Differentiating the Temperature Response of Soil Fungi and Bacteria

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Master of Science (Research) in Environmental Sciences

at

The University of Waikato

by

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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2022

Abstract

The decomposition of soil organic matter (SOM) may be accelerated under increasing temperatures from climate change, which could create feedback loops that result in the loss of soil carbon (C) stocks. Fungi and bacteria regulate the decomposition of SOM, each having their own unique adaptations that allow them to fulfill different roles in the decomposition process. However, fungi and bacteria may respond to temperature differently which could complicate soil C cycling dynamics. To date, few studies have attempted to differentiate the temperature response of fungal and bacterial respiration.

The aim of this research was to differentiate the temperature optima (T_{opt}) and inflection point temperatures (T_{inf}) of fungal and bacterial respiration. The T_{opt} is the temperature at which respiration is maximal whereas the T_{inf} is the point at which respiration is most sensitive to temperature.

Soils were collected from different locations along a geothermal temperature gradient (that had average temperatures between ~13-39 °C). The selective inhibition method was used to differentiate the temperature response of fungal and bacterial respiration by treating soils with streptomycin and cycloheximide, respectively. Soils were also treated with and without glucose to determine the non-substrate limited respiration response of fungal and bacterial respiration. Treated soils were incubated in a temperature block that allowed the incubation of soils at temperatures ranging between ~5-52 °C for five hours. Headspace samples were taken from each treatment tube and injected into an infrared gas analyser (IRGA) to measure carbon dioxide (CO_2) concentrations and respiration rates. Macromolecular rate theory (MMRT) was used to model the temperature response of the respiration curves to derive the T_{opt} and T_{inf} of fungal and bacterial respiration, allowing the comparison of the temperature responses of fungal and bacterial respiration.

An additional aim of this research was to measure changes in fungal and bacterial biomass along the geothermal temperature gradient using phospholipid fatty acid (PLFA) analysis. Soil samples were collected from the same sampling locations along the geothermal temperature gradient as those used in the selective inhibition experiments. A modified Bligh and Dyer method was used to extract phospholipids from soil samples, which were used as biomarkers to differentiate the biomass of different fungal and bacterial groups.

Results from selective inhibition experiments showed that respiration rates decreased from streptomycin and cycloheximide treatment, allowing an estimation of fungal and bacterial contributions to total soil respiration, respectively. Fungal contributions to soil respiration (~55%) were greater than bacterial contributions (~21%), suggesting that fungi contribute more to soil C decomposition than bacteria. Furthermore, the T_{opt} of fungal respiration was found to decrease with environmental temperature at a rate of -0.396 °C °C^{-1} whereas the T_{opt} of bacteria and the microbial community was not correlated with environmental temperature. Additionally, the T_{opt} and T_{inf} of fungal respiration (38.6 °C and 22.1 °C, respectively) was not significantly different from the T_{opt} and T_{inf} of microbial respiration (37.0 °C and 21.7 °C, respectively) but was significantly greater than the T_{opt} and T_{inf} of bacterial respiration (30.4 °C and 19.0 °C, respectively). Results from PLFA analysis showed that gram-positive bacteria and general bacteria dominated total microbial biomass and biomass from identified fungal and bacterial groups peaked at ~19-21 °C. Fungal-to-bacterial (F:B) ratios decreased with increasing environmental temperature, indicating a proportional decrease in fungal biomass relative to bacteria at higher temperatures. These results suggest that fungi were excluded with increasing environmental temperature as they were unable to adapt their metabolism and growth as environmental temperatures increased.

The selective inhibition method presented a range of limitations that may have constrained interpretation of the results. First, inhibitors were not equally effective across the range of incubation temperatures. Inhibition from cycloheximide showed a clear temperature optimum where inhibition was greatest within ~45-50 °C. In contrast, inhibition from streptomycin declined at temperatures above ~30 °C. Secondly, percent inhibition of microbial respiration from streptomycin increased as total bacterial biomass increased, providing evidence that streptomycin was effective in targeting bacteria. In contrast, percent inhibition of microbial respiration from cycloheximide was not correlated with fungal biomass, indicating that cycloheximide may not have been effective in selectively targeting fungi.

Overall, these results indicate that fungi may be more at risk from increasing temperatures under climate change, though it is unknown as to how this could translate to soil C stocks. This was likely the first study that attempted to differentiate the temperature response of fungi and bacteria by selectively inhibiting fungi and bacteria and incubating them across a range of temperatures. However, caution should be taken when interpreting results as a

range of methodological constraints were uncovered regarding the veracity of the selective inhibition method. While previous studies have reported some limitations of the selective inhibition method, these issues have likely been under-reported as researchers may not publish inconclusive or negative results. Therefore, this study also highlights the importance of critically assessing methods that may be ineffectual.

Acknowledgements

This thesis is the product of a lot of time and work from myself and various different people. I would like to acknowledge some of the people who have helped in the completion of this thesis or have otherwise provided support during this thesis.

I would first like to thank Professor Louis Schipper for giving me the opportunity for this thesis and supporting me along the way as my supervisor. I do not think I could have asked for a better supervisor. I am deeply grateful for all the time and effort you have invested into me and the success of this thesis, from letting me barge into your office to ask the simplest of questions, to reading over the final drafts of the thesis, and everything in between. I also appreciate your patient, calm, reassuring nature among my many frustrated moments when everything was going wrong. I am honoured to have been your student, and I know the valuable lessons and skills I have learnt under you will prove useful in the future.

I am very appreciative of Charlotte Alster for her insightful advice and feedback throughout this thesis. I appreciate your kind nature and the knowledge you have shared that helped make this thesis successful. I am also appreciative of Allycia van de Laar for all she has done, from showing me the field and lab methods to having lunch with me and listening to my various inane ramblings. I appreciate your caring, helpful nature, even when answering my random questions afterhours.

Thank you to Holly Harvey-Wishart and Dani McMonagle for helping me get set up in the lab and making the soils lab a warm, inviting environment. I would also like to thank Kerry Smith and Graeme Pullen at Arikikapakapa Golf Rotorua for giving me the permission and freedom to access the field site.

I would also like to extend my appreciation to Darby Moyle and Julie Deslippe at the Victoria University of Wellington for their help with the phospholipid fatty acid section of this thesis. I greatly appreciate your hospitality while I was down there, your enthusiasm for my work, and helping make my visit a very positive, memorable experience. Thank you, Darby, for helping me with the lab work and making it a very stress-free experience.

I would like to thank the WaiBER research group for welcoming me into the group, I look forward to working in the group in the future. In particular, I would like to thank Aaron Wall for the advice he gave me throughout this thesis and for giving me the opportunity to get out of the office to do some fieldwork when I needed it most. I would also like to give a big thank you to Jordan Goodrich for helping me with the MMRT code. I would also like to thank Tsitsi Chiwetu for listening to my sudden frustrated rants.

I am very grateful to have received funding from the New Zealand Agricultural Greenhouse Gas Research Centre and the Marsden Council. I am also thankful to have received the University of Waikato Masters Research Scholarship.

Finally, I would like to thank my mum, dad, and grandmother for dealing with me throughout this thesis, even during my moody moments. I appreciate your unconditional support over the last few years, even when you may not have been legally obliged to. I would also like to thank my brother Calum for helping me get my mind off my thesis when necessary by motivating me with music stuff.

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Chapter 1

Introduction

1.1 Background

The movement of carbon (C) between different earth reservoirs is essential for life on earth (Houghton, 2003). The largest quantity of terrestrial carbon (C) is stored in the soil and can be separated into different functional pools in many different ways (Jobbágy & Jackson, 2000; Lavallee et al., 2020). One way soil C can be conceptually partitioned is into two fractions: particulate organic matter (POM) and mineral-associated organic matter (MAOM). Particulate organic matter consists of organic fragments that have not undergone much decomposition or chemical transformation (e.g., fresh litter that has been fragmented). This fraction tends to be more readily available but can vary much more in quality. In contrast, MAOM generally consists of smaller, simpler organic compounds that have been chemically transformed by soil biota and adsorbed onto mineral surfaces, providing physical protection (Lavallee et al., 2020). Photoautotrophs, such as plants, contribute a large portion of MAOM and POM to soil in the form of root exudates and dead plant structures (Kuzyakov & Domanski, 2000; Janzen, 2004; Lavallee et al., 2020). In addition, microorganisms can contribute MAOM to soil as low molecular weight compounds such as sugars and organic acids. Fungi also contribute complex compounds, such as chitin, to soil (Lavallee et al., 2020). Heterotrophs, such as fungi and bacteria, are largely responsible for the decomposition of these organic fragments, releasing CO_2 upon respiration (Janzen, 2004). These inputs of organic matter to soil and subsequent release of CO_2 from soil drives soil C cycling.

Fungal and bacterial niches overlap to some degree, sharing similar roles in soil (Rousk et al., 2008), though they each have specialised roles. For example, fungi tend to produce a wider range of extracellular enzymes that allows them to degrade more complex POM, such as cellulose and lignin (Xu & Shang, 2016; Khatoon et al., 2017). In addition, they can also weather some minerals to release mineral-bound compounds (Jilling et al., 2018). In contrast, bacteria are generally thought to be more responsible for degrading simpler, labile compounds (Hunt et al., 1987; Khatoon et al., 2017; Lavallee et al., 2020). Further research is required to better understand the relative roles of fungi and bacteria in C cycling, particularly as climate changes (see more details in Chapter 2).

The roles of fungi and bacteria in soil have been differentiated using a range of methods, such as the selective inhibition method. In this method, soils are treated with a fungicide or a bactericide to reduce fungal and bacterial respiration. Substrate, such as glucose, is subsequently added to accelerate metabolic activity among remaining metabolically active cells. Fungal and bacterial respiration can then be quantified by measuring CO_2 accumulation from the treated and untreated soils. Rates of CO_2 production can be compared between treated and untreated soils, allowing a partial estimation of fungal and bacterial contributions to total substrate-induced soil respiration, highlighting their role in soil C cycling (Anderson & Domsch, 1973a; Anderson & Domsch, 1973b). Selective inhibition studies often find that fungal contributions to soil respiration are greater than that of bacteria, suggesting that fungi may contribute more to soil organic matter decomposition (Anderson & Domsch, 1973a; Bailey et al., 2002; Ananyeva et al., 2006). There are known limitations to this method where inhibitors may not be completely selective, resulting in incomplete inhibition of target organisms or inhibition of non-target organisms, leading to inaccurate estimations of fungal and bacterial contributions (Beare et al., 1990; Velvis, 1997). Resistant microorganisms may also utilise the inhibitors as a substrate, thereby stimulating respiration and potentially confounding results (Parkinson et al., 1971; Hu et al., 1997; Nakamoto & Wakahara, 2004). While these limitations are noted, these inhibition approaches have contributed valuable information on how fungi and bacteria contribute to soil C cycling (Rousk et al., 2009a; Chen et al., 2014).

The selective inhibition method is useful in estimating fungal and bacterial respiration, but biomass is also important in understanding the role of fungi and bacteria in soil. Phospholipid fatty acid (PLFA) analysis has previously been used to differentiate fungal and bacterial biomass in soil systems (Willers et al., 2015). Microbial cell membranes contain phospholipids that differ between microbial groups and has been used as a proxy of biomass of different fungal and bacterial groups (Willers et al., 2015). Consequently, phospholipids can be useful as an indicator of soil microbial community composition, particularly when expressed as a ratio of fungi to bacteria (Frostegård & Bååth, 1996; Willers et al., 2015). Both selective inhibition and PLFA analysis have proven to be useful in differentiating fungi and bacteria in soil (Chen et al., 2014; Willers et al., 2015; Swallow & Quideau, 2020).

Fungi and bacteria share similar, but also distinct, roles in mediating the flow of C into and out of soil. Soil warming under climate change could affect fungal and bacterial

activity, thereby altering soil C cycling dynamics (Davidson & Janssens, 2006). As such, understanding the temperature sensitivity of soil fungi and bacteria is important in predicting changes in soil C cycling under climate change. In the context of soil respiration, temperature sensitivity is the degree of change in respiration rate with temperature (Robinson et al., 2017). Previously, the Arrhenius equation and its derivatives (e.g., Lloyd & Taylor, 1994; Fang & Moncrieff, 2001) have frequently been used to characterise the temperature sensitivity of soil respiration. Despite its success in modelling the temperature sensitivity of chemical reactions, many of the existing equations do not fully capture the temperature response of biochemical reactions. Arrhenius-like equations predict a continuous exponential increase in reaction rate with temperature. In reality, biochemical reactions display a temperature optima (T_{opt}), or a temperature at which respiration is maximal. Macromolecular rate theory (MMRT) was developed to more accurately model reactions catalyzed by macromolecules such as enzymes (Hobbs et al., 2013). Macromolecular rate theory recognises the initial exponential increase in respiration, but curves to fit the T_{opt}. The subsequent decrease in respiration beyond the T_{opt} has previously been associated with enzyme denaturation. However, it has previously been found that this curvature is instead due to changes in enzyme heat capacity ($\Delta C_p^{\ddagger}$) as enzyme denaturation tends not to occur until much higher temperatures (Hobbs et al., 2013). In addition to the T_{opt}, MMRT also calculates the T_{inf}, or the temperature at which the respiration sensitivity to temperature is greatest (Robinson et al., 2017). Both the T_{opt} and T_{inf} have proven to be useful in describing the temperature sensitivity of respiration across soils of different moisture contents and soil types as well as geothermally warmed soils (Schipper et al., 2014; Robinson et al., 2017; Robinson et al., 2020; Numa et al., 2021; Van de Laar, 2021). Though MMRT has proven to be useful in characterizing the response of respiration to temperature, more research is required in understanding how microbial communities (e.g., fungal and bacterial groups) will respond to increasing temperatures under climate change.

The thermal adaptation of soil microbial communities in response to increasing soil temperatures from climate change is not fully understood (Bradford, 2013), let alone potentially differential thermal adaptation of fungi and bacteria, independent of one another. Thermal adaptation can be defined as the change in metabolic activity (such as respiration) in response to temperature (Bradford et al., 2008), which might include shifts in T_{opt} and T_{inf}. Though fungi and bacteria have been reported to adapt to increasing temperature (Bárcenas-Moreno et al., 2009; Stefansson et al., 2013), bacteria are thought

to be generally more capable of withstanding higher temperatures (Griffin, 1985; Casadevall et al, 2019). However, how these microbial adaptations then translate to soil C cycling remains uncertain. Temperature gradients may be of use for studying thermal adaptation in microorganisms. Previously, elevational temperature gradients (Wang et al., 2012), latitudinal temperature gradients (Dacal et al., 2019), and temperature gradients that occur across biomes, such as from natural to artificially managed systems have been used study thermal adaptation (Bradford et al., 2019). In addition, geothermal sites can produce a modest range of temperatures (e.g., 16 – 35 °C) across a relatively short distance with hotter temperatures closer to the geothermal source and cooler temperatures further away from the source. There is potential for these geothermal temperature gradients to be used as a proxy for soil warming to study thermal adaptation. A study conducted by Van de Laar (2021) used geothermal temperature gradients to study thermal adaptation of the whole microbial community but did not separate fungi and bacteria. The differential thermal adaptation of fungi and bacteria remains a relatively unexplored area of research that could be beneficial in further understanding how soil microbial communities and soil C cycling dynamics are affected by climate change.

1.2 Aims and objectives

The primary objective of this thesis was to differentiate the temperature responses of fungal and bacterial respiration. This was achieved by applying the selective inhibition method to soils collected from a geothermal temperature gradient and modelling the responses using MMRT. A secondary objective was to examine changes in microbial communities along the geothermal temperature gradient, specifically using fungal and bacterial biomass determined by PLFA analysis.

Overall, this thesis aimed to address two main questions:

1. Does the temperature response (e.g., T_{opt} and T_{inf}) of fungal respiration differ from that of bacterial respiration?
2. Does fungal and bacterial respiration and biomass show signs of thermal adaptation along a geothermal temperature gradient?

1.3 Thesis outline

Following this introduction, there are four further chapters:

Chapter 2 is a literature review that covers key concepts and background information relevant to the topic of the thesis. This has particular focus on carbon cycling and the role and significance of microorganisms, with emphasis on distinguishing fungi and bacteria, in soil environments. A review of the literature surrounding the temperature sensitivity of soil respiration and thermal adaptation among microbial communities is also presented.

Chapter 3 presents a description of the geothermal site that was used as the study site for assessing thermal adaptation among fungi and bacteria. Subsequently, this section presents a detailed description of the methods used to measure bacterial and fungal respiration and biomass. This also includes preliminary experiments undertaken during the development of the final experimental protocol.

Chapter 4 presents the results and discussion of the research written in the style of a journal article, including a more succinct version of the site description and laboratory methods.

Chapter 5 summarises the main conclusions and recommendations that can be drawn from this research. Included in this section are propositions for future research.

Chapter 2

Literature Review

2.1 Global carbon cycling

Carbon (C) is essential for life on earth as it is the atomic skeleton that allows organic molecules to form (Pace, 2001; Houghton, 2003). The storage and flow of C occurs between four primary earth reservoirs: the atmosphere, biosphere, hydrosphere, and lithosphere (Carlson et al., 2001). The pedosphere acts as an interface between these aforementioned reservoirs that allows soil to exist and soil formation processes to proceed (Laceby et al., 2019; Mikhailova et al., 2020). The flow of C between these reservoirs is bridged by a series of different processes, including biological processes, such as respiration and photosynthesis, as well as anthropogenic processes, such as fossil fuel combustion and land use activity (Janzen, 2004). Soil is the greatest terrestrial store of organic C, containing more C than C stored in plants and the atmosphere combined (Jobbágy & Jackson, 2000). A simplified diagram of the global carbon cycle is shown below (**Figure 2.1**). Cycling of C between soil and the atmosphere will be focussed on throughout subsequent sections, as it is the interaction that is most relevant to this thesis.

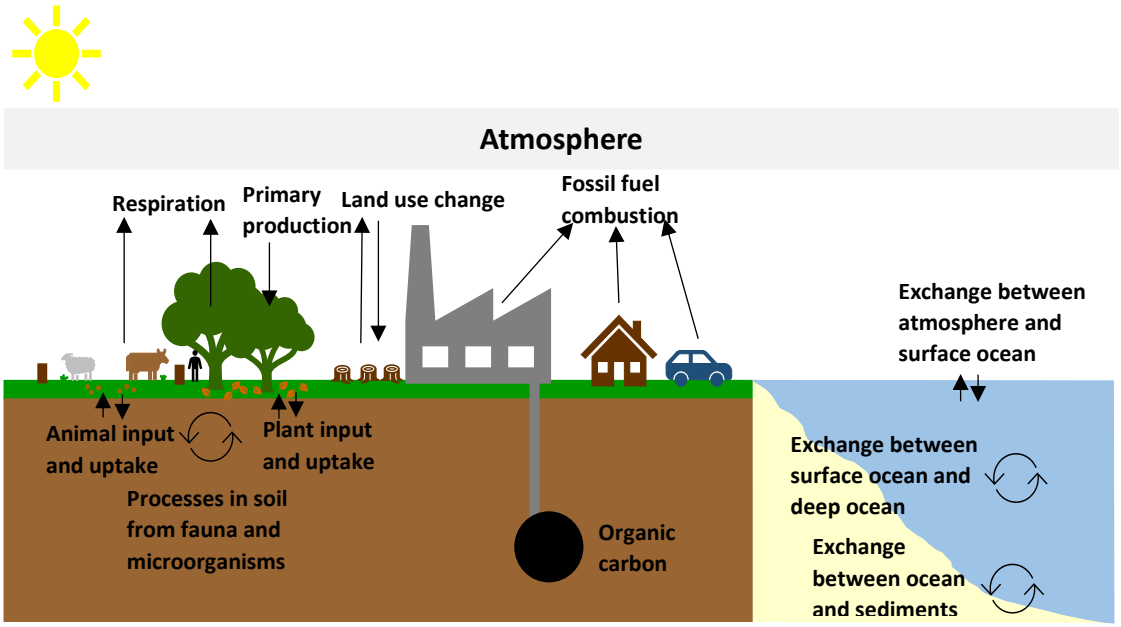


Figure 2.1 Simplified depiction of the main processes involved in the global carbon cycle. Arrows represent movement of CO_2 or organic matter between different reservoirs. Diagram adapted from Carlson et al. (2001) and Hannah (2011).

This literature review will discuss the storage and flow of C into and out of soil, particularly in the context of climate change. The roles of different organisms involved

in C cycling will be discussed, with particular focus on the relative roles of fungi and bacteria in soil systems and methods used to differentiate them. The temperature response of microbial respiration will be discussed, including models used to characterise the temperature sensitivity of microbial respiration and an overview of the relatively novel macromolecular rate theory (MMRT) model. The literature surrounding thermal adaptation of microorganisms will be covered, including how fungi and bacteria may adapt to increasing temperatures under climate change. Finally, this literature review will conclude by exploring potential avenues for future research.

2.1.1 Anthropogenic influence on climate

Greenhouse gases are radiatively active gases that are naturally present in the Earth's atmosphere (Jain, 1993). Atmospheric greenhouse gases, such as carbon dioxide (CO_2), nitrous oxide (N_2O), and methane (CH_4), absorb solar radiation and infrared radiation and reradiate this energy in all directions. Radiation redirected towards the Earth's surface causes a warming effect, known as the greenhouse effect, which is essential for keeping the Earth at a temperature that is hospitable for life (**Figure 2.2**) (Jain, 1993; Cassia et al., 2018). Greenhouse gases, such as CO_2, exist naturally at trace concentrations in the atmosphere and are produced through biological processes such as respiration and, for some greenhouse gases, geological processes such as rock formation and volcanic processes (IPCC, 1992). Carbon dioxide is most relevant to this thesis and will be the main focus of this literature review.

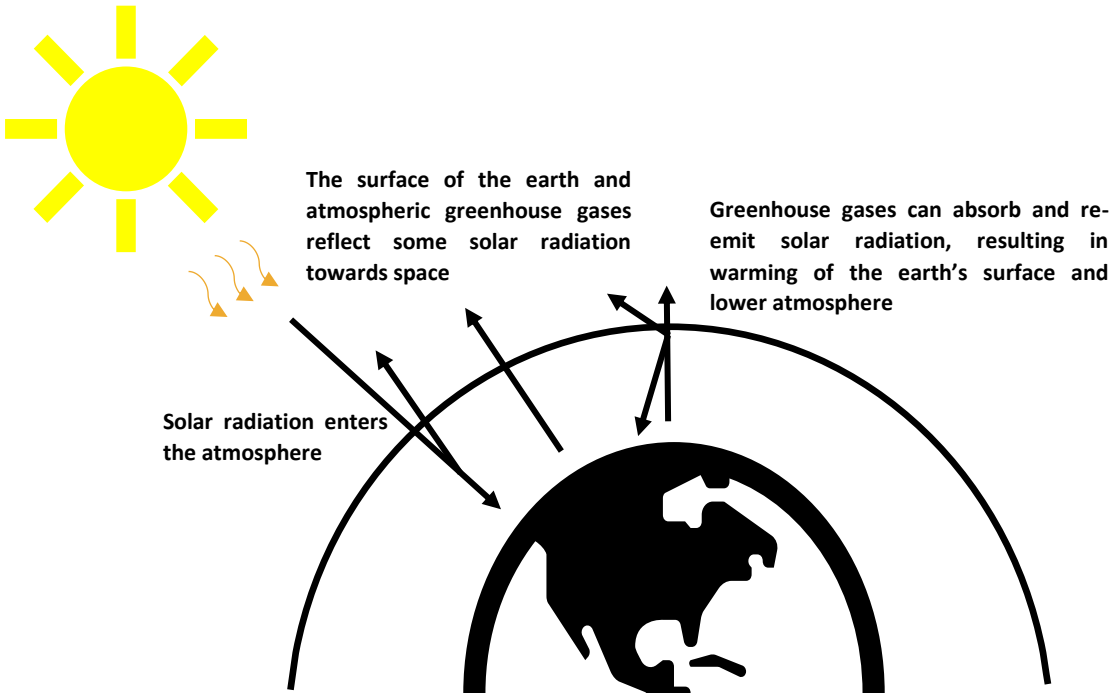


Figure 2.2 Simplified depiction of the greenhouse effect. Diagram adapted from IPCC (1992).

Anthropogenic activity has increased atmospheric greenhouse gas concentrations, thereby exacerbating the greenhouse effect. Global atmospheric CO_2 concentrations have increased by ~47% from 1750 to 2019, from approximately 280 to 412 ppm (Taylor & Lloyd, 1992; IPCC, 2021) (**Figure 2.3**). Globally, transport and energy production, industrial processes, and agriculture, forestry, and land use are the economic sectors that produce the most greenhouse gases, accounting for 48%, 24%, and 21% of total global greenhouse gas emissions in 2018, respectively (Lamb et al., 2021). Anthropogenic emission of greenhouse gases has caused the global temperature of the Earth to increase by ~1.07 °C between 1850 and 2010 (IPCC, 2021). Continued human population growth will likely lead to increased fossil fuel consumption and land modification, particularly urbanisation, resulting in greater greenhouse gas emissions and further warming of the climate (Janzen, 2004; Cohen, 2010).

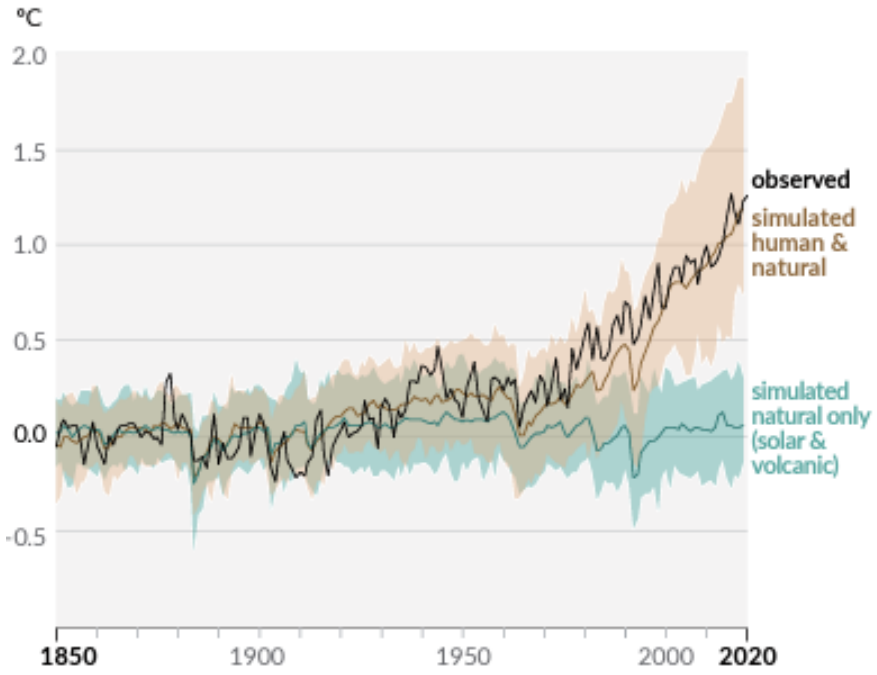


Figure 2.3 Observed and simulated change in annual average global surface temperature from human and natural and only natural forcing between 1850-2020. Graph taken from IPCC 2021.

Increasing global surface temperatures may increase C input to soil via enhanced photosynthesis and accelerate C outputs by increasing microbial respiration (Davidson & Janssens, 2006). As the average global surface temperature increases, CO_2 fluxes from soil are expected to increase, resulting in potentially significant shifts in soil C stocks and altering the stability of these C stocks, thereby creating a feedback loop (Trumbore, 1997; Davidson & Janssens, 2006). Increased soil warming has the potential to impact soil C stocks in a couple of different ways. Under one scenario, a positive feedback loop could occur, where a warming climate will accelerate soil decomposition processes, resulting in greater releases of CO_2 to the atmosphere and thereby further accelerating climate change. Alternatively, a negative feedback loop could occur, where increased temperatures enhances plant growth, resulting in increased soil C inputs from plants which may exceed rates of microbial decomposition, resulting in a net gain of soil C (Davidson & Janssens, 2006). Despite these potential scenarios, there is little consensus on what scenario is more likely to occur and it could be that negative or positive feedback loops vary across ecosystems across the globe. Some evidence supports the theory that warming-accelerated decomposition will increase soil CO_2 efflux into the atmosphere, creating a net decrease in soil C stocks (Cox et al., 2000; Kirschbaum, 2000; Bond-Lamberty et al., 2018). Other evidence supports the theory that a warmer climate and increased atmospheric CO_2 concentrations will accelerate plant photosynthesis and

respiration, thereby enhancing soil C sequestration and creating a net increase of C stocks in soil (Liski & Westman, 1997; Thornley & Cannell, 1997; Cao & Woodward, 1998).

2.2 Soil carbon cycling

Globally, soils cumulatively contain approximately 1500-2000 petagrams (Pg) of C, predominantly in the top 1 metre of soil in the form of soil organic matter (SOM). This soil C exists in different forms and can be separated into separate pools that are influenced by a variety of different factors, including anthropogenic activity, biological processes, climate, and other environmental factors (Janzen, 2004).

2.2.1 Soil carbon pools

Soil organic matter has frequently been conceptually partitioned into different pools to allow further exploration, however, it is important to remember that these pools are likely convenient constructs that may or may not represent reality. In one approach, SOM can be separated into three distinguishable C pools, including the active or labile pool, the passive or recalcitrant pool, and the intermediate pool (Trumbore, 1997). The availability of C in each pool varies, depending on its quality (see section 2.3.3.1) (Trumbore, 1997; Davidson & Janssens, 2006). The active pool of SOM is comprised of rapidly decomposable organic matter (turnover of <1 year), such as root exudates and soft plant tissues located at the top layers of soil. Decomposition of these sources often account for the majority of CO_2 fluxes at the soil surface (Townsend et al., 1995; Trumbore, 1997). In contrast, C can be retained in soil in a passive C pool for centuries to millennia in a mineralised, recalcitrant state, protected by mineral particles and resistant to microbial decomposition. Finally, there exists a poorly defined pool, the intermediate pool, which has a turnover rate in the range of years to centuries. This pool incorporates organic matter that is not as easily degradable as the active pool of C but is not as resistant as the passive C pool (**Figure 2.4**) (Trumbore, 1997).

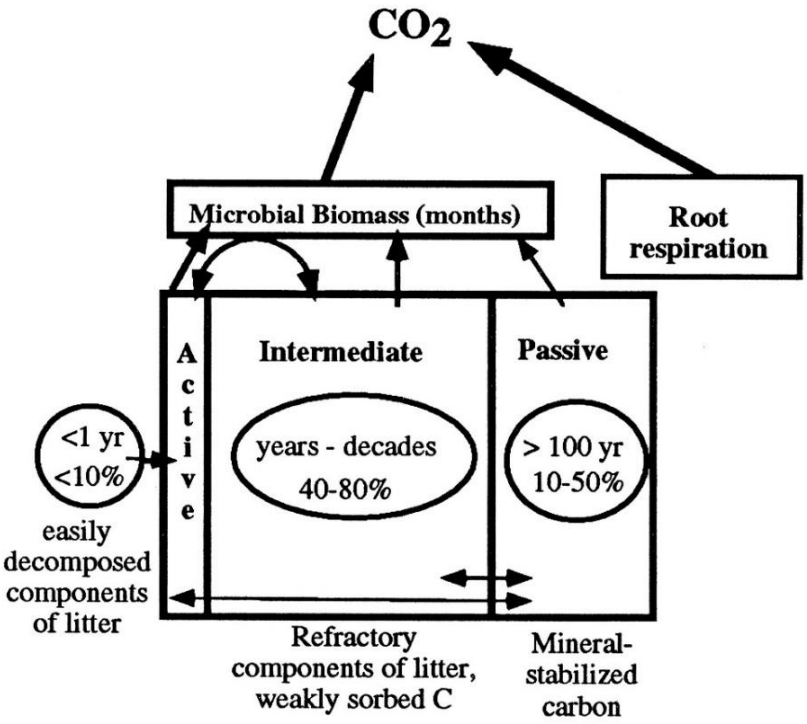


Figure 2.4 Diagram of the different conceptual soil C pools. Diagram taken from Trumbore (1997).

The stability and movement of C is highly dependent on factors such as temperature, soil texture, moisture, pH, and the composition, or 'quality' of the organic matter (Trumbore, 1997; Lehmann & Kleber, 2015). Biological processes, such as primary productivity and microbial decomposition, are likewise highly dependent on environmental factors such as temperature, moisture, aeration, pH, management practices, and organic matter accessibility, which, in turn, can influence SOM turnover (Linn & Doran, 1984; Trumbore, 1997; Six & Jastrow, 2002).

2.2.2 Soil carbon inputs and losses

The C cycle relies on inputs and outputs primarily from two types of organisms: photoautotrophs and heterotrophs. Photoautotrophic organisms also include cyanobacteria and algae, but plants will be focussed on here. Photoautotrophs capture energy through photosynthesis, where light is used to reduce atmospheric CO_2 to create organic C compounds in chloroplasts, as shown in equation (2-1) (Yiqi & Zhou, 2006; Chapin & Eviner, 2013).

$$6CO_{2(g)} + 6H_2O \xrightarrow{Photons} C_6H_{12}O_6 + 6O_2 \quad (2\text{-}1)$$

The remains of dead organisms and root exudates contribute organic matter to the soil, which is then incorporated into the C cycle. Plants leave behind organic debris including

shoots and roots upon their death, which accumulate in soil and act as a source of nutrients. In their lifespan, plants will also extend their roots into soil, releasing exudates into the soil and also leaving behind fragments of organic matter, such as root hairs (**Figure 2.5**) (Kuzyakov & Domanski, 2000). This organic C ultimately enters the soil C cycle where it is transformed through various processes, such as stabilization, degradation, mineralization, and recalcitrance (Sollins et al., 1996). Carbon can also enter soil environments through abiotic processes such as atmospheric deposition, where rainwater containing dissolved organic C may land on soil and permeate the soil profile, adding C to the soil (Dawson & Smith, 2007).

While plants are a source of C to soil systems, plant roots can also release CO_2 into the atmosphere during respiration, contributing to the overall output of CO_2 through soil respiration (Davidson et al., 2006). However, heterotrophs, such as fungi and bacteria, typically account for the majority of CO_2 fluxes from soil. Heterotrophs derive energy from consuming external substrates. This involves breaking down organic compounds, in the presence of oxygen for aerobic microorganisms, to produce CO_2 which can subsequently be released from soil, as shown in equation (2-2) (Khatoon, 2017; Gaedke, 2021). Heterotrophic respiration, primarily through microbial decomposition from fungi and bacteria, is largely responsible for the turnover of SOM and the release of CO_2 from soil (**Figure 2.5**) (Dawson & Smith, 2007; Khatoon, 2017). Soil C can also be lost through abiotic processes, such as through hydrological processes and soil erosion from water and wind (Dawson & Smith, 2007).

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_{2(g)} + 6H_2O + ATP \qquad (2\text{-}2)$$

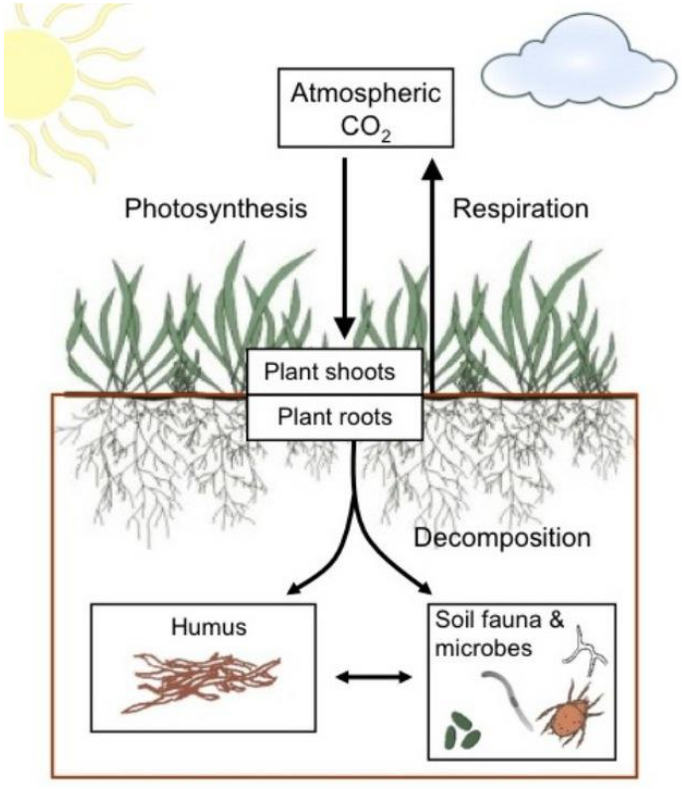


Figure 2.5 Illustration of primary inputs and losses from soil environments. Diagram taken from Ontl & Schulte (2012).

