Human Impact on the McMurdo Dry Valley Soils of Antarctica: Extending the Limits of DNA Detection

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Abstract

Antarctica is commonly regarded as the most pristine place on Earth and is safeguarded by the Antarctic Treaty System (ATS). The ATS designates the continent as a "natural reserve, devoted to peace and science" and sets out environmental principles regulating human activities. The obligations under the ATS include providing environmental impact assessments and leaving the environment minimally impacted after all operations. The associated human footprint inevitably increases as human activity increases in intensity and diversity on the continent. The human footprint is not clearly defined, and most investigation has focused on visible disturbances of physical (e.g., soil disturbance) and chemical (e.g., hydrocarbon spills) nature. The unseen human footprint remains to be investigated and quantified, which includes released genetic materials and human-associated microorganisms. Given the extremely low biomass in most of terrestrial Antarctica, these materials' presence can be a reliable indicator of human presence and activities.

As part of New Zealand's effort to carry out an evidence-based risk assessment of the McMurdo Dry Valley ecosystem (DryVER project), this research quantified human impact, in the form of DNA released from humans and human-associated microbiota, across spatial and temporal scales. This project was part of the MBIE grant entitled "Evidence-based Risk Assessment of the McMurdo Dry Valley Ecosystem". Areas of human activity, including seasonal field camps and facilities zones, were selected as sampling sites in the McMurdo Dry Valleys (MDVs), from which soil samples were collected. Human-specific genetic and microbial markers were used with digital PCR to quantify human presence at the lowest possible detection limit across spatial scales. A marker targeting a human *MT-CYB* gene region was the best marker for detecting and predicting human trace up to 1 km from a highly occupied site. Human impact derived from mtDNA was accumulative with occupancy and greatest closest to a campsite. A marker targeting 16S rRNA of *Bacteroides dorei,* a human-associated gut bacterium, was used to detect faecal trace. The faecal signal was generally localised to the toilet area of a camp and was too low to predict the distance of detectable signal, regardless of the intensity of occupancy. A DNA longevity experiment was established *in situ* in Miers Valley to investigate DNA persistence in a cold desert across temporal scales. The results from this experiment demonstrated that intracellular and extracellular DNA remained detectable with endpoint PCR for at least two years. DNA persistence was not significantly affected by soil sterility or UV exposure but did accumulate damage over time.

The direct outcome of this research will be a review of the environmental management systems in place, namely the McMurdo Dry Valleys Antarctic Specially Managed Area (ASMA) Management Plan. Better management will safeguard the unique MDV ecosystems by using the best practice to

minimise anthropogenic impact and strengthen New Zealand's stewardship over the Ross Dependency as one of the original 12 signatories to the Antarctic Treaty.

Statement of Authorship

I hereby declare that this is a submission of my own work, and to the best of my knowledge does not contain material that has been previously published by another individual, nor has it been used for the awarding of another degree.

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"The best view comes after the hardest climb."

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Abbreviations

aDNA	Ancient DNA
ASMA	Antarctic Specially Managed Areas
ASPA	Antarctic Specially Protected Areas
ATCM	Antarctic Treaty Consultative Meeting
ATS	The Antarctic Treaty System; comprises the Antarctic Treaty and related agreements
BacHum	16s rRNA marker for *Bacteroides dorei*
CEP	Committee for Environmental Protection
COMNAP	Council of Managers of National Antarctic Programmes
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
dPCR	Digital PCR
DryVER	Dry Valley Ecosystem Resilience Programme
eDNA	Environmental DNA
EP	Protocol on Environmental Protection to the Antarctic Treaty
FST	Faecal source tracking
HcytB	Human mitochondrial marker for *cytochrome b*
IAATO	International Association of Antarctic Tour Operators
ICTAR	International Centre for Terrestrial Antarctic Research
IGY	International Geophysical Year
kya	Thousands of years ago
LTER	Long-Term Ecological Research
MDV	McMurdo Dry valleys
mtDNA	Mitochondrial DNA
mya	Millions of years ago
NP	National Programme
NSF	National Science Foundation
PCR	Polymerase chain reaction
qPCR or rtPCR	Quantitative polymerase chain reaction or real-time PCR
SCAR	Scientific Committee on Antarctic Research
TSB	Tryptic soy broth
UHI	Unseen human impact
USAP	United States Antarctic Program
UV	Ultraviolet radiation

Chapter 1. Literature Review

1.1 Introduction

The Antarctic continent was the last frontier of human colonisation. Human activity commenced in the 19th century to exploit natural resources, such as seals, whales and penguins; the first recorded landing in Antarctica was in 1821 (Cowan *et al.,* 2011). The exploitation of resources was succeeded by the heroic age of fervent polar exploration during the late 19th-early 20th century. During this era there were numerous expeditions, including those led by Scott, Shackleton and Mawson, and the race to reach the south pole, which was successfully achieved by Amundsen. Most activity was around coastal areas near exploration huts and traverse routes to the continent's interior (Blanchette *et al.,* 2004). These early explorers paid little attention to their environmental footprint, and poor environmental practice was prevalent. The International Geophysical Year (IGY) of 1957-58 initiated a surge of international interest in Antarctica that coincided with the Cold War.

In response to an increasing presence on the continent, the Antarctic Treaty (1959) was signed to safeguard the continent (south of latitude 60°) against economic exploitation from mining and oil drilling, and nuclear testing, whilst permitting peaceful activities, which presently include research, commercial fishing, and tourism. However, the Treaty was not prescriptive of conduct to promote environmental protection when operating in the Antarctic, and a lack of environmental management produced a legacy of unreported landfills, an accumulation of human and animal wastes, and contamination of the surrounding terrestrial and marine environments (Stark *et al.,* 2006, Tin *et al.*, 2009).

The Treaty is central to the Antarctic Treaty System (ATS), which is comprised of the Protocol on Environmental Protection to the Antarctic Treaty (signed in 1991, ratified in 1998; thereafter referred to as the 'EP') and the Convention for the Conservation of Antarctic Marine Living Resources (CAMLR convention), and is also augmented by other resolutions and measures. The first proactive shift towards environmental protection occurred with the Agreed Measures on the Conservation of Antarctic Fauna and Flora in 1964, and with numerous recommendations arising from the Antarctic Treaty Consultative Meetings (ACTM) in 1964- 1991 with regard to environmental protection. The ATS is international legislation where all operators have an obligation to plan and conduct activities to limit adverse impacts on the Antarctic environment, where 'adverse' is not clearly defined, and to produce impact assessments. There are 56 signatory nations to the Antarctic Treaty and 42 to the EP, and NZ is one of the original 12 Treaty signatories. The EP consists of six annexes, five directly related to terrestrial environments (further expanded in section 1.3).

1.2 Human operations in the Antarctic

The primary human activities in the terrestrial Antarctic are science-driven and tourism via ship (Bender *et al.,* 2016, Hughes *et al.,* 2019, Frame *et al.,* 2022). Both activities are generally well regulated: science-led activities are coordinated through National Programmes (NPs), and tourist activities operate through the International Association of Antarctica Tour Operators (IAATO).

Both scientist and tourist groups are sources of environmental contamination through the dissemination of biological materials (skin, hair, epithelial cells, and microorganisms) and other wastes. Annex III of the EP outlines waste management and disposal, which should be an obligation from all countries. Article 1.2 states: "The amount of wastes produced or disposed of in the Antarctic Treaty area shall be reduced as far as practicable so as to minimise impact on the Antarctic environment and to minimise interference with the natural values of Antarctica, with scientific research and with other uses of Antarctica which are consistent with the Antarctic Treaty". The obligation to limit adverse impacts under the ATS is not clearly defined and the EP recognises that some activities will have more than a transitory impact. Anthropogenic impacts are not clearly defined between operators, and current environmental management focuses on visual footprint. Footprint can be defined as the spatial extent of human activities and their impacts. The human footprint in the Antarctic is diverse, encompassing "disturbance footprint" such as long-term modifications to ice-free areas and "building footprint", the buildings and infrastructure, along with the associated sewage, noise, visual and chemical contaminants (Brooks *et al.,* 2019). In contrast to the visible footprint is the "unseen" footprint, such as the release of human biological materials, human-associated microbes, and xenobiotic chemicals. This type of anthropogenic impact remains to be thoroughly investigated and quantified in a terrestrial Antarctic setting.

There are key differences between science and tourism-led activities, which result in a differing intensity of impact. Science-led activities have had a more significant presence on the continent, starting with the explorers of the heroic age in the late 19th century. The tourism industry has largely developed since the 1960's, which notably includes the tours offered by the pioneering operator Lars-Erik Lindblad from 1966 via ship (Liggett *et al.,* 2011), and brings a much larger volume of human visitation to the continent than NPs. The US National Science Foundation (NSF) began compiling tourist visitation data at the start of the tourist season of 1989/90. Subsequently, IAATO began to aid the compilation of tourist data, such as visitor numbers at specific landing sites (Bender *et al.*, 2016). Tourist numbers have steadily increased, from ~30,000 visitors during 2010 (Hall, 2010) to ~40,000 tourists during the 2017/2018 season (IAATO, 2018), to 104,000 visitors during the 2022/2023 season (IAATO, 2024), the majority of which will travel via cruise ship (IAATO, 2020). Although tourism brings more people to the region, tourism arguably has a lesser footprint than

scientists associated with one of the 30 NPs. The current trend for science-led activity is an increase in constructing more stations, bases and supporting infrastructure (Tin *et al.,* 2009). Compared to researchers, tourists spend a twentieth of the total time on the continent, and lack permanent infrastructure on land as a requirement under Annex I of the EP (Hughes *et al.,* 2011).

Currently, over 30 nations are active in scientific research in the Antarctic. Both the US and NZ National Programmes have had a presence in the McMurdo Dry Valleys (MDVs) for decades, McMurdo being the biggest base on the continent. During the austral summer period for 2-8 weeks annually, the population of both McMurdo (USA) and Scott Base (NZ) increases to ~1300 persons in a localised land area, primarily for research-driven activities (COMNAP, 2017). Base operations continue to impact the local environment today, typically confined to a few hundred metres around the base (Figure 1.1). However, much of the visible impact, e.g., large-scale hydrocarbon and metal contamination, are carry-over from historical practices (Klein *et al.,* 2014*)*. A sizable proportion of science personnel working will travel to the MDVs, the largest contiguous ice-free area in the Antarctic of 6861 km^2 (Levy, 2013), to conduct research, which requires temporary camping infrastructure to be set up during the field season (Figure 1.2).

Figure 1.1 Scott Base during the 2015/2016 austral summer, located on Ross Island, Ross Dependency (NZ). Soil disturbance is visible around the base.

Figure 1.2 An example of a temporary field camp established at Spaulding Pond in Taylor Valley during a field work season.

1.3 Current Terrestrial Environmental Management

Environmental management has come a long way since the signing of the Antarctic Treaty in 1959 and since the EP came into force in 1991. The EP provides a comprehensive framework for the protection of Antarctica, which most notably includes the indefinite mining ban, which ensures that all activities are conducted in Antarctica in a way that limits their environmental impact. Briefly, the annexes are: Annex I: Environmental Impact Assessment, Annex II) Conservation of Antarctic flora and fauna, Annex III) Waste Disposal and Treatment, Annex IV) Prevention of Marine Pollution and Annex VI) Responsibility arising from Environmental Emergencies. Annexes I to IV came into force in 1998 having been adopted by all the Consultative Parties, whilst Annex V was drafted later and came into force in 2002. The sixth annex not yet entered into force and remains to be adopted by all Consultative Parties. There is no end date to the Treaty or the EP, however, there is a provision for any Party to call for a review of the EP from 2048, 50 years after coming into force.

Under the EP, there is a ban on mining, the inadvertent and deliberate introduction of non-native species, disturbance of biota as well as requiring waste management and environmental impact assessments. The EP has identified Antarctica's scientific, environmental, aesthetic and wilderness values as worthy of protection. The Antarctic Treaty Consultative Meetings (ATCM) and the Committee for Environmental Protection (CEP) are the mechanisms to enact the legal agreements of the ATS. The CEP meets annually to provide advice on environmental protection to the ACTM, and has developed the 'Non-native species manual' (revised 2019). The aim of this manual is to mitigate the risks of non-native species unintentionally introduced to the Antarctic, and from the movement of species across biogeographic regions. The legislation has reduced local environmental impacts at some locations, but there is clear evidence that the rigour of application is inconsistent across the Parties. Conclusions from recent meetings were that protected areas don't accurately represent the full range of biodiversity (SCAR, 2017).

Despite the high level of environmental protection conferred by the EP, extensive impacts on Antarctic ecosystems have been documented near the site of intensive human activity, including the detection of microplastics (Sfriso *et al.,* 2020) and elevated hydrocarbon levels (Beseres Pollack *et al.,* 2022) near research stations. There are still shortcomings around EP implementation that need to be addressed, due to differences in cultural values and inconsistent environmental impact assessments (O'Neill, 2017). To extend effective environmental protection, improvements need to be made around long-term monitoring of the terrestrial environment by ATS signatories to mitigate environmental risk as much as possible (Brooks *et al.,* 2018).

A plethora of data on human activities in the MDVs has been collected for decades around visitation, movements, activities and impacts. For instance, 2.7 million records were collected over the last 200 years from publications, scientific databases and tourism (Leihy *et al.,* 2020). Many programmes have been established to monitor the human footprint on the continent (e.g., Pertierra *et al.,* 2017, Brooks *et al.,* 2019). The current trend for human activity in the Antarctic is an increase in intensity, diversification, and expansion of the associated footprint (Hughes *et al.,* 2018, Rintoul *et al.,* 2018). The duration of field seasons for science and tourism are projected to increase as the austral summer lengthens. In conjunction with the projected expansion of ice-free areas due to climate change (Lee *et al.*, 2017), this is an area of concern. There are already over 100 NP facilities across Antarctica (COMNAP, 2017) and with increasing presence comes more infrastructure and footprint.

A protected area system has been in place since the 'Agreed measures' of 1964. Annex V of the EP is concerned with "Area Protection and Management" and includes the current designations of Antarctic Specially Protected Areas (ASPA) and Antarctic Specially Managed Area (ASMA) classifications. An ASPA designation is the highest level of environmental protection under the Treaty. It requires a management plan to detail visitor guidelines and a permit for entry from a designated national authority. This designation is based on an area's environmental, scientific, historic, wilderness or aesthetic value. The creation of inviolate areas within the ASPAs is one tool of the EP to protect microbial habitats, requiring quarantine equipment in addition to obtaining a permit. There are currently 'prohibited zones' within ASPAs 140 and 175, both are geothermal. These zones are to be set aside for future research and will act as baselines for pristine habitats representative of native microbial diversity and their functions (Van de Putte, 2017).

ASMAs are where operations presently occur or may be conducted in future, and aim to assist in the planning and co-ordination of activities, avoid possible conflicts to improve co-operation between parties, and to minimise environmental impacts. The ASMA management plan is agreed by the ACTM and is applicable to all parties. The MDVs are classified as an Antarctic Specially Managed Area (ASMA), which was jointly proposed by the US and NZ in 2004. Scientists and other visitors who go to the MDVs with the New Zealand National Programme (Antarctica NZ) operate according to the recommendations outlined in the McMurdo Dry Valleys ASMA manual, which is mainly managed under the Code of Conduct (Antarctic New Zealand, 2015).

1.4 Antarctica as a model system for human impact research

Several factors make terrestrial Antarctica a tractable environment to study microbiological processes and hypotheses regarding human impact. Compared to other terrestrial areas, Antarctica lacks an indigenous human population and is commonly described as the last 'pristine' place on

Earth. The continent has the largest terrestrial tracts that have remained free from direct human impact than anywhere else on Earth (Hughes *et al.,* 2011). These areas, such as the Balham and Barwick Valleys (ASPA 123), are invaluable as baselines for comparison to human-impacted locations, particularly concerning environmental management. The benefits afforded to environmental management include the identification of areas at risk of 'alien' invasions by non-indigenous species (Hughes and Convey, 2010), identifying areas at risk of cumulative impact near research stations and tourist sites (Mahlon *et al.,* 2010) and in making informed decisions around the designation of protected areas to be managed under the Antarctic Treaty system.

Compared to temperate terrestrial systems, food webs are simple in the Antarctic, and most biological processes in the soil are driven by microorganisms (Lee *et al.,* 2012). Much of the continent lacks higher terrestrial animals and vascular plants (Magalhaes *et al.,* 2012). The trophic simplicity of the MDV soils provides the opportunity to test hypotheses relating to the effects of human impact *in situ*, such as changes to biodiversity, functionality, and the succession of invasive species (Huiskes *et al.*, 2014).

1.5 Characteristics of a microbe-dominated landscape in the McMurdo Dry Valleys

The MDVs comprise a unique ecosystem within a continent characterised by cold desert soils and an arid climate, classified as a cold, polar desert. The most extreme environmental conditions on Earth occur in the MDVs, which is the coldest, windiest, and driest place on Earth, along with high soil salinity, high UV radiation, katabatic winds, extreme temperature fluctuations and low water availability (Claridge and Campbell, 1977, Smith *et al.,* 1992, Nylen *et al.,* 2004, Cary *et al.,* 2010). Annual snowfall in the MDVs was observed to be between 3 to 50 mm water equivalent, with a gradient of being highest near the coast and decreasing inland and a mean annual air temperature of -15°C to -30°C (Doran *et al.,* 2002, Fountain *et al.,* 2010, Obryk *et al.,* 2020). Moisture evaporation generally exceeds precipitation in the MDVs, resulting in largely ice-free soils and salt accumulation like other desert soils (Balks and O'Neill, 2016).

The main abiotic drivers for microbial biodiversity are physiochemical, namely soil moisture, soil pH, soil salinity and temperature fluctuations, in conjunction with solar energy availability (Lee *et al.,* 2012, Convey *et al.,* 2014, Siciliano *et al.,* 2014, Chown *et al.,* 2015). The most important drivers are moisture availability and salinity (George *et al.,* 2021). The former becomes available to the soil communities during summer, with the melting of permafrost and ice deposits (Fountain *et al.*, 2014), when biological activity and productivity is at a maximum (Niederberger *et al.,* 2019, Sohm *et al.,* 2020).

These harsh environmental constraints have prevented many taxa from flourishing, leading to soils dominated by microbial communities characterised by low species richness and genetic structuring across spatial and temporal scales (Chown and Convey, 2007, Convey *et al.,* 2014, Pointing *et al.,* 2015), with well-adapted physiology (Lo Giudice *et al.,* 2007, Anesio and Laybourn-Parry, 2012, Mohit *et al.,* 2017). MDV soils were initially considered sterile in the 1970s, then later depauperate, where culture-based studies demonstrated low biomass and diversity (Boyd, 1966, Cameron, 1970, Horowitz *et al.,* 1972). In contrast, contemporary comparative studies have revealed a far greater microbial diversity than anticipated, based on next-generation sequencing and metagenomics, to the extent that much of Antarctica's biodiversity is microbe-dominated, primarily by bacterial and archaeal species (Cary *et al.,* 2010, Dreesens *et al.,* 2014, Chown *et al.,* 2015) whilst the macrofaunal species that thrive are of low diversity and include: springtails (Collembola) and mites (Acari), nematodes, diatoms, rotifers and tardigrades (Treonis *et al.,* 1999, Maslen and Convey, 2006, Stanish *et al.,* 2012), all of which demonstrate high endemicity (Boenigk *et al.,* 2006, Convey *et al.,* 2008, Vyverman *et al.,* 2010, Fraser *et al.,* 2014).

1.6 Ongoing anthropogenic threats to Antarctic Biota

Human activity elicits changes to the Antarctic terrestrial ecosystem (Mahlon *et al.,* 2010), including changes in biodiversity and community structures (Czechowski *et al.,* 2016). The long-term effects of these changes are not adequately understood regarding ecosystem resilience, diversity and community function (Kim *et al.,* 2015). This is a serious concern due to the unique environmental characteristics, which can amplify human impact on a local scale compared to temperate climes, presenting a severe threat to soil microbe communities (Hughes, 2014). The majority of human visitation occurs during the austral summer, the most biologically sensitive time for terrestrial biota, when biological processes peak in response to increased temperature and available moisture (Gröndahl *et al.,* 2009, Sohm *et al.,* 2020). There are similarities between science and tourism-based activities in the Antarctic. Both activities are seasonal and are heterogeneously concentrated in the coastal ice-free areas, due to accessibility via ship and plane landings. These ice-free areas comprise c. 0.18% of the total Antarctic landmass of c. 14 million km^2 yet are disproportionately impacted by human activities (Burton-Johnson *et al.,* 2016). For instance, 55% of tourist landings occur at one of eight sites around the Antarctic Peninsula (Lynch *et al.,* 2010), whilst 81% of buildings are on ice-free areas (Brooks *et al.,* 2019).

As human presence intensifies, the associated human footprint increases for science-driven and tourist activities, thereby reducing the number and size of "pristine" areas. A range of observed disturbances include: disruption of the soil surface (O'Neill *et al.,* 2012), release of sewage (Stark *et al.,* 2016), contamination by waste and detritus, release of xenobiotic chemicals, such as

hydrocarbons (McWatters *et al.,* 2016), microplastics (Waller *et al.,* 2017), pharmaceuticals (González-Alonso *et al.,* 2017) and heavy metals (Chu *et al.,* 2018), the introduction of exotic species (Bargagli, 2008, Dauner *et al.,* 2015), microbial human-borne contamination (Gröndahl *et al.,* 2009) and disturbance to wildlife (Coetzee and Chown, 2015).

Several routes enable the release of xenobiotic chemicals into the environment. Under Annex III to the Protocol, sewage must only be macerated before release into the ocean without requiring further processing. Raw human waste is discharged into the environment under certain circumstances. Xenobiotic chemicals and their metabolites are also released, through urine, such as field parties' disposing of human waste on the ice shelves, known as "tide-cracking", or tourist ships releasing waste into the ocean. There is no record of medication taken by scientists or tourists at the bases or camps. Water treatment does not remove these substances or their metabolites before treated water is released into the environment. A large proportion of bases do not adequately treat wastewater (Gröndahl *et al.,* 2009). As a result, pathogens, exotic microbes, caffeine, biologically active substances, including steroid hormones and endocrine disruptors and personal hygiene products (e.g., sunscreen, soaps, sanitisers), and pharmaceutical and illicit drugs, have been released into Antarctic waters at detectable levels (Emnet *et al.,* 2015, Esteban *et al.,* 2016). Such substances can have an adverse effect on the environment and aquatic animals (Kostich and Lazorchak, 2008, Santos *et al.,* 2010). The concern is due to the low ambient temperatures and biodiversity of Antarctic environments, making these ecosystems vulnerable to disruption from contaminants, as has been observed for Arctic ecosystems (Gunnarsdóttir *et al.,* 2013). Other synthetic wastes released by scientists and tourists include materials released from tents and clothing (González-Alonso *et al.,* 2017).

Out of all chemical contaminants, hydrocarbon release is the most significant source of environmental contamination in the Antarctic via the accumulation of aliphatic and aromatic compounds, which could alter soil chemical properties and biota (Aislabie *et al.,* 2004, Saul *et al.,* 2005). Unintentional release most often occurs by accidental spillage during fuel handling (Klein *et al.,* 2012). Due to the extreme cold of the Antarctic, these pollutants become trapped and persist in the environment (Corsolini, 2011). These pollutants are semi-volatile and are readily transported over long distances once they enter the environment via water or the atmosphere (Wania, 2003). Both tourist and science programmes rely heavily on hydrocarbon combustion to supply power for transport, heating, construction, and power generation (Hughes and Stallwood, 2006). For example, the United States Antarctic Program (USAP) uses c. 25 million litres of fuel (National Science Foundation, 2004). At McMurdo Station and Scott Base, both situated on MDV soils, hydrocarbon

contamination of the surrounding soils resulted in a change in soil microbial communities (Aislabie *et al.,* 2004, O'Neill *et al.,* 2013).

1.6.1 Physical disturbances

The soil surface is one of the most vulnerable environments in the Antarctic and provides a habitat for MDV soil biota. Severe ecological implications result from human soil disturbance, particularly biological processes. The MDV soil lacks plant roots, which allows water and nutrients to readily translocate down the soil profile (O'Neill *et al.,* 2013). As a result, the dominant food web of microorganisms is limited close to the soil surface (Wall and Virginia, 1999). Disturbance in the form of compaction from pedestrian trampling has been demonstrated to directly disturb abiotic features (Zak *et al.*, 1994). When the abiotic features of soil are compacted, vital terrestrial processes such as nutrient cycling are also impaired, thus, affecting overall functional diversity (Garcia-Pausas and Paterson, 2011, Hartmann *et al.,* 2014). Compaction from trampling also disturbs soil biota. As compaction from increased foot traffic intensifies, species abundance and richness decrease (Tejedo *et al.*, 2016). This pattern has been observed for Arthropod species (*Collembola*) (Ayres *et al.,* 2008) and plant species (mosses, lichens, bryophytes, algae and cyanobacteria) (Tejedo *et al.,* 2016).

The severity of soil disturbance and subsequent recovery depends on the soil type, disturbance intensity (e.g., the number of passes, foot vs vehicle), and regional climate. Although coastal tourist sites experience a large volume of foot traffic, coastal sites are more recoverable and resilient due to large differences in climatic conditions: warmer temperatures, higher humidity, and higher rainfall (O'Neill, 2017, Pershina *et al.,* 2018). In comparison, the arid MDV soil substrates are soft and are vulnerable to disturbances from pedestrian traffic that may recover after a few seasons or remain visible for 10s-100s years (O'Neill, 2017). The recovery of disturbed soils is aided by freeze-thaw cycles, wind to a lesser extent, and biological re-colonisation (Campbell *et al.,* 1993).

The protocol for field operations has changed markedly since New Zealand's early presence in the Antarctic. Vehicular use is limited to lake ice, except for a few sites in Taylor Valley, including New Harbor and Marble Point (Antarctic New Zealand, 2015). In previous decades, tractors were utilised in Wright Valley by the New Zealand programme. Tractor tracks in Wright Valley remain visible today from tractors operating in the 1970s (Figure 1.3). Unlike pedestrian traffic, soil disturbance from vehicular tracks is more severe and persistent (Tin *et al.,* 2009). Soil disturbance resulting from foot traffic in and around field camps can also be severe when the desert pavement is disturbed, often remaining visible decades after the event. The fine underlying material becomes exposed and easily dispersed by aeolian processes (Campbell *et al.,* 1993). In soft material, as few as 20 walking passes are sufficient to cause a disturbance still visible after 23 years (Campbell *et al.,* 1998). Remote

sensing is a possible solution to reduce disturbance to MDV substrates compared to more traditional ground-based field sampling. This approach is relatively new in an MDV setting and utilises unmanned aerial vehicles (UAVs) such as drones and fixed-wing aircraft. Potential applications for UAVs in the MDVs include spatial ecological and landscape surveys, such as mapping cyanobacterial mats (Bollard-Breen *et al.,* 2015).

Figure 1.3 Vehicular tracks in Wright Valley 2017 near Brownworth Hut, produced by a tractor in the 1970s.

1.6.2 Global warming and invasive species

Global warming is possibly the greatest anthropogenic threat to the Antarctic, affecting biotic and abiotic systems (González-Alonso *et al.,* 2017). Current climatic changes can be primarily attributed to increased solar radiation leading to increased permafrost and glacial melting (Fountain *et al.,* 2014). The flow-on effects of increased melting are serious and include a reduction in snow cover, an increase in soil moisture and nutrient mobilisation and a large release of organic carbon into the contemporary carbon cycle (Mann *et al.,* 2014). These changes primarily affect hydrology, geochemistry and biological communities, particularly dry-adapted microbial species in soil. It has been postulated that climate change has already weakened biogeographic boundaries, resulting in the homogenisation of ecological niches in the polar regions (Pointing *et al.,* 2015). The climate of the Antarctic region has been stable for millennia until recently (Denton *et al.,* 1993), having been covered in a permanent ice sheet ~34 mya. Southern Victoria Land has had ice-free regions, including the MDVs, for millions of years, which are on the verge of climate-induced change. These areas have acted as refugia for terrestrial taxa and have helped preserve local biodiversity amidst changes such as the last glacial maxima (McGaughran *et al.,* 2008).

Permafrost is an important feature of Antarctic soils, vulnerable to seasonal and longer-term climatic changes. The MDVs are situated in Victoria Land, an area unique from the rest of the continental Antarctic in several ways. This region undergoes dramatic climatic changes every year where the "active zone" of the permafrost undergoes freeze-thaw cycles annually (Guglielmin *et al.,* 2011). Victoria Land is also one of the few places in continental Antarctica where groundwater flow through the active layer has been documented (Papale *et al.,* 2018). This active layer is characterised by harsh conditions of extended periods of sub-zero temperatures, large fluctuations in soil temperature and moisture availability, long dry periods, low nutrient availability and high levels of UV radiation (Steven *et al.,* 2006). This environment is a distinct ecological niche, largely inhabited by cold-adapted psychrotolerant microorganisms (Wilkins *et al.,* 2013). The resident microbial communities carry out most of their physiological activities in the active layer, including carbon mineralisation from CO_2 and methane frozen in the permafrost, one of the most important feedback items in a terrestrial system (Mann *et al.,* 2014). These communities are also largely endemic and highly adapted to their environmental niches (Takacs-vesbach *et al.,* 2013, Kleinteich *et al.,* 2017). It is known that these microbial communities are sensitive to change and respond rapidly in the context of a changing environment (Buelow *et al.,* 2016, Niederberger *et al.,* 2019); it is predicted that the continent will continue to get warmer and wetter (Bracegirdle *et al.,* 2008).

The advent of global warming increases the likelihood of successful establishment of microbial species, coined as 'alien invasion' by Hughes and Convey 2014a. Soil surface temperatures fluctuate widely, ranging from -15°C to a maximum of 18°C in summer (Balks *et al.,* 2002), which falls well within the cardinal growth temperatures of mesophiles (0° T_{min}- 40°T_{max}). The result of such climatic change would be the replacement of terrestrial taxa adapted to cold, oligotrophic conditions by more cosmopolitan taxa from temperate climes (Hughes and Convey, 2014b). The high levels of endemism, isolation, and a lack of inter-species competition characteristic of Antarctic soil communities also make them vulnerable to being invaded and outcompeted by alien species (Chown and Convey, 2007). There is also a risk of homogenising Antarctic biota from different areas within the continent through the human-mediated transfer of native species, which can lead to a loss of genetic diversity in soil communities (Frenot *et al.,* 2005, Hughes and Convey, 2010, Convey and Peck, 2019). When alien species become established, they can dominate both environmental and biogeochemical processes and permanently change community function (Cowan *et al.,* 2011). It is largely unknown how Antarctic microbiota will respond to invasive microbes, regarding their resilience and whether overall ecological function will be changed (Hughes *et al.,* 2013).

1.6.3 Modes of transport of Invasive species to the Antarctic

Antarctica is constantly inundated by non-indigenous organisms, plant propagules, microbes and arthropods from the Southern Hemisphere (Muñoz *et al.,* 2004). The annual net movement of exotic biomass into the continent is an estimated >70,000 seeds and plant propagules, transported from northern clines by an estimated 20% of tourists and 45% of science workers visiting the region (Huiskes *et al.,* 2014). Natural processes that facilitate the travel of new species to the Antarctic are dominated by Aeolian processes (pollen and microbes) but can also include transportation by animals such as fish, migratory birds, and sea mammals, as well as oceanic currents (Vincent, 2000).

Compared to natural transport processes, humans are the primary facilitators of introducing non-indigenous species to the Antarctic by several orders of magnitude (Hughes and Convey, 2012). Human vectors, such as ballast water in ships, aeroplanes, Velcro, clothing, footwear, fresh food and building materials, have enabled species to colonise new territories (Vincent, 2000, Whinam *et al.,* 2005). The only two plants native to the Antarctic region are *Colobanthus quitensis* and *Deschampsia antarctica* (Molina-Montenegro *et al.,* 2014). In contrast, there are c. 200 recorded invasive species in the sub-Antarctic, some of which have altered island ecosystems (Frenot *et al.*, 2005). A well-documented invasion is the grass *Poa annua,* a cosmopolitan grass species found in areas of anthropomorphic soil disturbance and in microclimates near permanent structures built by humans (Galera *et al.,* 2015).

1.7 The "unseen" footprint: Human DNA and human-associated microorganisms

1.7.1 The shedding of biological materials

Hair, skin and gut cells and human-associated microbiota are continually disseminated from humans and are released into the environment (Caldwell *et al.,* 2007). The rate of release of cells and DNA into the environment varies between individuals, referred to as "shedder status", and is affected by factors such as gender (Tan *et al.,* 2019), use of moisturiser (Schwender *et al.,* 2021), and habits, e.g. touching one's face (Jansson *et al.*, 2022). DNA 'shedding' refers to the passive release of genetic materials instead of DNA deposition by intentional touch.

Human presence can also be identified indirectly by detecting human-associated microorganisms. Each part of the human body harbours a distinct microbial signature, as illustrated by the Human Microbiome Project (HMP) (Huttenhower *et al.,* 2012). For instance, compare the oral cavity, where *Streptococci* (*S. salivarius* and *S. mutans*) dominate (Nakanishi *et al.,* 2009), to the colon, where Bacteroidetes and Firmicutes dominate (Bik *et al.,* 2010, Jandhyala *et al.,* 2015). Faecal matter is composed of microorganisms by 60% weight (Stephen and Cummings, 1980), of which 28-44% are

Bacteroidales (Hong *et al.,* 2008). The microbiome comprises mutual and commensal species representative of the host's environment and habits. A microbiome differs between individuals based on their diet (David *et al.,* 2013), the presence of pets (Misic *et al.,* 2015), their health status (Casarin *et al.,* 2013), obesity (Boulangé *et al.,* 2016), with whom they live (Song *et al.,* 2013) and their location (Yatsunenko *et al.,* 2012). Microbial communities have a high degree of interindividual variability (Costello *et al.,* 2009) but are likely to remain stable within an individual over time (Fierer *et al.,* 2008).

1.7.2 Cell and DNA persistence in the environment

Cell viability is expected to rapidly diminish in the MDV environment due to the harsh climatic conditions. Ah Tow and Cowan (2005) demonstrated that *S. epidermidis,* a common human skin commensal, could be detected in highly impacted areas but not in low-impact or pristine areas. Although the *S. epidermidis* cells rapidly lost viability, the cells persisted for a long time as non-viable cells and/or naked DNA. Released skin microbes are persistent (Fierer *et al.*, 2010), possibly due to their high tolerance to environmental stressors, such as UV and temperature fluctuations, similar to the microbes native to MDV soils.

DNA molecules can be released into the environment by dead or decaying cells, compromised cells, viral particles and via excretion by living cells (Nielsen *et al.,* 2007). About 40% of detectable DNA is of extracellular origin (Carini *et al.,* 2016). Once released onto the soil, DNA has several fates: it can be consumed as a nutrient source, be incorporated into a biofilm or biocrust (Vorkapic *et al.,* 2016, Swenson *et al.,* 2018), be taken up by bacteria as part of horizontal gene transfer (Thomas and Nielsen, 2005) or persist in the environment when adsorbed to soil particulates (Morrissey *et al.,* 2015). DNA released into the environment is a significant nutrient source. It is chemically uniform and ubiquitous for heterotrophic organisms, where it can be utilised as a sole carbon source and provides bioavailable P and N for bacterial growth (Paraskova *et al.*, 2013). Adsorption of DNA to solid soil components promotes DNA persistence in soil, where bound DNA is more resistant to DNase degradation than free DNA (Crecchio and Stotzky, 1998). The adsorbed DNA remains available for uptake by competent bacteria and may contribute to horizontal gene transfer (Romanowski *et al.,* 1992).

