques to match individual stoats that have preyed on kiwi and to identify stoat stomach contents during the kiwi breeding season. 2018-2019.
Hasegawa M, Kishino H, Yano TA. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of molecular evolution. 22(2): 160-174.
Hocken A. 2000. Cause of death in blue penguins (*Eudyptula m. minor*) in North Otago, New Zealand. New Zealand Journal of Zoology. 27(4): 305-309.
Hogg ID, Banks JC, Woods SM. 2018. Biodiversity Genomics: Monitoring Restoration Efforts Using DNA Barcoding and Environmental DNA. Lake Restoration Handbook, Springer. Pp. 395-417.
Holdaway RJ, Wood JR, Dickie IA, Orwin KH, Bellingham PJ, Richardson SJ, Lyver POB, Timoti P, Buckley TR. 2017. Using DNA metabarcoding to assess New Zealand's terrestrial biodiversity. New Zealand Journal of Ecology 41(2): 251-262.
Hopken MW, Orning EK, Young JK, Piaggio AJ. 2016. Molecular forensics in avian conservation: a DNA-based approach for identifying mammalian predators of ground-nesting birds and eggs. BMC research notes 9(1): 1-9.
Horváth MB, Martínez-Cruz B, Negro JJ, Kalmár L, Godoy JA. 2005. An overlooked DNA source for non-invasive genetic analysis in birds. Journal of avian biology 36(1): 84-88.
Innes J, Kelly D, Overton JM, Gillies C. 2010. Predation and other factors currently limiting New Zealand forest birds. New Zealand Journal of Ecology 34(1): 86.
Jay M, Morad M, Bell A. 2003. Biosecurity, a policy dilemma for New Zealand. Land Use Policy 20(2): 121-129.

Jerde CL. 2021. Can we manage fisheries with the inherent uncertainty from eDNA? Journal of fish biology 98(2): 341-353.
Jerde CL, Mahon AR, Chadderton WL, Lodge DM. 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA. Conservation Letters 4(2): 150-157.
Jeunen G-J, Knapp M, Spencer HG, Lamare MD, Taylor HR, Stat M, Bunce M, Gemmell NJ 2019. Environmental DNA (eDNA) metabarcoding reveals strong discrimination among diverse marine habitats connected by water movement. Molecular Ecology Resources.. 19(2): 426-438.
Jeunen G-J, Urban L, Lewis R, Knapp M, Lamare M, Rayment W, Dawson S, Gemmell N. 2020a. Marine environmental DNA (eDNA) for biodiversity assessments: a one-to-one comparison between eDNA and baited remote underwater video (BRUV) surveys. Authorea Preprints.
Jeunen G-J, Lamare MD, Knapp M, Spencer HG, Taylor HR, Stat M, Bunce M, Gemmell NJ. 2020b. Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal. Environmental DNA 2(1): 99-111.
Jeunen GJ, Lamare MD, Knapp M, Spencer HG, Taylor HR, Stat M, Bunce M, Gemmell NJ. 2020c. Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal. Environmental DNA. 2(1): 99-111.
Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 28(12): 1647-9.
King C, Forsyth D. 2021. The handbook of New Zealand mammals, CSIRO Publishing.
Klomp N, Wooller R. 1991. Patterns of arrival and departure by breeding little penguins at Penguin Island, Western Australia. Emu. 91(1): 32-35.
Kress WJ, García-Robledo C, Uriarte M, Erickson DL. 2015. DNA barcodes for ecology, evolution, and conservation. Trends in ecology & evolution 30(1): 25-35.
Lacoursière-Roussel A, Deiner K. 2021. Environmental DNA is not the tool by itself. Journal of fish biology. 98(2): 383-386.
Lacoursière-Roussel A, Côté G, Leclerc V, Bernatchez L. 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. Journal of Applied Ecology. 53(4): 1148-1157.
Laroche O, Wood SA, Tremblay LA, Lear G, Ellis JI, Pochon X. 2017. Metabarcoding monitoring analysis: the pros and cons of using co-extracted environmental DNA and RNA data to assess offshore oil production impacts on benthic communities. PeerJ 5: e3347.
Laroche O, Wood SA, Tremblay LA, Ellis JI, Lear G, Pochon X. 2018. A cross-taxa study using environmental DNA/RNA metabarcoding to measure biological impacts of offshore oil and gas drilling and production operations. Marine Pollution Bulletin. 127: 97-107.
Laroche O, Wood SA, Tremblay LA, Ellis JI, Lejzerowicz F, Pawlowski J, Lear G, Atalah J, Pochon X. 2016. First evaluation of foraminiferal metabarcoding for monitoring environmental impact from an offshore oil drilling site. Marine Environmental Research .120: 225-235.
Lear G, Dickie I, Banks J, Boyer S, Buckley HL, Buckley TR, Cruickshank R, Dopheide A, Handley KM, Hermans S. 2018. Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. New Zealand Journal of Ecology 42(1): 10-50A.
Letunic I, Bork P. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic acids research. 47(W1): W256-W259.

Li J, Lawson Handley LJ, Read DS, Hänfling B. 2018. The effect of filtration method on the efficiency of environmental DNA capture and quantification via metabarcoding. Molecular Ecology Resources. 18(5): 1102-1114.
Lugg WH, Griffiths J, van Rooyen AR, Weeks AR, Tingley R. 2018. Optimal survey designs for environmental DNA sampling. Methods in Ecology and Evolution. 9(4): 1049-1059.
Luypaert T, Hagan JG, McCarthy ML, Poti M. 2020. Status of marine biodiversity in the Anthropocene. YOUMARES 9-The Oceans: Our Research, Our Future. Germany: Springer, Cham. Pp. 57-82.
Mace P, Ritchie P, Wellenreuther M, McKenzie J, Hupman K, Hillary R, Francis M, Bunce M. 2020. Report of the workshop on the utility of genetic analyses for addressing New Zealand fisheries questions. New Zealand fisheries science review 1.
Marchant S, Higgins PJ. 1990. Handbook of Australian, New Zealand & Antarctic birds. Vol. 1, Ratites to ducks, P. AB, UK: Oxford University Press.
Mariani S, Baillie C, Colosimo G, Riesgo A. 2019. Sponges as natural environmental DNA samplers. Current Biology. 29(11): R401-R402.
McColl-Gausden EF, Weeks AR, Tingley R. 2020. A field ecologist's guide to environmental DNA sampling in freshwater environments. Australian Zoologist 40(4): 641-651.
McColl-Gausden EF, Weeks AR, Coleman RA, Robinson KL, Song S, Raadik TA, Tingley R. 2020. Multispecies models reveal that eDNA metabarcoding is more sensitive than backpack electrofishing for conducting fish surveys in freshwater streams. Molecular Ecology. Molecular Ecology. Early view.
McNeill M, Phillips C, Young S, Shah F, Aalders L, Bell N, Gerard E, Littlejohn R. 2011. Transportation of nonindigenous species via soil on international aircraft passengers' footwear. Biological Invasions 13(12): 2799-2815.
Melbourne Water. (2019). Platypus census. [Accessed 2021 Mar 17] https://www.melbournewater.com.au/water-data-and-education/get-involved/be-citizen-scientist/platypus-census
Miskelly CM, Dowding JE, Elliott GP, Hitchmough RA, Powlesland RG, Robertson HA, Sagar PM, Scofield RP, Taylor GA. 2008. Conservation status of New Zealand birds, 2008. Notornis 55(3): 117-135.
Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. 2008. Database indexing for production MegaBLAST searches. Bioinformatics 24(16): 1757-1764.
Mumma MA, Soulliere CE, Mahoney SP, Waits LP. 2014. Enhanced understanding of predator–prey relationships using molecular methods to identify predator species, individual and sex. Molecular Ecology Resources 14(1): 100-108.
Neice AA, McRae SB. 2021. An eDNA diagnostic test to detect a rare, secretive marsh bird. Global Ecology and Conservation: e01529.
Niemi GJ, McDonald ME. 2004. Application of ecological indicators. Annu. Rev. Ecol. Evol. Syst. 35: 89-111.
Pan H, Cole TL, Bi X, Fang M, Zhou C, Yang Z, Ksepka DT, Hart T, Bouzat JL, Argilla LS. 2019. High-coverage genomes to elucidate the evolution of penguins. GigaScience 8(9): giz117.
Parkes J, Murphy E. 2003. Management of introduced mammals in New Zealand. New Zealand Journal of Zoology 30(4): 335-359.
Parlato EH, Armstrong DP. 2018. Predicting reintroduction outcomes for highly vulnerable species that do not currently coexist with their key threats. Conservation Biology 32(6): 1346-1355.

Pearman JK, Keeley NB, Wood SA, Laroche O, Zaiko A, Thomson-Laing G, Biessy L, Atalah J, Pochon X. 2020. Comparing sediment DNA extraction methods for assessing organic enrichment associated with marine aquaculture. PeerJ 8: e10231.
Peelle L. 2016. Predator species identification from saliva at kill sites with limited remains. [Master's thesis]. University of Washington.
Peters C, Nelson H, Rusk B, Muir A. 2020. A novel method to optimise the utility of underused moulted plumulaceous feather samples for genetic analysis in bird conservation. Conservation Genetics Resources 12(3): 457-467.
Peucker AJ, Dann P, Burridge CP. 2009. Range-Wide Phylogeography of the Little Penguin (*Eudyptula minor)*: Evidence of Long-Distance Dispersal. The Auk 126(2): 397-408.
Piaggio AJ, Shriner SA, Young JK, Griffin DL, Callahan P, Wostenberg DJ, Gese EM, Hopken MW. 2019. DNA persistence in predator saliva from multiple species and methods for optimal recovery from depredated carcasses. Journal of Mammalogy 101(1): 298-306.
Pickerell GA, O'Donnell CF, Wilson DJ, Seddon PJ. 2014. How can we detect introduced mammalian predators in non-forest habitats? A comparison of techniques. New Zealand Journal of Ecology. 38(1) 86-102.
Pochon X, Zaiko A, Fletcher LM, Laroche O, Wood SA. 2017. Wanted dead or alive? Using metabarcoding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. PloS one. 12(11): e0187636.
Pochon X, Wood SA, Keeley NB, Lejzerowicz F, Esling P, Drew J, Pawlowski J. 2015. Accurate assessment of the impact of salmon farming on benthic sediment enrichment using foraminiferal metabarcoding. Marine Pollution Bulletin. 100(1): 370-382.
Pochon X, Wood S, Atalah J, Fletcher L, Laroche O, Pearman J, Elvines D, Zaiko A, Biessy L, Thomson-Laing G. 2021. A validated protocol for fish farm monitoring using environmental DNA. ARPHA Conference Abstracts. Pp. e65421.
Ramón-Laca A, Gleeson D, Yockney I, Perry M, Nugent G, Forsyth DM. 2014. Reliable discrimination of 10 ungulate species using high resolution melting analysis of faecal DNA. Plos one 9(3): e92043.
Rawlence NJ, Wood JR, Armstrong KN, Cooper A. 2009. DNA content and distribution in ancient feathers and potential to reconstruct the plumage of extinct avian taxa. Proceedings of the Royal Society B: Biological Sciences 276(1672): 3395-3402.
Reilly P. 1994. Penguins of the world. New York (NY): Oxford University Press.
Robertson HA, Dowding JE, Elliott G, Hitchmough R, Miskelly C, O'Donnell CF, Powlesland R, Sagar PM, Scofield RP, Taylor GA 2013. Conservation status of New Zealand birds, 2012, Publishing Team, Department of Conservation.
Robinson NM, Scheele BC, Legge S, Southwell DM, Carter O, Lintermans M, Radford JQ, Skroblin A, Dickman CR, Koleck J. 2018. How to ensure threatened species monitoring leads to threatened species conservation. Ecological Management & Restoration. 19(3): 222-229.
Romero LM, Platts SH, Schoech SJ, Wada H, Crespi E, Martin LB, Buck CL. 2015a. Understanding stress in the healthy animal – potential paths for progress. Stress 18(5): 491-497.
Romero LM, Platts SH, Schoech SJ, Wada H, Crespi E, Martin LB, Buck CL. 2015b. Understanding stress in the healthy animal–potential paths for progress. Stress 18(5): 491-497.
Salter I, Joensen M, Kristiansen R, Steingrund P, Vestergaard P. 2019. Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. Communications biology 2(1): 1-9.

