PHYSIOLOGICAL CHARACTERISTICS OF FUNGI ASSOCIATED WITH ANTARCTIC ENVIRONMENTS

by

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DEDICATION

To my dearest Manjiri, Mumma and Pappa, for always believing in me and letting me follow my dreams.

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ABSTRACT

The permanent ice covers on the lakes of Antarctica's McMurdo Dry Valleys region harbor a diverse group of phototrophic and heterotrophic microorganisms that metabolize during the short summer months when solar radiation produces melt inclusions within the ice and provides energy to drive photosynthesis. Laboratory cultures of fungi were obtained from ice cores taken from Lakes Bonney (east lobe) and Chad, and sediments collected from Subglacial Lake Whillans (West Antarctica). Using molecular techniques, the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was sequenced to identify fungal types and to determine whether they may be unique to this region. Four axenic fungal cultures, *Tetracladium ellipsoideum*, *Lecythophora hoffmannii*, *Mucor* sp., and an unidentified *Ascomycota* were successfully isolated. These isolates are closely related to organisms that have been previously reported in Antarctica and other cold habitats. The isolates were tested for growth characteristics under various temperature and nutrient regimes. Temperature response experiments revealed that all the isolated fungi were psychrotolerant and growth rates were greatest at 25°C. Of major significance in evaluating the potential of Antarctic fungi as a bioresource is their ability to produce bioactive compounds. Two out of four isolated organisms exhibited antimicrobial activity against several plant pathogens. The metabolic potential and preferred substrate utilization was examined by exposing fungal isolates to a variety of substrates in a 96 well "Biolog" plate. A strong correlation was found among substrate utilization, isolates, temperature and the different carbon substrates. This experiment revealed that the isolated fungi have preferences for different labile carbon substrates at 4°C and 24°C which may imply different physiologies at different times of year in the lake ice-covers. Results from my studies will help understand the role of fungi in lake ice and subglacial lake sediment ecosystems, and the physiology of fungi living in cold environments.

INTRODUCTION

Background: The McMurdo Dry Valleys, Antarctica

The McMurdo Dry Valleys region (MDV) located in southern Victoria Land, Antarctica represents some of the most extreme microbial habitats present on the planet (Priscu et al., 1998). The MDV is the largest (~4800 km^2) ice free area on the continent of Antarctica (Levy, 2012) and is comprised of dry soils, ephemeral glaciers, streams and perennially ice-covered lakes (Moorhead & Priscu, 1998; Priscu et al., 1998). In winter, it experiences sub-zero temperatures ranging from near -20°C to -50°C (Doran et al., 2002) and low snowfall less than 50 mm (water equivalent) per year (Fountain et al., 2010) making the MDV region one of the driest and coldest deserts on Earth.

Ice-covered Lakes

There are more than 20 perennially ice-covered lakes and ponds in the MDV's (Doran et al., 1994; McKay et al., 1985) that are distinct in their physical parameters in terms of elevation, depth, lake and drainage type, and salinity (Doran 1994, and references therein). The thickness of the ice cover in the larger lakes is typically 3 to 6 m (Priscu et al., 1998) and limits the amount of light that that penetrates the water column (Palmisano & Simmons, 1987; Wharton et al., 1989; Lizotte and Priscu, 1992). The ice cover also limits gas exchange between the atmosphere and water column (Priscu et al., 1996; Wharton, et al., 1986) thus reducing the interaction between the surrounding atmosphere and the lake system. The ice cover represents a dynamic equilibrium between

the upward movement of ice due to ablation losses at the ice surface and new ice formation at the bottom, caused by seasonal freezing of lake water (Priscu et al. 1998) (Figure 1).

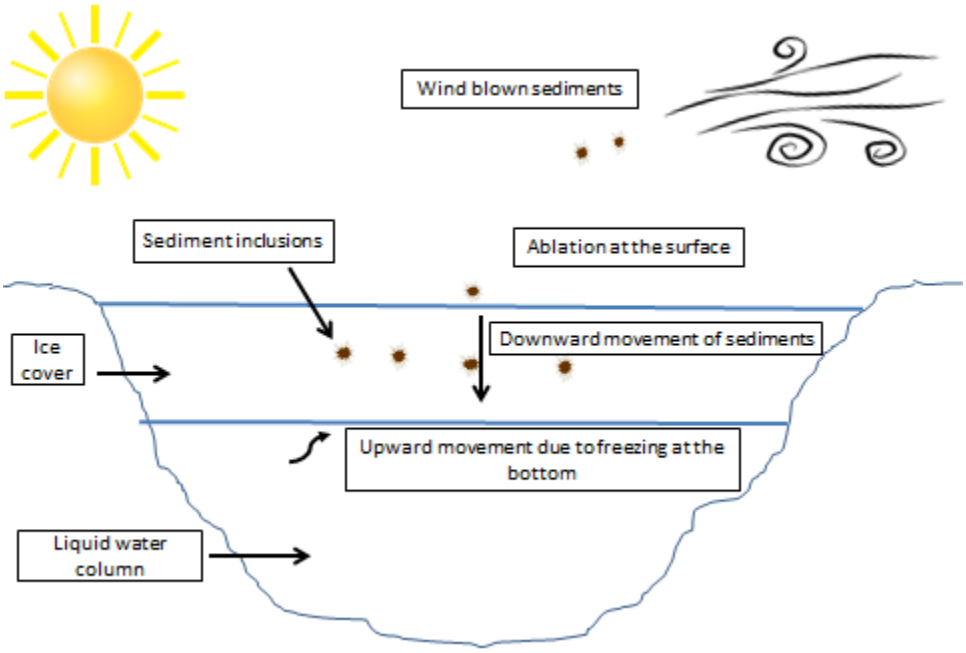

Figure 1. Conceptual model depicting the dynamic equilibrium of sediment inclusions in Antarctic lake ice. Modified from "Permanent Ice Covers of the McMurdo Dry Valley Lakes. Antarctica: Liquid Water Contents," by Fritsen, C. H., Adams, E. E., McKay, C. P. (1998).

The strong and persistent katabatic winds arising from the Polar Plateau result in aeolian deposition of sediments (sand and gravel) on the surface of the permanent ice covers (Fritsen et al., 1998; Šabacká et al., 2012). For about 9 months of the year, solar energy is inadequate to melt these sediments down into the ice (Fritsen et al., 1998). However during the austral summer, these dark sediments absorb solar radiation which

allows them to melt (Jepsen et al., 2010) eventually reaching a depth where solar radiation can no longer supply adequate energy to generate melt (Priscu et al., 2005). The downward movement of sediments from summer melting is balanced by the upward movement of the ice cover that results from the winter freeze-on of new ice at the bottom of the ice cover. The balance between downward melt and upward movement caused by new ice formation at the bottom results in a dynamic equilibrium where the sediment aggregates accumulate at a depth of ~ 2 m in the ice (Fritsen et al., 1998; Jepsen et al., 2010).

Subglacial Lakes

The subglacial lakes that were first speculated to exist in the 1960s (Robinson 1964) are another underexplored component of the microbial habitat. These subglacial lakes are located hundreds to thousands of meters beneath the surface of the ice sheet (Dowdeswell & Siegert, 2003). Currently, 379 perennial subglacial lakes have been identified beneath the Antarctic ice sheets (Wright & Siegert, 2012). I obtained samples of benthic sediments from Subglacial Lake Whillans (SLW), which lies 800 m beneath the surface of the Whillans Ice Stream in West Antarctica (Figure 2). SLW has recently been shown to harbor an active microbial ecosystem (Christner et al., 2014) that has been isolated from the atmosphere for many thousands of years (Priscu et al., 2010).

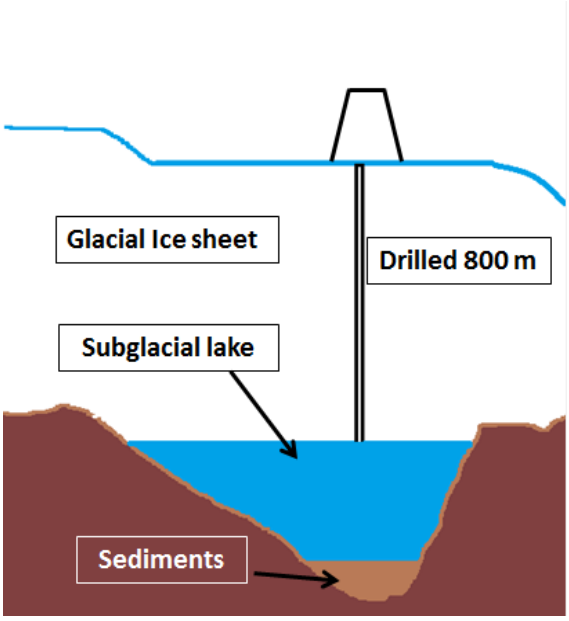

Figure 2. Conceptual model depicting the location of sediments in Subglacial Lake Whillans. Modified from www.antarcticglaciers.org/modern-glaciers/subglacial-lakes.

Overview of Antarctic Fungi

Lakes of the MDVs have been the focus of intensive studies on phytoplankton, Bacteria, and Archaea investigated primarily for the presence of bacteria and Archaea (Vick & Priscu, 2012), with little attention given to fungi (Broady & Weinstein, 1998; Ma et al., 1999). Although Antarctic fungi occupy various ecological niches, their significance in terms of physiology and ecosystem processes is not fully understood (Bridge & Spooner, 2012). After decades of investigations, it has been possible to at least partially identify the diversity of fungi for continental Antarctica which includes species belonging to phyla *Chytridiomycota*, *Ascomycota*, *Zygomycota* and *Basidiomycota* (Onofri et al., 2005; Onofri et al., 2006). A higher diversity of fungal species are recorded from maritime and sub Antarctic terrestrial habitats, mostly associated with particular

substrates such as mosses, mud, algae, microbial mats, rocks, lichens, lake sediments, glaciers, soil, and animals and bird droppings. In Continental Antarctica, around 250 fungal species have been reported, primarily anamorphic *Ascomycota* and *Basidiomycota* yeasts from terrestrial habitats (Onofri et al, 2007).

According to Vishniac (1996), fungi are low in abundance and widely spread across Antarctica. Research conducted by Connell et al. (2006) on the distribution and abundance of fungi present in the soils of MDV showed that filamentous fungi are most closely tied to habitats with a higher pH and soil moisture. These close associations were not observed for yeast and yeast-like fungi, demonstrating that the yeast-like fungi utilize a broader range of habitats. Fungi have been identified from a wide range of Antarctic habitats including cryptoendolothic (inside rock) communities at high elevations in Victoria Land to sub-Antarctic soils (Vishniac, 1996). An early study found 27 filamentous species comprising 17 genera in air and soil samples taken from different locations around Antarctica, particularly near the established campsites (Sun et al., 1978). Several mycorrhizal fungal species associated with mosses and liverworts have been found in Victoria Land (Tosi et al., 2002; Williams et al., 1994). Additionally, species of *Cadophora* that decay (soft rot) artifacts and wooden structures were found in the historic huts on Ross Island in Southern Victoria Land (Blanchette et al., 2004). According to Tosi et al. (2001), most of the yeasts and filamentous fungi reported are cosmopolitan species, but some are indigenous. The presence of cosmopolitan fungal species is likely a result of propagules transported to Antarctica; they have been isolated from the areas associated with human activity and from lakes of MDV (Baublis et al., 1991).

In the MDVs, many studies have focused on cryptoendolithic fungi that occur in microbial communities within rocks (Selbmann et al., 2005). These particular communities are thought to be the most psychrotolerant microorganisms on earth; therefore, they have been proposed as a model group of organisms for exobiological investigations (Onofri et al., 2004).

Diversity of Fungi in Icy Ecosystems

Icy ecosystems are defined as environments containing ice or snow crystals as a characteristic component that is in contact with liquid water, or liquid water in a supercooled state (Psenner et al., 2003). Icy ecosystems are present at altitudes and latitudes that experience permanent or seasonal existence of snow or ice (Psenner et al., 2003). Ma et al. (2000) and Taylor et al. (1997) detected and characterized fungi entrapped in glacial ice from Greenland. This work has been further extended to fungal recovery in Antarctic ice, demonstrating that most of the fungi recovered were similar to contemporary fungi and revealing the presence of a higher number of ascomycetes than basidiomycetes (Patel, 2006). Fungi have been reported from ancient glacial layers of Lake Vostok core where fungal mycelia and yeasts of genus *Rhodotorula* and *Cryptococcus* were identified using molecular methods (Abyzov et al., 2004). Fungi have been isolated and characterized from Lake Vostok accretion and glacial ice; a total of 270 fungal colonies were cultured from the accretion ice and 14 from the glacial ice present immediately above the accretion ice (Elia et al., 2009). The length of isolation from the atmosphere and the extreme conditions make results from this study interesting. Similar

studies have reported the presence of fungi in Lake Vostok accretion ice (Poglazova et al., 2001). While there are numerous studies on fungi in the lake water column, glaciers, and accretion ice, only Mosier et al., (2007) reported a diverse range of Eukarya present at all depths in the ~20 thick ice cover of Lake Vida in the MDV.

Role of Fungi in Icy Ecosystems

Fungi are regarded as one of the most diverse, resilient, and vital kingdoms of eukaryotes. They are major contributors to biodiversity and greatly influence the biogeochemical processes in many ecosystems. Some fungi can be mediators of primary production and are mutually symbiotic in nature (Dighton, 2003) and others are important decomposers and pathogens. Fungi can also greatly influence the dynamics of icy ecosystems (Godinho et al., 2013) by providing important ecosystem services, such as mineralization, nutrient immobilization and decomposition of organic matter (Cantrell et al., 2011). Therefore factors that affect the activity of these fungi consequently affect the entire ecosystem (Kerry, 1990).

Some Antarctic fungi have shown resistance to low temperatures, high UV and IR radiation, (Ruisi et al., 2007) and high salinity (Gunde-Cimerman et al., 2003). Several physiological survival mechanisms for withstanding freezing in fungi have been reported, and it is possible that Antarctic fungi employ a combination of strategies such as antifreeze production, anoxia tolerance, and high super cooling activity to survive (Robinson, 2001).

Fungi recovered from the permanently ice covered lakes ice of the MDVs have over time developed mechanisms to withstand the harsh environment and low nutrient availability (Onofri et al., 2007). A majority of microorganisms found in Antarctica are psychrotolerant (Azmi & Seppelt, 1997; Kerry, 1990; Zucconi et al., 1996). The optimum temperatures for growth of psychrotolerant organisms are typically between 20°C and 40°C but they are also capable of growth at 0°C. Some fungi are true psychrophiles and these do not grow when exposed to temperatures above 20°C and exhibit an optimum growth temperature (OGT) of ≤ 15°C and maximum growth temperature (MGT) of ≤ 20°C (Morita, 1975; Wang et al., 2015). In the studies conducted by Kerry (1990) on leaves and soil samples collected from subantarctic Macquarie Island and Australia's Casey Station, respectively, it was observed that temperature affected the rate at which fungal spores germinated and also the rate at which mycelium grows.