A range of intrinsic and extrinsic factors interact to affect DNA persistence in soil. The former includes factors such as the DNA source (Pietramellara *et al.,* 2009), G + C content (Vuillemin *et al.,* 2017) and DNA conformation (e.g., supercoiled plasmid) (Poly *et al.*, 2000). Extrinsic factors include soil mineralogy (Ogram *et al.,* 1988, Levy-Booth *et al.,* 2007), moisture (Widmer *et al.,* 1996), temperature (Widmer *et al.,* 1996, Gulden *et al.,* 2005), light intensity and soil pH (Levy-Booth *et al.,*

2007). Large variations in these factors have resulted in considerable differences in DNA persistence across studies (Ogram *et al.*, 1988, Barnes *et al.,* 2014).

Upon release, DNA degrades over time due to processes such as microbial enzymatic activity, mechanical shearing, hydrolysis and oxidation, nicking, and becoming chemically altered with cross-links, abasic sites and deaminated cytosines (Lindahl, 1993, Willerslev *et al.*, 2004, Briggs *et al.,* 2010, Gould *et al.,* 2010, Overballe-Petersen *et al.,* 2013). The extreme environmental conditions in the Antarctic of desiccation and freezing are like those used for DNA preservation (e.g., freeze-drying): rapid desiccation, low temperature, low pH, and high salt concentrations have all been shown to slow down DNA degradation processes (Pietramellara *et al.,* 2009), thus DNA can be expected to persist in MDV soils for a long time. Naked DNA is stable in soils and sediments and can persist at detectable levels for years in cold desert soils (Pietramellara *et al.*, 2009). The oldest recovered DNA was obtained from perennially frozen, dry environments, such as a horse specimen from Siberia ~1.28 mya BP (van der Valk *et al.,* 2021), and an environmental DNA (eDNA) sample from the Kap København Formation sediments in Northern Greenland from 2.4 mya (Kjær *et al.,* 2022).

Data around DNA persistence in terrestrial systems is comparatively scarce compared to aquatic environments. In a terrestrial system, the degradation of environmental DNA has also been observed as asymptotic exponential decay in soil (Morrissey *et al.,* 2015). An inverse relationship exists between the number of surviving DNA fragments and their length; as time progresses, a larger proportion of smaller fragments are recovered (Dabney *et al.,* 2013). The exact upper limit of successful amplification from an ancient DNA (aDNA) or eDNA is unknown. Theoretically, small amounts of DNA could persist in the environment as part of the exponential decay pattern (Sirois and Buckley, 2019). Realistically, given enough time, DNA will likely be too degraded to be amplified with PCR as it becomes increasingly fragmented because of hydrolysis and oxidation (Gould *et al.,* 2010).

1.8 Molecular Techniques

1.8.1 Selection of markers

There is an array of previously published genetic markers to detect human presence, which target the DNA of humans, or the DNA of human-associated microbe species. For this research, previously published markers and the methodology for their development were based on faecal source tracking (FST) studies, an area of research that aims to identify the origin of faecal pollution in water. Human DNA markers target mitochondrial DNA (mtDNA) (He *et al.,* 2016, Zhu *et al.,* 2020). MtDNA markers are selected due to having the greatest abundance in the human genome of 100s-1000s of copies of mtDNA, depending on the tissue type (Zhang *et al.,* 2015, D'Erchia *et al.,* 2015). For microbial DNA

markers, regions of the 16S rRNA are usually targeted. Most microbial DNA markers are derived from the dominant gut microorganisms, usually anaerobic bacteria of the *Bacteroidales* order (Bernhard and Field, 2000, Nakanishi *et al.,* 2013, Åström *et al.,* 2015), as well as bacteria belonging to the *Bifidobacterium* (Ballesté and Blanch Anicet, 2011) and *Lactobacillus* (Marti *et al.,* 2010) genera.

Microbial DNA markers are more abundant than human DNA markers (Nakanishi *et al.,* 2016). Estimates of bacterial cell abundance range from more than 10-fold than human cells (Savage, 1977) to a more recent estimate of human/bacterial cells of the same order of magnitude (1:1) (Sender *et al.,* 2016), thus microbial markers are perceived to provide greater sensitivity for detecting a target. A caveat with using a microbe target is that an individual is not guaranteed to harbour a certain commensal species. A human DNA marker is more geographically stable, as changes in diet do not alter the host DNA, whereas a gut commensal species could be affected (Roslev and Bukh, 2011). Microbial markers for FST must first be rigorously validated before being applied to a new geographic region, as performance can vary, resulting in lost sensitivity or specificity (Toledo-Hernandez *et al.,* 2013).

1.8.2 Target detection

The human and microbial DNA markers in this research were treated as eDNA samples, where the deposition of human materials was assumed to be passive via routine activities in the field. The samples will be soil collected along the transects from field camps and stations in the MDVs for the spatial component of this research. 'Environmental DNA' (eDNA) refers to the sum of DNA that can be extracted and analysed directly from environmental samples without requiring culturing methods (Taberlet *et al.,* 2012). Sources of released genetic materials can be from sloughed cells, hair, faeces, gametes, blood, and mucous secretions, obtainable from a range of samples like soils and sediments, water, and aerosols (Willerslev *et al.,* 2003, Thomsen *et al.,* 2012, Bohmann *et al.,* 2014). eDNA comprises a heterogeneous mixture of DNA, including genomic, plasmid and chromosomal DNA, from a range of organisms at various stages of degradation, from intact DNA to small fragments, that can be free-floating or adsorbed to environmental particulates (Siuda and Chrost, 2000). The detection of eDNA has proven useful in detecting low-abundance targets across various environments for both extant and extinct species (refer to Table 1. of Thomsen and Willerslev, 2015). Applications include microbial source tracking (Xi *et al.,* 2015), surveillance of water quality (Sinigalliano *et al.,* 2010, Liang *et al.,* 2015) and conservation (Turner *et al.,* 2015, Boothroyd *et al.,* 2016), species distribution (Matsuhashi *et al.,* 2016), biomass estimations (Takahara *et al.,* 2012, Doi *et al.,* 2017) and occupancy probability across surveying sites (Pilliod *et al.,* 2013, Schmidt *et al*., 2013).

There are several considerations for working with eDNA samples. The DNA recovered from environmental samples is characteristically low abundance, often fragmented, and in various stages of degradation (Ficetola *et al.*, 2015, Thomsen and Willerslev, 2015). The environmental conditions of temperature, sunlight exposure and marker origin affect the decay rate of an eDNA marker (Walters *et al.,* 2009, Dick *et al.,* 2010), in addition to DNA length and conformation (Barnes *et al.,* 2014). An environmental sample will contain a mixture of DNA species. The target is likely to be several orders of magnitude lower than the DNA of the soil communities (Taberlet *et al.,* 2018). The success of an eDNA assay relies on maximising sensitivity, i.e., the probability of detection. Increased sensitivity can be achieved by optimising the surveying method in both the field (e.g., the volume of sample collection) and laboratory (e.g., number of PCR replicates), and also relies on the dispersion of target DNA concentration at a site (Furlan *et al.,* 2015).

Real-time or quantitative polymerase chain reaction (rtPCR or qPCR) is often considered the gold standard for quantifying DNA, attributed to its high quantitative performance, high accuracy, precision, reduced handling time, dynamic range, use of closed tube assays and low cost (Morrison *et al.,* 2006, Navarro *et al.,* 2015). Applications requiring absolute DNA quantification using qPCR include functional genomics (Van Heetvelde *et al.,* 2017), quantification of genetically modified organisms (Cankar *et al.,* 2006), conservation (Doi *et al.,* 2015), copy number variation and gene quantification (Bharuthram *et al.,* 2014, Tanaka *et al.,* 2017, Demeke and Eng, 2018), human medicine (Hudecova, 2015, Ramírez *et al.,* 2015), forensics (Swango *et al.,* 2006, Swango *et al.,* 2007, Bruijns *et al.,* 2016) and titration of next-generation sequencing libraries (White *et al.,* 2009, Buehler *et al.*, 2010, Robin *et al.,* 2016).

qPCR uses either specifically designed primers and probes or an intercalating dye to amplify and quantify a targeted DNA region. The results obtained from qPCR can be qualitative, showing either the presence or absence of a DNA region, or quantitative. DNA quantification is achieved by detecting fluorescence at the logarithmic stage in PCR from the quantification cycle (C_q). The amplification signal is detected above the background level as the PCR product accumulates exponentially (Bustin *et al.,* 2009). Theoretically, each cycle would produce 2^n copies after *n* cycles if efficiency is 100%. More realistically, amplification saturates and reaches a plateau as PCR reagents are consumed and the PCR products self-anneal (Quan *et al.,* 2018). qPCR performance relies on external calibration with reference standards and endogenous controls to quantitate samples of unknown concentration. Variations in external calibration can significantly skew quantification accuracy and, thus, become a source of variation in analytical performance between laboratories (Hudecova, 2015, Svec *et al.,* 2015).

There are challenges and limitations with qPCR, particularly when quantifying low-copy number templates such as eDNA samples. qPCR is sensitive to PCR inhibitors often co-purified with environmental DNA that skews quantification by inhibiting *Taq* polymerase activity and primer annealing (Schrader *et al.,* 2012, Hunter *et al.,* 2017). Poor amplification efficiency affects the cycling threshold (C_T), affecting the accuracy of target DNA quantitation (Hoshino and Inagaki, 2012).

Digital PCR (dPCR) is a newer technology that can provide absolute quantification of DNA and overcome some of the limitations of qPCR. In the early stages of dPCR development, researchers identified that a combination of a limiting dilution of DNA, end-point amplification and Poisson statistics could provide absolute quantification of the target without the need for calibration (Vogelstein and Kinzler, 1999, Sykes *et al.*, 1992). dPCR signal is binary, where the sample is first diluted and partitioned. Partitioning concentrates target DNA into discreet reactions as technical replicates compared to a single-tube assay. Concentrating sample DNA into partitions reduces template competition, better enabling the detection of a rare target in the presence of other higher abundance DNA species (Quan *et al.,* 2018). Due to the fixed number of partitions, dPCR has a lower dynamic range than qPCR. A reduced dynamic range is a trade-off for superior precision, sensitivity, and reproducibility over qPCR (Sanders *et al.,* 2011, Sanders *et al.,* 2013). Precision and sensitivity are also increased with more partitions by enabling resolution between samples of minute DNA concentration differences (Dube *et al.,* 2008). The target quantification is not affected by delayed amplification or Cq variability as for qPCR (Whale et al., 2012). Endpoint detection of the target may be the reason for the higher precision observed in eDNA surveys compared with qPCR (e.g., Whale *et al.,* 2012, Doi *et al.,* 2015)

1.8.3 Principles of target quantification with digital PCR

The criteria to achieve absolution quantification with dPCR are i) the random distribution of DNA molecules across partitions follows a Poisson distribution and ii) the DNA target is present at a limiting concentration. The distribution of DNA molecules into the partitions before amplification is a random and independent process (Bhat *et al.,* 2009). After thermocycling, the probability that a partition will contain at least one target sequence corresponds to a binomial process: either 0 or ≥1 copies of target DNA per partition. The DNA target is typically detected using fluorophore-labelled oligonucleotides, such as TaqMan chemistry (Hindson *et al.,* 2011). Data interpretation is more simplistic than for qPCR; Poisson statistics are applied, which requires a proportion of partitions to be void of target DNA to produce an accurate quantification (Quan *et al.,* 2018). At the endpoint of thermocycling, there is an enumeration of binomial events (absence (0) OR presence (1) of detected fluorescence) for each partition, and a Poisson correction is applied during the analysis to account

for partitions with more than one target DNA molecule (Robin *et al.,* 2016). Quantification is calculated from the proportion of positive endpoint reactions using Poisson statistics:

$$\lambda = -\ln(1 - p)$$

Where λ is the average number of target DNA molecules per replicate reaction, and p is the fraction of positive end-point reactions. An estimate of the absolute quantification of the DNA target is then produced from λ, in conjunction with PCR reaction volume and the number of replicates analysed (Hindson *et al.,* 2011). In cases of 10,000 or more partitions, the maximal confidence is obtained for a λ value of about 1.6; the precision is poor for low values of λ, reaches an optimal for a λ of 1.6 before slowly declining with increasing values of λ, which corresponds to a saturation of the partitions (Majumdar *et al.,* 2015).

1.9 Summary, objectives, and hypotheses

This literature review outlined the dominant activities in the Antarctic, the associated anthropogenic impacts, their risks, the current environmental management practices, and the available contemporary genetic tools to quantify DNA. As human activity in the Antarctic increases and diversifies, the associated footprint is anticipated to increase. Visible environmental disturbances have been thoroughly investigated, but not the 'unseen' disturbances, which consist of human materials released during routine operations in the field.

Under the ATS, there are obligations to carry out environmental impact assessments and to leave the environment unimpacted to the maximum degree practicable, as outlined in the six annexes of the EP. These obligations need to be addressed regarding unseen human impact. Before the environmental risks can be identified, the unseen human impact must first be detected and quantified. The simplicity of the MDV soils, in conjunction with highly sensitive molecular detection tools, provides an opportunity to test hypotheses around DNA persistence on spatial and temporal scales.

This research endeavoured to investigate the unseen human impact (UHI) through the detection of human DNA and human-associated microbes on spatial and temporal scales, and to investigate DNA persistence in a McMurdo Dry Valley environment, by investigating the following objectives:

1) To determine if UHI is detectable and quantifiable in an MDV setting

An assay targeting a region of the human *MT-CYB* gene was applied to a field camp at Vanda Station in Wright Valley during the 2015/16 field work season to demonstrate that UHI is detectable and quantifiable in the MDVs as a proof of concept. Given the reported persistence of DNA in cold terrestrial systems, the mtDNA signal was expected to persist to detectable levels with a cumulative effect. The signal was expected to be highest closest to Vanda Station, where human activity was concentrated and more human materials were expected to be released passively from routine activity.

2) To assess which types of UHI are most prevalent at camp and investigate their spatial patterns of detection, and determine whether a site can recover from human occupation

Three dPCR assays targeting genetic markers were applied to a range of impacted sites in the MDVs to identify the predominant source of UHI. The assay targets were a region of the human *MT-CYB* gene, *Bacteroides dorei* 16S rRNA and a region of the *tuf* gene from *Staphylococcus epidermidis*, to detect human DNA and released faecal and skin materials, respectively. The mtDNA marker was anticipated to have the greatest signal compared to the bacterial markers, as reported by previous

FST studies. The faecal marker was included to investigate the current management of faecal impact, by comparing the enclosed and exposed toilet areas. The exposed toilet areas were expected to have the capacity to recover from faecal material by being exposed to environmental factors that promote DNA degradation, e.g., solar UV, and by the aeolian dispersal of released human materials and DNA-bound soil away from the site.

The highest detected UHI signal was anticipated to be in the areas within or in close vicinity to a campsite, where most of the foot traffic was anticipated, and subsequently, more human materials were expected to be released through routine activity.

3) To assess DNA longevity in an MDV setting

Soil from Miers Valley was inoculated with three DNA conformations, intracellular plasmid, extracellular plasmid, and a linear amplicon derived from plasmid, then placed in Miers Valley. The DNA was collected after one and two years *in situ*, and DNA was isolated and amplified with four different primer pairs. These results were compared to controls that were not placed in Miers Valley. The aim was to assess which factors promoted DNA persistence in soil, including DNA conformation, soil sterility and exposure to solar UV. Previous studies demonstrated that DNA readily binds to soil components and persists for years in cold desert environments. Microbial degradation and solar UV exposure were expected to produce noticeable DNA degradation compared to the sterile and 'dark' controls. DNA decay in the environment is a complex interaction between intrinsic and extrinsic factors; it can be challenging to quantify the effect of each factor on DNA degradation in a complex system like soil.

The core themes of this thesis are summarised in Chapter 5, which is intended to be an online 'Antarctic Environments Portal' publication. The purpose of the Portal is to provide a link between Antarctic-focused science and policy. This link enables policymakers (e.g., CEP and the ATCP) to make environmental management decisions and to highlight emerging issues, on the foundation of current evidence-based information that undergoes rigorous peer review.

Chapter 2. A new genetic tool for detecting and quantifying the remnants of human trace at field camps in terrestrial Antarctica

Abstract

The terrestrial environment of Antarctica is commonly described as the last pristine place on Earth, yet in ice-free areas, Antarctica experiences an intense human visitation from research scientists and tourism. Humans constantly disseminate biological materials containing human DNA and human-associated microorganisms. This unseen human impact (UHI) in the form of amplifiable DNA has not been quantified in a cold desert environment such as terrestrial Antarctica, where the longevity of this material is expected to be persistent and detectable. As a proof of concept, a TaqMan assay (HcytB) targeting human *MT-CYB* gene was adapted for a digital PCR (dPCR) platform to detect and quantify human traces in soil from Wright Valley, Antarctica. This assay proved to be highly sensitive and specific in a polar desert environment and provided an absolute quantification of DNA at a single-cell level. The ability to detect and quantify human DNA offers the opportunity to detect the presence and accumulated impact of human habitation at any site and, ultimately, with continued sampling, the longevity of this impact. This molecular tool's development applies to environmental forensics, much like point source tracking, or where human presence needs to be determined and quantified.

2.1 Introduction

The success of our species has enabled most of the globe to be inhabited or explored by humans. As a result, anthropogenic activities and their associated impacts have affected most ecosystems globally. In terrestrial systems, there is a range of environmental impacts, from the highly visible (e.g., deforestation, soil disturbance, the effects of agriculture) to the unseen impacts (e.g., the release of pesticides and other xenobiotic chemicals, foreign microbes, and genetic materials into the environment). The unseen human impact (UHI) in the form of environmental DNA (eDNA), derived from humans and human-associated microorganisms, can be detected and accurately quantified with PCR-based methodology supporting the emerging field of environmental forensics.

Since the International Geophysical Year (1957-58), Antarctic visitation has increased exponentially. The number of tourists was projected to be 78,520 in 2019-2020, twice that of a decade ago (IAATO, 2019), and at least 4000 tourists have visited the Taylor Valley Visitor Zone alone since the 1990s (Priscu & Howkins 2016). In response to increasing interest and presence, the Antarctic Treaty System (ATS) was established to ensure that Antarctica will be reserved for peaceful purposes, particularly science-driven endeavours, free from warfare and nuclear activity, and to prevent the continent from becoming a subject of international discord. New Zealand was one of the original signatories to the Antarctic Treaty in 1959 (it came into force in 1961). Under the ATS, the Environmental Protocol was signed in 1991 (Madrid) and enacted in 1998, requiring that the environment be left without adverse impact after all activities. “Impact” can be interpreted as remnants of human genetic materials and human-associated microorganisms, referred to as UHI. Unlike physical impacts (e.g., hydrocarbon release, soil disturbance), unseen impacts are not immediately apparent and are much less tractable. The unseen environmental risks must first be identified and quantified to determine if mitigation is possible or required. A small proportion of terrestrial Antarctica (<0.31%) is free of snow and ice (Terauds & Lee 2016), the largest contiguous area being the McMurdo Dry Valleys (MDVs) located in Victoria Land (Fox *et al.* 1994). These ice-free areas experience disproportionately high visitation due to the proximity of several research bases, including McMurdo Station (US), Scott Base (NZ), Zuchelli (Italy) and Jang Bogo (South Korea), that directly drive an increase in field operations during the austral summers (COMNAP 2017, Priscu & Howkins 2016), making them particularly vulnerable to direct anthropogenic impacts.

The unseen remnants of routine human activity, derived from human-associated bacteria and human DNA, have not been thoroughly investigated in any terrestrial ecosystem (Emmons *et al.* 2017), much less a cold desert such as the MDVs. The environmental conditions in the MDVs of low temperatures, low moisture availability, and high salt conditions (Cary *et al.* 2010) are ideal for the long-term preservation of DNA (Caputo *et al.* 2011). Terrestrial Antarctica has several advantages in

testing hypotheses concerning microbiology compared to a temperate environment. Food webs are simple and most of the biomass in Antarctic soils is microbial (Niederberger *et al.* 2008). The biomass turnover is much slower than in temperate or tropical terrestrial environments (Pan *et al.* 2013), thus any genetic material released is more likely to persist in the environment. Therefore, the MDVs provide a tractable environment to test hypotheses associated with the detection, distribution, and persistence of human genetic material in the environment on both spatial and temporal scales.

Highly sensitive methods capable of single-copy detection are required to detect and quantify a low-abundance target in an environmental DNA (eDNA) sample. Genetic markers used in conjunction with quantitative PCR (qPCR) or, increasingly, digital PCR (dPCR) are well suited for this purpose, with applications in quantifying faecal contamination (Cao *et al.* 2015, Monteiro & Santos 2017, Vadde *et al.* 2019), conservation (Coxon *et al.* 2019), detecting invasive species (Doi *et al.* 2015) and genetically modified organisms (Halter & Zahn 2017). Digital PCR has several advantages over qPCR in being more robust, as it does not require calibration standards and is less prone to inhibition (Huggett *et al.* 2008, Rački *et al.* 2014). These factors and its capability to outperform qPCR at the lowest detection levels in sensitivity and specificity (Quan *et al.* 2018, Salipante & Jerome 2019, Franke *et al.* 2020) make dPCR ideal for working with eDNA.

The environmental conditions of MDV soils of low moisture and constant low temperatures (Cary *et al.* 2010) emulate the conditions used in DNA preservation. Ancient eDNA studies have demonstrated that DNA persists extensively in cold environments, having been recovered from a range of materials, including soils, sediments, and permafrost (Willerslev *et al.* 2007, Orlando *et al.* 2013, Slon *et al.* 2017), with the oldest reported to be 1.2 million years old (van der Valk *et al.* 2021). The methodology in this study was derived from environmental forensics and ancient DNA approaches. A simple system, such as the MDVs, was required to test the effectiveness of this methodology as an environmental forensic tool. This research aimed to validate a molecular detection tool to quantify the unseen remnants of human activity at varying spatial scales. We present a new, highly sensitive tool capable of quantifying human DNA in Antarctic mineral soils.

2.2 Materials and methods

2.2.1 Procedures for sterility, DNA removal and an overview of eDNA controls

It was imperative throughout all procedures to maintain absolute sterility to ensure that any detected signal was attributable to an environmental source and not from contamination. This required sterilisation via the removal of contaminating DNA and microorganisms. Care was taken during surveying and sample handling to prevent the transfer of human materials to samples at the sampling site or during processing in the lab. A Tyvek suit and face mask were worn at all stages during sample handling, both impermeable to particles ≥0.1 µm, including skin, microbes, and hair. We predicted a low abundance of the DNA target in the soil, thus the methods in handling were derived from those used for ancient DNA and forensic samples and used biocidal UV radiation (254 nm) (Weber *et al.* 2016) and a solution of 30% H_2O_2 and 0.4% (v/v) sodium hypochlorite, as described by Champlot *et al.* (2010). All equipment, consumables, and glove surfaces were sterilised in the field with ChemGene HLD_4L (Medimark Scientific Ltd, United Kingdom) and 30% H_2O_2. All reagents prepared for DNA isolation were filter-sterilised (0.22 µM) and autoclaved. For each set of extractions, the required reagent volumes were aliquoted and then sterilised with UV radiation for 1 hr before use, together with consumables and equipment. All soil DNA extractions were isolated in a PC2 clean facility where no PCR products or other high-concentration DNA samples were processed.

It is recommended to incorporate a range of controls into eDNA surveying, to identify any source of contamination, and to establish a baseline to distinguish dPCR amplification from background noise (Takahashi *et al.,* 2023). A total of four types of controls were included during field work and laboratory handling, to ensure the complete removal of contaminating DNA and microorganisms, and to establish a baseline level of HcytB dPCR signal (Figure 2.1). Duplicate controls were collected for each transect during surveying, which included i) a no-target 'soil control' (SC) from an area far away from camp (e.g., from under a rock), and ii) a 'no soil control' (NSC) to monitor the sterility of the sampling equipment and consumables, where water was used in place of soil. In the laboratory, duplicate controls were included for iii) the extraction buffer during DNA isolation, and iv) no template controls (NTC) using ultrapure water were included for each PCR. The mean signal for each control type was used to establish a baseline of HcytB signal from a range of sources (e.g., reagents, dPCR instrument noise) to set a threshold for positive dPCR amplification.

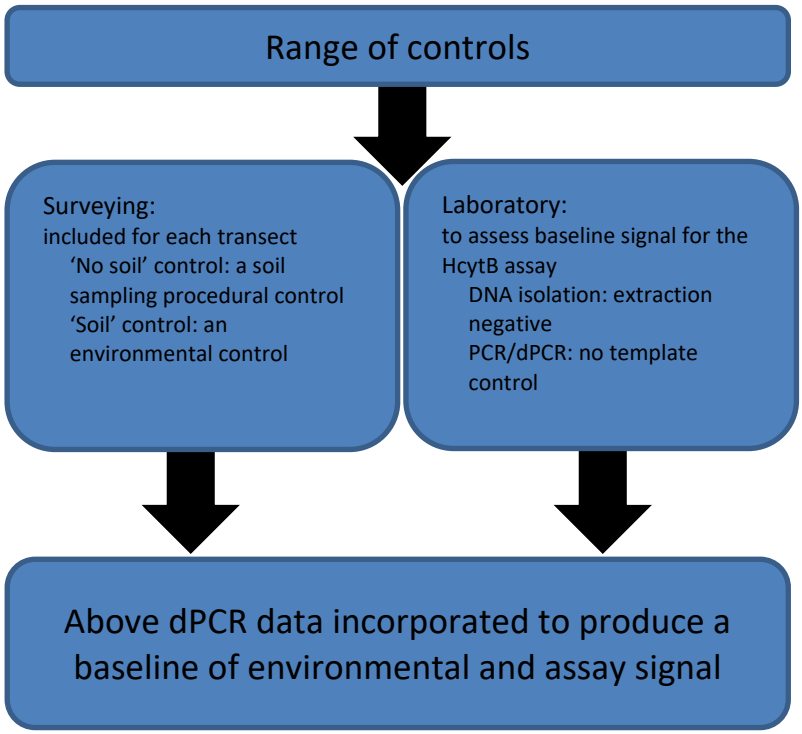


Figure 2.1 Pipeline for establishing an accurate level of background signal at each study location.

2.2.2 Study site and soil collection

Vanda Station (S 77.51667, E 161.66667) was a NZ Antarctic field camp consisting of four structures located 140 m southeast of Lake Vanda and 110 m from the Onyx River. Since the completion of construction in 1969, Vanda Station has been regularly occupied, with an average of 400 visitors per year (Taylor 2015) before being decommissioned in 1993 due to the threat of inundation from the shores of Lake Vanda (Harrowfield 1999). Some structures were relocated to the Vanda Hut Facilities Zone (S 77.52286, E 161.68901) and have been used by researchers since 1995, replacing Vanda Station. This more recently used site (hereafter referred to as Vanda) was selected as the primary study site because it is a permanent structure with regular human occupation since construction. Vanda is, therefore, a site where sufficient human microscopic remnants have likely been deposited, which could subsequently be detected and quantified on a spatial scale. Soil sampling occurred during the 2015-2016 and 2016-2017 field seasons.

During the 2015-2016 season, a single transect of samples was surveyed, extending from the Vanda huts in a south-easterly direction. There was no field camp during that season. During the 2016-2017 field season, three transects were sampled, radiating from the Vanda huts and away from Lake Vanda. There was a large field camp that season, ranging from 4 to 13 people over 17 days,

equivalent to ~145 person-days in the campsite's vicinity. Each transect converged near the entrance to the Vanda huts, where the highest volume of foot traffic was anticipated (see Figure 2.2). The transect direction was selected to be up-valley to increase the likelihood of detecting remnants of deposited human materials dispersed with the prevailing westerly wind during the austral summer (McKendry & Lewthwaite 1990). A total of three transects were included to increase the up-valley land area surveyed and to maximise the likelihood of detecting human traces (Figure 2.3).

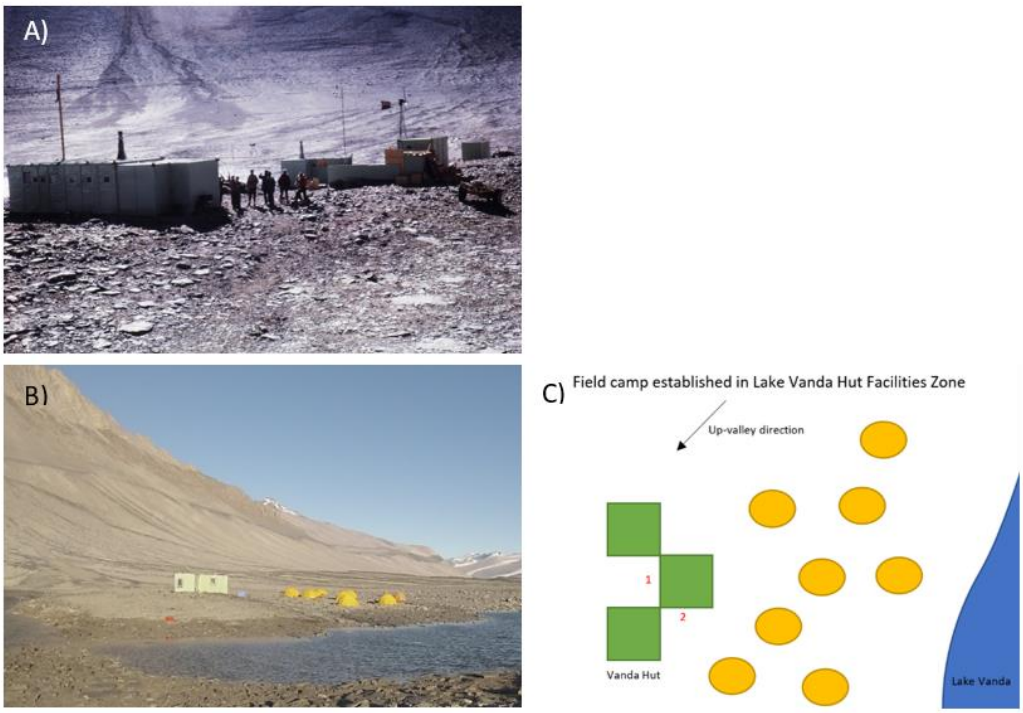


Figure 2.2 (A) The original Vanda Station in 1969 (B) Photograph of Vanda Hut during the 2017-18 field season (C) Schematic of the camp layout at Vanda Hut Facilities Zone. The point labelled 1 is the entrance to the Vanda huts, which experienced the highest foot traffic and where Transects 1 and 2 originated. Point 2 is where Transect 3 originated. The camp consisted of nine tents and a toilet bucket near the Vanda huts, a permanent structure.

(A) (B) (C)

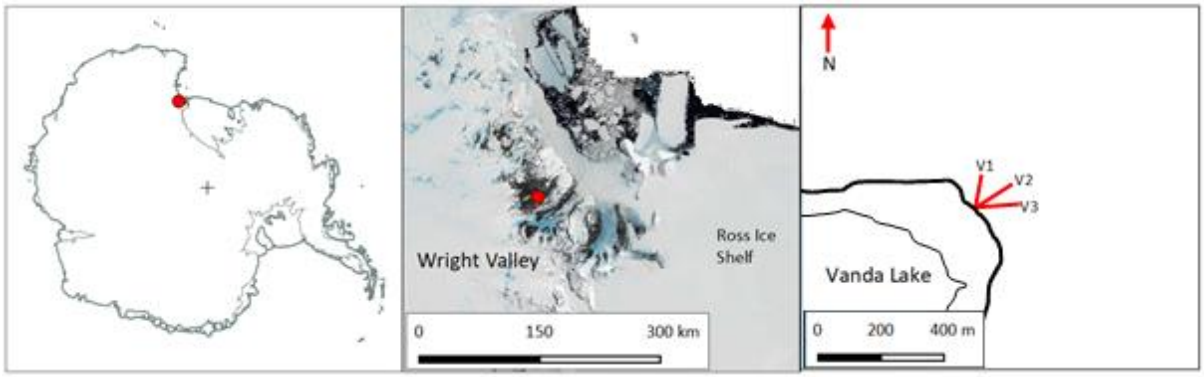


Figure 2.3 (A) Wright Valley in Antarctica, (B) Wright Valley in Victoria Land, (C) the locations of each transect radiating from Vanda in Wright Valley.

A total of 12 soil samples were collected for each transect at 0, 1, 2, 4, 8, 16, 32, 64, and 128 m from the Vanda huts entrance. Sampling was carried out on an exponential scale to capture the anticipated pattern of an exponential decrease of the target signal as distance increased from the Vanda huts entrance. 15 g of surface soil (0.5 cm depth) was collected for each sample and placed into a nucleic acid-free 50-mL Falcon tube. In some instances, sample collection was not possible due to a geographic feature (e.g., surface water). All soil samples were stored in a chiller in the field, then in a -20°C freezer at Scott Base before being transported back to NZ under dry ice. Once received in the laboratory, samples were stored at -80°C until processed.

2.2.3 DNA isolation

A cetyltrimethylammonium bromide (CTAB) protocol optimised for low-biomass soils was used to extract environmental DNA (Barrett *et al.* 2006), with the following modifications: 1) 5 g of soil was weighed into a 15-mL Falcon tube with a UV-sterilized extraction buffer consisting of 1,350 µL phosphate buffer (100 mM NaH_2PO_4) and 1,350 µL of SDS lysis buffer (10% SDS, 100 mM NaCl, 500 mM TRIS pH = 8.0); 2) after DNA precipitation, the DNA pellet was resuspended in 18 µL 1x TE buffer; 3) reagent controls were included with each lot of extractions.

2.2.4 Primer design and TaqMan analysis

A mitochondrial genetic marker was selected to detect human DNA from the Vanda soil samples using previously published primers and probes that amplify a region of the human mitochondrial *MT-CYB* gene (Baker-Austin *et al.* 2010) and conventional qPCR. The selection of a human mitochondrial marker ensured the greatest likelihood of detecting human genetic remnants, compared to human nuclear DNA or a microbial marker, and guaranteed universal distribution

across all individuals in abundance (Zhu *et al.* 2020). The probe length was optimised for a QuantStudio 3D digital PCR platform (QS3D; ThermoFisher, Massachusetts, USA) by truncating to 13 bp to increase specificity, following recommendations for the minor-groove binding chemistry as outlined by ThermoFisher (Waltham, Massachusetts, USA). TaqMan minor groove binding (MGB) probes are designed to have a higher melting temperature (T_m) and a shorter probe length to increase assay specificity. Careful assay design is paramount to preventing base-pair mismatches in the primer and probe regions between the assay and target species (Wilcox *et al.* 2013).

The assay region was amplified with endpoint PCR using the following conditions. Thermocycling was done on a QS3D ProFlex (ThermoFisher, Massachusetts, USA), with an initial denaturation at 94°C for 2 min, followed by 32 cycles of 94°C for 2 min, annealing at 58°C for 30 s, extension at 72°C for 30 s, followed by a final extension at 72°C for 2 min. The amplicons were visualised on a 1% agarose gel to confirm that a single amplicon of the correct size was amplified. To verify that the modified primer/probe-combination maintained specificity on the dPCR platform, initial control and sample amplifications were sequenced on an ABI Prism 3100 Genetic Analyzer (Waltham, Massachusetts, USA). The resultant nucleotide sequence was checked against GenBank entries (*Homo sapiens* KU686629.1, KU68674.1) to confirm correct gene amplification. A sequence alignment of the target region of human mitochondrial HcytB was made in Geneious (v7.1 by Biomatters) to ensure that the primer and probe regions were conserved. The primers were synthesised by Integrated DNA Technology (Coralville, Iowa, USA). Applied Biosystems synthesised the TaqMan probe (Foster City, CA, USA) labelled with a 6-FAM reporter dye at the 5' end and an MGB and a non-fluorescent quencher (NFQ) at the 3' end.