Sherwen SL, Magrath MJ, Butler KL, Hemsworth PH. 2015. Little penguins, *Eudyptula minor,* show increased avoidance, aggression and vigilance in response to zoo visitors. Applied Animal Behaviour Science 168: 71-76.
Sigsgaard EE, Jensen MR, Winkelmann IE, Møller PR, Hansen MM, Thomsen PF. 2020. Population-level inferences from environmental DNA—Current status and future perspectives. Evolutionary Applications 13(2): 245-262.
Speller CF, Nicholas GP, Yang DY. 2011. Feather barbs as a good source of mtDNA for bird species identification in forensic wildlife investigations. Investigative genetics 2(1): 1-7.
Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SS, Sigsgaard EE, Hellström M. 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods in Ecology and Evolution 8(5): 635-645.
Steffens KE, Sanders MD, Gleenson DM, Pullen KM, Stowe CJ. 2012. Identification of predators at black-fronted tern Chlidonias albostriatus nests, using mtDNA analysis and digital video recorders. New Zealand Journal of Ecology: 48-55.
Steinmetz K, Murphy S, Cadhla OÓ, Barnett J, Mirimin L. 2021. Enabling pinniped conservation by means of non-invasive genetic population analysis. Conservation Genetics Resources: 1-12.
Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular ecology 21(8): 2045-2050.
Taberlet P, Bonin A, Zinger L, Coissac E. 2018. Environmental DNA: For biodiversity research and monitoring. Oxford (UK): Oxford University Press.
Tablado Z, Jenni L. 2017. Determinants of uncertainty in wildlife responses to human disturbance. Biological Reviews 92(1): 216-233.
Takahashi S, Sakata MK, Minamoto T, Masuda R. 2020. Comparing the efficiency of open and enclosed filtration systems in environmental DNA quantification for fish and jellyfish. PloS one 15(4): e0231718.
Taylor G. 2000a. Action Plan for Seabird Conservation in New Zealand. Part A: Threatened Seabirds, Department of Conservation, Wellington. 234 p.
Taylor G. 2000b. Action plan for seabird conservation in New Zealand. Part B: Non-threatened seabirds. Department of Conservation, Wellington. Threatened Species Occasional Publication 17: 201.
Thomsen PF, Willerslev E 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation 183: 4-18.
Thomsen PF, Møller PR, Sigsgaard EE, Knudsen SW, Jørgensen OA, Willerslev E. 2016. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. PloS one 11(11): e0165252.
Tingley R, Greenlees M, Oertel S, van Rooyen A, Weeks A. 2019. Environmental DNA sampling as a surveillance tool for cane toad Rhinella marina introductions on offshore islands. Biological invasions 21(1): 1-6.
Tompkins DM 2018. The Research Strategy for a 'Predator Free'New Zealand. Proceedings of the 28th Vertebrate Pest Conference. 11-18
Ushio M, Murata K, Sado T, Nishiumi I, Takeshita M, Iwasaki W, Miya M. 2018. Demonstration of the potential of environmental DNA as a tool for the detection of avian species. Scientific Reports 8(1): 4493.
Valsecchi E, Bylemans J, Goodman SJ, Lombardi R, Carr I, Castellano L, Galimberti A, Galli P. 2020. Novel universal primers for metabarcoding environmental DNA

surveys of marine mammals and other marine vertebrates. Environmental DNA 2(4): e72.
van Bleijswijk JD, Begeman L, Witte HJ, IJsseldijk LL, Brasseur SM, Gröne A, Leopold MF. 2014. Detection of grey seal Halichoerus grypus DNA in attack wounds on stranded harbour porpoises Phocoena phocoena. Marine Ecology Progress Series 513: 277-281.
Vertigan C, McMahon C, Andrews-Goff V, Hindell M. 2012. The effect of investigator disturbance on egg laying, chick survival and fledging mass of short-tailed shearwaters (Puffinus tenuirostris) and little penguins (*Eudyptula minor)*. Animal Welfare 21(1): 101-111.
von Ammon U, Wood SA, Laroche O, Zaiko A, Lavery SD, Inglis GJ, Pochon X. 2019. Linking environmental DNA and RNA for improved detection of the marine invasive fanworm Sabella spallanzanii. Frontiers in Marine Science 6: 621.
von Ammon U, Wood SA, Laroche O, Zaiko A, Tait L, Lavery S, Inglis GJ, Pochon X. 2018. Combining morpho-taxonomy and metabarcoding enhances the detection of non-indigenous marine pests in biofouling communities. Scientific reports 8(1): 1-11.
Wallis GP. 2019. Thirty years of conservation genetics in New Zealand: what have we learnt? Journal of the Royal Society of New Zealand 49(3): 320-346.
Waterhouse BR, Boyer S, Wratten SD. 2014. Pyrosequencing of prey DNA in faeces of carnivorous land snails to facilitate ecological restoration and relocation programmes. Oecologia 175(2): 737-746.
Waugh J. 2016. DNA barcodes highlight two clusters within the little penguin (Eudyptula minor): time to reassess species delineation?
White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18(1): 315-322.
Whitehead E, Adams N, Baird KA, Bell E, Borelle S, Dunphy BJ, Gaskin C, Landers TJ, Rayner MJ, Russell JC. 2019. Threats to seabirds of northern Aotearoa New Zealand.
Wilson K-J, Mattern T. 2018. Little (blue) penguin/kororā. New Zealand penguins-current knowledge and research priorities. 19: 35.
Wood SA, Pochon X, Laroche O, von Ammon U, Adamson J, Zaiko A. 2019. A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. Molecular ecology resources. 19(6): 1407-1419.
Wood SA, Biessy L, Latchford JL, Zaiko A, von Ammon U, Audrezet F, Cristescu ME, Pochon X. 2020. Release and degradation of environmental DNA and RNA in a marine system. Science of the Total Environment 704: 135314.
Wu Q, Kawano K, Ishikawa T, Sakata MK, Nakao R, Hiraiwa MK, Tsuji S, Yamanaka H, Minamoto T. 2019. Habitat selection and migration of the common shrimp, *Palaemon paucidens* in Lake Biwa, Japan—An eDNA-based study. Environmental DNA. 1(1): 54-63.
Young MJ, Dutoit L, Robertson F, van Heezik Y, Seddon PJ, Robertson BC. 2020. Species in the faeces: DNA metabarcoding as a method to determine the diet of the endangered yellow-eyed penguin. Wildlife Research 47(6): 509-522.
Zaiko A, Pochon X, Garcia-Vazquez E, Olenin S, Wood SA. 2018. Advantages and limitations of environmental DNA/RNA tools for marine biosecurity: management and surveillance of non-indigenous species. Frontiers in Marine Science 5: 322.
Zaiko A, Wood SA, Pochon X, Biessy L, Laroche O, Croot P, Garcia-Vazquez E. 2020. Elucidating Biodiversity Shifts in Ballast Water Tanks during a Cross-Latitudinal

Transfer: Complementary Insights from Molecular Analyses. Environmental Science & Technology 54(13): 8443-8454.
Zemanova M. 2020. Towards more compassionate wildlife research through the 3Rs principles: Moving from invasive to non-invasive methods. Wildlife Biology. 2020.
Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. Journal of Computational biology. 7(1-2): 203-214.
Zinger L, Bonin A, Alsos IG, Bálint M, Bik H, Boyer F, Chariton AA, Creer S, Coissac E, Deagle BE. 2019. DNA metabarcoding—Need for robust experimental designs to draw sound ecological conclusions. Molecular Ecology. 28(8): 1857-1862

Chapter Five

Synthesis and conclusions

5.1 Summary

In this thesis, I discuss the value of environmental DNA (eDNA) surveys and highlight the potential they hold for both remote Antarctic studies and future monitoring in New Zealand. I first reviewed the literature on eDNA studies in Antarctica, the Subantarctic and the Southern Ocean, identifying a limited number of studies that applied eDNA surveys to Antarctic vertebrates (McInnes et al. 2016; Cowart et al. 2018; Ficetola et al. 2018; Mariani et al. 2019). None of the Antarctic vertebrate eDNA studies I reviewed utilised snow as a source of eDNA (Chapter Two). I identified potential applications for future eDNA studies in Antarctica. For example, advances in portable sequencing technology such as the MinION device (Oxford nanopore) present an opportunity for real-time eDNA analysis from the ice (Edwards et al. 2016; Goordial et al. 2017; Ducluzeau et al. 2018; Edwards et al. 2019; Gowers et al. 2019; Millán-Aguiñaga 2019, Johnson et al. 2017). Additionally, the increasing presence of base staff, researchers and tourists could be utilised for community sampling, as eDNA surveys require minimal training. Outsourcing samples could help tackle the spatial and temporal biases caused by the logistical challenges of a harsh climate and dark winter.

Having identified Weddell seals as a potential target species for Antarctic eDNA studies in the review, I used Antarctic snow samples taken from an imprint left behind by a Weddell seal to develop a protocol for the sampling, extraction, amplification and sequencing of environmental DNA (Chapter Three). While I had limited success in extracting and amplifying eDNA from the snow samples, with seven successful sequences out of our thirty-three samples, this study does represent the first extraction of vertebrate DNA from snow in

Antarctica. Looking at similar studies in the Northern hemisphere (Dalén et al. 2007; Franklin et al. 2019; Kinoshita et al. 2019), it is likely that sample size, filter removal and storage length limited the success of this study. While the length of time the samples spent in storage potentially affected our results, it does highlight the benefits of investigating portable sequencing technology on a continent renowned for harsh weather and travel delays. I also discuss the potential for future population genetic applications of eDNA in Antarctica. This study suggests there will be some challenges with population genetic approaches, given the samples were collected from a single seal imprint but displayed a high level of divergence in the phylogenetic tree. However, with improving technology and methods, there is potential for non-invasive sampling to complement current species monitoring and further increase sampling efforts across spatial and temporal scales. Thus eDNA has particular value for monitoring indicator species in priority areas such as the Ross Sea Marine Protected Area. While I highlight the value of eDNA surveys for informing conservation management in the Antarctic, this protocol requires further optimisation and standardisation before it can assist with these goals.