The potential of extremophilic fungi as a bioresource is not well known, although they have been researched for anti-freeze compounds (Tsuji et al., 2013). However, fungi are also capable of producing a wide spectrum of other complex molecules, including volatile organic compounds (VOCs) that belong to different chemical classes like alcohols, ketones, aldehydes, benzene and naphthalene derivatives and terpenoids (Mends et al., 2012; Strobel et al., 2008; Tomsheck et al., 2010; Ul-Hassan et al., 2012). These gaseous compounds can possess specific or non-specific antimicrobial activities that are capable of killing or inhibiting microorganisms (Ul-Hassan et al., 2012b; Wheatley, 2002).

MATERIAL AND METHODS

Study Sites

I isolated and studied fungi collected from two different regions in Antarctica. The MDV region is an ice-free polar desert in Antarctica with a combined area of approximately 4800 km^2 making it the largest ice-free area on the continent (Levy, 2012). The Taylor Valley (TV) located at 77° 45' – 77° 30' S– 163°40' E, lies in the middle of the MDVs (Figure 3). There are four main lakes in TV, the east and west Lobes of Lake Bonney, Fryxell and Hoare, along with other smaller lakes and ponds. The samples for the study were retrieved from east lobe Bonney (ELB) and Lake Chad, one of the smaller lakes. Lake Bonney is a narrow lake (7 km long) located at the head of Taylor Valley, southern Victoria Land, Antarctica (77° 43' S, 162° 23' E) and has a perennial ice cover with a thickness of approximately 4m. The ice thickness varies spatially and seasonally from ~3.5 m to ~ 4.5 m (Fritsen et al., 1998; Priscu et al., 1998)

The other study site was Subglacial Lake Whillans (SLW) that lies 800 m beneath the surface of the Whillans Ice Stream in West Antarctica (84.240 °S, 153.694 °W). SLW had a shallow water column at the time of sampling (~2 m deep; Christner et al., 2014) and is considered to be an 'active' lake that drains and refills regularly (Fricker et al., 2007). The surficial sediments of SLW contain active microorganisms that are phylogenetically distinct from the water column and drilling water (Christner et al., 2014)

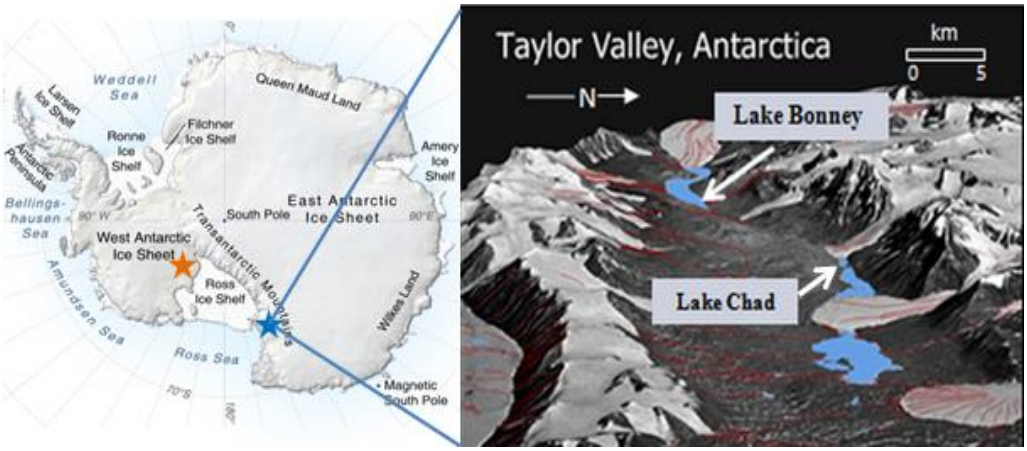

Figure 3. Map showing the location of Lake Bonney and Lake Chad in the Taylor Valley (TV) (Blue star = TV, 77° 43'S, 162° 23'E) and Subglacial Lake Whillans (Orange star = SLW, 84.237°S, 153.614°W) in West Antarctica.

Sample Collection and Processing

The sediment sample from Subglacial Lake Whillans (SLW) was collected in January 2013 using a microbiologically clean, hot water drilling system that created a ~0.6 m diameter borehole through the West Antarctic Ice Sheet (WAIS) above SLW and frozen immediately until further processing (Priscu et al., 2013; Tulaczyk et al., 2014). The ice core samples were collected during the 2012-2013 summer season from the permanent ice covers of ELB and Lake Chad in the McMurdo Dry Valleys (Figure 3). The ice cores were collected from 0.5- 2m depths using a SIPRE coring device which is 3-inch in diameter and is equipped with a Badger power head. The ice cores were placed into clean polyethylene bags, kept frozen (-20°C) and shipped via a sea going vessel to the United States, then over land to Montana State University. On arrival, they were placed in a -20°C freezer until processing. Contaminants originating from sources like

drilling, handling, transporting, storage and processing are always present on the outside of the ice core sections (Castello & Rogers, 2005), so ice cores were decontaminated in a cold clean room prior to processing. The ice cores were warmed at 4°C in the Cold Clean Room for at least an hour before starting the decontamination protocol. Surface sterilization of ice cores was carried out following the protocol developed by Christener et.al (2005) where scraped ice samples were cut using a band saw, and then transferred into a sterile funnel and thoroughly washed with 95% autoclaved ethanol and rinsed with MilliQ water inside a sterile Class II hood of a biologically-clean environment (Christner et al., 2005). Following surface sterilization, another ~5 mm layer of the ice core was removed using a band saw. Four agar plates containing potato dextrose agar, nutrient agar, yeast peptone dextrose agar and yeast extract agar were placed in the laminar flow hood as controls to monitor the air quality inside of the laminar flow hood during ice core handling. The ice cores were positioned on a sterile glass funnel and melted at room temperature (24°C) with controls set up alongside. The meltwater corresponding to the outer portion and sequentially interior portion of the ice core was collected. This meltwater along with the sedimentary inclusions were used to carry out culturing, microscopic and molecular studies.

Fungal Culturing

Lake ice sediments and meltwater were plated on a variety of agars, including potato dextrose agar (0.4% potato starch, 2% dextrose, 1.5% agar [pH 5.6]), nutrient agar (0.3% beef extract, 0.5% peptone, 1.5% agar [pH 6.8]), yeast extract agar (3% yeast

extract, 3% malt extract, 0.5% peptone, 1% dextrose, 2% agar [pH 6.2]) and yeast peptone dextrose agar (2% yeast extract, 3% peptone, 1% dextrose. 2% agar [pH 6.5]. All the media components were obtained from Difco, USA. In order to suppress bacterial growth, 0.25 mg ml^{-1} chloramphenicol and 0.1 mg ml^{-1}ampicillin antibiotics were added to the agar after autoclaving. The plates were incubated under ambient light at 24°C. During storage, the plates were examined every 24 hours for growth and bacterial contamination. The fungal isolates were preserved for the long-term at -80°C in cryovials with sterile 15% glycerol.

Microscopic Observations

Slides of each isolate prepared by mounting fungal hyphae in methylene blue were examined under a light microscope (Zeiss). In some cases, the fungal material was also stained using Calcoflour White Stain and observed under UV light to highlight chitin cell walls.

DNA Extraction

A pure culture of each isolate, grown on PDA, was used as a source of DNA after incubation at 25°C. Squares of the cultured mycelia (0.5cm^2, approximately 100 mg) were cut from 3-week-old-cultures. In cases where the mycelium was firmly attached to the agar, the topmost layer of mycelium was scraped to exclude as much agar as possible. The mycelium was rinsed thoroughly in sterile double distilled water and the excess water was drained off aseptically. The mycelium was added to a 2.0 mL screw cap

microcentrifuge tube containing two 5 mm diameter sterile glass beads. Next, 400 µL AP1 buffer and 4 µL RnaseA enzyme was added to this tube. The sample tubes were disrupted using a Mini-Beadbeater (Biospec Products, USA) at the lowest (2500 rpm) speed settings for one minute. This step was repeated once or twice if necessary to ensure complete homogenization of the tissue. Tubes containing the samples were incubated at 65°C in a water bath for 10 minutes. During incubation, the tubes were inverted 2 - 3 times. The lysate was then centrifuged for 5 min. at 14000 rpm. DNA was extracted following the manufacturer's guidelines provided in Dneasy Plant Mini Kit (Qiagen).

PCR Amplification of the ITS Region

The ITS regions of the fungal DNA were amplified using the universal ITS primers, ITS1-F and ITS4 (White et al. 1990). The ITS region is the most widely sequenced DNA locus and is considered to be a universal DNA barcode marker for fungi (Nilsson et al., 2008). The primer sequences used were: ITS1-F (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-30) (Integrated DNA Technologies). Polymerase Chain Reaction (PCR) was performed in a 25 µL reaction mix containing 1 µL template DNA extracted from the fungal culture, 1 µL primer ITS1-F (10 µM), 1 µL primer ITS4 (10 µM), and 12.5 µL REDtaq ReadyMix (Sigma-Aldrich). The final volume (25 µL) was adjusted using PCR- grade sterile ddH_2O. The PCR conditions used were as follows: initial denaturation at 94°C for 2 min., followed by 30 cycles of 94°C for 30 sec., 55°C for 60s, 72°C for 1 min. and a final extension at 72°C for 5 min. (Barge, 2015).

Visualization, Purification and Quantification of PCR Products

The amplified PCR products were examined using gel electrophoresis and visualized on 1.5% agarose gels with 0.003% ethidium bromide in order to confirm the presence of a single amplified band of approximately 600 bp. The amplicons (5 µL) were purified using QIAquick PCR Purification Kit (Qiagen) following the instructions provided by the manufacturer. DNA concentration in purified PCR products was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

The gels were run for 35 min at 140V with 1X TBE buffer in GelXLUltra V-2 (OWL EasyCast Separation Systems, Inc. Portsmouth, NH, USA) electrophoresis chamber. Gel imaging was performed under UV light in a bio-imaging system (UV Kodak High Performance Ultraviolet Transilluminator). Approximately 600-bp PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. DNA concentration in purified PCR products was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

DNA Sequencing

Purified products were sent to Functional Biosciences (Madison, WI) for direct PCR sequencing. The amplified products were sequenced and aligned with the sequences in GenBank by the BLASTN program (Altschul et al., 1997) in order to ascertain the sequence homology with closely related organisms. Phylogenetic trees were generated by Charles River (Newark, DE). The program called SeqTrace v.0.8.1 was used to

automatically align and calculate consensus sequences from matching forward and reverse reads. Unreliable base calls were eliminated manually throughout the sequences and replaced with N's and the ends of poor nucleotide sequences were trimmed (Stucky, 2012).

Temperature Experiments

Growth of isolates was tested on PDA medium (pH 5.6) because this medium yielded the highest fungal growth. Fungal isolates selected from Lake Chad, Lake Bonney and Subglacial Lake Whillans were placed on PDA plates. A square of the cultured mycelium ($0.5cm^2$) was cut from actively growing colonies of each test isolate and placed in the center of a fresh agar plate for growth. Triplicates of each plate were incubated at 0°C, 4°C, 7.5°C, 10°C, 15°C, 20°C, and 25°C under ambient light to check for temperature tolerance of the organisms.

Growth was calculated as the average increase in diameter (cm) for each day that fungal colonies were on the agar surface during the exponential phase of growth (Kerry, 1990). The average of three diameters, taken at right angles and passing through the point of inoculation at the center of the plate, was calculated. The Q_{10} values were calculated the factor by using the following formula:

$$Q_{10} = (R_2 / R_1)^{10/(T_2 - T_1)}$$

Where Q_{10} represents the factor by which the growth rate (R) increases when the temperature (T) is raised by 10 degrees. R_1 is the measured growth rate at temperature T_1 (where $T_1 < T_2$), R_2 is the measured growth rate at temperature T_2 (where $T_2 > T_1$), T_1 is

the temperature at which growth rate R_1 is measured, and T_2 is the temperature at which growth rate R_2 is measured. The Q_{10} values helped determine the adaptation of fungi to growth at low temperatures and also the amount of psychrotolerance exhibited by fungi. The Q_{10} values were the factor by which growth rate at 25°C exceeded that at 15°C and growth rate at 15°C exceeded that at 4°C. The method followed by Kerry (1990) is based on the assumption that the linear growth on the surface of the agar is proportional to the total volume of mycelium produced. Hence, careful observations were made to observe and record any aerial growth.

Test Organisms to Profile Bioactivity: Fungi and Bacteria

Plant pathogens that were used in the bioactivity profiling test were obtained from D. Mathre of the MSU Department of Plant Sciences. The plant pathogens used were *Pythium ultimum (Oomycota), Phytophthora cinnamonmi (Oomycota), Sclerotinia sclerotiorum (Ascomycota)* and *Fusarium solani (Ascomycota)*. Two bacterial strains were isolated from the meltwater of the ice core from ELB and lake sediments from Subglacial lake Whillans. These two bacterial strains that were tested against the fungal isolates from the same habitat for bioactivity did not show any growth on the control plates. All fungi and bacteria were grown on PDA at 25°C.

Bioactivity Profiling Test Organisms

Screening for antimicrobial activity was carried out using two techniques, dual culture assay and gas test. The gas test was conducted if the dual culture assay showed

positive results in which the fungal isolate inhibited the growth of pathogen or bacterial test strain.

Dual Culture Assay

All four fungal cultures were incubated at 25°C for 12 days or as mentioned otherwise on PDA media. A dual culture assay was carried out to detect any bioactivity against the plant pathogens by following protocols developed by (Jayaswal et al.,1990). In this technique, an agar block (5 mm diameter) of a 10 day old culture of the selected fungal isolate was placed in the center of the Petri plate containing PDA media. Plant pathogens were inoculated 2 cm away from the fungus and tested for bioactivity (Figure 4). A loopful of bacteria was streaked 3 cm away from the test fungus present on the same dish. Plates were wrapped with Parafilm and incubated at 25°C for 10 days. Appropriate controls were set up in which the plant pathogen or bacterium was subjected to the same procedure without the fungal isolate on the center of the Petri plate. The experiment was repeated twice to compare results with three replicates of each treatment. Percent growth inhibition (PGI) was determined after 48 hours incubation at 25°C by using the formula of Skidmore & Dickinson (1976):

$$PGI\ (\%) = \frac{K_r - r_1}{K_r} \times 100$$

Where K_r represents the distance (measured in cm) of the control pathogens growth from the point of inoculation to the edge of the colony on the plates, and r_1 the distance of pathogen's growth towards the test fungus.