An assay standard (864 bp) flanking the dPCR HcytB assay target (125 bp) was produced using newly designed primers (Table 2.1) and a PCR mixture that contained 0.2 µM dNTPs, 2.0 mM $MgCl_2$, 1U Platinum Taq and 1x buffer (ThermoFisher, Massachusetts, USA), 0.02 mg mL^{-1} bovine serum albumin, 0.4 µM of each forward and reverse primer, prepared to a final volume of 25 µL with Milli-Q H_2O. Thermocycling conditions were 94°C for 2 min, 30 cycles of 94°C 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and then a final extension at 72°C for 5 min. The purpose of the assay standard was to test assay sensitivity and determine the lowest detection dPCR limit, as well as acting as a positive control. The assay standard was serially diluted 10-fold over five orders of magnitude ranging from 32,000 (1.6 DNA molecules/partition) to 3.2 amplicon copies per chip and quantified with the dPCR HcyB assay following the thermocycling conditions stated below in section 2.2.5.

Table 2.1 Primers and probe used in this study.

	Sequence 5'-3'	Length (bp)	Melting temperature, T_m (°C)	Amplicon size (bp)
HcytB assay				
Forward Primer	CCTCCAAATCACCACAGGACTAT	23	59.8	125
Reverse Primer	CGTGAAGGTAGCGGATGATTC	21	58.9	
TaqMan Probe	FAM-TCGCCCACATCAC-MGB	13	69.0	
Assay standard				
Forward primer	TTCTCGCACGGACTACAACC	20	60.0	864
Reverse primer	GGGTGTTTAAGGGGTTGGCT	20	60.2	

2.2.5 Amplification and quantification with QuantStudio 3D dPCR

QS3D v2 dPCR chips were loaded with isolated DNA in the following preparation: 0.95 µM of each forward and reverse primer, 0.25 µM TaqMan probe, 1x QS3D master mix, 5.0 µL of extracted DNA, and ultrapure water to make 9.5 µL. The PCR master mix was prepared with a 10% excess to account for the volume lost from pipetting. Each QS3D v2 chip was loaded with 9.5 µL of the above master mix and 5.0 µL isolated eDNA to a final volume of 14.5 µL. The QS3D v2 chips consist of 20,000 partitions of 755 pL volume.

Thermocycling was carried out according to the manufacturer's recommended parameters: 96°C for 10 min, 39 cycles of 60°C for 2 min and 98°C for 20 s, followed by a final extension at 60°C for 2 min on a QS3D ProFlex thermocycler (ThermoFisher, Massachusetts, USA). The QS3D Digital PCR instrument (application version 3.1.2, algorithm version 4.4.10) measured the endpoint fluorescence. A Poisson Plus model (Majumdar *et al.* 2017) was used to transform the data as some samples exceeded 2,000 copies μL^{-1}, with a confidence interval of 95% and the desired precision set to 10%.

Absolute quantification of the HcytB amplicon was achieved using the QS3D Digital PCR system in conjunction with the QS3D AnalysisSuite™ Cloud software (v3.0). Firstly, the QS3D software produced fluorescence thresholds (distinguishing between amplification and no-amplification partitions), which were manually adjusted. Each sample's fluorescence threshold was reviewed in the scatter plot and histogram views before making a comparison to the positive and NTC chips. The fluorescence threshold varies according to signal intensity, so the scatterplots of samples of a similar signal were overlaid. Outlier data points were omitted from each chip in the scatterplot view. The quality control determined by the cloud software is based on the number of chip partitions that exceed a quality threshold based on loading, fluorescent signal, and noise. The quality threshold was set to the manufacturer's default 0.5, as no observed artefacts such as bubbles or incorrect PCR

mixture volumes were observed. The resultant QuantStudio 3D output produced an absolute quantification of the target as copies μL^{-1} in 5 g of soil.

2.2.6 Assay sensitivity and cross-reactivity

In any environmental sample, the target HcytB was expected to be at much lower concentrations than non-target DNA species. To test that the sensitivity of the HcytB dPCR assay remained constant under inundation by exogenous DNA, ~3 molecules of the assay standard amplicon (refer to Table 2.1) was spiked with DNA extracted from a New Zealand mollusc, the green-lipped mussel *Perna canaliculus*, in 0 ng, 250 ng, 500 ng, 750 ng, and 1,000 ng amounts. DNA from this organism was used to test cross-reactivity as this species is not present in MDV soils, and the isolated DNA had no mitochondrial crossover with the targeted human *MT-CYB*. Each spiked sample was then amplified with dPCR as described above, but only 1 µL of DNA was added to the PCR mix, with the remaining volume made up with Milli-Q water to 14.5 µL.

The specificity of the HcytB assay was tested *in silico* using the National Centre for Biotechnology (NCBI) tools BLASTn for the targeted HcytB region and Primer-BLAST (Ye *et al.* 2012) for the primer and probe sequences. To check for any cross-reactivity with Antarctic megafauna, an alignment of nine nucleotide sequences of the region amplified by the HcytB assay was produced in Geneious (v7.1; Biomatters). Nucleotide sequences were obtained from GenBank, including human, other primates, and Antarctic fauna (*H. sapiens* KU686629.1 and KU686674.1, *Pygoscelis adeliae* AB776021.1, *Leptonychotes weddellii* KU885358.1, *Gorilla gorilla* AY550982.1 and KJ193350.1, *Pan troglodytes* EF660849.1 and EF660850.1, *P. paniscus* JF727224.2) (Appendix A, Figure 7.1). The only mammal to frequent the Dry Valleys besides humans is the occasional Weddell seal (*L. weddellii*) that travels up the valleys from the coast and often dies in the process. Cross-reactivity was also tested with 3.2 ng of mummified seal DNA extracted from muscle tissue.

2.3 Results and discussion

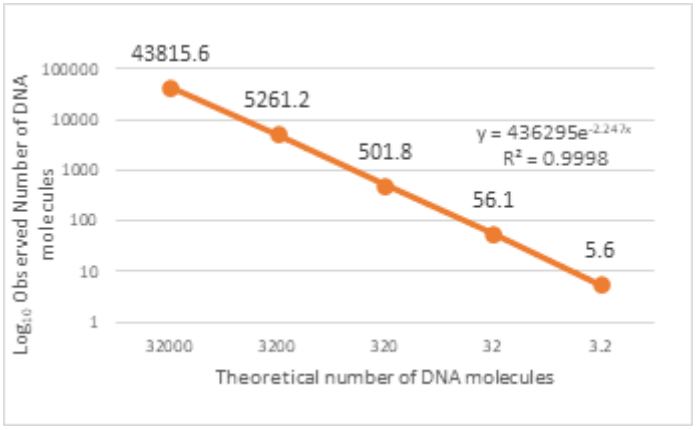
2.3.1 Target confirmation

The designed HcytB assay primers successfully amplified a 125 bp region of human mitochondrial *MT-CYB,* and the assay standard primers amplified an 864 bp region flanking the assay region (Table 2.1.) The correct size of both amplicons was confirmed with visualisation on agarose gel electrophoresis (data not shown). A nucleotide BLAST search (BLASTn) of the assay standard nucleotide sequence confirmed that the amplified region was the intended target of the *MT-CYB* gene of *Homo sapiens* (position 14,717 to 15,534 of the human mitochondrion, NC012920.1).

2.3.2 Limit of detection and sensitivity

In developing any dPCR quantification tool, the dynamic range and limit of detection with the native target (amplicon) must be determined in exogenous non-target DNA. The HcytB dPCR assay using the new primers and probe had a log-linear range of detection over five orders of magnitude using only the assay standard (Figure 2.4A). A dynamic range of five orders of magnitude is acceptable for dPCR, which is typically limited to four rather than seven orders of magnitude for standard qPCR (Jones *et al.* 2016). There are several constraints on the dPCR dynamic range compared to standard qPCR, including a fixed number of partitions on a chip (QuantStudio) and a requirement for the target to be present at a limiting concentration for maximal sensitivity and precision (Majumdar *et al.* 2015). dPCR produces positive and negative amplification across the 20,000 partitions per chip. To enumerate λ (target molecules per partition), an estimate is derived from the fraction of partitions without amplification over the total number of partitions. Poisson statistics are applied to account for the partitions with positive amplification where one, two, or more target molecules were amplified (Majumdar *et al.* 2017). A concentration of molecules in the environmental sample can then be obtained by dividing the above by the partition volume, assumed to be constant.

(A)



(B)

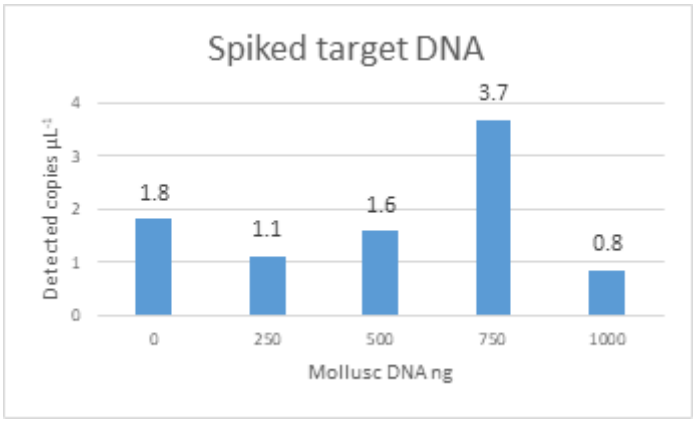


Figure 2.4 (A) Sensitivity of the HcytB assay for QuantStudio dPCR. The template was a serially diluted assay standard, an amplicon that flanked the assay region. (B) 3 copies of the assay standard were spiked with non-target DNA.

In this investigation, the limit of detection (LOD) of the HcytB assay was 5.6 copies μL^{-1} of the *MT-CYB* gene (5.22 ag of DNA) at a 95% confidence interval (Figure 2.4A). LOD was derived by serially diluting a known number of target molecules in a neutral solution. Detection of the target DNA species was not possible at further dilution (i.e., six orders of magnitude dilution) due to the amplification stochasticity and the difficulty in distinguishing between positive amplification or background noise of the TaqMan assay. Target quantification at one order of magnitude greater than 32,000 molecules (320,000 molecules) was not tenable due to positive amplification flooding the chip.

Forensic analysis of environmental DNA is challenging, with factors such as degradation and low DNA copy number limiting quality material to use in downstream analysis, such as short tandem repeat

(STR) typing and sequencing (Niederstätter *et al.* 2007, Thomsen & Willerslev 2015). It, therefore, requires high sensitivity to the target and stringent aseptic handling to prevent contamination during sample collection and processing, especially when detecting a human target. A range of factors throughout the sampling and analytical processes contribute to variation in DNA quantification at low copy number levels, including pipetting inconsistency, heterogeneous clumping of DNA, the presence of PCR inhibitors, the varying states of eDNA degradation, and even the type of plastic used in lab consumables (Ellison *et al.* 2006). Considering that a human somatic cell contains ~80 to ~2000 mitochondria, depending on the state of the cell (Robin & Wong 1988, Kukat *et al.* 2011, Bogenhagen 2012), the detection level attained here is equivalent to significantly less than a single human cell.

The result of spiking ~3 copies of the assay target standard with serially increasing exogenous non-target mollusc DNA did not affect the dynamic range or detection sensitivity of the HcytB assay (Figure 2.4B). Increasing the amount of exogenous non-target DNA from 0 ng to 1000 ng did not significantly increase positive amplification and, thus, no increase in false-positive signal (Figure 2.4B). Dry Valley Antarctic soils are characteristically extremely low in microbial biomass and typically yield low levels of extracted DNA (Goordial *et al.* 2016). A typical low yield of DNA isolated from MDV soil is unlikely to lead to non-target amplification or primer competition. As MDV soil is an environmental sample containing an array of DNA species, it was necessary to exclude the possibility of non-target amplification or primer competition in the presence of exogenous DNA, particularly when the target DNA is present at a much lower abundance.

2.3.3 Cross-reactivity and specificity of the HcytB assay

Both *in silico* testing and direct experimentation with non-target DNA produced no indication of the HcytB assay's cross-reactivity with any species other than the intended target, human HcytB (Appendix A, Figure 7.2). *In silico* specificity testing of the primers and probe using GenBank indicated they were highly specific for HcytB and would not cross-react with any known DNA sequence other than the intended target, i.e., a region of the human *MT-CYB* gene. 100% nucleotide identity of the assay amplicon was observed in the targeted *MT-CYB* gene region of *H. sapiens*. In addition, no cross-reactivity was observed when tested with DNA extracted from the mummified seal tissue at a concentration significantly higher than would ever be expected in any MDV soil. The amplification signal was comparable to the background signal produced in the NTC (4.5 cf. 2.1 copies μL^{-1}).

2.3.4 HcytB detection on a spatial scale

There was a pattern of HcytB signal across the three transects surveyed from Vanda during 2017 (Table 2.2). Human HcytB signal was greatest closest (0-4 m) to the main hut for all three transects, and the signal attenuated as the distance from the station increased. Target signal decreased to background signal levels for two of the three transects surveyed in 2016-2017 at 128 m for transect 1, and 8, 64, and 128 m for transect 3. Transect 2 remained above the background signal for all distances (Table 2.2). The variation in signal intensity across the three transects could be attributable to the aeolian dispersal of the deposited human materials. Wright Valley has a bimodal wind regime, where katabatic winds from the plateau dominate in winter, and lighter winds prevail from the ice shelf in summer (Doran *et al.* 2002). With this wind regime, Transect 2 should capture the most human materials dispersed around the Vanda huts in summer.

eDNA target detection is affected by the DNA shedding rate of each human, the length of accumulation time and the duration of eDNA persistence in the environment (Buxton *et al.* 2017). Previous field and laboratory studies have indicated that increased target organism abundance and organism density increase detectable eDNA (Klymus *et al.* 2015, Anaïs *et al.* 2016). Human signals around Vanda were not uniform across the transects surveyed, most probably because foot traffic was likely concentrated closer to the station where the transects converged near the station entrance.

2.3.5 Attenuation of signal

Transect 2 was surveyed over two austral summers: i) January 2016 (no field camp), and ii) January 2017 (a large field camp). Before sampling, the total human hours for the 2017 field camp at Vanda was estimated to be ~145 days, compared to the 0.1 person days (2 person-hours) spent at Vanda in 2016. Regular human occupation is known to have occurred in the vicinity of Vanda since Vanda Station was commissioned in 1969. At least 9285 person days have been recorded at Vanda Station from 1969 to 1995, while 479 person days were recorded at the newer Vanda hut from 1995-2007, according to available Antarctica NZ records.

For transect 2, we observed a level of HcytB signal that never diminished below that of background level for both 2016 and 2017. A greater signal level of more than two-fold was observed during 2017, compared to 2016 (a mean of 297.3 vs 132.9 copies μL^{-1}, Table 2.2). An ANCOVA test demonstrated that the difference between human hours (between transect 2 in 2016 and all three transects in 2017) did not significantly affect the detected signal ($p = 0.54$). Distance from the hut entrance had a significant effect on the detected signal, which attenuated as distance increased ($p < 0.01$), likely indicating that foot traffic affects the signal (Figure 2.5).

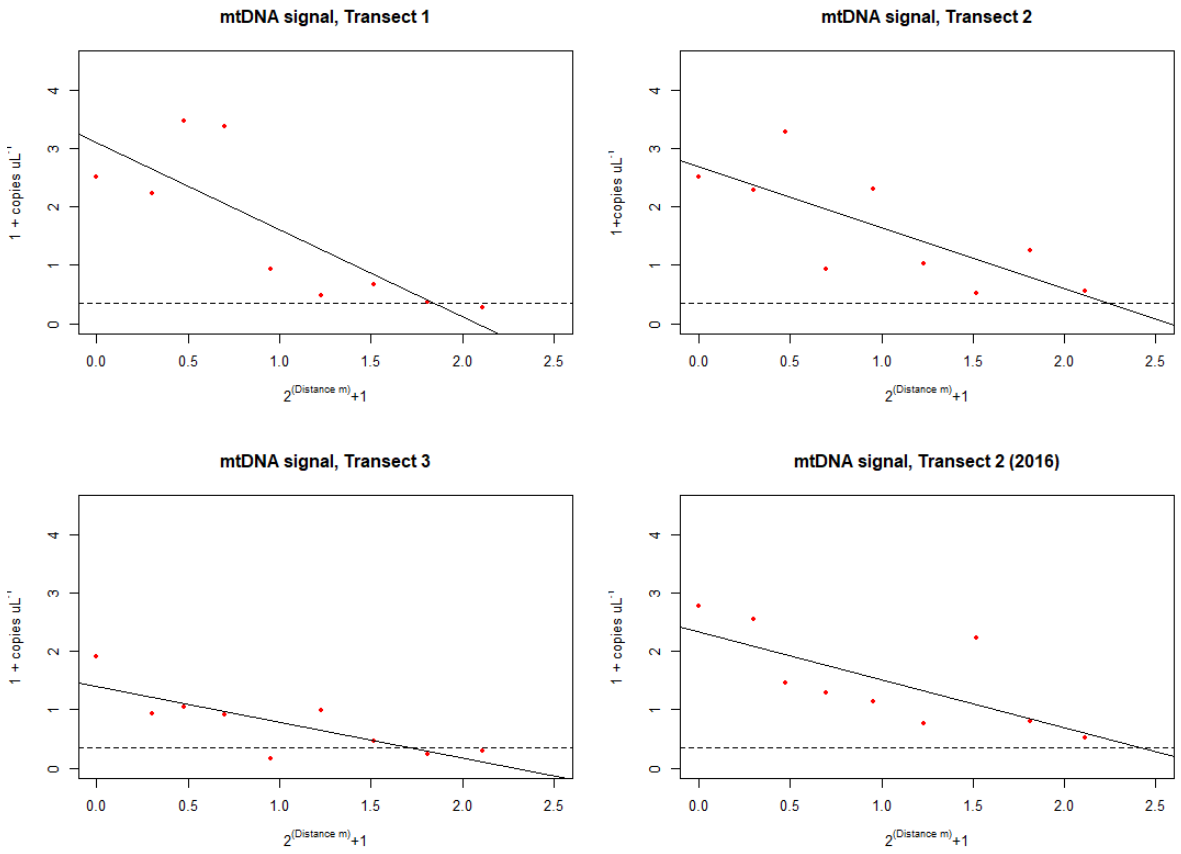


Figure 2.5 Linear regression of log_{10}-transformed transect data. The background level of the mtDNA (log_{10}(0.35 copies μL^{-1})) signal is shown as a dashed line. All transects originated at the entrance of the Vanda huts.

2.3.6 Factors affecting DNA persistence in McMurdo Dry Valley soil

The MDV land area comprises exposed soil primarily free of ice and snow during the austral summer. The soil matrices are highly heterogeneous in physicochemical properties and biotic content (Lee *et al.* 2012, Bottos *et al.* 2020). Both biotic and abiotic factors affect DNA persistence in soil. High temperature, high UV-B, and low pH are abiotic factors that have been identified in promoting DNA degradation (Strickler *et al.* 2015, Eichmiller *et al.* 2016). The mean soil temperature in the MDVs fluctuates greatly during the austral summer and throughout the year, ranging from -15°C to -40°C, depending on depth (Thompson *et al.* 1971). DNA degradation is accelerated at higher temperatures when the microbial communities flourish and microbial metabolism increases (Strickler *et al.* 2015, Tsuji *et al.* 2017).

Once released onto the soil, there are several outcomes for DNA, which can be degraded by nucleases, removed by horizontal gene transfer, consumed as a nutrient source by heterotrophs (Pietramellara *et al.* 2009), or adsorbed by soil particulates (Paget *et al.* 1992). Soil composition, such

as the proportion of clays and silts, will influence the number of surface reactive particles present and the resultant adsorption of genetic materials (Prosser & Hedgpeth 2018). DNA adsorption to soil colloids and high salt concentrations will protect against enzymatic degradation, promoting DNA persistence in the environment (Cai *et al.* 2006). Neither eDNA persistence nor mobility has been thoroughly investigated in a terrestrial environment, much less an MDV environment. Previous studies have suggested that DNA can persist at detectable levels beyond 100,000 kya in soils and sediments, particularly in cold desert soils (Ah Tow & Cowan 2005, Pietramellara *et al.* 2009, Andersen *et al.* 2012, van der Valk *et al.* 2021). Polar desert soils are characterised by low moisture availability throughout most of the year (Campbell *et al.* 1998, Wynn-Williams 1998), large fluctuations in surface temperature (Obryk *et al.* 2020), and often high conductivities (Geyer *et al.* 2014), which are all conducive to DNA preservation and long-term persistence.

The amount of biological material and DNA shed by each person varies between individuals. It is widely accepted in forensics that there is variation in "shedder status," i.e., the number of cells and resulting deposited DNA varies significantly between individuals (Goray *et al.* 2016). The variation in the number of cells released (Tan *et al.* 2019) and the varying states of DNA degradation affect HcytB detection levels. Human DNA is obtainable from cells and hair disseminated from the human body, often providing an obtainable genetic profile. An estimated 0.4 million exfoliated skin cells are released from the skin surface daily (Wickenheiser, 2002), most of which are keratinised and enucleated (Kita *et al.* 2008). Fragmented DNA is also sloughed off the cornified skin layer in sweat and can be utilised for individual identification with genetic markers (Kita *et al.* 2008, Akutsu *et al.* 2018). Hair is another source of DNA commonly encountered in forensic investigations. Both mitochondrial and nuclear DNA is obtainable from hair samples. The former is available from both the hair root and shaft, whereas the latter is usually limited to the hair root (Ottens *et al.* 2013, Linacre & Ottens 2016).

2.3.7 Considerations

While the dPCR methodologies are extremely powerful for our application, there must be consideration of potential biases. PCR-based technologies, including dPCR, can overestimate low DNA concentrations through false-positive background signal (artefacts) and non-specific amplification (Strain *et al.* 2013, Kiselinova *et al.* 2014), and from the "concentration plateau" in dPCR assays because of fixed instrument responses (Hunter *et al.* 2017), pipette errors and sample-related effects (Majumdar *et al.* 2015). Technical replication of PCR samples at low template concentrations may not produce greater consistency in quantification where samples are stochastic, containing zero, one, or multiple DNA molecules from the same solution. This makes samples with low template concentration difficult to distinguish as a true positive or as signal due to an artefact or

noise. In this study, it was paramount to establish a background level of signal from the mean of all combined negative controls, both environmental and procedural, throughout surveying and handling in the lab. dPCR is very sensitive to contamination, and forensic-level cleanliness is required when the targets are human DNA.

A qPCR/dPCR assay must be rigorously tested in a defined geographic location to detect eDNA (Ohad *et al.* 2015). An eDNA sample is likely to contain the target at a low concentration in various states of degradation amongst a range of other DNA targets and PCR inhibitors. An assay able to meet the preceding challenges robustly is a forensic application candidate, particularly in an environmental forensic context (Zubakov *et al.* 2018). The high sensitivity and lack of cross-reactivity with any Antarctic or non-Antarctic species suggest that the HcytB assay is a powerful tool to quantify UHI in an MDV environment.

Consistent periodic human occupation during Antarctic field seasons of both the original Vanda Station (since 1969) and the more recent Vanda Hut Facilities Zone (since 1995) provides a good start to estimate where human activity was likely to have been concentrated across field seasons, although both structures were constructed in slightly different locations. The exact movements of personnel could not be determined from field camp records, but sampling was planned based on the assumption that foot traffic was concentrated near the buildings. A more comprehensive record of foot traffic could be incorporated into future investigations, such as incorporating an individual's movements via GPS.

	Copies μL^{-1}											
	Transect 2 (Jan 2016)			Transect 1 (Jan 2017)			Transect 2 (Jan 2017)			Transect 3 (Jan 2017)		
Distance from camp (m)	Mean	Lower CL	Upper CL	Mean	Lower CL	Upper CL	Mean	Lower CL	Upper CL	Mean	Lower CL	Upper CL
0	591.0	567.3	615.7	321.3	305.0	338.4	327.2	305.0	338.4	82.1	74.2	90.9
1	362.5	344.8	381.1	173.1	160.8	186.2	194.8	160.8	186.2	7.4	5.3	10.5
2	27.7	23.0	33.3	3020.9	2960.5	3082.6	1912.8	2960.5	3082.6	10.3	7.7	13.8
4	19.0	15.2	23.7	2381.5	2327.3	2437.0	7.7	5.6	10.7	7.1	5.0	10.0
8	12.9	9.7	17.0	7.7	5.6	10.7	201.9	188.7	216.1	0.5	0.1	1.9
16	4.8	3.1	7.5	2.0	1.0	3.8	9.8	7.2	13.2	8.7	6.3	12.0
32	170.2	157.6	183.8	3.7	2.3	5.9	2.3	1.2	4.3	2.0	1.0	3.9
64	5.4	3.5	8.3	1.3	0.6	2.9	16.8	13.0	21.2	0.7	0.2	2.2
128	2.3	1.2	4.4	0.9	0.3	2.3	2.6	1.4	4.6	0.9	0.4	2.5
Mean copies μL^{-1}	132.9			656.9			297.3			13.3		

Key
Mean background signal 0.000-1.2 copies μL^{-1}
10X Background signal ≥1.2-12.1 copies μL^{-1}
100X Background signal ≥12.1-121.3 copies μL^{-1}
1000X Background signal ≥121.279 copies μL^{-1}

Table 2.2 Human DNA quantified with the HcytB assay from Vanda, Wright Valley. The DNA quantification is displayed as copies μL^{-1} obtained from 5 g soil at each sampling site, with a 95% C.I. Soil samples were collected during the austral summers of January 2016 and 2017.

2.4 Conclusions and further directions

Applying the HcytB assay to MDV soil demonstrated highly sensitive and specific target detection, where absolute target quantification was attained to attogram concentrations. There was no indication of cross-reactivity with Antarctic fauna or any other species by *in silico* or direct experimentation. This tool demonstrated that UHI is accumulative and dependent on distance from a campsite, where signal attenuated as the distance from a camp increased. The assay's sensitivity and specificity were rigorously tested and demonstrated to perform sufficiently in an MDV setting, giving the assay the potential to be used in other terrestrial systems across the continent and beyond.

The ability to accurately detect and quantify UHI in an Antarctic environment enables effective risk management of human activities in the MDVs by providing a validated tool to be utilised by National Programmes. This capability assists in implementing the best practice in field operations under the ATS obligations. To further test the capability of this tool, a range of impacted sites (from pristine to highly impacted) could be tested to determine if a human signal can be predicted with distance from a campsite and whether UHI is accumulative. To extend the investigation of UHI in the MDVs, it is necessary to test more assays *in situ* targeting a range of human materials (e.g., skin, faecal matter), to elucidate the primary source of human contamination. To date, there remains to be a thorough investigation of DNA persistence in MDV soils using a dPCR approach, particularly around the factors that promote DNA persistence and its duration.

Chapter 3. Detecting unseen human trace at field camps and established long-term national programme facilities in the McMurdo Dry Valleys, Antarctica

Abstract

Antarctica is commonly described as the last pristine environment on Earth, yet it has experienced large increases in human visitation since the International Geophysical Year (1957/58). The Antarctic Treaty System was established to reserve Antarctica for peaceful purposes, banning military and nuclear warfare, while promoting research-led activities without adverse effect on the environment. This study aimed to quantify the unseen remnants of continued human activity in the McMurdo Dry Valleys (MDVs) in the form of released DNA derived from humans and their associated microbes. Three molecular detection assays were applied to soil samples collected from temporary and permanent field camps located in the MDVs, Antarctica. Unseen human impact (UHI) in the form of eDNA was detected and quantified using highly sensitive digital PCR (dPCR). The targets were human mitochondrial DNA (HcytB), and bacteria associated with skin and faecal materials, which were 16S rRNA from *Bacteroides* (BacHum) and the *tuf* gene from skin *Staphylococcus epidermidis* (Sepi), respectively. Two assays, HcytB and BacHum, were sufficiently sensitive and subsequently applied to US research stations and NZ field camps. Overall, the human mitochondrial signal was cumulative with occupancy, and was highest closest to a campsite. The mitochondrial signal was predicted to be detectable from a moderately or highly occupied camp up to ~1000 m away using linear regression. The faecal signal was generally localised to within meters of the toilet area of a camp, and could not provide a significant pattern of detection, regardless of the intensity of impact. The faecal assay would thus be better applied to a site of known human occupancy as a threshold to detect faecal contamination. The Sepi assay was found to be insufficiently sensitive for quantifying UHI and was not applied to soil samples beyond the initial validation stages.

3.1 Introduction

Humans have extensively colonised or explored most of terrestrial Earth. The resultant anthropogenic impact of increasing population pressures has affected every ecosystem worldwide (Kareiva *et al.* 2007, Ellis *et al.* 2010, Ellis *et al.* 2013). Antarctica is commonly described as the last pristine area on Earth and was one of the last frontiers to be explored (Hughes *et al.* 2015). Activities are currently tourism-led, which saw 104,000 visitors during the 2022/2023 season (IAATO, 2024), or, increasingly, science-driven, which presently comprise at least 33 National Programmes (NP) and 100 facilities (COMNAP 2017). The impacts associated with these activities range from physical disturbances, such as soil trampling (Tejedo *et al.* 2012, O'Neill *et al.* 2013, O'Neill *et al.* 2014, Brooks *et al.* 2019) and the combustion of hydrocarbons (Lyons *et al.* 2018), to less visible impacts, such as the release of xenobiotic chemicals such as caffeine (Korekar *et al.* 2020), sterols (Isobe *et al.* 2002) and optical brighteners (Salas *et al.* 2019), increased antibiotic resistance in indigenous microbial populations (Miller *et al.* 2009, Segawa *et al.* 2013, Wang *et al.* 2016b), and the release of non-native microbial species (Huiskes *et al.* 2014).

Human presence in the Antarctic has markedly increased since the last International Geophysical Year (IGY) 1957-58 (Walton 2011). The Antarctic Treaty (1959) came into force in 1961, reserving the continent for peaceful endeavours, while promoting science-led activities and harmony between Parties, then later expanding into the Antarctic Treaty System (ATS). The ATS remains the primary international legislation regarding Antarctica (Hughes *et al.* 2018). The first protected area tools (specially protected areas, SPAs) were agree upon in the 'Agreed measures for the conservation of Antarctic Flora and Fauna' (1964), followed by 'The Protocol on Environmental Protection' (The Protocol) signed in 1991. The protocol has recognised the importance of safeguarding the fragile ecosystems on the continent (south of 60°S latitude), designating the continent a 'natural reserve, devoted to peace and science', to ensure that Antarctica's scientific, historical, environmental, and intrinsic values are protected. Later in 1991, Annex V to the ATS, titled 'Area Protection and Management', was agreed upon, which created the classifications of Antarctic Specially Managed Areas (ASMA) and Antarctic Specially Protected Areas (ASPA). ASPAs have the highest level of environmental protection and require a permit for entry. 'Prohibited zones' are located within some APSAs (e.g., 140 and 175), which will be invaluable as baseline comparisons to other areas in the future. In contrast, ASMAs contain currently conducted activities that require a management plan (Annex V, Article 5) and may include ASPAs (Hughes *et al.* 2013).

It is known that terrestrial Antarctica is vulnerable to human impacts after decades of research (Tin *et al.* 2009, Chown *et al.* 2015, Chown & Brooks 2019) and increasingly in the context of global warming (Turner *et al.* 2014, Lee *et al.* 2017). Under Annex I, ATS participants are obliged to

undertake Environmental Impact Assessments (EIAs) for all activities within the Antarctic Treaty area. EIAs are recognised as the most effective in implementing the best practice to mitigate or reduce impact (Convey *et al.* 2012, Pertierra *et al.* 2013, Hughes *et al.* 2019). They are essential as human activity intensifies and diversifies in the Antarctic and the associated footprint increases (Hughes *et al.* 2018). More visible impacts, such as waste generated during field operations, have been investigated and included in the relevant management plans established by participating NPs. The unseen human impact (thereafter referred to as UHI), in the form of released human DNA or the release of non-native microorganisms, have not been quantified in the Antarctic, even though EIAs have been recommended to extend beyond visual inspection (Tejedo *et al.* 2016). Under the ATS, there is an obligation to leave the environment without adverse impact as far as practicably possible after all operations. UHI has yet to be thoroughly investigated in the context of ATS obligations. Recent advances in sensitive methodology have made it feasible and cost-effective to quantify a rare DNA target in the environment (e.g., Hunter *et al.* 2017, Wood *et al.* 2019). Quantifying UHI can serve as a proxy to the geographic extent and recovery of human impact in environments required to be kept pristine, such as the 'prohibited zones' within certain ASPAs. Before determining if mitigation is required, the environmental risks of UHI must first be identified. Other forms of UHI (e.g., invasive non-native microorganisms, increased antibiotic resistance) could potentially be devastating to fragile terrestrial Antarctic biota, as their resilience to change remains largely unknown (Hughes *et al.* 2015, Cavicchioli *et al.* 2019, Hughes *et al.* 2020).

The ice-free areas of Antarctica constitute only a minute proportion of total continental landmass <1% (Convey *et al.* 2012) yet receive a disproportionate amount of human visitation compared to the rest of the continent during the austral summer. One of Antarctica's most visited terrestrial areas is the McMurdo Dry Valleys (MDVs) in Victoria Land, which at 4500 $km^{2,}$ comprise the most extensive contiguous ice-free area in Antarctica (Levy 2013). The MDVs are a designated ASMA and experience significant visitation impact for a terrestrial Antarctic area due to the proximity and accessibility of several permanent research stations in the Ross Dependency, including McMurdo Station (US), Scott Base (NZ), Jang Bogo (South Korea) and Zuchelli (Italy). McMurdo Station (US) and Scott Base (NZ) (COMNAP 2017) receive a total of c. 1300 (National Science Foundation) and c. 300 persons (Antarctica NZ), respectively, over the austral summer.

Several factors make the MDVs a tractable environment to investigate UHI to assess whether the Treaty obligations are being met. Like the rest of terrestrial Antarctica, MDV soils are dominated by microbial communities consisting of bacteria, protists, rotifers and tardigrades (Wynn-Williams 1996, Arenz & Blanchette 2011), all of which could be influenced by UHI. Access to the MDVs is well

supported due to the proximity of NZ and US bases. There are also rarely visited pristine areas within the MDVs, such as Barwick and Balham Valleys (ASPA no. 123), which will be available in the future as a comparison to valleys regularly visited. Microbial communities in the MDVs are highly localised and contain key heterotrophs that drive biogeochemical processes (Lee *et al.* 2012, Cowan *et al.* 2014, Wei *et al.* 2016) and have been reported to change in response to human physical disturbance (Jara *et al.* 2020), yet the long-term effect of releasing non-indigenous microorganisms and genetic materials remains unknown. A comprehensive understanding of how visitation impacts terrestrial systems in Antarctica is critical, particularly under current climate change predictions for a warmer and wetter continent with increasing ice-free areas (Lee *et al.* 2017).

An environmental forensic approach is well-suited to the objectives of this study in quantifying an unseen environmental contamination of human origin, much like microbial source tracking (e.g., Stange & Tiehm 2020, Edge *et al.* 2021), where genetic markers are measured using quantitative PCR (qPCR) or digital PCR (dPCR). dPCR is fast gaining traction for a wide array of applications and has several advantages over qPCR, such as a greater tolerance to environmental PCR inhibitors (Wang *et al.* 2016a) and greater sensitivity (Hunter *et al.* 2017). Poisson statistics are applied to dPCR data to provide an absolute quantification of a target, instead of standard curves for calibration and thus, dPCR has greater robustness, precision, and reproducibility for low target copies than qPCR (Brunetto *et al.* 2014, Cao *et al.* 2015, Whale *et al.* 2016, Rowlands *et al.* 2019) when using TaqMan chemistry (Duong *et al.* 2021). dPCR has successfully quantified low copy number DNA from a range of environmental samples, such as soil, water, permafrost, and bioaerosols (Folloni *et al.* 2012, Blaya *et al.* 2016, Cave *et al.* 2016, Monteiro and Santos, 2017), and can outperform qPCR in sensitivity and specificity at the lowest limits of detection in environmental samples (Doi *et al.* 2015, Nathan *et al.* 2014). The assay targets were anticipated to be present in MDV soil at relatively low concentrations compared to the magnitude of indigenous microbial DNA. To detect and quantify UHI targets TaqMan chemistry was used in conjunction with QuantStudio 3D digital PCR (QS3D, ThermoFisher Scientific, Waltham, MA, US).