To further develop the protocol, I shifted focus to eDNA in a New Zealand context (Chapter Four). I was interested in the kororā (little penguin, *Eudyptula minor*) for a number of reasons; firstly, a protocol developed for New Zealand penguins would be easier to adapt for their Antarctic relatives in the future. Secondly, I was able to partner with the International Antarctic Centre in Christchurch, home to a rescue population of kororā, allowing me to further develop my methods in an enclosed system. Thirdly, while the kororā is considered a species of least concern under the IUCN Red List (BirdLife International, 2020), this status is conservation dependent. The species faces a number of threats in both the terrestrial and marine environment (Wilson and Mattern 2018). Similarly, the taxonomy of the kororā is unclear, with suggestions that the New Zealand and Australian/Otago

populations should be split into two species (Banks et al. 2002; Peuker et al. 2009; Clarke et al. 2012; Waugh et al. 2016). The white flippered morphotype is also regularly referred to as a subspecies (*Eudyptula minor albosignata*), despite not being officially recognised as so (Baker et al. 2006; Pan et al. 2019) Should the taxonomy of the kororā change, so may the conservation status of populations. Thus, monitoring the species is important, and environmental DNA could inform future management. Having identified my filtration step as an issue in my Weddell seal study, I opted to use enclosed Sterivex filter units as they are benefitial for in-field filtration (Spens et al. 2017; Tingley et al. 2019). The Sterivex filters also dramatically reduced my extraction time (Fig. 5.1). While direct extraction from feathers was unsuccessful, seven out of nine of the self-made feather water samples were successfully extracted, amplified and sequenced, as were all eight of the penguin tank samples. All but one (unaligned) sequence was grouped within the *Eudyptula minor* clade on my phylogenetic tree. However, as with the Weddell seal study, the branch lengths indicated a high level of divergence. There was also some uncertainty with the alignments in the BLAST database. Low sequence quality, and potential contamination likeley impacted this result. A comparison of the changes made to the Weddell seal protocol is summarised in Fig. 5.1. The challenges faced in the methodological development highlight the need for extensive optimisation of protocols, considering each stage in the process. Likewise, eDNA methods require clear communication, which includes discussing the challenges that arise. The development of detailed, standardised protocols is important for comparability in the field (Spens et al. 2017; Dickie et al. 2018; Lear et al. 2018). With this considered, eDNA surveys are a promising tool for researchers interested in monitoring both the remote Antarctic environment and New Zealand's unique biodiversity.

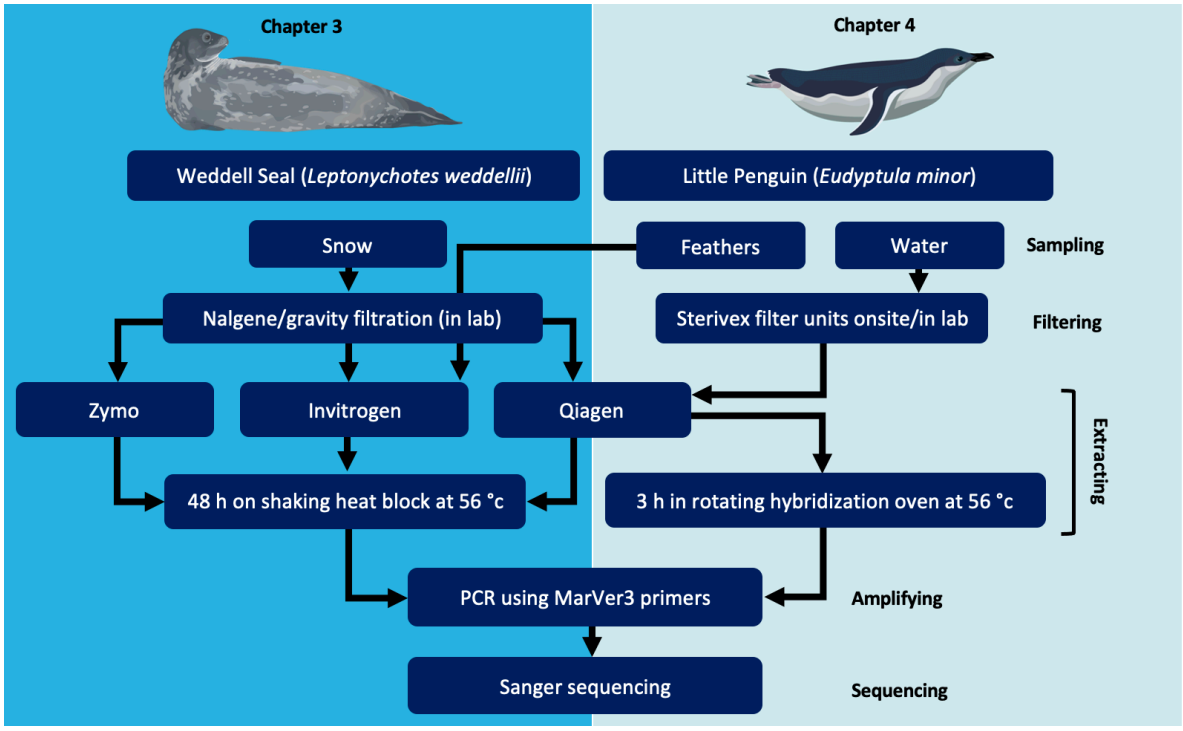

Figure 5.1. A summary of the methods used in the Weddell seal eDNA study (Chapter Three) compared to the kororā eDNA methods (Chapter Four). Highlighting the similarities and modifications across the stages of sampling, filtering, extracting, amplifying and sequencing eDNA.

5.2 Broader implications

Incorporating eDNA sampling into long-term environmental monitoring programs could support current bioassessment efforts (Barnes and Turner 2016; Pawlowski et al. 2020) in New Zealand and Antarctica. Investigating and incorporating novel, non-disruptive monitoring methods in Antarctic research contributes towards the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) mandate for providing the 'best available science' (Sylvester and Brooks, 2020). It is critical we continue to explore new tools to better coordinate and inform the monitoring and management of Antarctic fauna, and eDNA surveys are a promising development for polar research. By implementing long-term eDNA monitoring programs, we can construct a picture of biodiversity shifts over time, indicating disturbances within the ecosystem (Djurhuus et al. 2020). As eDNA technologies rapidly improve, the applications will continue to expand (Ruppert et al. 2019). One

promising area of expansion is assessing abundance and population genetics (Adams et al. 2019; Sigsgaard et al. 2019). Likewise, eDNA could be applied to monitoring anthropogenic disturbance both in Antarctica and New Zealand. For example, eDNA could be used to assess fisheries catches (Lacoursière-Roussel et al. 2015; Thompsen et al. 2016; Baillie et al. 2019; Jerde 2019; Gilbey et al. 2021). This data could be combined with metabarcoding surveys assessing trophic interactions to indicate the overlap between critical prey and commercially important species (Emami-Khoyi et al. 2016). In addition to targeting species of conservation concern, eDNA can potentially assess predators in the environment. For example, saliva from bite wounds can be barcoded to indicate the predator responsible, informing invasive species control (Steffens et al. 2012; Harmoinen 2019). This is particularly valuable in a New Zealand context and could be beneficial for informing the predator-free 2050 goals and contributing to the management and conservation of New Zealand's unique wildlife.

5.3 Final conclusions

Environmental DNA is a rapidly growing field, however, within Antarctic research, eDNA surveys remain limited to microbiology. While this has begun to shift in recent years, the application of environmental DNA to Antarctic vertebrates remains mostly unexplored. In this thesis, I aimed to address this gap by developing an eDNA protocol that utilised Antarctic snow to detect vertebrate eDNA, laying the foundation for future investigations into snow samples as a source of DNA, with a particular interest in future population genetic applications. I had limited success extracting Weddell seal DNA and highlight the challenges of optimising a protocol for remote research, with limited sample sizes and long storage periods potentially impacting DNA quality. Further development of the protocol was done from New Zealand, targeting a conservation dependant species, the kororā (*Eudyptula minor*). However, in both studies, I found a high level of divergence between eDNA samples,

which could be problematic for drawing population-level inferences in the future, and suggests further optimisation of our protocol is required. Despite these challenges, eDNA surveys show promise for future assessments of Antarctic and New Zealand, fauna, with progress being made in a number of key areas for conservation and management. There remains a lot of opportunity for using eDNA techniques, both on the ice and closer to home.

5.4 References

Adams CI, Knapp M, Gemmell NJ, Jeunen G-J, Bunce M, Lamare MD, Taylor HR. 2019. Beyond biodiversity: can environmental DNA (eDNA) cut it as a population genetics tool? Genes. 10(3): 192.

Baillie SM, McGowan C, May-McNally S, Leggatt R, Sutherland BJ, Robinson S. 2019. Environmental DNA and its applications to Fisheries and Oceans Canada: National needs and priorities. Canadian Technical Report of Fisheries and Aquatic Science. 3329

Baker AJ, Pereira SL, Haddrath OP, Edge KA. 2006. Multiple gene evidence for expansion of extant penguins out of Antarctica due to global cooling. Proceedings of the Royal Society B: Biological Sciences. 273(1582): 11-17.

Banks JC, Mitchell AD, Waas JR, Paterson AM. 2002. An unexpected pattern of molecular divergence within the blue penguin (Eudyptula minor) complex. Notornis. 49(1): 29-38.

BirdLife International. 2020. *Eudyptula minor*. The IUCN Red List of Threatened Species 2020: e.T22697805A184753545. https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T22697805A184753545.en. Downloaded on 17 March 2021.

Clark JA, Banks JC, Waas JR. 2013. Economical genotyping of little blue penguin (Eudyptula minor) clades from feather-based DNA. New Zealand Journal of Ecology. 37(1): 146-150.

Cowart DA, Murphy KR, Cheng C-HC. 2018. Metagenomic sequencing of environmental DNA reveals marine faunal assemblages from the West Antarctic Peninsula. Marine Genomics .37: 148-160.

Dickie IA, Boyer S, Buckley HL, Duncan RP, Gardner PP, Hogg ID, Holdaway RJ, Lear G, Makiola A, Morales SE. 2018. Towards robust and repeatable sampling methods in eDNA-based studies. Molecular Ecology Resources. 18(5): 940-952.

Djurhuus A, Closek CJ, Kelly RP, Pitz KJ, Michisaki RP, Starks HA, Walz KR, Andruszkiewicz EA, Olesin E, Hubbard K. 2020. Environmental DNA reveals seasonal shifts and potential interactions in a marine community. Nature communications. 11(1): 1-9.

Ducluzeau A-L, Tyson J, Collins R, Snutch T, Hassett B. 2018. Genome sequencing of sub-arctic mesomycetozoean Sphaeroforma sirkka strain B5, performed with the Oxford Nanopore minION and Illumina HiSeq systems. Microbiology resource announcements. 7(15): 7:e00848-18

Edwards A, Debbonaire AR, Sattler B, Mur LA, Hodson AJ. 2016. Extreme metagenomics using nanopore DNA sequencing: a field report from Svalbard, 78 N. BioRxiv: 073965.