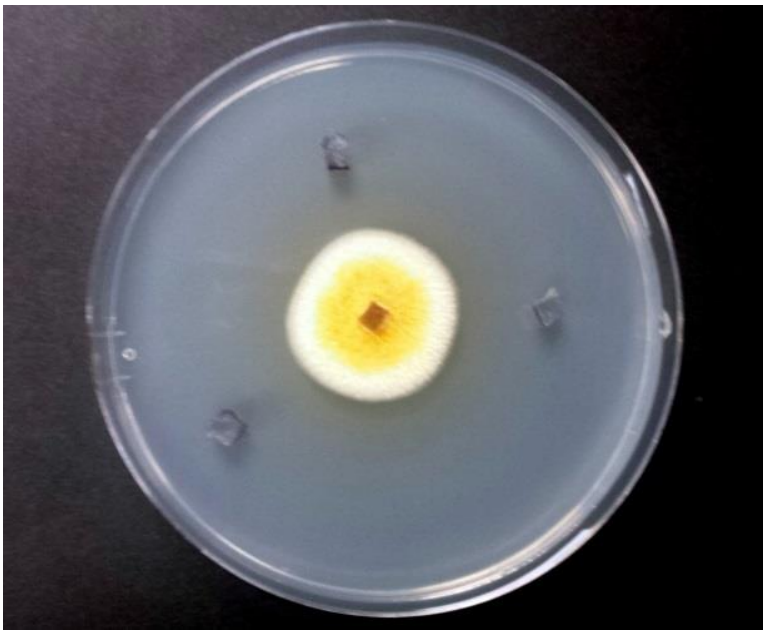

Figure 4. A 9 cm wide Petri dish plated with the fungus and test pathogens placed in periphery.

Test for Volatile Antimicrobials

Isolates were tested for the productions of antimicrobial VOCs (volatile organic compounds) by following protocols developed by (Strobel et al., 2001). Briefly, antifungal and antibacterial activities of each fungal isolate was assessed against all four plant pathogens, *Pythium ultimum, Phytophthora cinnamonmi, Sclerotinia sclerotiorum, Botrytis cinerea* and two bacterial strains isolated from the same lake ice cover and subglacial lake sediments as the respective fungus. To determine if VOCs were being produced by the fungal isolates from the ice cores, an agar strip of 2 cm was removed from the mid-portion of the plate. Removing the strip ensures that inhibitory compounds are VOCs and not diffusible secondary metabolites. The fungal isolate of interest was inoculated on the half-moon of the agar plate and cultivated for 10 days to optimize the

potential production of VOCs at room temperature (24°C). Plugs (5 mm) of test organisms consisting of plant pathogens were each placed on the other half of the plate (Figure 5).

Figure 5. A 9cm wide Petri dish plated with the fungi (left) and the pathogen (right) to check for inhibitory bioactivity of the fungal isolate.

Essentially the organisms are allowed to grow in the artificial atmosphere that is produced by any VOCs that may be present. This enables them to grow under the artificial atmosphere that is produced by the VOCs. The plates were wrapped with Parafilm and incubated for 12 days. Bacterial cultures were streak-inoculated on the other half of the same plate. The plates were wrapped in double layer of Parafilm to ensure that any VOCs produced did not escape from the plate and were incubated at 24°C. Controls were set up in which the plant pathogen or bacterium was subjected to the same procedure without the presence of fungal isolate in the plate.

Growth of test pathogens was recorded as the average increase in diameter (cm) after 48 hours of exposure to the pathogens. Growth was reported as percent inhibition as

compared to relevant control plates without the test organism. The bacterial and yeast cultures were visually estimated for the amount of colony growth and inhibition.

Measuring Substrate Utilization Using BIOLOG Plate Assay

The Biolog technique is a redox system where microorganisms, are incubated across a range of oxidize substrates present in a 96 well plate and respiratory potential is measured as the rate of reduction of a colorless tetrazolium dye to a violet formazan, which is then measured spectrophotometrically at 490 nm (Stefanowicz, 2006). The Biolog® substrate utilization test was first used by Garland and Mills to estimate the metabolic potential of isolates and to characterize the use of various substrates by filamentous fungi (Garland & Mills, 1991, Stefanowicz, 2006, FF Microplate™ Instructions for Use). Since then, this approach has been repeatedly applied in understanding community-level physiology (Weber & Legge, 2010). The FF (Filamentous Fungi) Microplates that have been recently introduced by Biolog contain a set of 95 varied carbon substrates and utilize reductions and/or turbidity of tetrazolium dye to indicate substrate utilization activity. It employs the same redox technique as seen in other Biolog plates where the colorless tetrazolium redox dye turns purple if the added microorganisms utilize that carbon substrate (Pohland & Owen, 2009). Different groups of microorganisms utilize substrates at different rates which results in variability in the rate of color development and the intensity that depends on the fungal isolate present (Stefanowicz, 2006).

The FF Microtiter plate contains 95 varied carbon substrates, one in each well, and a control well without any substrate. The plate was prepared by following the instructions provided in the FF Microplate (FF Microplate™ Instructions for Use). Pre-made FF Microplates (Biolog catalog # 1006) containing 95 different carbon substrates one in each well, and a control well without any substrate were purchased from Biolog (2114 Cabot Blvd, Hayward, CA) and stored at 4°C until needed. The MicroPlates were prewarmed for one hour before use. Pre-made FF-IF (Biolog catalog # 72106) sterile borosilicate test tubes containing 16 ml of sterile 'gelling' inoculating fluid 0.25%, Phytagel and 0.03% Tween 40 were used; the tubes were inverted to resuspend the gelling agent before use. Pure cultures of the fungi were first grown on PDA medium as it supported the best growth of the isolates. Cultures were incubated at 25°C under ambient light for 5-10 days. Turbidity range was established using the FF turbidity standard tube which gave 75% transmittance. The spectrophotometer (Milton Roy Spectronic 20) was blanked (100% transmittance) using a clean tube containing uninoculated FF fluid. Once the fungal colony had reached the desired size, the mycelium of each pure culture was lightly scraped using a sterile swab under a type II biological safety hood. This swab was then dipped into the FF-IF tube and gently twirled against the inside of the tube on the dry glass above the fluid line. This ensures the separation of any conidia present and release of cells to prepare a uniform suspension of the inoculum. The tube was gently but thoroughly mixed to ensure a homogenous mixture. At the same time, precaution was taken to ensure that bubbles were not introduced into the tube since they interfere with the absorbance reading.

Each cell suspension was inoculated into the microplate within 10 minutes of preparation. This step prevents strains from losing their metabolic activity because of being suspended in the inoculating fluid without nutrients. Next, each well of the Microplate was inoculated with 100 µL of the prepared suspension and incubated at 4°C and 24°C. The Microplates were set up in replicates of three for each isolate. Absorbance at 490 nm was measured on the FL 600 Microplate Fluorescence Reader at 24, 48, 72, 96, 120, 144 and 168 hours of incubation.

The substrate utilization profiles of the four isolates at 4°C and 24°C for the 92 carbon substrates were compared to investigate dependence of temperature on growth and to understand the preferred carbon substrate utilized by the organisms at the two temperatures. The optical density (OD_i) value from each well was corrected by subtracting the value for the least used substrate. The absorbance value of the control well was inexplicably higher than the absorbance values of some substrates and was not used as a subtraction value (Hitzl et al., 1997). In addition, carbon substrates were divided into 6 substrate guilds 1) amines/ amides 2) amino acids 3) carbohydrates 4) polymers 5) carboxylic acids and 6) other miscellaneous substrates, as suggested by Dobranic and Zak (1999) (Appendix A.1).

Statistical Analyses

Metabolic rate data from the 95 different carbon sources were examined simultaneously using the Biolog FF Microplate assay. The absorbance variables such as isolates, temperature, and substrate groups were managed and analyzed using Minitab 17

and R software package (http://www.r-project.org/). A Linear Model was used to investigate the relationships between temperature, isolates and groups. Analysis of Variance (ANOVA) F-tests were used to examine evidence of differences among levels of each factor. Tukey's Honest Significance Difference (HSF) test was conducted to examine pairwise differences within factors which showed evidence of a significant difference.

The statistical analyses were carried out as previously described by Buyer et al., (1999). Substrates divided into the 6 substrate guilds described above (Zak et al., 1994) and the average absorbance within each guild was calculated for all the wells. The integral approach helps detect difference among isolates in wells where growth responded- both slow and fast to the organic substrate (Garland, 1999).

HYPOTHESES

There is a great interest in discovering new species and their physiological capacity in extreme environments (Wang et al., 2015). My study addresses the physiology and function of fungi in the lake ice cover of east lobe Bonney and Lake Chad, and in subglacial lake sediments from Subglacial Lake Whillans to help provide a better understanding of the role of fungi in icy and sub-ice environments. The overarching theme of my study is fungi play important roles in the solid ice and sub-ice ecosystems. Within this overarching theme, I tested the following specific hypotheses which addressed selected aspects of the physiology, temperature response, bioactive properties, metabolic potential and substrate utilization of fungi present in the icy ecosystem.

Hypothesis 1: Viable fungi are present in the lake ice cover and subglacial lake sediments that can be cultured. To address this hypothesis, fungi retrieved from the lake ice cover and subglacial lake sediments were isolated and cultured using conventional practices. Molecular methods were used to identify the fungi and to help determine if they are novel.

Hypothesis 2: Fungi in lake-ice ecosystems and subglacial lake sediments are cold-adapted. To address this hypothesis, laboratory cultures obtained from ice cores taken from ELB, Lake Chad, and sediments from Subglacial Lake Whillans (SLW) were tested for growth and activity under various temperature and nutrient regimes. Growth and activity at different temperatures provides an insight into the ecological and

physiological mechanisms important in fungal survival in the lake ice and subglacial lake sediments.

Hypothesis 3: Fungi isolated from MDV and sediment sample from SLW are capable of producing bioactive volatile organic compounds. This hypothesis was tested by with an in-vitro dual culture assay. Briefly, each isolate was cultured and placed with plant pathogens or prokaryotes test organisms on the same petri plate for 48 hours to determine whether the isolate exhibited any bioactivity by inhibiting the growth of the test organisms. In a second assay, an agar strip was removed from the mid-portion of the plate to determine if VOCs were being produced.

Hypothesis 4: Fungal isolates have specific preferences for labile carbon substrates and this preference is a function of temperature. BIOLOG plates specific for fungi (FF Microplate^{TM}) were used to examine the preferred carbon substrates by each fungal isolate at 4°C and 24°C .

RESULTS

Isolation, Culturing, and Taxonomic Identification

Three fungal species were isolated from three different ice cores retrieved from the lake ice covers of ELB and Lake Chad, and one fungus was retrieved from a sediment sample taken from Subglacial Lake Whillans. Attempts were made at culturing meltwater from the ELB ice core on PDA, but this resulted in only bacterial growth on the petri plate. There was no fungal growth in the meltwater inoculation. Culturing the sediment rich ice cover resulted in fungal growth on petri plates with PDA medium (Figure 6).

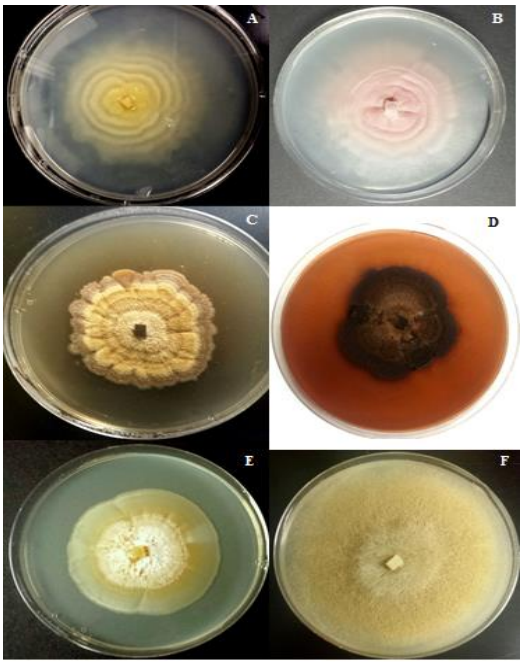

Figure 6. Mycelial colonies of 10 day old axenic cultures of fungi isolated from various sites. A) Younger culture of *Tetracladium ellipsoideum* from Lake Chad, B) Older culture of *Tetracladium ellipsoideum* from Lake Chad C) Younger culture of *Lecythophora hoffmannii* from SLW D) Older culture of *Lecythophora hoffmannii* from SLW E) Unidentified *Ascomycota (Helotiales)* isolate from ELB, and F) *Mucor* sp. from ELB

Sequence analysis of the ribosomal ITS region was conducted on four fungal cultures to identify them. The ITS regions of the isolates from Subglacial Lake Whillans and the isolate from Lake Chad were successfully amplified using Polymerase Chain Reaction (PCR) with fungal primers ITS 1-F and ITS 4 followed by DNA sequencing. Phylogenetic analysis of fungal isolates was performed after acquisition of the ITS- 5.8 S ribosomal gene sequence. BLAST searches of GenBank indicated that the two successfully amplified fungal cultures were members of the phylum *Ascomycota.* The isolate from of Sublglacial Lake Whilllans was identified as *Lecythophora hoffmanni* and the isolate from Lake Chad as *Tetracladium ellipsoideum.* Attempts to amplify the ITS regions for the other two isolates were unsuccessful. The isolate from east lobe Bonney was identified as a *Zygomycota* and a member of the *Mucor* sp. based on its morphology and the isolate from East Lobe Bonney remains unidentified to species but belongs to the phylum *Ascomycota (Helotiales)* (Table 1).

Table 1: Summary of fungal sequences from BLAST search of GenBank

Taxon	Location	Source	GenBank Accession number	Sequence similarity (%)	Score
Tetracladium ellipsoideum	Lake Chad	Ice cover	AB776689.1	99	987
Lecythophora hoffmannii	Subglacial Lake Whillans	Sediment	AB231012.1	100	920
Mucor sp.	East Lobe Bonney	Ice cover	NA	NA	NA
Unidentified *Ascomycota*	East Lobe Bonney	Ice cover	KC965738.1	93	431

Morphological Description Using Microscopic Observations

Morphological and microscopic observations were conducted on individual culture plates. Culturing the sediment inclusions from the ice core samples resulted in four isolates.

1. *Tetracladium ellipsoideum* (Wang et al., 2015)

(Lake Chad ice cover)

Description: Younger cultures pale yellow, flat to slightly raised with no aerial hyphae; makes concentric rings in ambient light on PDA; margin uneven; reverse of culture is yellow in color. Older cultures light pink (Figure 6 B). Hyphae narrow, hyaline, thin-walled, about 1-2 μm wide, some arranged in parallel strands of hyphae and coiled; immature conidiophores observed early on but not recorded in later stages.

Comments: DNA analysis was successful for this isolate and showed a 100% match in BLAST search of GenBank. This species has been reported earlier in polar regions (Wang et al., 2015). The first sequence mentioned in the phylogenetic tree (Figure 7) is the *T. ellipsoideum* isolate that was isolated and cultured in this study. The remaining were reference isolates used to determine the position of *T. ellipsoideum.* The taxon name for this isolate was determined by using the best GenBank accession number match. According to the phylogenetic tree, the isolate was closest match to *T. ellipsoideum*, however, there was 2.7% distance between the two isolates.