This study aimed to develop and validate molecular methods to quantify UHI across spatial scales in a terrestrial Antarctic setting. The research sought to answer three specific questions; which type of unseen human trace is most prevalent at a site, does a campsite recover over time following occupation, and what procedures can be used to minimise UHI source contamination in a field camp? UHI has multiple sources, including human-specific nuclear and mitochondrial DNA (mtDNA) and human-associated microbes, such as commensal gut and skin species. Three dPCR assays were trialled to answer these questions, targeting human mitochondrial DNA (HcytB), faecal bacteria

(BacHum) and skin bacteria (Sepi). Mitochondrial DNA (mtDNA) is the most abundant genetic material in humans and is present in every cell at 100-1000 copies depending on the tissue type (Veltri *et al.* 1990) and is conserved across all humans, providing higher sensitivity and specificity than microbe-associated markers (Zhu *et al.* 2020). A *Bacteroides* target was selected to detect faecal contamination as these organisms comprise 30-40% of human gut bacteria and are obligate anaerobes that do not proliferate in the environment (Bernhard & Field 2000). *Staphylococcus epidermidis* is a permanent and ubiquitous resident of the human skin microbiome (Otto 2009) and, thus, likely a good candidate to detect skin-derived UHI. The Sepi assay detects the *tuf* gene (encoding elongation factor Tu), which is more reliable than 16S rRNA for differentiating between *Staphylococci* (Martineau *et al.* 2001, Li *et al.* 2012, Khosravi *et al.* 2018). Multiple markers were trialled to provide a thorough insight into the type of UHI being left behind and to find the most suitable marker for an MDV environment (Åström *et al.* 2015).

3.2 Materials and methods

3.2.1 DNA decontamination and handling and an overview of controls

It was essential to maintain sterility throughout all procedures, from field sampling to laboratory handling, to ensure that any human-derived DNA and microorganisms were prevented from contaminating throughout the processing pipeline, as the gene targets were human-specific or human-associated microorganisms (overview shown in Appendix B, Figure 8.1). A Tyvek suit, face mask, sleeves, and gloves (PPE) were worn at all stages during sample handling since these are impermeable to particles ≥0.1 µm, including skin, microbes, and hair. During laboratory handling, we used biocidal UV radiation of 254 nm (Weber *et al.* 2016) for 30 min and 30% H_2O_2 and 0.4% (v/v) sodium hypochlorite solution to remove any contaminating DNA from exogenous microorganisms or laboratory personnel (Champlot *et al.* 2010). In the field, all equipment, consumables, and glove surfaces were sterilised in the field with ChemGene HLD_4L (Medimark Scientific Ltd, UK) and 30% H_2O_2. All reagents prepared for DNA isolation were filter-sterilised (0.22 µm) and autoclaved. For each lot of extractions, the required reagent volumes were aliquoted and then sterilised with UV radiation for 1 hr prior to use. All soil DNA extractions were conducted in a PC2 clean facility without other humans present in the lab in a biological safety cabinet (BSCII) where no PCR products or other high-concentration DNA samples had been processed.

A comprehensive range of negative controls were included throughout all stages of surveying and in the laboratory. This was to distinguish true UHI target signal aside from a range of environmental sources and to ensure the absence of contamination. This established a baseline level of background signal (see Appendix B, Figure 8.1 for an overview of all controls). During sample collection, controls included collecting ‘soil negatives’ (SC) from a distant area (1-2 km) from each sampled camp to

assess background signal at each site, 'no soil' controls (NSC) collected by following the sampling procedure described below without collecting soil, to assure the sterility of sampling equipment and consumables and good aseptic technique. No template controls (NTC) were also included during all PCR and dPCR amplifications to eliminate the possibility of reagent contamination and to determine the background level of signal for each assay.

3.2.2 Study locations

This study included a range of field camps and Facilities Zones used by the NZ and US NPs located in four of the McMurdo Dry Valleys: Wright Valley, Miers Valley, Victoria Valley, and Taylor Valley (Figure 3.1). These sites provided a broad range of human visitation: "High", continuous habitation near permanent facilities (US sites in Taylor Valley: F-6, New Harbor, and Fryxell Facilities zone); "moderate", in the vicinity (≤ 20 m) of previous field camp using recorded GPS coordinates (Wright Valley, Miers Valley, Spaulding Pond in Taylor Valley); and "pristine" (Victoria Valley), the only site visited that did not have a previous field camp in the vicinity (Figure 3.1). Records of human habitation obtained from Antarctica NZ were used to estimate human presence ("person days") at the NZ campsites (Table 3.1). Records of human habitation at US sites were not accessible, but these sites were included because they are long-term, well-established facilities with permanent infrastructure and yearly visitation.

(A)

(B)

(C)

(D)

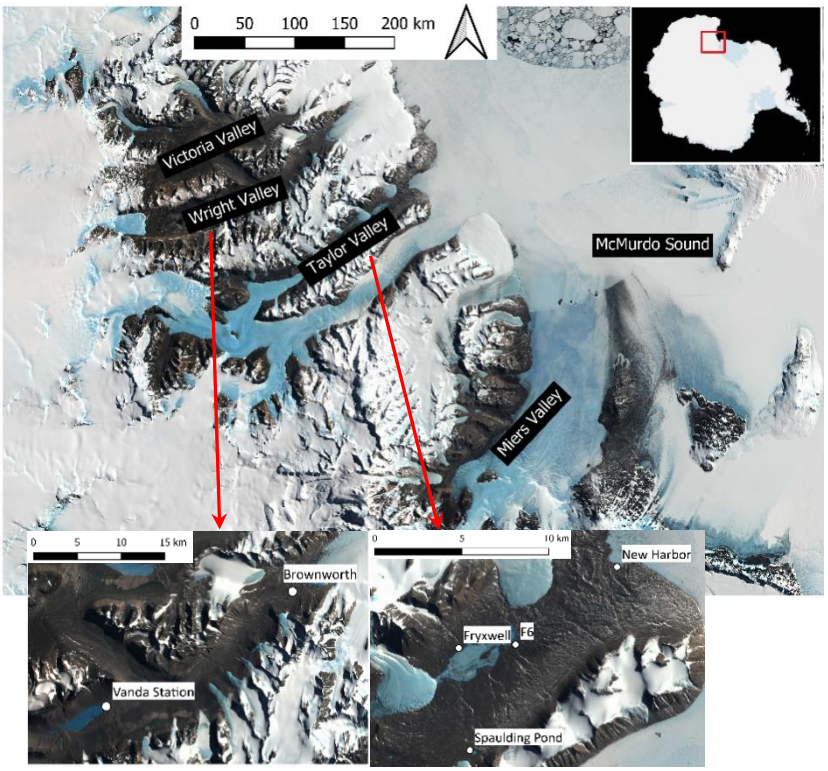


Figure 3.1 Examples of (A) a US research station, Lake Bonney Facilities Zone, with permanent infrastructure and (B) a NZ field camp in Taylor Valley. (C) The toilet area of a NZ camp is either exposed in a small camp (left) or enclosed in a tent (right) in a larger camp. (D) Map of the study sites in the McMurdo Dry Valleys. Multiple camp sites were surveyed in Wright and Taylor Valleys, shown in the inset panels.

Table 3.1 Human presence at NZ field camps during the austral summer. NS = not surveyed. Refer to the "McMurdo Dry Valleys ASMA Manual" for a description of the facilities zone. TFC = temporary field camp. *180.0 person days from a previous field party. ** Refers to three moveable huts established after Vanda Station was decommissioned.

Location	Person days per austral summer			Occupancy designation	Permanent Facilities Y/N	Year established	Number of Transects sampled	Location	National Programme
	2015-16	2016-17	2017-18						
Victoria Valley	30.0	NS	NS	Pristine	N	TFC	1	S 77.37690 E 161.79880	NZ
Lower Wright Refuge Hut (Brownworth)	NS *(180.0)	33.0	NS	Moderate	Y	1971	4	S 77.44247 E 162.65161	NZ
Lake Vanda Hut Facilities Zone (Vanda)	0.2	145.0	NS	Moderate	Y	1994**	3	S 77.52286 E 161.68901	NZ
Spaulding Pond, Taylor Valley	NS	0.2	256.0	Moderate	N	TFC	0	S 77.65714 E 163.10728	NZ
Miers Valley	104.2	NS	0.3	Moderate	N	TFC	1	S 78.09526 E 163.78845	NZ
New Harbor Camp Facilities Zone	Unknown	Unknown	Unknown	High	Y	Unknown	1	S77.57786 E 163.52014	US
F-6 Camp Facilities Zone	Unknown	Unknown	Unknown	High	Y	2001	2	S 77.60828 E 163.25520	US
Lake Fryxell Camp Facilities Zone	Unknown	Unknown	Unknown	High	Y	2001	2	S 77.60524 E 163.12192	US

3.2.3 Soil sampling

For the temporary NZ field camps, a total of 12 soil samples were collected for each transect (refer to Table 3.1 for transect details at each site) at 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 m distance from the centre of a camp. For each US field camp (Table 3.1), 11 soil samples were collected at 1, 3, 5, 10, 20, 50, 100, 200, 300, 400 and 500 m from the centre of the camp. Sampling was conducted on an exponential scale to capture the anticipated pattern of exponential decay of the target signal as distance increased from a campsite. Transect directions included up- and down-valley where possible to capture aeolian dispersal of UHI-bound soil.

15 g of surface soil (0.5 cm depth) was collected for each sample and placed into a nucleic acid clean 50 mL falcon tube. In some instances, sample collection was not possible due to a geographic feature (e.g., located underwater). All soil samples were frozen before transport back to NZ under dry ice and stored at -80°C until processed. For an overview of the entire pipeline of marker selection and sample collection, see Figure 3.2.

3.2.4 DNA isolation

A modified cetyltrimethylammonium bromide (CTAB) protocol was used (Barrett *et al.* 2006), where 5 g soil of soil was weighed into a 15 mL falcon tube containing an extraction buffer of 1.35 mL phosphate buffer (100 mM NaH_2PO_4) and 1.35 mL of SDS lysis buffer (10% SDS, 100 mM NaCl, 500 mM TRIS pH = 8.0). For samples that previously yielded no quantifiable DNA using the above extraction method, SwiftMag beads (MoBio, Qiagen) were used to capture whole genomic DNA with a 1:1 ratio of extraction buffer to 100% ethanol, instead of DNA precipitation with 2-propanol and ammonium acetate. The beads were subsequently washed with 500 µL 100% ethanol, and dried at 60°C before resuspending the DNA in 18 µL 1xTE. Negative controls were included with each lot of DNA extractions to ensure reagent and equipment sterility, and to confirm good aseptic technique in the laboratory. Other negative controls included NSC (no soil controls) to confirm sterility during soil sampling and SC (soil negatives), from a low-impact area far from camp to ascertain the baseline of environmental signal for dPCR.

3.2.5 Marker selection and assay design

The HcytB assay was derived from a previously published qPCR assay by Baker-Austin *et al.* (2010), with the modification of truncating the probe to 13 bp, following manufacturer recommendations for TaqMan minor-groove binding (MGB) chemistry (ThermoFisher; MA, US), to maximise probe sensitivity. The BacHum assay was previously published by Lee *et al*. (2010) as a qPCR assay, and no modifications were made to either the primers or MGB probe. The Sepi assay was developed during this study and targeted *Staphylococcus epidermidis* present on the skin, following the manufacturer guidelines for TaqMan assay design (ThermoFisher; MA, US).

A standard for each assay was developed to assist in dPCR detection and quantification testing. PCR primers that flanked each of the assay regions are listed in Table 3.2. Each standard was produced with endpoint PCR using the following conditions. The PCR mixture contained 0.2 µM dNTPs, 2.0 mM $MgCl_2$ for HcytB or 1.5 mM for BacHum and Sepi, 1U Platinum Taq and 1x buffer (Thermofisher), 0.02 mg mL^{-1} bovine serum albumin, 0.4 µM of each forward and reverse primer, prepared to a final volume of 25 µL with milliQ H_2O. Thermocycling conditions were 94°C for 2 min, 30 cycles of: 94°C 30 s, annealing at 58°C (HcytB) or 54°C (BacHum and Sepi) for 30 s, extension at 72°C for 1 min (HcytB and BacHum) or 2 min (Sepi), then a final extension at 72°C for 5 min. Gel electrophoresis was subsequently used to confirm that all amplicons were the expected size, and Sanger sequencing confirmed the assay primers amplified the correct gene target.

Sanger sequencing was carried out on an ABI Prism 3100 Genetic Analyzer (Waltham, Massachusetts, United States) for each assay. Sequence identities were confirmed with GenBank nucleotide sequences by using BLAST (blastn) for each of the HcytB (accession numbers KU686629.1, KU686674.1), BacHum (accession numbers CP072246.1 and LC515579.1 for *Bacteroides dorei*), and Sepi (accession numbers NZ_CP035288.1 for *S. epidermidis* and NZ_LR134242.1 for *Staphylococcus warneri*) assays. A sequence alignment of each target region was made in Geneious (v7.1 by Biomatters) to ensure the assay binding regions were conserved. The primers were synthesised by Integrated DNA Technology (Coralville, Iowa, US). Applied Biosystems synthesised the TaqMan MGB probe (Foster City, CA, US).

Table 3.2 Primers and probes used in this study

	Sequence 5'-3'	Length bp	Tm°C	Amplicon Size bp	Target	Publication
HcytB Assay					Human mtDNA *MT-CYB* gene	Baker-Austin *et al*. (2010)
Forward Primer	CCTCCAAATCACCACAGGACTAT	23	59.8	125		
Reverse Primer	CGTGAAGGTAGCGGATGATTC	21	58.9			
TaqMan Probe	FAM-TCGCCCACATCAC-MGB	13	69.0			
Assay Standard						This study
Forward Primer	TTCTCGCACGGACTACAACC	20	60.0	864		
Reverse Primer	GGGTGTTTAAGGGGTTGGCT	20	60.2			
BacHum Assay					16s rRNA *Bacteroides*	Lee *et al*. (2010)
Forward Primer	CGCGGTAATACGGAGGATCC	20	60.1	165		
Reverse Primer	CGCTACACCACGAATTCCG	19	59.0			
TaqMan Probe	FAM-AAGTTTGCGGCTCAAC-MGB	16	70.0			
Assay Standard						Bernhard & Field (2000)
Forward Primer	AACGCTAGCTACAGGCTT	18	55.9	696		
Reverse Primer	CAATCGGAGTTCTTCGTG	18	53.2			
Sepi Assay					*Staphylococcus epidermidis tuf* gene	This study
Forward Primer	ACGTTGGTGTACCAGCWTTAG	25	58.3	125		
Reverse Primer	CGTCACCTGGGAAGTCATATTC	22	58.5			
TaqMan Probe	FAM-ACATGGTAGACGACGAAGA-MGB	17	69.0			
Assay Standard						This study
Forward Primer	GGTGGTATGGGTGAATTACAC	21	53.2	1963		
Reverse Primer	ACGTCAGTAGTACGGAAATAGA	22	52.6			

3.2.6 Amplification and quantification with QuantStudio 3D (QS3D) dPCR

QuantStudio v2 dPCR chips were loaded with sampled DNA in the following preparation: 0.95 µM of each forward and reverse primer, 0.25 µM TaqMan probe, 1X QS3D master mix, 5.0 µL of extracted DNA, and ultrapure water to make 9.5 µL. The PCR master mix was prepared with a 10% excess to account for the volume lost from pipetting. Each QuantStudio v2 chip was loaded with 9.5 µL of the above mastermix and 5.0 µL of isolated eDNA to a final volume of 14.5 µL.

Thermocycling was according to the manufacturer's recommended parameters: 96°C for 10 min, 39 cycles of 60°C for 2 min and 98°C for 20 s followed by a final extension at 60°C for 2 min and a hold at 10°C on a QS3D Proflex thermal cycler (Thermofisher, MA, US). Endpoint fluorescence of the chip partitions was measured by QS3D Digital PCR instrument (application version 3.1.2, algorithm version 4.4.10). The selected algorithm was "Poisson Plus," as some samples exceeded 2000 copies μL^{-1}. The confidence interval was set to 95%, and the desired precision was set to 10%.

Absolute quantification of the targeted amplicon was achieved using the QS3D Digital PCR system in conjunction with the QS3D AnalysisSuite Cloud software (v 3.1). Each QuantStudio dPCR chip contains 20,000 reaction wells, equivalent to 20,000 technical replicates. The quality threshold was set to the manufacturer's default 0.5, as no artefacts, such as bubbles or incorrect PCR mixture volumes, were observed. The fluorescence thresholds produced by the QS3D cloud software were reviewed manually with comparison to positive and NTC chips, and by overlaying the scatterplots of samples of similar signal together (Appendix B, Figure 8.2). Outlier data points were also omitted from each chip in scatterplot view. The calculated quantities were reviewed to produce an absolute quantification of the target as copies μL^{-1} for 5 g of soil. For each dPCR run, NTC controls were included, along with the reagent, SC and NSC negative controls included during the DNA extraction procedure.

3.2.7 Assay sensitivity and cross-reactivity

The three assays were first tested *in silico* using the National Centre for Biotechnology (NCBI) tools BLASTn for each target region and Primer-BLAST (Ye *et al.* 2012) for all primer and probe sequences. An alignment of all appropriate nucleotide sequences for each assay region obtained from GenBank was produced using Geneious (v 7.1. Biomatters), which included humans and Antarctic fauna for HcytB (Appendix B Figure 8.3), or closely related microbial species to the BacHum and Sepi assay targets (see Appendix B, Figures 8.4 and 8.5, and Tables 8.1 and 8.2, for a complete list of species and accession numbers). Cross-reactivity was also tested with 3.2 ng of mummified seal DNA extracted from muscle tissue following the CTAB extraction method previously described.

Mummified seals are commonly encountered in the MDVs, and their remains may be present in MDV soil, thus, it was important to eliminate potential cross-reactivity.

A ten-fold serial dilution of each assay standard was done across four orders of magnitude to test sensitivity. The dilution series ranged from the maximum precision of ~1.6 DNA molecules per dPCR well (32,000 DNA molecules total) (Majumdar *et al.* 2017) down to a theoretical $1.6x10^{-4}$ DNA molecules per well (3.2 DNA molecules total). A challenge of using environmental DNA is that many DNA species are present within a sample, and the target DNA species is usually present at an extremely low concentration. A spiking experiment was carried out to assess potential sensitivity loss in the presence of increasing inundation by non-target DNA, where ~3 copies of target DNA were spiked with 0 ng, 250 ng, 500 ng, 750 ng and 1000 ng of mollusc (*Perna canaliculus*) DNA.

3.2.8 Data Analysis

Linear regression models were produced to provide an estimate of distance from a camp where the assay signal was equivalent to the background signal. Paired T-tests (unequal variances, alpha = 0.05) and linear regression analyses were carried out on target quantification obtained from dPCR in Microsoft Excel. A log (x + 1) transformation was applied to data generated from the QuantStudio software to achieve normality before applying linear regression models. A linear regression ($Y = b_0+b_1x+e$), where b_0 = intercept, b_1 = regression coefficient, x = distance and e = residual errors, was done for complete transects. Linear regression, model residuals, and Q-Q plots were performed in RStudio (v3.3.1) using the base and stats packages (http://www.r-project.org/). All analyses were performed with a 95% confidence interval and were considered significant if both the Y-intercept (b_0) and the regression (b_1) p-values were ≤ 0.05.

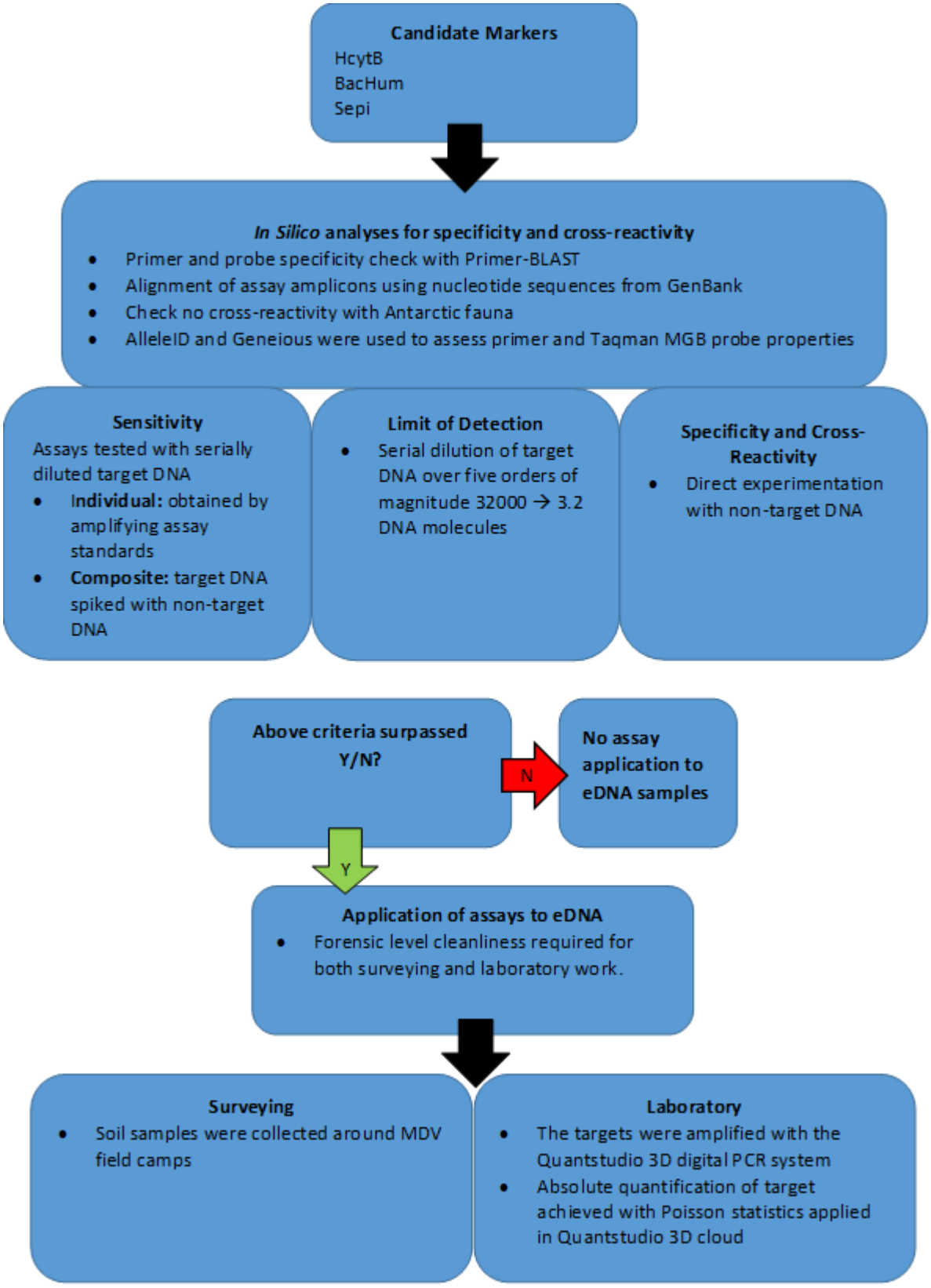


Figure 3.2 Overview of the pipeline from sample collection to absolute quantification.

3.3 Results

3.3.1 Assay Development and Validation

There was no indication of cross-reactivity with species other than the intended targets from both *in silico* testing and direct experimentation. An NCBI BLAST search for each assay amplicon yielded 100% sequence identity to the targeted nucleotide region. *In silico* testing of the Sepi assay primer and probe binding regions confirmed that the assay was specific to *S. epidermidis* and not other *Staphylococcus* species (Appendix B, Table 8.2). Direct experimentation with non-target DNA did not indicate significant cross-reactivity. All assays were tested with seal tissue DNA (4.51, 0.00 and 9.20 copies μL^{-1} for HcytB, BacHum, and Sepi, respectively). The result of spiking ~3 DNA molecules with an increasing gradient of non-target mollusc (*P. canaliculus*) DNA did not show any cross-reactivity for the three individual assays (Figure 3.3, D-E).

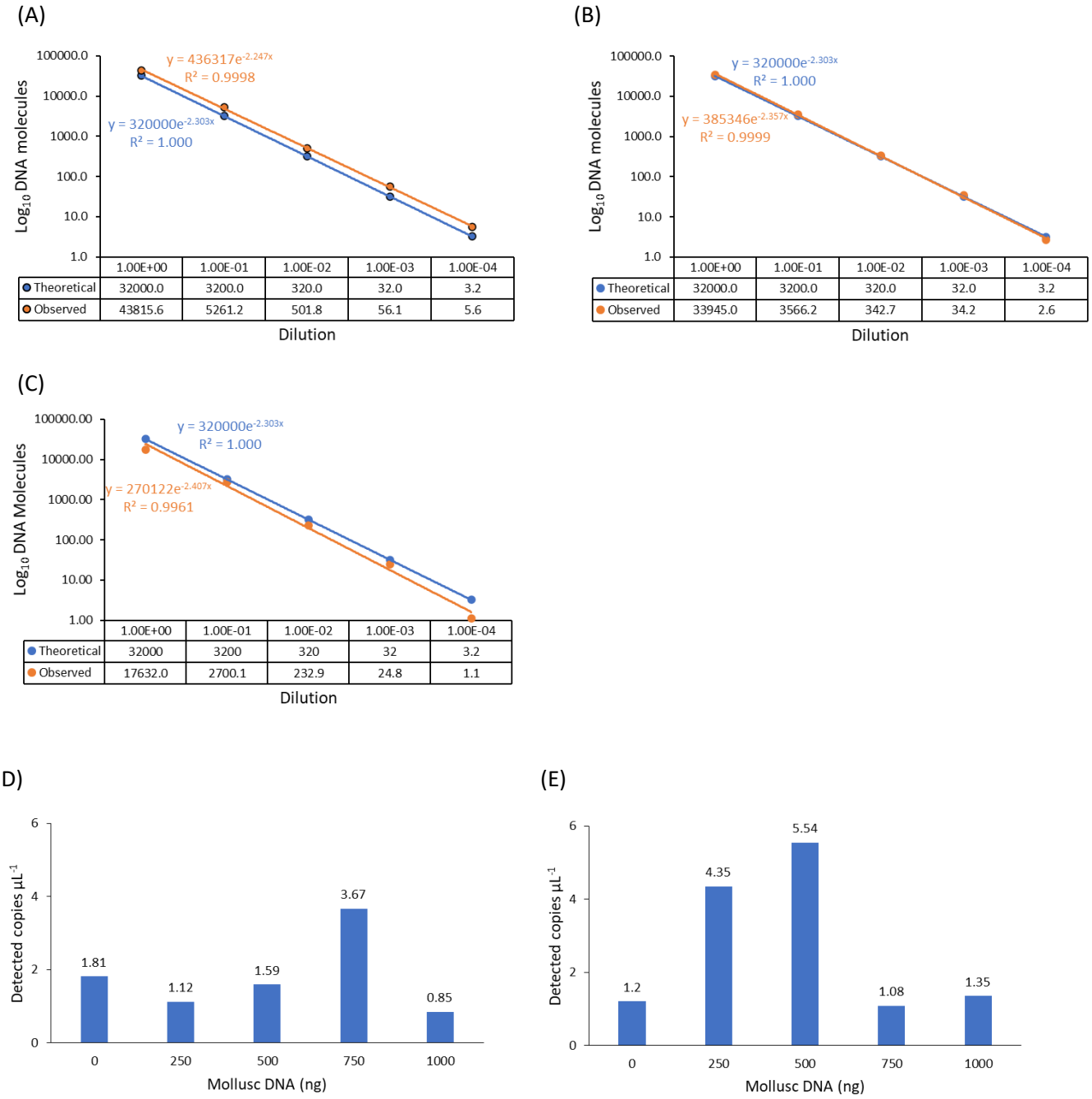


Figure 3.3 A five-fold serial dilution of DNA molecules derived from end-point amplification of each assay standard for (A) the HcytB assay, (B) the BacHum assay and (C) the Sepi assay. The result of spiking ~3 copies of target DNA with an increasing gradient of non-target mollusc DNA for (D) the HcytB assay and (E) the BacHum assay.

After a five-fold serial dilution of a known number of target molecules, the limit of detection for the HcytB, BacHum, and Sepi assays were 5.6 copies μL^{-1} (R^2 = 0.9998), 2.6 copies μL^{-1}, (R^2 = 0.9999) and 1.1 copies μL^{-1} (R^2 = 0.9926) respectively (Figure 3.3). All assays were sufficiently sensitive for single-cell detection. Mitochondrial DNA is the most abundant source of DNA in humans, where a single cell can contain tens to thousands of copies of mtDNA, depending on the tissue type (Cole 2016). For the microbe-associated assays, Bacteroidales species contain ~5 copies of 16s rRNA (Bernhard and Field, 2000), and *Staphylococci* contain a single copy of the *tuf* gene (Chen *et al.,* 2008).

Of the three assays, HcytB (mitochondrial) would be most suitable for quantifying and predicting UHI at a site. BacHum would be applicable to a site of known human presence to determine if faecal contamination is present. Although the Sepi was adequate at the validation stage, there was an inadequate separation between the amplification and no-amplification peaks (histogram view) during the dPCR data analysis with QS3D AnalysisSuite Cloud software (Appendix B, Figure 8.6). Due to the lack of confidence in assay sensitivity, Sepi was not subsequently applied to environmental samples.

3.3.2 Spatial patterns of UHI at campsites

Fourteen transects were sampled across all sites, with some including multiple time points where possible, to give a total of 29 transect samples (Table 3.3). All UHI data (in target copies μL^{-1}) for each transect were $\log_{10}(x + 1)$ transformed to better fit the exponential decay pattern observed as distance increased from a site. The linear regression models provided an estimate of distance from a site where the UHI signal would diminish to background levels normally observed in the environment, regardless of human presence and subsequent UHI. Of a total of 29 transects, 14 had a significant linear regression $y = b_0+b_1x$, where both the y-intercept (b_0) and regression (b_1) had a p-value ≤ 0.05 (see Figures 3.4 and 3.5). For the mitochondrial signal, all transects with a significant regression had a negative regression for mitochondrial signal as distance from a site increased. None of the transects displayed a significant regression for faecal signal; thus, no pattern of faecal signal was observed in relation to distance from a site.

Table 3.3 A total of 29 transects (14 transects including additional arrival and departure sampling where possible) were surveyed around field camps and research stations in the MDVs. '*' denotes statistically significant linear regressions ($p \leq 0.05$).

Site	Transect	Year sampled	Distance (m) of detectable UHI, derived from linear regression, $y = B_0+B_1x$	Person days	Transect direction
Victoria Valley	A) HcytB (T0)*	2016	-0.60	0.0	S
Victoria Valley	A) HcytB (TD)	2016	94.05	30.0	S
Victoria Valley	A) BacHum (TD)	2016	$7.81x10^9$	30.0	S
Wright Valley, Vanda Hut Facilities Zone	B) 1, HcytB	2017	384.01	145.0	NNE
Wright Valley, Vanda Hut Facilities Zone	C) 2, HcytB*	2017	173.90	145.0	NE
Wright Valley, Vanda Hut Facilities Zone	C) 2, HcytB *	2016	260.54	0.2	NE
Wright Valley, Vanda Hut Facilities Zone	C) 2, BacHum	2016	65.13	0.2	NE
Wright Valley, Vanda Hut Facilities Zone	D) 3, HcytB*	2017	50.04	145.0	NW
Lower Wright Refuge Hut (Brownworth)	E) 1, HcytB (TD)*	2017	76.42	33.0	SE
Lower Wright Refuge Hut (Brownworth)	E) 1, HcytB (T0)	2017	471.28	0.2	SE
Lower Wright Refuge Hut (Brownworth)	E) 1, BacHum (T0)	2017	-1.00	0.2	SE
Lower Wright Refuge Hut (Brownworth)	E) 1, BacHum (TD)*	2017	-0.74	33.0	SE
Lower Wright Refuge Hut (Brownworth)	F) 2, HcytB (TD)	2017	5.87	33.0	NE
Lower Wright Refuge Hut (Brownworth)	G) 3, HcytB (TD)*	2017	1032.23	33.0	NW
Lower Wright Refuge Hut (Brownworth)	G) 3, HcytB (T0)*	2017	92.30	0.2	NW
Lower Wright Refuge Hut (Brownworth)	G) 3, BacHum (TD)	2017	-0.96	33.0	NW
Lower Wright Refuge Hut (Brownworth)	H) 4, HcytB (TD)	2017	$1.85x10^5$	33.0	SW
Miers Valley	I) HcytB (T0)*	2016	193.32	8.3	SW
Miers Valley	I) HcytB (TD)*	2016	182.83	104.2	SW
Miers Valley	I) HcytB (Recovery)*	2018	50.09	0.3	SW
Miers Valley	I) BacHum (TD)	2016	$1.22x10^6$	104.2	SW
Miers Valley	I) BacHum (recovery)	2018	9.31	0.3	SW
Taylor Valley, F-6 Camp Facilities Zone	J) HcytB*	2018	271.65	Unknown	SW
Taylor Valley, F-6 Camp Facilities Zone	J) BacHum	2018	$1.72x10^6$	Unknown	SW
Taylor Valley, F-6 Camp Facilities Zone	K) HcytB	2018	8840.83	Unknown	NE
New Harbor Camp Facilities Zone	L) HcytB*	2018	189.29	Unknown	SW
Lake Fryxell Camp Facilities Zone	M) HcytB*	2018	258.74	Unknown	SW
Lake Fryxell Camp Facilities Zone	M) BacHum	2018	920.78	Unknown	SW
Lake Fryxell Camp Facilities Zone	N) HcytB*	2018	311.87	Unknown	NE

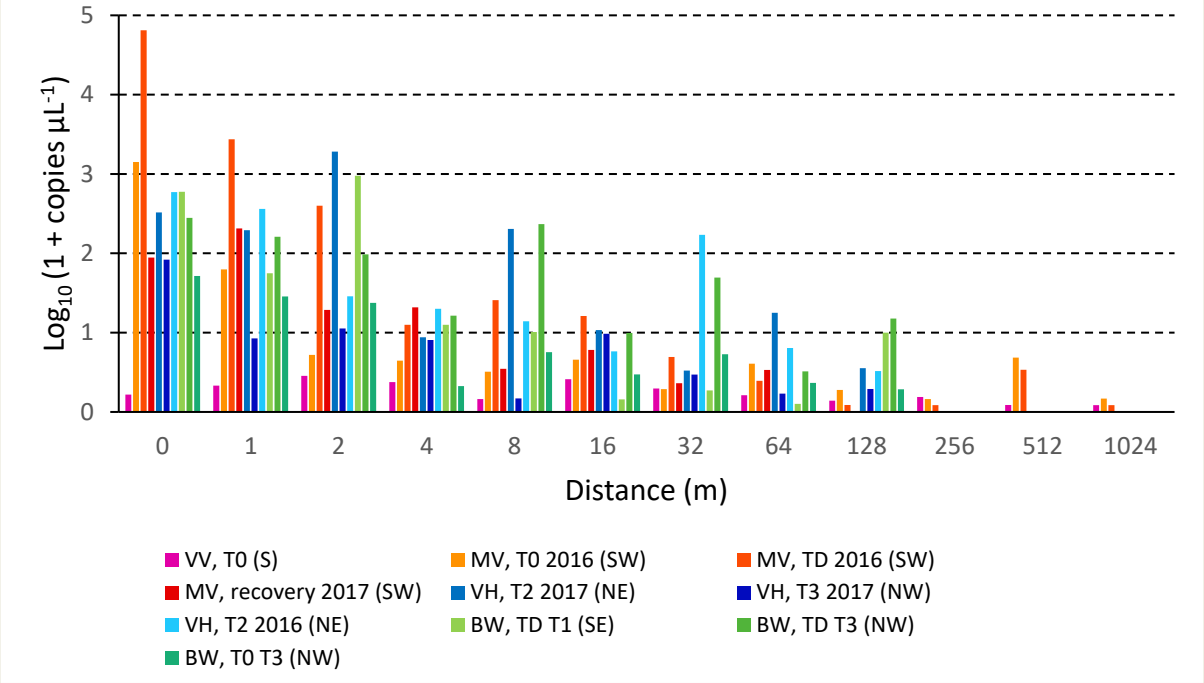


Figure 3.4 An overview of mitochondrial signal detected at NZ campsites, where the linear regression was significant ($p \leq 0.05$). VV = Victoria Valley, MV = Miers Valley, VH = Vanda Hut, BW = Brownworth.