Emami-Khoyi A, Hartley DA, Paterson AM, Boren LJ, Cruickshank RH, Ross JG, Murphy EC, Else TA. 2016. Identifying prey items from New Zealand fur seal (Arctocephalus forsteri) faeces using massive parallel sequencing. Conservation Genetics Resources. 8(3): 343-352.
Ficetola GF, Miaud C, Pompanon F, Taberlet P 2008. Species detection using environmental DNA from water samples. Biology letters 4(4): 423-425.
Gilbey J, Carvalho G, Castilho R, Coscia I, Coulson MW, Dahle G, Derycke S, Francisco SM, Helyar SJ, Johansen T. 2021. Life in a drop: Sampling environmental DNA for marine fishery management and ecosystem monitoring. Marine Policy. 124: 104331.
Goordial J, Altshuler I, Hindson K, Chan-Yam K, Marcolefas E, Whyte LG. 2017. In situ field sequencing and life detection in remote (79 26′ N) Canadian high Arctic permafrost ice wedge microbial communities. Frontiers in microbiology. 8: 2594.
Gowers G-OF, Vince O, Charles J-H, Klarenberg I, Ellis T, Edwards A. 2019. Entirely off-grid and solar-powered DNA sequencing of microbial communities during an ice cap traverse expedition. Genes. 10(11): 902.
Harmoinen K. 2019. Kiwi and introduced mammalian predators: A case study on the use of molecular techniques to match individual stoats that have preyed on kiwi and to identify stoat stomach contents during the kiwi breeding season 2018-2019. [Master's thesis] University of Helsinki
Jerde CL. 2021. Can we manage fisheries with the inherent uncertainty from eDNA? Journal of fish biology. 98(2): 341-353.
Johnson SS, Zaikova E, Goerlitz DS, Bai Y, Tighe SW. 2017. Real-time DNA sequencing in the Antarctic dry valleys using the Oxford Nanopore sequencer. Journal of Biomolecular Techniques. 28(1): 2.
Lacoursière-Roussel A, Côté G, Leclerc V, Bernatchez L. 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. Journal of Applied Ecology. 53(4): 1148-1157.
Mariani S, Baillie C, Colosimo G, Riesgo A. 2019. Sponges as natural environmental DNA samplers. Current Biology. 29(11): R401-R402.
McInnes JC, Emmerson L, Southwell C, Faux C, Jarman SN. 2016. Simultaneous DNA-based diet analysis of breeding, non-breeding and chick Adélie penguins. Royal Society Open Science. 3(1): 150443.
Millán-Aguiñaga N, Soldatou S, Brozio S, Munnoch JT, Howe J, Hoskisson PA, Duncan KR. 2019. Awakening ancient polar Actinobacteria: diversity, evolution and specialized metabolite potential. Microbiology. 165(11): 1169-1180.
Pan H, Cole TL, Bi X, Fang M, Zhou C, Yang Z, Ksepka DT, Hart T, Bouzat JL, Argilla LS. 2019. High-coverage genomes to elucidate the evolution of penguins. GigaScience 8(9): giz117.
Peucker AJ, Dann P, Burridge CP. 2009. Range-Wide Phylogeography of the Little Penguin (Eudyptula minor): Evidence of Long-Distance Dispersal. The Auk. 126(2): 397-408.
Ruppert KM, Kline RJ, Rahman MS. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. Global Ecology and Conservation. 17: e00547.
Sigsgaard EE, Jensen MR, Winkelmann IE, Møller PR, Hansen MM, Thomsen PF. 2020. Population-level inferences from environmental DNA—Current status and future perspectives. Evolutionary Applications .13(2): 245-262.
Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SS, Sigsgaard EE, Hellström M. 2017. Comparison of capture and storage methods for aqueous

macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods in Ecology and Evolution. 8(5): 635-645.
Steffens KE, Sanders MD, Gleenson DM, Pullen KM, Stowe CJ. 2012. Identification of predators at black-fronted tern Chlidonias albostriatus nests, using mtDNA analysis and digital video recorders. New Zealand Journal of Ecology. 36(1) 48-55.
Sylvester ZT, Brooks CM. 2020. Protecting Antarctica through Co-production of actionable science: lessons from the CCAMLR marine protected area process. Marine Policy. 111: 103720.
Thomsen PF, Møller PR, Sigsgaard EE, Knudsen SW, Jørgensen OA, Willerslev E. 2016. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. PloS one. 11(11): e0165252.
Tingley R, Greenlees M, Oertel S, van Rooyen A, Weeks A. 2019. Environmental DNA sampling as a surveillance tool for cane toad Rhinella marina introductions on offshore islands. Biological invasions. 21(1): 1-6.
Waugh J. 2016. DNA barcodes highlight two clusters within the little penguin (Eudyptula minor): time to reassess species delineation? Notornis. 63, pp.66-72.
Wilson K-J, Mattern T 2018. Little (blue) penguin/kororā. New Zealand penguins-current knowledge and research priorities. 19: 35. http://www.birdsnz.org.nz/wp-content/uploads/2019/06/1904-NZ-Penguin-Research-Priorities-Report-Mattern-Wilson.pdf. Accessed on Mar 17

Supplementary information

Table 1. Systematic review of the literature using Scopus. Search was done within the Title, Abstract, Keywords' field using 'Antarctic' with the terms 'e*DNA', 'environmental DNA' 'Next Generation Sequencing' (NGS), 'High Throughput Sequencing' (HTS), 'barcoding' and 'Metagenomics'. Papers were excluded where the sampling was not done in Antarctica, the sub-Antarctic or the Southern Ocean or where genetic data were not collected from environmental samples. Papers were assessed and divided by the environmental source of DNA (water, soil, sediment, ice/snow and other). References in bold are repeats within the same environmental source but different search terms. Studies that targeted vertebrate DNA are marked with *.

Sample		Search Term	Hits	References
Water	*Seawater*	**Total**	**38**	
		*e*DNA*	4	**Cowart et al. 2018*; Flaviani et al. 2018;** Fuentes et al. 2019; **Mariani et al. 2019***
		Environmental DNA	5	Rich et al. 2008; Rodríguez-Martínez et al. 2009; **Cowart et al. 2018*; Flaviani et al. 2018; Mariani et al. 2019***
		**barcoding*	2	**Zoccarato et al. 2016; Mariani et al. 2019***
		NGS	10	Ghiglione et al. 2012; Wolf et al. 2013a; Wolf et al. 2013b; **Lee et al. 2015;** Millard et al. 2016; **Moreno-Pino et al. 2016; Flaviani et al. 2018; De Corte et al. 2019;** Moss et al. 2020; **Sadaiappan et al. 2020**
		HTS	11	Alonso-Sáez et al. 2011; **Lee et al. 2015;** Yu et al. 2015; **Moreno-Pino et al. 2016; Zoccarato et al. 2016;** Lin et al. 2017; **Cowart et al. 2018*;** Gast et al. 2018; Lin et al. 2019; Liu and Jiang 2020; **Zhang et al. 2020**
		Metagenomics	19	Alonso-Sáez et al. 2012; Grzymski et al. 2012; Wilkins et al. 2013; Williams et al. 2013, 2014; Bertrand et al. 2015; Delmont et al. 2015; **Lee et al. 2015;** Brum et al. 2016; Gionfriddo et al. 2016; Miranda et al. 2016; Alcamán-Arias et al. 2018; **Cowart et al. 2018*;** Alarcón-Schumacher et al. 2019; **De Corte et al. 2019;** Kim et al. 2019; Yang et al. 2019a; Yang et al. 2019b; **Sadaiappan et al. 2020; Zhang et al. 2020**
	Terrestrial water bodies	**Total**	**27**	
		*e*DNA*	1	Fröls et al. 2012
		Environmental DNA	1	Karr et al. 2005
		**barcoding*	0	

		NGS	4	Lagkouvardos et al. 2014; Li et al. 2020b; Picazo et al. 2019; Weisleitner et al. 2019
		HTS	5	Archer et al. 2014; Quiroga et al. 2015 Archer et al. 2016; Schiaffino et al. 2016; Cho et al. 2020
		Metagenomics	17	Ng et al. 2010; Lauro et al. 2011; Yau et al. 2011; Durso et al. 2012; Demaere et al. 2013; Huang et al. 2013; Yau et al. 2013; **Lagkouvardos et al. 2014;** Lõpez-Bueno et al. 2015; Simmons et al. 2015; Tschitschko et al. 2015; de Cárcer et al. 2016; Cornet et al. 2018; Tschitschko et al. 2018; Hamm et al. 2019; Yang et al. 2019; Li et al. 2020b
Soil		**Total**	**43**	
		*e*DNA*	1	**Fraser et al. 2018**
		Environmental DNA	8	**Cieśliński et al. 2009b; Cieśliński et al. 2009a;** Rao et al. 2012; Teasdale et al. 2013; **Pansu et al. 2015; Czechowski et al. 2016b; Czechowski et al. 2016a; Fraser et al. 2018**
		**barcoding*	6	**Pansu et al. 2015; Czechowski et al. 2016a; Fraser et al. 2018; Khomich et al. 2018;** Rippin et al. 2018; Canini et al. 2020
		NGS	6	Bergstrom et al. 2015; Pessi et al. 2015; **Baeza et al. 2017;** Yan et al. 2017; **Borsetto et al. 2019;** Weisleitner et al. 2019
		HTS	12	van Dorst et al. 2014; Cox et al. 2016; **Czechowski et al. 2016a; Czechowski et al. 2016b;** Tahon et al. 2016; Wei et al. 2016; Cong et al. 2017; Kleinteich et al. 2017; Chua et al. 2018; **Khomich et al. 2018;** Han et al. 2019; Rego et al. 2019
		Metagenomics	25	Berlemont et al. 2009; **Cieśliński et al. 2009a; Cieśliński et al. 2009b;** Heath et al. 2009; Berlemont et al. 2011; Hu et al. 2012; Pearce et al. 2012; Bartasun et al. 2013; Berlemont et al. 2013; Chan et al. 2013; Winsley et al. 2014; Amos et al. 2015; Anderson et al. 2015; **Baeza et al. 2017;** Goordial et al. 2017b; Ji et al. 2017; Pulschen et al. 2017; Santamans et al. 2017; Donovan et al. 2018; Van Goethem et al. 2018; **Borsetto et al. 2019;** Li et al. 2019a; Molina-Montenegro et al. 2019; Oh et al. 2019; Yuan et al. 2019
Sediment		**Total**	**22**	
		*e*DNA*	0	
		Environmental DNA	7	Gordon et al. 2000; Habura et al. 2004; Karr et al. 2005; Pawlowski et al. 2005; Jaraula et al. 2010; Pawlowski et al. 2011; Ficetola et al. 2018*
		**barcoding*	3	**Fonseca et al. 2017;** Brannock et al. 2018; Vause et al. 2019
		NGS	1	Weisleitner 2019
		HTS	6	Emil Ruff et al. 2014; Archer et al. 2015; Fonseca et al. 2017; Li et al. 2019a; Cho et al. 2020; Li et al. 2020a
		Metagenomics	6	Huang et al. 2013; Hopkins et al. 2014; Matos et al. 2016; Vishnivetskaya et al. 2018; Centurion et al. 2019; Millán-Aguiñaga et al. 2019
Ice/Snow		**Total**	**12**	

		*e*DNA*	0	
		Environmental DNA	0	
		**barcoding*	2	Davey et al. 2019; Soto et al. 2020
		NGS	1	Weisleitner et al. 2019
		HTS	7	Webster-Brown et al. 2015; **Lopatina et al. 2016;** Sommers et al. 2018; Gast et al. 2018; Campen et al. 2019; Lutz et al. 2019; Sommers et al. 2019a
		Metagenomics	5	Shtarkman et al. 2013; Antony et al. 2016; Gionfriddo et al. 2016; **Lopatina et al. 2016;** Sommers et al. 2019b
Other	*Air*	**Total**	**3**	
		*e*DNA*	0	
		Environmental DNA	0	
		**barcoding*		
		NGS	2	Weisleitner et al. 2019; Kobayashi et al. 2016
		HTS	1	Bottos et al. 2014
		Metagenomics	1	
	Rock	**Total**	**13**	
		*e*DNA*	1	Vause et al. 2019
		Environmental DNA	1	De la Torre et al. 2003
		**barcoding*	4	Coleine et al. 2018; Coleine et al. 2019; Coleine et al. 2020a; **Coleine et al. 2020b**
		NGS	0	
		HTS	4	Archer et al. 2017; Lacap-Bugler et al. 2017; Rego et al. 2019; **Coleine et al. 2020b**
		Metagenomics	4	Chan et al. 2013; Le et al. 2016; Guerrero et al. 2017; Li et al. 2019b
	Microbial Mat	**Total**	**14**	
		*e*DNA*	0	
		Environmental DNA	1	Callejas et al. 2011

		**barcoding*	0	
		NGS	3	**Zawar-Reza et al. 2014; Koo et al. 2017a;** Koo et al. 2017b
		HTS	7	Tytgat et al. 2014; Pessi et al. 2016; Johnson et al. 2017; **Koo et al. 2017a;** Pessi et al. 2018; Almela et al. 2019; Lezcano et al. 2019
		Metagenomics	6	**Zawar-Reza et al. 2014;** Koo et al. 2016; Velázquez et al. 2016; **Koo et al. 2017a;** Zaikova et al. 2019; Dillon et al. 2020
	Bio film	**Total**	**1**	
		*e*DNA*	0	
		Environmental DNA	0	
		**barcoding*	0	
		NGS	0	
		HTS	0	
		Metagenomics	1	Tighe et al. 2017
	Faeces	**Total**	**7**	
		*e*DNA*	0	
		Environmental DNA	0	
		**barcoding*	0	
		NGS	2	Varsani et al. 2014; McInnes et al. 2016*
		HTS	4	Medeiros et al. 2016; Neira et al. 2017; Yew et al. 2017; Morandini et al. 2019
		Metagenomics	1	Grzesiak et al. 2020

References

Alarcón-Schumacher T, Guajardo-Leiva S, Antón J, Díez B. 2019. Elucidating viral communities during a phytoplankton bloom on the west Antarctic Peninsula. Frontiers in Microbiology. 10:1014.