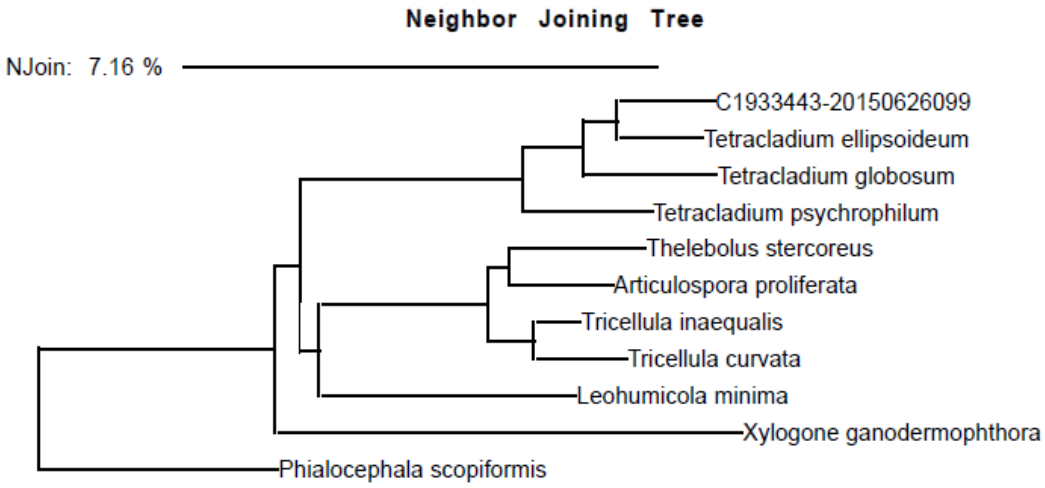

Figure 7: Phylogenetic tree showing the relationship of *T. ellipsoideum* to reference taxa generated by Charles River (Newark, DE).

2. *Lecythophora hoffmannii* (W. Gams and McGinnis, *Mycologia* 75:985, 1983)

(Subglacial Lake Whillans sediments)

Description: Younger cultures pale orange to salmon, relatively flat, less floccose with slight aerial mycelium; margin sharp and slightly lobed with a few radial folds; reverse of the culture is dark brown; exudates turn the agar pale orange to salmon (Figure 6 C). In older cultures, margins have tufts of brown hyphae reaching into agar, and exudates turn the agar pale brownish with slight orange tint (Figure 6 D). Reverse is then dark brown. Hyphae narrow, hyaline, about 2- 2.5 µm wide, regularly septate with some septa showing constrictions. Conidia present in masses. Conidia mostly long, elliptical, a few slightly curved and hyaline, approximately 3- 4 x 1.5 - 2.0 µm; Q = 1.8 cm. Many individual swollen end cells with a darker pigmented granular interior present. Although a few hyphal-like phialides with expanded lips were possibly observed projecting at right angles to main hyphal branches, these were not distinct.

Comments: Obsolete synonyms of this species are *Margarinomyces hoffmannii* and *Phialophora hoffmannii* (Weber, 2002). Conniochaeta is the sexual stage. DNA analysis was successful for this isolate and showed a percent similarity of 100% in BLAST search of GenBank. According to Weber et al. (2002), *L. hoffmannii* is phylogenetically distinct from the other species present in the genus. *Lecythophora lignicola* is the only species of *Lecythophora* that has been previously reported in Antarctica and was retrieved from the mud ponds in Victoria Land (Corte and Gestro, 1994). No slimy heads were evident for the *L. hoffmannii* conidia as mentioned by Gams & McGinnis (1983). The first sequence in the phylogenetic tree (Figure 8) is the *L. hoffmannii* isolate that was isolated and cultured in this study. The others are reference sequences used to determine the phylogenetic position of *L. hoffmannii*.

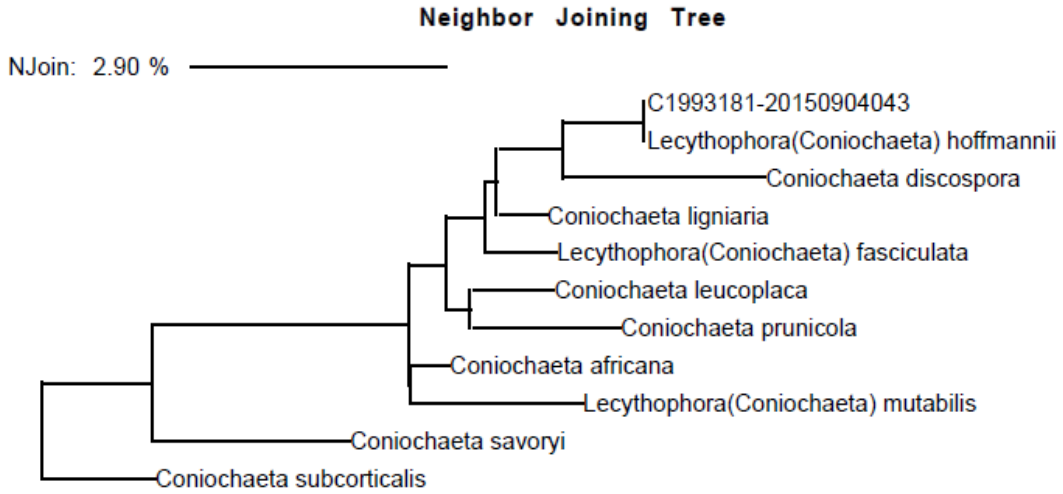

Figure 8: Phylogenetic tree showing the relationship of *L. hoffmannii* to reference taxa generated by Charles River (Newark, DE).

3. *Mucor* sp. (*Zygomycota, Mucorales)*

(Lake East Lobe Bonney)

Description: Colonies on PDA growing rapidly, covering the whole petri plate in 7 days at 24°C. Colony light greyish (Figure 6 E), a low turf, some aerial mycelium in center, composed of tall and short sporangiophores, with sympodial branching, up to 20 µm in diameter, with encrusted walls near the apex. Sporangia typically 40-60 µm in diameter but up to 80 µm and rarely to 100 µm. Sporangia yellow, pale yellow, or brown, and globose; walls of the sporangia are minutely echinulate; smaller sporangia are present on shorter slightly curved branches; columella are light-colored, slightly elliptical, and up to 50 µm each way; the small collarette indistinct; sporangiospores ellipsoidal, smooth, approximately 6-8 x 4-6 µm. Zygospores not present.

Comments: DNA analysis was not successful for this isolate which was identified to the order level on morphology alone. Several species of the *Mucorales* genus have been reported from Antarctica, including *Mucor circinelloides* Tiegham f. *circelloides*, *M. circinelloides* f. *janssenii* (Lendn.) Schipper, *M. flavus* Bainier, *M. heimalis* Wehmer, *M. mucedo* L., *M. piriformis* A. Fisch., *M. plumbeus* Bonord., *M. racemosus* Fresen, and *M. racemosus* f. *shaerosporus* (Hagem) and Schipper (Tosi). Most of these species can be ruled out on sporangiospore size and shape alone; however some such as *M. circinelloides* (including forme *circinelloides*) that possess small ellipsoid spores, pale sporangia, and minutely echinulate sporangia remain as possible taxa (Shipper 1976, 1978).

4. Unidentified *Ascomycota (Helotiales)*

(Lake East Lobe Bonney)

Description: Younger cultures white turning yellow, radially tufted; hyphae in bundles, hyaline, smooth-walled, about 1.5–2 µm wide, arranged in paralled hyphal strands, more aerial hyphae in the center, some densely coiled hyphae, some hyphae up to 50 µm wide.

Comments: DNA analysis did not give sequence information for definitive identification. It was identified on the basis of closest match in the BLAST search that showed a 99% match with an unidentified uncultured fungal clone that was collected from Arctic soil samples (Timling et al., 2014). However, it could be determined that this fungus is an *Ascomycete* and that it is the *Helotiales* clade. It is possibly another *Tetracladium* species.

Temperature Experiments

Growth rates were determined for fungal cultures growing on PDA plates incubated at 0°C, 4°C, 7.5°C, 10°C, 15°C, 20°C, and 25°C under ambient light to test for temperature tolerance. The maximum diameter (in cm) reached by each fungal isolate and the corresponding time required (in days) at each of the seven temperatures are presented in Appendix A.1. and figures 9-12. Standard deviations of the means were low, the highest being 0.156 for a mean colony diameter of 8.08 cm for *Mucor* sp.

Temperature response experiments revealed that isolated fungi were psychrotolerant and growth rates were greatest at 25°C (Figure 9 - 12). As compared to the other three isolates, the *Mucor* species isolated from East Lobe Bonney showed greatest dependence on temperature (Figure 9). At 25°C it covered the entire area of petri

plate (~ 8.05 cm) within a period of 3 days (Appendix A.1., Figure 9). *Lecythophora hoffmannii*, isolated from Subglacial Lake Whillans was the only isolate that showed no growth at 0°C (Appendix A.1., Figure 10). *Lecythophora hoffmannii* and the unidentified *Ascomycota sp.* from East lobe Bonney, showed very similar temperature preferences at 24°C with doubling time of 0.008 cm d^{-1} and 0.004 cm d^{-1}, respectively (Appendix A.1., Figure 11). Growth response for the two isolates from east lobe Bonney at 25°C showed different temperature responses. *Mucor* sp. and the unidentified *Ascomycota* isolate showed growth rates of 0.08 cm d^{-1} and 0.004 cm d^{-1} respectively. *Tetracladium ellipsoideum* showed an increase in growth rate at 7.5°C and a slight decrease in growth at 10°C (Appendix A.1., Figure 12).

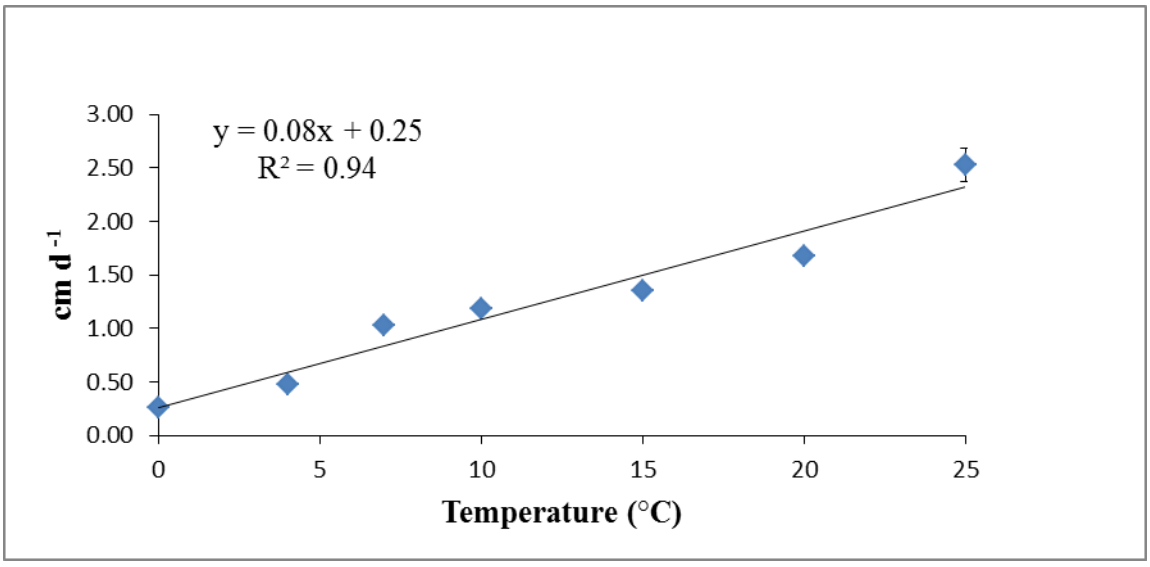

Figure 9. Effect of temperature on growth rates of *Mucor* sp. The error bars represent the average value of five replicated test plates.

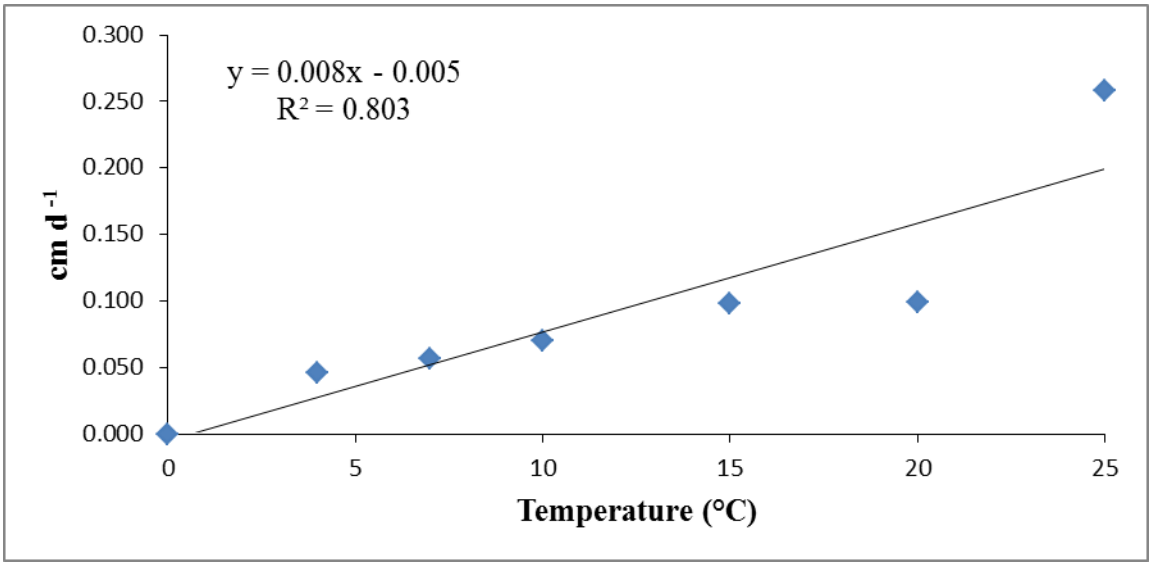

Figure 10. Effect of temperature on growth rates of *Lecythophora hoffmannii.* The error bars represent the average value of five replicated test plates.