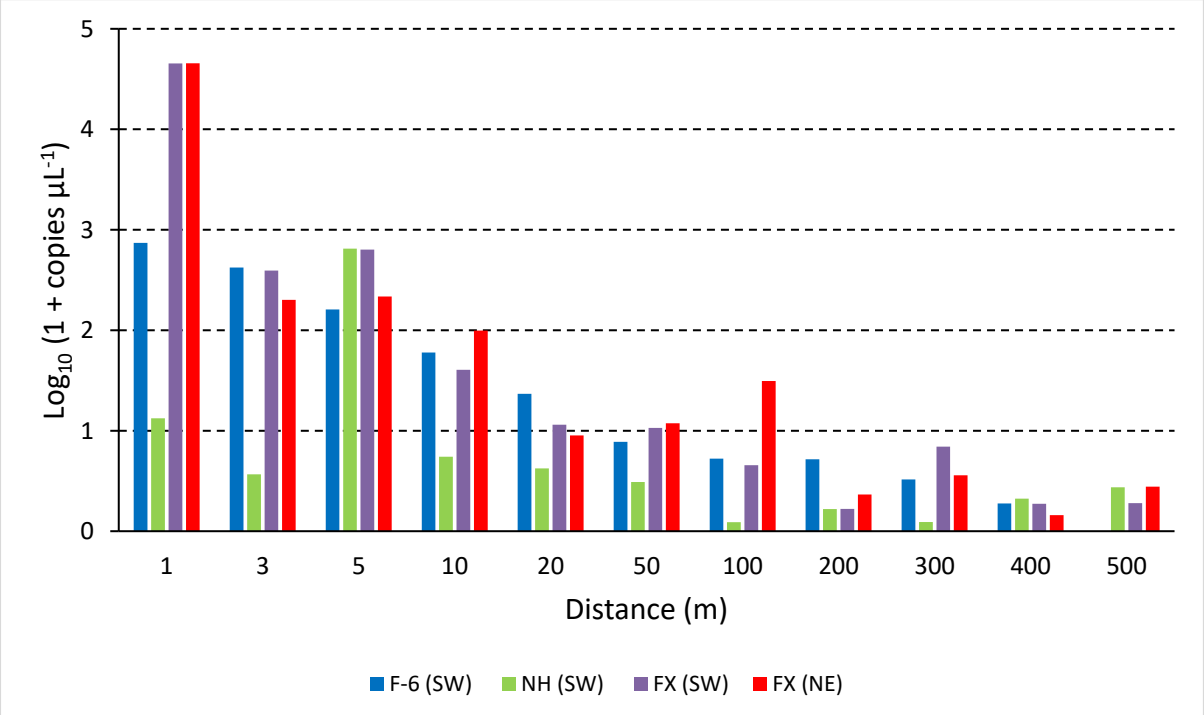


Figure 3.5 Mitochondrial signal detected at US Facilities Zones for transects with a statistically significant linear regression ($p \leq 0.05$).

Victoria Valley was a pristine site, where no field camp had been established before the 2015-16 field season. A single transect was sampled at arrival (for HcytB) after 0.0 person-days and at departure (for both HcytB and BacHum) after 30.0 person days. Upon arrival, the level of the mitochondrial signal was less than background levels and was predicted to diminish to a background level 94.1 m from camp after departure after 30.0 person days. Victoria Valley was the only site where the mean faecal signal at departure was greater than mitochondrial signal (20.6 vs 17.0 copies μL^{-1}, respectively), although the only statistically significant linear regression was for HcytB at arrival, which was not applicable since HcytB signal was below background levels.

Both Vanda Hut and Brownworth camps were established near permanent hut facilities in the moderately occupied sites of Wright Valley. All transects at Vanda Hut were down-valley towards McMurdo Sound and showed a negative linear regression in mitochondrial signal as distance increased from the campsite. Three transects were surveyed at Vanda Hut in 2017-18 (one of which was also sampled during the 2015-16 season), and four transects were surveyed at Brownworth in 2017-2018. A statistically significant linear regression, where the signal decreased with distance from the centre of a camp, could be derived from the majority of transects at both sites for mitochondrial signal. At Vanda Hut, the northeast (NE) and northwest (NW) transects (2017-18) had a significant regression (Figure 3.6). Interestingly, the NE transect at Vanda Hut had a greater mitochondrial signal in 2016 after 0.2 person days, compared to 2017 when there was a large field camp after 145.0 person days (Figure 3.6). Using the linear regression models, the distances from the camp where the mitochondrial signal was anticipated to diminish to background levels were estimated at 260.5 m (2016) and 173.9 m (2017) for the NE transect, and 50.0 m for NW transect.

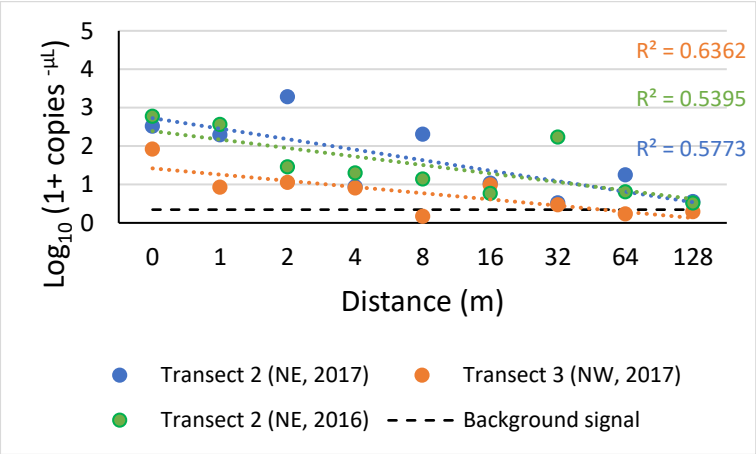


Figure 3.6 Three transects at Vanda Hut had a significant linear regression ($p \leq 0.05$); R^2 is displayed for each regression model.

Of the four transects surveyed at Brownworth, those with a significant linear regression for mitochondrial signal were southeast (SE) and NW transects at departure, after 33.0 person days, and a NW transect upon arrival (Figure 3.7). The linear regression models predicted mitochondrial UHI to be detectable 76.4 m from the SE transect and 1032.2 m from the NW transect at departure (after 33 person days), and 92.3 m along the NW transect at arrival after 0.2 person days (our field party) (Figure 3.7). Another field party had camped at Brownworth for ~180.0 person days, one month before our camp, releasing human materials to detectable levels on our arrival. No significant trend was observed for faecal signal along the NW, or SE transects at Brownworth.

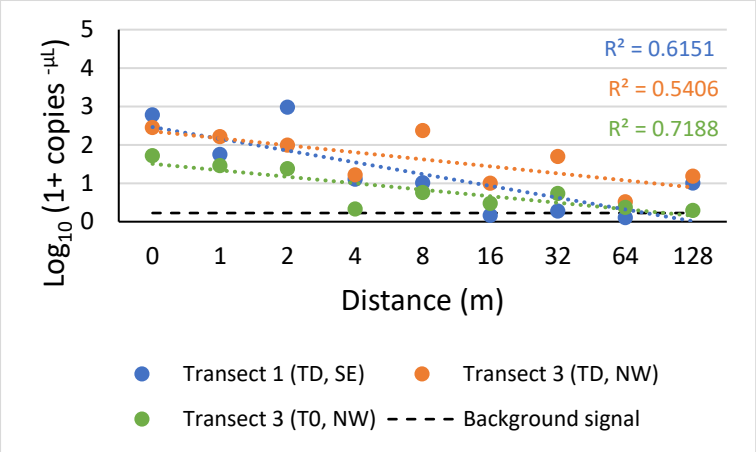


Figure 3.7 Three transects from Brownworth had a significant linear regression; R^2 is also shown.

We found the highest level of mitochondrial signal at the sites of a large field camp in Miers Valley and the US research facilities in Taylor Valley (F-6, New Harbor, and Fryxell). A single transect was surveyed at Miers on arrival and after departure for the 2015-16 season. The linear regression models predicted the mitochondrial signal to be detectable at 193.3 m from camp on arrival (T0) and 182.8 m after departure (TD). After a 10-fold increase in person days (T0 < 8.3 vs TD 104.2 person days) between arrival and departure, a greater increase in mitochondrial signal was expected after departure than was observed (Figure 3.8). The faecal signal remained low after departure compared to mitochondrial signal. As for all other Antarctica NZ field camps, no significant trend in faecal signal could be identified with linear regression at Miers camp.

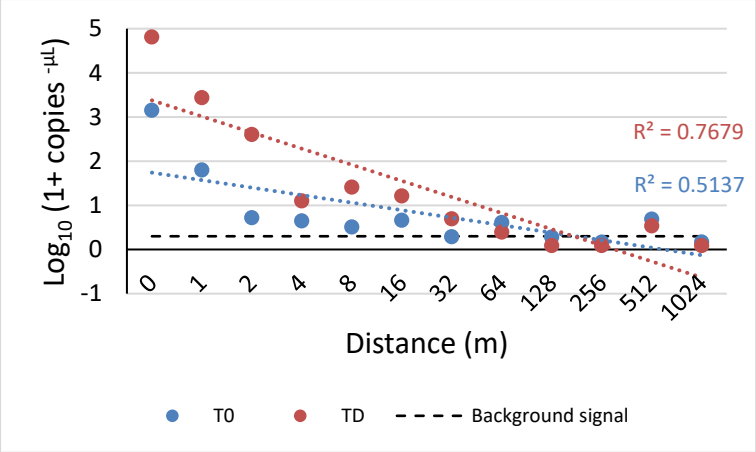


Figure 3.8 A comparison of the mitochondrial signal at arrival (T0) and departure (TD) at Miers camp, with linear regression displayed for both.

Of all the study sites, the US camps in Taylor Valley (F-6, New Harbor, and Fryxell Facility Zones) were the most heavily impacted and consistently had the highest mitochondrial and faecal signal. These sites were sampled at a single time point, as there was no available record of total occupancy. Two of the three US sites in Taylor Valley (all except New Harbor) had significant regressions for mitochondrial UHI. Of two transects (SW and NE) surveyed at F-6, the SW transect produced a significant regression, where mitochondrial signal was predicted to be detectable 271.7 m from camp (Figure 3.9). Both SW and NE transects surveyed at Fryxell produced significant regressions, where mitochondrial signal was predicted to be detectable 258.7 m along the SW transect and 311.9 m from the NE transect (Figure 3.10). A single SW transect was surveyed at New Harbor (Figure 3.11). The mitochondrial signal had a negative regression and was predicted to be detectable 189.3 m from camp but was only marginally significant (P = 0.05). In Taylor Valley, NE and SW were up- and down-valley directions, respectively.

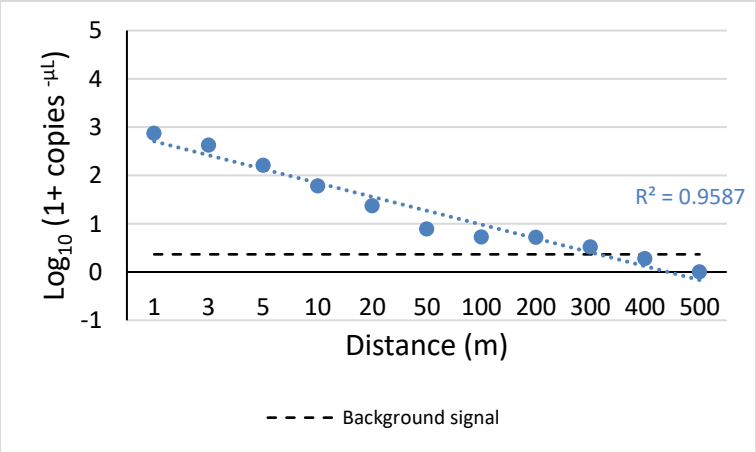


Figure 3.9 Linear regression produced for the SW transect at F-6 Facilities Zone.

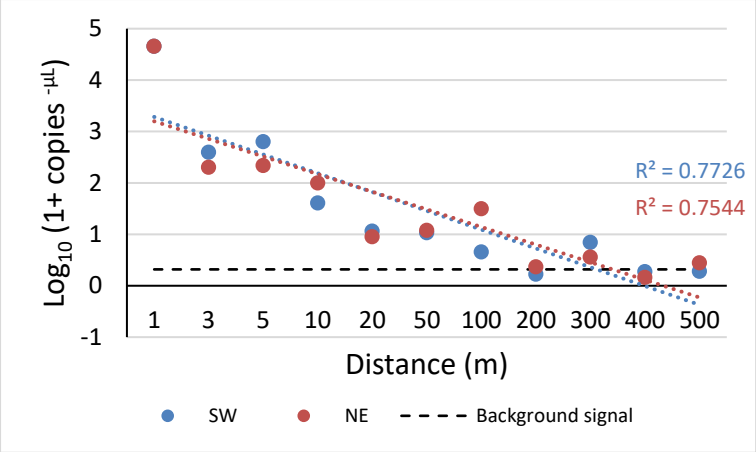


Figure 3.10 Linear regression produced for the SW and NE transects at Fryxell Facilities Zone.

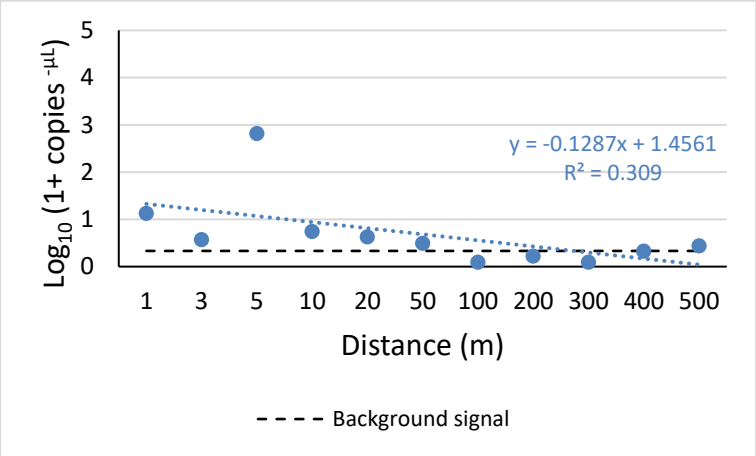


Figure 3.11 Linear regression produced for the SW transect at New Harbor Facilities Zone.

3.3.3 Effect of occupancy on detectable UHI

A greater mitochondrial signal was obtained from the one- and two-person tents compared with the control (a vacant tent site), but no clear difference could be seen when comparing the linear trends of T0 (arrival) and TD (departure) time points (Figure 3.12-A). No significant linear trend in faecal signal at TD was observed across tent occupancy (R^2 = 0.6674), or between the control and one- and two-person tents (Figure 3.12-B).

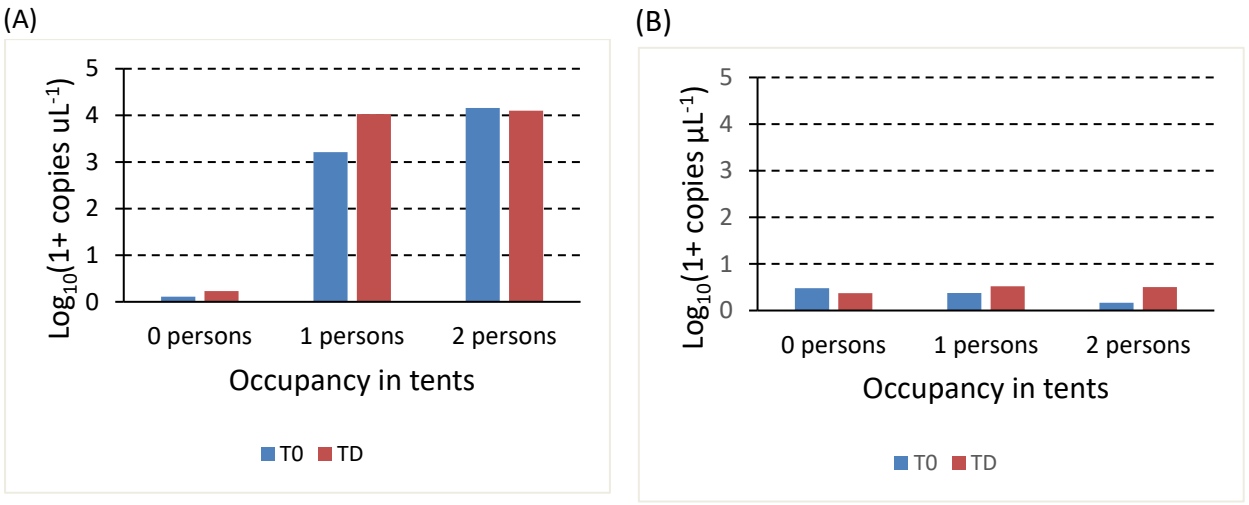


Figure 3.12 (A) Mitochondrial and (B) faecal UHI signal was measured in 0 (control, vacant tent site), one- and two-person tents upon camp arrival (T0), and before departure (TD), after 0, 3, and 6 person-days, respectively.

3.3.4 Type of UHI most prevalent: comparison of mitochondrial and faecal signal intensity

The mitochondrial and faecal signal intensity was compared across averaged data from a range of sites (Figure 3.13). A paired t-test (one-tailed) showed a significant difference between the intensity of mitochondrial (M = 3.04, SD = 1.33) and faecal (M = 2.10, SD = 1.13) signal; $t_{(11)} = 3.53$, $p = 0.002$, where mitochondrial signal was greater than faecal signal across all but two sites.

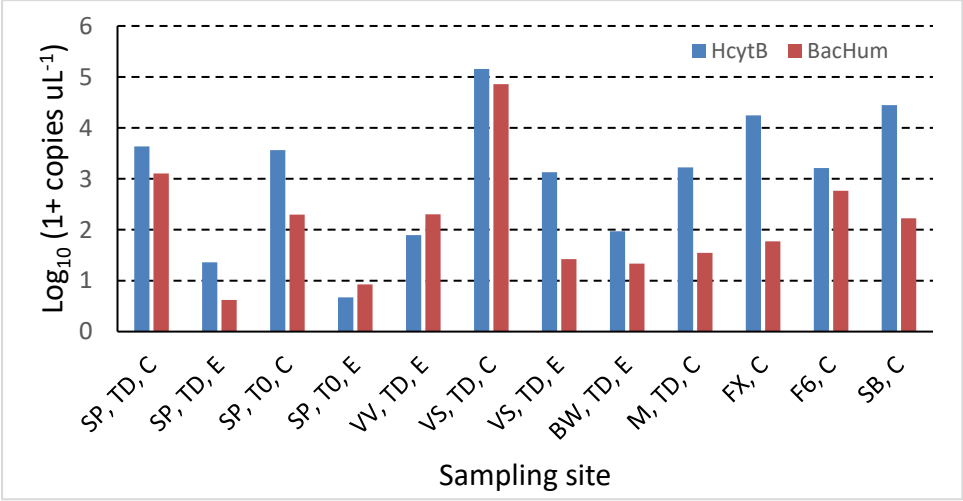


Figure 3.13 Comparison of total level of mitochondrial and faecal signal detected at a range of sites across toilet areas at field camps and Scott Base, including arrival (T0) and departure (TD) samples. SP = Spaulding Pond, VV = Victoria Valley, VS = Vanda Station, BW = Brownworth, M = Miers, FX = Fryxell, SB = Scott Base. C = Enclosed toilet area, E = Exposed toilet area.

3.3.5 Recovery of a site in Miers Valley from previous human presence

Both mitochondrial and faecal UHI levels diminished after 24 months when compared to UHI levels after departure in 2016 (Figure 3.14). A negative regression was observed for mitochondrial signal, which was predicted to be detectable 182.8 m after departure in 2016 (M = 1.96, SD = 1.43) and 50.1 m after recovery (M = 1.14, SD = 0.67) in 2018. Overall, the mitochondrial signal was greater than the faecal signal for both departure (M = 0.43, SD = 0.22) in 2016 and recovery (M = 0.22, SD = 0.32) in 2018 and linear regression did not produce significant models to predict faecal signal, which had a wide variance and low R^2 values (Appendix B, Table 8.4). This pattern suggests that given time, detectable remnants of UHI will diminish at a large camp.

(A) (B)

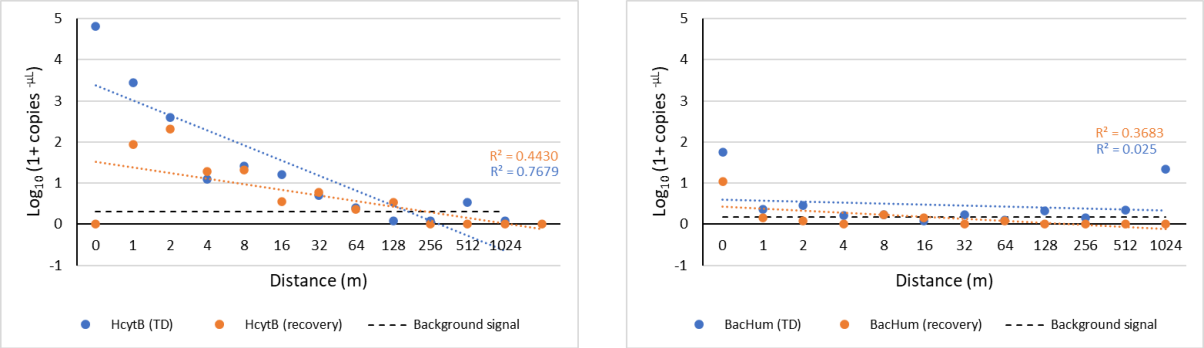


Figure 3.14 Linear regression of signal detected from a field camp in Miers Valley, comparing the signal detected at departure after 104.2 person days (blue) in 2016 against recovery after two years (orange) in 2018 for (A) mitochondrial and (B) faecal signal.

3.3.6 Managing and reducing faecal UHI in a camp

Elucidating ways to mitigate UHI was another objective of this study. The approach was to compare UHI in an open-air toilet area (i.e., exposed bucket) to a toilet in an enclosed area (e.g., in a portacom or tent) at two sites, Spaulding Pond and Vanda Hut. Enclosed toilet areas revealed higher mitochondrial and faecal signal compared to toilet areas exposed to the elements (Figure 3.15).

(A) (B)

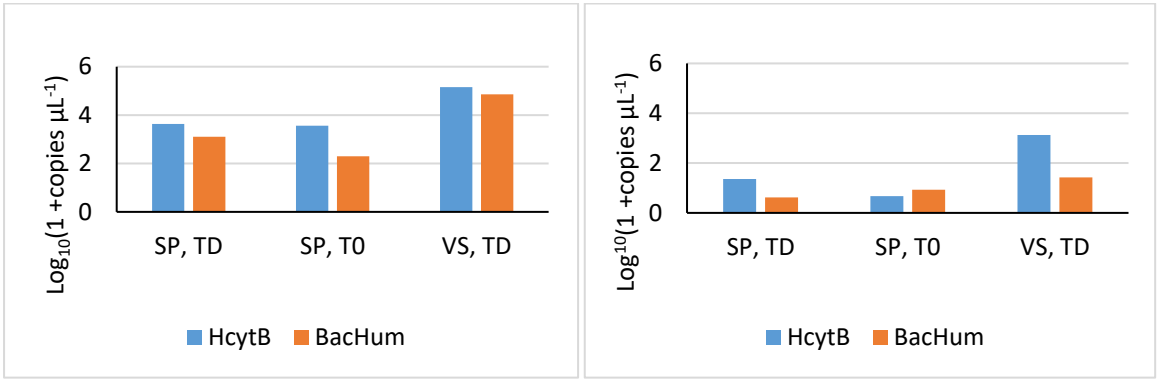


Figure 3.15 (A) HcytB and BacHum signal from an enclosed toilet area (toilet tent) at Spaulding Pond (SP) at arrival (T0) or departure (TD) and a portacom at Vanda Station (VS) and (B) for an uncovered toilet site consisting of an exposed bucket and barrel. Spaulding Pond was sampled at arrival and departure as a temporary field camp, whereas Vanda hut has permanent infrastructure and was sampled at a single time point.

Faecal signal accumulation was compared between arrival (M=0.20, SD=0.19) and departure (M=0.53, SD=0.43) at Brownworth along a transect near the toilet bucket in an outdoor toilet area (Figure 3.16). The mean accumulated signal (M = 0.33, SD = 0.40, n = 5) upon departure was not significantly greater than zero; one-tail p = 0.07, $t_{(4)}$ = -1.83.

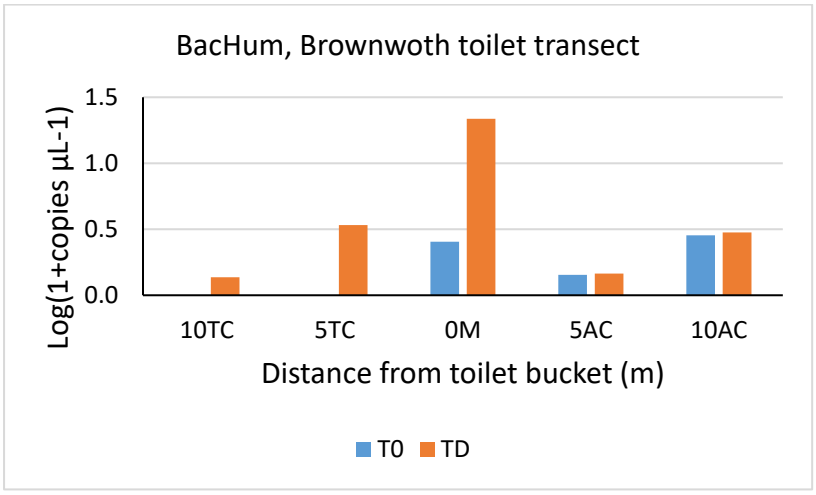


Figure 3.16 A transect including the uncovered toilet area was surveyed upon arrival (T0) and before departure (TD), after 145.0 person days at Brownworth. TC = towards camp, AC = away from camp, 0M was the location of the toilet bucket, 10 = 10 m and 5 = 5 m away from the toilet bucket.

3.4 Discussion

3.4.1 Factors affecting UHI detection in the MDVs

There were a range of potential signal stochasticity sources, both environmental and experimental, in investigating eDNA with PCR-based methodology. Most samples in this study were low copy number in the presence of a much greater magnitude of DNA species from indigenous soil microbial communities. The exponential nature of PCR amplification means that stochasticity and small biases with PCR-based methods can lead to large differences between a species' amplicon abundance relative to actual DNA concentrations present in the environment (Kelly *et al.* 2019). This is an important consideration when working with low copy templates characteristic of eDNA, ancient DNA, and forensic applications (Gill *et al.* 2009, Wilcox *et al.* 2016, Emery *et al.* 2020). Assay sensitivity can be influenced by a sample matrix's chemical and physical properties; thus, it was necessary to assess the intrinsic sensitivity in a neutral matrix such as a buffer solution (Harwood *et al.* 2009). For each assay, a 5-fold serial dilution of the assay standard was amplified with dPCR, starting at 1.6 copies per well (32,000 DNA molecules per chip), which provides maximal precision (Majumdar *et al.* 2017). In dPCR, background signal has been identified as a source of overestimation of target DNA abundance, which may arise through the fluorescence of unknown particles or instrumental artifacts (Strain *et al.* 2013, Kiselinova *et al.* 2014). Background signal in this investigation was considered by incorporating a range of negative controls across surveying and laboratory handling.

This study quantified human UHI as eDNA, an approach that uses intracellular and extracellular DNA released into the environment from a range of sources. Human materials released via routine activity were assumed to be deposited in the MDV environment passively. The gear stored in the tents and the tents themselves were not sterile and harboured human materials, thereby contributing to the overall UHI signal in addition to active occupancy; thus, human materials are likely to be introduced to a site upon arrival. In humans, DNA is sourced from skin cells (Kita *et al.* 2008), hair (Brandhagen *et al.* 2018), or bodily fluids such as sweat and urine (Valiere & Taberlet, 2000), as well as faeces (Hopwood *et al.* 1996). Shedding rates can vary between individuals, and the abundance of organisms and biomass are known to contribute to detected eDNA (Takahara *et al.* 2012, Sassoubre *et al.* 2016, Salter *et al.* 2019, Thalinger *et al.* 2021). Humans themselves have differing 'shedder status,' where the amount of deposited DNA varies between individuals (Kanokwongnuwut *et al.* 2018). Personal habits, such as handwashing, handedness, level of physical activity, also influence the amount of deposited DNA (Lowe *et al.* 2002, Goray *et al.* 2016). The volume of human materials and DNA deposited onto the collection site would have been stochastic, and more people at a site likely resulted in more deposited eDNA.

A range of environmental factors likely contributed to the observed patterns of UHI detection. eDNA persistence in soil is influenced by various physical and biological factors (Levy-Booth *et al.* 2007, Nielsen *et al.* 2007, Ivanova *et al.* 2017), the most decisive factors driving eDNA degradation are microbial activity, temperature, and available water (Tsuji *et al.* 2017, Sirois & Buckley 2019, Zulkefli *et al.* 2019). In the environment, MDV soil is highly heterogeneous in physicochemical properties, such as pH, particle size and mineral composition (Poage *et al.* 2008), which determine DNA-binding efficiencies across spatial zones and DNA preservation (Ivanova *et al.* 2017). In combination, environmental factors, such as pH and light intensity, accelerate the exponential decay of eDNA *in situ* in comparison to environmental factors in isolation (Zulkefli *et al.* 2019). The harsh MDV environmental conditions (cold, salty and dry) likely promote the preservation of DNA released in UHI.

Antarctic environmental conditions are more extreme in the summer, since the MDVs experience intense day-long solar radiation, warmer temperatures, and an increase in water availability from ephemeral streams (Fountain *et al.* 1999, McKnight *et al.* 2004). Intense UV radiation can either directly damage DNA or react with organic material to produce reactive oxygen species that damage DNA through oxidative stress (Lindahl, 1993, Strickler *et al.* 2015, Karran & Brem 2016). The increased water availability supports an increase in metabolic activity by microbes, and increased DNA degradation by heterotrophs (Sohm *et al.* 2020). Outside of summer, the environmental conditions in terrestrial Antarctica, such as constant low temperature, low moisture availability and high aridity (Cary *et al.* 2010), likely promote DNA preservation. Previous studies have demonstrated that the oldest DNA specimens were recovered from perennially frozen environments like terrestrial Antarctica, such as permafrost, cold sediments and surface soils, basal ice, ice cores, and tundra (Willerslev *et al.* 2007, Orlando *et al.* 2013, Christ *et al.* 2021, Crump *et al.* 2021, van der Valk *et al.* 2021). Even if environmental conditions are as close to ideal e.g., eDNA stored in permafrost (cold, low moisture, neutral pH, anaerobic), there is an upper time limit before PCR detection is no longer possible (Lydolph *et al.* 2005). Over time, eDNA becomes heavily fragmented and accumulates damage, including cross-linking, cytosine deamination and abasic DNA lesions (Willerslev *et al.* 2004, Deagle *et al.* 2006, Overballe-Petersen *et al.* 2013). The oldest recovered DNA to produce genomic data to date originated from mammoth molars in Siberian permafrost and was dated 1.2-1.1 mya (van der Valk *et al.* 2021).

Environmental DNA derived from a variety of sources persists after an organism has left or has died for a long time, depending on the environmental conditions (Mathieu *et al.* 2020). The shed biological tissue begins upon release to degrade to whole cells, organelles, and eventually free

extracellular DNA, sometimes referred to as 'relic DNA'. Once cells are released into the MDV environment, they rapidly lyse and lose viability (Ah Tow & Cowan 2005), but the resultant eDNA can persist at detectable levels for months to years (Levy-Booth *et al.* 2007, Pietramellara *et al.* 2009). The fate of free extracellular DNA upon release is varied, including utilisation by microbes as a nutrient source (Vorkapic *et al.* 2016), binding to soil matrix (Ogram *et al.* 1988), being incorporated into a biofilm (Jakubovics *et al.* 2013), or transformed by bacteria (e.g., by horizontal gene transfer) (Johnston *et al.* 2014, Dubnau & Blokesch 2019). eDNA detection is largely dependent on the ability of DNA to persist in soil long after an organism is absent or has died. DNA that is adsorbed to soil matrix components is more likely to persist in the environment as it is more stable than free DNA due to protection against DNase and climatic degradation (Nielsen *et al.* 2007, Torti *et al.* 2015, Emmons *et al.* 2017).

3.4.2 UHI detection by site

Aeolian dispersal of human materials and DNA-bound soil away from the site of deposition, up or down-valley, is dependent on the location and season. The MDVs experience extreme katabatic winds, predominantly Foehn winds year-round, where the prevailing wind direction during winter is from the East Antarctic Ice Sheet, through the MDVs to the coast (Nylen *et al.* 2004, Khan *et al.* 2018). During the austral summer, when fieldwork is conducted, the prevailing wind direction is up-valley from McMurdo Sound towards the East Antarctic Ice Sheet (Doran *et al.* 2002, Speirs *et al.* 2010).

The pattern of mitochondrial UHI detected generally corresponded to the predominant wind directions in the moderately (Wright Valley, Miers Valley) and highly impacted (the US facilities zones in Taylor Valley) sites. All transects at the sites of Miers Valley, and the US research facilities (Fryxell, F-6 and New Harbor) in Taylor Valley showed a clear negative regression pattern of mitochondrial UHI. Both Taylor Valley and Wright Valley have similar bimodal wind regimes, where strong katabatic winds descend from the plateau in winter and lighter winds originate from McMurdo Sound in summer (Doran *et al.* 2002). The transect sampled from the field camp in Miers Valley extended in an up-valley, south-west direction. The predominant wind direction is south to north (towards the Ross Ice Shelf) and, unlike Wright and Taylor Valleys, there is no strong topographic influence on wind direction in Miers Valley (Katurji *et al.* 2019). In Taylor Valley, aeolian material is primarily transported down-valley towards the coast (Šabacká *et al.* 2012, Diaz *et al.* 2018), but the direction can vary depending on location within the valley (Appendix B, Figure 8.7). Variation in wind direction and intensity over the austral summer could have resulted in greater aeolian dispersal of soil-bound genetic materials away from the campsite along transects in an up- or down-valley direction.

The short-term field camp in Victoria Valley was anticipated to have the lowest detected signal, having least number of reported person days across all sites, and was located at a site not previously visited. No discernible pattern of UHI was observed in Victoria Valley upon departure, likely due to a lesser deposition of human materials compared to the other sites surveyed. At the moderately impacted sites, the greatest mitochondrial signal was observed along transects that corresponded to down- and up-valley transects for both Brownworth (NW was up-valley and the SE transect was down-valley) and Vanda Hut (all transects were up-valley) in Wright Valley. At Brownworth, the NE and SW transects were perpendicular to the prevailing summer winds and thus, human materials were less likely to be dispersed in their direction.

In addition to aeolian dispersal, greater occupancy at the moderately and highly impacted sites likely resulted in a greater volume of shed human materials. Accumulation of UHI was indicated by a greater mitochondrial signal in sleeping tents occupied by one and two persons compared to the control (zero persons). The intensity of detected mitochondrial UHI corresponded to a site's impact designation. At NZ sites designated as moderately impacted, five out of eight transects had a significant pattern of mitochondrial UHI after departure, in Miers Valley, and at Brownworth and Vanda Hut in Wright Valley, after 104.2, 213.0, and 145.2 person days, respectively.