2.3 Microorganisms in soil environments

Soil C storage is reliant on SOM inputs from autotrophic organisms and C losses, driven primarily by heterotrophic microorganisms (Jobbágy & Jackson, 2000), including fungi and bacteria that decompose SOM. However, other organisms including nematodes and mites play an important role in breaking up more resilient organic matter, increasing its surface area and thereby facilitating microbial decomposition (Khatoon, 2017). Respiration from aerobic microorganisms usually accounts for the majority of soil decomposition and CO_2 fluxes (Walkiewicz et al., 2021), though anaerobic respiration can also play an important role in SOM decomposition. Anaerobic microsites are essentially pockets between and within soil peds and aggregates that are depleted in oxygen, facilitating the formation of colonies of anaerobic microorganisms. These microorganisms play important roles in soil decomposition and, in particular, in the decomposition of larger organic compounds (Keiluweit et al., 2017).

2.3.1 Differences between fungi and bacteria

The three-domain phylogenetic tree proposed by Woese et al. (1990) separates fungi as eukaryotes and places bacteria into their own classification, though the two-domain system proposed by Chatton (1938) classifies bacteria as prokaryotes (Mayr, 1998). Fungi are typically multicellular organisms (though yeasts are one notable exception) that have membrane-bound organelles and store their genetic information in a nucleus (Zabel & Morrell, 2020; Pesic, 2021). The cell walls of fungi are structured by chitin, an organic polymer that acts as a principal skeletal material. Fungi often form vegetative structures called hyphae that are used to produce sexual and asexual spores used in fungal reproduction. Furthermore, hyphae can aggregate into networks called mycelia, which can further develop to form fungal colonies (Lew, 2011; Zabel & Morrell, 2020). In contrast, bacteria are unicellular organisms that may cluster to form colonies. Bacterial organelles are not membrane-bound, so they store their genetic information in a nucleoid located in the cytoplasm of the cell. Cell walls contain peptidoglycan to help retain cell structure (Matsushita et al., 1993; Zabel & Morrell, 2020). Unlike fungi, bacteria are only capable of asexual reproduction, typically through binary fission (though there are some exceptions). The product of binary fission is two genetically identical bacterial cells (Chien et al., 2012). However, bacteria may still share advantageous adaptations with other cells by exchanging DNA through horizontal gene transfer (Aarestrup et al., 2008). A summary table is presented below to distinguish some key features between fungi and bacteria (**Table 2.1**).

Table 2.1 Summary table of some key differentiating features between fungi and bacteria (Matsushita et al., 1993; Cole, 1996; Pietikäinen et al., 2005; Robert & Casadevall, 2009; Rousk et al., 2010; Lew, 2011; Chien et al., 2012; Zabel & Morrell, 2020).

Feature	**Fungi**	**Bacteria**
Classification	Eukaryote	Prokaryote (two domain system); bacteria (three domain system)
Structures and growth	Hyphae are the key vegetative structure of fungi that form mycelial networks, which may form fungal colonies	Colonies form from individual cells
Location of genetic material	Nucleus	Nucleoid (located in cytoplasm)
Membrane-bound organelles?	Yes	No
Discerning cell wall properties	Chitin	Peptidoglycan
Motility	Typically immobile	Some have flagellum
Reproduction	Primarily through asexual and sexual spores	Asexual reproduction, primarily through binary fission. Other forms include hyphal growth (*Actinomycetes*) and budding (*Planctomycetes*)
Optimum pH for growth (see section 2.3.3.2 for further detail)	5-9	4-7
Optimum temperature for growth (see section 2.5 for further detail)	Typically ~25-30 °C; are quickly excluded beyond this optimum; grow better than bacteria at lower temperatures	Typically ~25-30 °C; are generally more capable of surviving elevated temperatures relative to fungi
Examples of particular groups or symbiotic relationships relevant to soil environments	Lichens, mycorrhizae	Rhizobia, rumen bacteria

Fungi and bacteria typically metabolise substrate by initially exuding extracellular enzymes into the environment. These enzymes break down the substrate to a size in which they can enter cells by diffusion, osmosis, or phagocytosis. These smaller particles subsequently undergo intracellular decomposition to utilize newly obtained nutrients and energy (Xu & Shang, 2016; Khatoon, 2017). Fungi tend to produce a wider range of extracellular enzymes relative to bacteria. This allows them to obtain nutrients from a wider range of sources that bacteria cannot access, such as cellulose and lignin (Boer et

al., 2005; Koranda et al., 2014; Xu & Shang, 2016; Khatoon et al., 2017; Stenholm 2021). In addition, some fungi can also accelerate the desorption of organic compounds from mineral surfaces via physical weathering (Jilling et al., 2018). In contrast, bacteria are thought to utilize a greater quantity of readily available, labile substrates (Hunt et al., 1987; Khatoon et al., 2017; Lavallee et al., 2020). However, certain groups of bacteria such as actinomycetes and anaerobic bacteria are capable of degrading some more complex substrates relative to other bacteria (Xu & Shang, 2016). For example, chitin-degrading bacteria, such as actinomycetes, may produce hydrolytic enzymes called chitinases to decompose chitin from dead fungi (Beier & Bertilsson, 2013).

Bacteria and fungi play important roles in soil aggregation, but for different reasons. Bacteria tend to enhance soil aggregation by releasing exopolysaccharides and lipopolysaccharides, which stabilize soil aggregates by bonding particles together (Cania et al., 2019). In contrast, fungi, in particular mycorrhizae, help stabilise aggregates by physically enmeshing soil particles, similar to plant roots, or by wetting and drying soil (Rillig & Mummey, 2006).

2.3.2 Methods of differentiating fungi and bacteria in soils

Fungi and bacteria have specialised roles in soil environments, though their niches overlap to some degree as both are important in soil decomposition processes (Rousk et al., 2008; Sun et al., 2017). Various methods have been employed in the past to differentiate fungal and bacterial activity in soil. Differentiating fungi and bacteria is important to better understanding how fungi and bacteria operate in their respective roles in soil systems. Furthermore, this helps in understanding how environmental changes may affect the processes undertaken by each group and how this may affect broader scale processes. This section will discuss some methods commonly used to differentiate soil fungi and bacteria. Particular focus will be taken towards the selective inhibition method and phospholipid fatty acid analysis (PLFA) as these methods are most relevant to this thesis.

2.3.2.1 Selective inhibition of soil respiration

Many studies that aim to differentiate fungal and bacterial respiration typically rely on inhibitors. The application of antibiotics to study soil microbial communities is not a novel idea (Martin, 1950; Williams & Davies, 1965), though the method as it is currently

known was established by Anderson and Domsch (1973b). This was one of the earliest studies to combine the substrate-induced respiration method and selective inhibition method to quantify fungal and bacterial roles in soil respiration, highlighting their respective roles in soil C cycling. The theory behind this method is that the addition of labile substrate accelerates anabolic and catabolic activity in metabolically active cells. Antibiotics are added to inhibit fungi or bacteria by selectively targeting a component of their metabolism. Respiration measurements can subsequently be taken by measuring the difference in gas exchange (typically either CO_2 flux or O_2 uptake) between uninhibited and inhibited samples. This theoretically provides an estimation of fungal and bacterial contributions to total soil respiration, independent from one another. This is typically expressed as a percent difference in total soil respiration between uninhibited and inhibited soils. This can be used to highlight the roles of fungi and bacteria in soil C cycling (Anderson & Domsch, 1974).

Initially, Anderson and Domsch (1973b) tested the effects of streptomycin bactericide and Euparen® fungicide on soil O_2 consumption as a proxy for soil microbial activity. Subsequent studies commonly used cycloheximide (also known as actidione) as a replacement fungicide, while soil CO_2 flux was used as a proxy for soil microbial respiration. Since these early inhibition studies, streptomycin and cycloheximide have become the most commonly used antibiotics in inhibition studies due to their selectivity (Anderson & Domsch, 1974; Anderson & Domsch, 1975; Chen et al., 2014). The effectiveness of this method in inhibiting substrate-induced soil microbial respiration is highly variable. Fungal contributions are typically estimated to be within the broad range of 15% to 45% (Bailey et al., 2002; Ananyeva et al., 2006; Chen et al., 2014), but can range from 0% to 79% (Susyan et al., 2005). Bacterial contributions normally range from 10% to 30% (Bailey et al., 2002; Susyan et al., 2005; Chen et al., 2014), but may range from 0% to 48.5% bacterial inhibition (Bailey et al., 2002; Rousk et al., 2009a).

Previous studies have reported respiration stimulation from soil following inhibitor addition (Ingham & Coleman, 1984; Bewley & Parkinson, 1985; West, 1986; Badalucco et al., 1994; Johnson et al., 1996; Hu et al., 1997; Nakamoto & Wakahara, 2004; Susyan et al., 2005). Added inhibitors will also not inhibit resistant microorganisms. It is possible for these resistant microorganisms to metabolise added inhibitors as substrate, thereby increasing respiration, which could confound respiration measurements (Parkinson et al., 1971; Nakamoto & Wakahara, 2004).

Different factors can introduce variability into selective inhibition results, including soil properties, soil and antibiotic preparation, substrate composition, and incubation time (Anderson & Domsch, 1978; Johnson et al., 1996; Bailey et al., 2002). However, CO_2 fluxes from streptomycin treatment tend to be much more variable than that from cycloheximide treated soil (Johnson et al., 1996; Hu et al., 1997). Abiotic factors of the soil may also affect inhibitor selectivity. For example, cycloheximide can weakly bind to soil but remain active as it has a neutral pH. In contrast, streptomycin is alkaline and can therefore be deactivated in acidic soils (Lin & Brookes, 1999; Ananyeva et al., 2006). Furthermore, inhibitors can change competition between microorganisms, which may indirectly affect respiration rates (Parkinson et al., 1971; Nakamoto & Wakahara, 2004). This method also overlooks the contributions of soil fauna, which can be approximately 10-15% of soil C flux (Hopkins & Gregorich, 2005). Another potential problem with this method is that inhibitors are not completely selective and may negatively affect non-target organisms (Beare et al., 1990; Velvis, 1997). For example, one previous study found that streptomycin can inhibit fungal growth and protists such as ciliates (Ingham & Coleman, 1984). Anderson and Domsch (1973b; 1975) recommended testing the selectivity of inhibitors such as by using plates to grow microbial communities from the soil then treating with inhibitor and incubating, then counting fungal and bacterial colonies. A clear limitation of this approach is that it is well established that the majority of microbes in soil have not been grown on agar or similar medium. Beare et al. (1990) instead proposed the inhibitor additivity ratio (IAR) to quantify overlap between selective inhibitors. The IAR can be calculated with equation (2.3).

$$\mathrm{IAR} = \frac{(A - B) + (A - C)}{A - D} \tag{2-3}$$

Where *A* represents respiration from soil treated with substrate, *B* represents soil treated with substrate and bactericide, *C* represents soil treated with substrate and fungicide, and *D* represents respiration from soil treated with substrate, bactericide, and fungicide.

The resulting IAR must be optimised to be as close to 1 as possible. A value greater than 1 indicates overlap between inhibitors (non-target inhibition) whereas a value less than 1 may suggest that inhibitors are not optimally effective (Beare et al., 1990; Chen et al., 2014). However, West (1986) argued that the original method assumes that antibiotic resistance and susceptibility is equal between fungi and bacteria in soil. As there is no evidence to support this, this means that the lower the proportion of inhibited soil respiration, the greater there is for potential error in estimating fungal and bacterial

contributions. West (1986) concluded that achieving maximal respiratory inhibition is more important than optimising inhibitor concentrations. Regardless, testing inhibitor overlap using the IAR tends to be the standard for inhibition studies (Nakamoto & Wakahara, 2004; Ananyeva et al., 2006; Chen et al., 2014).

Few other methods have been developed to differentiate the respiration response of fungi and bacteria. One other method to estimate differential fungal and bacterial respiration is to measure substrate uptake by isolated fungi or bacteria, then calculate a metabolic quotient as an indication of specific respiration rate (Di Lonardo et al., 2013; Pinzari et al., 2017). However, this method relies on estimating the metabolic quotient of individual fungal or bacterial isolates, which may overlook the complexity and dynamic nature of soil microbial communities. Nevertheless, this is a relatively new method and as such requires further testing to corroborate its effectiveness.

Selective inhibition is an interesting approach that has been successfully used in previous studies to differentiate the roles of fungi and bacteria in soil C cycling (Anderson & Domch, 1973b; Bailey et al., 2002). However, the selective inhibition method is not without its limitations and can require extensive preliminary experimentation to produce reasonable results. Despite this, inhibition studies can provide valuable information on the separate roles of fungi and bacteria in soil systems (Chen et al., 2014; Swallow & Quideau, 2020).

2.3.2.2 Phospholipid fatty acid analysis

Lipids are fatty molecules that are largely contained in cellular membranes. Different lipids have different roles in cells, though phospholipids will be of particular focus. Phospholipids are amphiphilic molecules, meaning that they have a hydrophilic head that interacts with water and a hydrophobic end that avoids interaction with water. The hydrophobic end of the molecule is comprised of two tails that are usually fatty acids (**Figure 2.6**).

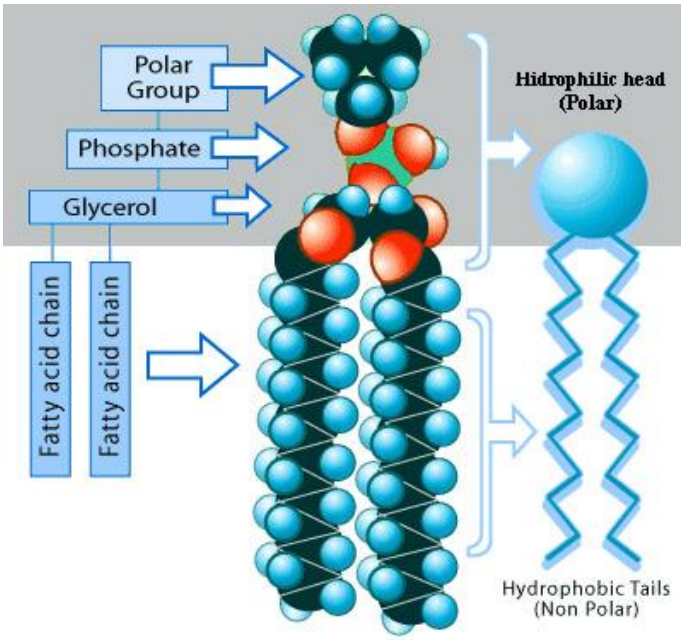


Figure 2.6 Representation of a phospholipid and its structure. Diagram taken from Shnyrova (2008).

This structure facilitates the arrangement of lipid bilayers, where the hydrophobic tails face inwards towards the cell while the hydrophilic heads face towards the external environment (**Figure 2.7**).

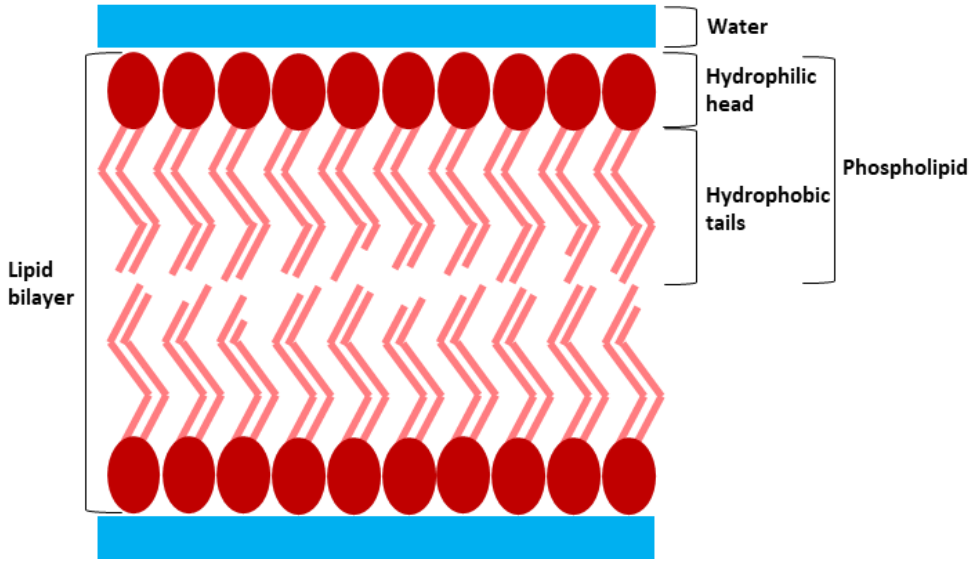


Figure 2.7 Simplified structure of the lipid bilayer. Diagram adapted from Alberts et al. (2002).

The tails of phospholipids may vary in length, which can affect how closely the phospholipid molecules are arranged in the membrane, thereby influencing membrane fluidity. Overall, phospholipids are very important structural components of cell walls (Alberts et al., 2002).

Lipids can be extracted from samples using a modified Bligh and Dyer (1959) method by adding a mixture of chloroform, methanol, and buffer. Lipids are then fractionated into neutral lipids, glycol-lipids, and phospholipids and analysed by gas chromatography (Willers et al., 2015; Lewe et al., 2021; Yu et al., 2021). As PLFAs are quickly degraded upon cell death, derived PLFAs are thought to be highly representative of living cells (Quideau et al., 2016).

Derived PLFAs can be used as biomarkers for particular microbial groups to assess their relative proportions in soil. This is typically of low taxonomic resolution and can normally only be used to identify microorganisms based on broad groupings, such as arbuscular mycorrhizal fungi, actinomycetes, and gram positive bacteria. However, this still provides a quantitative approximation of microbial community structure in the studied environment, which can be used to describe community composition (Willers et al., 2015). PLFAs can be expressed as a ratio of fungi to bacteria (F:B) by dividing the sum of the fungal PLFAs by the sum of the bacterial PLFAs (Frostegård & Bååth, 1996). As the F:B ratio increases, the proportion of fungi increases in the studied community. These ratios have been used to study the effects of different agricultural practices (de Vries et al., 2006), plant invasion, and the effect of climate change on microbial communities (Moyle, 2022), as well as soil C sequestration processes (Bailey et al., 2002).

Phospholipid fatty acid analysis and F:B ratios have been called into question as to how representative of microbial communities they actually are. Firstly, PLFAs only represent biomass at a given time from a given sample. In reality, natural systems can be highly complex and dynamic (Wang et al., 2019). Changes in lipid concentrations may occur as a result of changing environmental conditions and cell activity (Leckie, 2005; Wixon & Balser, 2013). For example, some studies have suggested that acidic pH conditions may favour fungal growth, resulting in greater fungal biomass and activity in acidic environments (Blagodatskaya & Anderson, 1998; Rousk et al., 2009b). Therefore, these metrics may not be completely representative of microbially mediated biogeochemical processes such as a nutrient cycling (Leckie, 2005; Wixon & Balser, 2013; Wang et al., 2019). Furthermore, pure culture studies have primarily been used to extract biomarkers from different groups and therefore may not represent samples from more complex environments (Watzinger, 2015). Fatty acid profiles have also not been established for every microorganism. Therefore, it is unknown as to how representative a biomarker is for a particular group (Watzinger, 2015; Willers et al., 2015). Lipids have also been

identified in multiple groups, meaning that PLFAs can inaccurately quantify different microbial groups (Olsson, 1999). Nevertheless, PLFA analysis remains a major tool for quantifying microbial communities at a broad level for comparisons between soils.

2.3.3 Controls of soil microbial respiration

Soil respiration is influenced by various proximal and distal factors. Proximal factors directly influence microbial activity and tend to vary on smaller scales. Distal factors tend to vary on broader scales and influence microbial activity indirectly by influencing proximal factors (Abnee et al., 2004; Stielstra et al., 2015; Nave et al., 2021). Five key proximal factors exert the most control on microbial respiration, including temperature, moisture, substrate availability (Davidson et al., 2006), pH (Wang et al., 2014), and oxygen availability (Neira et al., 2015). Distal controls of soil respiration can include factors such as land topography and land management (Malik et al., 2018; Zhang et al., 2021). The subsequent sections focus on proximal factors as they are directly influential in determining soil respiration. Distal factors are important but are beyond the scope of the current thesis. See Wang et al. (2016), Malik et al. (2018), and Zhang et al. (2021) for studies on the effects of land management on respiration.

2.3.3.1 Substrate availability

Heterotrophic microorganisms, comprising bacteria and fungi, are largely responsible for decomposition processes in soil environments. Positive relationships between substrate concentration and soil respiration are typically observed, resulting in increased CO_2 effluxes from soil (Wild et al., 2014; Zhang et al., 2020). This is because substrate provides microorganisms with an energy and C source along with nutrients essential for biological functioning, thereby boosting respiration from active microorganisms and potentially reactivating dormant microorganisms (de Nobili et al., 2001; Wild et al., 2014). As such, depleting available C sources will typically reduce respiration (Hartley et al., 2008). Substrate availability can be assessed by parameters such as the quality and the lability or recalcitrance of the substrate (Xu & Shang, 2016). In some ways, substrate availability and lability of C are effectively the same thing described from different viewpoints.

Carbon availability can be conceptually partitioned into two physical fractions: mineral-associated organic matter (MAOM) and particulate organic matter (POM). Mineral-

associated organic matter consists of organic compounds that have undergone decomposition by microorganisms or chemical transformation. These compounds tend to be low molecular weight compounds that often bind to mineral surfaces where they are physically protected and not readily available to biota (Lavallee et al., 2020). However, some fungi can overcome these physical bonds by weathering the mineral surface, releasing nutrients from mineral surfaces (Jilling et al., 2018). Sources of MAOM include exudates from plant roots and organic sugars and acids produced by soil microorganisms (Lavallee et al., 2020). In contrast, POM generally consists of organic fragments that have undergone little decomposition or chemical transformation. Particulate organic matter can vary much more in quality and so is not readily decomposed by many microorganisms. This fraction encompasses fresh litter that has not been fragmented, cellulose and lignin from plant structures, and chitin from fungi (Jilling et al., 2018; Lavallee et al., 2020). Soil animals, such as nematodes and earthworms, play an important role in breaking down POM into finer pieces, increasing its availability to other soil organisms (Xu & Shang, 2016; Khatoon, 2017).

Another way to describe the quality of a substrate is by its chemical composition (Melillo et al., 1982). Various indicators can be used to characterise substrate quality, including carbon-to-nitrogen (C:N) ratio, lignin content, and polyphenol content (Melillo et al., 1982; Tian et al., 1992; Brussard, 1994). Both C and nitrogen (N) are important elements in soil environments, allowing organisms to fulfil basic biological processes (Zhang et al., 2019). With regard to substrate quality, C:N ratios describe the elemental composition of SOM and can indicate its decomposability. A low C:N ratio often results in faster decomposition, greater respiration, and greater release of nutrients (Tian et al., 1992; Cornwell et al., 2008). Lignin content is thought to be as important an indicator of substrate quality as nitrogen content (Fogel & Cromack, Jr., 1977). Lignin is a complex organic molecule that plays an important structural role in the rigidity of cell walls of plants and the formation of wood (Liu et al., 2018). Decomposition of lignin therefore tends to be slower than that of other soil organic C fractions due to its complexity and resistance. This results in decomposition rates decreasing as lignin content of SOM increases (Whitaker et al., 2014; Hall et al., 2020). Decomposition of these complex C sources increases as more readily available substrates become less available (Hall et al., 2020). Finally, polyphenols are metabolites synthesised by plants to support various physiological functions such as metabolism, cell structure, or anti-predation mechanisms (Zak et al., 2019). The effect of polyphenols on soil microbial communities is highly

variable. They may serve as a labile C source to stimulate respiration (Talbot & Finzi, 2008), decrease respiration by inhibiting enzymes and/or precipitating nutritional proteins (Scalbert, 1991; Tian et al., 1992), or influencing nitrogen availability, potentially by inhibiting nitrification or by binding nitrogen to the surface of an insoluble compound (Baldwin et al., 1983; Sivapalan et al., 1985). Because of these various effects, polyphenols can select for or against certain microorganisms (Schmidt et al., 2013).

Microorganisms will preferentially metabolize substrate depending on its ease of access and lability, though the availability of substrate is partially dependent on environmental factors such as temperature and moisture (Luo et al., 2017; Pold et al., 2017). Labile substrate is typically consumed first due its ease of availability, whereas the breakdown of recalcitrant and more complex substrates tend to take longer (Xu & Shang, 2016). Labile substrate additions, such as glucose, tend to boost respiration much more efficiently than the addition of recalcitrant or more complex substrates (Jia et al., 2014).

2.3.3.2 Moisture content and oxygen availability

Due to the interdependent nature of soil moisture and oxygen availability, these two controls of microbial respiration will be discussed under one section.

Water and air move throughout soil through interconnected pores that form between soil particles and aggregates (Parlange, 1974). Water moves through soil pores by gravity, surface tension, osmosis, and capillarity, which is dependent on a variety of factors such as climate, topography, soil structure, vegetation, and climate and precipitation (Parlange, 1974; Gwak & Kim, 2016; Pepper & Brusseau, 2019). In contrast, oxygen moves through soil pores by diffusion (Parlange, 1974). The flow of water and air throughout soil can be limited when soil pore space is reduced, such as through compression from land management practices (Watson & Kelsey, 2006). Air typically poses little resistance to the movement of water in soil, with the exception of trapped air bubbles which can halt water flow between pores. Instead, water tends to slow oxygen movement through soil as oxygen diffuses much more slowly through water than through gas (Parlange, 1974).

Water is an essential solute and metabolite for all organisms and also acts as a buffer for harsh environmental conditions (Aung et al., 2018). Microorganisms under the stress of water limitation have greater energy requirements, nitrogen demand, and a lower growth

efficiency (Tiemann & Billings, 2011). When soil moisture content is low, overall microbial activity is diminished, and spore forming microorganisms such as fungi are favoured (Drenovsky et al., 2004). Furthermore, low moisture content can reduce substrate availability to microorganisms by reducing nutrient mobility by creating extra resistance for nutrients diffusing to the cell surface, thereby reducing nutrient diffusion (Papendick & Campbell, 1981; Stark & Firestone, 1995). However, studies have demonstrated that the activity of many enzymes varies as soil moisture content varies (Geisseler et al., 2012; Borowik & Wyszkowska, 2016). At an intracellular level, if water availability is insufficient, microbial enzyme activity reduces due to decreased enzyme hydration, which may change enzyme conformation (Stark & Firestone, 1995). In contrast, when the moisture content of a soil is too high, oxygen availability through diffusion can become limited, thereby restricting aerobic respiration and favouring anaerobic respiration (Le Bissonnais, 1996; Pepper & Brusseau, 2019).

The presence of oxygen is essential for aerobic microorganisms but can be useful, unnecessary, or toxic for anaerobic microorganisms (Pepper & Brusseau, 2019). Oxygen acts as a terminal electron acceptor in aerobic respiration and is therefore essential for aerobic microorganisms and plant roots. This permits the generation of ATP cellular energy and facilitates CO_2 production (Xu & Shang, 2016). Therefore, oxygen limitations that come with high moisture content selects against aerobic metabolic pathways and favours anaerobic metabolism (Drenovsky et al., 2004), giving rise to anaerobic microsites (Keiluweit et al., 2017).

In general, there tends to be a balance where soil moisture content and oxygen availability are optimal which provides favourable conditions for microbial activity to occur (**Figure 2.8**).

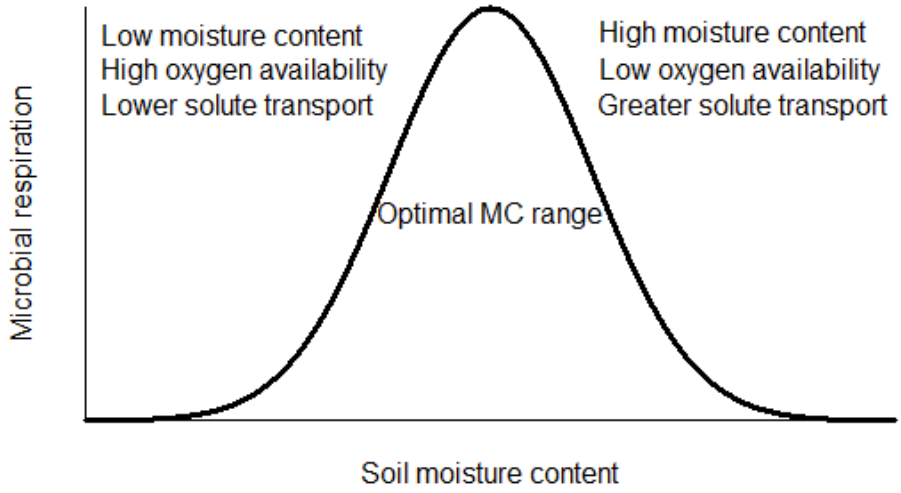


Figure 2.8 Relationship between soil moisture content and microbial respiration. Diagram adapted from Moyano et al. (2013).

2.3.3.3 pH

Soil pH measures the activity of hydrogen (H^+) ions in soil solution and is highly important in determining a range of biological and physiochemical properties of soil, such as C availability, nutrient availability, metal solubility, plant primary productivity, and microbial community composition (Kemmitt et al., 2006; Rousk et al., 2009b). The pH of a soil is influenced by various factors, including precipitation, irrigation (Simon et al., 2002; Li et al., 2021), mineral weathering (Schnoor & Stumm, 1986), and soil characteristics (such as composition and texture) (Gruba & Socha, 2016). Anthropogenic influence through management practices, such as the application of fertilisers, manure, and minerals (such as lime) can significantly alter pH (Parham et al., 2003; Abate et al., 2017). Furthermore, plants and microorganisms can drastically affect the pH of the soil they inhabit. Metabolites exuded by plant roots are metabolised by soil microorganisms, producing carboxylic groups and acidifying soil (Yan et al., 1996; Vives-Peris et al., 2020). Upon decomposition of SOM, protons are released from exchange sites and organic acids are mobilised in soil, also contributing to acidification (Simon et al., 2002; Hong et al., 2019). An important property of a soil is its buffering capacity, which, in relation to pH, can be defined as the ability of a soil to resist pH change in the presence of acidification or alkalisation (Curtin & Trolove, 2013). This buffering capacity is typically determined by cation exchanges and the presence of certain compounds and minerals such as carbonates and aluminosilicates, which can bind H^+ and hydroxide (OH^-) ions (Van Rensburg, 2009; Dvořáčková et al., 2022).

Changes in soil pH can have profound impacts on microbial communities. Studies have found that microbial richness and diversity were greatest in neutral pH soils (Schnittler & Stephenson, 2000; Fierer & Jackson, 2006). When the pH of an environment deviates too far from its typical pH, this can exert significant stress on residing microbial communities and thereby affect microbial community structure and the presence or absence of particular species (Schnittler & Stephenson, 2000; Fierer & Jackson, 2006). Bacteria tend to have a pH optima of 4-7 whereas fungi typically have a broader pH optima of 5-9 (Rousk et al., 2010). Microorganisms inhabiting pH environments outside of their pH optima will face adverse physiological stress that may have a drastic impact on community structure as well as essential biological processes such as growth and respiration. Microorganisms inhabiting an environment with a pH closer to their optimum will face less physiological stress (as well as associated stresses such as heavy metal concentration) and more normalised physiological function (Perrson & Wiren, 1989; Sitaula et al., 1995; Ellis et al., 1998; Hall et al., 1998). Some studies have found soil microbial respiration to generally increase as pH increases, though many studies do not test beyond approximately pH 8 (Persson & Wiren, 1989; Sitaula et al., 1995; Ellis et al., 1998; Reth et al., 2005; Aciego Pietri & Brookes, 2008).

Enzymes of microorganisms are also sensitive to pH and will irreversibly denature when pH is too high or too low (Frankenberger & Johanson, 1982). Enzymes tend to have their own pH optima, which can influence nutrient cycling. For example, the activity of urease, an enzyme responsible for catalysing urea into carbon dioxide and ammonia (Byrnes & Amberger, 1989), has been found to decrease in acidic environments (Frankenberger & Johanson, 1982). In contrast, β-glucosidase, an important enzyme in the hydrolysis of cellulose, tends to be most active in acidic soil environments, typically with an approximate pH optimum of 5 (Turner, 2010). Higher pH has also been associated with more efficient utilisation of low molecular substrates (Winding & Hendriksen, 2007; Wakelin et al., 2008), carboxylic acids (Creamer et al., 2016), and amino acid and carbohydrate based substrates (Sradnick et al., 2013).

Soil pH can influence the bioavailability and toxicity of different elements. The toxicity and concentration of heavy metals such as cadmium, lead, and zinc increase under acidic conditions as this tends to encourage the desorption of metal ions from minerals in soil (Jung, 2008; Kicińska et al., 2022) (**Figure 2.9**). Though some heavy metals are essential trace elements, they can be toxic to microorganisms in high concentrations or when in

certain derivative forms (Giller et al., 1998; Kicińska et al., 2022). This can also impose changes on community structure (Giller et al., 1998).

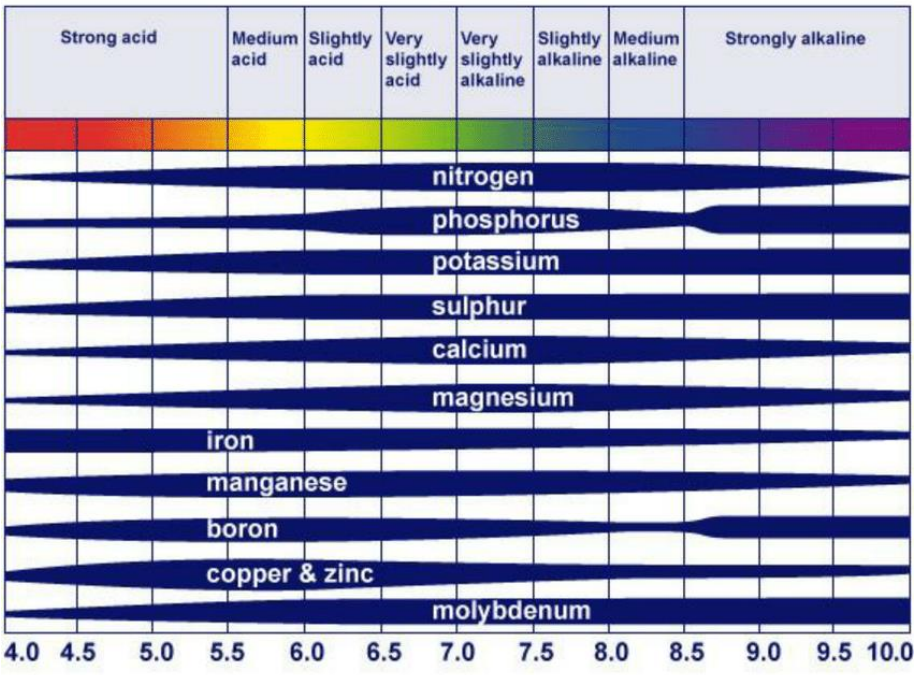


Figure 2.9 Effect of soil pH on availability of different nutrients and heavy metals. Diagram taken from Roques et al. (2013).