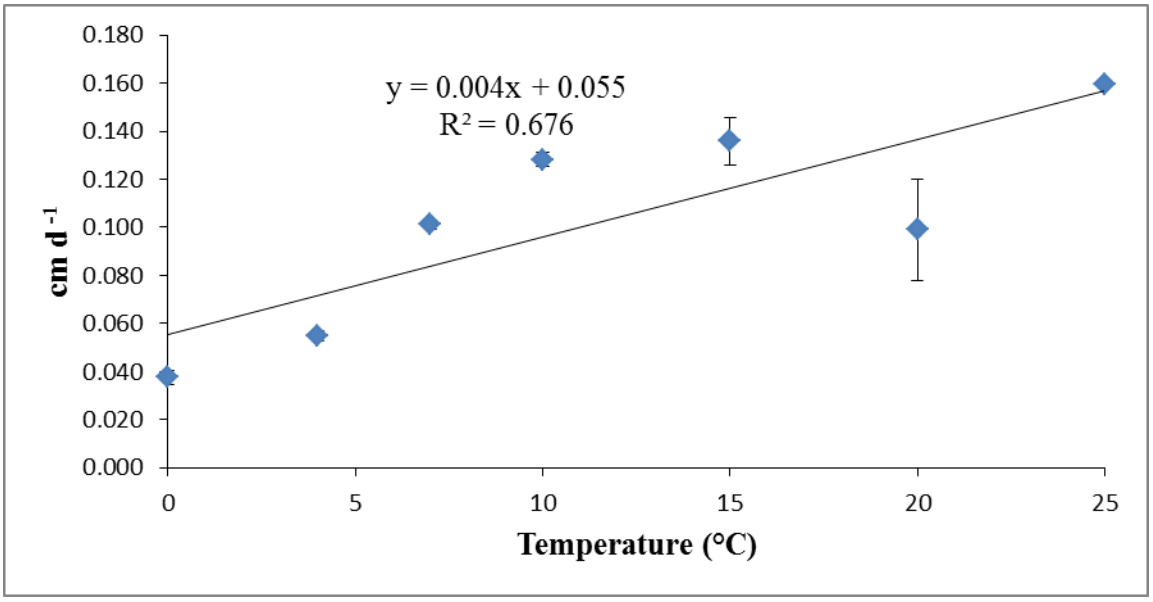

Figure 11. Effect of temperature on growth rates of Unidentified *Ascomycota*. The error bars represent the average value of five replicated test plates.

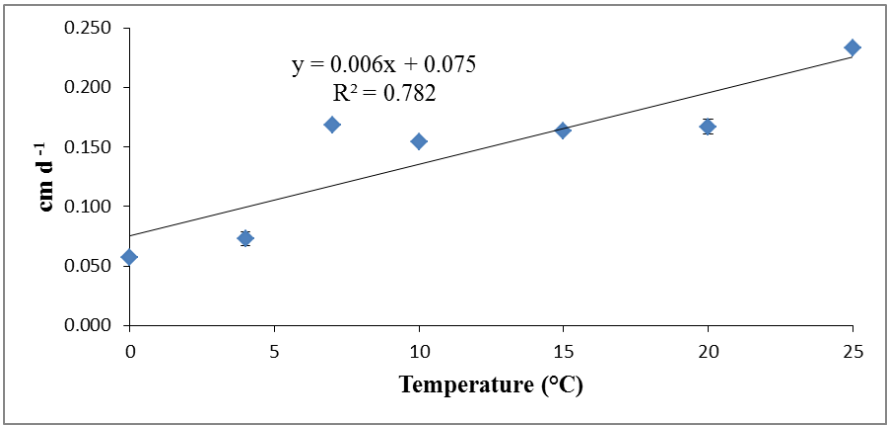

Figure 12. Effect of temperature on growth rates of *Tetracladium ellipsoideum*. The error bars represent the average value of five replicated test plates.

In the lower temperature range (from 4°C to 15°C), the Q_{10} values for *T. ellipsoideum, L. hoffmannii, Mucor* sp. and the unidentified *Ascomycota* all doubled. On the other hand, in the higher temperature range (15°C - 25°C), out of the four isolates only *L. hoffmannii* doubled when the temperature was increased by 10°C (Q_{10} = 2.63) (Table 2).

Table 2. Q_{10} values for four fungal isolates at three temperatures.

Isolate	**Q_{10} values**	
	Lower temperature 4°C - 15°C	Higher temperature 15°C - 25°C
Tetracladium ellipsoideum	2.07	1.42
Lecythophora hoffmannii	1.98	2.63
Mucor sp.	2.57	1.86
Unidentified *Ascomycota*	2.27	1.17

Biological Effects of Fungal Isolates on Test Organisms

Dual Culture Assay

Five plant pathogens obtained from Plant Bioscience department of MSU and two bacteria from the lake ice cover of ELB and sediment of SLW were used as test organisms in the dual culture assay. The test organisms selected were plant pathogens, *Pythium ultimum, Phytophthora palmivora, Sclerotinia sclerotiorum, Fusarium solani,* and *Botrytis cinera*. The Antarctic fungal isolates were first grown for 10 - 15 days at 25°C on PDA, except for *Mucor* sp. which was grown for 24 - 35 hours due to its greater dependence on temperature (Figure 9). The test organisms were then exposed to the fungal isolates for 48 hours to determine if the fungus inhibited the test organisms. Percent inhibition was measured relative to the growth of the control plate containing the pathogen alone. The unidentified *Acomycota* and *Mucor* sp. did not inhibit any of the test organisms (Table 3). *Lecythophora hoffmannii* inhibited the test organism *Pythium ultimum* 93% ($p < 0.05$), moderately inhibited both *Sclerotinia sclerotiorum* 30% ($p < 0.05$) and *Fusarium solani* 31% ($p < 0.05$), and *Phytophthora palmivora* 21% ($p < 0.05$). *Tetracladium ellipsoideum* inhibited both *Phytophthora palmivora* 96% ($p < 0.05$) and *Sclerotinia sclerotiorum* 97% ($p < 0.01$) (Table 3).

Table 3. Effects of the bioactive compounds produced by the fungal isolates on pathogens and bacteria from SLW and ELB. The inhibition values were calculated as percentage growth inhibition as compared to untreated control. The tests were carried out in triplicates.

Isolates	**Inhibition (%) after 48 h exposure to plant pathogens**			
	Pythium ultimum	***Phytophthora palmivora***	***Sclerotinia sclerotiorum***	***Fusarium solani***
L. hoffmannii	93.05 ($p < 0.05$)	20.65 ($p < 0.05$)	30.32 ($p < 0.05$)	30.41 ($p < 0.05$)
T. ellipsoideum	14.96 ($p < 0.05$)	95.67 ($p < 0.05$)	96.98 ($p < 0.01$)	0
Mucor sp.	0	0	0	0
Unidentified *Ascomycota*	0	0	0	0

0 indicates that no inhibition on test organism was observed.

Test for Volatile Organic Compounds

The fungal isolates that showed positive results for the dual culture assay were tested for production of VOCs. These were *L. hoffmannii* and *T. ellipsoideum*, and they were tested against the same five plant pathogens as in the first test. *Lecythophora hoffmannii* was the only isolate that produced volatile compounds. It inhibited the growth of *P. palmivora* by 62.35% ($p < 0.05$) and *S. sclerotiorum* by 30.05% ($p < 0.05$). No other test organisms were inhibited. The susceptibility of the plant pathogens to the VOCs of *L. hoffmannii* was dependent upon the age of the *L. hoffmannii* culture. The peak of effective VOC production by *L. hoffmannii* was between 11-15 days after inoculation of the test plate.

Substrate Profiling

The FF (Filamentous Fungi) Biolog Microplates were used to evaluate quantitatively the physiological profile of fungi present in the Antarctic lake ice cover and sediment samples. For each of the combinations of isolates, temperature, and substrate groups, the average values of metabolic activity (i.e. absorbance at 490 nm) across all of the 4 - 7 days, depending on the isolate, was taken as the response variable. Figures 13 and 14 show the average values of absorbance at 4°C and 24°C respectively.

ANOVA (Analysis of Variance) was used to analyze the carbon substrate utilization among the four isolates. The model incorporates main effects for isolate, temperature, replicates, and substrate guilds. Interactions between temperature and isolate, temperature and substrate guilds, and isolate and substrate guilds were also considered. Diagnostic plots showed an increase in variability of the residuals as the fitted values increase. Hence, a square-root transformation was performed on the response variable in order to normalize variability in the residuals. After transformation, other than a few observations with large residuals, the assumption of normally distributed errors was reasonable.

Results from ANOVA showed that all main effects such as isolate ($F_{3, 128} = 18.37$, p-value < 0.001), temperature ($F_{1, 128} = 102.34$, p-value < 0.001), substrate guilds ($F_{6, 128} = 15.70$, p-value < 0.001) and replicates ($F_{2, 128} = 3.56$, p-value < 0.05) had significant impact on the mean square-root absorbency. Also, there was strong evidence of an interaction between temperature and isolate ($F_{3, 128} = 9.26$, p-value < 0.001), and temperature and substrate guilds ($F_{6, 128} = 9.47$, p-value < 0.001). However, there was no

strong evidence of an interaction between the four isolates and substrate guilds ($F_{18, 128}$ = 0.9946, p-value = 0.47). Hence, the interaction between isolate and substrate guilds was not considered in the model.

Table 4. ANOVA for transformed response.

Source	df	Sum of Squares	Mean Square	F	p
Replicate	2	0.015	0.007	3.568	< 0.05
Temperature	1	0.225	0.225	102.341	< 0.001
Isolate	3	0.121	0.04	18.372	< 0.001
Group	6	0.207	0.034	15.707	< 0.001
Temperature : Isolate	3	0.061	0.02	9.265	< 0.001
Temperature : Group	6	0.125	0.002	9.472	< 0.001
Isolate : Group	18	0.039	0	0.994	0.4703
Residuals	128	0.282	0.002		

Results from the final ANOVA showed that after accounting for the effects of replications, temperatures, and isolates, a strong evidence ($F_{6, 146}$ = 15.71, p-value < 0.001) for differences in mean square-root absorbency across the different isolates was observed. After accounting for effects of replications, temperature, isolates, and substrate guilds, there was strong evidence ($F_{3, 146}$ = 9.27, p-value < 0.001) that the effects of isolates on mean square-root absorbency differ across temperature. Thus, all main effects such as isolate ($F_{3, 146}$ = 18.38, p-value < 0.001), temperature ($F_{1, 146}$ = 102.41, p-value < 0.001), substrate guilds ($F_{6, 146}$ = 15.71, p-value < 0.001) and replicates ($F_{2, 128}$ = 3.57, p-value < 0.05) have significant impact on the mean square-root absorbency.

Table 5. ANOVA for interactions between temperature and isolate, and temperature and group

Source	df	Sum of Squares	Mean Square	F	p
Replicate	2	0.015	0.007	3.568	< 0.05
Temperature	1	0.225	0.225	102.341	< 0.001
Isolate	3	0.121	0.04	18.372	< 0.001
Group	6	0.207	0.034	15.707	< 0.001
Temperature : Isolate	3	0.061	0.02	9.265	< 0.001
Temperature : Group	6	0.125	0.002	9.472	< 0.001
Residuals	146	0.321	0.002		

Carbon substrate utilization, assessed via Biolog FF Microplates, showed that at 4 °C, all four isolates showed maximum preference for polymers (Figure 13). The overall substrate utilization (absorbancy) was greater when the isolates were incubated at 24°C (Figure 14). At 24°C the isolates *T. ellipsoideum*, the unidentified *Ascomycota* and *Mucor* sp. showed maximum utilization of polymers (specifically a- cyclodextrin), amino acids, and carbohydrates as compared to other carbon guilds. All the four fungal isolates however, showed a preference for amino acids and carbohydrates over polymers at 24°C.

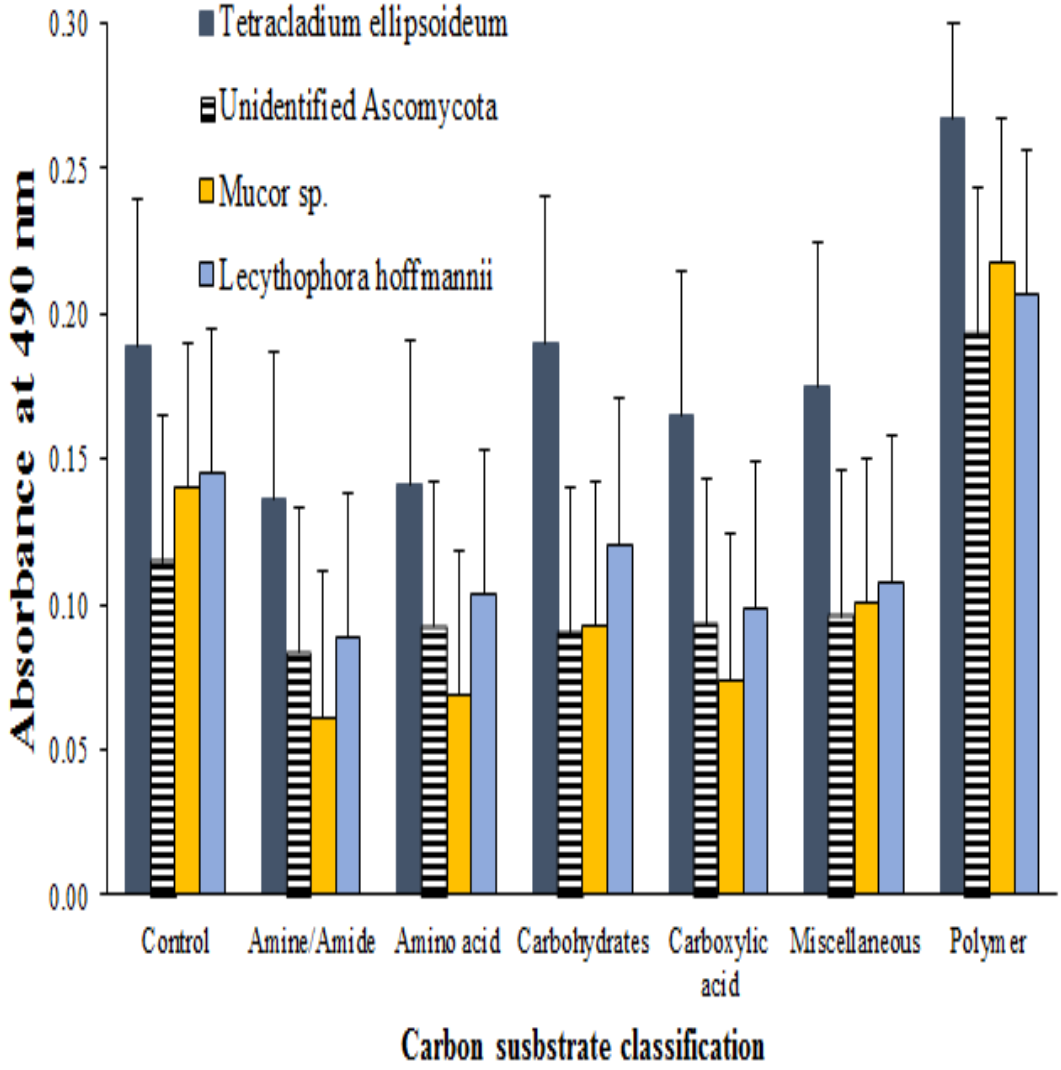

Figure 13. Carbon substrate utilization by all four isolates at 4°C. The error bars represent the average absorbance values for three replicates over a time period of 7 days.