The US field facilities were designated as 'high impact' due to consistent occupation over summer and having the largest areas of permanent facilities. Human presence could not be accurately quantified at the US sites but has been reported as an annual average of 100-600 person-days by the United States Antarctic Program (USAP) for Fryxell, F-6 and New Harbor Facility Zones (Penhale 2019). No clear accumulation of faecal signal was observed for the same experiment, which suggests that faecal contamination was not a significant source of UHI.

3.4.3 Type of UHI impact most prevalent at a site

Overall, mitochondrial UHI was the most abundant UHI in the environment and would be the best assay to first identify a site with UHI. This was demonstrated by a significant negative linear regression for the majority of transects from moderately and highly impacted sites for mitochondrial UHI. Given the low human trace detected compared to the other sites, linear regression models are not likely to be able to predict signals when applied at low impact sites, such as the field camp in Victoria Valley. Several factors influence mtDNA abundance in the environment. mtDNA has significantly lower decay rates than nuclear DNA when copy numbers are considered from cadaver and eDNA studies (Foran 2006, Emmons *et al.* 2017). The outer mitochondrial membrane may be protective and remains intact longer than other cellular structures (Henwood 1992), which may account for this. Mitochondrial DNA is present in hundreds to thousands of copies per cell,

depending on the tissue type (Wai *et al.* 2010), and it is guaranteed to indicate human presence, whereas a human-associated microorganisms, such as a *Bacteroidales* species, relies on the premise that a host harbours this microorganism.

In contrast to mtDNA, the faecal target *Bacteroidales* contains four to seven copies of the 16S rRNA gene (Kildare *et al.* 2007) and was observed to be in lower abundance in the environment. *Bacteroidales* species are a good aggregate marker for identifying the presence of human faecal matter. Although the gut microbiome is generally stable within adult individuals (Rinninella *et al.* 2019), the titre of bacterial species can vary significantly between individuals (Zitomersky *et al.* 2011, Backhed *et al.* 2012). That considered, a *Bacteroidales* marker would not be suitable for quantifying overall remnants of UHI. No significant pattern of faecal UHI was observed for any of the sites. This demonstrates that accumulation of faecal UHI is low and that the BacHum assay is not applicable as a linear regression at low or moderately impacted sites. The results suggest that the BacHum assay may be successfully applied to higher impact sites, such as the US campsites, or research stations such as Scott Base. In comparison with the mitochondrial assay, the BacHum may be better applied to detect faecal contamination in a binary manner (e.g., 'contaminated' or 'not contaminated') by setting a threshold for faecal contamination in sites of known human presence, or in conjunction with a mitochondrial assay. This study indicates that choosing to apply the BacHum assay would require prior knowledge of human occupancy, such as from Antarctica NZ camp journals.

3.4.4 Recovery of a site after occupation

At both Spaulding Pond and Vanda Hut, faecal UHI was lower in an exposed toilet area compared to an enclosed area. In enclosed areas, the released human materials are likely to remain at the site through protection from harsh environmental conditions, such as intense solar radiation and aeolian dispersal, resulting in a greater persistence of detectable DNA. None of the toilet areas of field camps produced sufficient faecal signal to produce a significant linear regression. If sites with permanent infrastructure and sheltered toilet areas (e.g., a portacom) were to be decommissioned, the preferred course of action might be to dispose of the soil likely to harbouring faecal materials to minimise footprint, such as the soil tracked into the portacom. If PCR testing indicates that the soil in immediate vicinity e.g., near an entrance, is contaminated with faecal material, this may be an additional consideration- depending on the extent.

3.4.5 Management of faecal UHI

There are currently no guidelines specific to faecal UHI, although the current recommendations outlined in the "McMurdo Dry Valleys ASMA Manual" (Antarctica NZ), appear adequate as indicated by the detection of very localised faecal signal. These management protocols are established by

National Programmes to ensure that all activities can be conducted in the MDVs whilst maintaining stewardship, to ensure that high quality data continues to be gathered from research-driven activities (e.g., the Long-Term Ecological Research, LTER). UHI appears to be accumulative over a field season, increasing with permanency and occupancy. Facilities zones with permanent structures cannot be moved; thus, human activity will remain concentrated at the same site. The results from this investigation indicate that sites can recover from UHI, given sufficient time; thus, the recommendation would be to establish smaller temporary field camps the same location with each successive field season, and to establish larger camps (e.g, Miers Valley) in the same location to contain the UHI footprint to a smaller area as possible, to minimise UHI dispersal from aeolian means and human foot traffic.

3.5 Conclusions and recommendations

In this study, we have shown that the mitochondrial marker was the best candidate for detecting, quantifying, and predicting human trace in an MDV setting. The results from the faecal assay targeting *Bacteroidales* did not produce a clear pattern of UHI, and faecal signal overall was much less than mitochondrial signal. This suggests human-associated markers may be better suited for identifying one type of impact, e.g., sewage, in conjunction with a marker to identify a site as having UHI, such as the mitochondrial marker. This is important because UHI remains to be thoroughly investigated and quantified, in an MDV environment. The genetic tools trialled for this study demonstrated the importance of testing sensitivity and cross-reactivity *in-situ* in the geographic location of interest and the challenges of working with low copy number eDNA samples, particularly regarding controls and contamination risk. A genetic marker must meet stringent sensitivity, specificity, and robustness standards to be a useful tool for environmental forensics.

The assays we validated for a dPCR platform could be used in the future by National Programmes (NPs) operating in the MDVs or any other Antarctic ice-free area to quantify UHI footprint left at a site after operations, or to assess recovery of a site from previous human occupation. This would be in accordance with the CEP's management plans and would ensure NPs are meeting the ATS obligation to leave the environment minimally impacted. To refine our methods more, future work could incorporate a wider range of UHI markers to produce a more comprehensive picture of the range of UHI. For instance, to better detect remnants of faecal signals, human-specific enteric viruses or protozoa could be targeted in addition to bacteria (Harwood *et al.* 2014). To better understand the spatial patterns of UHI detection at MDV camps, a range of other data could be integrated with transect data. For instance, wind data gathered from the Long-Term Ecological Research Project (LTER) and the Antarctic Science Platform would be valuable in elucidating where most deposited human materials are dispersed. Similarly, data for individual activity around a camp

to identify activity level could be incorporated with transect data. Moreover, the temporal persistence of DNA in an MDV environment has yet to be investigated in-depth**.**

Chapter 4. DNA longevity in Miers Valley soil, Antarctica

Abstract

Antarctica is commonly regarded as a pristine environment, relatively free from significant environmental degradation. The McMurdo Dry Valleys (MDVs) comprise a minute proportion of continental Antarctica, but have a significant human presence over the austral Summer, primarily for research-led activities where there is potential for environmental contamination. Unseen anthropogenic impact (e.g., human genetic materials) in field camps has not been investigated thoroughly in an MDV setting. To assess DNA persistence in an MDV setting, several DNA conformations derived from a pGEM-3Z plasmid were left *in situ* in Miers Valley: intracellular plasmid DNA, naked plasmid DNA, and linear DNA. A range of amplicon sizes were amplified from the recovered DNA and capillary electrophoresis was used to assess the size and quality of the amplicons. The relative recovery of a range of amplicon sizes was used to assess recovered DNA integrity, on the basis that DNA becomes increasingly fragmented as degradation progresses. DNA persists for at least two years in MDV soil, remaining detectable by endpoint PCR, with an indication of DNA fragmentation over time. Out of all the amplicons, the smallest was most easily amplified from the DNA recovered from Miers Valley. There was a general reduction in amplicon copy number as time progressed *in situ.* Soil sterility did not affect DNA recovery, which may suggest that abiotic factors have a greater effect on DNA persistence than the presence of heterotrophic organisms.

4.1. Introduction

Antarctica is regarded as the last pristine environment in the world. To promote peaceful activities and to prevent international discord, the Antarctic Treaty was signed in 1959. This has expanded to encompass 'The Protocol on Environmental Protection to the Antarctic Treaty' (Madrid, 1998), which designates the continent as a 'natural reserve, devoted to peace and science'. Under the Treaty system, there is the obligation to leave the Antarctic environment minimally impacted after all operations, which remains to be clearly defined. Like every other environment in the world, terrestrial Antarctica is vulnerable to the impacts from human activity, such as environmental degradation, wildlife disturbance and the introduction of exotic species. The ice-free areas are particularly vulnerable to the effects of human impact. These areas comprise a minute proportion (<0.2%) of the total continental land area (Burton-Johnson *et al.,* 2016) yet are biologically important and harbour the majority of terrestrial biodiversity, including bird and seal colonies, and soil microbial communities (Niederberger *et al.,* 2015, Niederberger *et al.,* 2019, Emslie, 2020). The McMurdo Dry Valleys (MDVs) are the largest contiguous snow and ice-free area of 4500 km^2, located in Victoria Land (Levy, 2013). During the austral summer, the ice-free areas near the coast experience disproportionate visitation, primarily through National Programmes (NPs). These areas contain the most supporting infrastructure, such as research stations, landing sites, and accommodation. As a result of human activity, these areas are constantly inundated with human materials (e.g., hair, skin, and epithelial cells) and human-associated microbes, which are disseminated in and around MDV field camps during routine activity as 'unseen impact'. It is unknown if such materials persist in a McMurdo Dry Valley (MDV) environment and whether they have a detectable impact on the environment, for example, by providing additional nutrients for soil microorganisms.

There is a paucity of knowledge around DNA persistence in cold desert soils, much less in an Antarctic setting. Several factors promote the MDVs as an ideal setting to test hypotheses around DNA persistence. The Dry Valley soils are characteristically low biomass, are microbe-dominated and have simple trophic systems (Willerslev *et al.,* 2004b, Cary *et al.,* 2010, Lee *et al.,* 2012, Chan-Yam *et al.,* 2019). Antarctica lacks forests, which are the dominant terrestrial ecosystems worldwide; thus, the rate of biomass turnover is much slower than that of temperate, tropical and agricultural terrestrial systems, and released DNA is more likely to persist in an Antarctic environment (Pan *et al.,* 2013, Carvalhais *et al.,* 2014, Erb *et al.,* 2016). A range of studies have isolated and sequenced DNA from environments of constant low temperatures, such as frozen soils and sediments, permafrost, and glacial ice (Shi *et al.,* 1997, Christner *et al.,* 2000, Yashina *et al.,* 2012, Yoccoz *et al.,* 2012, Legendre *et al.,* 2014, Maschenko *et al.,* 2017), including the oldest recovered complete

genome of ~700 kya (Orlando *et al.,* 2013) and genome-wide data obtained from mammoth molars <1,000,000 mya BP recovered from permafrost (van der Valk *et al.,* 2021). The upper limit for DNA preservation in a terrestrial environment under ideal conditions, such as permafrost, has been indicated to be between 400 kya to 1.5 mya (Willerslev *et al.,* 2004b). Beyond this time, the DNA is likely to be too severely degraded to be detectable by PCR through the accumulation of inhibitors, and DNA fragmentation, both of which lead to the production of short PCR products (Hansen *et al.,* 2006). The average soil temperatures of -40°C to -15°C (O'Neill *et al.,* 2013) in the Ross Sea region fall within the temperature range of previously recovered ancient DNA specimens.

DNA recovered from Antarctic soils is mainly microbial (including cyanobacterial) in origin, along with rotifer, tardigrade, and viral DNA (Zablocki *et al.,* 2016). Living cells rapidly lose viability once released into a Dry Valley environment and lyse upon death, releasing DNA and other cellular components into the environment. Ah Tow and Cowan (2005) demonstrated that the common skin commensal *S. epidermidis* is PCR detectable in highly impacted areas, as non-viable cells or naked DNA. The sum of detectable DNA in soil consists of extracellular DNA, either actively excreted or released after cell lysis, and intracellular DNA (Pietramellara *et al.,* 2009), the bulk of which originates from lysed microbial cells (Levy-Booth *et al.,* 2007, Pietramellara *et al.,* 2009, Carini *et al.,* 2016). Our understanding of the factors affecting DNA persistence in soil is expanding, yet there is a paucity of investigations providing a quantitative approach to DNA degradation dynamics in a natural terrestrial system, much less a cold desert environment such as Antarctica.

The aim of this research is to assess DNA persistence in Miers Valley soil *in situ* by comparing the recovery of different DNA fragment sizes. Sterile and non-sterile soil from Miers Valley was inoculated with several plasmid DNA conformations (intracellular, extracellular circular and linear DNA) and recovered at yearly intervals across two austral summers (2016-2018). Based on current knowledge of DNA persistence in cold environments, we hypothesised that smaller fragments would be recovered by endpoint PCR in greater relative abundance than longer fragments as time progresses *in situ*. DNA degrades into increasingly smaller fragments at a rate of exponential decay in a terrestrial environment (Morrissey *et al.,* 2015, Casanovas-Massana *et al.,* 2018, Wang *et al.,* 2019). We also anticipated that more amplifiable DNA would be recovered from sterile soil in the 'dark' treatment than non-sterile soil in anticipation of DNA damage from ultraviolet radiation (UV) exposure (Lindahl, 1993, Kciuk *et al.,* 2020). In Antarctic MDV soils, the main processes of DNA degradation are expected to be the result of microbial degradation, when water availability increases, and via cross-linking from intense 24-hour UV solar radiation during the austral summer.

4.2. Methods

4.2.1 *In situ* experimental design in Miers Valley

Dry exposed soil was taken from a site in Miers Valley (Figure 4.1) in 2016 and inoculated with the DNA conformations, in duplicate samples. For each sample, 1 g soil for both sterile and non-sterile treatments were inoculated with the following plasmid DNA conformations i) an *E. coli* cell pellet obtained from 1 mL of culture grown to OD_{600} and resuspended in 500 µL phosphate buffered saline (PBS), ii) pGEM-3Z plasmid DNA in 500 µL PBS, iii) or linear DNA derived from the pGEM-3Z plasmid amplicon in 500 µL PBS. The controls were inoculated with 500 µL PBS without DNA, and were included for each experiment. The soil was then passively dried for ~72 hrs at ambient temperature in a biosafety class II cabinet (BSCII), until the 'dry' weight was equivalent to the soil weight prior to inoculation. For the sterile soil treatment, the soil was first passively dried in a BSCII, to the initial weight prior to autoclaving, before inoculation with the DNA treatments. Soil sterility was achieved by autoclaving at 121°C for 15 minutes.

The samples were stored in sealed 24-well tissue culture plates which were described as having "poor resistance" to UV light by the manufacturer (Eppendorf, Hamburg, Germany), where 1g soil was to the depth of ~ 1cm per well. Both light and dark soil treatments were included for plates collected from Miers Valley at yearly intervals (T_1 and T_2), and the plates not placed in Miers Valley (T_0) were immediately placed in a -80°C freezer after passive drying, and were not exposed to natural light. Occlusive duct tape was used to exclude solar light for the 'dark' treatment.

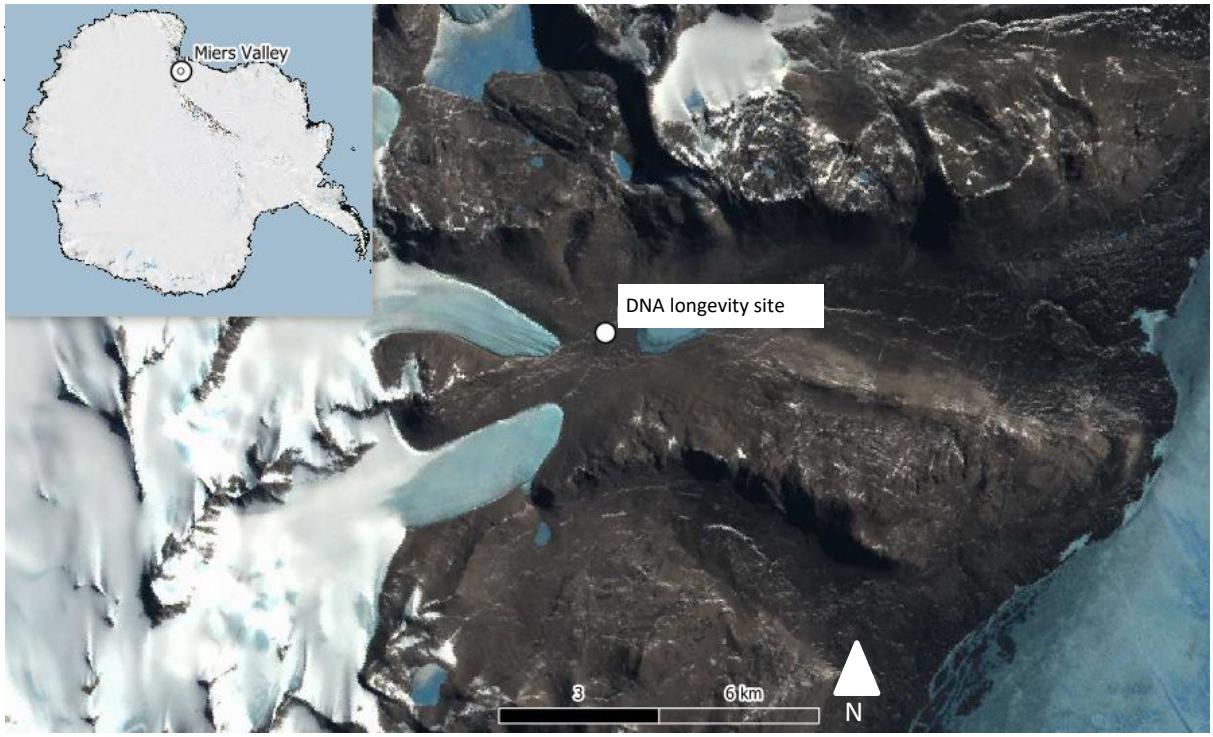


Figure 4.1: Location of the DNA longevity experiment in Miers Valley

4.2.2 *E. coli* growth, transformation, and alkaline lysis

Contamination is a high risk in microbial work, with microbial DNA being ubiquitous in all environments and in a laboratory setting. To minimise contamination, the pGEM-3Z (Promega, WI, US) plasmid was selected as a template because it is well characterised and not naturally occurring in the MDV environment. The pGEM-3Z plasmid was used for all three DNA conformations, which were:

i) Intracellular circular plasmid, killed *E. coli* transformed with plasmid
ii) Extracellular circular plasmid, isolated from *E. coli* by alkaline lysis
iii) Linear DNA derived from the pGEM-3Z plasmid, produced by endpoint PCR using primers which encompassed the plasmid, except for 4 bp between the 5' ends of the forward and reverse primers

E. coli cells transformed with plasmid were grown to OD_{600} 1.6 in Tryptic Soy Broth (TSB), with ampicillin added at a final concentration of 100 mg mL^{-1} to select for cells transformed with plasmid, at 37°C with agitation at 180 rpm. This culture was added at a 1/1000 dilution to TSB broth and grown overnight. Plasmid DNA was recovered from transformed *E. coli* using alkaline lysis as described by Birnboim and Doly (1988) and purified with gel excision using the PureLink Quick Gel Extraction Kit (ThermoFisher, MA, US).

4.2.3 *E. coli* kill methods

Sodium azide (NaN_3) and ethanol (EtOH) were tested as methods to kill transformed *E. coli* cells. *E. coli* grown to OD_{600}1.6 in TSB and incubated with NaN_3 at a final concentration of 2.0%, 5.0% and 10% (w/v) for one and two hours. To test 100% EtOH, the cells were pelleted with centrifugation, the TSB discarded, then resuspended with 5 mL 100% EtOH for 1 min at room temperature. The cells were washed twice with 5 mL phosphate buffer (NaH_2PO_4), pelleting each time with centrifugation. After the last wash, the cells were dried passively in a BSCII.

Serially diluted *E. coli* (10^{-3}- 10^{-8}) from each kill treatment was plated out onto Tryptic Soy Agar and incubated overnight at 37°C. The killed *E. coli* cells were stained with Syto13 and propidium iodide following the manufacturer's protocol (ThermoFisher, MA, US) and visualised with fluorescent microscopy to confirm the integrity of the cellular membrane. Live cells not subjected to a "kill treatment" were also plated as a control.

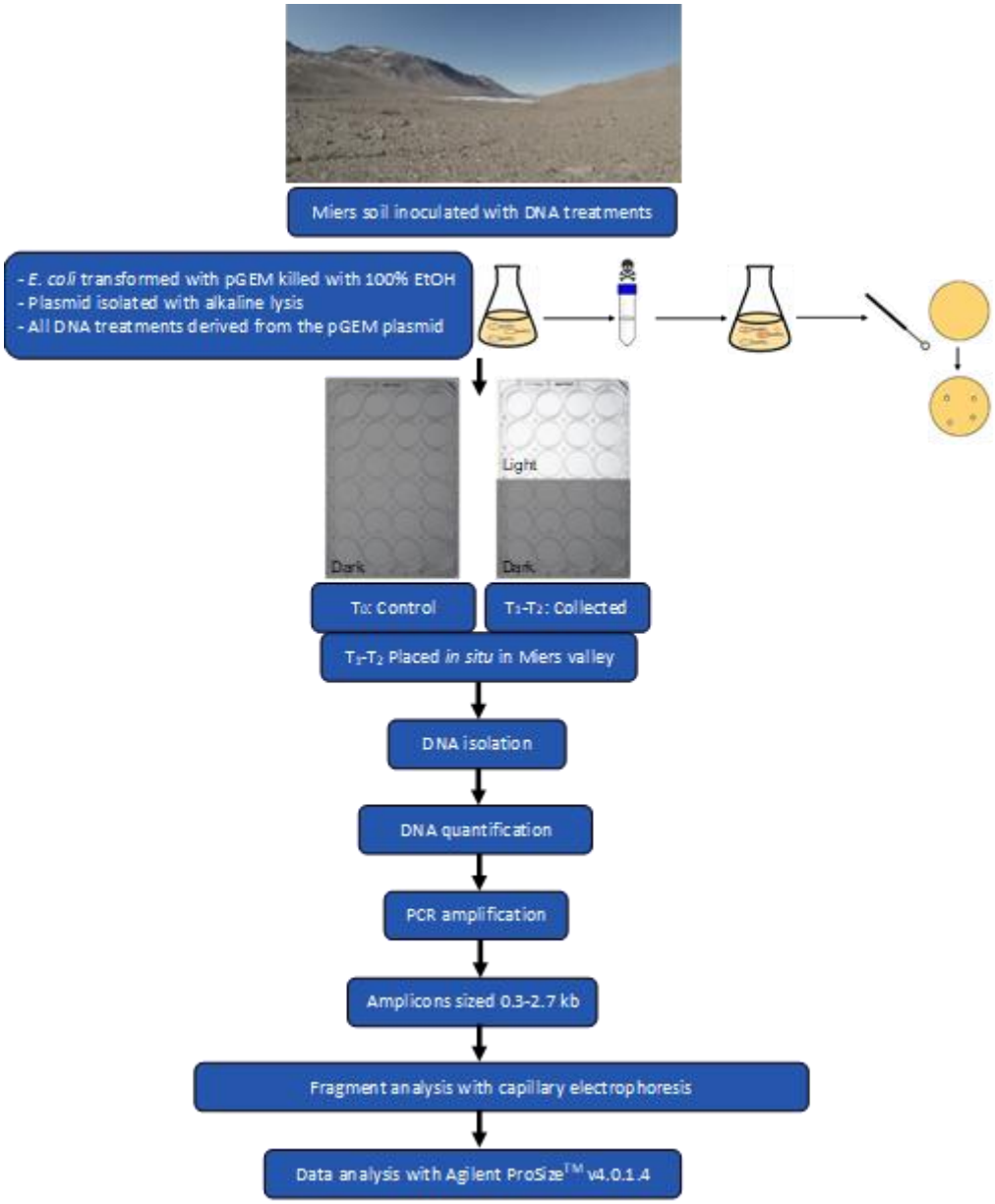


Figure 4.2: An overview of the workflow from field work to DNA recovery

4.2.4 DNA recovery from Miers Valley soil

A modified cetyltrimethylammonium bromide (CTAB) protocol optimised for Antarctic soils was used to isolate whole genomic DNA from each 1 g soil sample as described in the supplementary information of (Lee *et al.,* 2012), along with an additional 200 µL of phosphate buffer (NaH_2PO_4) added to the extraction buffer. After DNA precipitation, the DNA pellet was resuspended in 20 µL 1xTE. Regent controls were included to rule out contamination during DNA isolation.

4.2.5 Primer design and PCR amplification

Endpoint PCR on a QuantStudio Proflex (ThermoFisher, MA, US) system amplified four amplicons ranging from 2.7 kb to 0.3 kb from the DNA conformations and controls placed *in situ* in Miers Valley (Table 4.1).

The PCR mixture consisted of 0.2 µM dNTPs, 2.0 mM $MgCl_2$, 1U Platinum Taq and 1X buffer (Thermofisher, MA, US), 0.2 µM of each forward and reverse primer, prepared to a final volume of 20 µL with milliQ H_2O. All PCR reactions were done in triplicate to mitigate amplification bias. Thermocycling conditions were 94°C for 2 min, 32 cycles of: 94°C 30 s, followed by the optimised annealing temperatures and extension times (Table 4.2). To verify the correct size of the PCR amplicons, a high-resolution melt was performed at 0.2°C increments from 80 to 99°C on a Qiagen Corbett Rotor-Gene 6000 and the PCR products were visualised on a 2% agarose gel with SYBR safe (ThermoFisher, MA, US). Appropriate positive and negative controls were included.

Table 4.1: Primers used on DNA recovered from Miers soil. The reverse primer, BpGEMR, was labelled with a FAM fluorophore.

Primers	Sequence 5'-3'	Length bp	Tm °C	GC%	Amplicon size bp
M13F	CCCAGTCACGACGTTGTAAAACG	23	62.4	52.2	2689
1147F	CACGTTAAGGGATTTTGGTCATGAGA	26	61.5	42.3	1538
1998F	CCCACTCGTGCACCCAACTG	20	63.6	65.0	687
2353F	ACCTCTGACACATGCAGCTC	20	60.0	55.0	332
[FAM]-BpGEMR	CCCTGGCGTTACCCAACTTAATCG	24	63.7	54.2	NA

Table 4.2: Thermocycling conditions corresponding to each amplicon.

Amplicon size (bp)	Annealing Temperature (°C)	Extension time (min)	Final extension (min)
2689	60	3.0	10.0
1538	60	2.0	5.0
687	60	1.0	3.0
332	62	0.5	3.0

4.2.6 Fragment analysis with capillary electrophoresis

The recovered DNA produced PCR amplicons ranging in size from 332-2689 bp. Following endpoint PCR, the PCR triplicates were pooled, then diluted 1/10 before being run on a 5300 Fragment Analyzer (Agilent, CA, US). An Agilent NGS Fragment kit (1-6000 bp) was used with a 1200 LIZ size standard (ThermoFisher, MA, US). Peak assignment was made with several considerations. The correct peak size was assigned by running a positive control for each primer set, which was amplified with the BpGEM/M13F primers and cleaned and quantified post-PCR. All four amplicons were pooled and diluted 1/5 or 1/10 before being run as a single positive control. Peak assignment was then determined by peaks being the expected size, and the observed migration times being like those of the reference material. Any peaks below 100 bp were assessed to be left-over nucleotides and primers in the sample post-PCR, and excluded from subsequent analyses.

4.2.7 Data analysis

A Friedman's rank was performed on the three DNA conformations to compare the recovery of DNA from the sterile and non-sterile controls (T_0). Welch's ANOVA was used to compare the light and dark treatments for all DNA treatments for samples collected after one (T_1)- and two (T_2)- years *in situ*. Both datasets for the soil sterility and light treatments had unequal variances, thus non-parametric tests were selected in both instances. The controls (T_0) were not included in this analysis, as these were not exposed to a light treatment. To compare the recovery of different-sized DNA fragments, the yield of resultant amplicons was compared across T_1 and T_2 samples for each of the DNA treatments using a repeated measures analysis. The dark treatments were also compared across the T_0 controls and T_1 and T_2 samples. No significant difference was observed between the replicates for each of the above tests, thus replicates were nested for each experiment. All the analyses described above were done in Excel.

4.3 Results

4.3.1 *E. coli* kill methods

The efficacy of the *E. coli* kill methods was calculated as a percentage reduction:

$$\% \text{ Reduction of viable cells} = 100(A - B)/A$$

Where A= viable cells before a kill treatment and B= viable cells after a kill treatment.

For the sodium azide treatments, a 10% (w/v) concentration was the most successful at killing *E. coli.* Sodium azide at lower concentrations of 2.0% and 5.0% (w/v) killed 100% *E. coli* cells after a two-hour incubation. After a one-hour incubation, 2.0% NaN_3 was ineffective in killing cells and resulted in a 240% increase compared to the control (0% NaN_3), and 5.0% NaN_3 resulted in an 89.3% reduction in growth compared to the control (Table 4.3).

Washing pelleted cells with 100% EtOH successfully killed all cells and was the preferred method for obtaining intracellular plasmid enclosed within a cell membrane (Table 4.3). Subsequent staining of the *E. coli* with Syto13 and propidium iodide, and visualisation with fluorescent microscopy confirmed the integrity of cellular membranes (Figure 4.3).

Table 4.3: Mean colony forming units (CFU) for each kill treatment for triplicate plates. TMTC denotes 'too many to count'.

	Dilution					
Treatment	**10^{-3}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**CFU mL^{-1}**
Sodium azide, 1 hr incubation						
2.0% NaN_3	TMTC	TMTC	TMTC	102	12	1.0×10^9
5.0% NaN_3	TMTC	TMTC	32	10	0	3.2×10^7
10.0% NaN_3	0	0	0	0	0	0
0% NaN_3 (control)	TMTC	TMTC	TMTC	30	3	3.0×10^8
Sodium azide, 2 hr incubation						
2.0% NaN_3	0	0	0	0	0	0
5.0% NaN_3	0	0	0	0	0	0
10.0% NaN_3	0	0	0	0	0	0
0% NaN_3 (control)	TMTC	TMTC	TMTC	TMTC	102	1.0×10^{10}
100% EtOH	0	0	0	0	0	0

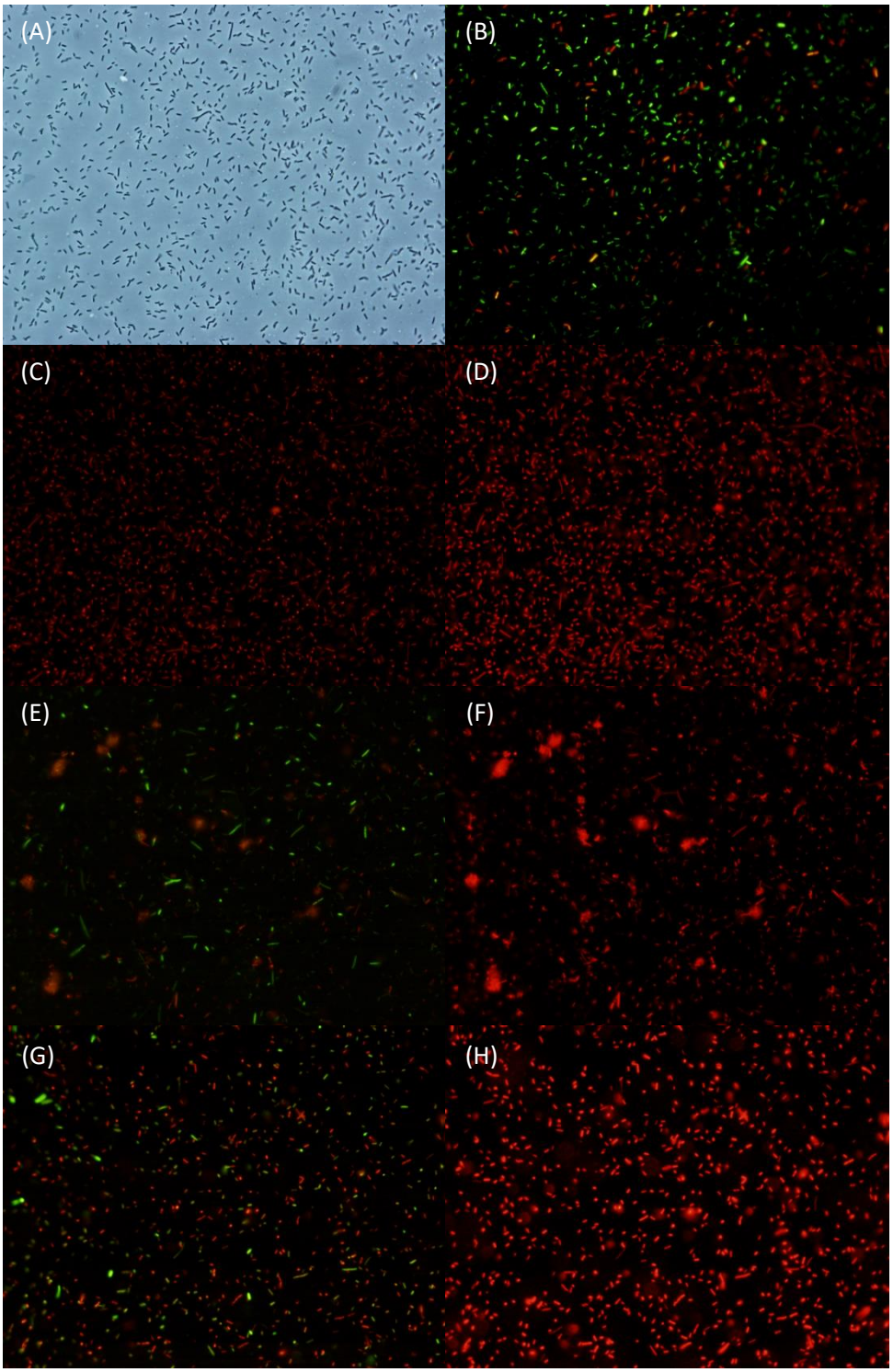


Figure 4.3: A wet mount of *E. coli* in (A) phase contrast and (B) stained with Syto13 and propidium iodide in the same field, show both living (green) and dead (red) cells. *E. coli* killed with 100% EtOH under (C) blue light and (D) green light. *E. coli* after 1-hour of incubation with 5.0% NaN_3 under (E) blue light and (F) green light. *E. coli* after 1-hour incubation with 10% NaN_3 under (G) blue light, and (H) green light.

4.3.2 DNA recovery from Miers soil

Quantifiable DNA was recovered from all soil samples, including the samples left *in situ* in Miers Valley, and the sterile and non-sterile soil controls. The average DNA yield was 16.3 ng μL^{-1} per sample across all replicates and controls, ranging from 0.1 ng μL^{-1} to 45.0 ng μL^{-1} (Table 4.4).

Table 4.4: A comparison of total DNA inoculated and recovered (mean) for both sterile and non-sterile soil controls (ng μL^{-1}).

	Intracellular plasmid		Plasmid		Linear		Control	
	Inoculated	**Recovered**	**Inoculated**	**Recovered**	**Inoculated**	**Recovered**	**Inoculated**	**Recovered**
Sterile control	3390.5*	35.2	150.24	0.4	342.4	1.4	0.0	0.1
Non-sterile control	3390.5*	45.0	150.24	12.7	342.4	15.1	0.0	20.1

* An average of DNA obtained from 1 mL *E. coli* grown to OD_{600} 1.0 in TSB.

4.3.3 Fragment analysis of recovered DNA

In this study, there were no clear effects of soil sterility, light availability, or DNA conformation (intracellular plasmid, naked plasmid and linear) on the amplification of recovered DNA. There was no significant difference between the yield of amplified DNA between the sterile and non-sterile controls (p= 0.2119). For a comparison of light exposure and light occlusion, there was no significant difference between the controls for any of the DNA conformations of intracellular plasmid in *E. coli* (t=0.4333, p=0.6881), extracellular plasmid (t= -0.2556, p= 0.8000) or linear amplicon (t= 1.2660, p= 0.2193), or any of the DNA conformations recovered after one and two years *in situ*, where all p values were > 0.05 for all amplicon sizes.