Soil pH can act as a barrier or a facilitator of microbial activity and can therefore affect soil C cycling dynamics (Malik et al., 2018). Some microorganisms have evolved adaptations to withstand extreme pH conditions. These groups are labelled as acidophiles (pH optima <5) and alkaliphiles (pH optima >9), relative to neutrophiles (pH optima ~7) (Driessen & Albers, 2007; Johnson, 2007; Yumoto, 2007). Acidophilic and alkaliphilic microorganisms have evolved adaptations to not only tolerate and grow, but also thrive, despite such extreme environmental pH conditions. Both groups tend to maintain an intracellular pH that is near neutral (or at least only slightly acidic/alkaline) in an extreme environment (Johnson, 2007; Horikoshi, 2011). Despite the high concentration of H^+ ions in their surrounding environment, acidophiles maintain a relatively low concentration of H^+ ions within the cell, thereby creating a proton gradient. This is achieved through an array of adaptations such as cellular membranes impermeable to H^+ and OH^-, proton sequestering enzymes, and systems that actively remove H^+ ions from the intracellular environment, such as using the enzyme F1Fo-ATPase (Johnson, 2007; Lopes & Lens, 2011; Chen, 2021). The issue facing alkaliphiles is the opposite of that of acidophiles, where a H^+ ion concentration gradient exists, but in this case the inside of the intracellular

environment is more acidic than the outside environment (Johnson, 2007; Horikoshi, 2011). Alkaline environments are rich in OH^- and often have an abundance of sodium ions (Na^+). Cell walls of alkaliphiles contain an abundance of polymers that prevent OH^- from seeping into the intracellular environment. Alkaliphiles also actively acidify their environment using Na^+/H^+ antiporters, which import sodium ions into the cell and export H^+ ions out of the cell (Horikoshi, 2011).

2.3.3.4 Effect of temperature on microbial respiration

Temperature is one of the most dominant controls of soil microbial respiration and therefore soil decomposition processes (Davidson & Janssens, 2006). Respiration generally increases with increasing temperature, but only up to a point. Shifts in environmental temperature because of seasonal changes or climatic variation can change the temperature stress imposed on soil microbial communities and alter respiration. Relatively small changes in surface temperature can enact relatively large changes in CO_2 efflux from soil by accelerating microbial decomposition of SOM (Fang & Moncrieff, 2001).

Temperature may also constrain other environmental factors, thereby affecting microbial respiration both directly and indirectly. Increasing soil temperature increases evaporation, decreasing availability of water to microorganisms (Trenberth, 2011). Soil pH may also fluctuate with temperature (Guoju et al., 2012), with climate change models predicting that soil pH may alter under increasing temperatures (Houle et al., 2020). As temperature can affect soil pH, it can also indirectly influence nutrient availability and heavy metal concentrations.

2.4 Temperature response models

To understand the effects of climate change on soil C cycling dynamics, it is important to understand the temperature sensitivity of microbial respiration. Temperature sensitivity has been defined in a number of different ways; however, in the context of respiration, temperature sensitivity can be defined as the change in respiration rate per degree change in temperature (Robinson et al., 2017). Modelling these temperature responses is important in identifying this change, quantifying the direction of change, and at what rate these changes may be occurring. Various models have been proposed to model the

temperature response of soil respiration. The Arrhenius model was one of the earliest developed models for characterising the temperature sensitivity of chemical reactions which subsequent models have since built on, including the model from Lloyd and Taylor (1994) as well as Fang and Moncrieff (2001). However, when applied to soil systems, these models predict a continuous increase in respiration with temperature, with no identification of a temperature optimum (T_{opt}), or a temperature at which respiration is maximal (Digel et al., 2008; Prentice & Arcus, 2017; Noll et al., 2020). In reality, all biological systems have a T_{opt}. The following sections will provide background on how temperature responses are described and modelled.

2.4.1 Absolute and relative temperature sensitivity

Quantifying the temperature sensitivity of SOM decomposition in absolute and relative terms is important in understanding the movement of C between soil and atmosphere and potential feedback loops of C in climate change (Davidson & Janssens, 2006). The following section covers the absolute and relative temperature sensitivities of SOM decomposition and is drawn mainly from Sierra (2012).

Absolute temperature sensitivity refers to the absolute change of a measure (for example, *k* for decomposition) for a given unit change in temperature. The absolute sensitivity of decomposition measures the change in decomposition rate with temperature, expressed as units of $time^{-1}\ K^{-1}$. The absolute temperature sensitivity of decomposition is described as the partial derivative of the decomposition rate relative to temperature, as shown in equation (2.4) (Sierra, 2012).

$$\frac{\partial k}{\partial T} = \frac{E_a A}{RT^2} \exp\left(-\frac{E_a}{RT}\right) = k\frac{E_a}{RT^2} \qquad (2\text{-}4)$$

Where E_a is activation energy, A is the pre-exponential factor, R is the universal gas constant, and T is absolute temperature (K).

In contrast, relative temperature sensitivity describes the change of a measure (k) relative to the actual value of k. This can be expressed as shown in equation (2-5).

$$\text{Relative temperature sensitivity} = \left(\frac{1}{k}\right)\frac{\partial k}{\partial T} \tag{2-5}$$

The relative temperature sensitivity refers to the change in decomposition as a proportion per unit change in temperature, expressed in units of K^{-1} (Sierra, 2012). The relative temperature sensitivity can be described by the following equation (2.6) (Sierra, 2012).

$$\frac{\partial \ln k}{\partial T} = \frac{E_a}{RT^2} = \frac{1}{k}\frac{\partial k}{\partial T} \tag{2-6}$$

Sierra (2012) showed that lower quality substrates tend to be more sensitive to temperature in relative terms, whereas higher quality substrates are typically more temperature sensitive in absolute terms. This means that for decomposition, the absolute and relative sensitivities move in opposite directions as substrate quality decreases; that is, absolute temperature sensitivity decreases at an exponential rate towards zero whereas relative temperature sensitivity increases linearly towards infinity. The absolute temperature sensitivity is shown in equation (2-7) and the relative temperature sensitivity is shown in equation (2-8) (Sierra, 2012).

$$\lim_{E \to \infty} \frac{\partial k}{\partial T} = 0 \tag{2-7}$$

$$\lim_{E \to \infty} \frac{1}{k}\frac{\partial k}{\partial T} = \infty \tag{2-8}$$

Figure 2.10a below shows the increase of relative temperature sensitivity of decomposition towards infinity whereas **Figure 2.10b** shows the decrease in absolute temperature sensitivity towards zero.

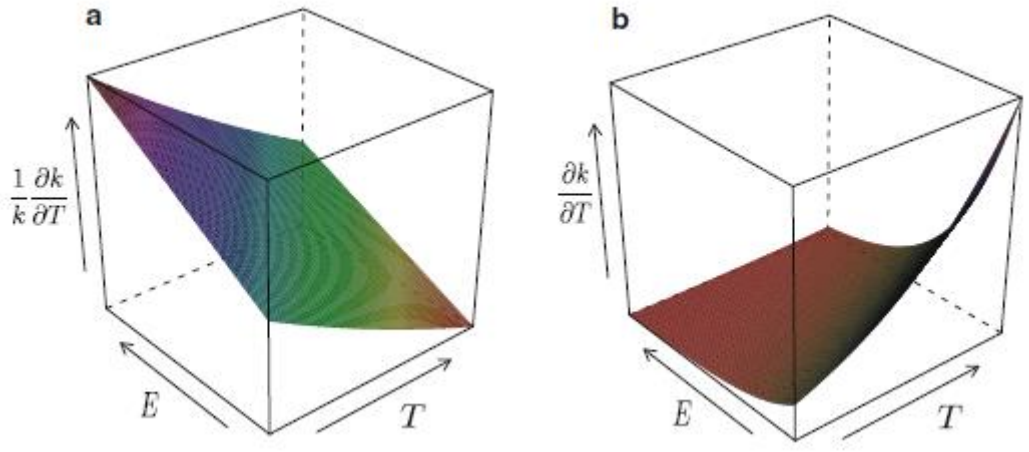


Figure 2.10 Graphical depictions of (**a**) the relative temperature sensitivity ((1/*k*) $\partial k/\partial T$) of SOM decomposition and (**b**) the absolute temperature sensitivity ($\partial k/\partial T$) of SOM decomposition. *E* represents activation energy, *T* represents temperature, and *k* represents rate of decomposition. Diagram taken from Sierra (2012).

This is a paradox embedded in the Arrhenius equation and based on the theory of enzyme kinetics, but reinforces the importance of differentiating relative and absolute temperature sensitivity in helping assess the temperature sensitivity of decomposition (Sierra, 2012). The research section of this thesis focuses on absolute temperature sensitivity (absolute change in respiration at a given temperature).

2.4.2 Intrinsic and apparent temperature sensitivity

In the context of SOM decomposition, intrinsic temperature sensitivity describes the decomposition of a substrate based on its molecular structure and environmental temperature (Davidson & Janssens, 2006; Schipper et al., 2014). Typically, the more complex the structure of a substrate is, the greater the activation energy and therefore the greater the temperature sensitivity of the substrate (Davidson & Janssens, 2006). Intrinsic temperature sensitivity is the partial derivative of the response variable (*X*, such as decomposition) to the explanatory variable (*Y*, such as temperature), thereby expressing the absolute change in the response variable in regard to the explanatory variable, as expressed in equation (2-9) (Sierra et al., 2015).

$$\text{Intrinsic temperature sensitivity} = \frac{\partial Y}{\partial X_i} \qquad (2\text{-}9)$$

In contrast, the apparent temperature sensitivity refers to the observed response of decomposition to temperature under environmental constraints. The apparent sensitivity takes into account the simultaneous change of the explanatory variables in a specific direction ($\boldsymbol{u}$), as expressed in equation (2-10) (Sierra et al., 2015).

$$\text{Apparent temperature sensitivity} = \nabla Y - \boldsymbol{u} \qquad (2\text{-}10)$$

Where $\nabla$is a vector of partial derivatives.

Environmental constraints may affect the apparent temperature sensitivity of decomposition temporarily or indefinitely (Davidson & Janssens, 2006). These constraints can come in different forms. This includes physical protection, where microbial access to substrate is reduced because substrate is physically out of reach of microbes and their enzymes, due to being trapped within soil aggregates. Secondly, substrate can be adsorbed to the surface of minerals or aggregates, making it inaccessible to microbes until the substrate is desorbed from the surface (Oades et al., 1988; Davidson & Janssens, 2006). Thirdly, low moisture conditions (as discussed in section 2.3.3.2) can reduce nutrient mobility to cells and create a substrate limitation for microbes (Papendick & Campbell, 1981; Stark & Firestone, 1995; Davidson & Janssens, 2006). Fourthly, high moisture conditions and flooding excludes oxygen availability in soil environments, thereby favouring anaerobic respiration over aerobic respiration (as also discussed in section 2.3.3.2) (Le Bissonnais, 1996; Davidson & Janssens, 2006; Pepper & Brusseau, 2019). Finally, freezing soil greatly reduces, but may not eliminate (at least initially), the diffusion of substrate to cells as well as enzyme activity (Elberling & Brandt, 2003; Davidson & Janssens, 2006). Overall, these environmental constraints obscure the intrinsic temperature sensitivity of decomposition, resulting in the apparent temperature sensitivity to be less than expected. As such, apparent temperature sensitivities are typically lower than intrinsic temperature sensitivities. However, environmental constraints of decomposition are often temperature dependent in themselves (Davidson & Janssens, 2006). **Figure 2.11** below describes the relationship between apparent temperature sensitivity and intrinsic temperature sensitivity with the effects of environmental constraints.

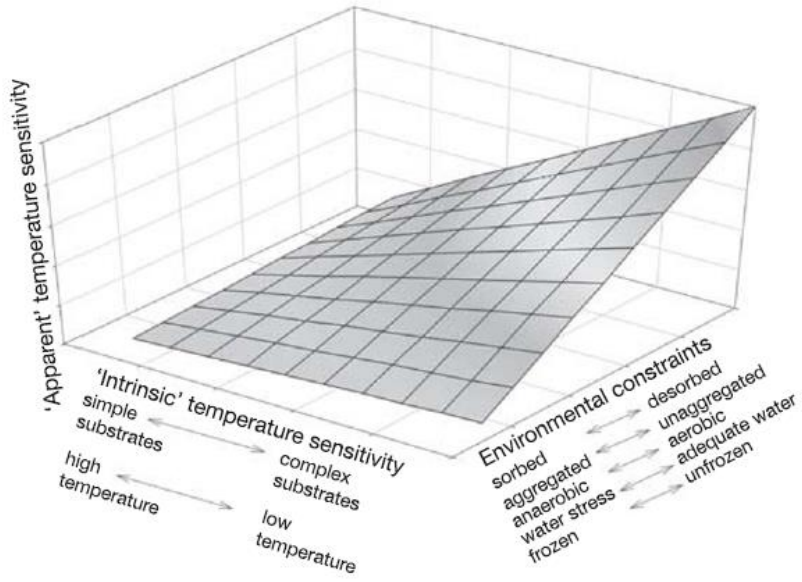


Figure 2.11 The influence of intrinsic temperature sensitivity and environmental constraints on apparent temperature sensitivity. Diagram taken from Davidson & Janssens (2006).

2.4.3 Arrhenius

The Arrhenius model was developed by Svante Arrhenius in 1889 to describe the temperature dependence of biochemical processes (Davidson et al., 2006). The model states that in order for chemical reactions to proceed, an activation energy is required. This activation energy is essentially an energy threshold that reactants must reach in order to break and/or create bonds to transform (Davidson et al., 2006; Schipper et al., 2014). The Arrhenius equation (2-11) has the formula as described in equation.

$$k = A\, e^{-E_a/RT} \tag{2-11}$$

Where k is the rate constant, A is a pre-exponential factor, e is an exponential term, E_a is the activation energy, R is the gas constant (8.314 J K^{-1} mol^{-1}), and T is temperature (Laidler, 1984).

The Arrhenius model has been successful in accurately characterising the temperature dependence of chemical reactions. However, when applied to biochemical reactions, these models tend to become problematic (Robinson et al., 2017). The Arrhenius model predicts that as temperature increases, there is a continuous, exponential increase in rate of reaction and does not allow the estimation of a T_{opt} of the reaction (Schipper et al.,

2014). In reality, all biochemical reactions have a T_{opt}, or a temperature at which the rate of reaction is maximal (Prentice & Arcus, 2017; Robinson et al., 2017). Furthermore, the Arrhenius model also underestimates respiration rates at low temperatures and overestimates at high temperatures (Lloyd & Taylor, 1994; Schipper et al., 2014). Regardless, the Arrhenius model, as well as similar derived models, have been applied to biological systems, such as soil decomposition processes (Sierra, 2012).

Lloyd and Taylor (1994) acknowledged that soil respiration is reliant on biological communities that may change over time, with each community potentially being uniquely sensitive to temperature. In their study, they found that activation energy increased as temperature decreased, potentially due to enzyme deactivation (Sharpe & De Michelle, 1977; Lloyd and Taylor, 1994). They proposed the following empirical equation (2-12) as an accurate way to estimate soil respiration rates.

$$R = R_{10} e^{E_{a0}\left(\frac{1}{283.15 - T_0} - \frac{1}{T - T_0}\right)} = R_{10} e^{308.56\left(\frac{1}{56.02} - \frac{1}{T - 227.13}\right)} \quad (2\text{-}12)$$

Where R is respiration, R_{10} is respiration at a standard temperature (10 °C), E_{a0} is activation energy (308.56 K), and T_0 is a temperature constant (227.13 K).

This model introduced a polynomial term based on data taken from soil respiration studies. However, much like the Arrhenius equation, the Lloyd and Taylor model does not predict a T_{opt} of respiration (Robinson et al., 2017). Other amendments have been proposed since, such as those proposed by Kirschbaum (2000) and Fang and Moncrieff (2001), but many either lack theoretical basis or possess similar flaws as the Arrhenius model (Robinson et al., 2017).

2.4.4 Q_{10}

Along with Arrhenius, Q_{10} has been one of the most widely used metrics to measure temperature sensitivity. Q_{10} is a ratio between response rates occurring at 10 ° incremental increases in temperature (Mundim et al., 2020). Therefore, Q_{10} is a ratio between two rates (Alster et al., 2020). The Q_{10} coefficient can be expressed with equation (2-13).

$$Q_{10} = \frac{K_{T+10}}{K_T} \quad (2\text{-}13)$$

Where *K* is decomposition rate, which can be interchanged with respiration (*R*).

In recent years, it has been suggested that Q_{10} is misleading. Similar to Arrhenius, it predicts an increase in response rate with increasing temperature, when in biochemical reactions, responses tend to be more dynamic and often decline at higher temperatures (Dell et al., 2011). Finally, as is also the case with the Arrhenius function, Q_{10} is dependent on the range of temperatures that the response was measured across. This means that Q_{10} values may not be an accurate metric for comparing temperature sensitivities between studies (Alster et al., 2020). Furthermore, substrate supply and quality can also affect derived Q_{10} values, which may confound accurate measurements and comparisons of Q_{10} between systems (Davidson & Janssens, 2006).

2.4.5 Macromolecular rate theory

Macromolecular rate theory (MMRT) was developed to more accurately model biochemical reactions catalysed by macromolecules, such as respiration (Hobbs et al., 2013). This theory takes the thermodynamic principles of these macromolecules into account to model the temperature response of biochemical reactions. It also suggests that activation energy is itself temperature dependent (Hobbs et al., 2013). When applied to soil respiration, MMRT captures the initial exponential increase in respiration and the increase in temperature sensitivity but curves to fit an optimum (at which point the temperature sensitivity falls to zero) (Schipper et al., 2014; Robinson et al., 2020).

Eyring and Polyani expanded upon the equation proposed by Arrhenius to produce a more theoretically accurate model, called the Transition State Theory. This model has had marked success in accurately describing the temperature dependence of chemical reactions, particularly across a broad range of temperatures (Schipper et al., 2014; Robinson et al., 2017). This equation proposed by Eyring and Polyani replaces the pre-exponential term *A* (see equation (2-8) above) for $k_B T/h$ and the activation energy E_a is substituted for Gibbs free energy. This equation is shown in equation (2-14).

$$k = \frac{k_B T}{h} e^{(\frac{-\Delta G^{\ddagger}}{RT})} \quad (2\text{-}14)$$

Where k_B is Boltzmann's constant, h is Planck's constant, and $\Delta G^{\ddagger}$ is Gibbs free energy. The natural log can be taken for both sides to simplify the equation, as shown in equation (2-15).

$$\ln(k) = \ln\left(\frac{k_B T}{h}\right) - \frac{\Delta G^{\ddagger}}{RT} \quad (2\text{-}15)$$

The Erying equation assumes that $\Delta G^{\ddagger}$, as well as its associated components such as enthalpy change ($\Delta H^{\ddagger}$) and entropy change ($\Delta S^{\ddagger}$), are independent of temperature. Though this is often true for smaller molecules, this is not the case for more complex systems that include macromolecules (Schipper et al., 2014; Arcus et al., 2016). Hobbs et al. (2013) showed that $\Delta G^{\ddagger}$ is temperature dependent through the change in heat capacity ($\Delta C_p^{\ddagger}$) associated with enzyme catalysis. Heat capacity is defined as the amount of heat per unit mass required to raise the temperature of a substance by 1 °C (Shrivastava, 2018). This is an important factor to consider as biochemical reactions tend to be dependent on macromolecules, such as enzymes, which tend to have large heat capacities (Cooper, 2005; Hobbs et al., 2013). The equation for Gibbs free energy is provided in equation (2-16).

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (2\text{-}16)$$
$$= \left[\Delta H^{\ddagger}_{T_0} + \Delta C_P^{\ddagger}(T - T_0)\right] - \mathrm{T}[\Delta S^{\ddagger}_{T_0} + \Delta C_P^{\ddagger}(lnT - lnT_0)]$$

Note that both $\Delta H^{\ddagger}_{T_0}$ and $\Delta S^{\ddagger}_{T_0}$ are at reference temperature T_0.

From this, MMRT was developed to more accurately describe the temperature dependence of reactions catalysed by enzymes (Hobbs et al., 2013). Macromolecular rate theory accounts for these changes in enzyme $\Delta C_p^{\ddagger}$ by incorporating the equation for Gibbs free energy into the Eyring equation. In doing so, MMRT accounts for the decline in enzymatic rates as temperatures extend beyond the T_{opt} (Schipper et al., 2014). The equation for MMRT is shown in equation (2-17) (Alster et al., 2022).

$$ln(R_s) = ln\left(\frac{k_B T}{h}\right) - \frac{\Delta H^{\ddagger}_{T_0}}{RT} - \frac{\Delta C^{\ddagger}_P(T - T_0)}{RT} + \frac{\Delta S^{\ddagger}_{T_0}}{R} + \frac{\Delta C^{\ddagger}_P(lnT - lnT_0)}{R} \quad (2\text{-}17)$$

Though MMRT was initially developed to model the temperature response of enzymes (Hobbs et al., 2013), it has been applied to a wider range of biochemical processes. This includes nitrification (Schipper et al., 2014), soil enzymes (Alster et al., 2016), the cycling of labile and recalcitrant C (Robinson et al., 2020; Numa et al., 2021; Van de Laar, 2021), different soil types (Numa et al., 2021), soils of varying moisture contents (Robinson et al., 2017; Schipper et al., 2019; Numa et al., 2021), and geothermal soils (Van de Laar, 2021).

The T_{opt} and T_{inf} (defined below; **Figure 2.12**) of soil respiration can be calculated as useful parameters to help characterise these biogeochemical processes (Robinson et al., 2020). The T_{opt} refers to the temperature at which respiration is maximal and the change in respiration equals zero (Robinson et al., 2020). Beyond this optimum, respiration decreases due to changes in enzyme heat capacity (C_p) from enzyme catalysis, resulting in large negative values, as opposed to enzyme denaturation that has been proposed in the past (though this does occur at higher temperatures) (Hobbs et al., 2013; Prentice & Arcus, 2017). In enzymes, the T_{opt} tends to increase as growth temperature of the parent organism increases (Elias et al., 2014), but are typically higher than the T_{opt} of growth as well as the environmental temperature (Prentice et al., 2020). In soil respiration, the T_{opt} for substrate-induced respiration tends to be approximately 37 °C (Numa et al., 2020).

After fitting MMRT in equation (2-17), the T_{opt} can be derived using equation (2-18) (Schipper et al., 2019).

$$T_{opt} = \frac{\Delta H^{\ddagger}_{T_0} + \Delta C^{\ddagger}_P T_0}{-\Delta C^{\ddagger}_P - R} \quad (2\text{-}18)$$

The inflection point (T_{inf}) is the steepest point of the temperature response curve where respiration rate changes most rapidly and is most sensitive to temperature (Schipper et al., 2019; Prentice et al., 2020; Robinson et al., 2020). Once MMRT is fitted using equation (2-17), equation (2-19) below can be used to calculate the T_{inf} (Schipper et al., 2019).

$$T_{inf} = \frac{\Delta H^{\ddagger}_{T_0} + \Delta C^{\ddagger}_{P.} T_0}{-\Delta C^{\ddagger}_{P} \pm \sqrt{-\Delta C^{\ddagger}_{P.} R}} \quad (2\text{-}19)$$

A visual representation of a temperature response curve fitted with MMRT (including T_{opt} and T_{inf}) compared with a curve fitted with Arrhenius and a mixed curve is shown below (**Figure 2.12**).

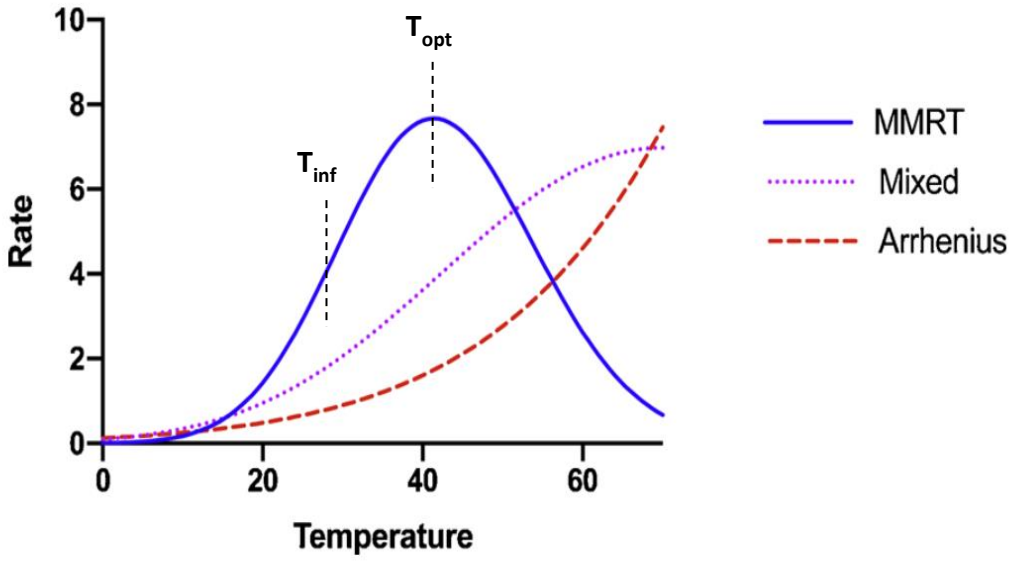


Figure 2.12 Graph of a temperature response curve fitted with MMRT (including T_{opt} and T_{inf}), Arrhenius, and a mix of both MMRT and Arrhenius. Diagram adapted from Schipper et al. (2019).

Though MMRT has been applied to soil microbial respiration, it has not yet been applied to fungal and bacterial respiration, separate from one another. Macromolecular rate theory could be useful in characterising the independent temperature responses of fungal and bacterial respiration. These respiration responses could be modelled using MMRT, which would allow the calculation of a T_{opt} and T_{inf} of respiration. Derived T_{opt} and T_{inf} values could be compared between fungi and bacteria, thereby differentiating their temperature responses.

2.4.5.1 Macromolecular rate theory and thermal adaptation

The application of the Arrhenius function to soil respiration has been shown to be problematic as it does not accurately characterise the rate of biochemical reactions, primarily because it does not account for a T_{opt} (Schipper et al., 2014). In contrast, MMRT has been shown to more accurately characterise the temperature response of various

different biochemical reactions (Schipper et al., 2014; Alster et al., 2016; Robinson et al., 2017; Schipper et al., 2019). Accurately modelling the temperature response of biochemical processes, such as respiration, is important in understanding how these biochemical systems will acclimate with warming under climate change. Alster et al. (2020) described thermal adaptation as the change in temperature response of an enzyme under shifts in temperature in the environment that an organism inhabits. This can be measured by $\Delta C_P^{\ddagger}$ and T_{opt}. Under this definition, Alster et al. (2020) posited three hypotheses of thermal adaptation among biochemical systems, all of which based around the principles of MMRT.

The enzyme rigidity hypothesis posits that with increasing temperature, $\Delta C_P^{\ddagger}$ becomes less negative, causing the response curve to become less steep, the T_{opt} to increase, and potentially leading the entire response curve to shift upwards. The optimum-driven hypothesis proposes that as environmental temperature increases, T_{opt} will acclimate to match these increases, though $\Delta C_P^{\ddagger}$ will remain steady. However, this may not be possible at highly negative $\Delta C_P^{\ddagger}$ and instead $\Delta C_P^{\ddagger}$ may have to increase as T_{opt} increases. Under the thermal breadth hypothesis, $\Delta C_P^{\ddagger}$ is partially dependent on the range of environmental temperatures. Enzymes exposed to greater temperature fluctuations may have less negative $\Delta C_P^{\ddagger}$ values and therefore flatten response curves, resulting in more consistent response rates in varying temperature regimen. These hypotheses so far have little experimental corroboration, but still present relatively novel avenues to study the thermal adaptation of biochemical systems under increasing temperatures (Alster et al., 2020).

Prentice et al. (2020) proposed the inflection point hypothesis. This states that the T_{inf} of an enzyme is more evolutionary relevant to thermal adaptation than T_{opt}. This is because enzymatic T_{opt} tends to sit above the T_{opt} of growth and therefore there is no clear selective pressure for T_{opt}. Prentice et al. (2020) also proposed that the T_{inf} of enzymes tends to regulate the optimal growth temperature by allowing microbes to coordinate enzyme activity over relatively small fluctuations in environmental temperature. This allows the activity of each enzyme to scale equally over increasing or decreasing environmental temperatures, thereby allowing the parent cell to maintain homeostasis (Prentice et al., 2020). This would be of importance to soil microorganisms as soil temperature can fluctuate greatly over short time scales. If the T_{inf} of microbial enzymes can scale with environmental temperature, this would prove to be advantageous in allowing the cell to maintain homeostasis in fluctuating temperatures (Prentice et al., 2020).

2.5 Thermal adaptation among soil microbial communities

Temperature is a selective force that excludes poorly adapted microorganisms and can thereby impose shifts on microbial community structure (Pietikäinen et al. 2005; Ali et al. 2018; Donhauser et al. 2020). Microorganisms have adapted to withstand and survive elevated temperatures. Thermophiles are a group of extremophiles that can inhabit habitats over ~50 °C (Lusk, 2019). In contrast, thermotolerant microorganisms can grow at temperatures up to 50 °C (Malashenko et al., 1975; de Oliveira et al., 2015). Thermophilic bacteria are speculated to have arisen as some of the earliest forms of life when earth's surface temperatures were over 50 °C (Lusk, 2019), though bacterial thermophily may have evolved on multiple different occasions (Hobbs et al., 2012). It is theorised that fungal thermophily arose to withstand high temperatures as a result of seasonal variation and particularly high daytime temperatures rather than to occupy free niches (Powell et al., 2012). Under increased environmental temperature, metabolic reactions accelerate, purely as a physical consequence (Bakar et al., 2020). As such, microorganisms must adapt their biological structures and metabolism to this increased temperature stress. Among the adaptations to survive temperature stresses include changes in membrane lipid composition (Russell, 1984), cell wall structure (Russell, 2003), or fatty acid structure (Russell, 1984; Suutari & Laakso, 1994), as well as increasing DNA heat stability (Maheshwari et al., 2000; Russell, 2003). However, the ability of proteins to withstand temperature has been identified as one of the most crucial properties in the evolution of organismal thermotolerance (Corkrey et al., 2012). It has been posited that thermal tolerance can occur relatively rapidly, achieved through mutations that alter protein folding and thereby increase protein stability under temperature stress (Riehle et al., 2001; Wallenstein et al., 2010).

The thermal adaptation of soil microbial respiration is not yet well understood. Soil warming is expected to increase microbial respiration over both short-term and long-term scales (Kirschbaum, 2006; Bradford et al., 2008). However, respiration rates tend to initially rise with increasing temperature but tend to drop to pre-warming rates within a few years (Luo et al., 2001; Melillo et al., 2002). In the context of soil respiration, Bradford et al. (2008) defined thermal adaptation as the change in soil microbial respiration in response to temperature change. In this same study, Bradford et al. (2008) found that mass specific respiration decreased in response to increasing temperatures from seasonal changes, as had previously been postulated by Hochachka and Somero (2002). The theory behind this is that high temperatures will eventually cause microbial

enzymes to lose their structure. Enzymes adapted to higher temperatures therefore tend to have reduced conformational flexibility, meaning that they are more rigid and do not change shape so easily. In contrast, enzymes in lower temperature environments are more stable and less likely to denature due to temperature stress. As a result, mass specific respiration rates from thermally adapted microorganisms tend to be lower than mass specific respiration rates from microorganisms inhabiting colder environments (Hochachka & Somero, 2002; Bradford et al., 2008).

2.5.1 Thermal adaptation in fungi and bacteria

Little research has been undertaken towards differentiating patterns of thermal adaptation in fungi and bacteria, particularly in the context of soil respiration. As such, the pool of literature relevant to this topic remains relatively small. From the available literature, patterns of local thermal adaptation among fungi have been previously identified (Fargues et al., 1997; Laine, 2008; Stefansson et al., 2013; Rangel et al., 2018; Szymczak et al., 2020; Bazzicalupo, 2022). Though these studies are useful in highlighting thermal adaptation among isolates and the potential direction of thermal adaptation, they tend to focus on individual strains as opposed to the numerous fungal species that typically comprise soil fungal communities. Regardless, other studies have identified possible acclimation of fungi to increasing temperature from climate change, resulting in the emergence of new fungal pathogens (de Crecy et al., 2009; Casadevall et al, 2019). In contrast, the bacterial domain is often seen as having a greater development toward thermophily than fungi, resulting in bacteria generally exhibiting greater thermotolerance than fungi and therefore having higher optimal temperatures for growth (Griffin, 1985). Thermal adaptation of bacteria has been reported in soil systems (Zogg et al., 1997; Pettersson & Bååth, 2003; Bárcenas-Moreno et al., 2009; Rinnan et al., 2009; Rousk et al., 2012; Cruz-Paredes et al., 2021; Rijkers et al., 2022), as well as other environments such as in laboratory cultures and glaciers (Bennett et al., 1992; Hassan et al., 2020). It has been suggested that fungi may have greater ability to grow more rapidly at lower temperatures, but are excluded much more quickly at elevated temperatures. A study by Robert & Casadevall (2009) suggested that of 4802 fungal strains grown in their study, 6% of fungal isolates were killed off per 1 °C increase in temperature in the range of 30-40 °C. In contrast, bacteria seem to be more capable of tolerating elevated temperatures but may be much slower growing at lower temperatures (Pietikäinen et al., 2005). Fungi may also have a lower minimum growth temperature (between -12.3 to -17.5 °C)

compared to bacteria (between -8.4 and -12.1 °C), reflecting the greater ability of fungi to grow at lower temperatures relative to bacteria (Pietikäinen et al., 2005; Nottingham et al., 2019).

Some bacterial adaptations to temperature may be different to those of fungi. DNA strands tend to become more tightly coiled due to increased supercoiling under elevated temperatures. However, in more thermotolerant bacteria and bacterial enzymes, DNA becomes more relaxed. Though this has been reported among fungi (Baldi et al., 2017), this has been reported more often in bacteria (Dorman et al., 1990; Grau et al., 1994; Mojica et al., 1994; Friedman et al., 1995; Marguet et al., 1996). This suggests that relaxation of DNA may be a physiological phenomenon that occurs as bacteria become more tolerant of increasing environmental temperatures (Tse-Dinh et al., 1997). Fungal adaptations to temperature may be different to those of bacteria. For example, fungi can accrue large quantities of the sugar trehalose in the cytoplasm of cells to aid in tolerating elevated temperatures and the accompanied decrease in water availability (Piper, 1993). Under temperature stress, fungi may also change enzyme production to maintain metabolic requirements, perhaps differently to that of bacteria (Krishnan et al., 2018; Bakar et al., 2020). However, it is likely that fungi and bacteria will have unique adaptations to survive temperature stress as they are markedly different in terms of cell physiology and community structure (see section 2.3.1).

2.5.2 Identifying thermal adaptation using geothermal temperature gradients

Currently, little is known about how microorganisms will adapt to increased temperatures under climate change. Temperature gradients present an interesting opportunity to study microbial thermal adaptation in response to gradual warming. Microbial thermal adaptation has previously been studied using different temperature gradients, including elevational temperature gradients (Wang et al., 2012), latitudinal temperature gradients (Dacal et al., 2019), and temperature gradients that span across different biomes, such as from natural to artificially managed systems (Bradford et al., 2019). Geothermal temperature gradients could also be useful in studying microbial thermal adaptation but remain relatively unexplored. Sufficiently heated geothermal ground will increase the temperature of its immediate vicinity, creating a declining temperature gradient leading away from the geothermal feature. If temperatures are high enough, this can exert a

selective force on microbial communities, selecting for thermally adapted microorganisms closer to the source of the geothermal activity, where it is presumably hotter. Geothermal temperature gradients could be used to study how microbial communities adapt to withstand the effect of natural warming in environments such as soil, which can be used to simulate soil warming under climate change.

One study by Marañón-Jiménez et al. (2018) tested the effects of natural warming from geothermal activity on microbial thermal adaptation in an Icelandic soil. Soils had been exposed to 7 years of soil warming which were then collected and incubated at a range of temperatures. They found higher metabolic rates in geothermally warmed soils, indicating a physiological change in microbial communities. However, they did not find any evidence of thermal adaptation of microbial respiration. A study conducted in Iceland by Walker et al. (2018) studied microbial respiration rates from a geothermal gradient and exposed them to temperatures ranging from ambient temperatures to 6 °C. Results from this study found that microorganisms did not adapt to warming in terms of respiration, growth, carbon use efficiency, or turnover rates. However, a study conducted by Maljanen et al. (2019) cautions that there are other, non-biological sources of CO_2 in geothermal environments. This may therefore confound respiration measurements conducted in geothermal environments. Finally, a New Zealand study conducted by Van de Laar (2021) studied patterns of thermal adaptation among geothermally warmed soils ranging from average annual temperatures of 16.1 °C to 35.4 °C. Soils were collected along the geothermal gradient and incubated along temperatures ranging from 1.8 °C to 53.0 °C with and without glucose to differentiate the labile C response. Overall, Van de Laar (2021) found that the T_{opt} of labile C respiration increased with environmental temperature at a rate of 0.157 °C $°C^{-1}$, indicating thermal adaptation of non-substrate limited microbial respiration.