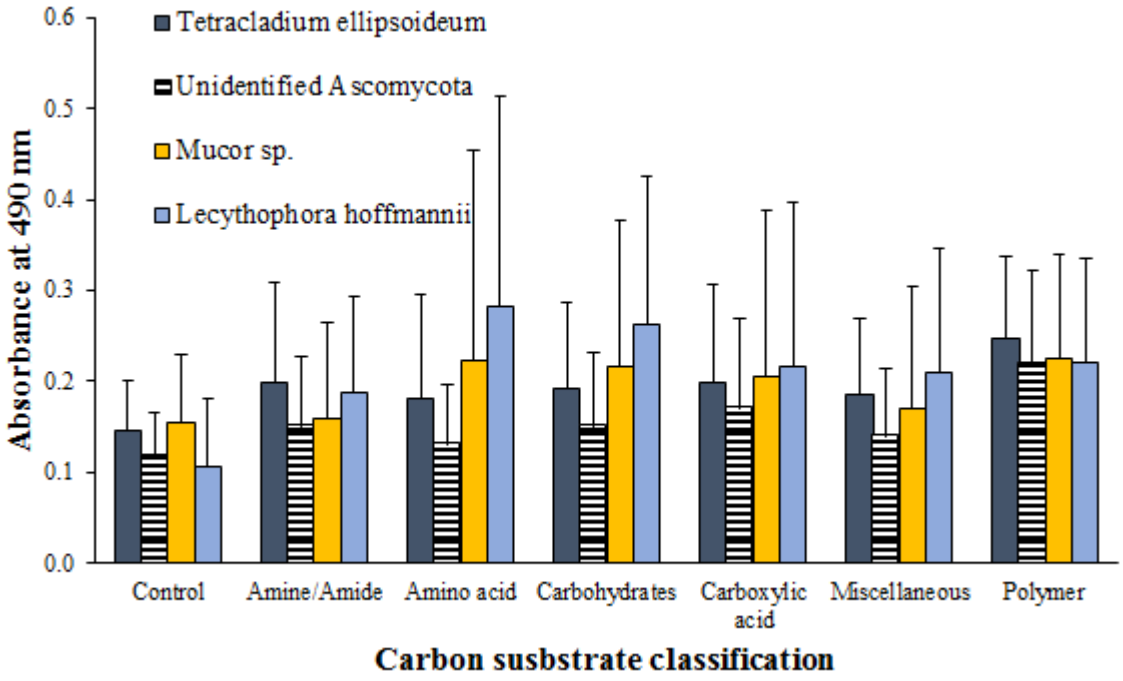

Figure 14. Carbon substrate utilization by all four isolates at 24°C. The error bars represent the average absorbance values for three replicates over a time period of 7 days.

The overall absorption, as noted earlier, was significantly higher ($p < 0.05$) at 24°C (0.43, $p < 0.05$) than at 4°C (0.35, $p < 0.05$) irrespective of the different substrate guilds, isolates and replicates. Effect plots for the model factors display the estimated effects on mean square-root absorbency across the different factors considered. One such plot (Figure 15) displays the effects of isolate and temperature conditioned on substrate groups and replication. By conditioning on replicates and substrate guilds, the effects of these variables were effectively averaged and deviations from this mean value for isolate and temperature combinations were considered. This makes it possible to consider deviations from this mean value for isolate and temperature interactions. From figure 15**,** it is evident that the unknown *Ascomycota*, *Mucor* sp. and *L. hoffmannii* have different effects on metabolic rate depending on the temperature at which they were inoculated.

However, *T. ellipsoideum* showed approximately similar substrate utilization response at both 4°C and 24°C without showing any apparent temperature dependence. Substrate utilization of all 95 substrates is higher when inoculated at 24°C whereas lower when inoculated at 4°C.

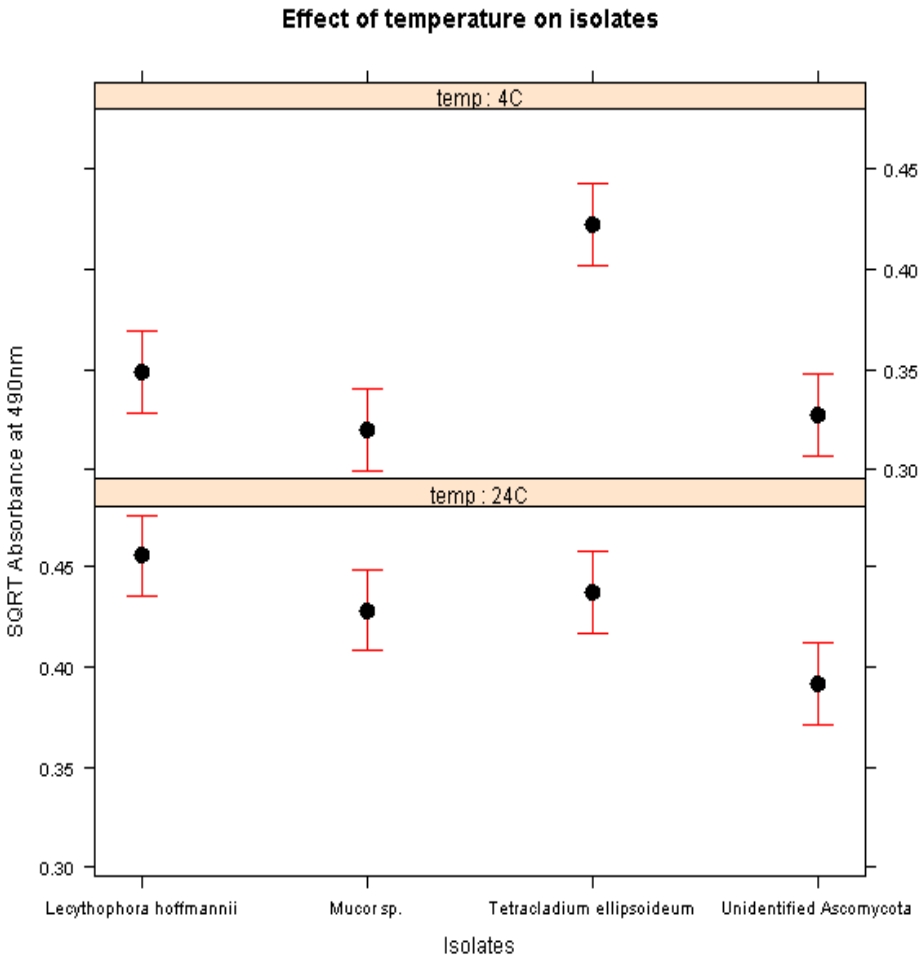

Figure 15: Effect of temperatures on the growth of isolates. The error bars represent the average absorbance values for three replicates over a time period of 7 days.

The post-hoc test was conducted using Tukey's HSD test to investigate if there was a significant difference between the substrate utilization of the four isolates at two

temperatures. At 4°C, substrate utilization for *T. ellipsoideum* was significantly higher ($p < 0.05$) than the unknown *Ascomycota*, *Mucor* sp. and *L. hoffmannii.* Substrate utilization for *Mucor* sp. and *L. hoffmannii* were also significantly higher ($p < 0.05$) than that of the unknown *Ascomycota* (Figure 15). At 24°C, the absorbance of the unknown *Ascomycota* and *L. hoffmannii* were significantly different than *T. ellipsoideum.* There was no significant difference between substrate utilization of *T. ellipsoideum* and *Mucor* sp. ($p > 0.05$) at 24°C (Figure 15).

Figure 16 displays the effect of temperature and substrate groups conditioned on isolates and replication. At both 4°C and 24°C, the polymers are the most utilized substrate guild amongst all six guilds across the four isolates and replicates (Figure 16). The post-hoc test was conducted using Tukey's HSD test to find whether temperature had a significant effect on different substrate guilds. At 4°C there was a significant difference ($p < 0.05$) among the square-rooted absorbance values of amines/amides and amino acid, carbohydrate, carboxylic acid, polymers and the miscellaneous group. At 24°C, there was no significant difference ($p > 0.05$) between absorbance of amino acid and carboxylic acid. There was a significant difference in substrate utilization among carbohydrates and carboxylic acid, polymers and the miscellaneous group.

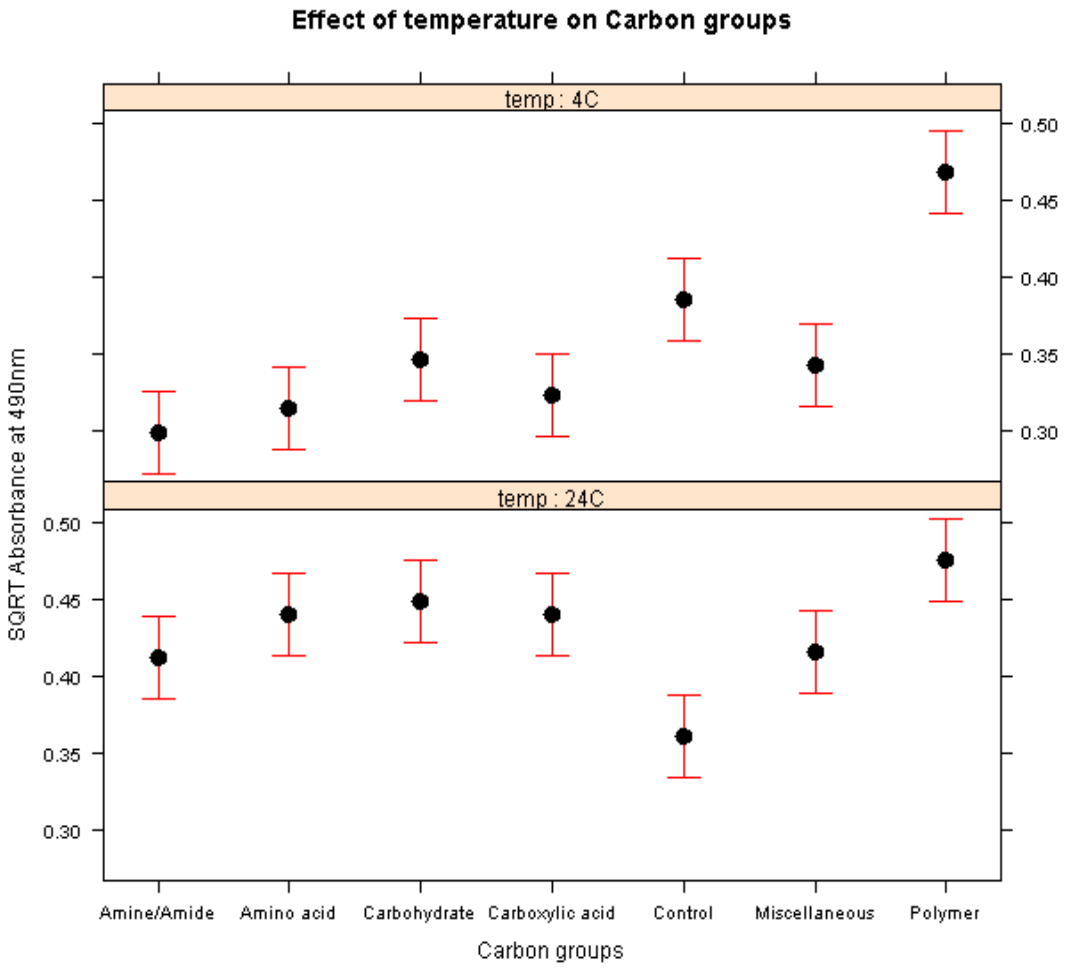

Figure 16: Effect of temperatures on the carbon guild utilization by the four fungal isolates. The error bars represent the average absorbance values for three replicates over a time period of 7 days.

A detailed comparison of the carbon substrates utilized by each of the four fungal isolates was investigated using heatmaps reflecting the absorbance values without statistical transformation (Appendix C). Results from heat map showed that at 4°C all four isolates had maximum substrate utilization of polymers (a- cyclodextrin) as shown in Appendix C. However, at 24°C the results from the heat map contradicted the Effect plots, where all four isolates had maximum preference for carbohydrates (Appendix C.1,

C.3, C.5, and C.7). At both the temperatures, *T. ellipsoideum* (Appendix C.1 and 2) and unidentified *Ascomycota* isolate (Appendix C.3 and 4) had maximum substrate utilization of sebacic acid. At 4°C, *Mucor* sp. had maximum substrate utilization for polymer a-cyclodextrin and poorly utilized N-acetyl-D-glucosamine (Appendix C.5). However at 24°C, the substrate utilization of N-acetyl-D-glucosamine was maximum (Appendix C.6). The overall substrate utilization of *L. hoffmannii* at 4°C was relatively low (Appendix C.7) compared to utilization at a higher temperature (Appendix C.8).

DISCUSSION

Fungi have been isolated from cold environments and have also been investigated to determine their adaptive strategies to withstand low nutrient availabilities and cold temperatures (Ruisi et al., 2007). They constitute a conspicuous portion of the microbiota in Antarctica. Lake ice cover and sediments from the subglacial environment provide a unique opportunity to investigate the microbiology of icy ecosystems that have remained isolated from the atmosphere for significant periods of time.

Diversity of Fungal Isolates from Antarctic Lake Ice and Subglacial Sediments

Fungal biodiversity in continental Antarctica has been widely investigated by isolating, culturing, and identifying fungal strains from samples collected in different habitats of the continent (Onofri, 2005b); Onofri et al. (2005b, 2006) reported that 99.4% of fungi present in continental Antarctica are true fungi that includes yeasts and filamentous fungi, which together comprise species belonging to the phyla *Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota.* In spite of the evidence that fungi comprise an important part of these habitats, the mycological component of ice communities is often overlooked (D'Elia, 2008). The findings from this section of the thesis targeted the isolation and characterization of fungi present in the permanent lake ice cover and sediments of subglacial Lake Whillans.

Phylogenetic analysis was carried out by sequencing the ITS 5.8S ribosomal gene sequence since this region has been successfully used in the past to resolve

phylogenetic relationships of fungi at inter- and intraspecific level (Egger & Sigler, 1993; Lee & Taylor, 1992). Axenic cultures of *Tetracladium ellipsoideum* from Lake Chad, *Mucor* sp. and an unidentified *Ascomycota* sp from East lobe Bonney were obtained from the permanent lake ice covers. An axenic culture of *Lecythophora hoffmannii* was also retrieved from the sediment sample of Subglacial Lake Whillans. Three of the four fungal cultures, *Tetracladium ellipsoideum, Lecythophora hoffmannii* and the unidentified *Ascomycota* isolate grouped phylogenetically with *Ascomycota. Mucor* sp.was identified based on its morphology and is included in phylum *Zygomycota***.**

The ITS sequence analysis of the fungal culture *Tetracladium ellipsoideum* that was retrieved from Lake Chad showed similarity with cultures that have been previously isolated from cold environments, such as permafrost soil and streams of alpine glacier (Kuhnert et al., 2012; Robinson et al., 2000), implying that it may be cold-adapted. Eight species of *Tetracladium* have been reported in the genus and are known to be distributed worldwide in lotic (Shearer, 2007) and aquatic habitats (Read et al.1992). An endophytic strain that was recovered from riparian plant roots from a freshwater stream of Nanital in Himalaya, India was recognized as a *Tetracladium* species called *T. nainitalense* (Sati, Arya, & Belwal, 2009)**.** In the samples obtained from soils and lake sediments in the Skarvsnes ice-free area of Antarctica, 9.9% of the dominant isolates belonged to the genera *Tetracladium* (Tsuji et al., 2013). In a study conducted by Arenz & Blanchette (2010), an uncultured *Tetracladium* clone was retrieved from soil samples collected from the Antarctic Peninsula. The *Tetracladium elliposideum* isolate that showed a 100% match in the BLAST search to my sample also matched up with a newly generated

sequence that was isolated from the soil samples of glacier on the Qinghai- Tibet Plateau (Wang et al., 2015).