There was indication of DNA fragmentation over time after recovery at one-year and two-year intervals, demonstrated by differences in the relative recovery of the different amplicon sizes (Figure 4.4). The total copy number for each amplicon size was greatest for the smallest amplicon size and decreased as the amplicon size increased (Table 4.5). A 1.5 kb fragment was the upper limit for PCR amplification; a 2.7 kb amplicon was not obtained from the recovered DNA after one- or two- years *in situ* (Table 4.5), and a relatively small amount of unfragmented DNA was obtained from both sterile and non-sterile soil controls, shown by relatively low copy number of the 2.7 kb fragment compared to smaller sized amplicons (Figure 4.4). For DNA recovered after one year, a significant difference was seen for the intracellular plasmid (F(3, 1)= 56.69, p= 0.0000) and extracellular plasmid (F(3, 1)= 7.24, p= 0.0115) treatments, but not the linear amplicon (F(1, 3)= 1.23, p= 0.3616). For DNA recovered after two years *in situ*, a significant difference was seen for intracellular plasmid (F(3, 1)=5.89, p= 0.0201) and linear amplicon (F(3, 1)= 7.69, p= 0.0096), but not extracellular plasmid (F(3,

1)= 3.78, *p*= 0.059). No amplification of a 2.7 kb fragment was obtained from recovered DNA after one or two years *in situ*.

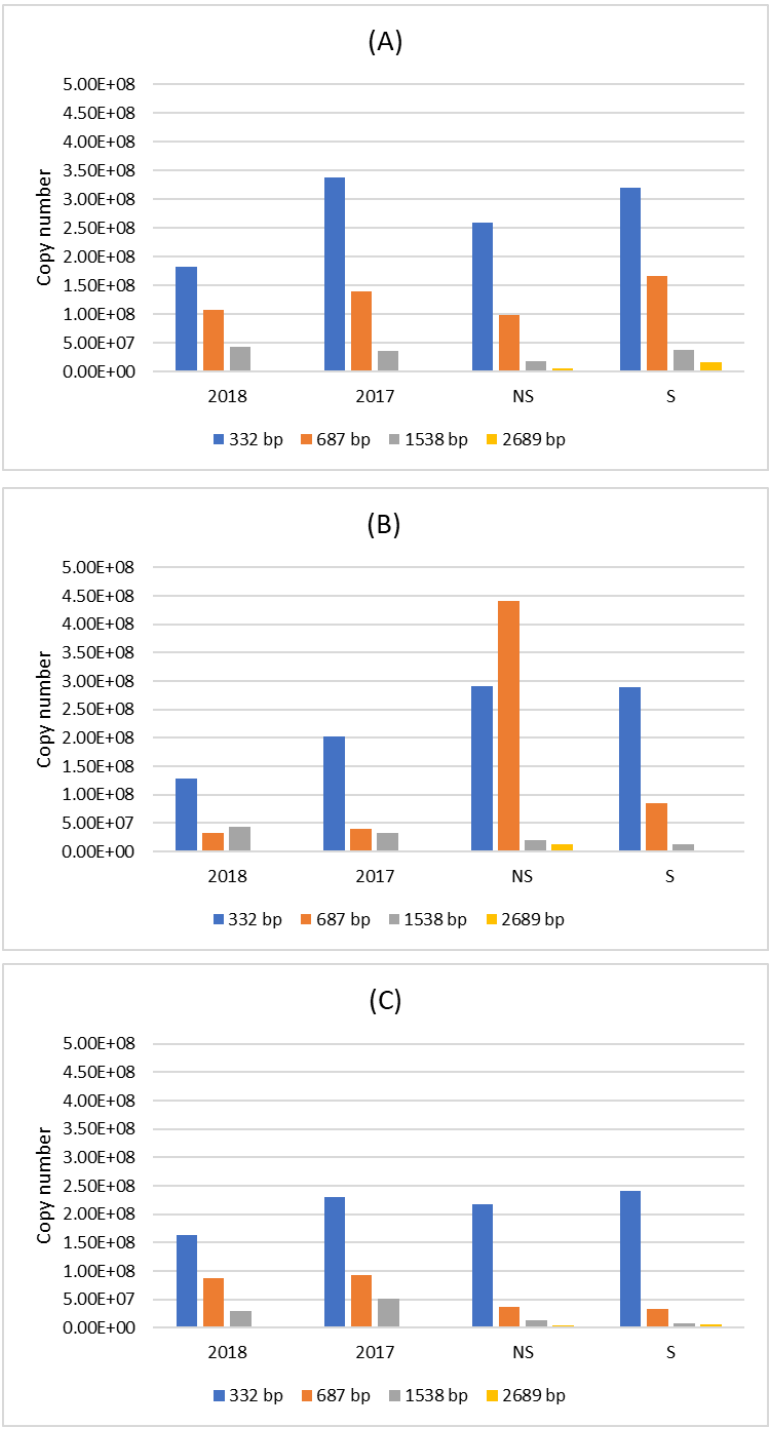


Figure 4.4: The copy number obtained for each amplicon size from DNA recovered for each DNA conformation: (A) intracellular plasmid, (B) naked plasmid and (C) linear DNA. The DNA was recovered from Miers Valley after one (2017) and two years (2018) *in situ*, and from non-sterile (NS) and sterile controls (C).

Table 4.5: The total DNA and copy number obtained for each amplicon size, across all DNA treatments recovered from Miers Valley (n= 4). To determine copy number, this formula was used: number of copies = (amount ng μL^{-1} * $6.022x10^{23}$) / (length bp * $1x10^{9}$ ng g^{-1}* 650 Da)

	Amplicon size							
	332 bp		687 bp		1538 bp		2689 bp	
DNA conformation	**ng μL^{-1}**	**Copy number**	**ng μL^{-1}**	**Copy number**	**ng μL^{-1}**	**Copy number**	**ng μL^{-1}**	**Copy number**
Sterile control								
Cell	0.11	3.94E+07	0.12	4.26E+07	0.06	2.17E+07	0.05	1.65E+07
Plasmid	0.10	3.57E+07	0.06	2.16E+07	0.02	6.98E+06	0.01	2.21E+06
Linear	0.09	2.97E+07	0.03	8.62E+06	0.01	5.12E+06	0.02	6.11E+06
Non-sterile control								
Cell	0.09	2.60E+08	0.07	9.81E+07	0.03	1.92E+07	0.02	6.40E+06
Plasmid	0.10	2.92E+08	0.18	2.48E+08	0.03	1.93E+07	0.04	1.24E+07
Linear	0.08	2.18E+08	0.03	3.75E+07	0.02	1.37E+07	0.02	5.32E+06
2017								
Cell	0.12	3.38E+08	0.10	1.40E+08	0.06	3.69E+07	0.00	0.00E+00
Plasmid	0.07	2.03E+08	0.03	4.03E+07	0.06	3.35E+07	0.00	0.00E+00
Linear	0.08	1.63E+08	0.07	9.33E+07	0.09	5.13E+07	0.00	0.00E+00
2018								
Cell	0.07	1.82E+08	0.04	5.03E+07	0.07	4.33E+07	0.00	0.00E+00
Plasmid	0.05	1.28E+08	0.02	3.32E+07	0.07	4.36E+07	0.00	0.00E+00
Linear	0.06	2.31E+08	0.06	8.71E+07	0.05	2.94E+07	0.00	0.00E+00

DNA degradation through fragmentation was demonstrated by a general reduction across the DNA conformations and amplicon sizes after one year in Miers Valley. The exception was intracellular and plasmid DNA for the 1538 bp amplicon, where there was an increase in copy number after one year (Figure 4.5). For all DNA conformations at other amplicon sizes, the time to complete recovery (100% reduction) was demonstrated to range from 2.5 to 16.7 years (Table 4.6). A linear regression model was used to estimate the recovery time, $Y= \beta_0 + \beta_1 X$, when Y=0, where Y= UHI signal in copy number, β_0 is the intercept, β_1 is the slope, and X is time in years. No estimate for duration of DNA persistence could be made for the 2689 bp amplicon, as no amplifiable DNA was obtained at this size for any DNA conformation (Table 4.6).

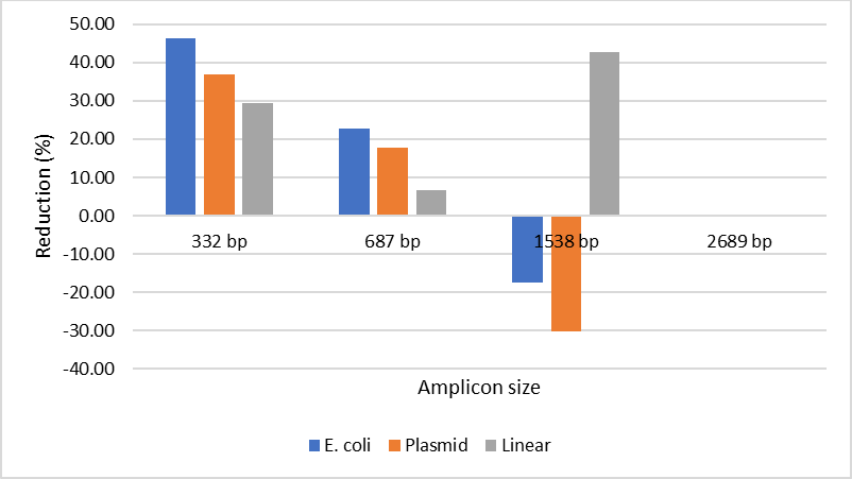


Figure 4.5: The change in amplicon copy number from DNA recovered between one (2017) and two years (2018) *in situ*.

Table 4.6: (A) A percentage change in copy number for amplicons from DNA recovered from 2017 to 2018, and (B) the number of years until recovery (100% reduction) using a linear regression model.

	332 bp		**687 bp**		**1538 bp**		**2689 bp**	
	(A)	**(B)**	**(A)**	**(B)**	**(A)**	**(B)**	**(A)**	**(B)**
E. coli	46.15%	2.50	22.66%	6.67	-17.34%	-5.00	0.00%	0.00
Plasmid	36.95%	3.75	17.62%	7.14	-30.15%	-4.29	0.00%	0.00
Linear	29.44%	4.29	6.65%	16.67	42.69%	3.50	0.00%	0.00

4.4. Discussion

4.4.1 The efficacy of the *E. coli* kill treatments

Sodium azide is acutely poisonous and is widely used as a biocide. In Gram-negative bacteria, such as *E. coli*, sodium azide inhibits cytochrome oxidase activity, which makes it useful as a bactericidal agent (Noumi *et al.,* 1987). This was confirmed by the inhibition of *E. coli* growth at all concentrations (2%, 5% and 10% w/v) after a two-hour incubation, whereas a 10% concentration inhibited growth after both one- and two-hour incubation periods. The efficacy of sodium azide as a biocide depends on its concentration and environmental matrix properties (Cabrol *et al.,* 2017). Previous studies have used a sodium azide concentration ranging from 1 to 150 mM, compared to 1.54 M, 0.77 M and 0.31 M (equivalent to 10%, 5% and 2% w/v, respectively) used in this investigation, where a much shorter incubation period of up to two hours was used, compared to much longer incubation times in other studies of multiple days (e.g., Wolf *et al.,* 1989, Cabrol *et al.,* 2017).

Ethanol is also a commonly used biocidal agent, with a rapid broad-spectrum activity against both Gram-negative and Gram-positive bacteria, viruses, and fungi (Kampf *et al.,* 2021). The most effective preparations for bactericidal activity are reported to be at a ~70% concentration (McDonnell and Russell, 1999), via cell membrane disruption, protein denaturation and RNA damage (Larson *et al.,* 1991, Al-Sayah, 2020). A concentration of 100% ethanol was demonstrated to kill *E. coli* cells in 10 seconds (Morton, 1950). Compared with sodium azide, ethanol had the best efficacy in killing *E. coli* cells, without requiring an incubation period, while maintaining the cell membrane integrity. At concentrations above 70%, the cell membrane becomes impermeable to ethanol (Ambrosino *et al.,* 2022). At 100% concentration, the ethanol was most likely not able to enter the *E. coli* cells nor solubilise the cell membrane.

4.4.2 Factors affecting the DNA persistence Miers Valley soil

DNA has several fates upon release into the environment, including degradation by DNases (Blum *et al.,* 1997), transformation by competent soil microorganisms (Overballe-Petersen *et al.,* 2013), consumption as a nutrient source by microorganisms (Morrissey *et al.,* 2015, Mulcahy *et al.,* 2010), leaching through the soil column (Poté *et al.,* 2007), incorporation into biofilms and biocrusts (Vorkapic et al., 2016), or persistence in the environment when adsorbed to the soil matrix (Crecchio and Stotzky, 1998). The predominant DNA damage mechanisms are determined by the interaction of physical, chemical, and biological properties of the microenvironment, such as available water, pH, salt, and temperature, in conjunction with biotic factors, such as degradation by microbial nucleases (Lindahl, 1993, Willerslev *et al.,* 2004a, Pedersen *et al.,* 2015).

Soil sterility was anticipated to have a greater effect on DNA fragmentation than found in this investigation. Relatively greater DNA degradation was expected for the non-sterile controls via microbial digestion of DNA, as the degradation of biological materials occurs more quickly in non-sterile soil compared to sterile soil (Lauber *et al.,* 2014). Although no significant difference in DNA fragmentation was seen between the sterile and non-sterile control groups, the heterotrophs present in MDV soil have the capacity to use DNA as a nutrient source for carbon, phosphorus and energy (Pinchuk *et al.,* 2008, Emmons *et al.,* 2017). During the austral summer MDV soils are hotspots for biological processes with primary productivity and heterotroph growth increasing markedly (Sohm *et al.,* 2020) when water availability increases (Fountain *et al.,* 2010, Pointing *et al.,* 2015). In contrast, a drier environment results in slower DNA degradation by limiting heterotroph growth and by slowing the hydrolytic damage of DNA (Willerslev *et al.,* 2004a). Antarctic soils are characteristically cold and oligotrophic, with limited moisture availability for most of the year. These soils subsequently support low biological activity and low biomass, where heterotrophic bacteria predominate (Niederberger *et al.,* 2008). Communities in MDV soils also have a relatively higher

abundance of stress- and dormancy- related genes compared to other soils (Wei *et al.,* 2016). These results indicate that abiotic factors could have a greater impact on DNA degradation than biotic factors, such as the microbial digestion of DNA.

Light did not appear to have a significant effect on the size of DNA recovered in this study, despite the prediction that light availability would have a greater effect on DNA fragmentation than other factors. The DNA treatments exposed to light were anticipated to accumulate UV-mediated damage, through the production of cross-linking, pyrimidine dimers, generating oxidised bases, and by inducing single-stranded breaks (Fuentes-León *et al.,* 2020), and subsequently produce a higher ratio of smaller amplicons to larger amplicons. The MDV soils receive intense solar radiation during the austral summer from November to January, and experience low light conditions for the remainder of the year (Dana *et al.,* 1998). Outside of summer, conditions of constant low temperature and desiccation in the MDV environment are close to ideal for the long-term preservation of biological material such as cells and nucleic acids (Goordial *et al.,* 2017). Other Antarctic environmental conditions of a mostly neutral soil pH, and low moisture also promote DNA preservation in soil and permafrost (Thompson *et al.,* 1971, Willerslev *et al.,* 2004b, Willerslev *et al.,* 2004a). Solar radiation intensity in the MDVs is highly variable, due to variations in atmospheric conditions (Obryk *et al.,* 2020). The site for this *in situ* experiment may have been sufficiently shaded to prevent some of the DNA damage induced from solar UV radiation, to be comparable to the light-occluded controls.

DNA persistence in soil is largely due to DNA adsorption onto soil components, such as clay minerals, sand particles and humic compounds (Khanna and Stotzky, 1992, Paget *et al.,* 1992, Crecchio and Stotzky, 1998). DNA sorbed to soil minerals is better protected against nuclease degradation than free DNA, and thus is more likely to persist for longer in the environment (Romanowski *et al.,* 1991, Demanèche *et al.,* 2001, Pietramellara *et al.,* 2009). A range of factors influence the adsorption of DNA to soil components, including DNA conformation and size. Of the three DNA conformations (intracellular plasmid, extracellular plasmid, and linear plasmid) compared in this investigation, intracellular plasmid was anticipated to be better protected against DNA degradation by abiotic factors such as UV radiation than extracellular plasmid. A comparison of the different DNA conformations over two-years *in situ* demonstrated that both extracellular and intracellular DNA can persist for years in MDV soils. In a previous study by Ah Tow and Cowan (2005), both non-viable cells and naked DNA showed measurable persistence in the environment over time, remaining detectable after seven weeks using culturing methods.

The common processes of DNA degradation, such as cross-linking, hydrolysis and oxidation are known to inhibit downstream PCR by preventing amplification, producing short PCR products or by

incorporating the wrong base (Hansen *et al.*, 2006). DNA damage occurs randomly across the genome (Hanssen *et al.,* 2017) and results in greater DNA fragmentation, where increasingly smaller DNA fragments are recoverable as degradation progresses with time (Dabney *et al.,* 2013). Fragmentation was observed for all DNA treatments left *in situ*. Upon recovery of the DNA treatments, the best amplification was consistently obtained from the 0.3 kb amplicon after one- and two-year intervals, whereas no amplification was obtained from the largest amplicon size of 2.7 kb. This is consistent with previous studies in which smaller fragments had greater amplification success in degraded DNA samples (Butler *et al.,* 2003, van Oorschot *et al.,* 2010, Machida and Kibayashi, 2020).

4.5 Conclusions and further directions

DNA persistence remains to be thoroughly investigated in an Antarctic setting as part of the unseen anthropogenic footprint, which is a pertinent consideration for environmental management under the Antarctic Treaty System obligations. Released human materials in the form of genetic materials and microorganisms are known to persist in cold soils and permafrost. This investigation demonstrates that Antarctic environmental conditions are favourable for the preservation of DNA, which remains detectable in MDV soils using endpoint-PCR beyond two years. Successful amplification was obtained from all the DNA conformations tested, namely intracellular plasmid, extracellular plasmid, and linear DNA. There was an indication of DNA degradation over time, demonstrated by a reduction in copy number of most amplicon sizes and DNA conformations from one to two years *in situ* in Miers Valley, and by amplification success relative to amplicon size; the greatest amplification was obtained from a 0.3 kb fragment to no amplification of a 2.7 kb fragment. Amplification to a maximum of the 1.5 kb fragment was obtained from both intracellular and extracellular DNA left *in situ* in Miers Valley.

This investigation was completed *in situ* in Miers Valley, and the DNA conformations would have been exposed to fluctuations in temperature and intensity of solar radiation due to seasonal and temporal variation. This could explain the large variation in detectable degradation for samples collected after one or two years *in situ* for any of the experiments. UV exposure did not have a discernible effect on DNA degradation after one or two years of exposure, which was unexpected. The wavelength of solar UV radiation transmittable through the soil container could not be determined for this investigation and therefore could not be identified as being germicidal. A cell culture plate with UV transparent properties, such as a fluorocarbon plate, would have been preferable to the polystyrene plate used in this study. In some instances, the recovered DNA yielded greater amplification after two years compared to one year *in situ*. Surprisingly, soil sterility did not have a significant effect on DNA persistence in this investigation. These results suggest that abiotic

factors may have a greater influence on DNA persistence in MDV soils than microbial degradation. However, it could not be ascertained whether sufficient moisture was available for heterotroph growth in these *in situ* experiments, to enable DNA degradation by microbial consumption. Further investigation is required to elucidate how biotic and abiotic factors interact to affect DNA persistence. The extremely heterogeneous physiochemical properties of MDV soils are an important consideration. Variations in soil particle size, composition and pH would likely have contributed to variation in DNA adsorption to soil particles and subsequent protection from degradation.

Given the possibility of DNA persistence for years after release in an MDV environment, considerations must be made around minimising human footprint regarding unseen impact. For instance, it would be valuable for national programs to consider the impact of the accumulation of unseen impact as a result of large field camps, and the duration of occupancy at a site, as part of their environmental management protocols. Further investigation is required to identify which factors have the greatest affect DNA persistence in an MDV setting. The overall impact of releasing genetic material and non-indigenous microorganisms (e.g., those associated with the human microbiome) remain unknown. The abiotic factors identified in previous studies as significantly affecting DNA persistence, such as light intensity and duration, moisture availability, and temperature could not be controlled or quantified in this *in situ* investigation. It would be beneficial to repeat the DNA longevity experiments in a more controlled setting, such as an environmental chamber, to maintain consistent environmental conditions, and as a comparison to this *in situ* study.

5. Conclusions

This chapter synthesises information from the preceding chapters to investigate unseen human impact (UHI) on spatial and temporal scales. Policymakers require easy access to evidence-based information on a range of topics relevant to the governance and management of the Antarctic, which is facilitated by the Antarctic Environments Portal. The key purpose of this investigation was to identify the amount and spatial impact of UHI at campsites and the duration of the impact. It is hoped that this information will support the decision-making bodies of the Antarctic Treaty System (ATS), such as the Committee for Environmental Protection (CEP), during the Scientific Committee on Antarctic Research (SCAR) meetings. This chapter will provide a concise overview of current scientific information to provide a bridge between science and policy and will be submitted for publication online under 'The Antarctic Terrestrial Environment' on the Antarctic Environments Portal (https://environments.aq/).

5.1 Overview

Humans have visited Antarctica for the last c.200 years. Under the Antarctic Treaty System (ATS), there is an obligation for operators (science or tourism) to minimise adverse environmental impacts as far as practicably possible. The unseen human impacts, such as the release and longevity of human-derived genetic materials, remain to be thoroughly investigated, and quantified. The McMurdo Dry Valley (MDV) soils provide an exceptional opportunity to test hypotheses around the detection, quantification, and persistence of released human biological materials. Routine research activity in the field camps will deposit human-derived materials, such as the shedding of skin cells and hair, and has the potential to be detected as unseen human impact (UHI) using sensitive molecular tools. This UHI has yet to be considered under current environmental management guidelines. In a series of recent studies, new genetic approaches for the detection and quantification of UHI have been developed and tested demonstrating that significant UHI accumulation does occur and should be considered when designing field camp logistics. It is important to consider UHI in the context of environmental management plans and to make the necessary amendments if required to meet ATS obligations.

5.2 Detailed Overview

The Antarctic Treaty System (ATS) was established by signing the Antarctic Treaty in 1959 to preserve Antarctica for peaceful endeavours, and to prevent international discord, which later included the Protocol on Environmental Protection to the Antarctic Treaty (hereafter referred to as the 'EP'). Under the ATS, the EP designates the Antarctic region south of latitude 60° as a "natural reserve, dedicated to peace and science", and requires Antarctica to be left without adverse impact after all operations. Terrestrial areas are known to be vulnerable to anthropogenic impacts and

require continual monitoring and assessment as human presence increases and diversifies on the continent (Brooks *et al.,* 2018). The risks associated with anthropogenic activities must first be identified for mitigation to occur through incorporation into evidence-based environmental management policies.

Human impact in terrestrial Antarctica can be broadly categorised as 'visible' or 'unseen'. The visible impacts have been investigated, such as physical disturbances (Kennicutt *et al.,* 2010, O'Neill *et al.,* 2013, Brooks *et al.,* 2019) and chemical contamination (Webster *et al.,* 2003), and are thus managed as prescribed by the National Programmes. In contrast, there remains a paucity of knowledge around unseen impacts, which includes the introduction of 'alien' species (Huiskes *et al.,* 2014), the homogenisation of terrestrial soil microorganisms through human movements, and the release of foreign genetic materials (e.g., human nucleic acids). The unseen impacts remain to be clearly defined in environmental policy, much less investigated. By not addressing unseen impacts and their associated risks, ATS obligations are unlikely to be fully met.

The majority of terrestrial Antarctica is characterised by simple trophic systems and highly oligotrophic soils of low biomass and low diversity (Cary *et al.,* 2010). The ice-free areas are a unique environment within terrestrial Antarctica worth preserving and comprise <1% of continental Antarctica. These areas experience intense human activity due to accessibility, particularly over the austral summer, as they are often associated with research infrastructure and tourist landing sites (Convey and Peck, 2019). For instance, the dominant activity is science-related and includes supporting logistics (Hughes *et al.,* 2018), with over 100 facilities supporting the 33 countries that operate in the Antarctic (COMNAP, 2017). Human presence in the Antarctic is projected to increase further and diversify (Hughes *et al.,* 2018, Tejedo *et al.,* 2022), and this ever-increasing human presence in the Antarctic prolongs and increases anthropogenic environmental pressure (Bender *et al.,* 2016). Most of the terrestrial biodiversity is soil microorganism communities (e.g., bacteria, archaea) which drive biological processes of nutrient cycling, including N_2 and CO_2 fixation (Sohm *et al.,* 2020). Direct experimentation has demonstrated that soil microbial communities respond directly to change (Tiao *et al.,* 2012), an important consideration in the context of climate change, which is likely to intensify the human environmental impact. For instance, increasing soil surface temperatures and available moisture increases the likelihood of introduced non-native species establishing and becoming invasive (Convey and Peck, 2019), and the expansion of ice-free areas (Lee *et al.,* 2017) will likely result in the expansion of human activity and its associated impacts. Thus, the long-term effects of unmitigated UHI in the form of released human materials are unknown and could directly impact the resilience of soil communities and greater ecology in terrestrial Antarctica.

Until recently, UHI has not been quantified, due to constraints in methodology and a lack of monitoring, leading to an underestimation of the invasion rate (Hughes *et al.,* 2020). Recent major advances in genetic tools, such as digital PCR (dPCR), now provide the means to detect extremely low abundance genetic targets (as low as a single copy of a target gene) as proxies for UHI from environmental samples. dPCR can quickly provide the absolute quantification of a DNA target, which has several advantages in detecting low abundance targets. This method is well-suited to amplifying environmental DNA (eDNA) samples, being highly robust and tolerant to PCR inhibitors (Te *et al.,* 2015). In Antarctic soil samples, the eDNA target is anticipated to be swamped by many orders of magnitude by DNA from indigenous soil communities.

To detect and quantify the remnants of UHI in a field study, we used human-associated genetic targets and applied them to sites of human activity in the McMurdo Dry Valleys (MDV). The gene targets included a human mitochondrial DNA marker targeting a region of the *MT-CYB* gene (HcytB assay) and a human gut-associated faecal microbe targeting a region of the 16S rRNA gene of *Bacteroidales* (BacHum assay). The genetic marker assays validated for a dPCR platform in this investigation were highly sensitive and robust, capable of single-cell detection down to attogram (10^{-18} g) concentrations of target DNA. The sensitivity of either assay did not decrease under inundation from non-target DNA, an important consideration for eDNA samples (Bowers *et al.,* 2021).

The MDVs provide an ideal environment to test methods and quantify UHI in the form of remnants of genetic materials derived from shed skin cells and hair after different durations of routine human activity. This terrestrial area is accessible from several bases in close proximity, including Scott Base (NZ) and McMurdo Station (US). The climatic conditions in the valleys mimic those utilised in routine DNA preservation, such as low moisture and constant low soil temperatures (Cary *et al.,* 2010). Biological materials such as DNA are thus likely to persist in the environment after being released. In addition, the MDV soils support low biomass, primarily dominated by microbial communities and a few select invertebrates (e.g., tardigrades, rotifers, nematodes) (Treonis *et al.,* 1999, Cary *et al.,* 2010, Lee *et al.,* 2012), and thus, food webs are simple in the absence of higher animals and plants making detection and quantification of human occupation possible.

A range of varying human impact sites located in four valleys (Victoria, Wright, Miers and Taylor Valleys) was incorporated into this investigation. These sites included temporary field camps (NZ) of differing sizes and the larger permanent US Facilities Zones in Taylor Valley (Figure 5.1). UHI from released DNA was assessed at points along transects extending from the centre of the site (maximum impact), radiating out into areas unlikely to be impacted. Where possible, new camps

were sampled at both arrival and departure. The most pristine site was in Victoria Valley (NZ), where there was no record of a previous camp and which had the smallest footprint in terms of total human days. A UHI signal, both mitochondrial and faecal, could be detected upon departure, demonstrating the sensitivity of applying this method to detect UHI. Mitochondrial signals could be detected and predicted from the moderately impacted sites, such as Lower Wright Hut Facilities Zone (Brownworth) and Vanda Hut Facilities Zone (Vanda Hut) in Wright Valley (both NZ facilities). Miers Valley had the greatest occupancy and footprint of all the NZ sites and a comparatively high UHI signal, including the greatest faecal signal. The US Facility Zones in Taylor Valley (Fryxell, F-6 and New Harbour Facility Zones) experience a greater intensity of visitation and a greater footprint, with a reported annual average of 100-600 person-days by the United States Antarctic Program (USAP). As expected, most of the large US Facility Zones showed the greatest extent of detectable UHI radiating from a station (Table 5.1). Mitochondrial UHI signals could be detected up to ~300 m from the US sites and were generally significant enough to provide a prediction of the distance of detectable signal.

In general, UHI was detected in all sites and the greatest level of signal was always detected closest to a site. Across all sites, regardless of the intensity of presence, human-mitochondrial DNA was detected at a greater level than the marker for faecal contamination, and generally had a pattern of exponential decay as distance increased from a site. The total occupancy at a site and the prevailing wind direction affected UHI detection. The UHI signal was cumulative, increasing with increased occupancy, demonstrated by a comparison of UHI at arrival and after departure and a comparison of tent sites ranging from zero (control) to three occupants. Aeolian dispersal of human materials and genetic materials bound to soil occurred in relation to the prevailing winds, which was evident in Wright and Taylor Valleys, where a predominant bimodal wind regime occurs in up- and down-valley directions (Doran *et al.,* 2002).

An important question remains regarding UHI persistence in a cold and dry environment such as the MDVs. Miers camp was visited over multiple seasons to see if the original measured UHI signals diminished over time, illustrating the capacity for natural recovery. The repeat visit after two years showed a significant reduction in UHI signal but not complete recovery. To better understand the longevity of UHI, a two-year experiment was conducted *in situ* in Miers Valley to assess the natural rate of UHI degradation under Antarctic conditions. This study demonstrated MDV conditions are favourable for preserving UHI, which remains detectable for at least two years. UHI is more likely to be dispersed by Aeolian means than degrade to below detectable levels.

When considering the immediate impact of UHI and the capacity for a site to recover, the current recommendations outlined by the ASMA Management Plan (2015) address the need to minimise footprint at field camps and recommend the use of existing or current field camps to limit the establishment of new camps. It remains unknown whether these recommendations are adequate regarding the long-term management of UHI, especially where sites must be repeatedly visited. Thus, protocols and procedures that limit the release of UHI should be developed and added to the ASMA Management Plan for the MDVs and any other impacted terrestrial area in Antarctica, as a priority by the National Programmes. To prevent the distribution of UHI over a larger area, from both human foot traffic and aeolian dispersal, the evidence from this study suggests that smaller camp locations should be more widely dispersed, whereas larger camps (e.g., Miers Valley) should be routinely maintained in the same location. The appropriate NP should record the GPS coordinates and occupancy at a field camp, in order to facilitate future monitoring if required. A suitable comparison could be made in future with areas earmarked as a baseline for impact, such as an area of the Barwick and Balham Valleys which is designated as an Antarctic Specially Protected Area (ASPA), the highest designated level of environmental protection under the ATS assigned to areas with outstanding environmental, scientific, historic or intrinsic values.

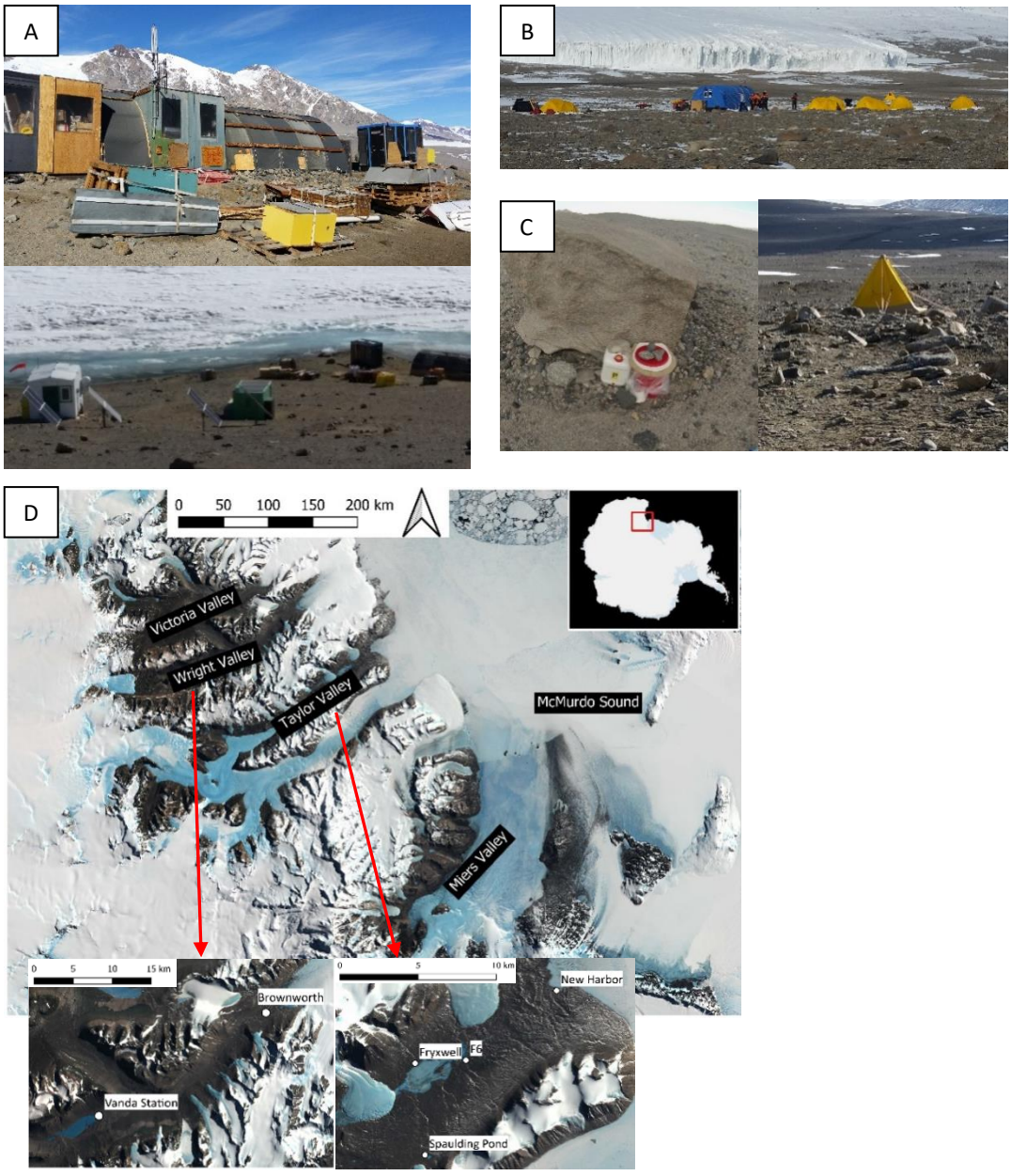


Figure 5.1 Examples of (A) a US research station, Lake Bonney Facilities Zone, with permanent infrastructure and (B) a NZ field camp in Taylor Valley. (C) The toilet area of a NZ camp is either exposed in a small camp (left) or enclosed in a tent (right) in a larger camp. (D) Map of the study sites in the McMurdo Dry Valleys. Multiple camp sites were surveyed in Wright and Taylor Valleys, shown in the inset panels.

Table 5.1 The mitochondrial and faecal TaqMan assays were applied to a range of occupied sites in the MDVs. An estimated distance of detectable mitochondrial signal from a camp site was derived from $y=B_0+B1_x$.*An estimated additional 180.0 human hours from a previous NZ field party, prior to sampling in 2016.