Few studies have utilised geothermal sites as a way of assessing thermal acclimation among microbial communities. Geothermal temperature gradient studies present an interesting way of examining how microbial communities may adapt to warming temperatures, simulating conditions under climate change scenarios. Reports from previous studies suggest that there have been mixed results. Regardless, more research into this topic is required before any confident judgements can be made.

2.6 Chapter review

This chapter has presented an overview of relevant background literature leading up to the research focus of this thesis. The presented literature review can be summarised into the following key points:

- Soil holds the largest store of terrestrial C, of which its flow between soil and other earth reservoirs is essential for life on earth. Increasing surface temperatures under climate change may alter C cycling dynamics by influencing microbial respiration.
- There are key differences between fungal and bacterial roles in soil. The selective inhibition method provides a potentially viable way to differentiate fungal and bacterial respiration, despite its limitations. Phospholipid fatty acid analysis could be used to accurately distinguish microbial biomass and population structure.
- Arrhenius and its derived models have previously been used to characterise soil biochemical processes. Macromolecular rate theory may present a more accurate way of modelling respiration as it allows the identification of a T_{opt} and T_{inf}. Macromolecular rate theory presents a relatively novel way of studying thermal adaptation among soil microorganisms.
- Thus far, little research has been conducted into differentiating thermal responses and adaptation among fungi and bacteria. Fungi and bacteria may respond differently to increasing environmental temperature, complicating the effects of increasing global temperatures on soil C cycling.
- Geothermal temperature gradients could be used to study how soil microbial communities adapt to increasing temperatures. This could provide insight into identifying patterns of thermal adaptation among microbial communities.

2.7 Future work

Though there is a certain degree of overlap between fungal and bacterial niches, each group is distinctly different from one another, both in terms of physiology and their roles in soil environments. So far, little research has been conducted on differentiating the

response of fungal and bacterial respiration to temperature. It is possible that under warming from climate change, fungi and bacteria will respond differently, which may complicate soil C cycling dynamics. Geothermal temperature gradients present an opportunity to study patterns of thermal adaptation among soil microbial communities by simulating soil warming under climate change. However, this approach has received little attention so far.

Van de Laar (2021) found that the T_{opt} of microbial respiration increased with environmental temperature at a rate of 0.157 °C °C^{-1}. Further study is required to corroborate these findings. Furthermore, this finding overlooks the individual responses of fungal and bacterial respiration to temperature. While MMRT has been applied to soil microbial respiration (Robinson et al., 2017; Schipper et al., 2019; Numa, 2020; Van de Laar, 2021), it has not yet been applied to fungal and bacterial respiration, separate from one another. A primary goal of this thesis was to differentiate the temperature responses of fungal and bacterial respiration. The selective inhibition method could be used as a viable method to partition fungal and bacterial respiration. Their respiration responses could be modelled using MMRT, which would allow the calculation of a T_{opt} and T_{inf} of respiration. Derived T_{opt} and T_{inf} values could be compared between fungi and bacteria, thereby differentiating their temperature response.

In addition, this thesis aimed to study the differential thermal adaptation of fungal and bacterial biomass in geothermal soils. Biomass of different fungal and bacterial groups can be separated using PLFA analysis. Soils collected at different locations along a geothermal temperature gradient may host different assemblages of fungi and bacteria. This would allow the examination of whether fungi and bacteria display different patterns of thermal adaptation.

Chapter 3
Methodology

3.1 Overview

The overarching objective of these experiments was to determine whether the temperature response of soil fungal and bacterial respiration differed, and whether this changed with soil warming. Soils collected along a geothermal temperature gradient allowed for examination of patterns of thermal adaptation among soil bacterial and fungal communities (O'Gorman et al., 2014; Marañón-Jiménez et al., 2018, Walker et al., 2018; Van de Laar, 2021). To differentiate the contributions of fungi and bacteria to total soil respiration, the respiration inhibition method developed by Anderson and Domsch (1973a; 1973b) was used. This method was paired with a temperature block, developed by Robinson et al., (2017), to assess the response of bacterial and fungal respiration to a range of temperatures. Phospholipid fatty acids (PLFAs) derived from fatty acid methyl ester (FAME) analysis were used to estimate changes in fungal and bacterial biomass along the geothermal temperature gradient (Lewe et al., 2021).

This chapter provides a full description of the sample site and the underpinning methods used during method development and in the final research design. A summarised version is provided in chapter 5 which is written in the format of a paper potentially ready for journal submission.

3.2 Site description

The Arikikapakapa Reserve is a natural geothermal zone that contains a large proportion of geothermal activity in the southern region of the Rotorua Geothermal Field, along with the neighboring Whakarewarewa Valley geothermal zone (Scott & Cody, 2000; Ratouis et al., 2015). Contained within the Rotorua geothermal field is the Rotorua Caldera, which was formed approximately 220,000 years ago from a single volcanic eruption (Wilson et al., 1995). The settlement of Rotorua is situated around the Rotorua Caldera. Geothermal activity in the Arikikapakapa geothermal zone consists primarily of geysers, hot springs, hot pools, mud pools, and thermal ground (Reeves & Rae, 2016). The Rotorua geothermal system is more broadly encompassed within the Taupō Volcanic Zone.

The study site of this research was a surface geothermal feature located on the southern end of the course of the Rotorua Golf Club, located in the Arikikapakapa Reserve (**Figure 3.1**). This geothermal feature has been measured at approximately 5.1 m long and 3.8 m wide and has been classified as thermal ground (Seward et al., 2015; Van de Laar, 2021). Aerial photographic evidence suggests that this feature has been present since at least the 1970s (Van de Laar, 2021), though no recorded evidence of its existence before this time period exists as the area was covered in native bush. Other geothermal features exist around the golf course, but few produced a temperature gradient as distinct as the one used in this study, which was initially used by Van de Laar (2021).

Figure 3.1 Pictures of the geothermal feature used in this study, taken 4th November 2022.

The soil of this site was previously mapped by Landcare Research, with the data being made available on their S-MAP Online tool. This soil was described as an inactive hydrothermal recent soil, and as such had a weakly developed structure but a distinct topsoil. The rhyolitic parent material was formed from tephra from local volcanic activity. Further characterization of this soil described it as sandy in texture and well drained with very low vulnerability to water logging (Manaaki Wehnua, 2019).

The vegetation surrounding the geothermal feature consisted mainly of grasses, in particular *Axonopus affinis* (carpet grass) and *Elymus repens* (couch grass), and various unidentified mosses (Van de Laar, 2021). Small patches of bush and trees were in the general vicinity of the geothermal feature, which provided habitat to Pūkeko that often frequented the geothermal site. Management of the area consisted only of a regular mowing regimen, with no addition of fertilisers or lime as the area was adjacent to the driving range rather than part of the playing area of the golf course.

3.3 Characterisation of the geothermal temperature gradient

The temperature of the geothermal temperature gradient was first described by Van de Laar (2021). The temperature gradient extended 16 m east from the geothermal feature and was quantified by Van de Laar (2021) by separating temperatures along the gradient into different temperature ranges along the gradient (**Figure 3.2**). These temperature ranges were used to guide the determination of sample locations for the current study. This ensured that collected soil samples captured the range of temperatures along the gradient.

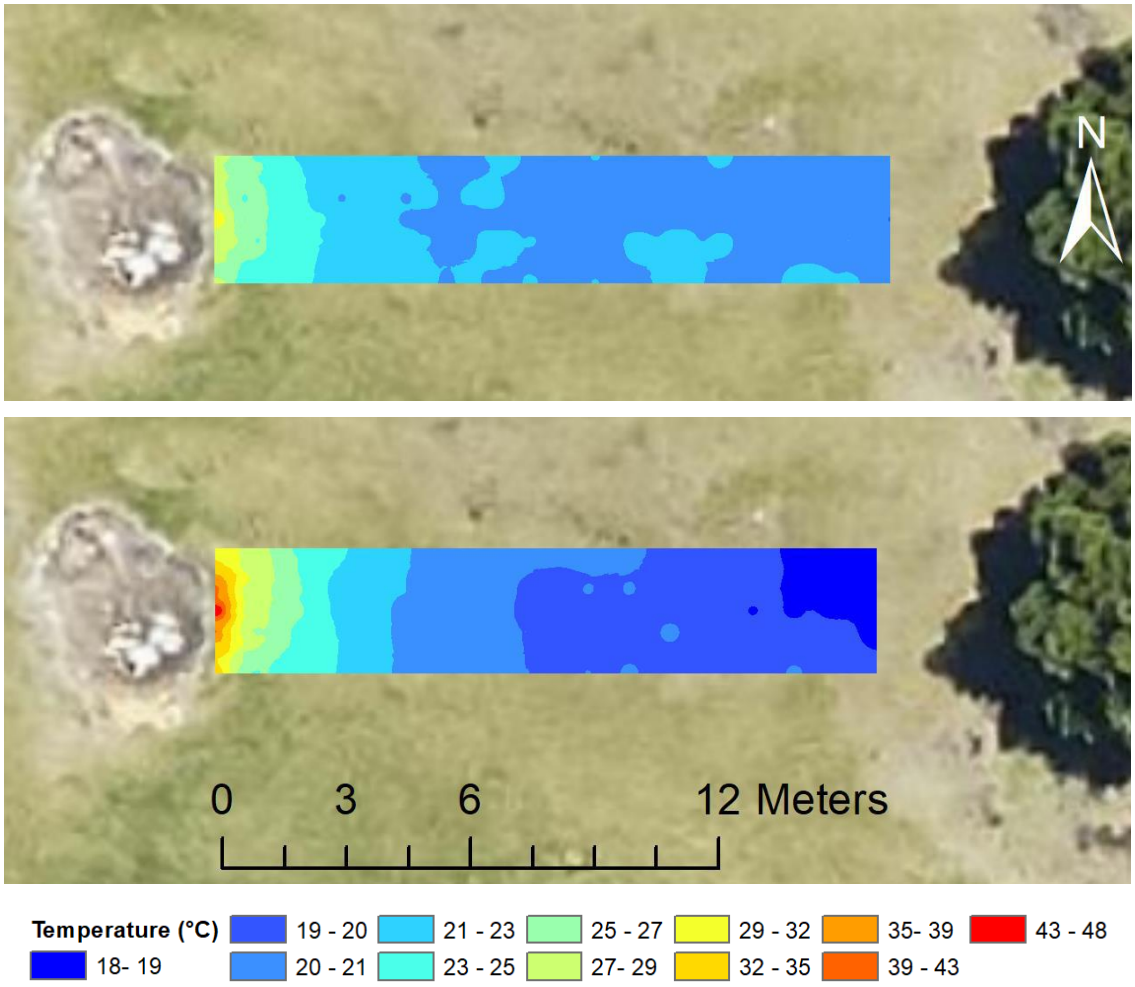


Figure 3.2 Average temperature data from Van de Laar (2021), collected using their grid method between 26th August 2020 to 22nd June 2021. The temperature profile was characterised at 2 cm depth (top) and 10 cm depth (bottom) (Van de Laar, 2021). Temperatures at 10 cm depth were used to guide sample locations used in the current study.

In this research, the temperature profile of the gradient was characterised in a manner similar to that of Van de Laar (2021). Monitoring of the geothermal temperature gradient for this study occurred from 1st September 2021 to 8th June 2022. Temperatures along the temperature gradient were monitored using thermochrons, small temperature logging devices manufactured by iButtonLink Technology (**Figure 3.3**). These iButtons have been found to be relatively reliable, particularly due to their water resistant design. The DS1922L model of iButton was used in this study, which were programmed using 1-Wire software by Maxim Integrated. These iButtons were programmed to take one temperature measurement every hour for approximately 4-8 weeks before they were collected and replaced.

Figure 3.3 Schematic of iButton (left) and appearance (right). Images taken from iButtonLink (2022).

Prior to arriving at the field site, five iButtons were programmed to take temperature measurements once every hour and were put on a two hour start delay to account for the time it takes to get to the field site. The iButtons were sprayed with a waterproofing spray (CRC Spray Seal Leak Stop) approximately one hour prior to being placed in the ground. Spraying iButtons with waterproofing spray has been found to have little influence on the ability of the iButton to record temperature (Roznik & Alford, 2012). Upon arrival at the field site, iButtons were placed approximately 7.5 cm deep in the ground following removal of a soil core using a bucket sampler (7.5 cm depth, 2.5 cm diameter) at 10 cm, 30 cm, 60 cm, 300 cm, and 1600 cm out from the geothermal feature. The soil core was replaced after the iButton was placed at the bottom of the hole.

Long term monitoring of the temperature gradient between 1st September 2021 to 8th June 2022 showed that each of the iButton locations were each relatively distinct in temperature, allowing the identification of a distinct temperature gradient (**Figure 3.4**), similar to that of Van de Laar (2021). The temperature gradient identified in the current study ranged from ~13-39 °C across 16 m similar to the range (~16-35 °C) measured by Van de Laar (2021). The greatest temperature changes occurred within the first 3 m of the geothermal feature, where the hottest temperatures were measured. Long-term temperature data revealed that temperatures fluctuated following daily and seasonal cycles and there were occasional drops in temperature associated with rainfall events. These drops in temperature were much steeper closer to the geothermal feature. On occasion, the iButtons were damaged or would disappear, resulting in data loss and gaps in the long-term temperature profile. This was particularly common at 10 cm out from the geothermal feature.

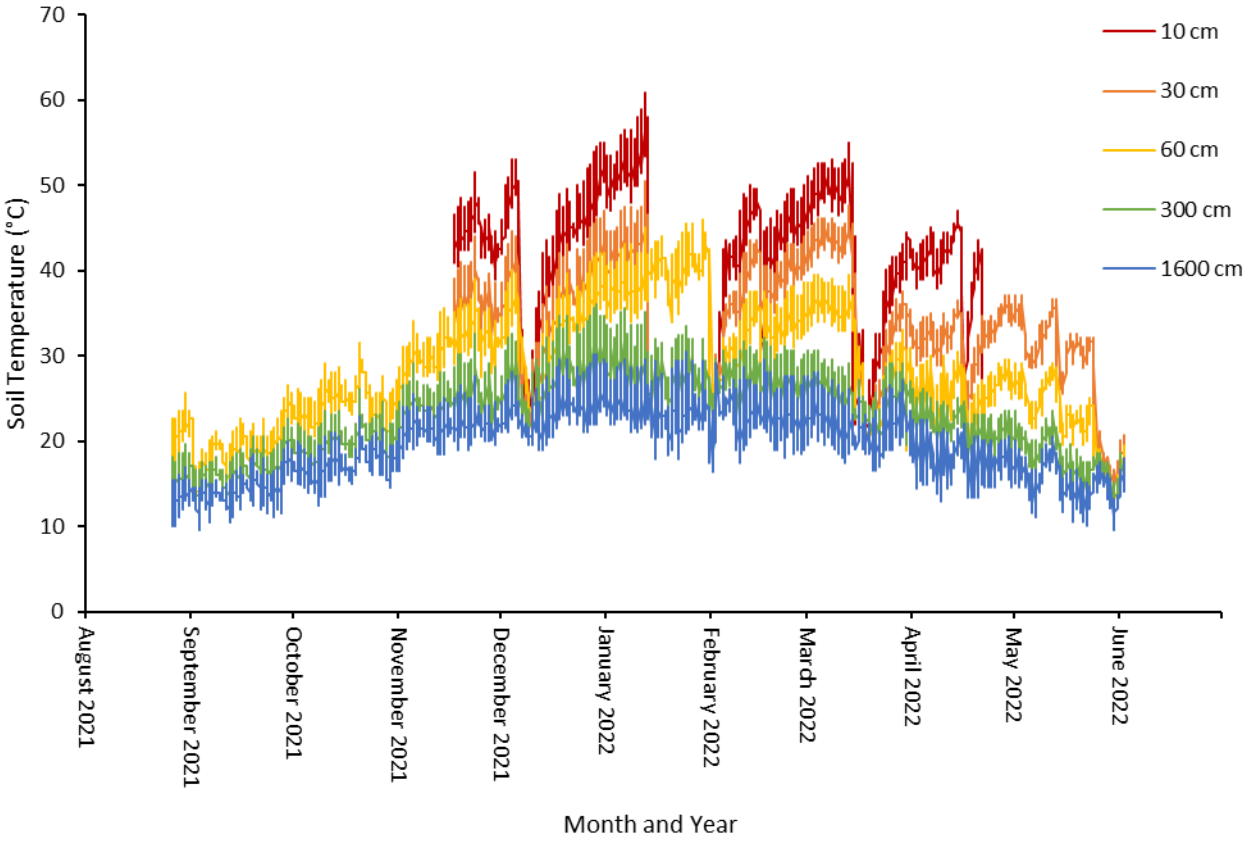


Figure 3.4 Temperature data collected from iButtons located at 10 cm (red), 30 cm (orange), 60 cm (yellow), 300 cm (green), and 1600 cm (blue) out from the edge of the geothermal feature. This graph shows temperature data collected from 1st September 2021 to 8th June 2022.

3.4 Method development

A number of preliminary experiments were undertaken to develop a final, optimised protocol for the respiration inhibition experiments. The objective for many of these preliminary experiments was to maximize inhibitor efficacy. Soil used for these preliminary experiments was collected adjacent to the geothermal temperature gradient (not from the gradient itself). Due to cycloheximide supply constraints, all preliminary studies were undertaken using streptomycin and the resulting conclusions were assumed to be the same for cycloheximide. Finally, glucose solution used in these preliminary experiments was made by dissolving 0.2702 g of glucose powder in 20 mL of water. Some studies have applied inhibitors as a solution (Anderson & Domsch, 1973a; Anderson & Domsch, 1973b; Vančura & Kunc, 1977; Nakas & Klein, 1980; West, 1986; Velvis, 1997; Nakamoto & Wakahara, 2004), but this was not a viable option for this study as both streptomycin and cycloheximide are soluble in water at approximately 20 mg mL^{-1} (Merck Index Online, 2022). This would mean that 125 mg of

inhibitor would have to be dissolved in 6.25 mL of water (100 mg g^{-1}; 1.25 g soil), at which point the volume of water would be far greater than the quantity of soil. As such, both streptomycin and cycloheximide were applied in powder form throughout all inhibition experiments. A more detailed description of the final optimised method is provided below (see section 3.5).

3.4.1 Developing a standard method

The literature differs a great deal in how samples are pretreated prior to measurement, which could contribute to differing results reported in the literature. A description of how soils were prepared in this study is presented in section 3.5.2.

The aim of this experiment was to develop a standard method to establish soil treatments on the day of incubation. An additional aim was to maximise rates of inhibition from added inhibitors. This experiment tested three different methods which were referred to as the 'direct method', the 'bag method', and the 'tray method'. The purpose of the bag and tray methods were to test whether inhibition was greater when soil was treated with inhibitor in bulk while also allowing different methods of mixing streptomycin throughout soil to be tested. The direct method was the standard method of testing soil respiration following glucose treatment (Numa, 2020; Van de Laar, 2021), but adapted for the selective inhibition method. The following methods were all set up on the same day and the experiment was run five times in total.

In the direct method, soil (1.25 g ± 0.01 g) was added to nine Hungate tubes (15 mL), with three treatment tubes per treatment. Soils were dosed with two different concentrations of streptomycin (40 mg g^{-1} and 80 mg g^{-1}) plus an uninhibited treatment, which were then treated with glucose solution (0.125 mL g^{-1}). Tubes were subsequently sealed with a rubber septum, screw cap, and left to incubate at 30 °C for five hours before CO_2 concentrations were measured from each treatment tube.

For the bag method, bulk soil (11.25 g) was added to three different plastic zip lock bags. Bagged soil was treated with streptomycin at two different concentrations (40 mg g^{-1} and 80 mg g^{-1}) plus an uninhibited treatment, followed by the addition of glucose solution to each bag (0.125 mL g^{-1}). Following streptomycin and glucose addition, bags were sealed and mixed via shaking. Treated soils were then added to nine Hungate tubes at quantities equivalent to that of

the direct method (i.e., soil (1.25 g) plus accounting for the extra weight from glucose addition and different concentrations of streptomycin). After soils were added to tubes, tubes were sealed and left to incubate at 30 °C for five hours prior to CO_2 accumulation.

Finally, for the tray method, bulk soil (11.25 g) was added to three different aluminium trays. Bulk soil was treated with streptomycin at two different concentrations (40 mg g^{-1} and 80 mg g^{-1}), plus an uninhibited treatment. Glucose solution (0.125 mL g^{-1}) was then applied to bulk soil and thoroughly mixed throughout the soil. Treated soils were then added to nine Hungate tubes at quantities equivalent to that of the direct method (i.e., soil (1.25 g) plus the extra weight from glucose addition and different concentrations of streptomycin). Tubes were subsequently sealed and left to incubate at 30 °C for five hours before quantities of CO_2 were measured.

Results suggested that there was little difference in respiration between the direct method and the bag method (P = 0.823), the direct method and the tray method (P = 0.756), and the bag method and the tray method (P = 0.749) (**Figure 3.5**). As there was no significant difference between the different methods, the direct method was subsequently used as it was the quickest to set up.

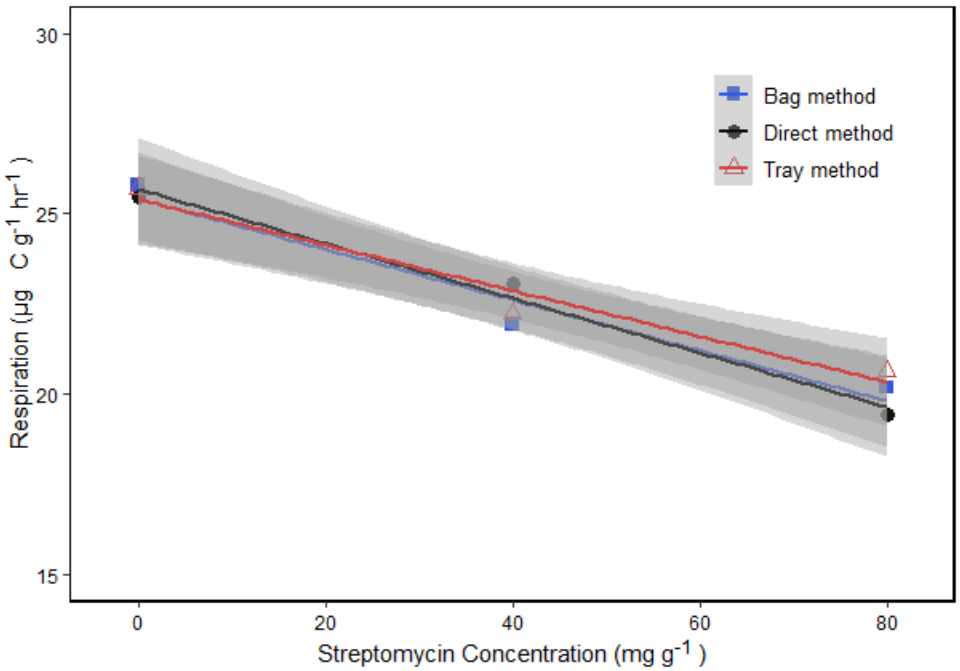


Figure 3.5 Respiration (µg C hr^{-1} g^{-1}) from soils treated with streptomycin (40 mg g^{-1} and 80 mg g^{-1}, plus an uninhibited treatment) using three different methods: the bag method, the regular method, and the tray method. Shaded areas represent standard error of the slope.

3.4.2 Fungal and bacterial contributions to soil organic matter decomposition

This experiment tested whether specific inhibitors could be used to distinguish fungal and bacterial respiration in the decomposition of SOM, in the absence of glucose addition.
To test this, soil (1.25 g ± 0.01 g) was added to nine Hungate tubes. Three tubes were treated with streptomycin at 60 mg g^{-1}, three were treated with streptomycin at a much higher concentration of 160 mg g^{-1}, and an additional three tubes received no streptomycin treatment. Tubes were treated with distilled water (0.125 mL g^{-1}), vortex mixed, sealed with a rubber septum and screw cap, and left to incubate for five hours at 30 °C. After incubation, CO_2 accumulation was measured. Overall, this experiment was repeated three times.

Unexpectedly, results showed that streptomycin at 60 mg g^{-1} significantly stimulated respiration relative to soil not treated with streptomycin ($P < 0.001$) (**Figure 3.6**). However, there was not a significant difference in respiration between soil treated with streptomycin at 60 mg g^{-1} and streptomycin treatments at 160 mg g^{-1}. It is possible that after bacteria had been eliminated following streptomycin addition, this released nutrients from dead bacteria which stimulated respiration from fungi, resistant bacteria, and other soil organisms (Parkinson et al., 1971; Nakamoto & Wakahara, 2004). Overall, it was not possible to differentiate fungal and bacterial respiration in SOM decomposition. Normally, studies in the literature also add an available C substrate to stimulate microbial activity when adding inhibitors (see below) (Anderson & Domsch, 1973b).

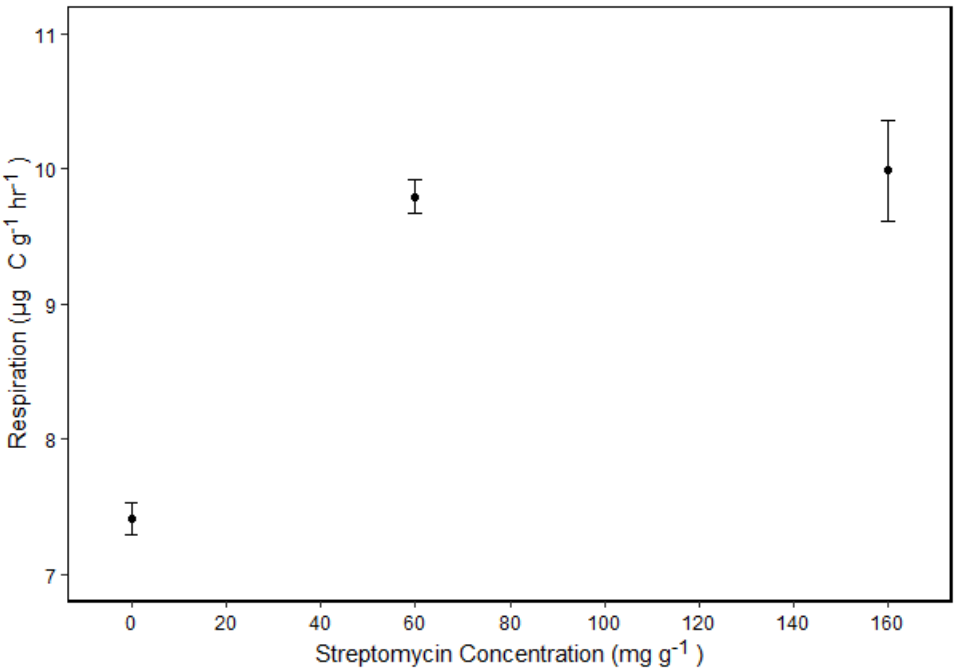


Figure 3.6 Respiration (with standard error bars) (µg C hr^{-1} g^{-1}) from soil treated with streptomycin at different concentrations.

3.4.3 Effect of glucose timing on substrate-induced soil respiration inhibition

West (1986) stated that if inhibitors were applied to soil after (rather than before) glucose addition, the inhibitors would be less effective. This was tested to determine whether inhibitors were more effective if added before or after substrate addition. To test this, soil (1.25 g ± 0.01 g) was added to nine Hungate tubes: three tubes to test the effect of glucose before substrate addition, three tubes to test the effect of glucose after substrate addition, and three tubes for treatment of glucose only. Streptomycin (60 mg g^{-1}) was added to appropriate treatment tubes before and after addition of glucose solution (0.125 mg g^{-1}). Soils were sealed and left to incubate for five hours at 30 °C prior to measurement of CO_2 concentrations. This experiment was repeated five times in total.

Respiration was found to be significantly lower when glucose was added after streptomycin ($P < 0.001$), indicating higher rates of inhibition from streptomycin, in agreement with West (1986). In contrast, when glucose was added to soil first followed by streptomycin addition, streptomycin did not decrease respiration, and may have stimulated respiration relative to the glucose only treatment. However, this was found to be marginally non-significant ($P = 0.056$). Additionally, respiration from soil treated with glucose first followed by streptomycin was much more variable (**Figure 3.7**).

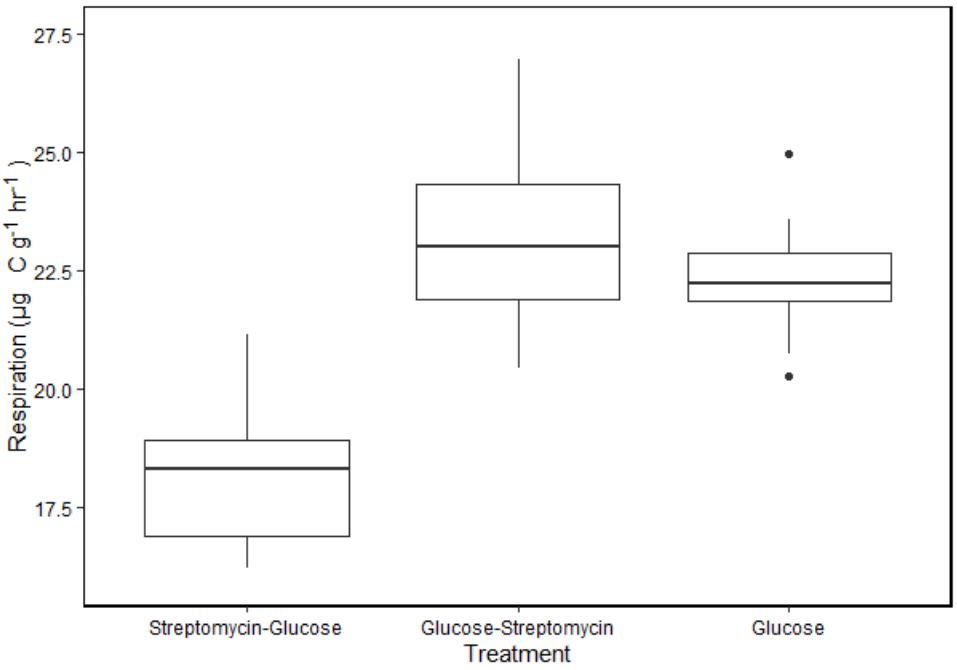


Figure 3.7 Respiration (µg C hr^{-1} g^{-1}) from soil treated with glucose and streptomycin in different orders, as well as soil treated with glucose for comparison. Streptomycin-glucose means streptomycin was added to soil first, followed by glucose. Glucose-streptomycin means soil was treated with glucose first, followed by streptomycin.

These results showed that adding glucose before streptomycin negates the inhibitory effects of streptomycin. This may have been because due to added glucose revitalizing metabolically inactive cells, thereby increasing metabolism in soil microbial communities (Anderson & Domsch, 1973a). It is possible that cells that are more metabolically active have greater resistance to streptomycin, meaning that the soil microbial community as a whole has a greater level of resistance. In contrast, adding streptomycin first may eliminate cells that are less metabolically active and more vulnerable, hindering the ability of microbial communities to resist inhibitory effects from streptomycin. Following the results from this experiment, glucose was applied to soil after streptomycin treatment. This effect is not often reported in the literature and might have contributed to inconclusive results in previous studies.

3.4.4 Mixing streptomycin with talcum powder

Previous studies have claimed to increase rates of inhibition by mixing inhibitors with talcum powder, improving inhibitor distribution throughout soil (Anderson & Domsch, 1978; Bewley & Parkinson, 1985; Lin & Brookes, 1999; Nakamoto & Wakahara, 2004; Ananyeva et al., 2006; Rousk et al., 2008). The following experiment was conducted to test whether mixing

streptomycin with talcum powder at two different concentrations could improve the distribution of inhibitors throughout soil and thereby increase inhibition.

Treatments were tested at a ratio of 1:2 (talcum powder-to-streptomycin) following the methods of Susyan et al. (2005) and Ananyeva et al. (2006), as well as at a ratio of 1:1. Ultimately, treatments consisted of soil treated with glucose (0.125 mL g^{-1}) and talcum powder at three different concentrations (30 mg g^{-1} and 60 mg g^{-1} as well as no talcum powder addition) and soil treated with streptomycin (60 mg g^{-1}) and talcum powder at the three different concentrations.

Soil (1.25 g ± 0.01 g) was weighed into 18 Hungate tubes. Streptomycin (60 mg g^{-1}) was weighed and mixed with talcum powder at prescribed quantities. Mixtures of streptomycin and talcum powder were subsequently added to soils, with three tubes per treatment. Treatments were subsequently vortex mixed, sealed with a rubber septum and screw cap, and left to incubate at 30 °C prior to measurement of CO_2 accumulation. This experiment was repeated three times in total.

Results found that mixing streptomycin with talcum powder reduced the inhibitory effects of streptomycin and stimulated soil respiration (**Figure 3.8**). Respiration was significantly boosted in talcum powder treatments ($P < 0.001$) as well as in the glucose and streptomycin treatment ($P < 0.001$).

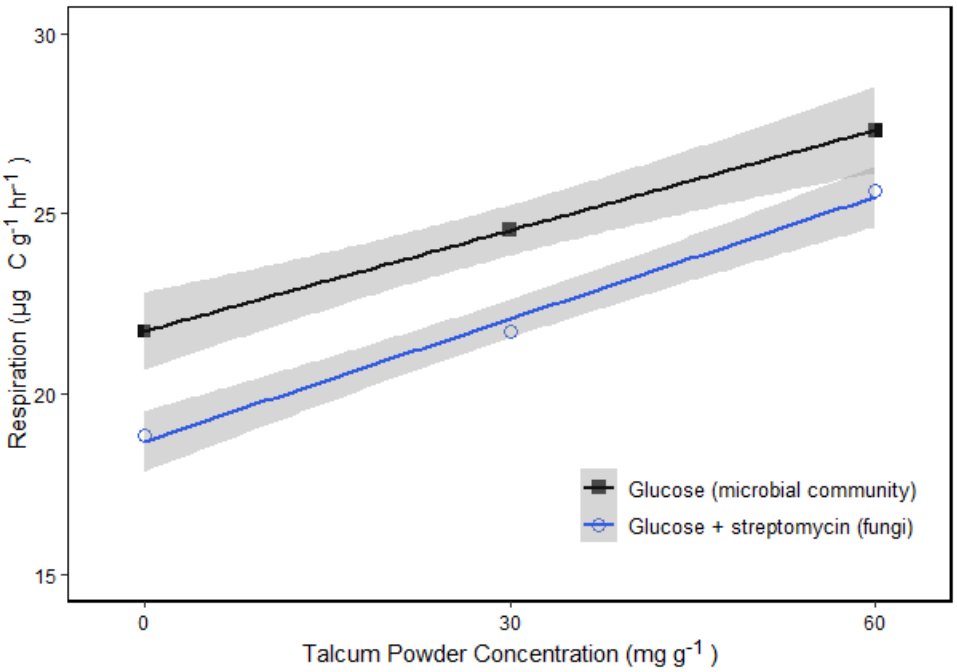


Figure 3.8 Respiration (µg C hr^{-1} g^{-1}) following addition of streptomycin mixed with varying quantities of talcum powder. Shaded areas represent standard error of the slope.

Overall, results from this experiment revealed that mixing streptomycin with talcum powder and adding this mixture to soil stimulated respiration, as has also been reported in previous studies (Lin & Brookes, 1999; Susyan et al., 2005). Talcum powder may increase CO_2 concentrations by accelerating the release of CO_2 from organo-mineral complexes (Anderson & Domsch, 1978). It was concluded that mixing inhibitors with talcum powder would not be useful in the final developed method.

3.4.5 Inhibitor concentration optimisation

The objective of this experiment was to find the concentration of streptomycin that caused the greatest inhibition, following the recommendations of West (1986). To test this, soil (1.25 g ± 0.01 g) was added to 21 Hungate tubes, with three tubes per treatment. Tubes were treated with one of six different streptomycin concentrations, ranging from 20 mg g^{-1} ± 1 mg soil up to 120 mg g^{-1} ± 1 mg soil in 20 mg intervals. Additionally, there was an uninhibited treatment that only received a dosage of glucose. All tubes were treated with glucose solution (0.125 mL g^{-1} soil) following streptomycin addition, sealed with a rubber septum and screw cap, vortex mixed, then left to incubate for five hours at 30 °C. Accumulated CO_2 was measured to determine differences in respiration between concentrations. This experiment was tested for a total of six times.