Alcamán-Arias ME, Farías L, Verdugo J, Alarcón-Schumacher T, Díez B. 2018. Microbial activity during a coastal phytoplankton bloom on the Western Antarctic Peninsula in late summer. FEMS Microbiology Letters. 365(10):1-51.

Almela P, Velázquez D, Rico E, Justel A, Quesada A. 2019. Carbon pathways through the food web of a microbial mat from Byers Peninsula, Antarctica. Frontiers in Microbiology. 10:628.

Alonso-Sáez L, Andersson A, Heinrich F, Bertilsson S. 2011. High archaeal diversity in Antarctic circumpolar deep waters. Environmental Microbiology Reports. 3(6):689-697.
Alonso-Sáez L, Waller AS, Mende DR, Bakker K, Farnelid H, Yager PL, Lovejoy C, Tremblay JE, Potvin M, Heinrich F, Estrada M, Riemann L, Bork P, Pedrós-Alió C, Bertilsson S. 2012. Role for urea in nitrification by polar marine Archaea. Proceedings of the National Academy of Sciences of the United States of America. 109(44):17989-17994.
Amos GCA, Borsetto C, Laskaris P, Krsek M, Berry AE, Newsham KK, Calvo-Bado L, Pearce DA, Vallin C, Wellington EMH. 2015. Designing and implementing an assay for the detection of rare and divergent NRPS and PKS clones in European, Antarctic and Cuban soils. PLoS ONE. 10(9):e0138327.
Anderson D, Ferreras E, Trindade M, Cowan D. 2015. A novel bacterial water Hypersensitivity-like protein shows in vivo protection against cold and freeze damage. FEMS Microbiology Letters. 362(15):fvn110.
Antony R, Sanyal A, Kapse N, Dhakephalkar PK, Thamban M, Nair S 2016. Microbial communities associated with Antarctic snowpack and their biogeochemical implications. Microbiological Research. 192:192-202.
Archer SDJ, McDonald IR, Herbold CW, Cary SC. 2014. Characterisation of bacterioplankton communities in the meltwater ponds of Bratina Island, Victoria Land, Antarctica. FEMS Microbiology Ecology. 89(2):451-464.
Archer SDJ, McDonald IR, Herbold CW, Lee CK, Cary CS. 2015. Benthic microbial communities of coastal terrestrial and ice shelf Antarctic meltwater ponds. Frontiers in Microbiology. 6:485.
Archer SDJ, McDonald IR, Herbold CW, Lee CK, Niederberger TS, Cary C. 2016. Temporal, regional and geochemical drivers of microbial community variation in the melt ponds of the Ross Sea region, Antarctica. Polar Biology. 39(2):267-282.
Archer SDJ, de los Ríos A, Lee KC, Niederberger TS, Cary SC, Coyne KJ, Douglas S, Lacap-Bugler DC, Pointing SB. 2017. Endolithic microbial diversity in sandstone and granite from the McMurdo Dry Valleys, Antarctica. Polar Biology. 40(5):997-1006.
Baeza M, Barahona S, Alcaíno J, Cifuentes V. 2017. Amplicon-metagenomic analysis of fungi from Antarctic terrestrial habitats. Frontiers in Microbiology. 8:2235.
Bartasun P, Cieśliński H, Bujacz A, Wierzbicka-Woś A, Kur J. 2013. A study on the interaction of Rhodamine B with methylthioadenosine phosphorylase protein sourced from an Antarctic soil metagenomic library. PLoS ONE. 8(1):55697.
Bergstrom DM, Bricher PK, Raymond B, Terauds A, Doley D, McGeoch MA, Whinam J, Glen M, Yuan Z, Kiefer K, Shaw JD, Bramely-Alves J, Rudman T, Mohammed C, Lucieer A, Visoiu M, Jansen van Vuuren B, Ball MC. 2015. Rapid collapse of a sub-Antarctic alpine ecosystem: The role of climate and pathogens. Journal of Applied Ecology. 52(3):774-783.
Berlemont R, Delsaute M, Pipers D, D'Amico S, Feller G, Galleni M, Power P. 2009. Insights into bacterial cellulose biosynthesis by functional metagenomics on Antarctic soil samples. ISME Journal. 3(9):1070-1081.
Berlemont R, Pipers D, Delsaute M, Angiono F, Feller G, Galleni M, Power P. 2011. Exploring the Antarctic soil metagenome as a source of novel cold-adapted enzymes and genetic mobile elements. Rev Argent Microbiol. 43(2):94-103.
Berlemont R, Jacquin O, Delsaute M, La Salla M, Georis J, Verté F, Galleni M, Power P. 2013. Novel cold-adapted esterase MHlip from an Antarctic soil metagenome. Biology. 2(1):177-188.

Bertrand EM, McCrow JP, Moustafa A, Zheng H, McQuaid JB, Delmont TO, Post AF, Sipler RE, Spackeen JL, Xu K. 2015. Phytoplankton–bacterial interactions mediate micronutrient colimitation at the coastal Antarctic sea ice edge. Proceedings of the National Academy of Sciences. 112(32):9938-9943.

Borsetto C, Amos GCA, Da Rocha UN, Mitchell AL, Finn RD, Laidi RF, Vallin C, Pearce DA, Newsham KK, Wellington EMH. 2019. Microbial community drivers of PK/NRP gene diversity in selected global soils. Microbiome. 7(1):78.

Bottos EM, Woo AC, Zawar-Reza P, Pointing SB, Cary SC. 2014. Airborne bacterial populations above desert soils of the McMurdo Dry Valleys, Antarctica. Microbial Ecology. 67(1):120-128.

Brannock PM, Learman DR, Mahon AR, Santos SR, Halanych KM. 2018. Meiobenthic community composition and biodiversity along a 5500 km transect of Western Antarctica: A metabarcoding analysis. Marine Ecology Progress Series. 603:47-60.

Brum JR, Hurwitz BL, Schofield O, Ducklow HW, Sullivan MB. 2016. Seasonal time bombs: Dominant temperate viruses affect Southern Ocean microbial dynamics. ISME Journal. 10(2):437-449.

Callejas C, Gill PR, Catalán AI, Azziz G, Castro-Sowinski S, Batista S. 2011. Phylotype diversity in a benthic cyanobacterial mat community on King George Island, maritime Antarctica. World Journal of Microbiology and Biotechnology. 27(6):1507-1512.

Campen R, Kowalski J, Lyons WB, Tulaczyk S, Dachwald B, Pettit E, Welch KA, Mikucki JA. 2019. Microbial diversity of an Antarctic subglacial community and high-resolution replicate sampling inform hydrological connectivity in a polar desert. Environmental Microbiology. 21(7):2290-2306.

Canini F, Geml J, D'Acqui LP, Selbmann L, Onofri S, Ventura S, Zucconi L. 2020. Exchangeable cations and pH drive diversity and functionality of fungal communities in biological soil crusts from coastal sites of Victoria Land, Antarctica. Fungal Ecology. 45:100923.

Centurion VB, Delforno TP, Lacerda-Júnior GV, Duarte AWF, Silva LJ, Bellini GB, Rosa LH, Oliveira VM. 2019. Unveiling resistome profiles in the sediments of an Antarctic volcanic island. Environmental Pollution. 255:113240.

Chan Y, Van Nostrand JD, Zhou J, Pointing SB, Farrell RL. 2013. Functional ecology of an Antarctic Dry Valley. Proceedings of the National Academy of Sciences of the United States of America. 110(22):8990-8995.

Cho H, Hwang CY, Kim JG, Kang S, Knittel K, Choi A, Kim SH, Rhee SK, Yang EJ, Lee S, Hyun JH. 2020. A unique benthic microbial community underlying the phaeocystis Antarctica-dominated Amundsen Sea polynya, Antarctica: A Proxy for Assessing the Impact of Global Changes. Frontiers in Marine Science. 6:797.

Chua CY, Yong ST, González MA, Lavin P, Cheah YK, Tan GYA, Wong CMVL. 2018. Analysis of bacterial communities of King George and Deception Islands, Antarctica using high-throughput sequencing. Current Science. 115(9):1701-1705.

Cieśliński H, Długołcka A, Kur J, Turkiewicz M. 2009a. An MTA phosphorylase gene discovered in the metagenomic library derived from Antarctic top soil during screening for lipolytic active clones confers strong pink fluorescence in the presence of rhodamine B. FEMS Microbiology Letters. 299(2):232-240.

Cieśliński H, Białkowska A, Tkaczuk K, Długołęcka A, Kur J, Turkiewicz M. 2009b. Identification and molecular modelling of a novel lipase from an Antarctic soil metagenomic library. Polish Journal of Microbiology. 58(3):199-204.

Coleine C, Zucconi L, Onofri S, Pombubpa N, Stajich JE, Selbmann L. 2018. Sun exposure shapes functional grouping of fungi in cryptoendolithic Antarctic communities. Life. 8(2):19.
Coleine C, Stajich JE, Pombubpa N, Zucconi L, Onofri S, Canini F, Selbmann L. 2019. Altitude and fungal diversity influence the structure of Antarctic cryptoendolithic Bacteria communities. Environmental Microbiology Reports. 11(5):718-726.
Coleine C, Stajich JE, Pombubpa N, Zucconi L, Onofri S, Selbmann L. 2020a. Sampling strategies to assess microbial diversity of Antarctic cryptoendolithic communities. Polar Biology. 43(3):225-235.
Coleine C, Pombubpa N, Zucconi L, Onofri S, Stajich JE, Selbmann L. 2020b. Endolithic fungal species markers for harshest conditions in the McMurdo Dry Valleys, Antarctica. Life 10(2):13.
Cong B, Wang N, Liu S, Liu F, Yin X, Shen J. 2017. Isolation, characterization and transcriptome analysis of a novel Antarctic *Aspergillus sydowii* strain MS-19 as a potential lignocellulosic enzyme source. BMC Microbiology. 17(1):1-14.
Cornet L, Bertrand AR, Hanikenne M, Javaux EJ, Wilmotte A, Baurain D. 2018. Metagenomic assembly of new (Sub)polar cyanobacteria and their associated microbiome from non-axenic cultures. Microbial Genomics. 4(9):212.
Cowart DA, Murphy KR, Cheng CHC. 2018. Metagenomic sequencing of environmental DNA reveals marine faunal assemblages from the West Antarctic Peninsula. Marine Genomics. 37:148-160.
Cox F, Newsham KK, Bol R, Dungait JAJ, Robinson CH. 2016. Not poles apart: Antarctic soil fungal communities show similarities to those of the distant Arctic. Ecology Letters. 19(5):528-536.
Czechowski P, White D, Clarke L, McKay A, Cooper A, Stevens MI. 2016b. Age-related environmental gradients influence invertebrate distribution in the prince Charles mountains, east Antarctica. Royal Society Open Science. 3(12):160296.
Czechowski P, Clarke LJ, Cooper A, Stevens MI. 2017. A primer to metabarcoding surveys of Antarctic terrestrial biodiversity. Antarctic Science. 29(1):3-15.
Davey MP, Norman L, Sterk P, Huete-Ortega M, Bunbury F, Loh BKW, Stockton S, Peck LS, Convey P, Newsham KK, Smith AG. 2019. Snow algae communities in Antarctica: metabolic and taxonomic composition. New Phytologist. 222(3):1242-1255.
De Cárcer DA, López-Bueno A, Alonso-Lobo JM, Quesada A, Alcamí A. 2016. Metagenomic analysis of lacustrine viral diversity along a latitudinal transect of the Antarctic Peninsula. FEMS Microbiology Ecology. 92(6):1-10.
De Corte D, Martínez JM, Cretoiu MS, Takaki Y, Nunoura T, Sintes E, Herndl GJ, Yokokawa T. 2019. Viral communities in the global deep ocean conveyor belt assessed by targeted viromics. Frontiers in Microbiology. 10:1801.
De la Torre JR, Goebel BM, Friedmann EI, Pace NR. 2003. Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. Applied and Environmental Microbiology. 69(7):3858-3867.
Delmont TO, Murat Eren A, Vineis JH, Post AF. 2015. Genome reconstructions indicate the partitioning of ecological functions inside a phytoplankton bloom in the Amundsen Sea, Antarctica. Frontiers in Microbiology. 6:1090.
Demaere MZ, Williams TJ, Allen MA, Brown MV, Gibson JAE, Rich J, Lauro FM, Dyall-Smith M, Davenport KW, Woyke T, Kyrpides NC, Tringe SG, Cavicchioli R. 2013. High level of intergenera gene exchange shapes the evolution of haloarchaea in an isolated Antarctic lake. Proceedings of the National Academy of Sciences of the United States of America. 110(42):16939-16944.