Lecythophora hoffmanni has been previously isolated from weathered wood and results in soft-rot decay in historic huts and artifacts (Bugos, Sutherland, & Adler, 1988**);** it has also been isolated as an endophytic fungus from Bryophytic samples collected on Barton Peninsula of King George Island in maritime Antarctica (Yu et al., 2013). Some species of this genus also are known to be pathogens of woody hosts (Damm et al., 2010) and have also been involved in human diseases associated with cases of sinusitis, peritonitis, subcutaneous infections, and canine osteomyelitis (Perdomo et al., 2011). *Lecythophora lignicola* has been reported from mud ponds in Victoria Land (Corte & Gestro, 1994),

The *Mucor* species retrieved from ELB in this study was identified based on its morphology. Amongst the *Mucorales*, *Mortierellaceae* and *Mucoraceae* are the most frequently recorded families in Antarctica. Genera in these families are known to produce a large number of mitotic spores that are resistant reproductive structures that enhance survival opportunities (Onofri et.al, 2006). They have a worldwide distribution and are regarded as psychrotolerant organisms (Onofri et.al, 2006).

Isolation, culturing and identification of fungi from the lake ice cover and subglacial lake sediments add new evidence to range of the existing microbial diversity and support the likelihood of the existence of a complex ecosystem within subglacial lakes. This analysis further supports the hypothesis stated in this study that at least some

fungi present in the lake ice cover and subglacial lake sediments are viable and can be cultured.

Growth Temperature Preference of Fungal Isolates

Antarctica exhibits some of the most extreme conditions on Earth. Cold adaptation is the most investigated aspect of fungi in Antarctica (Zucconi et al., 1996). The objective of this experiment was to investigate and compare the effects of temperature on fungal growth. The results of the growth experiments inform the discussion of fungal ecology in ice and sub-ice environments. Fungi have evolved mechanisms that enable them to withstand the low temperatures and nutrient availabilities (D'Amico et al., 2006; D'Elia et al., 2009; Ruisi et al., 2007), which allow them to persist in the Antarctic. Fungi that are capable of growing at or below 0°C have optimum growth temperatures (OGT) of ≤ 15°C and maximum growth temperatures (MGT) of ≤ 20°C are considered psychrophilic; those capable of growing close to 0°C, have OGT >15°C and MGT > 20°C are considered psychrotolerant (Morita, 1975).

All four fungal isolates grew well in the 15 - 25°C range on PDA medium. They also grew at temperature near 4°C identifying them as psychrotrophs (*L. hoffmannii, T. ellipsoideum* and unknown *Ascomycete* isolate) or cold tolerant mesophiles (*Mucorales* sp.) that are capable of growing at low temperatures but have OGT of 15°C and MGT of 20°C, respectively. *Lecythophora hoffmannii* isolated from lake sediments of SLW grew to 0.04 cm in diameter at 4°C in a period of 13 days and showed no growth at 0°C. The

Mucor species that was isolated from ELB grew the fastest with doubling time of 0.085 cm d^{-1} at 25°C on PDA medium. Previous studies have recorded *Mucor* species to have rapidly growing colonies and also regarded as a psychrotolerant thermophile with OGT being 35 - 37°C and MGT at 45°C (Domsch, 1980).

The Q_{10} values for *T. ellipsoideum, Mucor* sp., the unidentified *Ascomycota* and *L. hoffmannii* doubled between 4°C – 15°C. Results of this study were in agreement with the growth studies at different temperatures where *L. hoffmannii* showed no growth at 0°C (Figure 12). This shows that an increase in temperature by ± 10°C at a low temperature could have a greater effect on the growth of an organism as compared to increasing the temperature by 10°C in the higher temp range. Environmental temperatures in the area from where these fungal isolates were collected fluctuate between - 4°C to 7°C. Hence it is difficult to relate these Q_{10} values to the environment.

None of the isolates tested could be defined as psychrophilic because they were all capable of growth at temperatures > 20°C (Morita, 1975), but the growth temperature experiments showed that all four isolates in this study were psychrotolerant. This is in agreement with the previous studies that state microfungi from Antarctica are mostly psychrotolerant mesophiles capable of growing over wide temperature ranges (Kerry, 1990; Zucconi et al., 1996). All four isolates had optimum growth temperature of 20°C or 25°C which is a lower range than most mesophiles. The four psychrotolerant fungal isolates reported in this study have been collected from Antarctica and some other cold habitats in the past.

Biological Inhibition of Fungi by Antarctic Fungal Isolates

Antarctica is characterized by some of the main environmental stressors found on Earth, such as low temperatures, UV-radiation, desiccation, varying light conditions, salinity, nutrient concentration and freeze thaw-cycles. This infrequent combination of selection pressures has resulted in the evolution of novel biochemical and novel adaptations, and possibility of autochthonous species (Ellis-Evans & Walton, 1990; Vincent, 2000). In recent decades, there has been an increase in studies investigating the metabolic potential of Antarctic microbes in production of secondary antimicrobial compounds (Bull et al., 2000; Nichols et al., 1999). In a study conducted by Marinelli et al. (2004), 29% of the microfungal species collected from benthic mats of lakes in Antarctica showed antimicrobial activities. Brunati et al. (2009) reported new antibiotics produced by fungi that were also from the microbial mats from Antarctic lakes.

This study reports bioactivity in fungi associated with the Antarctic lake ice cover and sediments from a subglacial lake. Four fungal isolates were screened for antagonistic activity against five plant pathogens. Neither the *Mucor* sp. nor the unidentified *Ascomycota* isolated from ELB inhibited the growth of the pathogens in dual culture assay. *Lecythophora hoffmannii* inhibited radial growth of the plant fungal pathogen *Pythium ultimum* (*Oomycota)* by establishing a clear zone of inhibition in a dual culture test. It significantly inhibited ($p < 0.05$) the growth of *Phytophthora cinnamomi* (*Oomycota), Sclerotinia sclerotiorum (Ascomycota)* and *Fusarium solani (Ascomycota),* by 20.65%, 30.32%, and 30.41% , respectively. *Tetracladium ellipsoideum* isolated from

Lake Chad showed approximately 97% inhibition against *P. palmivora* and *S. sclerotiorum.*

VOCs were only produced by *L. hoffmannii*, and these VOCs significantly inhibited two of the pathogens *Phytophthora palmivora* by 62.35% ($p < 0.05$) and *Sclerotinia sclerotiorum* by 30.05% ($p < 0.05$). *L. hoffmannii* produced maximum VOCs after 12 days of growth; however as the culture grew older, the percent growth inhibition for the pathogens started to decline (data not shown).

Two fungal isolates, *T. ellipsoideum* from Lake Chad and *L. hoffmannii* from SLW possessed antifungal activity against known plant pathogens which has not been previously reported for fungi isolated from Antarctic lake ice. This could have promising applications in biotechnology, and possibly relates to the isolates ability to compete with other fungi in the environment.

Carbon Substrate Utilization Using Biolog Assay

The Biolog method was used to compare the substrate utilization of fungal isolates collected from the ice cores and lake sediments of McMurdo Dry Valley and Subglacial Lake Whillans region, respectively. This method was initially introduced to obtain physiological profiles of the entire microbial communities (Garland, 1997). Most research carried out using the Biolog Microplate focuses on community analyses, taxonomic richness or diversity (Zak, Willing, Moorhead, & Wildman, 1994). However, this study uses the Biolog plate to investigate the physiology of individual fungal isolates based on the respiratory potential and utilization of specific carbon guild by each fungus.

Each substrate on the FF Biolog plate was characterized on the basis of its chemical nature thus making it possible to sort the 95 different substrates into six main chemical groups such as amines/amides, carbohydrates, carboxylic acids, polymers and miscellaneous. The types of substrates utilized by the fungal isolates, as well as the levels of activities on various substrates assessed the ecological diversity of the isolates.

The FF MicroPlate profile revealed that there was a significant increase ($p < 0.05$) in substrate utilization patterns at 24°C as compared to 4°C (Figure 14) across all carbon guilds, isolates and replicates. This result is in agreement with the results from the temperature experiment where all four isolates were psychrotolerant and optimal growth was observed at 25°C as compared to 4°C (Figure 9-12). This indicates that both, growth rates and respiratory potential of these four isolates were dependent on temperature. Interestingly, the *Mucor* species which showed the fastest growth rate (0.85cm d^{-1}) did not show a higher utilization of carbon substrates in the BiologPlate assay at either temperature. Although, it has a faster growth rate, it does not produce a dense mycelium.

Conclusions

Within the Antarctic environment, the lake ice cover represents a relatively unexplored habitat in terms of fungal diversity and ecology. This study addresses the physiology of fungi present in lake ice cover and subglacial lake sediments. According to Ellis-Evans (1996), not much is known about the fungal communities in Antarctic lakes and, until now, only a few genera have been described. Based on the molecular characterization, the results showed that axenic cultures of *Tetracladium ellipsoideum*

from Lake Chad, *Mucor* sp. and an unidentified *Ascomycota* isolate from East lobe Bonney were successfully obtained from the lake ice cover. *Lecythophora hoffmannii* was obtained from the sediments of Subglacial Lake Whillans. All the isolates that were identified have been previously reported in Antarctica and are related to taxa from several different cold habitats. Most studies report fungi in soil, lake water column, and glaciers only Mosier et al., (2007) reported Eukarya present in the lake ice cover of Lake Vida.

Temperature experiments revealed that these organisms were psychrotolerant and showed an optimum growth at 25°C. All the fungal isolates grew optimally in the 15°C - 25°C range. From the ITS sequence analysis, morphology, and psychrotolerant characteristics of these four isolates, it can be confirmed that at least three of these isolates are likely members of the Antarctic mycoflora and not modern contaminants. The *Mucor* sp. remains unsubstantiated. The isolation of the fungi from the lake ice covers and sediments of subglacial lakes further supports that these habitats may harbor a diverse set of microorganisms and complex ecosystems.

Two out of four isolates, *T. ellipsoideum* and *L. hoffmanni* showed antifungal activity against some of the most detrimental plant pathogens such as *Pythium ultimum, Phytophtora palmivora* and *Sclerotinia sclerotiorum. L. hoffmanni* inhibited *P. ultimum* (94%, $p < 0.05$) and *T. ellipsoideum* inhibited *P. palmivora* (96%, $p < 0.05$) and *S. scletiorum* (97%, $p < 0.01$). *Lecythophora hoffmannii* produced bioactive organic compounds that inhibited the growth of *S. sclerotiorum* by 30.05% ($p < 0.05$) and *P. palmivora* by 62.35% ($p < 0.05$). It can be concluded that isolates from Antarctic lakes

are a potentially rich source of fungal strains that produce novel bioactive metabolites with promising applications in the field of agriculture and medicine.

To date, no extensive physiological investigations have been conducted on fungal isolates present in the solid ice or sub-ice habitats. Results from Biolog experiment confirm that the isolates have a preference for labile carbon substrates. The rationale behind conducting the BiologPlate assay at 4°C and 24°C was to understand the metabolism and substrate utilization of the fungal isolates at the given temperatures. At 4°C, the two isolates, *L. hoffmannii* and *T. ellipsoideum* which both belong to phylum *Ascomycota* showed differences in their substrate utilization profiles. *Lecythophora hoffmannii* is a slow growing isolate that did not grow at 0°C. For the BiologPlate assay, *L. hoffmannii* at 4°C showed lower absorbance (0.34) than *T. ellipsoideum* (0.43). At 24 °C, however, the difference between the absorbance values of the two isolates was not significant ($p > 0.05$). At 24°C, *L. hoffmannii* showed higher substrate utilization for amino acids followed by carbohydrates (Figure 14). In fungi, carbohydrates are primarily present as polysaccharides (Griffin, 1996). Also, it should be noted that although the substrate utilization was highest for polymers, there is a broad range of carbon sources that are utilized especially at 24°C by these four fungal isolates. This might contribute to the reason why some of these isolates can be found on varied substrates and cold habitats.

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APPENDICES

APPENDIX A

GROWTH OF FUNGAL ISOLATES

Table A 1. Growth of four fungal isolates at seven different temperatures. Average (+/- s.d.; n= 4) maximum diameter (cm) and the number of days required to reach this size are shown for each isolate.

Isolate	Temperature													
	0°C		4°C		7.5°C		10°C		15°C		20°C		25°C	
	Average (SD)	Days	Average (SD)	Days	Average (SD)	Days	Average (SD)	Days	Average (SD)	Days	Average (SD)	Days	Average (SD)	Days
T. ellipsoideum	1.56 (0.02)	14	3.14 (0.006)	25	5.21 (0.001)	25	5.45 (0.001)	25	2.8 (0.001)	11	2.7 (0.06)	12	6.92 (0.002)	25
L. hoffmannii	No growth	NA	0.04 (0.008)	13	2.13 (0.001)	24	2.98 (0.013)	17	2.93 (0.001)	20	4.06 (0.006)	26	5.62 (0.001)	19
Mucor sp.	3.41 (0.003)	10	5.91 (0.002)	12	8.47 (0.007)	7	8.30 (0.003)	6	2.64 (0.004)	10	8 (0.006)	4	8.08 (0.156)	3
Unidentified Acomycota	1.31 (0.003)	14	1.55 (0.002)	21	3.16 (0.002)	24	3.39 (0.003)	21	2.24 (0.01)	10	1.78 (0.021)	12	4.72 (0.001)	24

APPENDIX B

CARBON SUBSTRATE GUILDS

Table B.1. Carbon substrates found in the FF microtitre plates from BIOLOG, divided into the substrate guilds suggested by Dobranic and Zak.