Increasing streptomycin concentrations from 0 mg g^{-1} to 120 mg g^{-1} generally caused a significant decrease in respiration (P = < 0.001). However, this pattern was not consistent across the range of tested concentrations, as respiration appeared to remain relatively steady from 20 mg g^{-1} to 80 mg g^{-1} before respiration decreased further at 100 mg g^{-1} (**Figure 3.9**). Results from t-tests showed that respiration from streptomycin concentrations of 100 mg g^{-1} was significantly lower than at 80 mg g^{-1} (P = 0.014) but was not significantly different from respiration at 120 mg g^{-1} (P = 0.472). This suggested that 100 mg g^{-1} was the approximate threshold that minimised respiration. As such, the inhibitor concentration used in the final method was 100 mg g^{-1}.

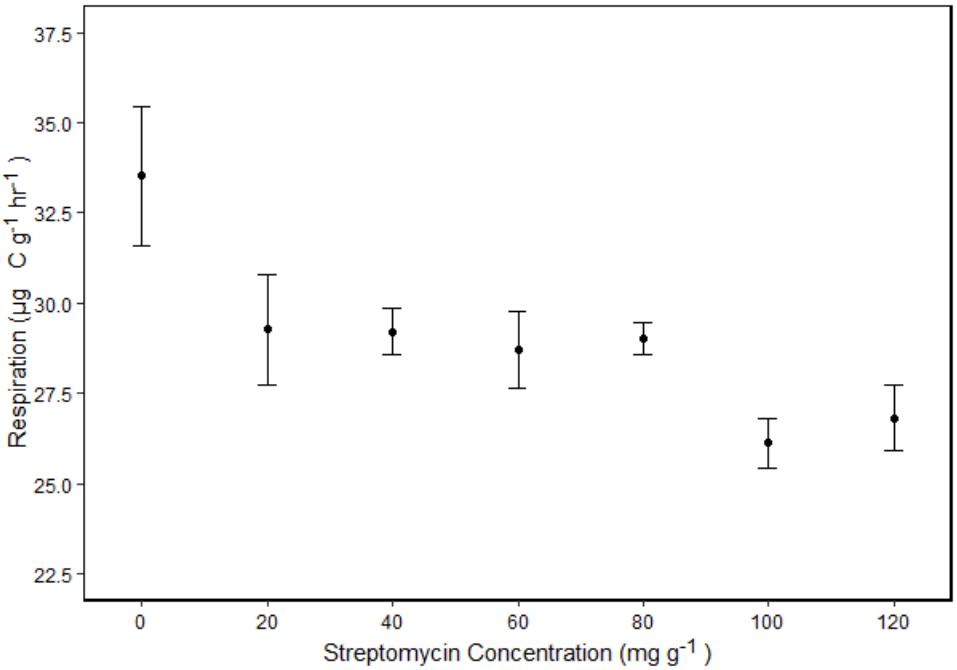


Figure 3.9 Effect of increasing streptomycin concentration (mg g^{-1}) on substrate-induced soil respiration (µg C hr^{-1} g^{-1}) with standard error bars.

This concentration was greater than previous studies, which typically used concentrations in the range of 1 to 10 mg/g soil (Ingham & Coleman, 1984; Badalucco et al., 1994; Hu et al., 1997; Blagodatskaya & Anderson, 1998; Chen et al., 2014). Few studies have tested concentrations above 10 mg/g soil (Bewley & Parkinson, 1985; Scheu & Parkinson, 1994), let alone up to 100 mg/g soil (Ananyeva et al., 2006). It is likely that optimal inhibitor concentrations are dependent on soil type and its capacity to sorb added inhibitors.

3.4.6 Testing change in substrate-limited respiration after 45 days

The aim of this experiment was to test how much substrate-limited soil respiration (i.e., no added glucose) diminished after 45 days. This experiment was completed before the final respiration inhibition experiments to test whether soil respiration changed significantly 45 days after soil collection. This was taken as a precaution in case the final inhibition experiments took longer than anticipated.

To test this, respiration measurements were run on three different bags of soil collected adjacent to the geothermal temperature gradient. Within days after collection of soils (referred to as day 0), tests were run to determine the substrate-limited temperature response of soil respiration. Soils were left at room temperature for 45 days before being retested to determine changes in soil respiration over this time period (day 45).

Soil (1.25 g ± 0.01 g) was added to 54 Hungate tubes, with each of the three soils being added to one set of 18 Hungate tubes. All soils were amended with distilled water (0.125 mL g^{-1}) and vortex mixed. Soils were sealed and placed in the temperature block to incubate for five hours (see section 3.5.3 below). After incubation, CO_2 accumulation was measured from each tube to establish a respiration curve along incubation temperatures (see section 3.5.6 below). This protocol was repeated on the same bags of soil after 45 days.

Initial observations when comparing respiration curves between day 0 and day 45 indicated that there may have been a slight decrease in substrate-limited soil respiration after 45 days (**Figure 3.10**). Simple t-tests were conducted between respiration rates from day 0 and day 45 at each temperature. Across all comparisons, there was found to be no significant difference between respiration rates at any point in the curve between the two different dates ($P > 0.05$ for all comparisons).

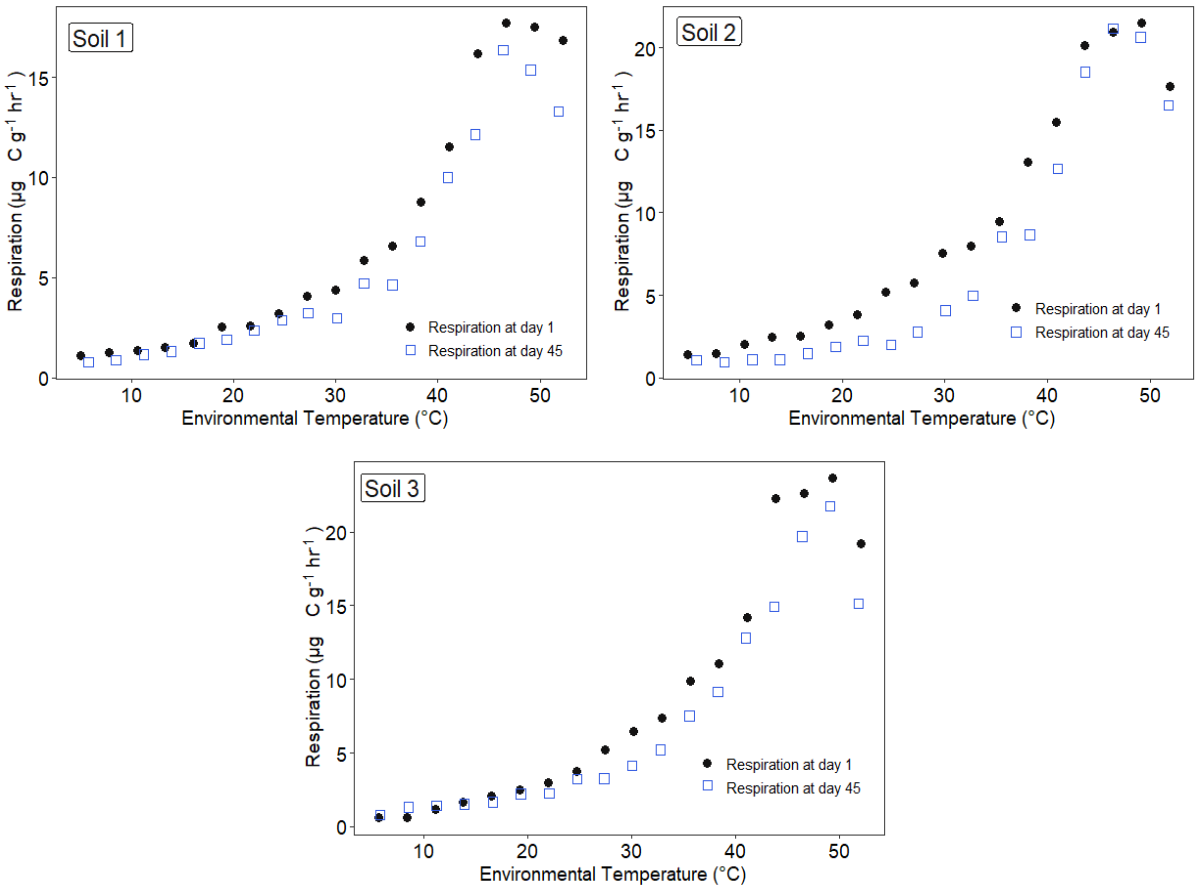


Figure 3.10 Substrate-limited respiration (µg C hr^{-1} g^{-1}) from soils at day 0 and after 45 days of storage prior to analysis.

3.5 Respiration inhibition

After previous preliminary experiments, the final optimised method is described in the following sections.

3.5.1 Selection of sample sites

The profile of the geothermal temperature gradient was previously characterised by Van de Laar (2021), who identified different temperature ranges along the gradient (**Figure 3.2**). This profile was used as the basis for determining sample locations (**Figure 3.11**) and was monitored year round using iButtons (**Figures 3.3**, **3.4**). Collected samples needed to be reflective of the range of temperatures of the gradient; however, the first 85 cm of the gradient had been heavily sampled. This reduced the number of available higher temperatures as samples were not taken within 5 cm of previous core locations. The undisturbed sections of the gradient were divided into 5 cm intervals and grouped based on temperature to provide replication at similar

temperatures. This was done to ensure that sampling was evenly spread across the range of available temperatures. Two sampling distances from each temperature range were randomly selected, totalling 14 samples (**Figure 3.11**). These distances were first sampled for PLFA analysis to confirm distribution of fungi and bacteria biomass along the gradient. Sites were subsequently resampled for the respiration inhibition experiments to provide fresh samples with the assumption that fungal and bacterial biomass had not changed substantially between sampling dates. This prevented samples from being stored for long periods before respiration analysis.

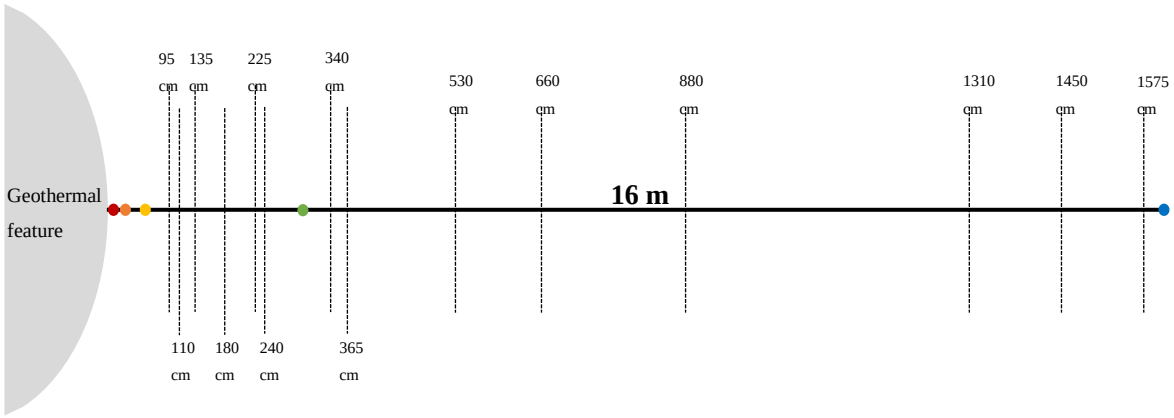


Figure 3.11 Diagram of sampling distances from the geothermal temperature gradient. Sampling locations were first cored for collection of PLFA samples on 4th April 2022 and cored a second time on 8th June 2022 to collect samples for respiration inhibition experiments. Coloured dots represent iButton locations: 10 cm, 30 cm, 60 cm, 300 cm, 1600 cm out from the geothermal feature (from left to right).

3.5.2 Collection of respiration inhibition samples

Collection of samples for respiration inhibition experiments took place on the morning of 8th June 2022. A bucket sampler (7.5 cm depth, 2.5 cm diameter) was used to collect soil cores along transects at each of the 14 sampling distances. Twenty cores were collected at each sampling distance and placed in plastic zip lock bags, one bag for each sampling distance. The bucket sampler was cleaned with ethanol and thoroughly rinsed with distilled water and wiped dry between collecting each sample from different distances.

The soil samples were sieved to 2 mm over the span of three days. Soil was sieved and placed in new plastic zip lock bags, removing any noticeable plant biomass (such as grass and large root clumps) and stones in the process. The sieve and collection tray were cleaned with ethanol and thoroughly rinsed with distilled water and wiped dry prior to the sieving of each sample.

After sieving, each bag was sealed and plugged with a cotton ball to facilitate gaseous exchange between the bag and the outside atmosphere.

The soil moisture content was measured for each sample. The moisture content of some samples was unusually high (compared to 0.35-0.80 gg^{-1} found in Van de Laar, 2021), likely because there had been heavy rain the days prior to collection, saturating the soils. Soils that were unusually moist were dried until they were within a typical field moisture range (0.35-0.80 gg^{-1}) (Van de Laar, 2021). Respiration inhibition samples were stored at room temperature over the eight-week period during which the incubations were run. Sample details, such as incubation dates, calculated temperatures, and soil moisture contents can be found in Appendix A.

3.5.3 Incubation methods

To determine the temperature response of soil respiration, soils were incubated at a range of temperatures using a temperature block (**Figure 3.12**) (Robinson et al., 2017). Soils from each sampling location were incubated on different days, meaning that one soil sample could be run per day.

A temperature block is an insulated aluminium block that is heated on one end and cooled on the opposite end using an ice bath and recirculating antifreeze, creating a temperature gradient along the length of the block. The cold end of this block produced a minimum average temperature of ~5.2 °C while the hot end produced a maximum average temperature of ~52.3 °C. This temperature block consisted of 18 cells that each had a distinct temperature that were strongly linearly related along the length of the block. Three Hungate tubes (15 mL) were able to fit in each cell, allowing three different soil treatments to be incubated concurrently, totalling 54 tubes maximum. Temperatures along the temperature block were monitored using iButtons, which were placed in the cells 1 (~5.2 °C), 7 (~21.8 °C), 13 (~38.5 °C), and 18 (~52.3 °C) to record the temperature of the cell throughout the incubation. A regression was used to calculate the temperature along the rest of the block using these measured temperatures.

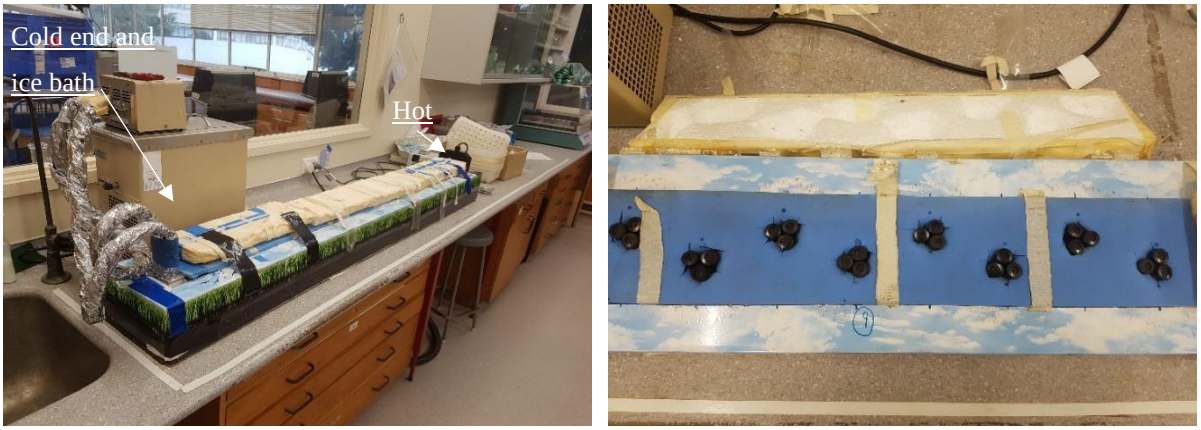


Figure 3.12 Image of the temperature block, note labelled cold and hot ends. Image on the right shows the treatment tubes inserted into the temperature block prior to being covered with polystyrene during incubation.

3.5.4 Treatment groups

Soils were incubated with and without added glucose ($C_6H_{12}O_6$) to determine the non-substrate limited temperature response. Soils without added glucose had an equivalent volume of distilled water added in order to keep moisture contents consistent between tubes. Glucose and streptomycin ($C_{21}H_{39}N_7O_{12} \cdot 1.5\ H_2SO_4$) were added to soil to reduce the bacterial response relative to the total substrate-induced respiration response (glucose only), allowing estimation of the fungal respiration response. Soil treated with glucose and cycloheximide ($C_{15}H_{23}NO_4$) partly inhibited the fungal response from the total substrate-induced respiration response, leaving the bacterial respiration response. Glucose was used as it is readily soluble in water and can be easily dispersed in soil. In addition, the majority of soil microorganisms are capable of utilising glucose (Anderson & Domsch, 1973b). It has also been shown to be an effective C source in both substrate-induced respiration experiments as well as substrate-induced respiration inhibition experiments (Anderson & Domsch, 1973b; Numa, 2020). Streptomycin is a bactericide that operates by binding to 16S rRNA on the 30S component of the bacterial ribosome, thereby inhibiting protein synthesis by interrupting the formation of the peptide bond. This specifically targets aerobic bacteria (Waters & Tadi, 2022), inhibiting a large portion of the bacterial component of soil respiration. Cycloheximide is a fungicide that blocks the elongation phase of eukaryotic translation by binding to the ribosome and inhibiting eEF2-mediated translocation (Schneider-Poetsch et al., 2010), thereby inhibiting fungal respiration. These inhibitors have been widely used in selective inhibition studies due to their supposed selectivity (Anderson & Domsch, 1974; Anderson & Domsch, 1975; Nakas & Klein, 1980;

Chen et al., 2014). However, as with all inhibitors, specificity and degree of inhibition varies between soils and their properties (Lin & Brookes, 1999; Ananyeva et al., 2006).

Due to limited supplies of cycloheximide, a full set of 18 tubes could not be treated with cycloheximide. Therefore, a partial set of 14 out of 18 tubes were treated with cycloheximide for seven of the 14 samples collected along the geothermal gradient. When placed in the temperature block, cycloheximide treatments were absent in cells 3, 6, 9, and 12. The seven samples to receive glucose and cycloheximide treatment were decided by randomly selecting one of the two samples from each of the seven temperature ranges identified along the gradient.

Consequently, seven samples (those that were randomly selected) received treatments of (i) glucose, (ii) glucose and streptomycin, and (iii) glucose and cycloheximide, with distilled water treatment being run on a separate date as only three treatments could be run concurrently (**Figure 3.13**). A further seven samples received treatments of (i) glucose, (ii) glucose and streptomycin, and (iii) distilled water (**Figure 3.14**). Incubation dates and details are available in Appendix A.

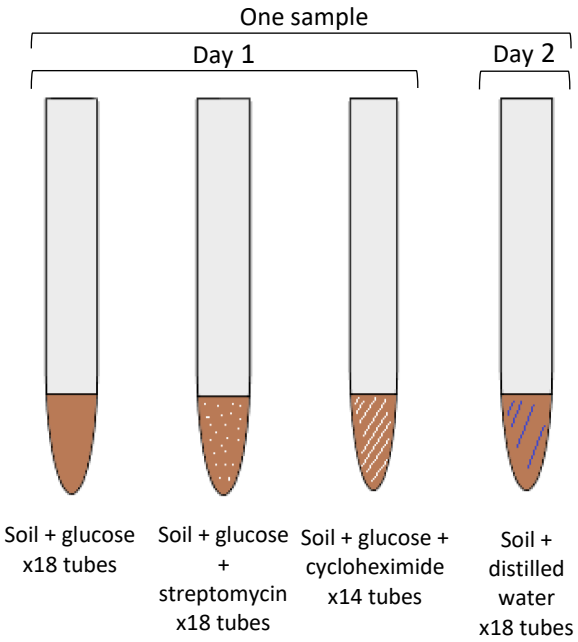


Figure 3.13 Treatment arrangements used for sampling distances 110 cm, 135 cm, 240 cm, 340 cm, 660 cm, 880 cm, and 1575 cm. As only three treatments could be run at the same time, two separate incubations had to be run for these samples. Soil treated with distilled water was run under a separate incubation at a later date (see Appendix A for details). Note that it was only possible to treat 14 tubes per sample with cycloheximide due to supply constraints.

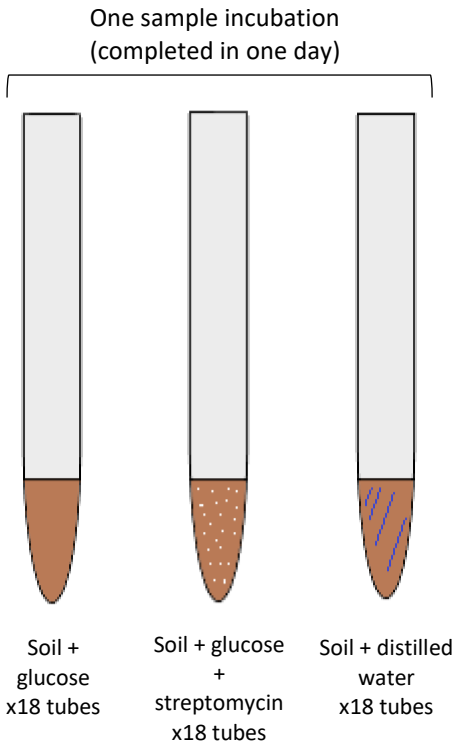


Figure 3.14 Treatment arrangement for one incubation used for the following sampling distances: 95 cm, 180 cm, 225 cm, 365 cm, 530 cm, 1310 cm, and 1450 cm. These experiments received streptomycin treatment but not cycloheximide treatment due to insufficient supplies of cycloheximide.

3.5.5 Inhibition methods

For sample incubations following the treatment arrangement displayed in **Figure 3.14**, soil (1.25 g, ± 0.01 g) was added to 54 Hungate tubes on the morning of the incubation: 18 to be treated with glucose, 18 for glucose and streptomycin, and 18 for distilled water. For samples with the treatment arrangement shown in **Figure 3.13**, soil was added to 50 Hungate tubes: 18 for glucose, 18 for glucose and streptomycin, and 14 for glucose and cycloheximide. On the second incubation date for these latter samples, soil was added to 18 Hungate tubes for distilled water treatment.

One sample incubation was run per day. Streptomycin (AppliChem) (100 mg g^{-1} soil ± 1 mg) was added to one set of 18 tubes of soil. For samples receiving cycloheximide treatments, cycloheximide (AG Scientific; Sigma-Aldrich (≥95% HPLC)) (100 mg g^{-1} soil ± 1 mg) was added to 14 tubes of soil. Inhibitors were added to soils in a fume hood. For long-term storage, inhibitors were stored in a refrigerator (~6 °C) and were taken out and left at room temperature two days prior to use. Directly after inhibitor addition, tubes were briefly sealed and vortexed to disperse inhibitors throughout the soil. An Eppendorf 20-200 µL pipette was used to apply

glucose solution to all treatment tubes following the ratio recommended by Numa et al. (2021) (0.125 mL g^{-1} soil). Glucose solution was prepared the day prior to the experiment and stored in a container at room temperature. To make the solution, 0.2702 g of glucose powder (D-GLUCOSE, Ajax Chemicals UNIVAR 783-500G) was dissolved in 20 mL of distilled water (Numa et al., 2021). This solution was divided into aliquots, one for each treatment, in order to reduce the chance of contamination between treatments when pipetting. The same volume of distilled water was added to tubes receiving only distilled water. Tubes were sealed with a rubber septum and a screw cap and vortexed to distribute the glucose solution or distilled water throughout the soil. Four blanks (tubes with no soil) were sealed at the same time as the other tubes. Tubes were subsequently inserted into the 18 cells of the temperature block, one tube from each of the three treatment groups into each cell. Cycloheximide treatment tubes were absent from cells 3, 6, 9, and 12 for the seven selected samples. Blank tubes were put aside and left at room temperature. Tubes were left to incubate for five hours prior to headspace gas sampling and analysis for CO_2 (see section 3.5.6 below).

3.5.6 Headspace sampling and carbon dioxide analysis

Once the 5-hour incubation was complete, the temperature block was turned off, sample tubes were removed and placed on ice to minimise respiration past the incubation period. Measurement of CO_2 concentrations in the headspace was completed within approximately 30 minutes of the incubation period by taking gas samples (1 mL) from the headspace of each tube using insulin syringes (Becton-Dickinson and co.) and injecting them into the IRGA (see below for CO_2 analysis). The headspace of the sample was mixed before the final 1 mL gas sample was taken by pumping the syringe up and down four times. Samples from blank tubes were injected last to account for background CO_2 concentrations.

An infrared gas analyser (IRGA; LI-COR, LI-7000 CO_2/H_2O Analyser) was used to measure CO_2 concentrations from the headspace of the Hungate tubes. A nitrogen carrier gas cylinder was turned onto 20 psi, sending nitrogen gas through a flow manifold into the IRGA (**Figure 3.15**). The IRGA was turned on, left to warm up for approximately 10 minutes, calibrated, and used to run CO_2 gas standards approximately 30 minutes before the end of the incubation period. Insulin syringes were used to take triplicate 0.08% CO_2 gas standards of known volumes and inject them through a septum of the flow manifold tubing, into the carrier nitrogen gas. The needle of these syringes was welded onto the barrel, reducing the chance of gas leakage from

the syringe (Robinson, 2016). Gas flowed into the IRGA, which was analysed using LI7000 software (version 2.0.3) and Matlab code (R2019b) (see section 3.5.7 below).

Figure 3.15 Images of tools used for gas sampling. Nitrogen carrier gas flows from the gas cylinder (bottom right) through the flow manifold (left) into the IRGA (top right).

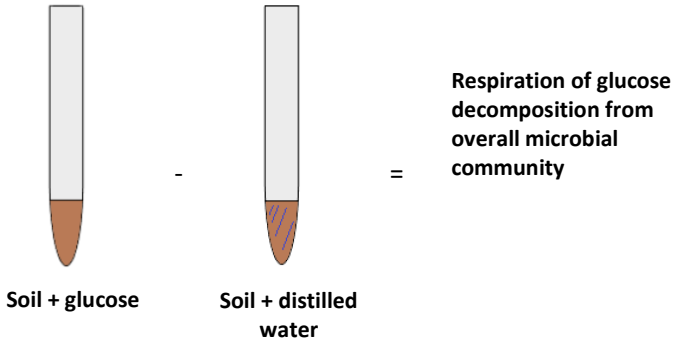
3.5.7 Data analysis

A calibration curve was generated by averaging the area from each of the CO_2 standard injection volumes. The largest and smallest areas of the headspace samples dictated the range of standards used. A polynomial fit was used to fit the standard curve ($R^2 > 0.997$). Respiration rates (R_S) (µg C g^{-1} hr^{-1}) were calculated for each sample on Microsoft Excel 2016 using equation (3-1).

$$R_s = \left[\left(\left(\frac{H_s/V_i}{H_{st}/V_i}\right) - \left(\frac{H_b/V_i}{H_{st}/V_i}\right)\right) \times S \times V \times 10^3\right] \div (ODW \times t) \qquad (3\text{-}1)$$

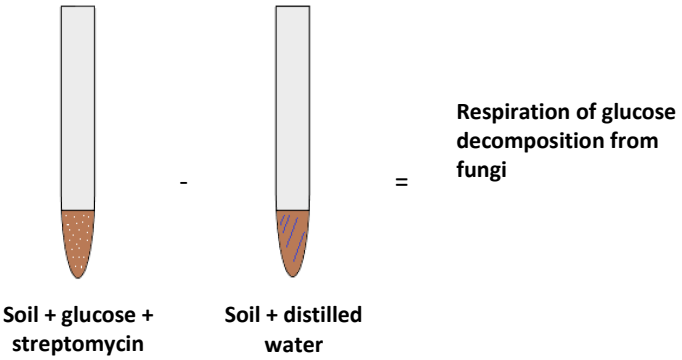
Where H_s, H_{st}, and H_b are curve areas of the sample (mV s^{-1}), CO_2 standard (0.08% CO_2; mV s^{-1}), and background concentrations from blank tubes (mV s^{-1}), respectively. V_i is the volume of the standard injection (mL). S is the concentration of CO_2 in the standard (0.08% = 0.0008 µg CO_2 mL^{-1} gas), V is the headspace volume (mL), ODW is the oven dried soil weight (g), and t is the incubation length (hr) (Robinson, 2016; Numa, 2020; Van de Laar, 2021).

By taking the difference in respiration rate between soil treated with and without added glucose, respiration from glucose decomposition from the overall microbial community could be separated from SOM decomposition, as shown in equation (3-2).



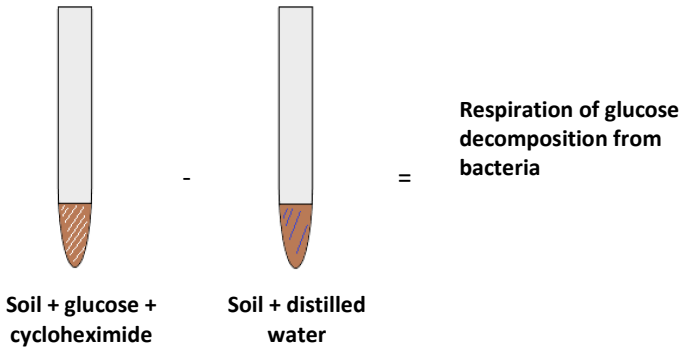
(3-2)

Subtracting the respiration of soil treated with distilled water from soil treated with glucose and streptomycin allows estimation of substrate-induced respiration, with greater contribution from the fungal community as a proportion of the bacterial community was inhibited. This is shown in equation (3-3).



(3-3)

Similarly, subtracting the respiration of soil treated with distilled water from soil treated with glucose and cycloheximide derived the non-substrate limited bacterial respiration response as a proportion of the fungal community was inhibited. This is shown in equation (3-4).



(3-4)

Contributions from each microbial group to total soil respiration was expressed as the percent difference between uninhibited substrate-induced respiration and substrate-induced respiration inhibited by streptomycin or cycloheximide, as shown in equation (3-5) (Chen et al., 2014). Negative inhibition was occasionally calculated when respiration from inhibitor treatment was greater than respiration from soil treated with glucose. These occasional values that indicated stimulation of respiration (negative inhibition values) were excluded from the dataset.

$$Inhibition\ (\%) = \frac{(A - B) \times 100}{A} \tag{3-5}$$

Where A is represents respiration from soil treated with glucose and B represents respiration from soil treated with glucose and streptomycin or cycloheximide.

3.5.8 Curve fitting

Macromolecular rate theory (MMRT) was used to model the respiration response of the different sample treatments with temperature. From the parameters produced, the temperature optima (T_{opt}; the point where respiration rate is maximal) and inflection point (T_{inf}; temperature where change in respiration rate is greatest) were of greatest interest for making comparisons between treatments (Robinson, 2020; Numa, 2020).

The model fits from MMRT 1.0 did not fully characterise the temperature response of the collected respiration data, resulting in an overestimation of T_{opt} and T_{inf} values. An updated version of MMRT, MMRT 2.0, is under development, so in the meantime an intermediary

version, MMRT 1.5, was used to fit the respiration data, as shown in equation (3-6), providing a better fit than MMRT 1.0.

$$ln(R_s) = ln\left(\frac{k_B T}{h}\right) - \frac{\Delta H^{\ddagger}_{T_0}}{RT} - \frac{\Delta C^{\ddagger}_P (T - T_0)}{RT} + \frac{\Delta S^{\ddagger}_{T_0}}{R} + \frac{\Delta C^{\ddagger}_P (lnT - lnT_0)}{R} \quad (3\text{-}6)$$

Where R_s is the respiration rate, k_B is Boltzmann's constant, T is temperature (K), h is Planck's constant, R is the universal gas constant, $\Delta H^{\ddagger}_{T_0}$ is the change in enthalpy (J mol^{-1}), $\Delta S^{\ddagger}_{T_0}$ is the change in entropy (J mol^{-1} K^{-1}), $\Delta C^{\ddagger}_P$ is the change in heat capacity (J mol^{-1} K^{-1}), T_0 is the reference temperature (at T_0; 300 K), ‡ represents the transition state, and R is the universal gas constant (Alster et al., 2022).

Additionally, MMRT 1.5 allows $\Delta C^{\ddagger}_P$ to vary linearly with temperature using a non-linear least-squares regression, as shown in equation (3-7), giving model curves more flexibility to better fit the data.

$$\Delta C^{\ddagger}_p = A(T - T_0) + B \quad (3\text{-}7)$$

Where A is the slope and B is the intercept. While allowing $\Delta C^{\ddagger}_P$ to vary with temperature adds complexity to the model equation, the primary purpose of filling was to determine the T_{opt} and T_{inf} (Alster et al., 2022).

3.5.9 Statistical analysis

Paired, two-tailed t-tests were used to determine if the T_{opt} and T_{inf} varied between treatment groups. Linear regressions were used between derived T_{opt} values and environmental temperature as well as derived T_{inf} values and environmental temperature. Paired, two-tailed t-tests were also used to compare T_{opt} and T_{inf} values to those found by Van de Laar (2021). Additionally, the efficacy of streptomycin and cycloheximide was tested by plotting average streptomycin inhibition and cycloheximide inhibition across all samples with incubation temperatures, allowing the estimation of the temperature sensitivity of inhibitors.

3.6 Microbial biomass

3.6.1 Collection of samples for phospholipid fatty acid analysis

Collection of soil samples for PLFA analysis took place on the morning of 4th April 2022, using a similar procedure of that used to collect the respiration inhibition samples. Soil cores were collected along transects from each of the 14 sampling distances using a bucket sampler (7.5 cm depth, 2.5 cm diameter). Collected cores from each sampling distance were placed into two separate plastic zip lock bags. Ethanol was used to clean the bucket sampler between samples, which was rinsed with distilled water and wiped dry. The soil samples were sieved to 2 mm and placed in new bags on the day of collection, removing large roots and stones in the process. The sieve and collection tray were cleaned with ethanol, rinsed with distilled water, and wiped dry prior to the sieving of each sample. On 6th April 2022, these samples were flash frozen using liquid nitrogen (-196 °C) and lyophilized overnight using a Buchi Lyovapor L-200. These samples were processed at Victoria University of Wellington for FAME (fatty acid methyl ester) analysis between 11th April and 14th April 2022.

3.6.2 Phospholipid fatty acid analysis

Fatty acid methyl ester analysis was used to estimate microbial biomass in geothermal soil sub-samples. The PLFA fraction from this analysis was also used to differentiate fungal and bacterial biomass in the sampled soils. A modified Bligh & Dyer method was used to extract lipids from geothermal soils (Lewe et al., 2021).

For each sample, soil (0.50 g ± 0.05) was weighed into 10 mL screw cap glass tubes. To extract the lipids, a mixture of chloroform, methanol, and phosphate-buffer (1:2:0.8; 4 mL) was added to each tube. The phospholipids 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (20 nmol) and neutral 19:0 Trinonadecanoin (20 nmol) (Avanti Polar Lipids, Inc., US) were added to tubes as internal standards. Samples were sonicated in an ultrasonic bath before being rotated in the dark for 2 hours. Samples were then placed in a centrifuge for 10 minutes at 3500 rpm. The liquid phase from these tubes was transferred into new glass tubes containing chloroform (1 mL) and water (1 mL) which were subsequently vortexed. Tubes containing the liquid phase were centrifuged for 15 minutes at 4500 RPM before the lower phase was pipetted into a new tube, which was then evaporated under nitrogen in a sample concentrator.

The lipid extracts were resuspended in chloroform (1 mL) and vortexed. Methanol (1 mL) and chloroform (1 mL) were passed through the silica wells (50 mg, Thermo Fisher Scientific) of SPE plates to condition them. Samples were passed through these wells, separating the phospholipids from the neutral lipids and glycolipids. Chloroform (1 mL) was passed through the wells to elute the neutral lipids which were collected by a collection plate. Wells on the SPE plates were washed with acetone (1 mL) to elute the glycolipids. Wells were washed with elution solution (0.5 mL) to elute phospholipids, which were collected in collection tubes placed below the wells. The neutral lipid and phospholipid samples were evaporated with nitrogen using a sample concentrator.