Dillon ML, Hawes I, Jungblut AD, MacKey TJ, Eisen JA, Doran PT, Sumner DY. 2020. Environmental control on the distribution of metabolic strategies of benthic microbial mats in Lake Fryxell, Antarctica. PLoS ONE. 15(4):e0231053.
Donovan PD, Gonzalez G, Higgins DG, Butler G, Ito K. 2018. Identification of fungi in shotgun metagenomics datasets. PLoS ONE. 13(2):e0192898.
Durso LM, Miller DN, Wienhold BJ. 2012. Distribution and quantification of antibiotic resistant genes and bacteria across agricultural and non-agricultural Metagenomes. PLoS ONE. 7(11):e48325.
Emil Ruff S, Probandt D, Zinkann AC, Iversen MH, Klaas C, Würzberg L, Krombholz N, Wolf-Gladrow D, Amann R, Knittel K. 2014. Indications for algae-degrading benthic microbial communities in deep-sea sediments along the Antarctic Polar Front. Deep-Sea Research Part II: Topical Studies in Oceanography. 108:6-16.
Ficetola GF, Poulenard J, Sabatier P, Messager E, Gielly L, Leloup A, Etienne D, Bakke J, Malet E, Fanget B, Støren E, Reyss JL, Taberlet P, Arnaud F. 2018. DNA from lake sediments reveals long-term ecosystem changes after a biological invasion. Science Advances. 4(5):4292.
Flaviani F, Schroeder DC, Lebret K, Balestreri C, Highfield AC, Schroeder JL, Thorpe SE, Moore K, Pasckiewicz K, Pfaff MC, Rybicki EP. 2018. Distinct oceanic microbiomes from viruses to protists located near the Antarctic Circumpolar current. Frontiers in Microbiology. 9:1474.
Fröls S, Dyall-Smith M, Pfeifer F. 2012. Biofilm formation by haloarchaea. Environmental Microbiology. 14(12):3159-3174.
Fraser CI, Connell L, Lee CK, Cary SC. 2018. Evidence of plant and animal communities at exposed and subglacial (cave) geothermal sites in Antarctica. Polar Biology. 41(3):417-421.
Fuentes S, Arroyo JI, Rodríguez-Marconi S, Masotti I, Alarcón-Schumacher T, Polz MF, Trefault N, De la Iglesia R, Díez B. 2019. Summer phyto- and bacterioplankton communities during low and high productivity scenarios in the Western Antarctic Peninsula. Polar Biology. 42(1):159-169.
Gast RJ, Fay SA, Sanders RW. 2018. Mixotrophic activity and diversity of Antarctic marine protists in austral summer. Frontiers in Marine Science. 5:13.
Ghiglione JF, Galand PE, Pommier T, Pedrós-Alió C, Maas EW, Bakker K, Bertilson S, Kirchman DL, Lovejoy C, Yager PL, Murray AE. 2012. Pole-to-pole biogeography of surface and deep marine bacterial communities. Proceedings of the National Academy of Sciences of the United States of America. 109(43):17633-17638.
Gionfriddo CM, Tate MT, Wick RR, Schultz MB, Zemla A, Thelen MP, Schofield R, Krabbenhoft DP, Holt KE, Moreau JW. 2016. Microbial mercury methylation in Antarctic sea ice. Nature Microbiology. 1(10):16127.
Goordial J, Davila A, Greer CW, Cannam R, DiRuggiero J, McKay CP, Whyte LG. 2017b. Comparative activity and functional ecology of permafrost soils and lithic niches in a hyper-arid polar desert. Environmental Microbiology. 19(2):443-458.
Gordon DA, Priscu J, Giovannoni S. 2000. Origin and phylogeny of microbes living in permanent Antarctic lake ice. Microbial Ecology. 39(3):197-202.
Grzesiak J, Kaczyńska A, Gawor J, Żuchniewicz K, Aleksandrzak-Piekarczyk T, Gromadka R, Zdanowski MK. 2020. A smelly business: Microbiology of Adélie penguin guano (Point Thomas rookery, Antarctica). Science of the Total Environment. 714:136714.
Grzymski JJ, Riesenfeld CS, Williams TJ, Dussaq AM, Ducklow H, Erickson M, Cavicchioli R, Murray AE. 2012. A metagenomic assessment of winter and summer bacterioplankton from Antarctica Peninsula coastal surface waters. ISME Journal. 6(10):1901-1915.

Guerrero LD, Vikram S, Makhalanyane TP, Cowan DA 2017. Evidence of microbial rhodopsins in Antarctic Dry Valley edaphic systems. Environmental Microbiology. 19(9):3755-3767.
Habura A, Pawlowski J, Hanes SD, Bowser SS. 2004. Unexpected foraminiferal diversity revealed by small-subunit rDNA analysis of Antarctic sediment. Journal of Eukaryotic Microbiology. 51(2):173-179.
Hamm JN, Erdmann S, Eloe-Fadrosh EA, Angeloni A, Zhong L, Brownlee C, Williams TJ, Barton K, Carswell S, Smith MA, Brazendale S, Hancock AM, Allen MA, Raftery MJ, Cavicchioli R. 2019. Unexpected host dependency of Antarctic Nanohaloarchaeota. Proceedings of the National Academy of Sciences of the United States of America. 116(29):14661-14670.
Heath C, Xiao PH, Cary SC, Cowan D. 2009. Identification of a novel alkaliphilic esterase active at low temperatures by screening a metagenomic library from Antarctic desert soil. Applied and Environmental Microbiology. 75(13):4657-4659.
Hopkins M, Kailasan S, Cohen A, Roux S, Tucker KP, Shevenell A, Agbandje-Mckenna M, Breitbart M. 2014. Diversity of environmental single-stranded DNA phages revealed by PCR amplification of the partial major capsid protein. ISME Journal. 8(10):2093-2103.
Hu XP, Heath C, Taylor MP, Tuffin M, Cowan D. 2012. A novel, extremely alkaliphilic and cold-active esterase from Antarctic desert soil. Extremophiles. 16(1):79-86.
Jaraula CMB, Brassell SC, Morgan-Kiss RM, Doran PT, Kenig F. 2010. Origin and tentative identification of tri to pentaunsaturated ketones in sediments from Lake Fryxell, East Antarctica. Organic Geochemistry. 41(4):386-397.
Ji M, Greening C, Vanwonterghem I, Carere CR, Bay SK, Steen JA, Montgomery K, Lines T, Beardall J, Van Dorst J, Snape I, Stott MB, Hugenholtz P, Ferrari B. 2017. Atmospheric trace gases support primary production in Antarctic desert surface soil. Nature. 552(7685):400-403.
Johnson SS, Zaikova E, Goerlitz DS, Bai Y, Tighe SW. 2017. Real-time DNA sequencing in the Antarctic Dry Valleys using the Oxford nanopore sequencer. Journal of Biomolecular Techniques. 28(1):2-7.
Karr EA, Sattley WM, Rice MR, Jung DO, Madigan MT, Achenbach LA. 2005. Diversity and distribution of sulfate-reducing bacteria in permanently frozen Lake Fryxell, McMurdo Dry Valleys, Antarctica. Applied and Environmental Microbiology. 71(10):6353-6359.
Khomich M, Cox F, Andrew CJ, Andersen T, Kauserud H, Davey ML. 2018. Coming up short: Identifying substrate and geographic biases in fungal sequence databases. Fungal Ecology. 36:75-80.
Kim SJ, Kim JG, Lee SH, Park SJ, Gwak JH, Jung MY, Chung WH, Yang EJ, Park J, Jung J, Hahn Y, Cho JC, Eugene ML, Rodriguez-Valera F, Hyun, JH
Rhee, Sung-Keun. 2019. Genomic and metatranscriptomic analyses of carbon remineralization in an Antarctic polynya. Microbiome. 7(1):15p.
Kleinteich J, Hildebrand F, Bahram M, Voigt AY, Wood SA, Jungblut AD, Küpper FC, Quesada A, Camacho A, Pearce DA, Convey P, Vincent WF, Zarfl C, Bork P, Dietrich DR. 2017. Pole-to-Pole connections: Similarities between arctic and Antarctic microbiomes and their vulnerability to environmental change. Frontiers in Ecology and Evolution. 5:137.
Kobayashi F, Maki T, Kakikawa M, Noda T, Mitamura H, Takahashi A, Imura S, Iwasaka Y. 2016. Atmospheric bioaerosols originating from Adélie penguins (*Pygoscelis adeliae*): Ecological observations of airborne bacteria at Hukuro Cove, Langhovde, Antarctica. Polar Science. 10(1):71-78.

Koo H, Hakim JA, Fisher PRE, Grueneberg A, Andersen DT, Bej AK. 2016. Distribution of cold adaptation proteins in microbial mats in Lake Joyce, Antarctica: Analysis of metagenomic data by using two bioinformatics tools. Journal of Microbiological Methods. 120:23-28.

Koo H, Mojib N, Hakim JA, Hawes I, Tanabe Y, Andersen DT, Bej AK. 2017a. Microbial communities and their predicted metabolic functions in growth laminae of a unique large conical mat from Lake Untersee, East Antarctica. Frontiers in Microbiology. 8:1347.

Koo H, Hakim JA, Morrow CD, Eipers PG, Davila A, Andersen DT, Bej AK. 2017b. Comparison of two bioinformatics tools used to characterize the microbial diversity and predictive functional attributes of microbial mats from Lake Obersee, Antarctica. Journal of Microbiological Methods. 140:15-22.

Lacap-Bugler DC, Lee KK, Archer S, Gillman LN, Lau MCY, Leuzinger S, Lee CK, Maki T, McKay CP, Perrott JK, de Los Rios-Murillo A, Warren-Rhodes KA, Hopkins DW, Pointing SB. 2017. Global diversity of desert hypolithic cyanobacteria. Frontiers in Microbiology. 8:867.

Lagkouvardos I, Weinmaier T, Lauro FM, Cavicchioli R, Rattei T, Horn M. 2014. Integrating metagenomic and amplicon databases to resolve the phylogenetic and ecological diversity of the Chlamydiae. ISME Journal. 8(1):115-125.