Site	Impact designation	Prevailing summer wind direction	Transect direction	Year Sampled	Person days	Predicted distance of mitochondrial signal (m)	Average mitochondrial signal across all transects (copies μL^{-1})	Average faecal signal across all transects (copies μL^{-1})
NZ Campsites (Hut facilities Zones)								
Victoria Valley	Pristine	SE	S	2016	0.0 (Arrival) 30.0 (Departure)	0.0 94.05	17.04	20.63
Wright Valley, Vanda Hut	Moderate	W	NE NE NW	2016 2017 2017	0.2 145.0 145.0	260.54 173.90 50.04	322.52	3.99
Lower Wright, Brownworth	Moderate	SW/NE	NW NW SE	2017 2017 2017	0.2* (Arrival) 33.0 (Departure) 33.0 (Departure)	92.30 1032.23 76.42	76.21	3.70
Miers Valley	High	S	SW SW SW	2016 2016 2018	8.3 (Arrival) 104.2 (Departure) 0.3 (Recovery)	193.32 182.83 50.09	5665.02	7.12
US Facilities Zones in Taylor Valley								
F-6 Camp	High	NE	SW	2018	Unknown	271.65	249.84	68.40
New Harbor	High	NE	SW	2018	Unknown	189.29	61.62	NA
Lake Fryxell	High	NE	SW NE	2018 2018	Unknown Unknown	258.74 311.87	4198.15	5.99

5.3 Challenges

One of the McMurdo Dry Valley ASMA Management Plan objectives is to improve understanding of environmental processes and human impacts, where knowledge has been identified as incomplete. This includes any long-term effects of recent human impact, such as the effects on microbial communities and their resilience. UHI should be considered as an important component to impact, as it has been predicted to increase with increasing human presence on the continent, especially in the context of global warming, where ice-free areas are projected to increase (Hughes *et al.,* 2018).

This study provides a preliminary insight into spatial patterns of UHI around sites of interest. However, a full investigation of DNA persistence in MDV soil has not been conducted long-term across multiple field seasons. The MDVs experience extreme climatic conditions, including constant low temperatures and high aridity, which promote DNA preservation. Biological activity is known to increase during the austral summer, when heterotrophic bacteria are most metabolically active (Sohm *et al.,* 2020), thus increasing the turnover rate of biological material present in soil. Questions remain around the duration of DNA persistence to quantifiable levels, the environmental and biotic factors, and their interactions that promote persistence of unseen human impact in the MDVs. To better understand detection in relation to patterns of human activity, personal GPS data should be required to produce maps of impact at a site, in conjunction with records of visitation (Antarctica NZ) that provide an estimate of 'human hours' at a site. Also, subsequent dispersal mechanisms should be taken into consideration when assessing spatial patterns of signal detection. Released environmental DNA readily binds to soil, and it can be assumed that aeolian dispersal (e.g., Foehn winds) redistributes UHI from the deposition site. It would also be useful to consider seasonal variations in wind direction to assess where UHI is likely to be redistributed, given a predominant bimodal wind regime in the MDVs.

5.4 Conclusion

This research endeavoured to investigate UHI in the form of detectable DNA disseminated from released human materials on spatial and temporal scales in an MDV setting.

Firstly, UHI was demonstrated to be detectable and quantifiable at Vanda in Wright Valley as a proof of concept. An assay targeting human mtDNA was validated for dPCR and was sufficiently sensitive and specific to be applied to UHI detected as eDNA. UHI detection with this described methodology has shown that the deposition of human materials after routine research operations in the MDVs is occurring, and that human-derived materials are transported a considerable distance from the site of deposition.

Secondly, a broader application of UHI detection was conducted across a wider range of MDV sites, from pristine to highly impacted. This identified human mtDNA (HcytB assay) as the most suitable marker to first identify an impacted site and to then predict the distance of impact. A human-microbial marker would be applicable to identify a specific source of UHI, as demonstrated by faecal detection using a *Bacteroidales* 16s marker (BacHum assay), but was highly localised to an impacted site. UHI was accumulative over a field season, increasing with permanency and occupancy at a site. Broadly, spatial patterns were observed for UHI detection, which were more evident at highly impacted sites, such as the US Facilities Zones, and at moderately impacted sites, such as the large field camp in Miers Valley. As anticipated, the greatest UHI signal was detected closest to a campsite, and attenuated as distance increased, in an exponential decay pattern. Greater UHI signal was detected over a larger distance from the highly impacted sites, compared to the smaller NZ field camps, which lacked permanent infrastructure. A site had the capacity to recover, demonstrated by diminishing UHI detection over time, where UHI levels were compared during a field season and after a year of recovery.

The final component of this research investigated DNA longevity in Miers Valley. The MDV conditions likely facilitated DNA persistence, which remained detectable for at least two years. There was indication of DNA degradation through fragmentation, shown by a greater recovery of smaller DNA fragments of up to 1.5 kb size. Further investigation is required to better elucidate the factors that affect DNA persistence in MDV soil, particularly with regard to moisture availability for heterotroph activity, and the intensity and duration of solar UV radiation. This could be achieved by repeating the DNA longevity experiments in a controlled setting, to quantify the abiotic factors that significantly affect DNA persistence, and as a comparison to the results obtained for the *in situ* experiments conducted in this investigation.

The findings from this research suggest that UHI must be recognised as a source, or at least an indicator, of anthropogenic impact in terrestrial Antarctica. It should be acknowledged in future management recommendations, as it remains detectable after habitation at a site irrespective of field camp size or location. Other Management Plan objectives aim to improve understanding of environmental processes and human impacts in terrestrial Antarctica, along with long-term protection of scientific values and minimising both the accumulative anthropogenic impacts on the environment and the footprint of a field camp. UHI should be thoroughly investigated in keeping with evidence-based policy produced by the Committee for Environmental Protection (CEP) for advising the Antarctic Treaty Consultative Meeting (ATCM), and to determine whether future mitigate is likely required. Evidence-based research is required to determine best practices when

operating in the field and designating protected areas (Hughes and Grant, 2018). As one of the original signatories to the ATS, it is important to maintain NZ's stewardship as kaitiaki over the Ross Dependency, including the MDVs.

Key Events

1959

NZ becomes a signatory to the Antarctic Treaty.

1961

The Antarctic Treaty comes into force and the first Antarctic Treaty Consultative Meeting (ATCM) is held.

1991

The EP signing was agreed upon at the ATCM in Madrid and came into force in 1998. Under the EP, environmental impact assessments are required for all activities to minimise and mitigate any impacts.

1998

The first meeting for the Committee for Environmental Protection (CEP) meeting is held.

2016

On the 25th anniversary of the EP, Antarctic Treaty Parties reaffirmed their obligations to EP objectives at the ATCM held in Santiago through Resolution 6.

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7. Appendix A

Chapter 2: Supplementary figures

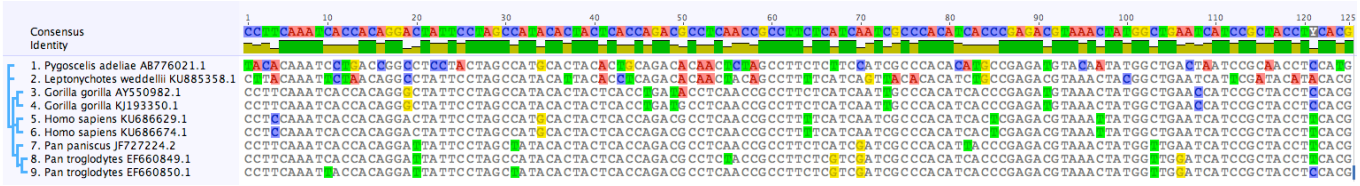

Figure 7.1 Alignment of the HcytB assay region.

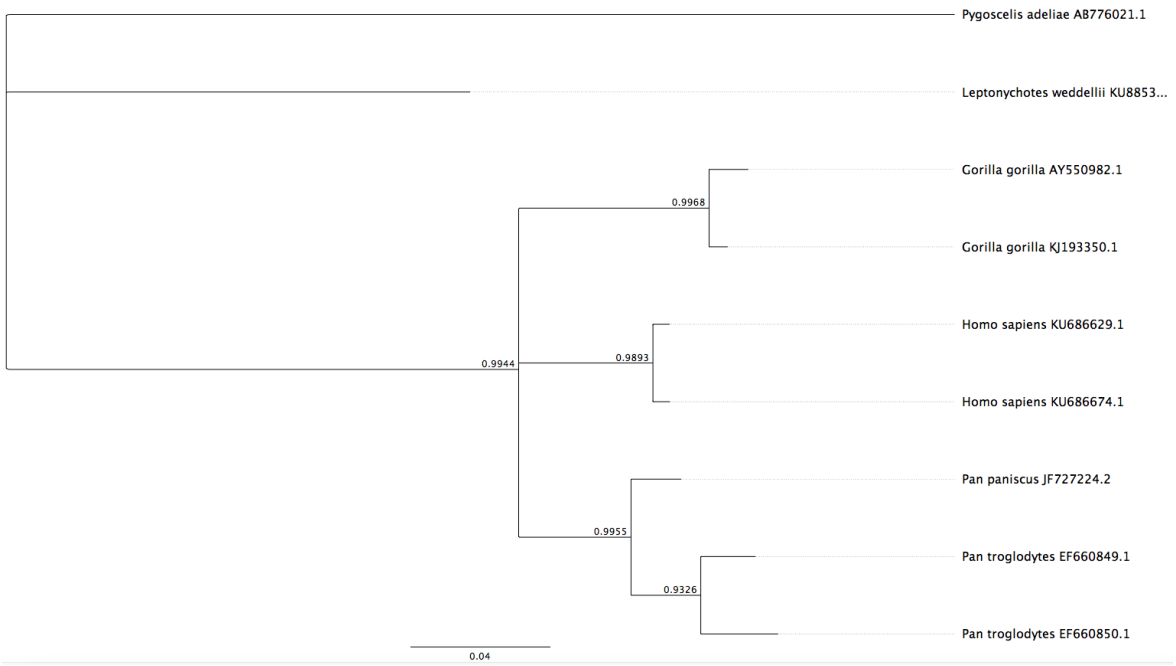

Figure 7.2 Alignment of the HcytB assay region for humans, closely related taxa, and Antarctic fauna. The substitution model JC69 with invariance and gamma was run with MrBayes. The chain length was 5,000,000 chain length; 1,000,000 generations were discarded as burn-in. There were three heated chains with a 0.2 heated chain temperature and one cold chain. Subsampling frequency was set to 2000 and the outgroup was "*P. adeliae*".

8. Appendix B

Chapter 3: Supplementary figures and tables

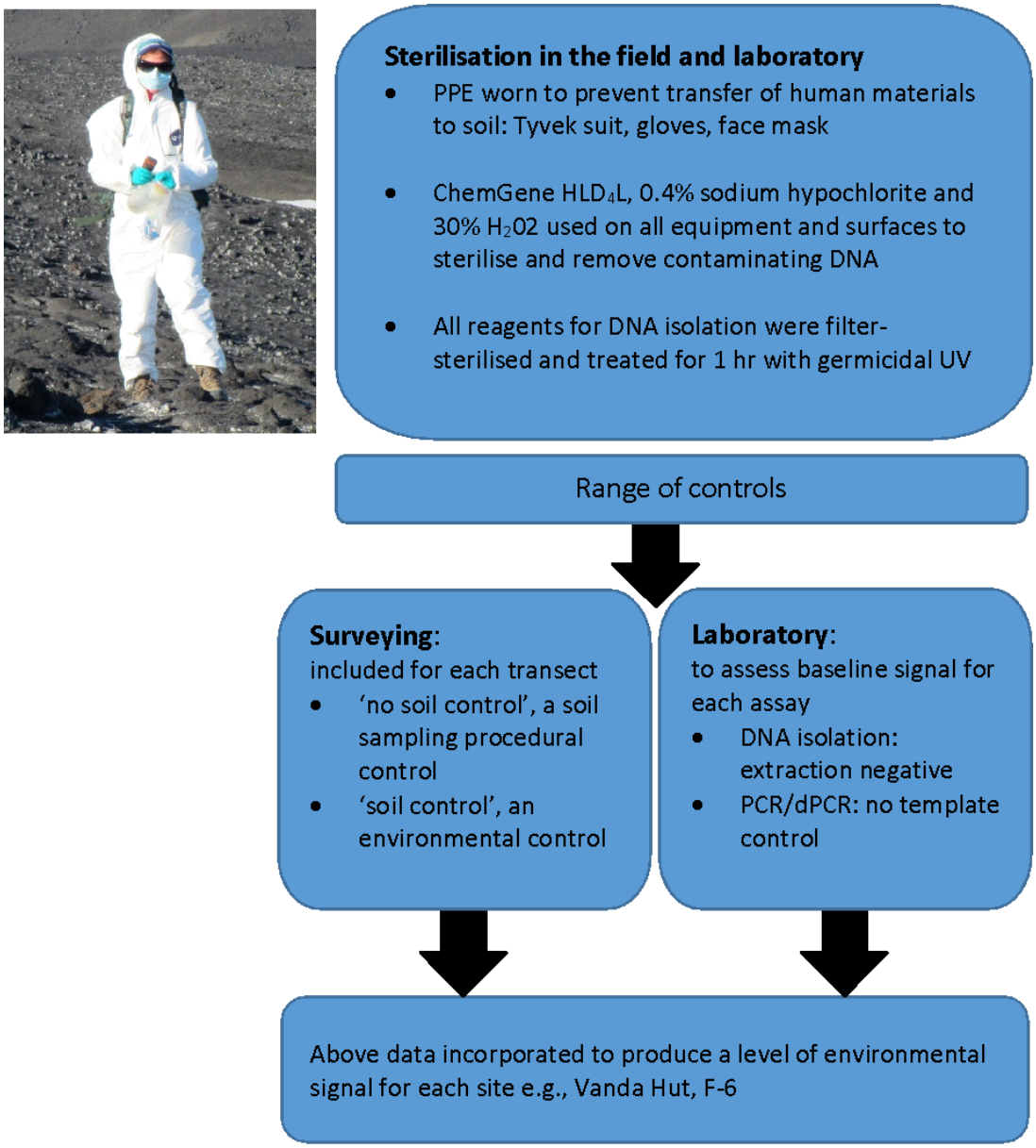

Figure 8.1 An overview of sterilisation and cleanliness precautions in the field and laboratory. A total of four types of controls were included throughout the entire process from field work, to processing samples in the laboratory.

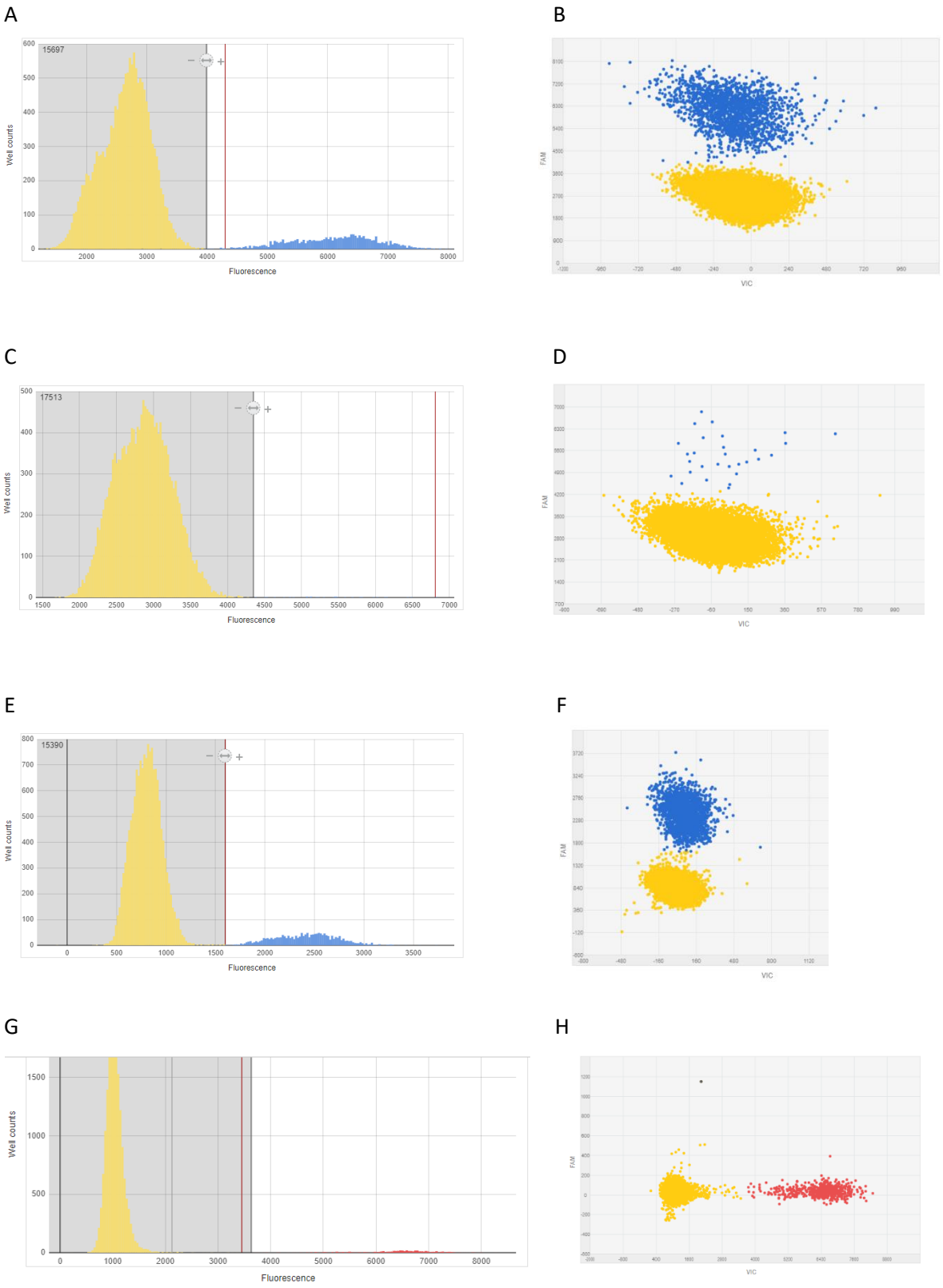

Figure 8.2 An example of the graphical output of dPCR with Quantstudio 3D software. (A) Histogram view of amplified HcytB target labelled with FAM (blue) from an eDNA sample, along with wells with negative amplification (yellow). Here, the threshold can be manually adjusted between the target

and no amplification histograms. (B) The corresponding scatterplot. (C) and (D) are different samples from the same run as (A) and (B), showing a lower target concentration. They illustrate the importance of having positive controls within a dPCR run to accurately establish a threshold, which can be done by overlapping the samples, along with positive and negative controls, in the scatterplot view, such as (B) and (D). (E) and (F) show BacHum target amplified from an eDNA sample. (G) and (H) show Sepi target amplified, labelled with VIC.

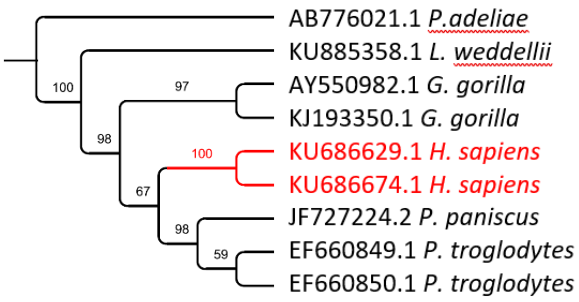

Figure 8.3 Phylogenetic tree (Jukes-Cantor) of an alignment made from HcytB nucleotide sequences targeting human mtDNA. Bootstrapping values are displayed on the nodes.

Figure 8.4 An alignment made from the amplicon (primers trimmed) generated from the BacHum assay using nucleotide sequences obtained from GenBank. The BacHum probe is shown in green

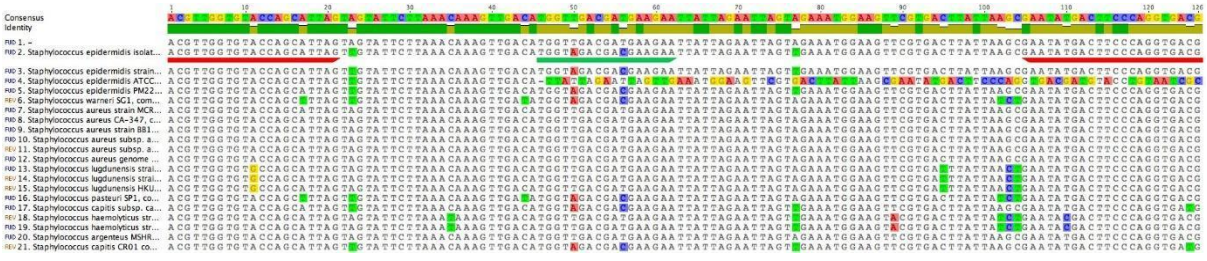

Figure 8.5 An alignment made from the amplicon generated by the Sepi assay using nucleotide sequences obtained from GenBank. The primer binding regions are shown in red, and the probe is shown in green.

(A) (B)

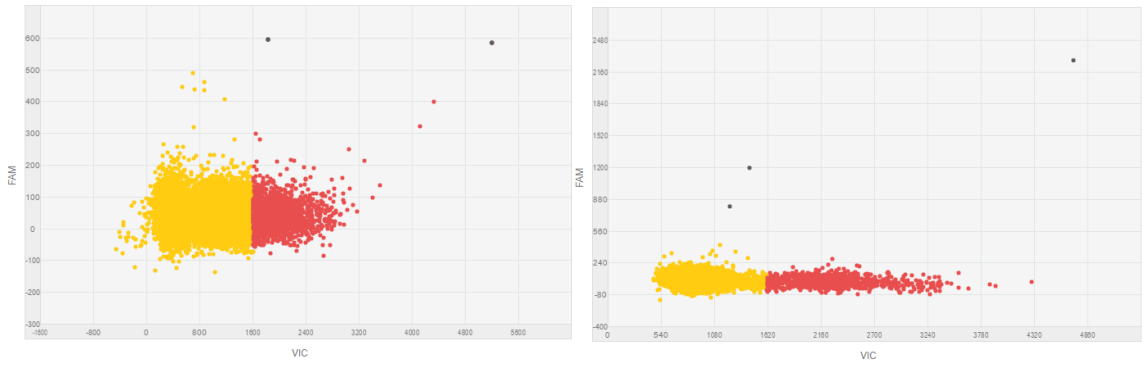

Figure 8.6 True amplification could not be distinguished from no amplification and background signal for the Sepi dPCR assay, demonstrated by a lack of separation for 'amplification' and 'no amplification' scatterplots for (A) 32000 and (B) 3200 DNA molecules of a positive control.

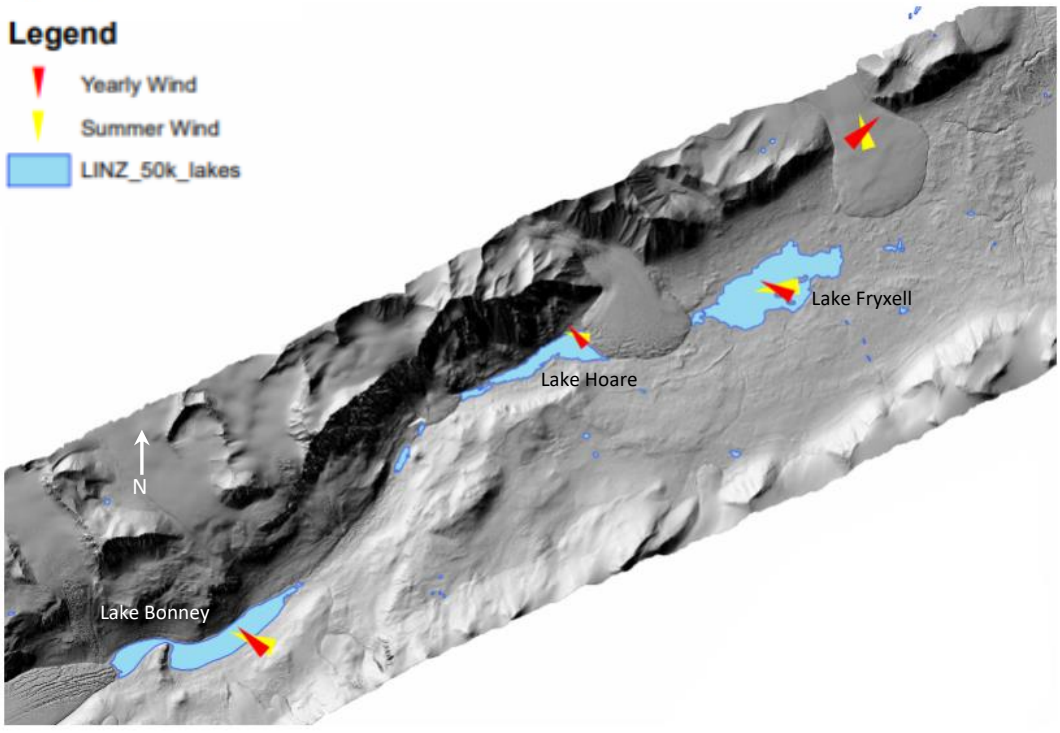

Figure 8.7 Average wind direction across three years (red) and three austral summers (yellow) in Taylor Valley. The data was obtained from LTER, 2015-2016.

Table 8.1 A nucleotide alignment of the primer and probe binding regions 5'-3' for the BacHum assay, with primer and probe sequences shown in the first row.

Name	Forward Primer	Probe	Reverse Primer
BacHum Assay	**CGCGGTAATACGGAGGATCC**	**AAGTTTGCGGCTCAAC**	**CGCTACACCACGAATTCCG**
***B. dorei*, CP072246**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LC515579**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR699004**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR999535**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR999538**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR999552**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR999559**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR999582**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR999585**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, LR999587**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, MN055948**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, MT464306**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, MT464394**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***B. dorei*, MT749285**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***P. dorei*, CP046176**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***P. dorei*, CP046424**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***P. dorei*, CP046425**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***P. dorei*, CP046426**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***P. dorei*, CP046427**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***P. dorei*, CP046428**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
***P. dorei*, MT902966**	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
Uncultured, MH515535	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
Uncultured, MH515536	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
Uncultured, MH515537	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
Uncultured, MH515538	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG
Uncultured, MH515539	CGCGGTAATACGGAGGATCC	AAGTTTGCGGCTCAAC	CGGAATTCGTGGTGTAGCG

Table 8.2 A nucleotide alignment of primer and probe binding regions for the Sepi assay for target and related *Staphylococci* with GenBank accession numbers. The probe binds without mismatches to *S. epidermidis*.

Name	Forward Primer	Probe	Reverse Primer
Sepi Assay	**ACGTTGGTGTACCAGCWTTAG**	**ACATGGTAGACGACGAAGA**	**CGTCACCTGGGAAGTCATATTC**
***S. argenteus*, NZ_CP015758.1**	ACGTTGGTGTACCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. arlettae*, NZ_UGZE01000001.1**	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. aureus*, NC_007795.1**	ACGTTGGTGTACCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. caeli*, NZ_FMPG01000001.1**	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. caprae*, NZ_AP018587.1**	ACGTTGGTGTACCAGCATTAG	ACATGGTTGACGACGAAGA	CGTCACCTGGGAAGTCATATTC
***S. cohnii*, NZ_WIVH01000006.1**	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. epidermidis*, NZ_CP035288.1**	ACGTTGGTGTACCAGCATTAG	ACATGGTAGACGACGAAGA	CGTCACCTGGGAAGTCATATTC
***S. haemolyticus*, NZ_CP013911.1**	ACGTTGGTGTACCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. hominis*, NZ_CP033732.1**	ACGTTGGTGTACCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. lugdunensis*, NZ_CP014022.1**	ACGTTGGTGTGCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
S. nepalensis, NZ_UHDS01000001.1	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. pasteuri*, NC_022737.1**	ACGTTGGTGTACCAGCTTTAG	ATATGGTAGACGACGAAGA	CGTCACCTGGGAAGTCATATTC
***S. pseudoxylosus*, NZ_JABXHL010000002.1**	ACGTTGGTGTACCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. saprophyticus*, NZ_LR134089.1**	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. succinus*, NZ_CP018199.1**	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. ureilyticus*, NZ_JAFFJY010000001.1**	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC
***S. warneri*, NZ_LR134242.1**	ACGTTGGTGTACCAGCTTTAG	ATATGGTAGACGACGAAGA	CGTCACCTGGGAAGTCATATTC
***S. xylosus*, NZ_CP008724.1**	ACGTTGGTGTTCCAGCATTAG	ACATGGTTGACGATGAAGA	CGTCACCTGGGAAGTCATATTC

Table 8.3 The background level of signal was calculated for each assay at each site from a range of controls included during surveying and laboratory handling. The mean signal for mitochondrial and faecal signal at each site at departure were determined from data generated from the QuantStudio software. 'NS' denotes not sampled.

Location	Background level of signal, Log_{10} (1+ copies μL^{-1})		Mean signal across all transects, copies μL^{-1}	Mean signal across all transects, copies μL^{-1}
	mtHum	BacHum	Mitochondrial	Faecal
Victoria Valley	0.407	0.188	17.04	20.63
Miers Valley	0.300	0.175	5665.02	7.12
Brownworth	0.226	0.283	76.21	3.70
Vanda Hut	0.345	0.579	322.52	3.99
F6	0.366	0.300	249.84	68.40
New Harbor	0.332	NS	61.62	NS
Fryxell	0.318	0.318	4198.15	5.99

Table 8.4 Linear regression for all transects for both the HcytB and BacHum assays. A total of 14 transects out of 29 had statistically significant ($p \leq 0.05$) B_0 and B_1.

Transect	Linear regression model	Intercept (b_0)	b_0 p-value	95% C. I.	Regression (b_1)	B_1 p-value	95% C. I.	Distance from site where signal diminishes to background level (m)	Person days	Transect direction
Victoria Valley										
HcytB (T0)	Y=0.37271 + -0.08729x	0.37	2.18E-05*	0.26-0.48	-0.09	1.33E-02*	-0.15- -0.02	-0.60	0.00	S
HcytB (TD)	Y=0.6313 + -0.1134x	0.63	5.53E-03*	0.23-1.03	-0.11	3.02E-01	-0.35-0.12	94.05	30.00	S
BacHum (TD)	Y=0.74269 + -0.05607x	0.74	7.53E-3*	0.25-1.24	-0.06	6.74E-01	-0.34-0.23	$7.81x10^9$	30.00	S
Wright Valley, Vanda Hut Facilities Zone										
1, HcytB (2017)	Y=3.0999+ -1.4911x	3.10	4.23E-04*	1.93-4.27	-1.49	8.15E-03*	-2.46- -0.53	384.01	145.00	NNE
2, HcytB (2017)	Y=2.6905+ -1.0458x	2.69	3.4E-04*	1.71-3.67	-1.05	1.83E-02*	-1.85- -0.24	173.90	145.00	NE
3, HcytB (2017)	Y=1.3935+ -0.6139x	1.39	3.83E-04*	0.88-1.91	-0.61	1.14E-02*	-1.04- -0.19	50.04	145.00	NW
2, HcytB (2016)	Y=2.3414+ -0.8258x	2.34	4.03E-04*	1.46-3.22	-0.83	3.06E-02*	-1.55- -0.10	260.54	0.17	NE
2, BacHum (2016)	Y=1.1102+ -0.2918x	1.11	7.59E-04*	0.65-1.57	-0.29	1.13E-01	-0.67-0.09	65.13	0.17	NE
Wright Valley, Brownworth										
1, HcytB (TD)	Y=2.4029 + -1.1523x	2.40	9.64E-04*	1.36-3.45	-1.15	1.57E-02*	-2.01- -0.29	76.42	33.00	SE
1, HcytB (T0)	Y=0.5338+ -0.1151x	0.53	0.1.4E-02*	0.15-0.92	-0.12	4.23E-01	-0.43-0.20	471.28	0.21	SE
2, HcytB (TD)	Y=0.5377+ 0.3724x	0.54	2.36E-01	-0.44-1.52	0.37	3.12E-01	-0.44-1.18	5.87	33.00	NE
3, HcytB (TD)	Y=2.3278+ -0.6973x	2.33	1.08E-04*	1.62-3.03	-0.70	2.53E-02*	-1.28- -0.12	1032.23	33.00	NW
3, HcytB (T0)	Y=1.4696+ -0.6313x	1.47	1.48E-04*	1.00-1.94	-0.63	6.16E-03*	-1.02- -0.25	92.30	0.21	NW
4, HcytB (TD)	Y=0.9093+ -0.1297x	0.91	1.98E-02*	0.19-1.63	-0.13	6.19E-01	-0.72-0.46	$1.85x10^5$	33.00	SW
1, BacHum (T0)	Y=0.44156 + 0.02465x	0.44	2.86E-02*	0.06-0.82	0.02	8.59E-01	-0.29-0.34	-1.00	0.21	SE
1, BacHum(TD)	Y=0.1826 + 0.1740x	0.18	3.76E-01	-0.27-0.64	0.17	3.10E-01	-0.20-0.55	-0.74	33.00	SE
3, BacHum (TD)	Y=0.4325+ 0.1073x	0.43	1.28E-01	-0.16-1.03	0.11	6.19E-01	-0.38-0.60	-0.96	33.00	NW
Miers Valley										
HcytB (T0)	Y=1.658+ -0.5934x	1.66	6.81E-04*	0.89-2.42	-0.59	1.38E-02*	-1.04- -0.15	193.32	8.33	SW
HcytB (TD)	Y=3.2267+ -1.2923x	3.23	2.23E-05*	1.85-4.60	-1.29	4.52E-04*	-2.09- -0.49	182.83	104.17	SW

Transect	Linear regression model	Intercept (b_0)	b_0 p-value	95% C. I.	Regression (b_1)	B_1 p-value	95% C. I.	Distance from site where signal diminishes to background level (m)	Person days	Transect direction
HcytB (recovery)	Y=2.010201 7+ -1.0014x	2.01	1.43E-04*	1.43-2.59	-1.00	4.29E-03*	-1.55- -0.45	50.09	0.25	SW
BacHum (TD)	Y=0.55768+ -0.06287x	0.56	8.03E-02	-0.08-1.20	-0.06	7.10E-01	-0.44-0.31	$1.22x10^6$	104.17	SW
BacHum (recovery)	Y=0.5069+ -0.3275x	0.51	0.037*	0.04-0.97	-0.33	1.20E-01	-0.77-0.11	9.31	0.25	SW
Taylor Valley, F6 Facilities Zone										
HcytB	Y=3.07089+ -1.11056x	3.07	1.84E-09*	2.78-3.36	-1.11	7.41e-08*	-1.27- -0.95	271.65	Unknown	SW
HcytB	Y=2.7284+ -0.5986x	2.73	5.64E-04*	1.54-3.92	-0.60	6.80E-02	-1.25-0.05	8840.83	Unknown	NE
BacHum	Y=1.0639+ -0.1225x	1.06	6.52E-03*	0.38-1.75	-0.12	4.80E-01	-0.50-0.25	$1.72x10^6$	Unknown	SW
Taylor Valley, New Harbor Facilities Zone										
HcytB	Y=1.5524+ -0.5354x	1.55	5.85E-03*	0.57-2.53	-0.54	5.10E-02	-1.07-0.00	189.29	Unknown	SW
Taylor Valley, Fryxell Facilities Zone										
HcytB	Y=3.7860+ -1.4363x	3.79	1.06E-05*	2.81-4.76	-1.44	1.92E-04*	-1.97-0.90	258.74	Unknown	SW
HcytB	Y=3.6538+ -1.3368x	3.65	1.64E-05*	2.66-4.65	-1.34	3.69E-04*	-1.88- -0.79	311.87	Unknown	NE
BacHum	Y=1.0135+ -0.2346x	1.01	2.47E-03*	0.46-1.57	-0.23	1.14E-01	-0.54-0.07	920.78	Unknown	SW