Transesterification reagent (0.2 mL) was added to all neutral lipid and phospholipid samples, which were then vortexed and left to incubate for 15 minutes at 37 °C. To extract the lipids, acetic acid (0.2 mL) was added to the samples and vortexed, followed by chloroform (0.4 mL) and vortexed. For each sample, the bottom 0.3 mL of this solution was pipetted into new microtubes. The chloroform extraction was then repeated, except the bottom 0.4 mL of the solution was pipetted into the new microtubes with the rest of the extracts. The chloroform in these microtubes was evaporated with nitrogen using a sample concentrator, leaving the extracted samples. The samples were dissolved in hexane (75 µL) and transferred into gas chromatograph vials. A Shimadzu Gas Chromatograph and Mass Spectrometer was used to separate the FAMEs within the sample extracts contained within the gas chromatograph vials.

3.6.3 Data analysis

Lipid concentrations from FAME analysis were calculated using equation (3-8).

$$\text{Lipid content } (nmol\ g^{-1}) = \left(\left(A_{PLFA} \times \left(\frac{0.267}{A_{IS}}\right)\right) \times \left(\frac{1}{RRF}\right)\right) \times \left(\frac{V}{sample\ weight}\right) \quad (3\text{-}8)$$

Where A_{PLFA} is the area of the phospholipid and A_{IS} is the area of the internal standard. RFF is the relative response factor (the ratio of the response factor of a given FAME to the response factor of the FAME 19:0 internal standard). *V* represents the volume of hexane (in this case 75

µL) the sample lipids were dissolved in, and sample weight refers to the weight of the soil sub-sample taken at the beginning of the procedure (Lewe et al., 2021; Moyle, 2022).

The FAMEs collected from the analyzed soils can be used as biomarkers for different microbial groups. A summary table for each FAME found in the soils and the microbial group they are indicative of is shown below (**Table 3.1**). Fungal to bacterial ratios were calculated by dividing the biomass of fungi by the cumulative biomass of all bacterial groups.

Table 3.1 Extracted FAMEs and their associated microbial groups (Bentley, 2021; Moyle, 2022).

Microbial group biomarker	FAME	Reference
Actinomycetes	10Me16:0, 10ME17:0, 10ME18:0	Vestal & White, 1989; Francisco et al., 2016
Arbuscular mycorrhizae	16:1ω5c, 16:1ω5t	Olsson et al., 1995
Bacteria	15:0, 16:0, 17:0, 18:0	Zelles, 1997
Fungi	18:2ω6, 18:3ω3, 18:1ω9c	Ahlgren et al., 1992; Zelles, 1997
Gram negative bacteria	2OH10:0, 2OH12:0, 3OH12:0, 2OH14:0, 3OH14:0, 16:1ω7c, 16:1ω7t, 16:1ω5t, delta17:0, 2OH16:0, 3OH16:0, 18:1ω7c, 18:1ω7t, 19:1ω9c, delta19:0	Parker et al., 1982; Wilkinson & Ratledge, 1988; Zelles, 1997
Gram positive bacteria	i15:0, a15:0, i16:0, a16:0, i17:0, a17:0	Vestal & White, 1989; Francisco et al., 2016

3.6.4 Statistical analysis

Van de Laar (2021) measured the C content of soils taken from along the geothermal temperature gradient, different from the sampling locations of the current study. A linear regression was used on the C content of soils measured by Van de Laar (2021) to estimate the C content of soils used in the current study. Total microbial biomass was calculated by summing the lipid contents of all identified microbial groups. A linear regression was used to test this relationship between calculated C content and total microbial biomass. The

relationship between F:B ratio and environmental temperature was also assessed using linear regressions. To test the efficacy of streptomycin and cycloheximide, a linear regression was used to test the relationship between total microbial biomass and inhibition from streptomycin and cycloheximide.

Chapter 4

Differentiating the Temperature Response of Soil Fungi and Bacteria

4.1 Abstract

Increasing temperatures from climate change could increase soil organic matter (SOM) decomposition, releasing more carbon dioxide (CO_2) into the atmosphere and further enhancing climate change. Fungi and bacteria mediate SOM decomposition but each have unique roles in soil environments. It is possible that fungi and bacteria will respond differently to increasing temperatures, which could affect soil carbon (C) cycling dynamics under climate change. The objective of this study was to differentiate the temperature sensitivity and thermal adaptation of fungi and bacteria in soils collected along a geothermal temperature gradient that ranged in temperature from ~13-39 °C. Collected soil samples were incubated for five hours at 18 distinct incubation temperatures ranging from 5 °C to 52 °C. The non-substrate limited respiration responses of fungi and bacteria were derived by treating soil with streptomycin or cycloheximide, respectively, and glucose. Carbon dioxide accumulation was measured from each treatment using an infrared gas analyser (IRGA). The temperature sensitivities of fungal and bacterial respiration were derived by fitting respiration response curves with macromolecular rate theory (MMRT) and calculating the temperature optimum (T_{opt}) and inflection point (T_{inf}) for each response curve. Microbial biomass was measured by phospholipid fatty acid (PLFA) analysis and derived phospholipids were used as biomarkers to distinguish the biomass of fungal and bacterial groups. Respiration inhibition results showed that the T_{opt} of fungal respiration decreased with environmental temperature at a rate of -0.396 °C °C^{-1} whereas there was no observable trend in T_{opt} of bacteria or the whole community. The T_{opt} and T_{inf} of fungal respiration (38.6 °C and 22.1 °C, respectively) was greater than that of bacteria (30.4 °C and 19.0 °C, respectively) and the microbial community (37.0 °C and 21.7 °C, respectively). Results from PLFA analysis showed that fungal biomass decreased with environmental temperature relative to bacterial biomass. These results suggest that fungi and bacteria respond differently to temperature, which could complicate soil C cycling dynamics under climate change. However, a number of limitations were identified with the selective inhibition method that may constrain interpretation of the results.

4.2 Introduction

The cycling of carbon (C) between the soil and atmosphere is an integral component of global C cycling and climate regulation. This process is dependent on C inputs from plant photosynthesis and the decomposition of these inputs by fungi and bacteria (Janzen, 2004). While there are many proximate controls of microbial respiration, temperature is of particular interest in the context of climate change (Davidson et al., 2006). Increasing surface temperatures from climate change has been postulated to increase fungal and bacterial respiration, which could create feedback loops that could affect soil C stocks. However, the effect of temperature on microbes and their role in soil C cycling is not yet fully understood and requires further research.

Soil contains the largest stock of terrestrial C (Jobbágy & Jackson, 2000). Soil C can be conceptually partitioned based on its quality and availability, such as into particulate organic matter (POM) and mineral-associated organic matter (MAOM). Particulate organic matter consists of more complex organic matter (e.g., fresh plant litter that has been fragmented) that is often less energetically favourable to microorganisms (Lavallee et al., 2020). In contrast, MAOM consists of compounds that are physically protected through adsorption to mineral surfaces (Lavallee et al., 2020). However, fungi and bacteria do not decompose both POM and MAOM equally. Fungi are capable of producing a wider range of extracellular enzymes that allows them to degrade compounds that are more resistant to decomposition, such as POM (Xu & Shang, 2016; Khatoon et al., 2017). In addition, fungi can obtain nutrients by accelerating the release of compounds from mineral surfaces (Jilling et al., 2018). In contrast, bacteria are generally not able to metabolise as wide a range of substrates and tend to consume more readily available substrates (Hunt et al., 1987; Khatoon et al., 2017). It is important to understand the relative roles of fungi and bacteria in soil C cycling, particularly as global surface temperatures increase, which could affect fungi and bacteria differently.

Different methods have been used to separate the activity of fungi and bacteria, such as the selective inhibition method, which partitions fungal and bacterial respiration by treating soil with fungicide or bactericide. The subsequent amendment of an additional labile substrate (e.g., glucose) boosts the respiration of remaining metabolically active cells. Carbon dioxide (CO_2) measurements can be taken from these treated samples, providing an approximation of fungal and bacterial contributions to total soil respiration (Anderson & Domsch, 1973a; Anderson &

Domsch, 1973b). Selective inhibition studies often find that fungal contributions are greater than bacterial contributions (Anderson & Domsch, 1973a; Bailey et al., 2002; Ananyeva et al., 2006). However, the selective inhibition method has some limitations that should be considered. One of the most common limitations of these inhibition studies is that inhibitors do not completely inhibit target organisms and may also inhibit non-target organisms (Beare et al., 1990; Velvis, 1997). Secondly, inhibitors can stimulate respiration, potentially from resistant microorganisms metabolising added inhibitors (Parkinson et al., 1971; Hu et al., 1997; Nakamoto & Wakahara, 2004). Finally, these studies can overlook the contributions of other soil organisms to total soil respiration, such as protists and soil animals (Hopkins & Gregorich, 2005). Nevertheless, selective inhibition studies have provided valuable information on the roles of fungi and bacteria in soil environments, provided these limitations are carefully considered.

Differentiating the microbial biomass of fungi and bacteria is also important in understanding the response of soil microbial communities to changing environments (Bailey et al., 2002; de Vries et al., 2006; Moyle, 2022). Phospholipids contained within cell membranes can be extracted using phospholipid fatty acid (PLFA) analysis, which can be used as a proxy for microbial biomass (Willers et al., 2015). This is frequently expressed as a ratio of fungi to bacteria (F:B) (Frostegård & Bååth, 1996), which is useful for describing the composition of soil microbial communities, particularly in response to changing environmental factors such as pH or temperature (Bååth & Anderson, 2003; Feng & Simpson, 2009). However, this method also has some constraints as some phospholipids have been reported in multiple different microbial groups (Olsson, 1999), complicating full interpretation. Despite these limitations, both selective inhibition and PLFA analysis have been frequently used as viable methods to differentiate soil fungi and bacteria (Chen et al., 2014; Swallow & Quideau, 2020).

The niches of fungi and bacteria overlap to an extent (Rousk et al., 2008), but each group play important differentiable roles in soils. It is likely that increasing temperatures under climate change will affect fungi and bacteria differently, along with their contributions to soil C cycling. As such, it is important to understand the independent temperature responses of fungi and bacteria. The temperature response of soil respiration has previously been characterised by the Arrhenius equation and its derivatives, such as Lloyd and Taylor (1994) as well as Fang and Moncrieff (2001). The Arrhenius equation predicts a continuous exponential increase in reaction rate. Though this accurately describes chemical reactions, this becomes questionable

when applied to biochemical reactions (Schipper et al., 2014; Robinson et al., 2017; Alster et al., 2020). Macromolecular rate theory (MMRT) was developed to describe the temperature response of biochemical reactions more accurately. Macromolecular rate theory recognises that the activation energy of reactions mediated by enzymes is temperature dependent and do not increase continuously (Hobbs et al., 2013). Instead, biochemical reactions often display a temperature optimum (T_{opt}), or a point at which respiration is maximal and subsequently decreases due to changes in enzyme heat capacity (Hobbs et al., 2013; Robinson et al., 2017). In addition, MMRT also allows the calculation of an inflection point (T_{inf}), or a point at which respiration is most sensitive to temperature (Robinson et al., 2017). These parameters have previously been used to describe the temperature sensitivity of respiration from different soils (Schipper et al., 2014; Robinson et al., 2017; Robinson et al., 2020; Numa et al., 2021; Van de Laar, 2021). However, while MMRT has been applied to soil respiration, it has not yet been applied to fungal and bacterial respiration in soil, independent from one another. One of the aims of this research is to characterise the temperature sensitivity of fungal and bacterial respiration with MMRT and compare derived T_{opt} and T_{inf} between the two groups.

Another component of this study was to investigate thermal adaptation in fungi and bacteria across modest temperatures (~13-23 °C) rather than at temperatures that would favour thermophilic microorganisms. Microbial thermal adaptation is not yet fully understood (Bradford, 2013), particularly the differential thermal adaptation of fungi and bacteria. Local thermal adaptation has been identified in soil fungi and bacteria (Laine, 2008; Bárcenas-Moreno et al., 2009; Rinnan et al., 2009; Stefansson et al., 2013), as well as in response to climate change (Zogg et al., 1997; de Crecy et al., 2009; Casadevall et al, 2019). Furthermore, it has been suggested that fungi may be more sensitive to higher temperatures but are favoured at lower temperatures, whereas bacteria are more tolerant of higher temperatures (Pietikäinen et al., 2005; Robert & Casadevall, 2009; Nottingham et al., 2019). However, many studies of thermal adaptation have focused on isolated strains, separated from natural soil environments or on more extreme thermophilic systems that may not have been representative of the broader terrestrial soil environment. It is important to study fungi and bacteria in natural soil systems as these environments are dynamic and may display more complex temperature responses.

In the context of soil respiration, thermal adaptation can be defined as the change in soil respiration with changing temperatures, which generally increases with increasing temperature (Bradford et al., 2008). Microbial thermal adaptation has previously been examined along

elevational (Wang et al., 2012), latitudinal (Dacal et al., 2019), and biome temperature gradients (Bradford et al., 2019). Additionally, a few studies have examined thermal adaptation along modest geothermal temperature gradients (Marañón-Jiménez et al., 2018; Walker et al., 2018; Van de Laar, 2021). Geothermal activity can warm soil within relatively short distances, which could be used as a proxy for comparative soil warming under climate change. A study by Van de Laar (2021) examined microbial thermal adaptation along a geothermal temperature gradient (ranging from 16 – 35 °C within 16 m) and showed that the T_{opt} of microbial respiration increased slightly with increasing environmental temperature at a rate of 0.157 °C °C^{-1}. However, this work did not separate fungal and bacterial contributions to respiration. These geothermal soil temperature gradients present an opportunity to study microbial thermal adaptation and further research is required to corroborate findings from previous studies. This study aims to examine patterns of fungal and bacterial thermal adaptation along a geothermal temperature gradient to improve our understanding of how increasing temperatures affect soil C cycling dynamics.

The primary objective of this study was to differentiate the temperature response (i.e., T_{opt} and T_{inf}) of fungal and bacterial respiration from soils collected from a geothermal temperature gradient in Rotorua, New Zealand, which ranged in temperature from ~13-39 °C. The selective inhibition method was used to partition the non-substrate limited respiration response from fungi and bacteria in collected soils, which were then incubated at temperatures ranging from ~5-52 °C using a temperature block. It was hypothesised that both the fungal and bacterial respiration response (T_{opt} and T_{inf}) would adapt with increasing environmental temperature and that fungal T_{opt} and T_{inf} would be lower than that of bacteria. A secondary objective of this research was to examine changes in fungal and bacterial biomass, as determined by PLFA analysis. It was expected that fungal to bacterial (F:B) ratios would decrease with increasing environmental temperatures.

4.3 Methods

4.3.1 Study site and sample collection

The field site of this study was a naturally occurring geothermal temperature gradient located on the southern end of Rotorua Golf Course. This golf course is located on the Arikikapakapa Reserve in Rotorua, New Zealand; a geothermal area located in the southern region of the

Rotorua geothermal field, which is more broadly encompassed within the Taupō Volcanic Zone. The geothermal feature of this study was classified as thermal ground (Seward et al., 2015), measuring approximately 5.1 m long and 3.8 m wide (Van de Laar, 2021) (**Figure 4.1**).

Figure 4.1 Pictures of the geothermal feature used in this study, taken 4th November 2022.

The soil around the site was described as an inactive hydrothermal recent soil, with a weakly developed structure and a distinct topsoil (Manaaki Whenua, 2019). The vegetation surrounding the geothermal feature consisted primarily of grasses with nearby shrubs, bushes,

and trees. Management of the area consisted only of a regular mowing regimen, with no inputs of fertiliser or lime.

The temperature gradient extended out 16 m east from the geothermal feature. The temperature profile of the gradient was characterised using iButton thermocrons (model DS1922L). These iButtons were placed at five different locations along the temperature gradient, taking one temperature measurement every hour (**Figure 4.2**). Some iButtons were lost on occasion, resulting in gaps in the temperature profile. The temperatures of this gradient fluctuated following rainfall events as well as daily and seasonal cycles where inputs of water rapidly cooled the soil but there was a gradual subsequent recovery of temperature after major rainfall events. Temperatures along the gradient ranged from ~13-39 °C, but samples were collected from soils that had average temperatures in the range of ~13-23 °C (see **Table A1** under Appendix A for further details).

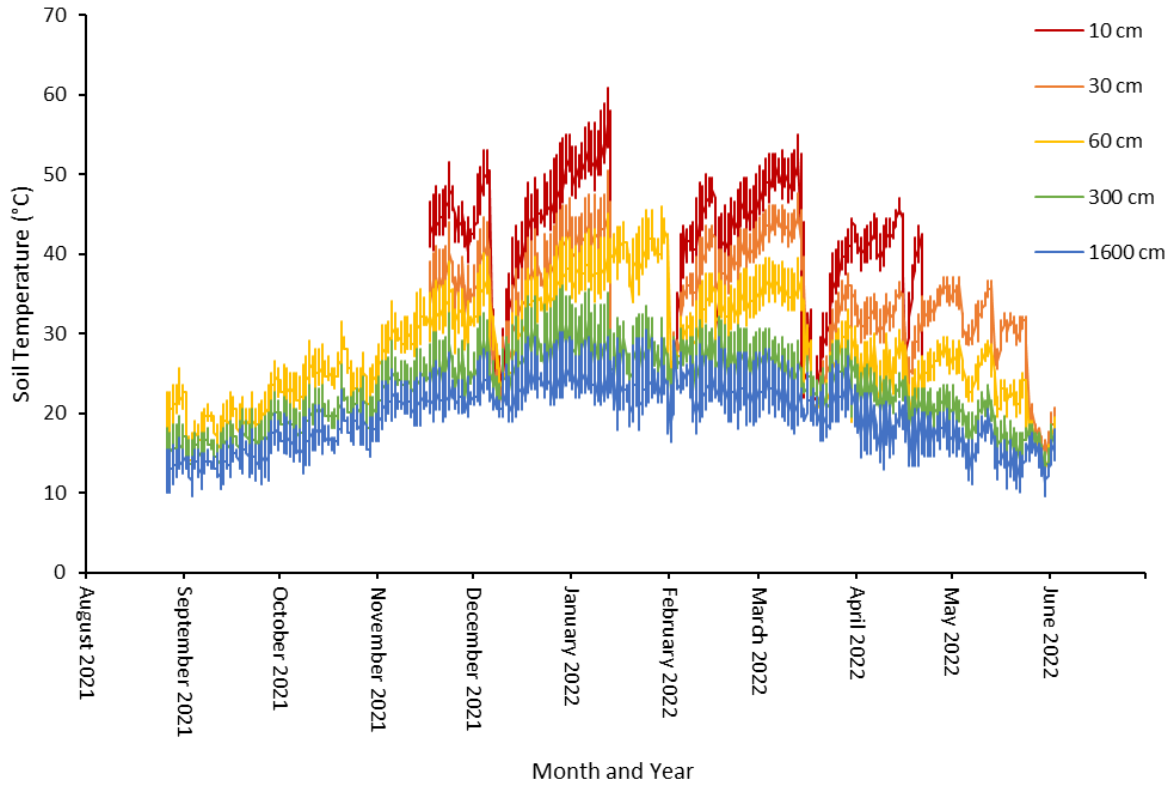


Figure 4.2 Collected temperature data from 1st September 2021 to 8th June 2022 for iButton locations at 10 cm (red), 30 cm (orange), 60 cm (yellow), 300 cm (green), and 1600 cm (blue) away from the edge of the geothermal feature.

The geothermal temperature gradient was first described by Van de Laar (2021) who quantified different temperature ranges along the gradient (**Figure 4.3**). These temperature ranges guided the determination of sample locations for the current study to ensure that samples captured the full range of available temperatures. Samples were not taken within 5 cm of prior coring sites to avoid disturbed sites.

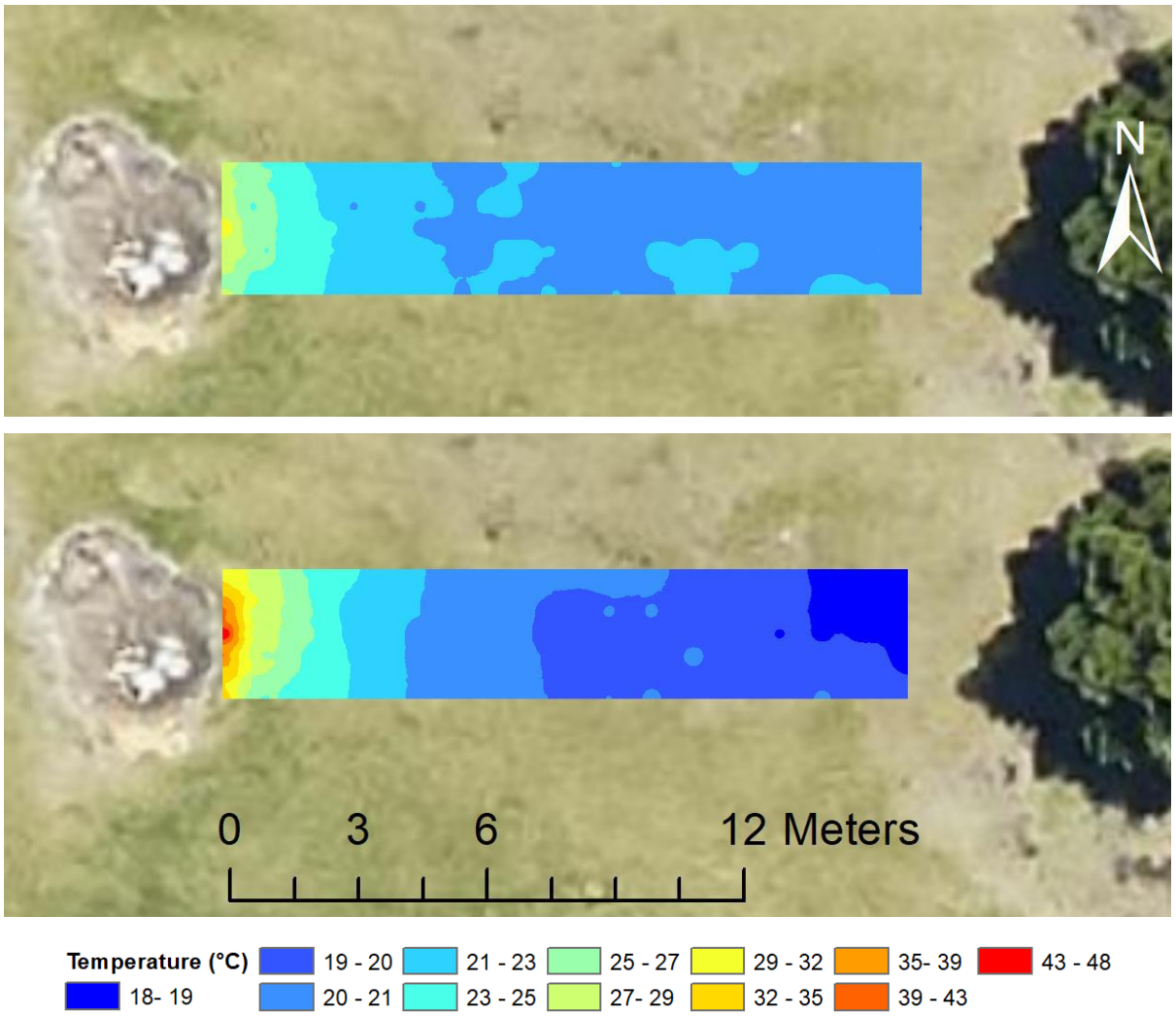


Figure 4.3 Average temperature data from 26th August 2020 to 22nd June 2021 at 2 cm (top) and 10 cm (bottom) depths from Van de Laar (2021).

Sample locations were first sampled for PLFA analysis on 4th April 2022, and resampled for respiration inhibition experiments on 8th June 2022. Soil was collected from 14 different sampling distances along the geothermal temperature gradient (**Figure 4.4**).

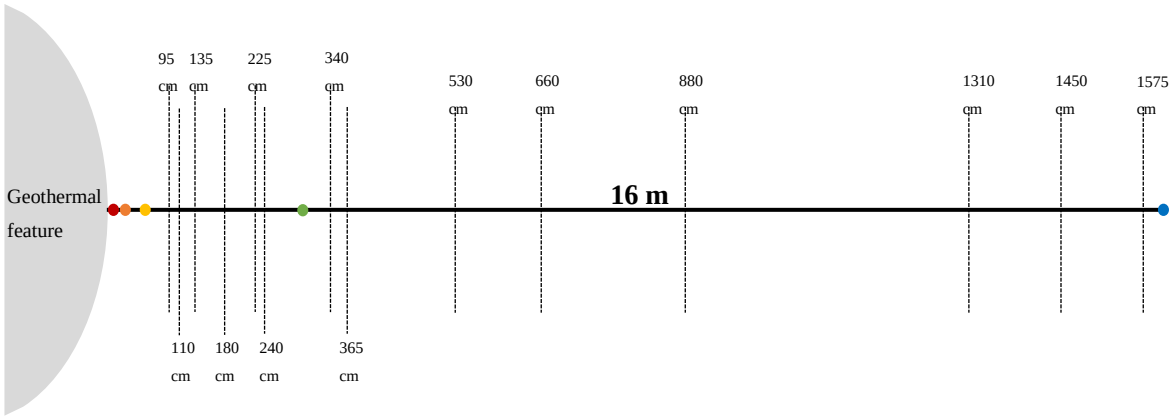


Figure 4.4 Sampling locations along the geothermal temperature gradient. These locations were first cored for collection of PLFA samples on 4th April 2022, then cored again for collection of respiration inhibition samples on 8th June 2022. Coloured dots represent iButton locations: 10 cm, 30 cm, 60 cm, 300 cm, 1600 cm out from the geothermal feature (from left to right).

For both respiration measurements and PLFA analysis, twenty soil cores were collected from each sampling distance using a bucket sampler (7.5 cm depth, 2.5 cm diameter), cleaning the bucket sampler with ethanol and water between collection of samples to reduce cross-contamination. Collected soils were sieved, removing stones and plant matter (i.e., root clumps and grass) and cleaning the sieve and collection tray between samples.

Samples taken for PLFA analysis were flash frozen with liquid nitrogen and lyophilized overnight the week prior to PLFA analysis. For respiration inhibition samples, the soil moisture content was measured for each sample. Samples that exceeded 0.80 gg^{-1} soil moisture were dried at room temperature until they were within a more typical field moist range (0.35-0.80 gg^{-1}) (Van de Laar, 2021). Over the period that incubations were run, soils were left at room temperature in zip lock plastic bags plugged with a cotton ball to allow gas exchange. See **Table A1** under Appendix A for further sample details, including sample temperatures and moisture contents.

4.3.2 Inhibition of soil respiration

A number of preliminary experiments were conducted to optimise the final laboratory method (see section 3.4). The following section describes a more succinct version of the final optimised method.

4.3.2.1 Laboratory methods

A temperature block was used to incubate soils at 18 distinct temperatures ranging from 5.2 °C to 52.3 °C. Each cell of the temperature block could fit three Hungate tubes (15 mL), allowing three different treatments to be incubated simultaneously for each soil sample.

The selective inhibition method was used to differentiate the substrate-induced respiration of fungi and bacteria. Soils were incubated with and without glucose to derive the non-substrate limited temperature response along with the temperature response of respiration from existing SOM where substrate limitation was evident (Robinson et al., 2020). Soils incubated without glucose were treated with an equivalent volume of distilled water to determine the respiration from soil organic matter (SOM) decomposition. To derive the substrate-induced respiration response of fungi, soil was treated with glucose and streptomycin to partially inhibit bacterial respiration. The non-substrate limited respiration response of bacteria was determined by treating soil with glucose and cycloheximide to decrease the fungal response.

For all 14 samples collected along the gradient, the substrate-induced microbial response (glucose), substrate-induced fungal response (glucose and streptomycin), and response from soil organic matter decomposition (distilled water) was determined. Cycloheximide was in limited supply and so the bacterial response (glucose and cycloheximide) was determined for only seven of the 14 samples. Furthermore, cycloheximide treatments were only incubated across 14 of the 18 temperatures along the temperature block, providing a partial temperature response of bacteria. For these select seven samples, distilled water treatment was run on a separate date, with the other three treatments being run concurrently on one incubation day (see **Table A1** under Appendix A and section 3.4.6).

At the start of the day, soil (1.25 g, ± 0.01 g) from one sample was weighed into 54 Hungate tubes. Eighteen tubes were treated with glucose, 18 were treated with glucose and streptomycin, and 18 tubes had distilled water added. For the seven samples receiving cycloheximide treatment, soil was added to 50 Hungate tubes on the first incubation day: 18 for glucose, 18 for glucose and streptomycin, 14 for glucose and cycloheximide. For the second incubation day, soil was added to 18 tubes for distilled water treatment.

Streptomycin (AppliChem) was added at 100 mg g^{-1} ± 1 mg (see section 3.4.5). Cycloheximide (AG Scientific; Sigma-Aldrich (≥95% HPLC)) was added to appropriate treatments at 100 mg g^{-1} ± 1 mg (see section 3.4.5). Glucose solution (0.125 mL g^{-1}) was added to all tubes (except those receiving distilled water) after inhibitor addition (see section 3.4.3). Glucose solution was made by dissolving 0.2702 g of glucose powder (D-GLUCOSE, Ajax Chemicals UNIVAR 783-500G) in 20 mL of distilled water (Numa et al., 2021). An equivalent volume of distilled water was applied to tubes receiving only distilled water. Tubes were sealed with a rubber septum and screw cap. Tubes were vortexed after inhibitor and glucose/distilled water addition to disperse substrate or inhibitor throughout soil. Tubes were then placed into the temperature block and left to incubate for five hours prior to CO_2 analysis.

After incubation, sample tubes were placed on ice to minimise further respiration. Headspace samples (1 mL) were extracted from each tube using a syringe and then injected into an infrared gas analyser (IRGA; LI-COR, LI-7000 CO2/H2O Analyser). For quantification of CO_2, a standard curve was also created by injecting known volumes of 0.08% CO_2 standard into the IRGA to allow the calculation of CO_2 in the headspace of treatment tubes. Addition of inhibitors and measurement of CO_2 accumulation were completed on the same day of incubation.

4.3.2.2 Data analysis and model fitting

Respiration rates measured from distilled water treatment was subtracted from all substrate-induced respiration treatments to separate the non-substrate limited respiration response from the respiration derived from decomposition of SOM. This difference is considered to isolate the temperature response of microbes that are not limited by substrate supply constraints such as sorption/desorption and diffusion. Each respiration response curve was then modelled using MMRT 1.5 in R software version 4.0.2 with equation (4-1). This allowed the calculation of the T_{opt} and T_{inf} of each respiration curve, representing the temperature sensitivity of each treatment.

$$ln(R_s) = ln\left(\frac{k_B T}{h}\right) - \frac{\Delta H^{\ddagger}_{T_0}}{RT} - \frac{\Delta C^{\ddagger}_P (T - T_0)}{RT} + \frac{\Delta S^{\ddagger}_{T_0}}{R} + \frac{\Delta C^{\ddagger}_P (lnT - lnT_0)}{R} \quad (4\text{-}1)$$

Where R_s is the respiration rate, k_B is Boltzmann's constant, T is temperature (K), h is Planck's constant, R is the universal gas constant, $\Delta H^{\ddagger}_{T_0}$ is the change in enthalpy (J mol^{-1}), $\Delta S^{\ddagger}_{T_0}$ is the change in entropy (J mol^{-1} K^{-1}), $\Delta C^{\ddagger}_P$ is the change in heat capacity (J mol^{-1} K^{-1}), T_0 is the reference temperature (at T_0; 300 K), ‡ represents the transition state, and R is the universal gas constant (Alster et al., 2022).

Additionally, $\Delta C^{\ddagger}_P$ was able to vary linearly with temperature using a non-linear least-squares regression, presented in equation (4-2) (Alster et al., 2022).

$$\Delta C^{\ddagger}_p = A(T - T_0) + B \tag{4-2}$$

Where A is the slope and B is the intercept.

Following the fitting of MMRT 1.5, the T_{opt} and T_{inf} was calculated for each respiration curve using equations (4-3) and (4-4), respectively.

$$T_{opt} = \frac{\Delta H^{\ddagger}_{T_0} + \Delta C^{\ddagger}_P T_0}{-\Delta C^{\ddagger}_P - R} \tag{4-3}$$

$$T_{inf} = \frac{\Delta H^{\ddagger}_{T_0} + \Delta C^{\ddagger}_{P.} T_0}{-\Delta C^{\ddagger}_P \pm \sqrt{-\Delta C^{\ddagger}_{P.} R}} \tag{4-4}$$

4.3.2.3 Statistical analysis

Significance between derived T_{opt} and T_{inf} values for each treatment group was calculated using paired, two-tailed t-tests. Linear regressions were used to assess the relationship between derived T_{opt} and T_{inf} values from each treatment and environmental temperature.

4.3.3 Phospholipid fatty acid analysis

4.3.3.1 Identification of fatty acid methyl esters

Fungal and bacterial biomass from sub-samples of each geothermal soil sample was estimated using fatty acid methyl ester (FAME) analysis. Lipids were extracted from geothermal soils

using a modified Bligh and Dyer method (Lewe et al., 2021), of which the PLFA fraction was used to differentiate fungal and bacterial biomass in these soil samples.

Soil (0.50 ± 0.05) was added to glass tubes (10 mL) to which a mixture of chloroform, methanol, and phosphate-buffer (1:2:0.8; 4 mL) was added to extract lipids, along with the internal standard phospholipids 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (20 nmol) and neutral 19:0 Trinonadecanoin (20 nmol) (Avanti Polar Lipids, Inc., US). Samples were sonicated, rotated for 2 hours, and centrifuged for 10 minutes at 3500 rpm. The liquid phase was decanted into new tubes containing chloroform (1 mL) and water (1 mL), which were then dried using nitrogen gas. Lipid extracts were redissolved in chloroform (1 mL) and phospholipids, neutral lipids, and glycolipids were separated by passing samples through silica wells and washing wells with elution solution. Acetic acid was added to derived lipids which were then extracted twice with chloroform (0.4 mL) and dried under nitrogen. Samples were dissolved in hexane (75 µL) and analysed via gas chromatography.

4.3.3.2 Data analysis

Lipid contents from each sample were calculated using equation (4-5).

$$\text{Lipid content } (nmol\ g^{-1}) = \left(\left(A_{PLFA} \times \left(\frac{0.267}{A_{IS}}\right)\right) \times \left(\frac{1}{RRF}\right)\right) \times \left(\frac{V}{sample\ weight}\right) \quad (4\text{-}5)$$

Where A_{PLFA} is the area of the phospholipid and A_{IS} is the area of the internal standard. RFF is the relative response factor (the ratio of the response factor of a given FAME to the response factor of the FAME 19:0 internal standard). *V* represents the volume of hexane (in this case 75 µL) the sample lipids were dissolved in, and sample weight refers to the weight of the soil sub-sample taken at the beginning of the procedure (Lewe et al., 2021; Moyle, 2022).

Derived phospholipids can be used as biomarkers for different fungal and bacterial groups, thereby indicating the biomass of different microbial groups (**Table 4.1**). Fungal-to-bacterial (F:B) ratios were calculated by dividing fungal biomass by bacterial biomass.

Table 4.1 Extracted FAMEs and their associated microbial groups (Bentley, 2021; Moyle, 2022).

Microbial group biomarker	FAME	Reference
Actinomycetes	10Me16:0, 10ME17:0, 10ME18:0	Vestal & White, 1989; Francisco et al., 2016
Arbuscular mycorrhizae	16:1ω5c, 16:1ω5t	Olsson et al., 1995
Bacteria	15:0, 16:0, 17:0, 18:0	Zelles, 1997
Fungi	18:2ω6, 18:3ω3, 18:1ω9c	Ahlgren et al., 1992; Zelles, 1997
Gram negative bacteria	2OH10:0, 2OH12:0, 3OH12:0, 2OH14:0, 3OH14:0, 16:1ω7c, 16:1ω7t, 16:1ω5t, delta17:0, 2OH16:0, 3OH16:0, 18:1ω7c, 18:1ω7t, 19:1ω9c, delta19:0	Parker et al., 1982; Wilkinson & Ratledge, 1988; Zelles, 1997
Gram positive bacteria	i15:0, a15:0, i16:0, a16:0, i17:0, a17:0	Vestal & White, 1989; Francisco et al., 2016

4.3.3.3 Statistical analysis

The C content of soils taken from different locations along the gradient was measured by Van de Laar (2021). The soil samples collected in the current study were not taken at the same locations and so soil C was estimated using a liner interpolation of total C in soils measured by Van de Laar (2021). Total microbial biomass was calculated by adding together the lipid contents of all identified microbial groups. The relationship between calculated C content and total microbial biomass was assessed using a linear regression. Additionally, linear regressions were used to assess the relationship between F:B ratio and environmental temperature. Finally, linear regressions were used to assess the relationship between streptomycin inhibition and bacterial biomass as well as cycloheximide inhibition and fungal biomass to provide an indication of inhibitor efficacy.