Lauro FM, Demaere MZ, Yau S, Brown MV, Ng C, Wilkins D, Raftery MJ, Gibson JA, Andrews-Pfannkoch C, Lewis M, Hoffman JM, Thomas T, Cavicchioli R. 2011. An integrative study of a meromictic lake ecosystem in Antarctica. ISME Journal. 5(5):879-895.

Le PT, Makhalanyane TP, Guerrero LD, Vikram S, De Peer YV, Cowan DA. 2016. Comparative metagenomic analysis reveals mechanisms for stress response in hypoliths from extreme hyperarid deserts. Genome Biology and Evolution. 8(9):2737-2747.

Lee J, Lee HT, Hong WY, Jang E, Kim J. 2015. FCMM: A comparative metagenomic approach for functional characterization of multiple metagenome samples. Journal of Microbiological Methods. 115:121-128.

Lezcano MA, Moreno-Paz M, Carrizo D, Prieto-Ballesteros O, Fernández-Martínez MÁ, Sánchez-García L, Blanco Y, Puente-Sánchez F, De Diego-Castilla G, García-Villadangos M, Fairén AG, Parro V. 2019. Biomarker profiling of microbial mats in the geothermal band of Cerro Caliente, Deception Island (Antarctica): life at the edge of heat and cold. Astrobiology. 19(12):1490-1504.

Li AZ, Han XB, Zhang MX, Zhou Y, Chen M, Yao Q, Zhu HH. 2019a. Culture-dependent and -independent analyses reveal the diversity, structure, and assembly mechanism of benthic bacterial community in the Ross Sea, Antarctica. Frontiers in Microbiology. 10:2523.

Li Y, Cha QQ, Dang YR, Chen XL, Wang M, McMinn A, Espina G, Zhang YZ, Blamey JM, Qin QL. 2019b. Reconstruction of the functional ecosystem in the high light, low temperature Union Glacier region, Antarctica. Frontiers in Microbiology. 10:2408.

Li J, Gu X, Gui Y. 2020a. Prokaryotic diversity and composition of sediments from Prydz Bay, the Antarctic Peninsula Region, and the Ross Sea, Southern Ocean. Frontiers in Microbiology. 11:783.

Li W, Dore JE, Steigmeyer AJ, Cho YJ, Kim OS, Liu Y, Morgan-Kiss RM, Skidmore ML, Priscu JC. 2020b. Methane production in the oxygenated water column of a perennially ice-covered Antarctic lake. Limnology and Oceanography. 65(1):143-156.

Lin Y, Cassar N, Marchetti A, Moreno C, Ducklow H, Li Z. 2017. Specific eukaryotic plankton are good predictors of net community production in the Western Antarctic Peninsula. Scientific Reports. 7(1):14845.
Lin Y, Gifford S, Ducklow H, Schofield O, Cassara N. 2019. Towards quantitative microbiome community profiling using internal standards. Applied and Environmental Microbiology. 85(5):e02634-18.
Liu Q, Jiang Y. 2020. Application of microbial network analysis to discriminate environmental heterogeneity in Fildes Peninsula, Antarctica. Marine Pollution Bulletin. 156:111244.
Lopatina A, Medvedeva S, Shmakov S, Logacheva MD, Krylenkov V, Severinov K. 2016. Metagenomic analysis of bacterial communities of Antarctic surface snow. Frontiers in Microbiology. 7:398.
Lõpez-Bueno A, Rastrojo A, Peirõ R, Arenas M, Alcamí A. 2015. Ecological connectivity shapes quasispecies structure of RNA viruses in an Antarctic lake. Molecular Ecology. 24(19):4812-4825.
Lutz S, Ziolkowski LA, Benning LG. 2019. The biodiversity and geochemistry of cryoconite holes in Queen Maud Land, East Antarctica. Microorganisms. 7(6):160.
Mariani S, Baillie C, Colosimo G, Riesgo A. 2019. Sponges as natural environmental DNA samplers. Current Biology. 29(11):401-402.
Matos MN, Lozada M, Anselmino LE, Musumeci MA, Henrissat B, Jansson JK, Mac Cormack WP, Carroll J, Sjöling S, Lundgren L, Dionisi HM. 2016. Metagenomics unveils the attributes of the alginolytic guilds of sediments from four distant cold coastal environments. Environmental Microbiology. 18(12):4471-4484.
Medeiros AW, Giongo A, Valdez FP, De Amorin DB, Tavares M, D'Azevedo PA, Franco AC, Frazzon J, Frazzon APG. 2016. Characterization of the faecal bacterial community of wild young South American (*Arctocephalus australis*) and Subantarctic fur seals (*Arctocephalus tropicalis*). FEMS Microbiology Ecology. 92(3):fiw029.
Millán-Aguiñaga N, Soldatou S, Brozio S, Munnoch JT, Howe J, Hoskisson PA, Duncan KR. 2019. Awakening ancient polar actinobacteria: Diversity, evolution and specialized metabolite potential. Microbiology. 165(11):1169-1180.
Millard AD, Pearce D, Zwirglmaier K. 2016. Biogeography of bacteriophages at four hydrothermal vent sites in the Antarctic based on g23 sequence diversity. FEMS Microbiology Letters. 363(7):fnw043.
Miranda JA, Culley AI, Schvarcz CR, Steward GF. 2016. RNA viruses as major contributors to Antarctic virioplankton. Environmental Microbiology. 18(11):3714-3727.
Molina-Montenegro MA, Ballesteros GI, Castro-Nallar E, Meneses C, Gallardo-Cerda J, Torres-Díaz C. 2019. A first insight into the structure and function of rhizosphere microbiota in Antarctic plants using shotgun metagenomic. Polar Biology. 42(10):1825-1835.
Morandini V, Dugger KM, Ballard G, Elrod M, Schmidt A, Ruoppolo V, Lescroël A, Jongsomjit D, Massaro M, Pennycook J Kooyman GL, Schmidlin K, Kraberger S, Ainley DG, Varsani A. 2019. Identification of a novel Adélie penguin circovirus at Cape Crozier (Ross Island, Antarctica). Viruses. 11(12):1088.
Moreno-Pino M, De la Iglesia R, Valdivia N, Henríquez-Castilo C, Galán A, Díez B, Trefault N. 2016. Variation in coastal Antarctic microbial community composition at sub-mesoscale: Spatial distance or environmental filtering? FEMS Microbiology Ecology. 92(7):fiw088.
Moss JA, Henriksson NL, Pakulski JD, Snyder RA, Jeffrey WH. 2020. Oceanic microplankton do not adhere to the latitudinal diversity gradient. Microbial Ecology. 79(2):511-515.

Ng C, Demaere MZ, Williams TJ, Lauro FM, Raftery M, Gibson JAE, Andrews-Pfannkoch C, Lewis M, Hoffman JM, Thomas T, Cavicchioli R. 2010. Metaproteogenomic analysis of a dominant green sulfur bacterium from Ace Lake, Antarctica. ISME Journal. 4(8):1002-1019.
Oh HN, Park D, Seong HJ, Kim D, Sul WJ. 2019. Antarctic tundra soil metagenome as useful natural resources of cold-active lignocelluolytic enzymes. Journal of Microbiology. 57(10):865-873.
Pansu J, Winkworth RC, Hennion F, Gielly L, Taberlet P, Choler P. 2015. Long-lasting modification of soil fungal diversity associated with the introduction of rabbits to a remote sub-Antarctic archipelago. Biology Letters. 11(9):20150408.
Pawlowski J, Fahrni JF, Guiard J, Conlan K, Hardecker J, Habura A, Bowser SS. 2005. Allogromiid foraminifera and gromiids from under the Ross Ice Shelf: Morphological and molecular diversity. Polar Biology. 28(7):514-522.
Pawlowski J, Fontaine D, da Silva AA, Guiard J. 2011. Novel lineages of Southern Ocean deep-sea foraminifera revealed by environmental DNA sequencing. Deep-Sea Research Part II: Topical Studies in Oceanography. 58(19-20):1996-2003.
Pearce DA, Newsham KK, Thorne MAS, Calvo-Bado L, Krsek M, Laskaris P, Hodson A, Wellington EM. 2012. Metagenomic analysis of a southern maritime Antarctic soil. Frontiers in Microbiology. 3:403.
Pessi IS, Osorio-Forero C, Gálvez EJ, Simões FL, Simões JC, Junca H, Macedo AJ. 2015. Distinct composition signatures of archaeal and bacterial phylotypes in the Wanda Glacier forefield, Antarctic Peninsula. FEMS Microbiology Ecology. 91(1):1-10.
Pessi IS, Maalouf PDC, Laughinghouse HDI, Baurain D, Wilmotte A. 2016. On the use of high-throughput sequencing for the study of cyanobacterial diversity in Antarctic aquatic mats. Journal of Phycology. 52(3):356-368.
Pessi IS, Lara Y, Durieu B, de Maalouf PC, Verleyen E, Wilmotte A. 2018. Community structure and distribution of benthic cyanobacteria in Antarctic lacustrine microbial mats. FEMS Microbiology Ecology. 94(5):fiy042.
Picazo A, Rochera C, Villaescusa JA, Miralles-Lorenzo J, Velázquez D, Quesada A, Camacho A. 2019. Bacterioplankton community composition along environmental gradients in lakes from Byers Peninsula (Maritime Antarctica) as determined by next-generation sequencing. Frontiers in Microbiology. 10:908.
Pulschen AA, Bendia AG, Fricker AD, Pellizari VH, Galante D, Rodrigues F. 2017. Isolation of uncultured bacteria from antarctica using long incubation periods and low nutritional media. Frontiers in Microbiology. 8:1346.
Quiroga MV, Valverde A, Mataloni G, Cowan D. 2015. Understanding diversity patterns in bacterioplankton communities from a sub-Antarctic peatland. Environmental Microbiology Reports. 7(3):547-553.
Rao S, Chan Y, Lacap DC, Hyde KD, Pointing SB, Farrell RL. 2012. Low-diversity fungal assemblage in an Antarctic Dry Valleys soil. Polar Biology. 35(4):567-574.
Rego A, Raio F, Martins TP, Ribeiro H, Sousa AGG, Séneca J, Baptista MS, Lee CK, Craig Cary S, Ramos V, Carvalho MF, Leão PN, Magalhães C. 2019. Actinobacteria and cyanobacteria diversity in terrestrial Antarctic microenvironments evaluated by culture-dependent and independent methods. Frontiers in Microbiology. 10:1018.
Rich VI, Konstantinidis K, DeLong EF. 2008. Design and testing of 'genome-proxy' microarrays to profile marine microbial communities. Environmental Microbiology. 10(2):506-521.
Rippin M, Lange S, Sausen N, Becker B. 2018. Biodiversity of biological soil crusts from the Polar Regions revealed by metabarcoding. FEMS Microbiology Ecology. 94(4):fiy036.

Rodríguez-Martínez R, Labrenz M, Del Campo J, Forn I, Jürgens K, Massana R. 2009. Distribution of the uncultured protist MAST-4 in the Indian Ocean, Drake Passage and Mediterranean Sea assessed by real-time quantitative PCR. Environmental Microbiology. 11(2):397-408.

Sadaiappan B, Kannan S, Palaniappan S, Manikkam R, Ramasamy B, Anilkumar N, Subramanian M. 2020. Metagenomic 16S rDNA amplicon data of microbial diversity and its predicted metabolic functions in the Southern Ocean (Antarctic). Data in Brief. 28:104876.

Santamans AC, Boluda R, Picazo A, Gil C, Ramos-Miras J, Tejedo P, Pertierra LR, Benayas J, Camacho A. 2017. Soil features in rookeries of Antarctic penguins reveal sea to land biotransport of chemical pollutants. PLoS ONE. 12(8):e0181901.