Number of substrates	**Wells**	**Chemical guild**	**Substrate**	**Chemical formula**
1	H8	**Amines/amides**	2-Amino ethanol	C_2O_7NO
2	B11		D-Glucosamine	$C_6H_{13}NO_5$
3	C2		Glucuronamide	$C_6H_{11}NO_6$
4	G7		L-Alaninamide	$C_3H_8N_2O$
5	H9		Putrescine	$C_4H_{12}N_2$
6	G3		Succinamic acid	$C_4H_7NO_3$
7	F1	**Amino acids**	γ-Amino butyric acid	$C_4H_9NO_2$
8	H1		Glycyl-L-glutamic acid	$C_7H_{12}N_2O_5$
9	G8		L-Alanine	$C_3H_7NO_2$
10	G9		L-Alanyl-glycine	$C_5H_{10}N_2O_3$
11	G10		L-Asparagine	$C_4H_8N_2O_3$
12	G11		L-Aspartic acid	$C_4H_7NO_4$
13	G12		L-Glutamic acid	$C_5H_9NO_4$
14	H2		L-Ornithine	$C_5H_{12}N_2O_2$
15	H3		L-Phenylalanine	$C_9H_{11}NO_2$
16	H4		L-Proline	$C_5H_9NO_2$
17	H5		L-Pyroglutamic acid	$C_5H_7N_{O3}$
18	H6		L-Serine	$C_3H_7NO_3$

Table B.1. Continued

19	H7		L-Threonine	$C_4H_9NO_3$
20	B12	**Carbohydrates**	α-D-Glucose	$C_6H_{12}O_6$
21	C8		α-D-Lactose	$C_{12}H_{22}O_{11}$
22	D5		α-Methyl-D-galactoside	$C_7H_{14}O_6$
23	D6		β-Methyl-D-galactoside	$C_7H_{14}O_6$
24	D7		α-Methyl-D-glucoside	$C_7H_{14}O_6$
25	D8		β-Methyl-D-glucoside	$C_7H_{14}O_6$
26	A6		Adonitol	$C_5H_{12}O_5$
27	A11		Arbutin	$C_{12}H_6O_7$
28	A8		D-Arabinose	$C_5H_{10}O_5$
29	A10		D-Arabitol	$C_5H_{12}O_5$
30	A12		D-Cellobiose	$C_{12}H_{22}O_{11}$
31	B5		D-Fructose	$C_6H_{12}O_6$
32	B7		D-Galactose	$C_6H_{12}O_6$
33	D1		D-Mannitol	$C_6H_{14}O_6$
34	D2		D-Mannose	$C_6H_{12}O_6$
35	D3		D-Melezitose	$C_{18}H_{36}O_{16}$
36	D4		D-Melibiose	$C_{12}H_{22}O_{11}$
37	D10		D-Psicose	$C_6H_{12}O_6$
38	D11		D-Rafinose	$C_{18}H_{32}O_{16}$
39	E1		D-Ribose	$C_5H_{10}O_5$
40	E4		D-Sorbitol	$C_6H_{14}O_6$

Table B.1. Continued

41	E8		D-Tagatose	$C_6H_{12}O_6$
42	E9		D-Trehalose	$C_{12}H_{22}O_{11}$
43	E12		D-Xylose	$C_5H_{10}O_5$
44	B9		Gentiobiose	$C_{12}H_{22}O_{11}$
45	B4		i-Erythritol	$C_4H_{10}O_4$
46	C9		Lactulose	$C_{12}H_{22}O_{11}$
47	A9		L-Arabinose	$C_5H_{10}O_5$
48	B6		L-Fucose	$C_6H_{12}O_5$
49	D12		L-Rhamnose	$C_6H_{12}O_5$
50	E5		L-Sorbose	$C_6H_{12}O_6$
51	C10		Maltitol	$C_{12}H_{24}O_{11}$
52	C11		Maltose	$C_{12}H_{22}O_{11}$
53	C12		Maltotriose	$C_{18}H_{32}O_{16}$
54	C6		m-Inositol	$C_6H_{12}O_6$
55	A3		N-Acetyl-D-galactosamine	$C_8H_{15}NO_6$
56	A4		N-Acetyl-D-glucosamine	$C_8H_{15}NO_6$
57	A5		N-Acetyl-D-mannosamine	$C_8H_{15}NO_6$
58	D9		Palatinose	$C_{12}H_{22}O_{11}$
59	E3		Sedoheptulosan	$C_7H_{12}O_6$
60	E6		Stachyose	$C_{24}H_{42}O_{21}$
61	E7		Sucrose	$C_{12}H_{22}O_{11}$
62	E10		Turanose	$C_{12}H_{22}O_{11}$

Table B.1. Continued

63	E11		Xylitol	$C_5H_{12}O_5$
64	F4	**Carboxylic acids**	β-Hydroxy butyric acid	$C_4H_8O_3$
65	F5		γ-Hydroxy butyric acid	$C_4H_8O_3$
66	F7		α-Keto glutaric acid	$C_5H_8O_5$
67	C7		2-Keto-D-gluconic acid	$C_6H_9O_7$
68	B8		D-Galacturonic acid	$C_6H_{10}O_7$
69	B10		D-Gluconic acid	$C_6H_{12}O_7$
70	C3		D-Glucuronic acid	$C_6H_{10}O_7$
71	F10		D-Malic acid	$C_4H_6O_5$
72	G1		D-Saccharic acid	$C_6H_{10}O_8$
73	F3		Fumaric acid	$C_4H_4O_4$
74	F9		L-Lactic acid	$C_3H_6O_3$
75	F11		L-Malic acid	$C_4H_6O_5$
76	G6		N-Acetyl-L-glutamic acid	$C_7H_{11}NO_5$
77	F6		p-Hydroxy phenylacetic acid	$C_8H_8O_3$
78	F12		Quinic acid	$C_7H_{12}O_6$
79	G2		Sebacic acid	$C_{10}H_{18}O_4$

Table B.1. Continued

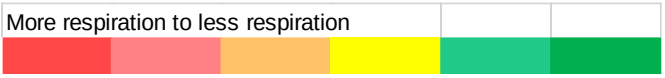
80	G4		Succinic acid	$C_4H_6O_4$
81	H10	**Miscellaneous**	Adenosine	$C_{10}H_{13}N_5O_4$
82	A7		Amygdalin	$C_{20}H_{27}NO_{11}$
83	H12		Adenosine-5P-monophosphate	$C_{10}H_{14}N_5O_7P$
84	F2		Bromo succinic acid	$C_4H_5O_4Br$
85	F8		D-Lactic acid methyl ester	$C_4H_8O_3$
86	C1		Glucose-1-phosphate	$C_6H_{13}O_9P$
87	C4		Glycerol	$C_3H_8O_3$
88	E2		Salicin	$C_{13}H_{18}O_7$
89	G5		Succinic acid mono-methyl ester	$C_5H_8O_4$
90	H11		Uridine	$C_9H_{12}N_2O_6$
91	B1	**Polymers**	α-Cyclodextrin	$C_{36}H_{60}O_{30}$
92	B2		β-Cyclodextrin	$C_{42}H_{70}O_{35}$
93	B3		Dextrin	$C_6H_{10}O_5$
94	C5		Glycogen	$(C_6H_{10}O_5)n$
95	A2		Tween 80	

APPENDIX C

HEAT MAPS OF FUNGAL ISOLATES

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.189	0.231	0.211	0.283	0.256	0.324	0.280	0.349	0.322	0.288	0.138	0.175
B	0.419	0.165	0.285	0.189	0.223	0.311	0.317	0.227	0.115	0.080	0.088	0.171
C	0.197	0.155	0.166	0.185	0.202	0.181	0.245	0.226	0.265	0.140	0.135	0.132
D	0.153	0.162	0.146	0.152	0.133	0.143	0.126	0.241	0.249	0.084	0.083	0.135
E	0.292	0.157	0.140	0.175	0.160	0.149	0.148	0.096	0.147	0.130	0.118	0.219
F	0.146	0.147	0.148	0.143	0.143	0.127	0.140	0.135	0.129	0.092	0.109	0.112
G	0.194	0.420	0.158	0.174	0.135	0.150	0.128	0.143	0.148	0.123	0.119	0.118
H	0.163	0.154	0.196	0.152	0.143	0.124	0.105	0.198	0.094	0.312	0.114	0.084

Figure C.1. Heat map for *T. ellipsoideum* at 4°C at 490nm



	1	2	3	4	5	6	7	8	9	10	11	12
A	0.146	0.228	0.223	0.210	0.187	0.166	0.155	0.230	0.206	0.129	0.103	0.018
B	0.238	0.223	0.276	0.186	0.202	0.215	0.178	0.160	0.165	0.166	0.128	0.097
C	0.186	0.224	0.223	0.248	0.254	0.229	0.240	0.212	0.192	0.188	0.158	0.118
D	0.162	0.230	0.253	0.219	0.213	0.222	0.195	0.194	0.177	0.163	0.135	0.109
E	0.262	0.197	0.238	0.287	0.262	0.237	0.262	0.229	0.233	0.184	0.173	0.183
F	0.168	0.207	0.201	0.205	0.195	0.219	0.224	0.194	0.179	0.172	0.144	0.104
G	0.155	0.428	0.216	0.178	0.208	0.195	0.173	0.184	0.173	0.172	0.130	0.098
H	0.146	0.121	0.247	0.232	0.287	0.182	0.205	0.279	0.173	0.210	0.144	0.100

Figure C.2. Heat map for *T. ellipsoideum* at 24°C at 490nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.127	0.157	0.150	0.133	0.116	0.077	0.070	0.123	0.094	0.079	0.074	0.101
B	0.369	0.124	0.147	0.068	0.075	0.095	0.083	0.033	0.069	0.028	0.018	0.115
C	0.147	0.115	0.134	0.124	0.133	0.106	0.107	0.083	0.097	0.086	0.043	0.079
D	0.102	0.088	0.092	0.109	0.077	0.082	0.096	0.101	0.069	0.053	0.032	0.064
E	0.185	0.123	0.100	0.115	0.099	0.074	0.070	0.077	0.063	0.067	0.061	0.085
F	0.107	0.083	0.073	0.095	0.076	0.065	0.084	0.058	0.057	0.064	0.045	0.048
G	0.163	0.309	0.097	0.105	0.116	0.084	0.059	0.081	0.076	0.070	0.055	0.058
H	0.139	0.097	0.146	0.109	0.105	0.081	0.072	0.141	0.060	0.096	0.076	0.067

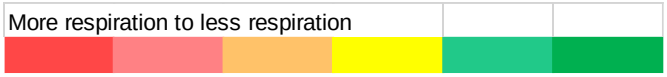
Figure C.3. Heat map for Unknown *Ascomycota* at 4°C at 490nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.121	0.155	0.181	0.186	0.156	0.126	0.120	0.176	0.170	0.095	0.081	0.019
B	0.242	0.147	0.258	0.155	0.158	0.157	0.140	0.119	0.146	0.123	0.101	0.090
C	0.176	0.186	0.180	0.197	0.236	0.197	0.228	0.179	0.164	0.151	0.109	0.073
D	0.150	0.179	0.142	0.177	0.168	0.191	0.169	0.143	0.154	0.134	0.113	0.062
E	0.204	0.190	0.178	0.172	0.210	0.180	0.176	0.187	0.190	0.172	0.172	0.090
F	0.119	0.166	0.176	0.171	0.174	0.175	0.187	0.130	0.147	0.135	0.121	0.050
G	0.180	0.393	0.168	0.161	0.178	0.175	0.156	0.150	0.135	0.124	0.095	0.027
H	0.128	0.148	0.198	0.145	0.157	0.133	0.135	0.193	0.098	0.118	0.072	0.048

Figure C.4. Heat map for Unknown *Ascomycota* at 24°C at 490nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.140	0.152	0.152	0.184	0.089	0.085	0.070	0.130	0.111	0.099	0.101	0.120
B	0.345	0.119	0.173	0.050	0.098	0.085	0.078	0.043	0.090	0.059	0.031	0.158
C	0.135	0.065	0.078	0.114	0.233	0.100	0.167	0.117	0.095	0.080	0.087	0.101
D	0.071	0.145	0.062	0.101	0.079	0.089	0.048	0.058	0.057	0.053	0.041	0.093
E	0.153	0.097	0.069	0.100	0.099	0.076	0.097	0.077	0.084	0.062	0.038	0.082
F	0.079	0.070	0.053	0.068	0.076	0.082	0.072	0.035	0.033	0.031	0.032	0.055
G	0.097	0.213	0.059	0.050	0.057	0.053	0.074	0.056	0.046	0.062	0.048	0.056
H	0.069	0.078	0.141	0.081	0.059	0.059	0.057	0.059	0.078	0.266	0.061	0.067

Figure C.5. Heat map for *Mucor* at 4°C at 490nm



	1	2	3	4	5	6	7	8	9	10	11	12
A	0.154	0.316	0.222	0.683	0.135	0.165	0.187	0.164	0.212	0.199	0.337	0.351
B	0.257	0.118	0.275	0.091	0.214	0.136	0.308	0.053	0.279	0.117	0.074	0.386
C	0.310	0.161	0.137	0.264	0.248	0.164	0.305	0.166	0.173	0.148	0.260	0.155
D	0.189	0.306	0.151	0.190	0.150	0.165	0.097	0.193	0.147	0.126	0.094	0.150
E	0.265	0.271	0.136	0.273	0.275	0.197	0.151	0.136	0.346	0.128	0.228	0.358
F	0.140	0.128	0.261	0.108	0.200	0.140	0.373	0.149	0.268	0.211	0.197	0.130
G	0.196	0.401	0.166	0.230	0.116	0.167	0.197	0.445	0.304	0.199	0.213	0.333
H	0.270	0.150	0.248	0.155	0.101	0.139	0.170	0.148	0.201	0.080	0.092	0.115

Figure C.6. Heat map for *Mucor* at 24°C at 490nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.145	0.248	0.176	0.181	0.142	0.107	0.083	0.177	0.131	0.091	0.091	0.099
B	0.340	0.112	0.228	0.073	0.119	0.091	0.077	0.025	0.098	0.027	0.044	0.167
C	0.148	0.090	0.115	0.195	0.144	0.105	0.161	0.117	0.118	0.093	0.120	0.117
D	0.111	0.174	0.152	0.116	0.091	0.117	0.085	0.115	0.110	0.057	0.043	0.112
E	0.197	0.112	0.095	0.143	0.131	0.108	0.130	0.127	0.147	0.080	0.073	0.170
F	0.112	0.096	0.100	0.079	0.080	0.060	0.102	0.074	0.091	0.050	0.050	0.093
G	0.165	0.286	0.123	0.089	0.082	0.105	0.101	0.104	0.085	0.046	0.070	0.075
H	0.125	0.130	0.169	0.134	0.108	0.104	0.082	0.104	0.068	0.152	0.077	0.061

Figure C.7. Heat map for *L. hoffmannii* at 4°C at 490nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.106	0.571	0.161	0.509	0.125	0.114	0.208	0.235	0.357	0.148	0.398	0.494
B	0.109	0.168	0.286	0.112	0.268	0.085	0.213	0.168	0.269	0.152	0.135	0.419
C	0.197	0.083	0.248	0.428	0.321	0.219	0.173	0.206	0.127	0.243	0.383	0.499
D	0.282	0.307	0.288	0.134	0.116	0.239	0.118	0.287	0.265	0.180	0.070	0.296
E	0.266	0.303	0.151	0.335	0.350	0.161	0.167	0.163	0.489	0.165	0.199	0.595
F	0.231	0.150	0.171	0.170	0.307	0.176	0.189	0.184	0.193	0.131	0.106	0.591
G	0.148	0.355	0.274	0.184	0.237	0.194	0.247	0.465	0.183	0.266	0.335	0.519
H	0.141	0.205	0.233	0.206	0.518	0.155	0.220	0.208	0.172	0.139	0.134	0.117

Figure C.8. Heat map for *L. hoffmannii* at 24°C at 490nm