4.4 Results

4.4.1 Inhibition of soil respiration

Respiration rates (µg C g^{-1} hr^{-1}) from soil treated with glucose increased with increasing incubation temperature until it reached the T_{opt} before subsequently decreasing (**Figure 4.5**). Streptomycin and cycloheximide generally reduced respiration across tested incubation temperatures, though to different degrees. Visually, inhibition shifted the T_{opt} of the respiration curve in many samples, occasionally causing a degree of flattening in the curvature of the respiration response. Negative respiration was observed at some of the higher temperatures for glucose and cycloheximide treatment and these results were excluded from the final calculations and additional analysis. These negative values occurred when respiration from SOM decomposition (distilled water treatment) exceeded respiration from glucose and cycloheximide treatment. Respiration from SOM decomposition was subtracted from all glucose treatments to differentiate the non-substrate limited response from the substrate limited response of fungal, bacterial, and microbial respiration. Furthermore, in some of the response curves, MMRT did not appear to fully capture the curvature of some of the respiration responses. Response curves of all 14 samples can be found in Appendix B.

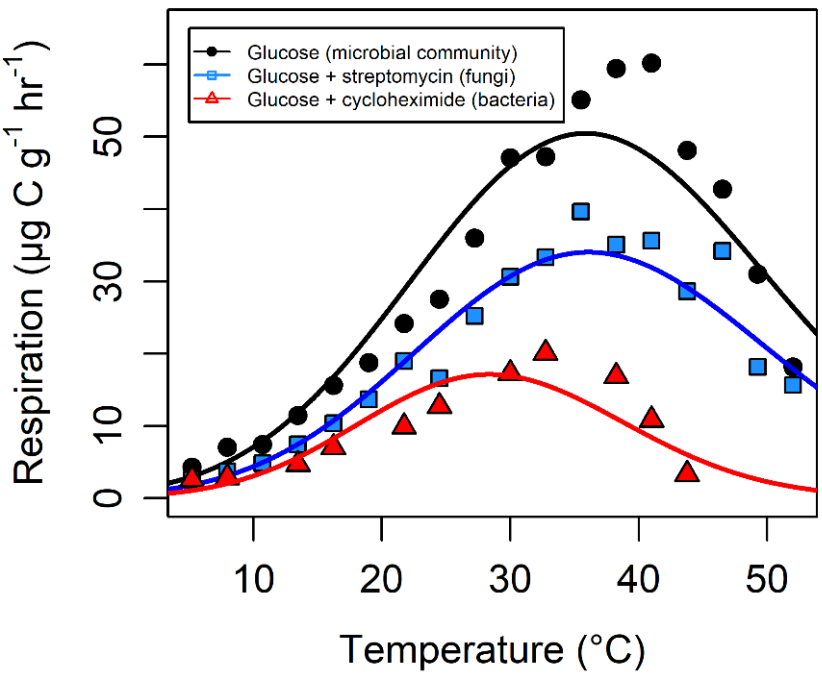


Figure 4.5 Example of temperature response measurements (symbols) and fitted curves of the microbial community (glucose treatment; circles), the fungal community (glucose and streptomycin treatment; squares), and the bacterial community (glucose and cycloheximide treatment; triangles). Curves shown here are from sample 7 (135 cm).

Percent inhibition was calculated as the percent difference between inhibited and uninhibited respiration. Streptomycin inhibition of substrate-induced respiration ranged from 1% to 59% inhibition, averaging 21% inhibition across all temperatures. Average inhibition from cycloheximide treatment was 55%, ranging from 1% to 79% inhibition.

The non-substrate limited respiration response from the soil microbial community was estimated by subtracting the respiration from distilled water treatment from the respiration from soils amended with glucose. Respiration rates from soil treated with distilled water represent the temperature response of SOM decomposition. The T_{opt} (°C) of non-substrate limited respiration along the geothermal gradient was not dependent on environmental temperature (R^2 = 0.037, P = 0.519) (**Figure 4.6**), in contrast to the linear increase observed by Van de Laar (2021).

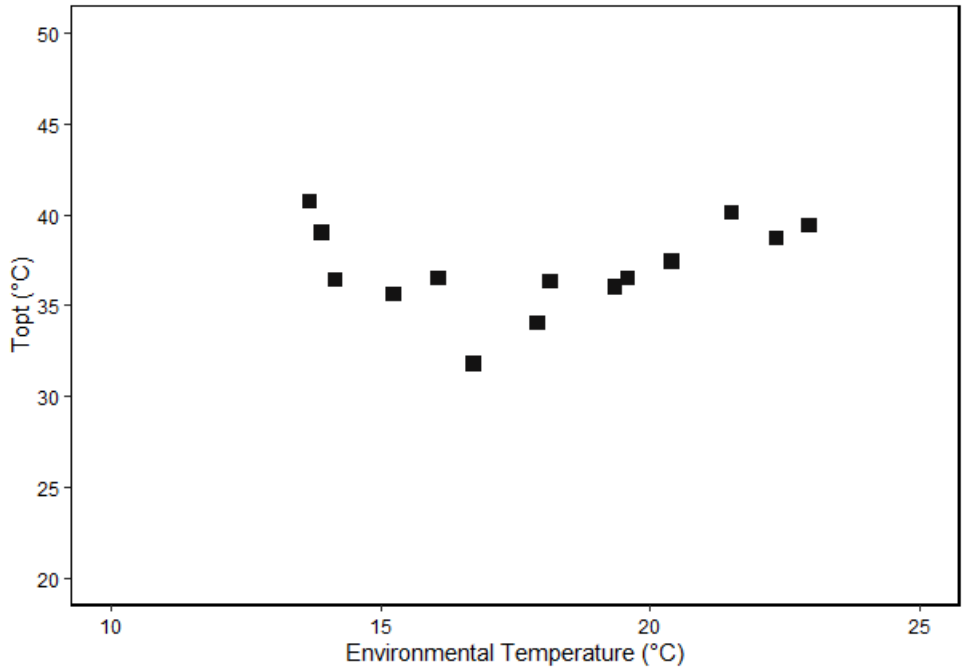


Figure 4.6 Derived T_{opt} (°C) of respiration for the microbial community plotted against environmental temperature (°C). A linear fit was not significant (P = 0.519).

Similarly, the non-substrate limited response of fungi was derived by treating soil with glucose and streptomycin, then subtracting the effect of SOM decomposition. A regression was fit to the relationship between fungal T_{opt} and environmental temperature, resulting in a slope of -0.396 °C $°C^{-1}$ ± 0.319 (standard error of the slope) (R^2 = 0.114, P = 0.040) (**Figure 4.7**).

Lastly, the bacterial temperature response was estimated by treating soil with glucose and cycloheximide and subtracting the respiration from SOM decomposition. Bacterial T_{opt} increased with environmental temperature at a rate of 0.210 °C °C^{-1} ± 0.177, but this relationship was found to be non-significant (R^2 = 0.221, P = 0.065) (**Figure 4.7**).

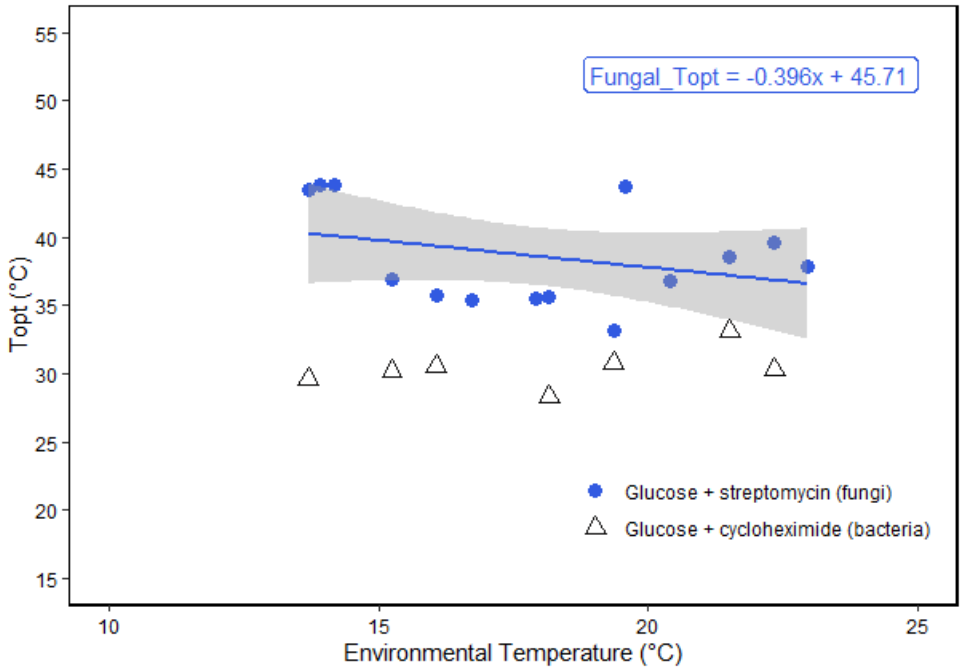


Figure 4.7 Derived T_{opt} (°C) of respiration from fungi and bacteria plotted against environmental temperature (°C). A linear fit between the T_{opt} of fungal respiration was significant (P = 0.040), whereas a linear fit between the T_{opt} of bacterial respiration was marginally non-significant (P = 0.065). Shaded areas represent standard error of the slope.

The average T_{opt} of the non-substrate limited respiration response from the total microbial community was 37.0 °C ± 0.65 (standard error of the mean) and not significantly different from the average fungal T_{opt} of 38.6 °C ± 0.99 (P = 0.095). The T_{opt} of the microbial community was significantly greater than the bacterial T_{opt} of 30.4 °C ± 0.55 (P < 0.001). The T_{opt} of bacteria was significantly lower than that of fungi (P = 0.002).

The T_{inf} (°C) of non-substrate limited respiration from the microbial community along the geothermal gradient was not significantly correlated with environmental temperature (R^2 = 0.050, P = 0.441) (**Figure 4.8**).

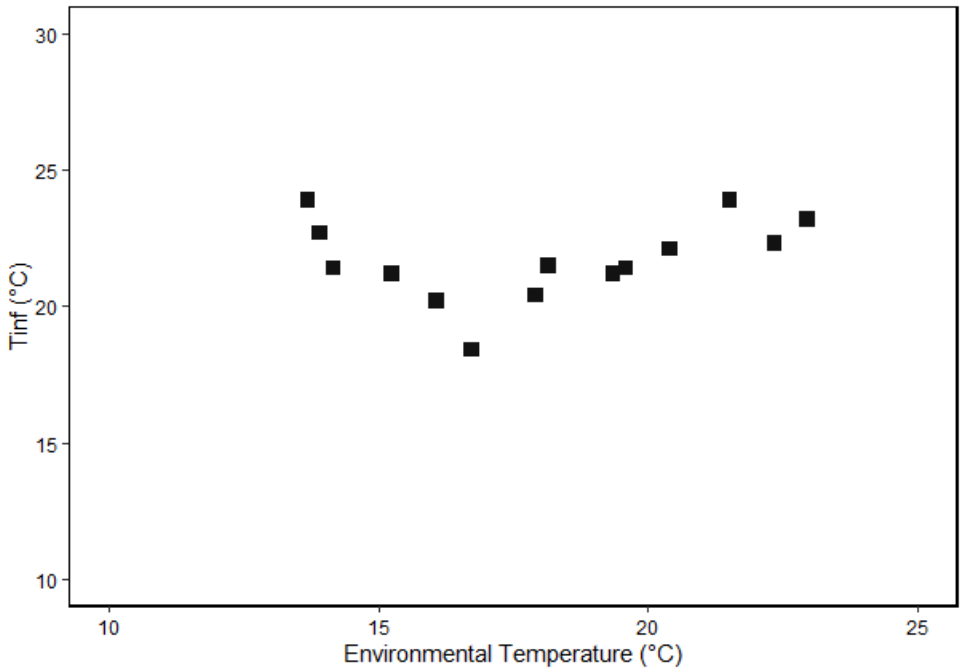


Figure 4.8 Derived T_{inf} (°C) of respiration from the microbial community plotted against environmental temperature (°C). A linear fit was not significant (P = 0.441).

Similarly, the T_{inf} from non-substrate limited fungal or bacterial respiration also had non-correlations with environmental temperature (R^2 = 0.002; 0.099, respectively; P = 0.504; 0.491, respectively) (**Figure 4.9**).

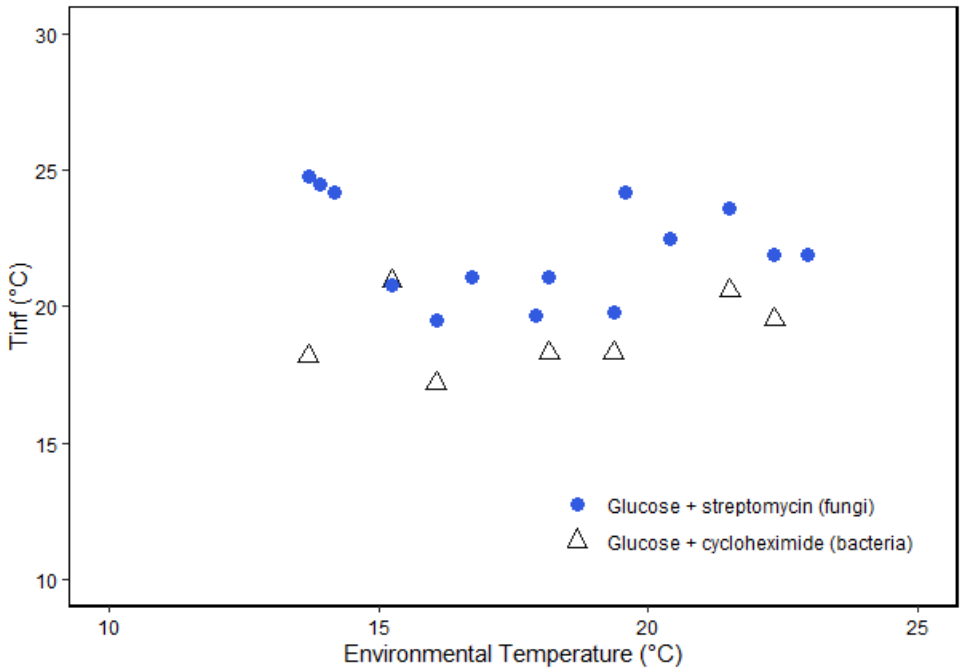


Figure 4.9 Derived T_{inf} (°C) of respiration from fungi and bacteria plotted against environmental temperature (°C). Linear fits were not significant (P = 0.504; 0.491, respectively).

The T_{inf} of the microbial community was 21.7 °C ± 0.40 and was not significantly different from that of the fungal community 22.1 °C ± 0.50 (P = 0.326). Microbial T_{inf} was significantly greater than the bacterial T_{inf} of 19.0 °C ± 0.52 (P = 0.002). The bacterial T_{inf} was significantly lower than the fungal T_{inf} (P = 0.015). A summary table of all temperature response results is shown below (**Table 4.2**).

Table 4.2 Summary table of calculated T_{opt} and T_{inf} values from the microbial, fungal, and bacterial communities from each sampling location.

Sample distance (cm)	Calculated environmental temperature (°C)	pH	Microbial community		Fungi		Bacteria	
			T_{opt}	T_{inf}	T_{opt}	T_{inf}	T_{opt}	T_{inf}
95	22.95	4.58	39.4	23.2	37.9	21.9		
110	22.34	4.59	38.7	22.3	39.6	21.9	30.4	19.6
135	21.52	4.62	40.1	23.9	38.6	23.6	33.1	20.6
180	20.41	4.47	37.4	22.1	36.8	22.5		
225	19.59	4.37	36.5	21.4	43.7	24.2		
240	19.35	4.35	36.0	21.2	33.2	19.8	30.8	18.3
340	18.15	4.72	36.3	21.5	35.6	21.1	28.3	18.3
365	17.92	4.58	34.0	20.4	35.5	19.7		
530	16.73	4.63	31.8	18.4	35.4	21.1		
660	16.07	4.57	36.5	20.2	35.8	19.5	30.6	17.2
880	15.24	4.58	35.6	21.2	36.9	20.8	30.2	21.0
1310	14.16	4.75	36.4	21.4	43.8	24.2		
1450	13.90	4.62	39.0	22.7	43.8	24.5		
1575	13.69	4.45	40.7	23.9	43.5	24.8	29.6	18.2

4.4.2 Microbial biomass

Biomass of different microbial groups identified in soils all displayed similar responses to increasing environmental temperature. The biomass of all groups generally increased with environmental temperature, peaking approximately within the range of 19-21 °C, before subsequently decreasing (**Figure 4.10**).

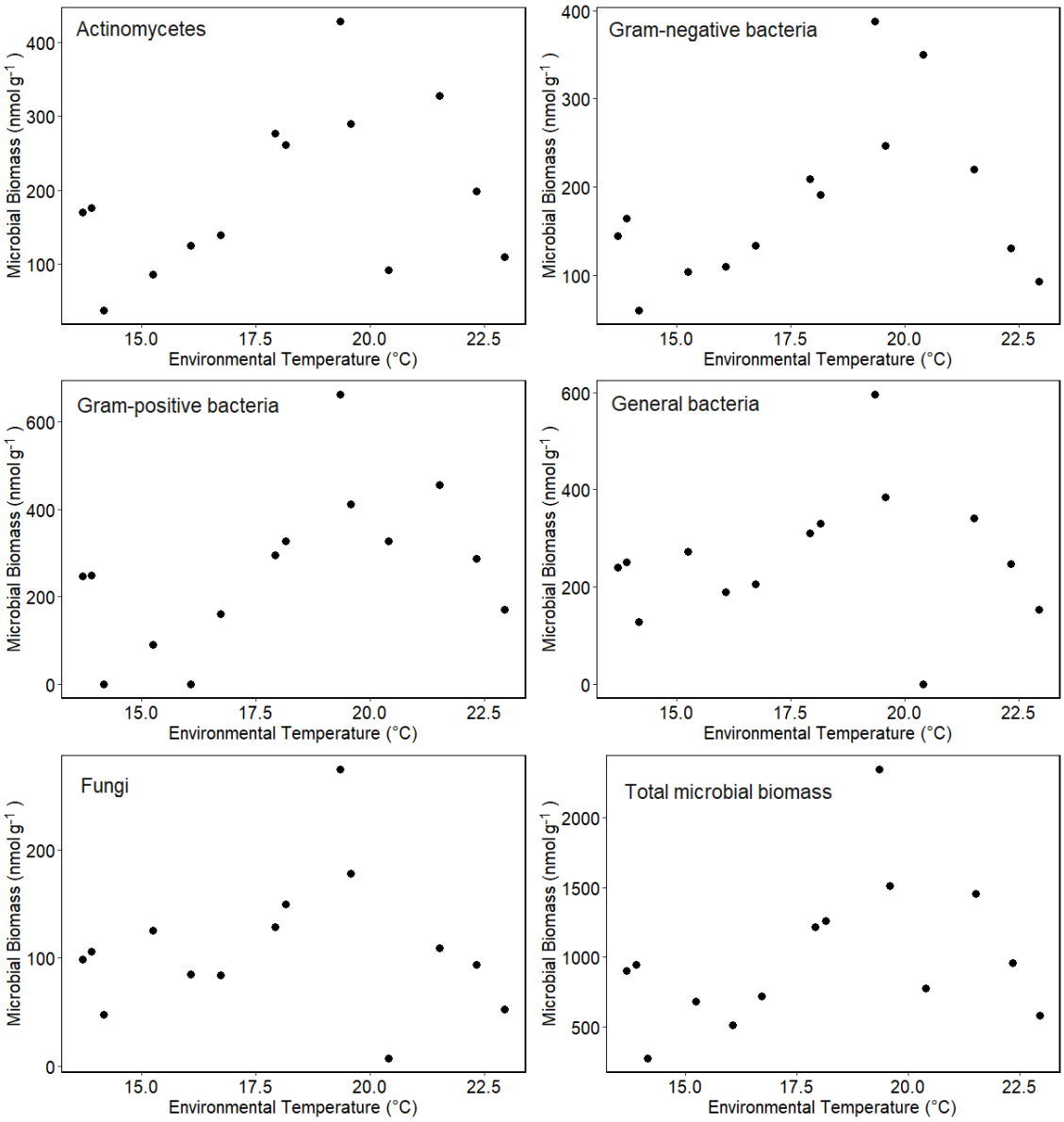


Figure 4.10 Effect of environmental temperature (°C) on microbial biomass (nmol g^{-1}) of actinomycetes, gram-negative bacteria, gram-positive bacteria, general bacteria, fungi, and total microbial biomass.

Results from PLFA analysis showed that F:B ratio was negatively correlated with environmental temperature ($R^2 = 0.366$; $P < 0.001$). The slope of the relationship between F:B ratio and environmental temperature was -0.011 ± 0.002 per °C change in environmental temperature (**Figure 4.11**), suggesting that fungi were more dominant at lower temperatures.

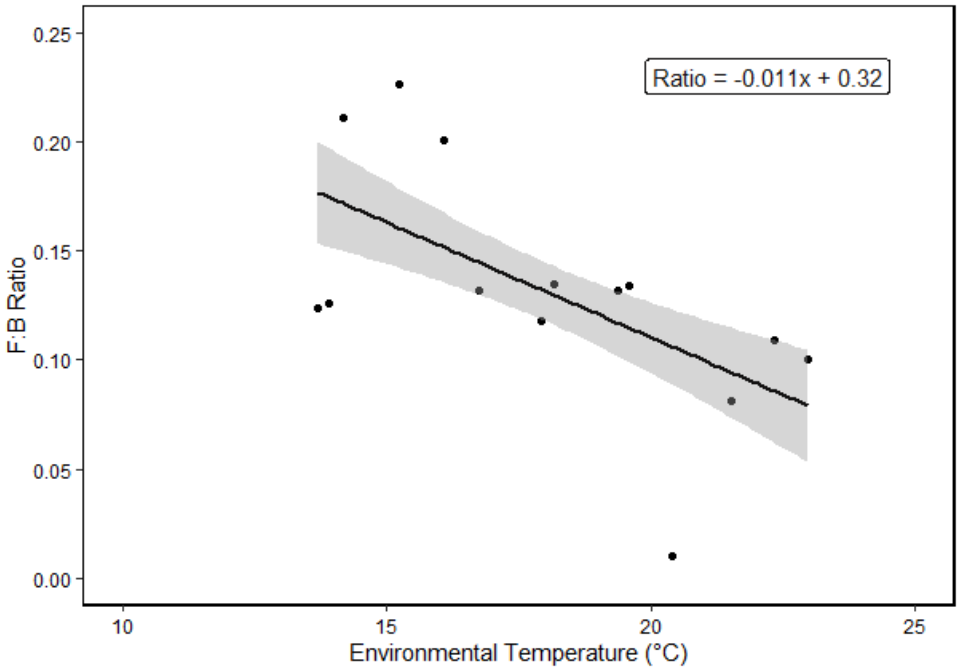


Figure 4.11 Effect of environmental temperature (°C) on F:B ratio. A linear fit was significant ($P < 0.001$).

Total microbial biomass and total C are often correlated. Carbon measurements were previously made at different locations along the geothermal temperature than where current soil samples were taken to characterise the nutrient profile of these soils (Van de Laar, 2021). This data from Van de Laar (2021) was interpolated to calculate total C at the sampling sites used in the current study. This percent C content was then correlated with total microbial biomass (nmol g^{-1}) which was calculated as the sum of PLFAs, but this relationship was not significant ($R^2 = 0.162$, $P = 0.153$) (**Figure 4.12**).

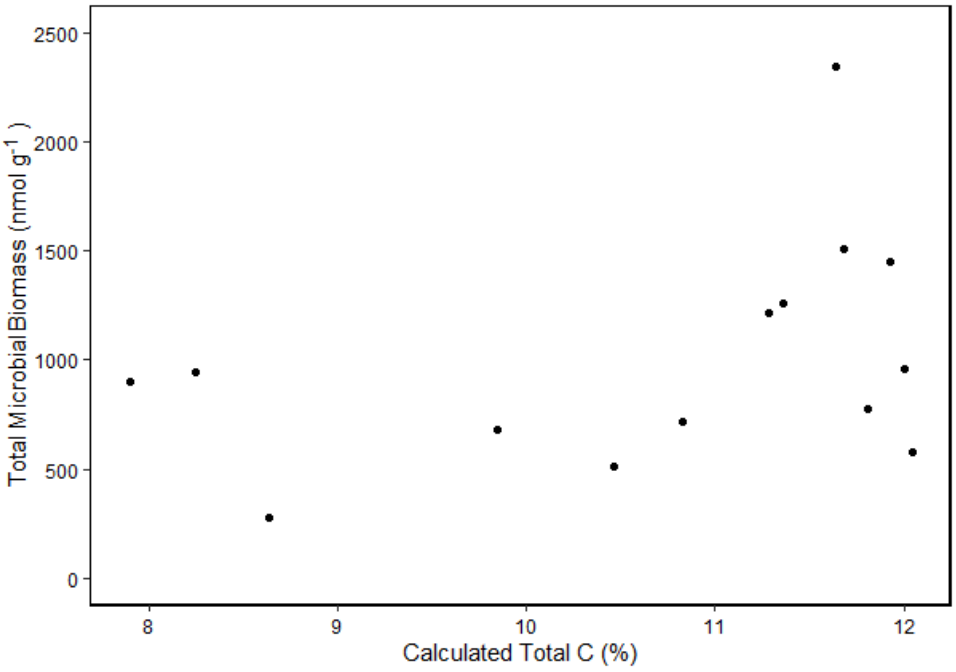


Figure 4.12 Relationship between total microbial biomass (nmol g^{-1}) and percent calculated total C content. A linear fit was not significant (P = 0.153).

4.4.3 Inhibitor efficacy

Inhibitor effectiveness was tested by comparing percent bacterial inhibition from streptomycin to bacterial biomass. If streptomycin was effective in inhibiting bacteria, percent bacterial inhibition would increase as total bacterial biomass increased. There was a significant positive correlation between percent bacterial inhibition and total bacterial biomass, with a slope of 38.04 ± 15.35 (R^2 = 0.338, P = 0.029) (**Figure 4.13**), supporting the argument that bacterial inhibition had been at least partially successful.

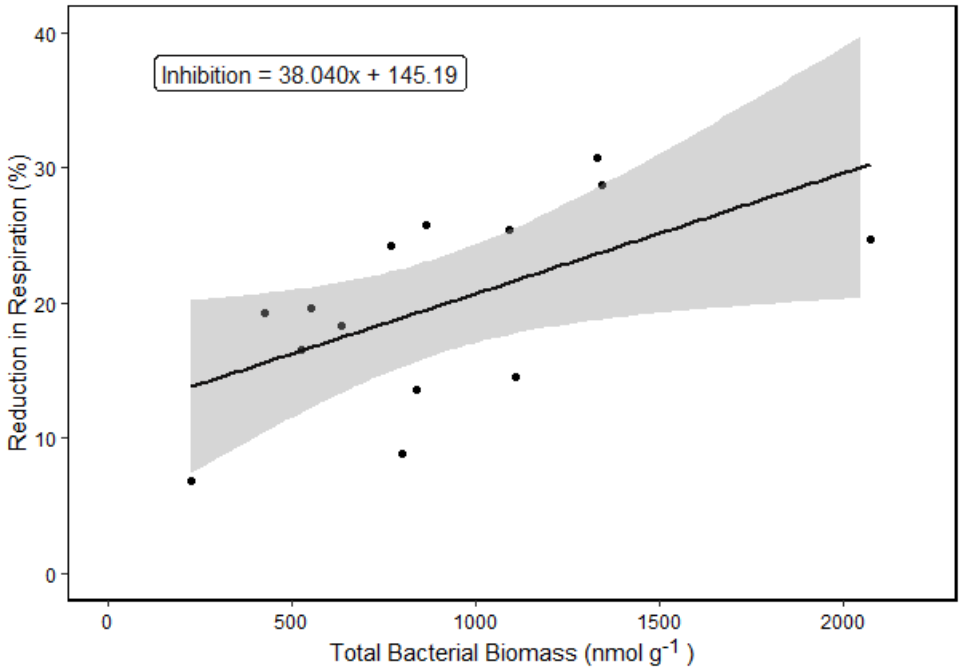


Figure 4.13 Relationship between percent reduction of respiration following streptomycin application and total bacterial biomass (nmol g^{-1}). A linear fit was significant (P = 0.029).

Similarly, if cycloheximide was effective at inhibiting fungi, it would be expected that percent fungal inhibition would increase as total fungal biomass increased. However, there was less evidence to suggest that fungal inhibition from cycloheximide was successful as percent fungal inhibition and fungal biomass were not correlated (R^2 = 0.070, P = 0.567) (**Figure 4.14**).

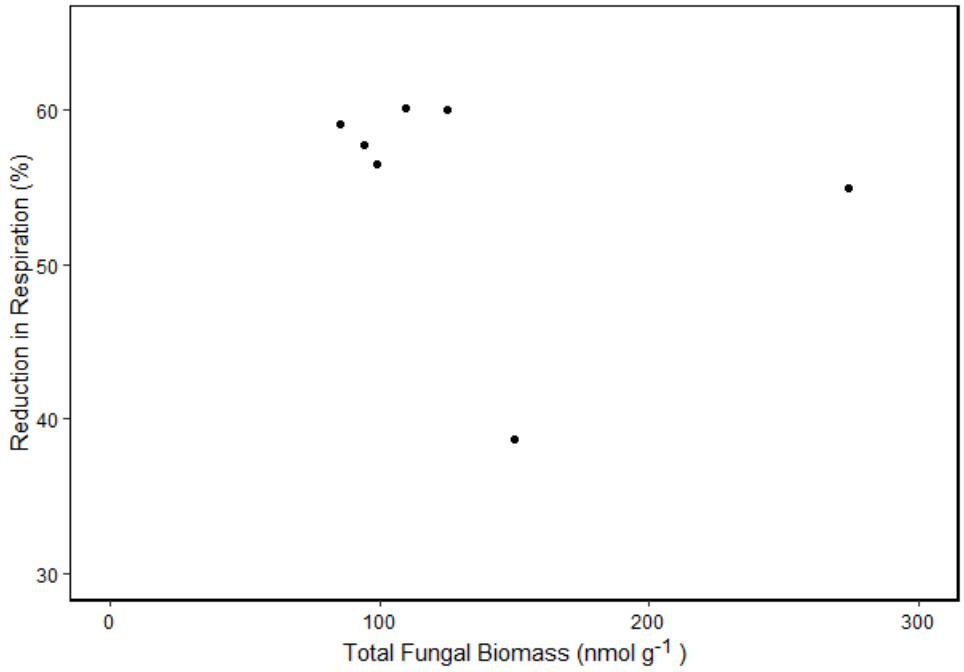


Figure 4.14 Relationship between percent reduction of respiration following cycloheximide application and total fungal biomass (nmol g^{-1}). A linear fit was non-significant (P = 0.567).

The effectiveness of inhibitors across different incubation temperatures was assessed by taking the average bacterial inhibition from streptomycin treatment across all 14 samples and the average fungal inhibition from cycloheximide treatment from the seven samples that received cycloheximide treatment. Average rates of streptomycin and cycloheximide inhibition were plotted against average incubation temperatures from all 14 samples. Results showed that cycloheximide inhibition was greatest at approximately 45-50 °C whereas streptomycin inhibition was greatest within the range of approximately 15-25 °C (**Figure 4.15**).

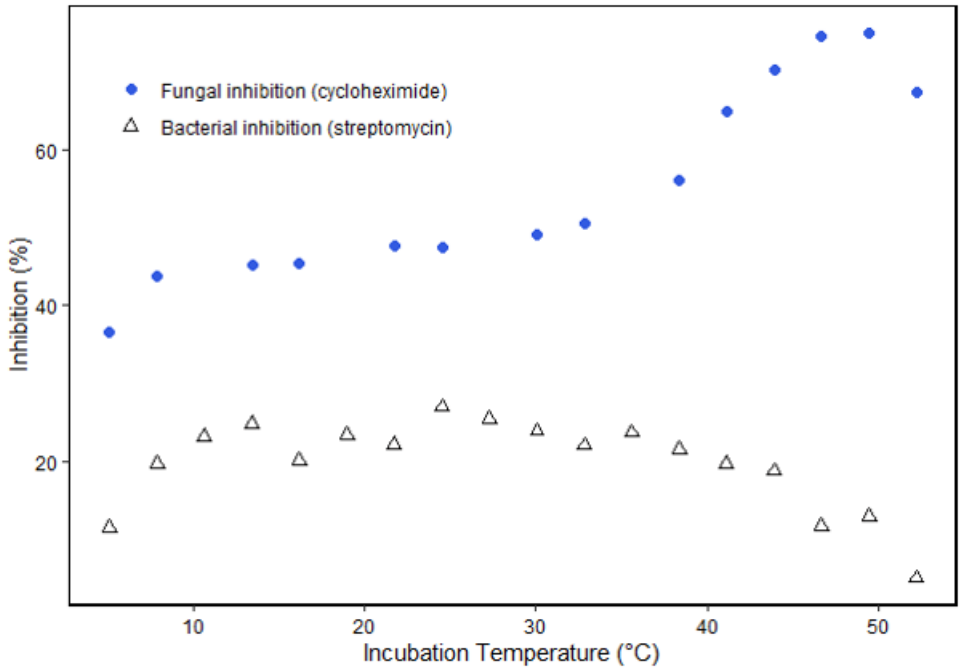


Figure 4.15 Effect of incubation temperature (°C) on average percent inhibition of respiration rates from soil amended with streptomycin and cycloheximide.

4.5 Discussion

4.5.1 Selective inhibition of soil respiration

Overall, results from selective inhibition experiments showed that fungal contributions to total soil respiration were greater than bacterial contributions. The T_{opt} of bacterial respiration increased with environmental temperature, though this relationship was marginally non-significant. In contrast, the T_{opt} of fungal respiration decreased with increasing environmental temperature, contrary to our hypothesis. The temperature response (T_{opt} and T_{inf}) of fungal respiration was not significantly different from the temperature response of microbial respiration but was greater than the temperature response of bacterial respiration, also in contrast to our expectations.

4.5.1.1 Fungal and bacterial contributions to soil respiration

Fungal contributions to total soil respiration were found to be greater than bacterial contributions, in agreement with previous studies (Anderson & Domsch, 1973a; Johnson et al., 1996; Bailey et al., 2002; Susyan et al., 2005). These results suggest that fungi were more efficient at metabolising soil C, particularly given that fungal biomass was considerably lower than bacterial biomass (see section 4.5.2). However, in the development of this method, enough glucose was added to ensure that substrate depletion

did not occur (Numa, 2020). These results suggest that fungi are more efficient at decomposing labile soil C, contrary to classical soil food web models that suggest that bacteria consume labile C twice as fast as fungi (Hunt et al., 1987; de Vries & Caruso, 2016; Khatoon et al., 2017).

4.5.1.2 Temperature response changes along the geothermal temperature gradient

The T_{opt} of bacterial respiration increased with environmental temperature at a rate 0.210 °C °C^{-1}. While this was not a significant increase (partially due to insufficient replication), this value was similar to the finding by Van de Laar (2021), who found that the T_{opt} of microbial respiration increased with temperature at a rate of 0.198 °C °C^{-1}. Together, this suggests that bacteria adapt to environmental temperature, as has been previously reported (Reischke, 2013). In contrast, the T_{opt} of fungal respiration showed a significant negative correlation with environmental temperature, decreasing at a rate of -0.396 °C °C^{-1}. This indicated that fungal respiration was sensitive to increasing temperature and were unable to adapt their metabolism to increasing temperatures. Hypothetically, if soil temperatures continued to increase, the T_{opt} of fungal respiration would eventually match the temperature of the soil environment, according to this result. It is unclear how decreasing fungal respiration T_{opt} would affect soil C cycling and ultimately soil C stocks, though this is assuming that inhibitors were fully effective in differentiating fungi and bacteria (further discussed in section 4.5.3). This finding was unexpected, as it has previously been reported that fungal respiration adapts to increasing temperatures (Lange & Green, 2005; Bárcenas-Moreno et al., 2009). It has also been reported that total microbial respiration adapts to increasing temperatures (Bradford et al., 2010; Dacal et al., 2019; Ye et al., 2020; Li et al., 2021a; Van de Laar, 2021). Therefore, we had hypothesized that in this study, fungal respiration would also adapt to increasing temperatures. However, there have also been reports where fungal respiration has not adapted to increasing temperature (Müller et al., 2011; Allison et al., 2018). As such, the degree of fungal thermal adaptation is not yet resolved and points to the need for further research.