Schiaffino MR, Lara E, Fernández LD, Balagué V, Singer D, Seppey CC, Massana R, Izaguirre I. 2016. Microbial eukaryote communities exhibit robust biogeographical patterns along a gradient of Patagonian and Antarctic lakes. Environmental Microbiology. 18(12):5249-5264.

Shtarkman YM, Koçer ZA, Edgar R, Veerapaneni RS, D'Elia T, Morris PF, Rogers SO. 2013. Subglacial Lake Vostok (Antarctica) Accretion Ice Contains a Diverse Set of Sequences from Aquatic, Marine and Sediment-Inhabiting Bacteria and Eukarya. PLoS ONE. 8(7):e67221.

Simmons MP, Bachy C, Sudek S, Van Baren MJ, Sudek L, Ares M, Worden AZ. 2015. Intron invasions trace algal speciation and reveal nearly identical Arctic and Antarctic micromonas populations. Molecular Biology and Evolution. 32(9):2219-2235.

Sommers P, Darcy JL, Gendron E, Stanish LF, Bagshaw EA, Porazinska DL, Schmidt SK. 2018. Diversity patterns of microbial eukaryotes mirror those of bacteria in Antarctic cryoconite holes. FEMS Microbiology Ecology. 94(1):fix167.

Sommers P, Porazinska DL, Darcy JL, Zamora F, Fountain AG, Schmidt SK. 2019a. Experimental cryoconite holes as mesocosms for studying community ecology. Polar Biology. 42(11):I973-1984.

Sommers P, Fontenele RS, Kringen T, Kraberger S, Porazinska DL, Darcy JL, Schmidt SK, Varsani A. 2019b. Single-stranded DNA viruses in Antarctic cryoconite holes. Viruses. 11(11):1022.

Soto DF, Fuentes R, Huovinen P, Gómez I. 2020. Microbial composition and photosynthesis in Antarctic snow algae communities: Integrating metabarcoding and pulse amplitude modulation fluorometry. Algal Research. 45:101738

Tahon G, Tytgat B, Willems A. 2016. Diversity of phototrophic genes suggests multiple bacteria may be able to exploit sunlight in exposed soils from the Sør Rondane Mountains, East Antarctica. Frontiers in Microbiology. 7:2026

Teasdale SE, Beulke AK, Guy PL, Orlovich DA. 2013. Environmental barcoding of the ectomycorrhizal fungal genus Cortinarius. Fungal Diversity. 58(1):299-310.

Tighe S, Afshinnekoo E, Rock TM, McGrath K, Alexander N, McIntyre A, Ahsanuddins S, Bezdan D, Green SJ, Joye S, Stewart Johnson S, Baldwin DA, Bivens N, Ajami N, Carmical JR, Herriott IC, Colwell R, Donia M, Foox J, Greenfield N, Hunter T, Hoffman J, Hyman J, Jorgensen E, Krawczyk D, Lee J, Levy S, Garcia-Reyero N, Settles M, Thomas K, Gómez F, Schriml L, Kyrpides N, Zaikova E, Penterman J, Mason CE. 2017. Genomic methods and microbiological technologies for profiling novel and extreme environments for the extreme microbiome project (XMP). Journal of Biomolecular Techniques. 28(1):31-39.

Tschitschko B, Williams TJ, Allen MA, Páez-Espino D, Kyrpides N, Zhong L, Raftery MJ, Cavicchioli R. 2015. Antarctic archaea-virus interactions: Metaproteome-led analysis of invasion, evasion and adaptation. ISME Journal. 9(9):2094-2107.

Tschitschko B, Erdmann S, DeMaere MZ, Roux S, Panwar P, Allen MA, Williams TJ, Brazendale S, Hancock AM, Eloe-Fadrosh EA, Emiley A, Cavicchioli R. 2018. Genomic variation and biogeography of Antarctic haloarchaea. Microbiome. 6(1):113.

Tytgat B, Verleyen E, Obbels D, Peeters K, De Wever A, D'Hondt S, De Meyer T, Van Criekinge W, Vyverman W, Willems A. 2014. Bacterial diversity assessment in Antarctic terrestrial and aquatic microbial mats: A comparison between bidirectional pyrosequencing and cultivation. PLoS ONE. 9(6):e97564.

Van Dorst J, Bissett A, Palmer AS, Brown M, Snape I, Stark JS, Raymond B, McKinlay J, Ji M, Winsley T, Ferrari BC. 2014. Community fingerprinting in a sequencing world. FEMS Microbiology Ecology. 89(2):316-330.

Van Goethem MW, Pierneef R, Bezuidt OKI, Van De Peer Y, Cowan DA, Makhalanyane TP. 2018. A reservoir of 'historical' antibiotic resistance genes in remote pristine Antarctic soils. Microbiome. 6(1):40.

Varsani A, Kraberger S, Jennings S, Porzig EL, Julian L, Massaro M, Pollard A, Ballard G, Ainley DG. 2014. A novel papillomavirus in Adélie penguin (Pygoscelis adeliae) faeces sampled at the Cape Crozier colony, Antarctica. Journal of General Virology. 95(6):1352-1365.

Vause BJ, Morley SA, Fonseca VG, Jazdzewska A, Ashton GV, Barnes DKA, Giebner H, Clark MS, Peck LS. 2019. Spatial and temporal dynamics of Antarctic shallow soft-bottom benthic communities: Ecological drivers under climate change. BMC Ecology. 19(1):27.

Velázquez D, López-Bueno A, Aguirre De Cárcer D, De Los Ríos A, Alcamí A, Quesada A. 2016. Ecosystem function decays by fungal outbreaks in Antarctic microbial mats. Scientific Reports. 6:22954.

Vishnivetskaya TA, Buongiorno J, Bird J, Krivushin K, Spirina EV, Oshurkova V, Shcherbakova VA, Wilson G, Lloyd KG, Rivkina EM. 2018. Methanogens in the Antarctic Dry Valley permafrost. FEMS Microbiology Ecology. 94(8):fiy109.

Webster-Brown JG, Hawes I, Jungblut AD, Wood SA, Christenson HK. 2015. The effects of entombment on water chemistry and bacterial assemblages in closed cryoconite holes on Antarctic glaciers. FEMS Microbiology Ecology. 91(12):fiv144.

Wei STS, Lacap-Bugler DC, Lau MCY, Caruso T, Rao S, de los Rios A, Archer SK, Chiu JMY, Higgins C, Van Nostrand JD, Zhou J, Hopkins DW., Pointing SB. 2016. Taxonomic and functional diversity of soil and hypolithic microbial communities in Miers Valley, McMurdo Dry Valleys, Antarctica. Frontiers in Microbiology. 7:1642.

Weisleitner K, Perras A, Moissl-Eichinger C, Andersen DT, Sattler B. 2019. Source environments of the microbiome in perennially ice-covered lake Untersee, Antarctica. Frontiers in Microbiology. 10:1019.

Wilkins D, Lauro FM, Williams TJ, Demaere MZ, Brown MV, Hoffman JM, Andrews-Pfannkoch C, McQuaid JB, Riddle MJ, Rintoul SR, Cavicchioli R. 2013. Biogeographic partitioning of Southern Ocean microorganisms revealed by metagenomics. Environmental Microbiology. 15(5):1318-1333.

Williams TJ, Wilkins D, Long E, Evans F, Demaere MZ, Raftery MJ, Cavicchioli R. 2013. The role of planktonic Flavobacteria in processing algal organic matter in coastal East Antarctica revealed using metagenomics and metaproteomics. Environmental Microbiology. 15(5):1302-1317.

Williams TJ, Allen MA, DeMaere MZ, Kyrpides NC, Tringe SG, Woyke T, Cavicchioli R. 2014. Microbial ecology of an Antarctic hypersaline lake: genomic assessment of ecophysiology among dominant haloarchaea. The ISME journal. 8(8):1645-1658.

Winsley TJ, Snape I, McKinlay J, Stark J, van Dorst JM, Ji M, Ferrari BC, Siciliano SD. 2014. The ecological controls on the prevalence of candidate division TM7 in polar regions. Frontiers in Microbiology. 5:345.
Wolf C, Frickenhaus S, Kilias ES, Peeken I, Metfies K. 2013a. Regional variability in eukaryotic protist communities in the Amundsen Sea. Antarctic Science. 25(6):741-751.
Wolf C, Frickenhaus S, Kilias ES, Peeken I, Metfies K. 2013b. Protist community composition in the Pacific sector of the Southern Ocean during austral summer 2010. Polar Biology. 37(3):375-389.
Yan W, Ma H, Shi G, Li Y, Sun B, Xiao X, Zhang Y. 2017. Independent shifts of abundant and rare bacterial populations across East Antarctica glacial foreland. Frontiers in Microbiology. 8:1534.
Yang Q, Gao C, Jiang Y, Wang M, Zhou X, Shao H, Gong Z, McMinn A. 2019a. Metagenomic characterization of the viral community of the South Scotia Ridge. Viruses. 11(2):95.
Yang Y, Li Z, Song W, Du L, Ye C, Zhao B, Liu W, Deng D, Pan Y, Lin H, Cao X. 2019b. Metagenomic insights into the abundance and composition of resistance genes in aquatic environments: Influence of stratification and geography. Environment International. 127:371-380.
Yau S, Lauro FM, DeMaere MZ, Brown MV, Thomas T, Raftery MJ, Andrews-Pfannkoch C, Lewis M, Hoffman JM, Gibson JA, Cavicchioli R. 2011. Virophage control of Antarctic algal host-virus dynamics. Proceedings of the National Academy of Sciences of the United States of America. 108(15):6163-6168.
Yau S, Lauro FM, Williams TJ, Demaere MZ, Brown MV, Rich J, Gibson JAE, Cavicchioli R. 2013. Metagenomic insights into strategies of carbon conservation and unusual sulfur biogeochemistry in a hypersaline Antarctic lake. ISME Journal. 7(10):1944-1961.
Yew WC, Pearce DA, Dunn MJ, Samah AA, Convey P. 2017. Bacterial community composition in Adélie (*Pygoscelis adeliae*) and Chinstrap (*Pygoscelis antarctica*) Penguin stomach contents from Signy Island, South Orkney Islands. Polar Biology. 40(12):2517-2530.
Yu Z, Yang J, Liu L, Zhang W, Amalfitano S. 2015. Bacterioplankton community shifts associated with epipelagic and mesopelagic waters in the Southern Ocean. Scientific Reports. 5:12897.
Zaikova E, Goerlitz DS, Tighe SW, Wagner NY, Bai Y, Hall BL, Bevilacqua JG, Weng MM, Samuels-Fair MD, Johnson SS. 2019. Antarctic relic microbial mat community revealed by metagenomics and metatranscriptomics. Frontiers in Ecology and Evolution. 7:1.
Zawar-Reza P, Argüello-Astorga GR, Kraberger S, Julian L, Stainton D, Broady PA, Varsani A. 2014. Diverse small circular single-stranded DNA viruses identified in a freshwater pond on the McMurdo Ice Shelf (Antarctica). Infection, Genetics and Evolution. 26:132-138.
Zhang W, Cao S, Ding W, Wang M, Fan S, Yang B, McMinn A, Wang M, Xie BB, Qin QL, Chen XL, He J, Zhang YZ. 2020. Structure and function of the Arctic and Antarctic marine microbiota as revealed by metagenomics. Microbiome. 8(1):77.
Zoccarato L, Pallavicini A, Cerino F, Umani SF, Celussi M. 2016. Water mass dynamics shape Ross Sea protist communities in mesopelagic and bathypelagic layers. Progress in Oceanography. 149:16-26.