If bacterial T_{opt} did truly increase with environmental temperature, this result, as well as the results from Van de Laar (2021), would support the optimum-driven hypothesis proposed by Alster et al. (2020). This hypothesis suggests that the T_{opt} of microbial

respiration increases to match changing environmental temperatures (Alster et al., 2020). As the temperature response curve shifts, this could result in lower soil C losses with increasing environmental temperatures (Alster et al., 2022). In contrast, the decline of the fungal respiration T_{opt} with temperature does not fit in with this hypothesis, which assumes that increases in microbial respiration would occur. If fungal respiration does not adapt to increasing temperatures, the optimum-driven hypothesis may need to be reconsidered for the fungal community.

Overall, it remains unclear as to whether fungal and bacterial respiration adapt to increasing environmental temperatures. The results from this study are uncertain as the ability of inhibitors to selectively eliminate target organisms is questionable (see section 4.5.3).

4.5.1.3 Comparing the temperature response of fungal, bacterial, and microbial respiration

This study found that the T_{opt} of fungal respiration (38.6 °C) was not significantly different from the microbial respiration T_{opt} (37.0 °C), but both were significantly greater than the bacterial respiration T_{opt} (30.4 °C). Similarly, the fungal respiration T_{inf} (22.1 °C) was not significantly different from the microbial respiration T_{inf} (21.7 °C), but were both significantly greater than the bacterial respiration T_{inf} (19.0 °C). This suggests that fungi might have a higher T_{opt} of respiration to avoid damage at higher temperatures. This could also suggest that as temperatures increase, fungi that are poorly adapted to survive these temperature changes are excluded, leaving behind thermotolerant or thermophilic fungi which may have higher respiration T_{opt} compared to mesophilic and less thermotolerant fungi. In contrast, bacteria may generally be more thermotolerant and capable of withstanding temperature related stress.

The microbial respiration T_{opt} (37.0 °C) and T_{inf} (21.7 °C) from this study were significantly different from the values reported by Van de Laar (2021) (34.5 °C and 14.9 °C, respectively). In their study, Van de Laar (2021) tested 20 soil samples across 40 different incubation temperatures, whereas the current study tested 14 soil samples across 18 different incubation temperatures (14 different incubation temperatures for glucose and cycloheximide treatments). Furthermore, Van de Laar (2021) also tested soils from a wider range of temperatures along the geothermal gradient, particularly at higher temperatures (18-36 °C in Van de Laar compared to 13-23 °C from the current study). A

wider range of sample site temperatures would likely make trends more detectable. Additionally, Robinson et al. (2017) found that at least 20 incubation temperatures are required to have sufficient confidence in model fits, meaning that the model fits used in the current study may not have been as accurate as Van de Laar (2021). If the current study were to be repeated, more samples should be taken at different soil temperatures and tested across a wider range of incubation temperatures.

Results reported in the current study indicate that fungi are more sensitive to temperature and therefore may be at greater risk of being impacted by increasing temperatures, such as from climate change. Results from this study could be used to roughly calculate how much the fungal respiration T_{opt} has decreased since pre-industrial times and its further projected decline. Global surface temperatures have increased 1.0 °C above pre-industrial temperatures as of 2022 and is expected to increase an additional 0.5 °C between 2030 and 2052 (IPCC 2022). The current study reported that the T_{opt} of fungal respiration was 38.6 °C and decreased with temperature at a rate of -0.396 °C $°C^{-1}$. This means that the T_{opt} of fungal respiration at pre-industrial temperatures was approximately 39.0 °C, which will further decline to approximately 38.4 °C between 2030 and 2052. However, this is assuming that climate projections and the T_{opt} of fungal respiration and its rate of change with temperature can be reasonably extrapolated (see section 4.5.3). It is not clear as to how this decrease in fungal respiration T_{opt} with increasing temperatures under climate change would alter the relative contributions of fungi to soil decomposition processes and ultimately changes in soil C stocks.

The temperature response of fungi and bacteria could be further investigated using other study sites to test the findings at the geothermal site used in the current study. Compost piles may be useful in providing further insight into the differential thermal adaptation of fungi and bacteria as compost piles tend to cycle between different phases. Compost piles tend to start off at a mesophilic phase (10-42 °C) where microorganisms rapidly consume labile substrate. Secondly, there is a thermophilic phase where temperatures increase to 45-70 °C, selecting for thermotolerant and thermophilic microorganisms that degrade complex organic matter like cellulose and lignin. As temperatures increase, fungi produce spores while bacteria, particularly actinomycetes, tend to dominate the metabolically active biomass. Thirdly, another mesophilic phase occurs where temperatures drop to approximately 40 °C, allowing fungi to form from spores and increasing the activity of mesophilic microorganisms. During this phase, degradation of complex organic matter

continues. Finally, a maturation phase occurs, where temperatures fall to ambient temperature and fulvic and humic acids start to form. Throughout these stages, pH can fluctuate, particularly in the thermophilic phase as nitrogen is converted to ammonia (Ryckeboer et al., 2003; Meena et al., 2021). However, this rise in pH is more likely to negatively affect bacteria because fungi are able to survive a wider pH range, particularly at higher pH values (Rousk et al., 2010; Meena et al., 2021). Compost piles could be used to study fungal and bacterial responses to temperature and to test findings from the current study and previous studies that suggest that fungi are excluded with increasing environmental temperature more quickly than bacteria (Pietikäinen et al., 2005; Bárcenas-Moreno et al., 2009; Robert & Casadevall, 2009; Nottingham et al., 2019). This could provide further insight into how temperature shapes microbial communities and how fungi and bacteria differentially respond to temperature.

The idea that fungi are more temperature sensitive than bacteria has previously been reported in the literature (Pietikäinen et al., 2005; Bárcenas-Moreno et al., 2009; Robert & Casadevall, 2009; Nottingham et al., 2019). Currently, this increased temperature sensitivity contributes to a prominent theory as to why there are relatively few fungal pathogens that target endotherms, particularly mammals. Mammals typically have an internal body temperature of approximately ~35-38 °C (Kwon-Chung & Bennett, 1992; Bergman & Casadevall, 2010), which coincides with the T_{opt} of fungal respiration reported in our study. It has been theorized that there are relatively few fungal pathogens because fungi are poorly adapted at surviving temperature stress and cannot compete with other organisms at typical mammalian body temperatures (Kwon-Chung & Bennett, 1992; Bergman & Casadevall, 2010). This postulation is supported by a study by Robert and Casadevall (2009) who found that of the 4802 isolated fungal strains grown in their study, 6% of fungal isolates were excluded per 1 °C increase in temperature in the range of 30-40 °C. If these findings are true, why is it that fungi are more temperature sensitive than bacteria? Furthermore, why is it that since the evolution of endothermy, more fungal pathogens have not evolved to survive these internal body temperatures?

The T_{opt} of microbial respiration (37.0 °C) was approximately 19 °C greater than the average environmental temperature along the gradient. Furthermore, the T_{opt} of fungal and bacterial respiration (38.6 °C and 30.4 °C, respectively) were approximately 20 °C and 12 °C greater than average environmental temperatures, respectively. Previous studies have suggested that the T_{opt} of microbial growth tends to be approximately 5 to

15 °C greater than soil temperatures (Rinnan et al., 2009; Donhauser et al. 2020). Furthermore, Bronikowski et al. (2001) suggested that enteric bacteria of sea turtles had an average growth T_{opt} of approximately 34 °C, approximately 11 °C greater than the average internal body temperature of their sea turtle hosts which was approximately 23 °C. It seems to be a common occurrence that many microorganisms inhabit environments that typically do not reach their T_{opt} of growth or activity. However, this is not always true as some microorganisms inhabit environments that do match their T_{opt}, such as *E. coli*, which have a T_{opt} of 37 °C (Doyle & Schoeni, 1984), inhabiting homeotherms, which typically maintain their internal body temperatures at ~35-38 °C (Kwon-Chung & Bennett, 1992; Bergman & Casadevall, 2010). This suggests that microorganisms can have different strategies to survive different thermal environments. Alternatively, microbes could adapt their T_{opt} to sit above the temperature of their environment. One plausible theory is that when environmental temperatures exceed an organism's temperature optimum, the risk of temperature related damage increases. Therefore, having a T_{opt} that exceeds typical environmental temperatures could reduce the probability of damage from temperature stress, perhaps at the trade-off of slower growth or metabolism (Bárcenas-Moreno et al., 2009; Rinnan et al., 2009). Furthermore, the T_{opt} of microbial respiration tends to sit approximately 10 °C greater than the T_{opt} of microbial growth (Ali et al. 2018; Liu et al. 2018). Why is it that the T_{opt} of microbial respiration tends to be greater than the T_{opt} of growth? This question remains relatively unexplored and may be a potential avenue of research that could be beneficial in further understanding microbial temperature sensitivity.

4.5.2 Microbial biomass

The biomass of fungal and bacterial groups identified in soil from PLFA analysis generally increased with environmental temperature up to approximately 20 °C before subsequently decreasing. Furthermore, F:B ratios decreased with increasing environmental temperature, indicating a proportional decrease in fungal biomass, supporting our hypothesis that fungi are less suited to warmer soils. Finally, total microbial biomass was not correlated with soil C content, contrary to our expectations.

The biomass of fungal and bacterial groups peaked at environmental temperatures of ~19-21 °C before decreasing at higher temperatures. Gram-positive bacteria and general bacteria appeared to be the most common groups in soil, though different bacterial groups

displayed similar temperature responses to one another. Fungal biomass was found to be lower in soil overall, even at lower temperatures where fungi were expected to be most abundant (Pietikäinen et al., 2005). While individual hypotheses could be established for each bacterial group, this was beyond the scope of this study, but presents a potential avenue of research that investigates the temperature response of different fungal and bacterial groups.

This study found that F:B ratios decreased with increasing environmental temperature along the geothermal temperature gradient, indicating that fungal biomass decreases with temperature more rapidly than bacteria. This corroborates the previous finding that fungal T_{opt} decreased with environmental temperature, therefore suggesting that both fungal activity and biomass are negatively affected by temperature and decrease more rapidly relative to bacteria. This supports the notion that increasing temperature can affect microbial community composition by favouring bacteria and thermotolerant fungi while more mesophilic fungi are quickly excluded due to their susceptibility to temperature, particularly in the range of 30-40 °C (Pietikäinen et al., 2005; Robert & Casadevall, 2009; Ali et al., 2018; Whitney, 2019; Nottingham et al., 2019). These decreases in fungal biomass and activity could be due to various factors, including physiological damage, metabolic constraints, changes in community composition, and/or competitive stresses (Griffin, 1985; Pietikäinen et al., 2005; Ali et al. 2018; Nottingham et al., 2019; Donhauser et al. 2020).

Finally, soil C content was not correlated with soil microbial biomass, contrary to trends generally observed in soil (Liddle et al., 2020; Bastida et al., 2021). Carbon contents from studied soils were not measured, but instead calculated based off measurements conducted by Van de Laar (2021). It is possible that insufficient C measurements were taken to make accurate predictions. Perhaps a stronger correlation could have been identified had C measurements been conducted on soils at the time of the study.

4.5.3 Caveats

There was evidence that streptomycin inhibition of bacteria had been at least partially successful. However, there was less evidence indicating that cycloheximide was successful in inhibiting fungi. The selective inhibition method presented a range of limitations that must be considered when interpreting results. While some of these issues

might be noted in the literature, these issues would likely be unreported due to publication bias where inhibitor failure is not easily publishable. This was likely the first study that employed the selective inhibition method to inhibit fungi and bacteria from soils across a range of incubation temperatures.

The efficacy of inhibitors was shown to vary across incubation temperatures. In particular, cycloheximide displayed its own temperature sensitivity curve, showing remarkably steady patterns of inhibition as it increased gradually throughout the incubation temperature range of ~10-35 °C before rapidly increasing at ~40 °C and reaching maximal inhibition at ~45-50 °C. This could suggest that either fungi were most sensitive to inhibition at ~45-50 °C or that cycloheximide operated optimally at ~45-50 °C. This could mean that greater inhibition led to a greater than real reduction in respiration and an under-estimation of bacterial respiration rate. In contrast, percent streptomycin inhibition remained steady throughout the temperature range of ~10-30 °C before inhibition rates subsequently started to decline. This finding is corroborated by a previous study that found that streptomycin was most stable at 28 °C (Regna et al., 1946). However, these patterns of inhibition from streptomycin and cycloheximide were relatively consistent across tested soil samples. This would suggest that these inhibitors, particularly cycloheximide, may not be effective at distinguishing fungal and bacterial inhibition across a range of temperatures. Instead, inhibitors that are more temperature stable would be required.

Streptomycin inhibition increased as bacterial biomass increased, providing evidence that streptomycin was effective in inhibiting bacterial respiration. In contrast, cycloheximide inhibition was not correlated with fungal biomass, suggesting that cycloheximide may not have been selective in inhibiting fungi, but instead may have inhibited other soil organisms as well. This evidence must be considered when interpreting results from this study. These inhibitors were chosen due to their selectivity, as reported by other studies (Anderson & Domsch, 1974; Anderson & Domsch, 1975; Nakas & Klein, 1980; Chen et al., 2014). However, streptomycin has previously been reported to inhibit fungal biomass growth (Ingham & Coleman, 1984; Badalucco et al., 1994), while cycloheximide has reportedly inhibited bacterial biomass (Badalucco et al., 1994; Swallow & Quideau, 2020). These conflicting reports suggest that the selectivity of streptomycin and cycloheximide remains uncertain. In the current study, the selectivity of inhibitors was not tested as recommended by Anderson and Domsch (1973a; 1975) who suggested using

plate counts to test inhibitor selectivity. However, this approach is limited as few soil microbes have been grown on agar. Beare et al. (1990) also introduced the inhibitor additivity ratio to provide an indication of inhibitor selectivity. Neither of these approaches were taken due to resource and time limitations. Furthermore, for this same reason, cycloheximide concentrations were not optimised, instead streptomycin concentrations were tested until maximal inhibition was achieved and this same concentration was used for cycloheximide in the final method, hence why concentrations used in this study are far greater than those used in other studies (see section 3.4.5). This was in accordance with West (1986), who suggested that it is more important to achieve maximal respiratory inhibition than it is to optimise inhibitor concentrations. For these reasons, it is questionable as to how effective streptomycin and cycloheximide were in inhibiting target organisms.

Other limitations have been noted in many previous studies that question the efficacy of inhibitors in soil. One issue is the potential for inhibitors to be deactivated in soil environments. Streptomycin is an alkaline antibiotic and therefore may be deactivated in acidic soils. In contrast, cycloheximide is neutral and so is not so easily deactivated by soil pH (Lin & Brookes, 1999; Ananyeva et al., 2006). Furthermore, selective inhibition studies overlook the contributions of soil fauna, which may contribute 10-15% of contributions to soil respiration (Hopkins & Gregorich, 2005). Additionally, protists often get overlooked, which often predate on bacteria, which may indirectly influence respiration rates (Parkinson et al., 1971; Ingham & Coleman, 1984; Nakamoto & Wakahara, 2004).

Overall, there are a range of caveats with the selective inhibition method which require extensive preliminary investigation prior to employment of the method. These issues are further complicated when inhibitors are exposed to different temperatures as streptomycin and cycloheximide are not temperature stable up to ~50 °C. While this method has had reported success in estimating fungal and bacterial roles in soil (Anderson & Domsch, 1973a; Beare et al., 1990; Susyan et al., 2005; Chen et al., 2014), few studies provided extensive criticism of this method. It is likely that past researchers have trialled this method and discarded the findings without publishing the results and uncovering the issues with this method. This further perpetuates the problem as it prompts unknowing researchers to trial the method. In their study, Badalucco et al. (1994) concluded that streptomycin and cycloheximide could not be used to distinguish fungal and bacterial

contributions to soil processes, claiming, “the use of both biocides creates more problems than it solves” (p. 339).

4.6 Conclusion

Overall, this study suggested that fungal contributions to total soil respiration were greater than bacterial contributions, which could be used as an indicator of their relative roles in soil C cycling. Fungal respiration T_{opt} and biomass decreased with increasing environmental temperature, suggesting that fungi were more sensitive to temperature than bacteria and as such fungi may have a higher respiration T_{opt} to help buffer the negative effects of higher temperature on fungal metabolic activity. In contrast, neither the bacterial nor microbial temperature response showed a strong correlation with environmental temperature and could not fully corroborate findings from Van de Laar (2021). Additionally, fungal and bacterial groups identified in geothermal soils responded similarly to environmental temperature. Finally, soil C content was not significantly correlated to total microbial biomass. These findings suggest that fungi are sensitive to temperature and will be disadvantaged as environmental temperatures increase under climate change, indicating that soil microbial communities may shift towards bacterial dominance. However, it is not clear how these findings will translate to soil C dynamics. Results from respiration inhibition should be interpreted with care due to a number of methodological limitations, particularly as incubation temperature complicated the efficacy of the inhibitors. While limitations have been acknowledged in previous studies, the method generally remains in a positive light due to its published successes. This may be because the selective inhibition method has not received proper criticism. It is likely that researchers have trialled this method in the past and discarded unreliable results when these results could be useful in warning other researchers of the numerous constraints included in the selective inhibition method.

Overall, this study highlights the importance of differentiating the temperature response of fungi and bacteria. Rising temperatures under climate change could have serious implications for soil C cycling and soil C stocks. This study also highlights the importance of communication in research and fairly criticising methods that may be ineffective.

Chapter 5
Conclusions

5.1 Conclusions

The primary objective of this thesis was to differentiate the temperature response of fungal and bacterial respiration from soils collected along a geothermal temperature gradient using streptomycin and cycloheximide to selectively inhibit bacteria and fungi, respectively (Anderson & Domsch, 1973a; West, 1986).

Respiration response curves were modelled using MMRT to quantify the temperature response (T_{opt} and T_{inf}) of fungal and bacterial respiration. Fungal contributions to total soil respiration exceeded bacterial contributions, indicating that fungi accounted for the majority of soil C decomposition processes. The T_{opt} of fungal respiration decreased with increasing environmental temperature at a rate of -0.396 °C °C^{-1}, suggesting that fungal respiration adapted negatively to increasing temperatures. In contrast, the T_{opt} of bacterial respiration increased with increasing environmental temperature at a rate of 0.210 °C °C^{-1}. Although this was not significant, this was similar to results found by Van de Laar (2021) which showed that microbial respiration increased with environmental temperature at a rate of 0.198 °C °C^{-1}.

The T_{opt} and T_{inf} of fungi (38.6 °C and 22.1 °C, respectively) was greater than that of bacteria (30.4 °C and 19.0 °C, respectively) possibly because mesophilic fungi are excluded with increasing temperature, leaving behind thermotolerant fungi. Finally, the T_{opt} and T_{inf} values from the total microbial community (37.0 °C and 21.7 °C, respectively) were found to be significantly different from Van der Laar (2021) (34.5 °C and 14.9 °C, respectively). It is likely that the current study was underpowered compared to Van der Laar (2021) as fewer soil samples and incubation temperatures were tested, making it more difficult to identify statistically significant trends.

Phospholipid fatty acid analysis allowed different microbial groups in the geothermal soils to be identified, including actinomycetes, gram-positive bacteria, gram-negative bacteria, general bacteria, and fungi. Biomass of all groups peaked when environmental temperatures were ~19-21 °C, with gram-positive bacteria and general bacteria accounting for the majority of microbial biomass. Additionally, F:B ratios declined with

increasing environmental temperature, indicating that fungi were relatively less capable of withstanding increasing environmental temperatures than bacterial communities.

The selective inhibition method presented a range of limitations that complicated interpretation of the results. This study found evidence that streptomycin and cycloheximide were not equally effective across the range of incubation temperatures (~5-52 °C) as inhibition from cycloheximide was greatest at ~45-50 °C whereas inhibition from streptomycin was greatest at ~30 °C. Furthermore, inhibition of soil respiration from streptomycin application increased with increasing total bacterial biomass, providing evidence that streptomycin may have been effective in targeting bacteria. In contrast, inhibition of soil respiration from cycloheximide addition was not correlated with fungal biomass, suggesting that cycloheximide may not have selectively inhibited fungi. Overall, these results indicate that streptomycin and cycloheximide may not be reliable in accurately differentiating fungal and bacterial respiration, calling to question previous studies that have employed the selective inhibition method (Anderson & Domsch, 1973a; Bailey et al., 2002; Ananyeva et al., 2006). As such, it is harder to publish studies when there are methodological limitations, which can lead to a perpetuation of ineffective methods.

The effect of increasing temperatures on fungi and bacteria, independent from one another, remains uncertain. If the temperature response of fungi and bacteria differ, this could complicate soil C cycling dynamics because fungi and bacteria hold different roles in SOM decomposition. Further research is required as this could have potentially significant consequences for soil C stocks. Finally, this study emphasises the importance of communicating reasonable criticism towards methods that are outdated or ineffectual.

5.2 Future research

Further work is required to corroborate findings presented in this study to help better understand the relative roles of fungi and bacteria in soil environments. As such, some potential avenues for future work are provided below:

- Attempts were made to differentiate the temperature response of fungal and bacterial respiration. However, due to methodological constraints, these results may not be reliable, and care should be taken when considering results. As such, the temperature response of fungi and bacteria remain to be differentiated. To

achieve this, a new method may need to be developed to more accurately differentiate fungal and bacterial respiration. The inhibition method could be further developed if more powerful selective inhibitors could be identified. Alternatively, other methods could be employed, such as metabolic profiling (Di Lonardo et al., 2013; Pinzari et al., 2017), the acetate incorporation into ergosterol method to measure fungal growth (Suberkropp & Gessner, 2005), or the leucine incorporation technique to determine bacterial growth rates (Lekfeldt et al., 2014).

- The selective inhibition method requires a critical review to assess whether it is veracious enough to produce reliable results given its range of limitations.
- A review or meta-analysis is required to clarify fungal and bacterial roles in soil C cycling as it was difficult to source reliable information. Furthermore, a review or meta-analysis of thermal adaptation among microbial, fungal, and bacterial communities is also required.
- Geothermal temperature gradients still pose an interesting opportunity to study the thermal adaptation of microbial respiration or the differentiated temperature response of fungal and bacterial respiration. To date, few studies have tested microbial adaptation along geothermal temperature gradients (Marañón-Jiménez et al., 2018; Walker et al., 2018; Van der Laar, 2021). While the current study adds to this body of research (considering the aforementioned limitations), further research is required to better understand the potential in using geothermal temperature gradients to study microbial thermal adaptation.
- This study briefly explored the temperature responses of different microbial groups, including actinomycetes, gram-positive bacteria, gram-negative bacteria, general bacteria, and fungi. It was beyond the scope of this research to establish individual hypotheses for each of these groups, but this could provide a future opportunity to explore thermal adaptation at a finer taxonomic resolution.
- Compost piles could be used to differentiate the temperature response of fungi and bacteria as temperatures often fluctuate considerably between different stages, thereby excluding and favouring different microorganisms.
- If fungi are sensitive to warmer temperatures (Robert & Casadevall, 2009), why have they not yet adapted mechanisms to survive these warmer temperatures like bacteria have? This discrepancy remains relatively unexplored.
- While some microorganisms have T_{opt} considerably higher than the temperature of their environment (Rinnan et al., 2009; Donhauser et al. 2020), other microorganisms have T_{opt} that are on par with environmental temperatures (Doyle

& Schoeni, 1984). This suggests that microorganisms can have different strategies to survive different thermal niches and that each strategy may confer different advantages. This remains to be explored to provide a better understanding of how microorganisms can adapt to tolerate different thermal environments.

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Appendices

Appendix A: Details of respiration inhibition samples

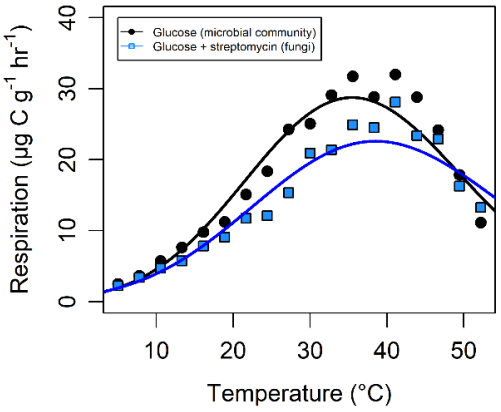
Table A.1 Summary of sample details and properties used for respiration inhibition experiments. Sample order is the same as the order in which incubations were conducted. A second incubation consisting of only distilled water treatments had to be run only for the seven samples receiving cycloheximide treatment.

Sample distance (cm)	Date of incubation		Calculated environmental temperature (°C)	Temperature range (°C)	Moisture content (gg^{-1}) (before drying)	Moisture content (gg^{-1}) (after drying)	pH
	First set	Second set*					
1450	21/6/22	N/A	13.90	13-14	0.628	N/A	4.62
365	22/6/22	N/A	17.92	17-19	0.642	N/A	4.58
180	23/6/22	N/A	20.41	20-22	0.854	0.698	4.47
110	27/6/22	25/7/22	22.34	22-23	0.927	0.771	4.59
240	28/6/22	25/7/22	19.35	19-20	0.949	0.793	4.35
1575	29/6/22	25/7/22	13.69	13-14	0.813	0.657	4.45
135	30/6/22	5/8/22	21.52	20-22	0.890	0.734	4.62
225	1/7/22	N/A	19.59	19-20	0.724	N/A	4.37
880	4/7/22	5/8/22	15.24	14-16	0.573	N/A	4.58
660	6/7/22	5/8/22	16.07	16-17	0.831	0.675	4.57
1310	7/7/22	N/A	14.16	14-16	0.637	N/A	4.75
530	11/7/22	N/A	16.73	16-17	0.908	0.752	4.63
95	12/7/22	N/A	22.95	22-23	0.839	0.683	4.58
340	18/7/22	8/8/22	18.15	17-19	0.663	N/A	4.72

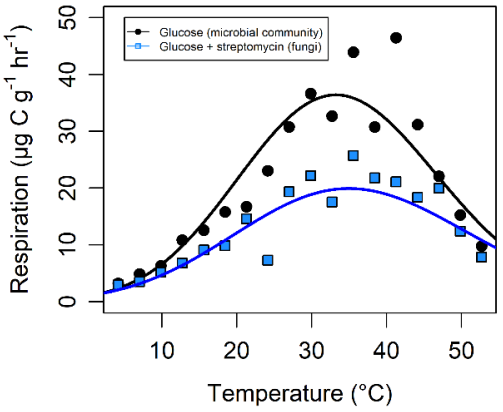
* See section 3.4.6 for more details

Appendix B: Respiration response curves fitted with MMRT 1.5 from different treatments

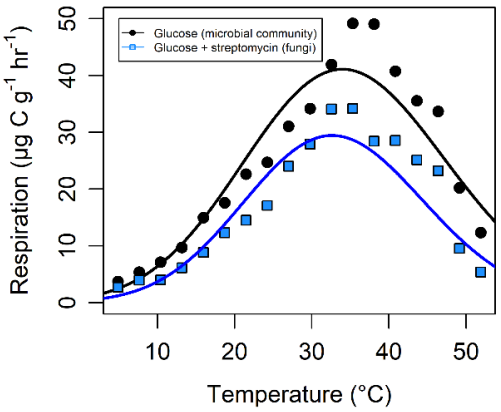
Run 1 - 1450 cm



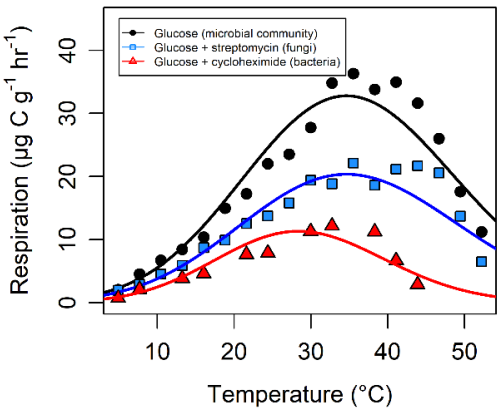
Run 2 - 365 cm



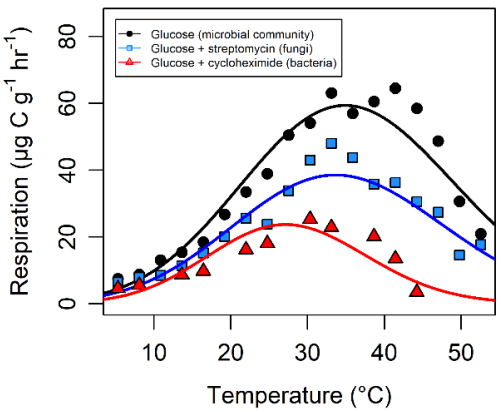
Run 3 - 180 cm



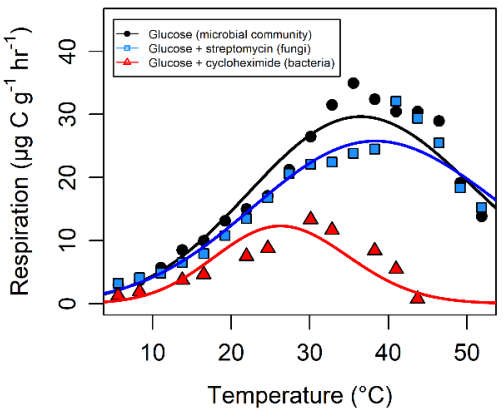
Run 4 - 110 cm



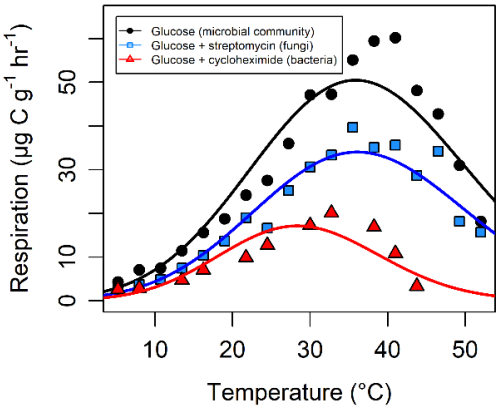
Run 5 - 240 cm



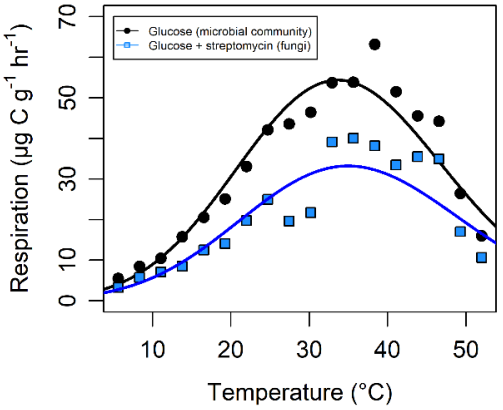
Run 6 - 1575 cm



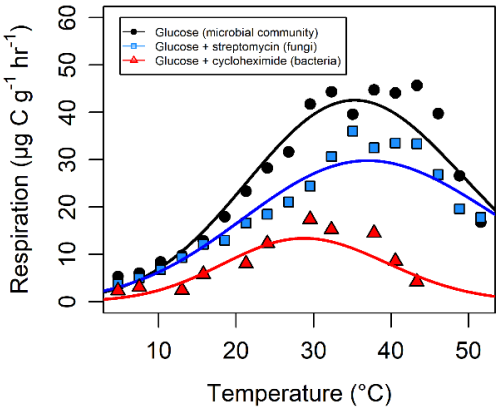
Run 7 - 135 cm



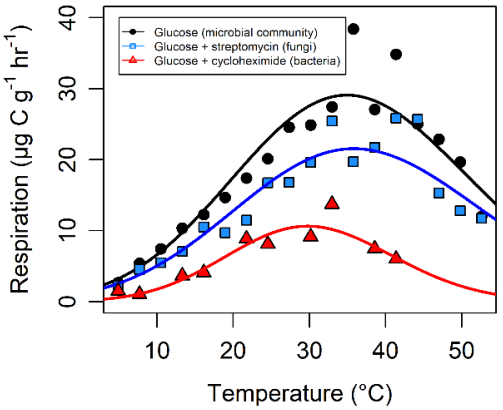
Run 8 - 225 cm



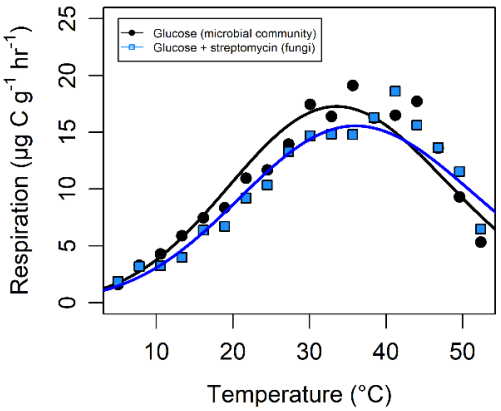
Run 9 - 880 cm



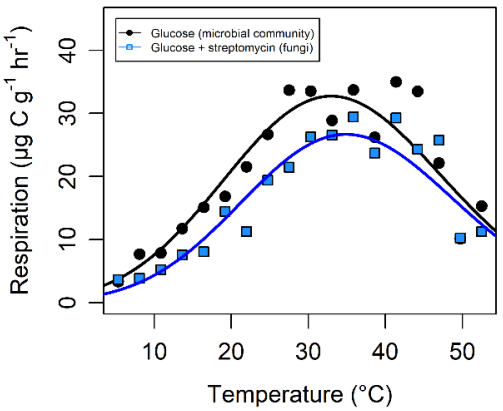
Run 10 - 660 cm

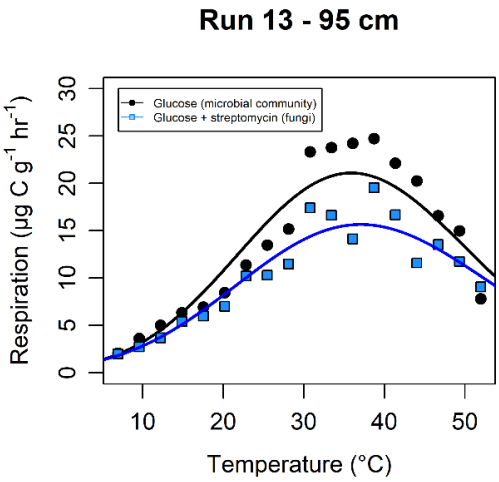


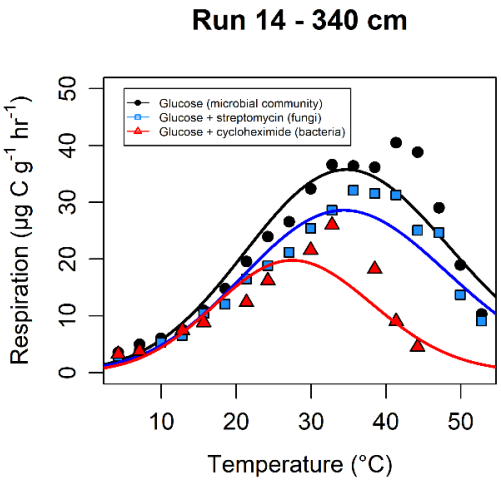
Run 11 - 1310 cm



Run 12 - 530 cm




Run 13 - 95 cm
Glucose (microbial community)
Glucose + streptomycin (fungi)
Respiration (µg C g-1 hr-1)
0
5
10
15
20
25
30
10
20
30
40
50
Temperature (°C)


Run 14 - 340 cm
Glucose (microbial community)
Glucose + streptomycin (fungi)
Glucose + cycloheximide (bacteria)
Respiration (µg C g-1 hr-1)
0
10
20
30
40
50
10
20
30
40
50
Temperature (°C)